UNIVERSITY OF NEUCHÂTEL
FACULTY OF SCIENCES

INSTITUTE OF BIOLOGY
LABORATORY OF ECOEPIDEMIOLOGY

Biology of *Borrelia burgdorferi*-infected and non-infected *Ixodes ricinus* ticks in the context of climate change

THESIS PRESENTED TO THE FACULTY OF SCIENCES OF THE UNIVERSITY OF NEUCHÂTEL FOR THE DEGREE OF DOCTOR OF SCIENCES BY

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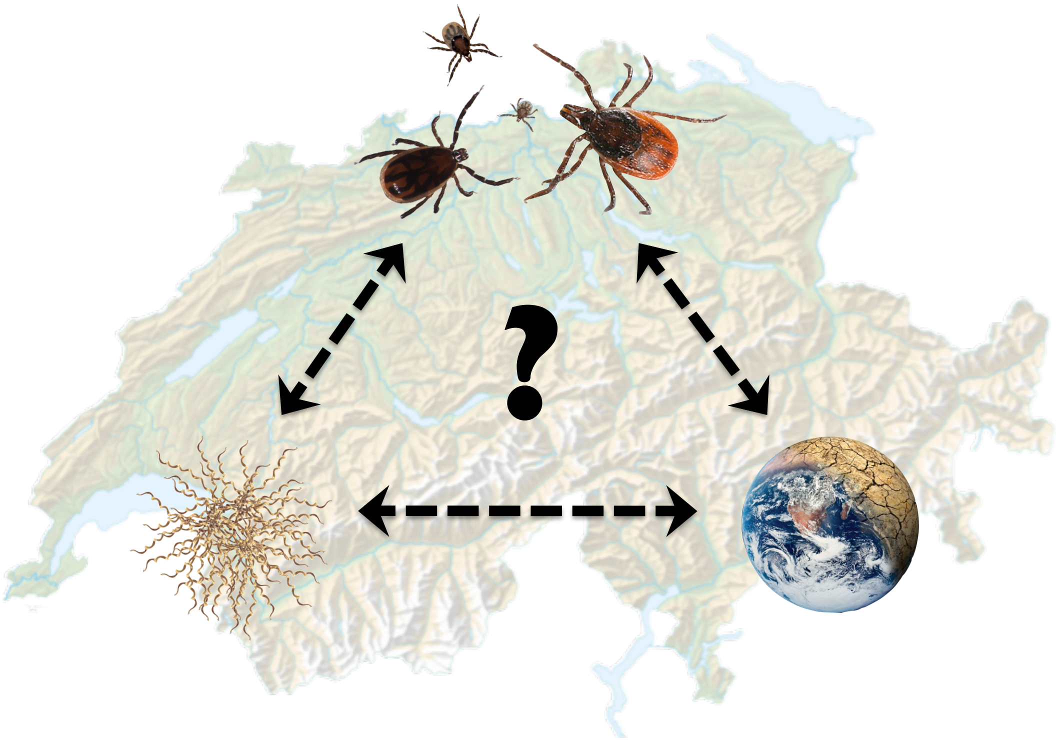

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“Biology *of Borrelia burgdorferi*-infected and non-infected *Ixodes ricinus* ticks in the context of climate change”

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Neuchâtel, le 28 juin 2013

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Success is a journey, not a destination. The doing is often more important than the outcome

Arthur Ashe

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1 ABSTRACT

I ABSTRACT

Ixodes ricinus is known to display little resistance to desiccation. This tick is particularly exposed to desiccating conditions while questing for a host on the vegetation. Therefore, survival under hot and dry conditions in field-collected *I. ricinus* nymphs and adults was studied by exposing ticks to desiccating conditions created in the laboratory using saturated salt solutions producing various relative humidity conditions at different temperatures. We observed that *I. ricinus* nymphs and adults infected with *Borrelia burgdorferi* sensu lato (s.l.) survived better under hot and dry conditions than uninfected ticks. These findings suggest that *Borrelia* spirochetes might change the physiology and/or metabolism of organs involved in water sorption, storage, and/or loss, resulting in enhanced water storage in infected ticks, thereby modifying *I. ricinus* need for humidity. To test this hypothesis, field-collected *I. ricinus* nymphs were allowed to walk within a humidity gradient in a two-choice arena, and their position (indicating their overall movement) was recorded. *I. ricinus* nymphs harbouring spirochetes walked less within the humidity gradient than uninfected nymphs. *Borrelia*-infected individuals showed a lower need to move to an environment that was favourable for maintaining water balance since they preferentially stayed in a moderately dry area, compared to uninfected ticks, which preferentially moved towards a moister area. This suggests that *B. burgdorferi*-infected ticks might tolerate desiccating conditions differently from uninfected individuals. Since *I. ricinus* ticks go down the vegetation more frequently when desiccation increases in nature (Randolph and Storey, 1999; Perret et al., 2004), which increases energy consumption, this higher tolerance to desiccation in infected ticks might be due to higher energy reserves (fat content) in the latter. Fat content was quantified in field-collected *I. ricinus* nymphs and we observed that nymphs infected with *B. burgdorferi* s.l. possessed higher energy reserves than uninfected nymphs. These findings imply that infected *I. ricinus* nymphs with higher energy reserves may quest for a longer time than uninfected individuals. Higher energy reserves allow more movements up and down the vegetation before depletion of energy reserves, which allows longer survival. Prolonged questing time increases chances for infected ticks to find hosts and thereby to transmit *Borrelia*. This is thought to be a manipulating strategy of *B. burgdorferi* spirochetes to enhance their transmission to other hosts by modifying phenotypic traits of their vector.

Survival during winter months is also a challenge for *I. ricinus*. Therefore, we exposed field-collected *I. ricinus* nymphs to cold temperatures at different frequencies of exposition. Results showed that younger (autumn) nymphs were more likely to survive under cold conditions than older (spring) nymphs. Moreover, nymphs died faster when they were exposed to temperature variations at high frequency than low frequency. This probably reflected higher energetic costs due to metabolic adaptations that temperature variations generated when experienced at higher frequency. Besides, *Borrelia*-infected nymphs survived slightly better than uninfected nymphs.

Borrelia infections in field-collected *I. ricinus* nymphs used in behavioural or physiological tests were combined in one data set to analyse statistically the associations between *Borrelia* genospecies. We observed that *Borrelia* genospecies that were specialised for the same reservoir hosts frequently occurred together, while *Borrelia* genospecies that were specialised for different reservoir hosts rarely co-occurred. Furthermore, infections involving *Borrelia* genospecies frequently co-occurring consisted of high spirochete numbers, whereas infections involving *Borrelia* genospecies rarely co-occurring consisted of low spirochete numbers. We suggest that the vertebrate immune system plays a role in shaping both the frequency of infections involving two *Borrelia* genospecies and the spirochete load inside the tick harbouring two genospecies.

This thesis has contributed to improve significantly our knowledge on the biology of *Borrelia*-infected and uninfected *I. ricinus* ticks in the context of climate change, in particular by revealing that *Borrelia*-infected ticks are more likely to survive and find hosts under desiccating conditions in spring and summer, and that *I. ricinus* ticks are likely to survive better in winter if temperature variations are less frequent.

Key words: *Ixodes ricinus*, *Borrelia burgdorferi* s.l., climate change, survival, humidity attraction, motor activity, energy reserves, genospecies interactions

Résumé

La tique *Ixodes ricinus* est très sensible à la dessiccation et elle est particulièrement exposée à des conditions desséchantes lorsqu'elle quête sur la végétation en attente d'un hôte. Nous avons donc étudié la survie de cette tique à la dessiccation en exposant des nymphes et des adultes à des conditions desséchantes créées en laboratoire à l'aide de solutions salines saturées produisant différentes conditions d'humidité relative à différentes températures. Nous avons observé que lorsque les tiques sont infectées par *Borrelia burgdorferi* sensu lato (s.l.), elles survivent mieux à la dessiccation que les tiques non infectées. Ces résultats suggèrent que les borrélies pourraient modifier la physiologie et/ou le métabolisme des organes impliqués dans l'absorption, le stockage, et/ou la perte d'eau résultant en une capacité à stocker l'eau améliorée chez les tiques infectées, modifiant leur besoin d'humidité. Nous avons vérifié cette hypothèse en exposant des nymphes *I. ricinus* récoltées dans la nature à un gradient d'humidité dans une arène proposant deux choix (sec ou humide), puis nous avons relevé leur position dans l'arène (indiquant leur mouvement général). Les nymphes infectées se sont moins déplacées le long du gradient d'humidité que les nymphes non infectées et sont restées préférentiellement dans un endroit modérément sec, comparé aux tiques non infectées qui, elles, se sont déplacées préférentiellement vers un endroit plus humide. Ceci laisse penser que les tiques infectées par *B. burgdorferi* s.l. tolèrent mieux des conditions desséchantes que celles qui ne sont pas infectées.

Dans la nature, quand l'atmosphère est très sèche, *I. ricinus* doit descendre au sol plus fréquemment pour se réhydrater (Randolph and Storey, 1999; Perret et al., 2004), ce qui augmente sa consommation d'énergie. La survie accrue à la dessiccation observée chez les tiques infectées pourrait être due à des réserves d'énergie (contenu en gras) plus grandes chez ces dernières. Nous avons donc quantifié le contenu en gras chez des nymphes récoltées dans la nature et nous avons observé que les nymphes infectées par *B. burgdorferi* s.l. possédaient de plus grandes réserves d'énergie que les nymphes non infectées. Ces résultats impliquent que les nymphes infectées ont la possibilité de quêter plus longtemps, car elles peuvent effectuer un plus grand nombre mouvements de haut en bas sur la végétation avant épuisement de leurs réserves d'énergie et donc survivre plus longtemps. Un temps de quête plus long augmente les chances de trouver des hôtes et ainsi de transmettre *Borrelia*. On peut donc parler d'une stratégie de manipulation de la part des spirochètes *B. burgdorferi* pour optimiser leur transmission à d'autres hôtes en modifiant les traits phénotypiques de leur vecteur.

La survie durant les mois d'hiver représente également un défi pour *I. ricinus*. C'est pourquoi nous avons testé la survie de nymphes *I. ricinus* à des températures froides et à des variations de température plus ou moins fréquentes. Nous avons observé que les nymphes plus jeunes (récoltées en automne) survivent mieux aux conditions hivernales que les nymphes plus âgées (récoltées au printemps). De plus, plus la fréquence de variation de température augmente, plus le taux de mortalité augmente chez les nymphes. Cette augmentation du taux de mortalité reflète probablement l'augmentation des coûts énergétiques des adaptations métaboliques que les variations de température génèrent à une fréquence croissante. En outre, les nymphes infectées par *Borrelia* survivent légèrement mieux aux conditions hivernales que les nymphes non infectées.

Nous avons combiné les données concernant l'infection par *Borrelia* des nymphes *I. ricinus* récoltées dans la nature et précédemment testées dans des tests comportementaux ou physiologiques. Cet important jeu de données nous a permis d'analyser statistiquement les associations entre les génoespèces de borrélies. Nous avons observé que les génoespèces de borrélies spécialisées pour les mêmes hôtes réservoirs co-infectaient fréquemment *I. ricinus*, alors que celles spécialisées pour des hôtes réservoirs différents étaient rarement détectées dans les mêmes tiques. De plus, les infections impliquant des génoespèces fréquemment en co-infection comprenaient un nombre élevé de spirochètes dans la tique, alors que les infections impliquant des génoespèces rarement en co-infection consistaient en des nombres faibles de spirochètes. De tels résultats laissent supposer que le système immunitaire des hôtes vertébrés joue un rôle dans la détermination à la fois de la fréquence des infections impliquant deux génoespèces de borrélies et du nombre de spirochètes dans la tique infectée par deux génoespèces.

Cette thèse contribue de manière significative à améliorer nos connaissances concernant la biologie des tiques *I. ricinus* infectées par *Borrelia* et non infectées dans un contexte de changement climatique, en révélant notamment que les tiques infectées par *Borrelia* risquent de mieux survivre et de trouver des hôtes plus aisément dans des conditions desséchantes au printemps et en été, et que les tiques *I. ricinus* sont susceptibles de mieux survivre en hiver si les écarts de température se font plus rares.

Mots clés: *Ixodes ricinus*, *Borrelia burgdorferi* s.l., changement climatique, survie, attraction à l'humidité, activité motrice, réserves d'énergie, interactions entre génoespèces

2 INTRODUCTION

2 INTRODUCTION

2.1 Aim of the Thesis

The purpose of the present thesis originated from an unexpected result of burning vegetation in a North American forest. The aim of the burning was to destroy tick habitat in order to reduce *I. dammini* (later known as *I. scapularis*) prevalence and subsequently the risk for human infection with Lyme disease, linked to the abundance of ticks infected with spirochetes causing the disease (Mather et al., 1993). Unsurprisingly, the abundance of *I. dammini* nymphs was lowered by 49 % in the burnt woods (Mather et al., 1993). However, the risk of encountering ticks infected with *B. burgdorferi* remained identical in the burnt and the unburnt area, since Lyme disease spirochete prevalence was significantly higher in nymphs collected from the devastated section of the woodlot (35 %) than the undamaged section (18 %) (Mather et al., 1993). Such disproportionate death rate among selected subpopulations of *I. dammini* remained unexplained.

As an increasing number of studies show that parasites influence the biology of their arthropod vectors and in the context of global warming in which environmental conditions change gradually, it appeared interesting to focus on the following questions:

- **Might *I. ricinus* survival under hot and dry conditions be influenced by infection with *B. burgdorferi* s.l. spirochetes?**
- **Might *I. ricinus* behaviour within a humidity gradient be influenced by infection with *B. burgdorferi* s.l. spirochetes?**
- **Might *I. ricinus* level of energy reserves be influenced by infection with *B. burgdorferi* s.l. spirochetes?**
- **Might *I. ricinus* survival under cold conditions be influenced by infection with *B. burgdorferi* s.l. spirochetes?**

Finally, the last chapter of the present thesis was dedicated to the **associations and interactions between *B. burgdorferi* s.l. genospecies present in questing *I. ricinus* ticks**.

2.2 Vector: *Ixodes ricinus*

2.2.1 Scientific Classification

Ixodes ricinus is a hard tick that belongs to the phylum *Arthropoda*, which also contains insects, the class *Chelicerata*, the subclass *Arachnida*, which ticks share with spiders, the order *Acari*, which also comprises mites, and the suborder *Ixodida* (*Metastigmata*) (Table 2.1) (Hoogstraal and Aeschlimann, 1982; Keirans and Robbins, 1999; Horak et al., 2002; Barker and Murrel, 2004). The *Ixodida* are divided into three families: *Ixodidae*, i.e. hard ticks accounting for the majority of known tick species (approximately 700 species), *Argasidae*, i.e. soft ticks consisting of approximately 200 species, and *Nuttalliellidae*, a monospecific family containing only 1 species, *Nuttalliella namaqua*, the closest relative to the ancestral tick lineage (Sonenshine et al., 2002; Guglielmone et al., 2010; Mans et al., 2011). The genus *Ixodes* is the largest, with an estimated 243 species, among which 14 species belong to the *I. ricinus* complex: *I. pavlovskyi*, *I. nipponensis*, *I. gibbosus*, *I. jellisoni*, *I. pararicinus*, *I. affinis*, *I. muris*, *I. minor*, *I. granulatus*, *I. nuttallianus*, *I. pacificus*, *I. persulcatus*, *I. ricinus*, and *I. scapularis* (the last four species being the most prominent ones) (Keirans et al., 1999; Sonenshine et al., 2002; Xu et al., 2003; Guglielmone et al., 2010).

Table 2.1 Systematics of *I. ricinus* (Hoogstraal and Aeschlimann, 1982; Keirans and Robbins, 1999; Horak et al., 2002; Barker and Murrel, 2004).

Phylum	*Arthropoda*
Class	*Chelicerata*
Subclass	*Arachnida*
Order	*Acari*
Suborder	*Ixodida* (*Metastigmata*)
Family	*Ixodidae*
Genus	*Ixodes*
Species	*Ixodes ricinus*

2.2.2 Biology

2.2.2.1 Morphology

Ixodid ticks are quite large, their body length ranging from two to 20 mm (Wall and Shearer, 2001). Their body is divided into two parts: the anterior gnathosoma (or capitulum), which carries the mouthparts, and the posterior idiosoma (Wall and Shearer, 2001). In turn, the idiosoma is divided into two parts: the anterior podosoma, which bears the legs and the genital pore, and the posterior opisthosoma, which harbours the stigmata and the anus (Fig. 2.1) (Sonenshine et al., 2002).

I. ricinus ticks develop in three stages: larva, nymph and adult (among which a sexual dimorphism distinguishes male from female) (Fig. 2.3). Ixodid ticks feature a sclerotised dorsal plate called scutum, which covers entirely the dorsal surface in males and partially in females to allow the expansion of the alloscutum occurring during feeding (body weight increases up to 100 times) (Wall and Shearer, 2001; Capinera et al., 2008).

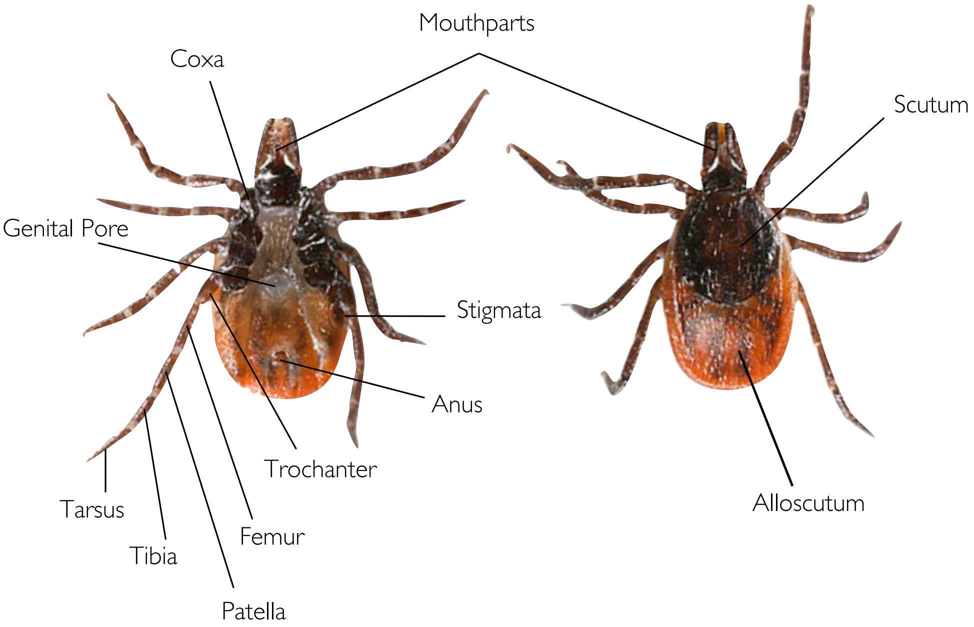

Fig. 2.1 External morphology of female *I. ricinus*: ventral view (left) and dorsal view (right) (Picture: S. Sollfors [http://www.eurospiders.com/Ixodes_ricinus.htm], modified).

Ixodid larvae possess three pairs of legs but nymphs and adults possess four (Sonenshine, 1991). Each leg consists of six segments: coxa, which attaches the leg to the body, trochanter, femur, patella, tibia, and tarsus, on which small claws help the tick grasp a passing host (Sonenshine et al., 2002). Located on the tarsi of the first leg are numerous sensory hairs and a pit known as Haller's organ (Wall and Shearer, 2001). Haller's organ is a chemoreceptor that enables the tick to detect potential host stimuli (such as temperature and smell) while searching for hosts (Lees, 1948; Sonenshine et al., 2002). Other chemoreceptors are present on the palps, chelicerae and scutum (Wall and Shearer, 2001).

In Ixodid ticks, the eyes are situated on the lateral margin of the scutum (Phillis and Cromroy, 1977), unlike other arthropods where the eyes are on the head. *I. ricinus*, in contrast to other hard ticks such as *Amblyomma americanum*, *A. variegatum* or *Hyalomma dromedarii*, does not have eyes with corneas (Phillis and Cromroy, 1977; Kaltenrieder et al., 1989). Instead, *I. ricinus* possesses photosensitive cells positioned dorsoventrally on each side of the tick behind the coxae of the second pair of legs (Perret et al., 2003).

The mouthparts consist of a pair of segmented pedipalps, which are sensory organs of touch, two segmented appendages known as chelicerae, which move back and forth in order to pierce and cut through the skin of the vertebrate host during feeding, and the toothed hypostome, which anchors the tick in the host skin during feeding (Wall and Shearer, 2001; Sonenshine et al., 2002)

The internal organs of Ixodid ticks are bathed in a watery medium, the hemolymph, which is rich in salts, amino acids, soluble proteins, and other dissolved substances. The hemolymph also contains hemocytes, among which are numerous phagocytes (Sonenshine et al., 2002). The hemolymph is filtered by a simple heart, positioned middorsally (Fig. 2.2) (Sonenshine et al., 2002).

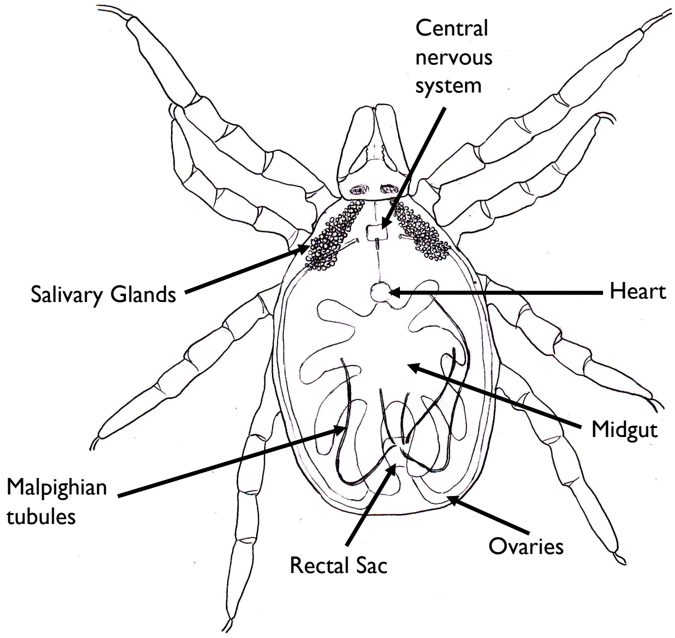

Fig. 2.2 Drawing of the internal anatomy of a female *Ixodes* tick (Drawing: C. Herrmann).

The most prominent internal organ is the midgut, a spider-like structure with many lateral diverticuli (Sonenshine et al., 2002). While larvae lack a tracheal system and stigmata (water loss and transpiration take place directly through the integument), nymphal and adult ticks possess a complex branching system of tracheae that opens to the exterior at the stigmata, a pair of respiratory openings located posterior to the coxae of the fourth pair of legs (Wall and Shearer, 2001). Paired salivary glands, which look like clusters of grapes, are positioned anterolaterally and are connected to the mouthparts via the salivary ducts (Sonenshine et al., 2002). The central nervous system is a synganglion, a mass formed by the fusion of the brain ganglia and the abdominal nerve cord, which is situated antero-ventrally (Sonenshine et al., 2002; Mehlhorn, 2008). Excretion is accomplished by the Malpighian tubules, which are a pair of long and coiled structures that empty into the rectal sac, where nitrogenous wastes are stocked in the form of snow-white guanine (Sonenshine et al., 2002). Reproductive organs include the ovary, paired oviducts, uterus, vagina and seminal receptacle in females and the testes, vasa deferentia, seminal vesicle, the ejaculatory duct and the spermatophore in males, and are prominent internal structures positioned posteriorly (Sonenshine et al., 2002; Mehlhorn, 2008).

2.2.2.2 Life Cycle

I. ricinus is a three-host tick of which each stage, i.e. larva, nymph and adult female (adult male may take a small blood meal but does not engorge), parasitises a different vertebrate host (Aeschlimann, 1972) (Fig. 2.3). *I. ricinus* ticks feed on a wide range of hosts, i.e. more than 300 different vertebrate species (Anderson, 1991), some of them acting as reservoir hosts of Lyme disease spirochetes (see section 2.3.5). Rodents such as *Apodemus flavicollis* or *Myodes glareolus* are primarily infested by immature ticks, mainly larvae (Matuschka et al., 1991; Humair et al., 1999; Randolph and Storey, 1999; Hanincova et al., 2003a). Adults usually feed on larger mammals (Aeschlimann, 1972), but are also outnumbered by immature ticks on such hosts (Jeanson and Tälleklint, 1992). *I. ricinus* does not show host specificity and the most important determinants of host choice are host habitat and behaviour (Aeschlimann, 1972), microclimate conditions (Randolph and Storey, 1999), and questing height of the different tick stages. Immature instars, larvae and nymphs, usually stay closer to the ground, i.e. they are predominantly observed between 0 to 30 cm and 30 to 70 cm, respectively (Gigon, 1985; Mejlon and Jaenson, 1997), probably because they are more sensitive to ambient humidity than mature stages due to their high surface area to volume ratio (see section 2.2.3.3) (Randolph and Storey, 1999). Male and female adults are observed higher up on the vegetation, usually 60 to 80 cm above the ground, but they may be found higher than 1.5 m depending on the surrounding vegetation (Gigon, 1985; Mejlon and Jaenson, 1997).

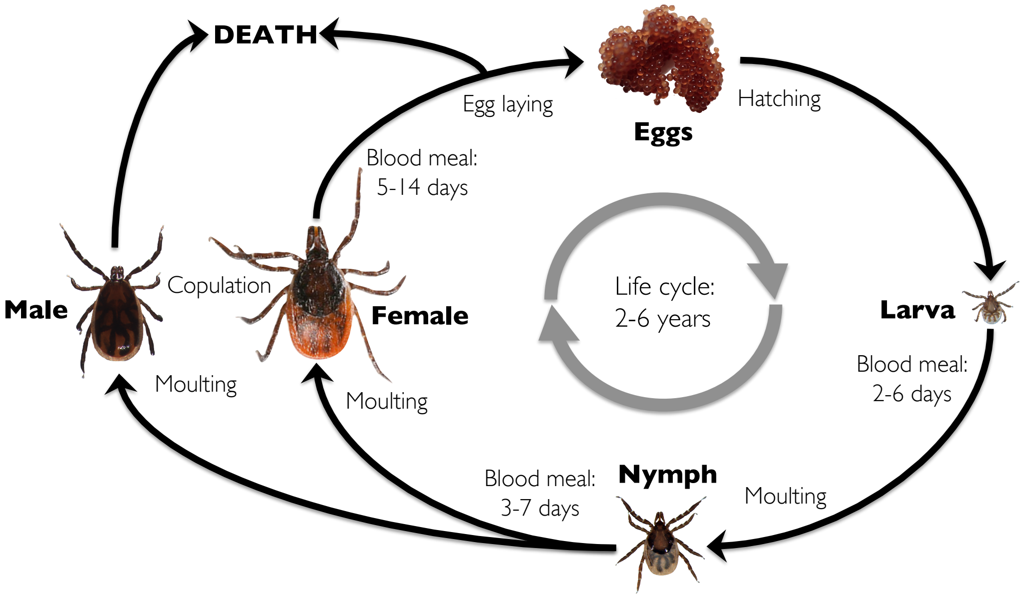

Fig. 2.3 *I. ricinus* life cycle displaying its three developmental stages: larva (0.5 to 0.7 mm), nymph (1.3 to 1.5 mm), and adult male (2.5 to 3 mm) and female (3 to 4 mm) ticks (Aeschlimann, 1972; Graf, 1978c; Gray, 1991; Wall and Shearer, 2001) (Pictures: N. Tonetti, S. Sollfors [http://www.eurospiders.com/Ixodes_ricinus.htm], J. K. Ty [http://www.astrophotophils.com/images/091012tick-d.jpg], modified).

Ticks belonging to the *I. ricinus* complex have long been considered to move essentially in the vertical plane to seek the two resources that are necessary to their survival, i.e. water vapour (down the vegetation, in the litter layer) and hosts (up the vegetation) (Goddard, 1993). However, horizontal movements, though first described as being minimal or inexistent (Lees and Milne, 1951; Goddard, 1993), have in fact revealed to be considerable in ticks of the *I. ricinus* complex in more recent studies. Hence, horizontal movements in the vegetation in colour-marked adult *I. ricinus* may reach up to 2 m (Gigon, 1985). Horizontal walking in a natural setting may be triggered in *I. ricinus* (Gray, 1985) and *I. dammini* (Falco and Fish, 1991) with carbon dioxide traps. In such cases, *I. ricinus* nymphs and adults, and *I. dammini* adults have been found as far as 1 m, 3.5 m, and 1.8 m, respectively, from their release point. Nymph and adult *I. scapularis* have been observed to disperse more than 4.5 m at the woods-pasture interface (Carroll and Schmidtmann, 1996). More recently, Lane et al. (2009) have reported that *I. pacificus* nymphs disperse up to 2.97 m in litter. In a laboratory setting, *I. ricinus* nymphs may walk great distances, up to 9.56 m (although the authors could not distinguish vertical from horizontal movements) (Perret et al., 2003).

In order to find vertebrate hosts, ticks such as *I. ricinus* climb up the vegetation and wait on the tips of grasses and shrubs with their first pair of legs outstretched, a behaviour known as questing. Sites up the vegetation are not chosen randomly by questing ticks. In fact, *I. ricinus* adults reuse extensively stems previously and recently used (Healy and Bourke, 2008). Moreover, *I. scapularis* adults exploit kairomones left by hosts on the vegetation in order to select questing sites (Carroll et al., 1995, 1996, 1998; Carroll, 2002). While questing, ticks detect host stimuli (such as temperature, moisture, smell, carbon dioxide or vibrations) with various sensory receptors (Wall and Shearer, 2001). The most important chemoreceptor used by questing ticks is Haller's organ, located on the tarsi of the first pair of legs (Lees, 1948; Sonenshine et al., 2002). When vertebrate hosts brush along spots where ticks are waiting, ticks immediately grab the passing hosts and climb onto them.

Once the tick has found a suitable feeding site on a vertebrate host, it starts blood feeding. Blood meal duration lasts 2 to 6 days for larvae and 3 to 7 days for nymphs (Fig. 2.3) (Gray, 1991), female nymphs feeding longer than male nymphs (Graf, 1978a; Mbow et al., 1994; Dusbabek, 1996). Sexual dimorphism in *I. ricinus* nymphs is most pronounced when comparing engorgement weight, which ranges from 3.7 to 8.7 mg in female nymphs compared to 2.4 to 4 mg in male nymphs (Graf, 1978a; Mbow et al., 1994; Dusbabek, 1996). It appears that the presence of a parasite, *Babesia microti*, in tick host blood increases feeding success in *I. trianguliceps* (Randolph, 1991) and *I. scapularis* (Hu et al., 1997) immature stages. After moulting from the nymphal stage, adult females take a blood meal for 5 to 14 days (Fig. 2.3) (Gray, 1991). Copulation occurs either on the vegetation before or on the host during the female blood meal. Males usually die after repeated mating (generally 2 to 3 times). In fact, males that have mated die earlier than those that have not copulated yet (Graf, 1978b). Once females are fully engorged, they let themselves fall on the ground and lay thousands of eggs. Females die rapidly after egg laying.

Each stage takes approximately one year to develop to the next, so that the *I. ricinus* life cycle usually takes three years to complete (Fig. 2.3) (Gray, 1991). However, it may vary from two to six years depending on host availability and climate conditions, in particular temperature (Van Es et al., 1998) and humidity (Gray, 1991; Randolph and Storey, 1999; Randolph et al., 2002), which influence development duration and energy consumption (see sections 2.2.2.3, 2.2.3.2, and 2.2.3.3).

2.2.2.3 Energy Reserves

In haematophagous arthropods, fat content is a source of energy derived from each blood meal (Lehane, 1991). Although the blood meal primarily consists of proteins (up to 95 %), it appears that the digestive products obtained during feeding are largely converted to and stored as fat (lipids) (Lehane, 1991). Hence, lipids increase dramatically during the blood meal in the salivary glands in *A. americanum* (Shipley et al., 1993). In *I. ricinus* ticks, blood digestion is intracellular, occurring largely within the midgut and gut epithelium (Akov, 1982; Franta et al., 2010). Moreover, mitochondrial cells of the salivary glands are involved in lipid production during feeding in *A. cajennense* (Denardi et al., 2006). In ticks, lipids are stored in epithelial cells of the midgut, as shown in unfed *Dermacentor variabilis* (Tarnowski and Coons, 1989) and *Haemaphysalis longicornis* (Umemiya-Shirafuji et al., 2010), and in the fat body (Sonenshine, 1991). Unlike that of insects, the fat body of ticks is a diffuse organ of highly dispersed strands of cells (primarily trophocytes) adhering to the branches of the tracheal system and, occasionally, to other internal organs (Sonenshine, 1991). Fat content can be metabolised to produce energy as well as water since the latter is a by-product of metabolism. After emergence, ticks consume energy (i.e. fat) while locating and ascending stems from which they quest, searching for a blood meal, and descending to moist conditions at the base of the vegetation where they restore their fluid content by passive and active uptake of atmospheric water (Lees and Milne, 1951; Knülle and Rudolph, 1982; Needham and Teel, 1991; Kahl and Alidousti, 1997). Body lipid content appears to decline with age in *A. americanum* (although variation is large) (Jaworski et al., 1984) and *I. ricinus* nymphs (Steele and Randolph, 1985). Steele and Randolph (1985) have suggested a minimum fat content threshold of approximately 2 µg before starvation in nymphs and estimated consumption of fat content at a rate of 0.18 µg per day. Later, Randolph and Storey (1999) have demonstrated that ticks kept under dry conditions consume their fat twice as fast as those kept under wet conditions (0.28 µg per day and 0.14 µg per day, respectively) and that ticks can survive with lipid contents as low as 0.4 µg. In addition, a linear relationship between temperature and lipid consumption has been observed in male and female *I. ricinus* (Van Es et al., 1998). Hence, in *I. ricinus*, the rate of lipid consumption increases under unfavourable conditions of humidity (Randolph and Storey, 1999) and temperature (Van Es et al., 1998). As ticks have no other energy sources, their body fat content declines between blood meals and thus fat content reflects both the history of the tick and the energy reserves remaining for future use (Randolph and Storey, 1999; Randolph et al., 2002).

To date, lipid content has been exclusively quantified in ticks sampled in the United Kingdom (Wales and England). In Wales, fat content in nymphal *I. ricinus* ranges from 1.4 to 24 µg, with means decreasing from 8 to 2 µg over the spring (Steele and Randolph, 1985). In southern England, nymphal fat content seems to be slightly lower, ranging from 1.6 to 18.2 µg, with a mean of 4.7 µg in May and June (Randolph and Storey, 1999; Crooks and Randolph, 2006). Randolph et al. (2002) have reported that lipid content is quite low at the end of summer, down to 0.2 µg, but that in the autumn, a large proportion of ticks possess higher lipid contents, up to 15 µg, sometimes even more. Moreover, fewer ticks with high lipid contents quest from November onwards, this trend persisting throughout the following spring (Randolph et al., 2002). Since a much smaller proportion of high-fat ticks are observed during the spring than the autumn, Randolph et al. (2002) have concluded that ticks emerge from the previous stage and are recruited (i.e. start questing) in autumn, before returning to inactivity over winter. However, Walker (2001) has reported two annual cohorts (one in early spring and one in autumn) of both nymphs and adults in *I. ricinus* when age is assessed by dissection of gut and Malpighian tubules. According to Uspensky (1995) and Uspensky et al. (2006), characteristics such as histological and histochemical changes in tick gut and Malpighian tubules, first described by Balashov (1961), or the evaluation of the ratios between sizes of the scutum and the alloscutum are more reliable in determining tick physiological age than fat content. More recently, Schmidt et al. (2011) have suggested that C and N isotopes may be also used to estimate the age of unfed ticks.

2.2.3 Environmental Influence

2.2.3.1 Light

I. ricinus belongs to the group of ticks that do not possess eyes with corneas (Phillis and Cromroy, 1977; Kaltenrieder et al., 1989) but instead have photosensitive cells (Perret et al., 2003). These cells may perceive changes in light intensity, which allow the tick to perceive changes in day length (i.e. photoperiodicity). Changes in photoperiodicity (occurring when winter comes) elicit developmental (or morphogenetic) diapause in fed ticks, a physiological state during which metabolic processes (and therefore development) are slowed down (Belozerov, 1982). Moreover, *I. ricinus* photosensitive cells are considered to be responsible for triggering mobility in ticks when darkness sets in. Hence, in an experimental setting locomotor activity occurs primarily during darkness in both *I. scapularis* (Carroll et al., 1998) and *I. ricinus* (Perret et al., 2003). In fact, an immediate questing response is observed in *I. ricinus* nymphs when the light stimulus disappears (Lees, 1948). Similarly, *I. scapularis* adults show predominantly nocturnal questing activity in a natural setting (Durden et al., 1996). Such behaviour, i.e. increased motor and questing activity during darkness, has been considered to be a way to minimise water loss (and energetic costs to reabsorb it) by undertaking locomotion and questing during less desiccating conditions coinciding with darkness (Perret et al., 2003). In addition, many tick hosts show peak activity just after dark (Hausser, 1995), which increases chances for ticks to find a host if they are more active during that time period.

2.2.3.2 Temperature

Cold temperatures are temperatures at which ticks are not active. *I. ricinus* ticks in an inactive state overwinter in the leaf litter or in the upper layers of the soil (usually 5 to 7 cm deep) where temperatures are milder than on the vegetation (Dusbabek et al., 1971; Daniel et al., 1972; Gigon, 1985; Daniel et al., 1976, 1977). The state of inactivity in unfed ticks that occurs during winter has been classically called behavioural diapause (Belozerov, 1982). According to Belozerov (1982), behavioural diapause is a category of dormancy that regulates the seasonality of life cycle; it is a pre-adaptive behaviour that precedes unfavourable environmental conditions and that is regulated by inherent mechanisms rather than being a direct response to unfavourable conditions; it is triggered by changes in photoperiodicity. However, it has recently been shown that questing *I. ricinus* ticks may be collected during winter when temperature rises high enough (Randolph et al., 2002; Dautel et al., 2008; Gray, 2008). Although photoperiodicity is considered to be the dominant stimulus to trigger diapause, temperature may have a secondary but important influence on *I. ricinus* activity during winter. Depending on how Belozerov's terminology of diapause is interpreted, it may be argued whether the inactive state observed in overwintering ticks should be called a behavioural diapause or a facultative quiescence (i.e. a kind of inactivity triggered by adverse conditions and coming to an end as soon as favourable conditions are back), either of which can be triggered by autumnal conditions (Gray, 1991; Randolph et al., 2002).

I. ricinus survival decreases when the temperature falls below 0°C but also when the frequency of temperature changes (between cold and warm temperatures) increases (Gigon, 1985). *I. ricinus* adults, especially females, survive better under cold conditions than nymphs, which in turn survive better than larvae (Gigon, 1985; Dautel and Knülle, 1997). Furthermore, engorged larvae and nymphs are less resistant to cold than unfed individuals in *I. ricinus* (Dautel and Knülle, 1997), and in *I. scapularis* (Vandyk et al., 1996). Finally, colony-reared ticks are more cold tolerant than field-collected ticks (Dautel and Knülle, 1997).

Ticks belonging to the *I. ricinus* complex may resist temperatures well below 0°C for some time. Hence, most *I. persulcatus* and *I. nipponensis* nymphs survive when exposed to -5°C for 50 days (Fujimoto, 1994) and 50 % of *I. ricinus* nymphs survive a -10°C exposure for one month (Dautel and Knülle, 1997). Fifty percent mortality has been observed in *I. scapularis* nymphs exposed to -18°C for 2 h (Vandyk et al., 1996). Several days of acclimation at 3 to 4°C lowers the lethal temperature of a few degrees in *I. ricinus* (Dautel and Knülle, 1997) and *I. scapularis* (Burks et al., 1996). Interestingly, it has been reported that a bacterium, *Anaplasma phagocytophylum*, that is harboured by *I. scapularis* and *I. ricinus* increases tick ability to survive the cold by inducing them to express a gene coding for antifreeze proteins (Neelakanta et al., 2010).

Warm temperature mainly determines tick development rate and questing activity. Hot temperature (25°C and above) alone usually does not result in tick death but it may do so in association with unfavourable humidity, as increasing temperature increases water loss (MacLeod, 1935; Needham and Teel, 1986) (see section 2.2.3.3). Daily rate of interstadial development is increased when *I. ricinus* is exposed to increasing temperatures, from 15°C to 30°C for instance (MacLeod, 1934; Aeschlimann, 1972; Randolph, 2004), larvae being more affected by increasing temperature than nymphs and adults (Randolph, 2004). Similarly, preeclosion period for eggs, premoult period of engorged larvae and nymphs decreases with increasing temperature in *I. scapularis* (Ogden et al., 2004).

Threshold temperatures of questing activity vary according to developmental stages in ticks. It appears that this variation is size-dependent, so that the larger the body size of the tick, the lower the activity threshold temperature (Clark, 1995). Clark (1995) has found that the mean activity threshold temperature in *I. scapularis* females is 6.2°C (±3.6°C), while that in males and nymphs is 8.5°C (±3.0°C) and 9.8°C (±3.4°C), respectively. In line with the latter results is another study that has observed activity in *I. scapularis* adults when temperature reaches 4°C (Duffy and Campbell, 1994). Interestingly, Schulze et al. (2001) has reported that adult *I. scapularis* may be questing at temperatures as low as -0.6°C. In contrast, *I. ricinus* has been shown to be negatively geotropic, i.e. to climb up the vegetation, at 12°C (MacLeod, 1935) but sheep infestation is detected when daily maximal temperature has reached 7°C over a week (MacLeod, 1936). More recently, it was found that questing *I. ricinus* ticks may be occasionally sampled when daily maximal temperature is between 1.9°C and 3.8°C, but are always collected when temperature reaches 5.2°C (Perret et al., 2000). *I. ricinus* ticks are active when the daily maximal temperature has reached 7°C over 5 days (Perret et al., 2000).

The optimal range of temperature at which *I. ricinus* is negatively geotropic, i.e. climbs up the vegetation, is found between 7 and 24°C (MacLeod, 1935; Perret et al., 2000). The upper temperature activity threshold for *I. ricinus* is around 30°C, at which ticks tend to be positively geotropic (MacLeod, 1935), i.e. return to the litter layer where they enter a quiescent state. In *I. scapularis*, temperature affects mean distance moved and percentage of time in questing posture, with 25°C being the optimal temperature for such behaviours (Vail and Smith, 2002). In *I. ricinus*, questing duration is reduced by temperature increasing from 15°C to 25°C at 85 % relative humidity (RH) (Perret et al., 2003).

2.2.3.3 Humidity and Saturation Deficit

Like other terrestrial arthropods, the biggest challenge that ticks face during their life is to maintain water balance while living in a relatively dry environment, i.e. below the critical equilibrium humidity (Needham and Teel, 1986). The critical equilibrium humidity is the threshold below which RH causes ticks to continuously lose water and above which ticks may actively uptake atmospheric water (Knülle and Wharton, 1964). Under conditions that are unfavourable to maintain water balance, ticks therefore need to minimise water loss and actively uptake water vapour from the atmosphere (Knülle and Rudolph, 1982).

In ticks, water may be lost through two phenomena: transpiratory water loss and respiratory water loss (Knülle and Rudolph, 1982). Transpiratory water loss is the loss of water through the integument of the general body surface when ticks are exposed to humidity conditions below their critical equilibrium humidity (Knülle and Rudolph, 1982). Such phenomenon occurs when ticks are waiting for a passing host on the vegetation, which is a relatively dry environment. The rate of water loss through the integument varies between tick species and the difference is mainly due to the permeability of the integument to water (Lees, 1946). For instance, *I. ricinus* adults rapidly lose water at the rate of 10 % of their original body mass per day when they are on grass tips, exposed to dehydrating conditions (50 % RH, 25°C) (Lees, 1946). Moreover, water loss of 15 to 20 % of their original body mass is the critical threshold for death in nymph and adult *I. ricinus* (Kahl and Alidousti, 1997). Different waxy lipids deposited in the epicuticle of the integument provide variable efficiency at minimising the loss of water into the atmosphere from the general body surface (Lees, 1946). In contrast, respiratory water loss is achieved in the course of respiratory gas exchange via the tracheal system (Knülle and Rudolph, 1982). Respiratory water loss is particularly important during mobility since respiration - and thus spiracle opening - accelerates in ticks that move (Rudolph and Knülle, 1979; Pugh et al., 1988; Lighton et al., 1993). Hence, water loss increases in ticks when the latter climb up and down the vegetation in nature.

To compensate water loss due to transpiration and respiration, ticks need to acquire water from the surrounding environment. However, ticks such as *I. ricinus* do not drink liquid water (Lees, 1946; Kahl and Alidousti, 1997; Kröber and Guerin, 1999). Instead, they retrieve water from the atmosphere (Lees, 1946; Knülle and Rudolph, 1982). In nature, ticks must return periodically to moist surroundings such as the litter layer (Lees, 1946; Lees and Milne, 1951) where they can actively extract water vapour from the atmosphere (Lees, 1946). Ticks may extract water vapour from the atmosphere even if humidity level is below the critical equilibrium humidity, but at a higher energetic cost. In fact, *I. ricinus* absorbs water vapour faster when atmospheric RH reaches 85 to 90 % (Lees, 1946). Such phenomenon is called active water sorption and costs energy (O'Donnell and Machin, 1988; Needham and Teel, 1991). Active water sorption from the atmosphere is achieved through the production of hyperosmotic fluids by the type I agranular alveoli of the salivary glands, followed by the secretion of the hygroscopic solution on the hypostome and subsequent reabsorbtion of the latter fluids in the mouth by the suction of the pharynx (Rudolph and Knülle, 1974, 1979; Knülle and Rudolph, 1982; Gaede and Knülle, 1997). Water deficient ticks recover body water faster at higher humidity conditions (Knülle and Rudolph, 1982). Hence, adult *I. ricinus* may uptake water at a rate of more than 20 % of their original body mass per day (Kahl and Alidousti, 1997). Furthermore, higher ambient RH conditions correspond with higher body water levels in ticks (Knülle and Rudolph, 1982). Interestingly, nymph and female *I. ricinus* previously exposed to a saturated atmosphere (i.e. harbouring high body water levels) avoid high humidity and seek drier surroundings (Lees, 1948). However, dehydrated nymphs are more likely to move towards fully saturated than drier air (Crooks and Randolph, 2006).

I. ricinus is extremely sensitive to temperature and humidity compared with other tick species (MacLeod, 1935; Lees, 1946; Aeschlimann, 1972; Knülle and Rudolph, 1982; Sonenshine, 1991). In fact, the most important limiting factor in *I. ricinus* survival and activity has been reported to be humidity and the different developmental stages display little resistance to desiccation (MacLeod, 1935; Lees, 1946; Aeschlimann, 1972). Moreover, immature stages, i.e. larvae and nymphs, are more sensitive to ambient humidity than mature stages due to their high surface area to volume ratio (Randolph and Storey, 1999). RH needs to be above 70 to 80 % in order to allow *I. ricinus* questing activity and survival (MacLeod, 1935). Similarly, it has been observed that nymphal *I. scapularis* questing height (Vail and Smith, 2002) and survival (Stafford, 1994) are reduced when humidity decreases. Interestingly, *I. scapularis* nymphs exposed to dry air (40 % RH) for 4 to 6 h may recover if they are exposed to humid air (95 % RH) afterwards (Rodgers et al., 2007).

Since temperature and humidity are often dependent on each other, saturation deficit (SD), a measure of the drying power of the atmosphere depending on both temperature and RH (Randolph and Storey, 1999), is probably the most accurate parameter to mention when tick activity and survival are concerned. Calculated in mmHg, low SD values are conditions that ticks need to quest and survive in nature. Low SD values usually correspond to high RH and low temperature conditions, but may also be obtained with very high RH and relatively high temperature or relatively low RH and very low temperature (see section 3.2.1.2). In fact, SD is the most important factor that affects questing nymph activity in *I. ricinus* (Tagliapietra et al., 2011). Increased SD reduces *I. ricinus* duration of questing (Perret et al., 2003, 2004) and the abundance of ticks questing (Burri et al., 2007; Morán Cadenas et al., 2007a; Gern et al., 2008; Knap et al., 2009) but does not influence duration of quiescence (Perret et al., 2003). More precisely, *I. ricinus* ticks demonstrate positive geotropism, i.e. go down the vegetation, when SD reaches 4.4 mmHg, which represents 80 % RH at 24°C (MacLeod, 1935). SD values that are lower than 5 mmHg are favourable for *I. ricinus* questing activity and development (MacLeod, 1935; Perret et al., 2003; Burri et al., 2007). Activity is no longer observed when SD exceeds 15 mmHg (Perret, 2002). Increased SD reduces *I. ricinus* survival in nature (Perret, 2002; Perret et al., 2000, 2004) and in a laboratory setting (Perret, 2002).

2.2.3.4 Seasonal Activity

Host-seeking seasonal activity of ticks of the *I. ricinus* complex varies greatly depending on the surrounding environment and climate. While the different developmental stages of *I. scapularis* in northeastern North America are active at different times of the year (nymphs from spring to late summer, larvae from summer to autumn, and adults from autumn to winter), the different developmental stages of *I. ricinus* in Europe are active simultaneously (Fig. 2.4) (Kurtenbach et al., 2006). However, *I. ricinus* ticks questing in southern England and Ireland distinguish themselves from individuals questing in Central Europe, northern England and Scotland. The different developmental stages of *I. ricinus* in southern England and Ireland are active during a single activity peak (from early spring to late autumn) whereas two distinct activity peaks are usually observed in Central Europe, northern England and Scotland (one in spring and one in autumn) (Fig. 2.4) (Aeschlimann, 1972; Gray, 1991; Randolph et al., 2002; Randolph, 2004; Kurtenbach et al., 2006; Morán Cadenas et al., 2007a).

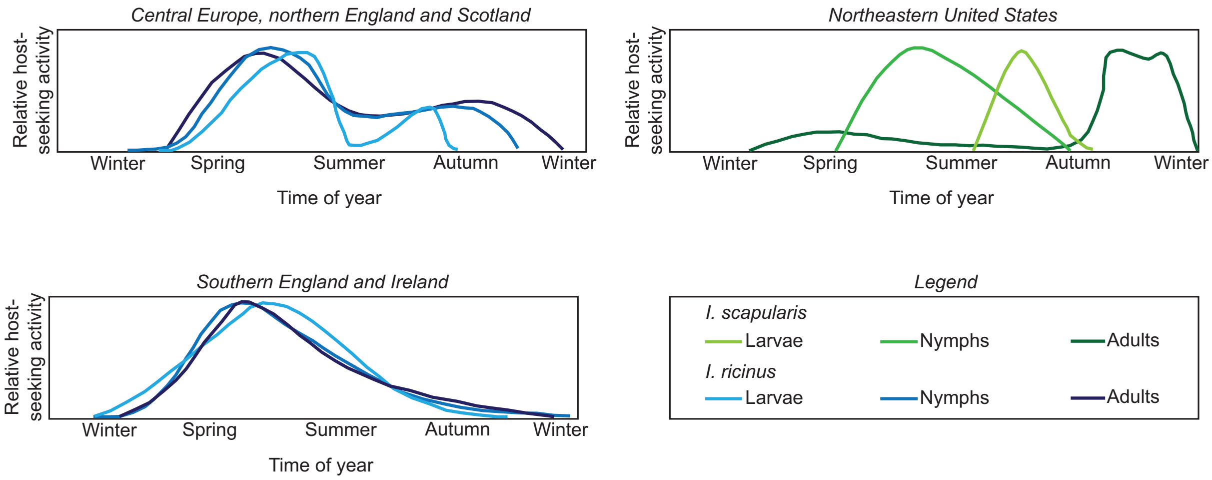

Fig. 2.4 Relative host-seeking activity of *I. ricinus* and *I. scapularis* different developmental life stages in various parts of the world: northeastern United States (bottom right), southern England and Ireland (top right) and Central Europe, northern England and Scotland (top right) (Figure: B. Gertsch, based on Kurtenbach et al., 2006).

In Switzerland, the highest density of questing in *I. ricinus* is recorded in spring (April through June) but questing individuals may be found as early as mid-February to early March, depending on weather conditions (Aeschlimann, 1972; Morán Cadenas et al., 2007a). Adults and nymphs usually emerge first, followed by larvae (Aeschlimann, 1972). In summer, questing *I. ricinus* ticks are rarely collected, except at higher altitudes where climate is milder (Burri et al., 2007; Morán Cadenas et al., 2007a). However, in autumn, when favourable conditions of temperature and humidity are back, a second activity peak is often reported (Aeschlimann, 1972; Morán Cadenas et al., 2007a). The autumn peak is of lower intensity than the one observed in spring and may be absent if weather conditions are not favourable (Aeschlimann, 1972; Gray, 1991; Perret et al., 2000, 2004; Morán Cadenas et al., 2007a). The last questing individuals are usually sampled in October to early November (Aeschlimann, 1972; Morán Cadenas et al., 2007a). In winter, *I. ricinus* ticks are not active but may re-emerge periodically when the temperature warms up (Dautel et al., 2008) (see section 2.2.3.2).

2.2.4 Habitat and Geographical Distribution

I. ricinus has a wide distribution throughout Europe. It occurs from Iceland (sparse and scattered populations) as far south as Morocco, Algeria, Tunisia and the Atlas Mountains in North Africa and from the Atlantic coast to as far east as 70 to 80° longitude in Central Asia. There, its distribution overlaps that of *I. persulcatus*, another tick species belonging to the *I. ricinus* complex, which is restricted to eastern Europe and Asia (Gern and Humair, 2002; Stanek et al., 2012) (Fig. 2.5). *I. pacificus* and *I. scapularis*, two other major ticks species belonging to the *I. ricinus* complex, are found in western and eastern North America, respectively (Piesman, 2002; Stanek et al., 2012).

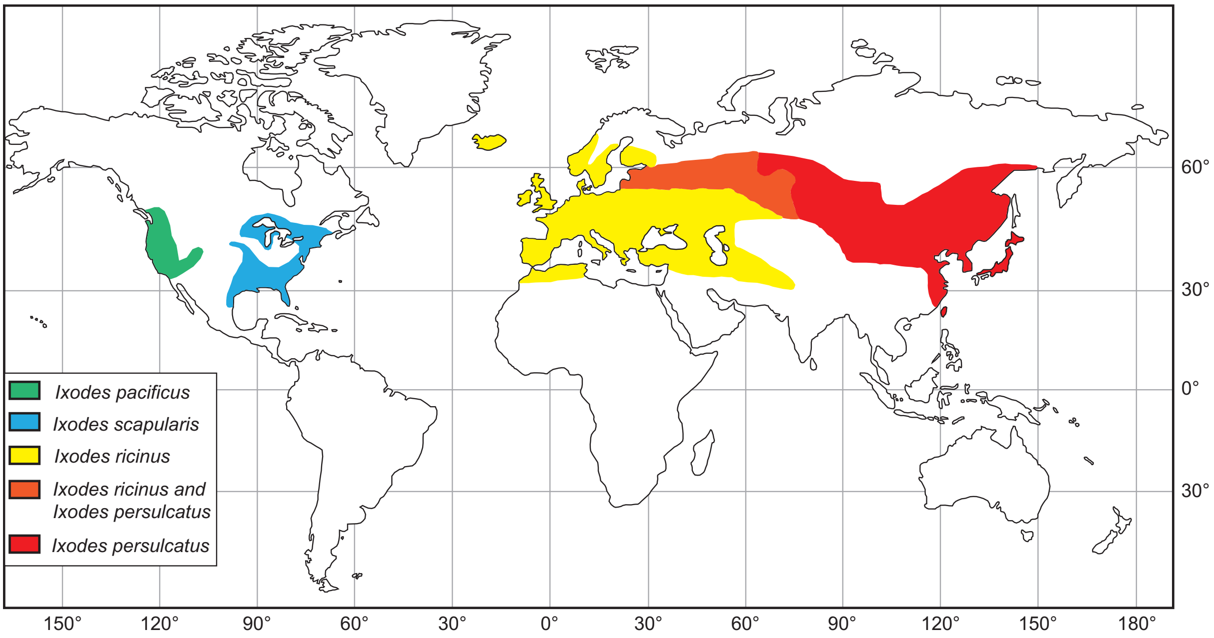

Fig. 2.5 Distribution of the four major tick species belonging to the *I. ricinus* complex: *I. ricinus* (yellow and orange), *I. pacificus* (green), *I. scapularis* (blue) and *I. persulcatus* (red and orange) (Figure: B. Gertsch, based on Swanson et al., 2006; Stanek et al., 2012).

A shift towards higher latitudes in the northern distributions of *I. ricinus* has been recorded in Sweden (Lindgren et al., 2000; Jaenson et al., 2012) and Norway (Jore et al., 2011), of *I. persulcatus* in Russia (Yasyukevich et al., 2009;

Tokarevich et al., 2011), and of *I. scapularis* in the northeastern United States (Dennis et al., 1998) and southern Canada (Leighton et al., 2012), presumably due to climate change.

I. ricinus survives in various habitats but thrives in woodlands to grasslands, with a slight preference for deciduous compared to coniferous woodlands (Aeschlimann, 1972; Gray, 1998; Gray et al., 1998; Gern and Humair, 2002; Piesman and Gern, 2004). Moreover, deciduous and mixed deciduous forests dominated by deciduous trees such as oak (*Quercus* spp) and beech (*Fagus* spp) trees are favoured habitats for *I. ricinus* (Gray, 1998; Gray et al., 1998; Lindström and Jaenson, 2003). Also, increasing shrub cover in such environments appears to enhance *I. ricinus* presence (Tack et al., 2012a, 2012b). A higher abundance of ticks in those environments is related to the fact that such forests provide a cooler, moister microclimate (especially due to the leaf litter producing high humidity microenvironments) as well as forage and shelter for a diverse fauna (and potential hosts for a blood meal) (Gray, 1998). *I. ricinus* habitat is located in the leaf litter and the low strata of vegetation, where humidity is high (Gern and Humair, 2002). However, *I. ricinus* may also occur at high densities in coniferous forests with thick litter layer and high rainfall, as well as in open areas and meadows with rough vegetation and high rainfall (major hosts being livestock such as cattle and sheep in this case), notably in the British Isles (Gray, 1998; Gray et al., 1998; Walker et al., 2001). Although *I. scapularis* and *I. pacificus* live under similar environmental conditions in eastern and western North America, respectively, as *I. ricinus* in Europe, i.e. deciduous-dominated forest (Piesman, 2002), *I. persulcatus* inhabits variants of taiga, i.e. boreal coniferous forests (Korenberg et al., 2002).

I. ricinus density usually decreases when altitude increases (Aeschlimann, 1972; Perret, 2002; Jouda et al., 2004b; Morán Cadenas et al., 2007a), but it may increase when altitude increases in particular areas. Hence, tick density has been observed to be higher at altitudes over 1000 m than at lower altitudes in two studies where ticks were collected on the north-facing slopes of the Rhône Valley (Burri et al., 2007) and of Chaumont Mountain (Morán Cadenas et al., 2007a) in Switzerland. This phenomenon has been explained by local microclimatic conditions favouring better maintenance at higher than lower altitudes, notably high SD values impairing the tick population at lower altitudes (Burri et al., 2007; Morán Cadenas et al., 2007a). Recently, a shift in *I. ricinus* distribution towards higher altitudes has been observed due to global warming (Daniel et al., 2003; Morán Cadenas et al., 2007a; Materna et al., 2005, 2008; Jore et al., 2011). Hence, increased temperatures, especially during the summer months, make higher altitudes suitable for *I. ricinus* as they reach values encountered by this tick at lower altitudes in the past (Gern et al., 2008). Moreover, higher temperatures in spring and autumn that extend the seasons during which ticks may develop allow the establishment of tick populations at higher altitudes (Danielova et al., 2006). In Switzerland, *I. ricinus* has been encountered up to 1650 m above sea level (Perret, 2002), but *I. pacificus* has been collected as high as 2345 m above sea level in Arizona where climate conditions are favourable (Olson et al., 1992).

2.2.5 Vector of Pathogenic Agents

Worldwide, ticks transmit a greater variety of infectious agents than any other group of blood-feeding arthropods. They are only surpassed by mosquitoes in terms of public health and veterinary importance (Sonenshine et al., 2002). Three conditions need to be fulfilled for an arthropod species to be considered a vector of a pathogenic agent. In the first place, the arthropod needs to be able to acquire the pathogen when feeding on an infective host. Secondly, the arthropod has to be able to transmit the pathogen transstadially, i.e. maintain the infection through blood digestion, and through subsequent moulting. Finally, the arthropod needs to transmit efficiently the pathogenic agent to susceptible hosts during its later blood meals (Eisen and Lane, 2002). Arthropods belonging to non-vector species fail to perform or do the latter poorly.

Different modes of pathogen infection allow ticks to acquire, maintain and transmit pathogens efficiently. Direct transmission from an infected host to a tick (or from an infected tick to a host), transovarial transmission from an infected female tick to its eggs, transstadial transmission from a developmental stage to the next (i.e. larva to nymph or nymph to adult), and co-feeding transmission from an infected tick to another are ways by which uninfected ticks acquire and maintain *Borrelia* spirochetes in nature (see section 2.3.3).

Borrelia burgdorferi sensu lato (s.l.) spirochetes are the most abundant and widely distributed infectious agents that are vectored by *I. ricinus* ticks (see section 2.3). However, several other pathogens of veterinary and public health importance are harboured and transmitted by these ticks. In Switzerland, these are Tick-Borne Encephalitis (TBE) virus, spotted fever rickettsiae, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, babesiae, and *Francisella tularensis*.

TBE virus, the causative agent of tick-borne encephalitis, is a small (about 50 nm), lipid-enveloped virus with a spherical structure, whose genome consists of single-stranded, positive-sense RNA (Lindenbach et al., 2007). It belongs to the genus *Flavivirus*, within the family Flaviviridae and occurs in three subtypes: the Siberian subtype, the Far Eastern subtype, and the European subtype (Ecker et al., 1999). The first two subtypes are closely associated with *I. persulcatus* whereas the last one is closely associated with *I. ricinus* and is the one that is found in Switzerland (Mansfield et al., 2009). TBE virus prevalence is very low in Switzerland (0.1 to 0.95 %) and is restricted to endemic regions or foci (Wicki et al., 2000; Gäumann et al., 2010; Burri et al., 2011b; Lommano et al., 2012a). However, infection prevalence may reach 14 % in a known endemic focus (Casati et al., 2006b). TBE virus distribution was limited to eastern Switzerland until 2006, when it started to spread to the western parts of the country (De Vallières et al., 2006), a phenomenon that is poorly understood so far, although migratory birds have been suspected to play a role (Lommano et al., 2012a). TBE virus reservoir hosts are primarily small mammals such as rodents (especially *Apodemus* spp and *Myodes* spp.) (Labuda et al., 1993b; Süss, 2011). Ticks get infected while feeding on these hosts, mainly by co-feeding transmission (Labuda et al., 1993a; Burri et al., 2011b). If infected with TBE virus, ticks remain infected throughout their life cycle (Gritsun et al., 2003) and may transmit the virus to human beings when blood feeding. In human patients, the central nervous system is usually impaired (Mansfield et al., 2009), but this is rarely followed by a fatal outcome (1 % of cases) (Gritsun et al., 2003). TBE virus is transmitted very early during the tick blood meal since it is located in tick salivary glands and therefore early tick removal does not prevent infection (Nuttall and Labuda, 2003). TBE virus is not curable (only treatable) but an efficient vaccine is available to prevent infection (Heinz et al., 2007).

Rickettsiae of the spotted fever group, the causative agents of tick-borne rickettsioses, are gram-negative intracellular bacteria (0.8 to 2 µm in length and 0.3 to 0.5 µm in width) that are rod-shaped and whose genome consists of a single circular chromosome (Yu and Walker, 2005). They are members of the genus *Rickettsia*, within the family Rickettsiaceae and the order Rickettsiales (Raoult and Roux, 1997; Yu and Walker, 2005). Two human pathogenic *Rickettsia* species have been detected in questing *I. ricinus* ticks in Switzerland, *R. helvetica* and *R. monacensis* (Parola et al., 2005; Boretti et al., 2009; Lommano et al., 2012b). While *R. helvetica*, which was initially discovered in Switzerland (Burgdorfer et al., 1979), is observed in 9 to 12 % of questing *I. ricinus* in Switzerland, *R.*

monacensis appears to be rare (0.1 %) (Boretti et al., 2009; Lommano et al., 2012b). Ticks act as vectors and reservoirs in the life cycle of rickettsiae, acquiring them transovarially or during a blood meal on rickettsiemic hosts, such as rodents, roe deer or boars (Sprong et al., 2009; Schex et al., 2011), and remaining infected throughout their life cycle (Raoult and Roux, 1997; Parola et al., 2005). Rickettsiae may be transmitted to human beings via tick saliva during blood feeding, resulting in infection causing clinical symptoms such as high fever, headache and rash, which is curable with antibiotics (Raoult and Roux, 1997).

A. phagocytophilum, the causative agent of human granulocytic anaplasmosis or ehrlichiosis, is a gram-negative intracellular bacterium (up to 6 µm in diameter), consisting of a coccoid or ellipsoidal cell, usually parasitising granulocytes, and whose genome is located on a single circular chromosome (Dumler et al., 2005). *A. phagocytophilum* belongs to the genus *Anaplasma*, within the family Anaplasmataceae and the order Rickettsiales (Dumler et al., 2005). *A. phagocytophilum* prevalence in questing *I. ricinus* ticks is quite low (0.9 to 2.2 %) (Pusterla et al., 1998a, 1999; Liz et al., 2000; Wicki et al., 2000; Burri et al., 2011a), revealing a patchy distribution (Lommano et al., 2012b). Various mammals including rodents, insectivores, foxes, boars, chamois, and roe deer (Liz et al., 2000, 2002; Petrovec et al., 2003), may harbour these bacteria, which *I. ricinus* ticks ingest during blood feeding and retain transstadially. *I. ricinus* ticks transmit *A. phagocytophilum* to human beings via saliva while feeding, sometimes causing infection resulting in high fever accompanied by leukopenia and thrombocytopenia, which is curable with antibiotics (Dumler et al., 2005). Not all strains of *A. phagocytophilum* seem to be pathogenic for humans (De la Fuente et al., 2005) and to date, no human cases of infection by *A. phagocytophilum* have been reported in Switzerland, although evidence of contacts of Swiss residents with bacteria causing human granulocytic ehrlichiosis has been demonstrated (Liz et al., 1997; Pusterla et al., 1998b).

Candidatus Neoehrlichia mikurensis is a gram-negative intracellular bacterium (0.5 to 1.2 µm in diameter), consisting of a coccoid cell, preferentially infecting endothelial cells (Kawahara et al., 2004). *Candidatus* Neoehrlichia mikurensis is a member of the cluster *Candidatus* Neoehrlichia (no recognized genus), within the family Anaplasmataceae and the order Rickettsiales (Kawahara et al., 2004; Rar and Golovljova, 2011). Since *Candidatus* Neoehrlichia mikurensis was first differentiated from other *Anaplasma* and *Ehrlichia* spp in 2004 (Kawahara et al., 2004), its presence in Switzerland has only been documented recently. However, *Candidatus* Neoehrlichia mikurensis prevalence in questing *I. ricinus* ticks is of moderate importance, reaching more than 6 % (Lommano et al., 2012b). Reservoir hosts of *Candidatus* Neoehrlichia mikurensis seem to be small rodents (Rar and Golovljova, 2011). As far as *Candidatus* Neoehrlichia mikurensis pathogenicity is concerned, the bacterium has been found in the blood samples of febrile patients in Europe (Von Loewenich et al., 2010; Welinder-Olsson et al., 2010), including in Switzerland (Fehr et al., 2010). Clinical symptoms are high fever and septicaemia and can be treated with antibiotics (Fehr et al., 2010; Von Loewenich et al., 2010; Welinder-Olsson et al., 2010), but infection may be lethal (Von Loewenich et al., 2010).

Babesiae, the causative agents of babesiosis or piroplasmosis, are pear-shaped protozoan parasites that parasitise erythrocytes (Homer et al., 2000). They belong to the genus *Babesia*, within the family Babesiidae, the order Piroplasmida, and the phylum Apicomplexa (Levine, 1971; Homer et al., 2000). Babesiae are grouped into the small *Babesia* (including *B. microti*) measuring 1 to 2.5 µm and the large *Babesia* (including *B. divergens*) measuring 2.5 to 5 µm, based on nuclear small subunit-ribosomal DNA sequences (Homer et al., 2000). There is one exception to this classification: *B. divergens* is genetically related to the large babesiae, but is actually small (0.4 to 1.5 µm)

(Homer et al., 2000). The two primary *Babesia* species that have been shown to infect humans, *B. divergens* in Europe and *B. microti* in North America (Gray et al., 2010), have been detected in questing *I. ricinus* ticks in Switzerland (Casati et al., 2006a; Gigandet et al., 2011; Lommano et al., 2012b). Moreover, a third species, *B. venatorum* (formerly called EU1), recently described as pathogenic for humans in Europe (Herwaldt et al., 2003; Häselbarth et al., 2007), has been reported in Switzerland (Casati et al., 2006a; Gigandet et al., 2011; Lommano et al., 2012b). *Babesia* prevalence is very low in Switzerland, *B. divergens*, *B. microti*, and *B. venatorum* infecting 0.2 to 0.3 %, 0.7 %, and 0.7 to 1.2 %, respectively, of questing *I. ricinus* ticks (Casati et al., 2006a; Gigandet et al., 2011; Lommano et al., 2012b). *I. ricinus* ticks ingest babesiae while feeding on reservoir hosts of these pathogenic agents, such as rodents, roe deer and cattle, and remain infected throughout their life cycle (Homer et al., 2000; Chauvin et al., 2009). The infection is transmitted to humans during blood feeding, resulting in malaria-like symptoms that may include malaise, chills, anaemia, fatigue, fever, haemoglobinuria, jaundice, and respiratory, renal, hepatic, and cardiac failure, infections due to *B. divergens* appearing to be more severe than those by *B. venatorum* and *B. microti* (Homer et al., 2000; Gray et al., 2010). Babesiosis is successfully treated with antibiotics, but immunocompromised patients are at risk for a fatal outcome (Homer et al., 2000; Gray et al., 2010).

F. tularensis, the causative agent of tularaemia, is a gram-negative facultative intracellular bacterium (0.2 to 0.7 µm in length and 0.2 µm in width), consisting of a rod-shaped or coccoid cell, specialised to infect macrophages but also observed in fibroblasts, endothelial cells, hepatocytes, and muscle cells, whose genome is located in a single circular chromosome (Sjöstedt, 2005; Keim et al., 2007). It is a member of the genus *Francisella*, within the family Francisellaceae and the order Thiotrichales (Sjöstedt, 2005). *F. tularensis* has rarely been investigated in Switzerland, probably because no human cases of infection have ever been documented in the country. In fact, a single study has documented *F. tularensis* presence in questing *I. ricinus* ticks in Switzerland, reporting a very low prevalence of 0.12 % (Wicki et al., 2000). Lagomorphs and rodents are considered to be the major reservoirs of *F. tularensis*, and ticks are the main vectors of this pathogen in Central Europe (mites, tabanid flies, and mosquitoes being major vectors in northern Europe) (Keim et al., 2007). Arthropod vectors ingest *Francisella* bacteria while feeding on reservoir hosts and may infect human beings via saliva during a later blood meal. Clinical symptoms of tularaemia are plague-like, including chills, fever, headache, joint stiffness, muscle pains, occasionally leading to osteomyelitis, pericarditis, meningitis, and pneumonia, and are usually cured by antibiotics, although a fatal outcome may occur (Schaffner, 2007; Penn, 2009).

2.3 Pathogenic Agent: *Borrelia burgdorferi* sensu lato

2.3.1 Scientific Classification

B. burgdorferi s.l. spirochetes are gram-negative bacteria, which belong to the phylum *Spirochaetes*, the class *Spirochaetes* and the order *Spirochaetales*, which contains a few families of veterinary and medical importance such as the *Leptospiraceae* (leptospirosis), the *Brachyspiraceae* (brachyspirosis), and the *Spirochaetaceae* (Table 2.2) (Dworkin and Falkow, 2006; Paster, 2010). *B. burgdorferi* s.l. are members of the *Spirochaetaceae* family, which comprises the causative agent of syphilis (*Treponema pallidum*), and those of the genus *Borrelia* (Wang et al., 1999b). The genus *Borrelia* is divided into three major pathogenic groups, all of which are transmitted by arthropods: the relapsing fever borreliae (such as *Borrelia recurrentis*, *B. hermsii*, *B. duttoni* or *B. parkeri*), Lyme disease borreliae (i.e. the *B. burgdorferi* complex), and the etiological agent of avian spirochaetosis, *Borrelia anserina* (Bergström et al., 2002).

Table 2.2 Systematics of *B. burgdorferi* (Paster, 2010).

Phylum	*Spirochaetes*
Class	*Spirochaetes*
Order	*Spirochaetales*
Family	*Spirochaetaceae*
Genus	*Borrelia*
Species	*Borrelia burgdorferi* sensu lato

2.3.2 Morphology and Genetics

B. burgdorferi spirochetes are flexible helically shaped cells that measure 4 to 30 µm in length and 0.18 to 0.25 µm in width (Fig. 2.6) (Johnson et al., 1984a). The cells are motile with both rotational and translational movements (Barbour and Hayes, 1986).

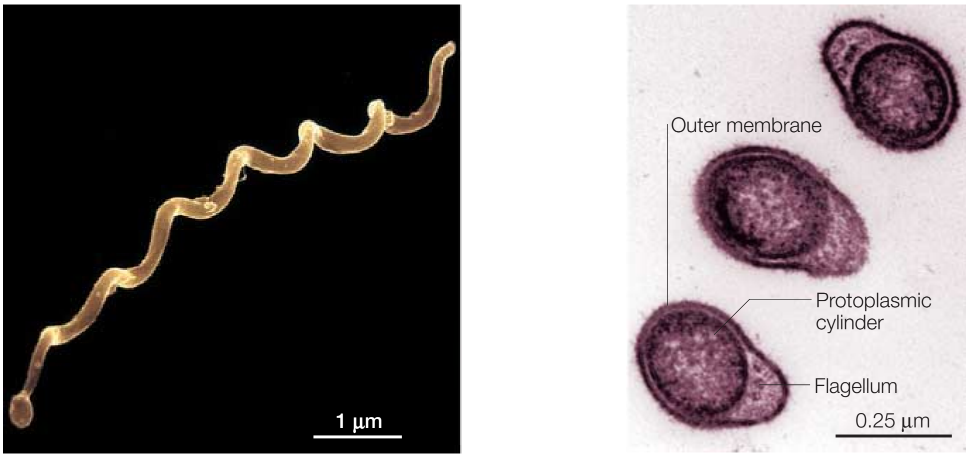

Fig. 2.6 Scanning (left) and transmission (right) electron micrographs of B. burgdorferi (adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Rosa et al., 2005), copyright (2013)).

A multilayered outer membrane sheaths borrelial cells. The outer membrane surrounds the protoplasmic cylinder, which consists of the cytoplasm, the inner cell membrane, the peptidoglycan layer (whose main diamino acid is ornithine), and the cytoplasmic membrane (Johnson et al., 1984a; Rosa et al., 2005). *B. burgdorferi* spirochetes possess seven to eleven flagella that are not located at the spirochete surface but in the periplasmic space situated between the outer cell membrane and the protoplasmic cylinder. The periplasmic flagella insert at the termini of the protoplasmic cylinder and overlap in the central region of the cell. They wind around the protoplasmic cylinder, imparting both motility and shape to the spirochete. Movement of the flagella produces a screw-like motion that propels the spirochete forward (Johnson et al., 1984b; Barbour and Hayes, 1986).

B. burgdorferi genome size is relatively small in contrast to that of free-living bacteria, possibly reflecting its lifestyle as an obligate parasite. It consists of approximately 1.5 Mb, comprising one linear chromosome of 950 kb (Baril et al., 1989) and at least 21 extrachromosomal elements or plasmids (620 kb among 9 linear and 12 circular plasmids) (Casjens et al., 2000). *B. burgdorferi* possesses the largest number of plasmids observed in a bacterium to date (Pal and Fikrig, 2003). Some of the plasmids may be lost when spirochetes are grown *in vitro*, usually resulting in reduction of infectivity in mice (Schwan et al., 1988). The entire genome of *B. burgdorferi* s.s. strain B31 was sequenced at the end of the 20th century. The chromosome holds 853 genes coding for basic proteins (similar to those of many other bacteria) required for cell cycle, growth and metabolism, while plasmids contain another 535 genes (often multi-copy genes), most of which have no homology to genes outside the *Borrelia* genus and seem to be associated with spirochete pathogenicity (Fraser et al., 1997). Unlike other pathogenic bacteria, a large portion of *B. burgdorferi* genome is devoted to producing lipoproteins (Casjens et al., 2000), such as outer surface proteins (Osp) that are contained in the borrelial outer membrane (Wilske et al., 1988; Bergström et al., 1989; Norris et al., 1992; Lam et al., 1994; Zhang et al., 1997). The latter are highly variable and differentially expressed at different stages of the *B. burgdorferi* life cycle within vertebrates or ticks and are used by the spirochetes to modify their surface structure, supposedly facilitating host adaptation and immune evasion (Wilske et al., 1988; De Silva and Fikrig, 1997; Kenedy et al., 2012). For example, OspB is critical for *B. burgdorferi* adherence and survival within *Ixodes* ticks (Neelakanta et al., 2007). OspA serves an antibody-shielding role during tick blood meal uptake from immune hosts (Battisti et al., 2008) but is also essential for colonisation of and survival within tick midgut (Anguita et al., 2003; Yang et al., 2004). In contrast, OspC is crucial for colonisation of the vertebrate host, facilitating *Borrelia* migration from tick midgut to salivary glands (Gilmore and Piesman, 2000; Pal et al., 2004; Fingerle et al., 2007a) and dissemination to host tissues (Seemanapalli et al., 2010). In fact, when a tick starts feeding, spirochetes tend to downregulate OspA and upregulate OspC (Montgomery et al., 1996; Schwan and Piesman, 2000; Anguita et al., 2003; Pal and Fikrig, 2003).

2.3.3 Life Cycle Within the Vector

The most common way by which spirochetes are acquired by ticks is direct (horizontal) transmission. During direct transmission, *Borrelia* spirochetes are transmitted from a systemically infected vertebrate host to feeding ticks along with the blood meal (Fig. 2.7a).

Another way by which spirochetes are acquired by ticks is co-feeding transmission. During co-feeding transmission *Borrelia* spirochetes are directly transmitted from an infected tick to an uninfected individual feeding simultaneously, spatially close to each other, without a systemic infection of the host (Fig. 2.7b). Spirochetes transmitted by the infected tick remain localised at the site of inoculation and are ingested by the uninfected individual from there, without dissemination. Co-feeding usually involves immature stages, i.e. nymphs and larvae, since they often share vertebrate hosts due to their similar questing height (see section 2.2.2.2), but adults may be involved as well. Co-feeding transmission of *B. burgdorferi* spirochetes has first been described in *I. ricinus* (Gern and Rais, 1996) and has later been reported to occur in *I. persulcatus* (Sato and Nakao, 1997) and *I. scapularis* (Patrican, 1997). The exact mechanism of co-feeding transmission of Lyme disease spirochetes has not been solved yet, but it seems that *B. burgdorferi* migration is mediated by tick salivary gland extracts since spirochetal migration is increased by salivary gland extracts of fed *I. scapularis* ticks (Shih et al., 2002). Although co-feeding transmission has not been recognized to contribute as much as direct transmission to the amplification of *B. burgdorferi* s.l. prevalence in *I. ricinus* (Randolph et al., 1996; Randolph and Gern, 2003) and *I. scapularis* (Piesman and Happ, 2001) tick populations, it is sufficient to maintain enzootic cycles of Lyme disease involving *I. ricinus* and non-reservoir hosts of *B. burgdorferi* spirochetes such as sheep (*Ovis aries*) (Ogden et al., 1997). Furthermore, co-feeding transmission is suspected to occur in enzootic cycles involving wild sika deer (*Cervus nippon yesoensis*), which are non-reservoir hosts of *B. burgdorferi* spirochetes, and *I. persulcatus* (Kimura et al., 1995).

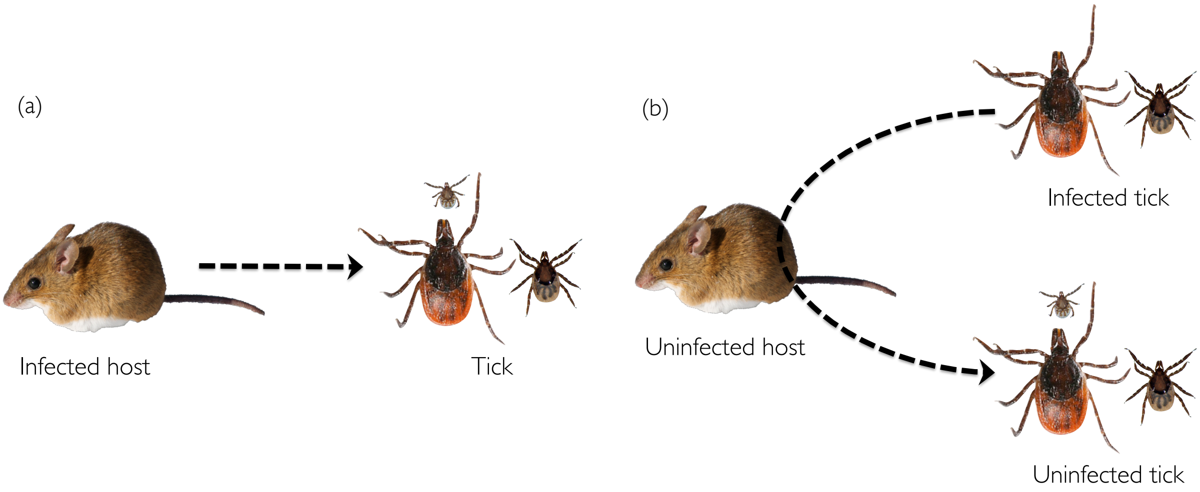

Fig. 2.7 Lyme disease spirochetes acquisition (dashed arrows) in ticks of the *I. ricinus* complex by (a) direct transmission and (b) co-feeding transmission. Hosts are pictured as mice but may represent other tick host, i.e. more than 300 different vertebrate species (Anderson, 1991) (Pictures: N. Tonetti, S. Sollfors [http://www.eurospiders.com/Ixodes_ricinus.htm], O. Schumann, modified).

In the tick midgut, *B. burgdorferi* spirochetes are tolerated, i.e. their tick vector is immunotolerant (Johns et al., 2001), but they are recognized by tick immune system when they become systemic. Hence, spirochetes have been found associated with tick hemocytes that possibly ingest them by phagocytosis in hemolymph (Kuhn et al., 1994; Coleman et al., 1997). However, the host immune system accompanying the blood meal is a more challenging threat to borreliae residing in the tick midgut. When the tick begins feeding, one of the first immune components encountered by *B. burgdorferi* spirochetes within the midgut are natural antibodies, i.e. immunoglobulins belonging to the early host defence against invading pathogens, found in mammalian serum in the absence of exposure to environmental pathogens (Greenberg, 1985; Casali and Schettino, 1996). These molecules have a borreliacidal effect and limit the spirochete burden within feeding ticks, prior to the development of adaptive immune responses (Ochsenbein et al., 1999; Belperron and Bockenstedt, 2001). In addition, in hosts that have not previously been in contact with *I. ricinus* ticks, serum complement has been shown to be the major component of the host innate immune system that destroys particular *B. burgdorferi* species (or strains) *in vitro* (Kurtenbach et al., 1998a). In fact, when sensitive borreliae encounter serum complement, they are lysed in the tick midgut, preventing any transmission to the vertebrate host (Papatheodorou and Brossard, 1987; Kurtenbach et al., 2002a).

In unfed *Ixodes* ticks, *B. burgdorferi* s.l. spirochetes are primarily restricted to the midgut (Lebet and Gern, 1994). However, borreliae may be observed in other tissues such as hemolymph and salivary glands (Burgdorfer, 1984; Burgdorfer et al., 1982, 1988, 1989; Lebet and Gern, 1994; Leuba-Garcia et al., 1994). In starved *I. scapularis* nymphs, low numbers of nonmotile spirochetes (about 500 to 1000 per tick) are detected (De Silva and Fikrig, 1995; Piesman et al., 2001). Spirochetal dissemination within feeding ticks appears to be biphasic, consisting of a replicating nonmotile and a motile phase (Dunham-Ems et al., 2009). When nymphs attach to the host and start feeding, borreliae multiply rapidly by binary fission (i.e. double in numbers after 4 h) in the midgut in *I. ricinus* (Gern et al., 1990) and in *I. scapularis* (De Silva and Fikrig, 1995). During the first phase of rapid growth in *I. scapularis* (lasting up to 36 h after attachment), spirochetes form networks of nonmotile organisms (Dunhams-Ems et al., 2009). Between 36 h and 48 h after attachment, spirochetes transition to motile single organisms that leave the midgut by penetrating the basement membrane and entering the hemocoel. They migrate through the hemolymph and enter *I. scapularis* salivary glands (Dunhams-Ems et al., 2009). In *I. ricinus*, the migration process appears to be slower as spirochetes are only observed in the salivary glands after 2 days of feeding (Gern et al., 1996). The period of most rapid increase in spirochetal numbers in the salivary glands occurs between 48 to 60 h post-attachment in *I. scapularis* (Piesman et al., 2001). Spirochetes in the salivary glands are transmitted to the host with injected saliva in *I. ricinus* (Gern et al., 1990) and *I. scapularis* (De Silva and Fikrig, 1995). Interestingly, *Borrelia* genospecies appear to be transmitted to hosts at different rates, *B. afzelii* being transmitted earlier than *B. burgdorferi* s.s. spirochetes by *I. ricinus* (Crippa et al., 2002). The peak of spirochete number per nymph is observed around 72 h after attachment in *I. scapularis* (up to more than 17-fold) (De Silva and Fikrig, 1995; Piesman et al., 2001). However, soon after completion of feeding and detachment from the host (96 h), the mean number of borreliae decreases steadily and all organs but the midgut seem to be cleared of spirochetes (De Silva and Fikrig, 1995). Furthermore, spirochete number decreases dramatically during the subsequent premoulting period in *I. scapularis* (up to 10-fold drop) (Piesman et al., 1990). This decrease in spirochetes has been attributed to a lack of necessary resources such as N-acetylglucosamine (Piesman et al., 1990).

Different carbohydrates are selectively available to *B. burgdorferi* during its enzootic cycle. During the tick blood meal, high concentrations of carbohydrates including glucose, the dominant carbohydrate in mammalian blood (Young et al., 1971; Welton et al., 1973), are available to spirochetes. *B. burgdorferi* uses mainly glucose to support growth during that phase, although other carbohydrates present in lower quantities, such as mannose, N-acetylglucosamine, maltose, chitobiose may also be used (Von Lackum and Stevenson, 2005). Nutrients present in the blood meal are rapidly depleted (hexose sugars permeate the tick peritrophic membrane that covers the midgut epithelial cells during feeding while spirochetes remain trapped on the other side) and are non-existent in an unfed tick midgut (Zhu et al., 1991; Terra, 2001). Spirochetes residing in the midgut therefore need to find an alternative energy source until the next blood meal. An alternative and readily available energy source is glycerol, a small carbohydrate, which is produced by *I. uriae* for example (Lee and Baust, 1987). Hence, *B. burgdorferi* switches carbohydrate utilisation during the mammal-to-tick transition, and glycerol is essential for maximum spirochetal fitness during the tick phase of the enzootic cycle (Pappas et al., 2011). More recently, it has been shown that *B.*

burgdorferi may also use trehalose (which may be found in tick tissues according to Barker and Lehner, 1976), N-acetylglucosamine and chitobiose (both available during tick moulting) during persistence in the tick since the bacteria grow well in these media *in vitro* (Tilly et al., 2001; Hoon-Hanks et al., 2012).

Borrelia spirochetes may be transstadially maintained from one tick developmental stage to the next (Fig. 2.8a), a phenomenon called transstadial transmission (Monin et al., 1989). This mode of transmission means that the ingested spirochetes need to survive through blood meal digestion and moulting, two processes during which tick internal physiology changes drastically. This changing environment is often harsh on colonising pathogens and many of them are destroyed during these events. The latter is what happens to pathogens in ticks that are incompetent vectors. For instance, *I. persulcatus*, *Haemaphysalis concinna* and *Dermacentor silvarum* ticks can all acquire *Borrelia* spirochetes while feeding on infected mice, but borreliae do not survive the time period of digestion and moulting in *H. concinna* and *D. silvarum*, two non-vector species, whereas they survive in *I. persulcatus*, a known vector of *B. burgdorferi* s.l. (see section 2.3.6) (Sun and Xu, 2003). Even when ticks are vectors of certain microorganisms, the latter may suffer during digestion and subsequent moulting as mentioned previously.

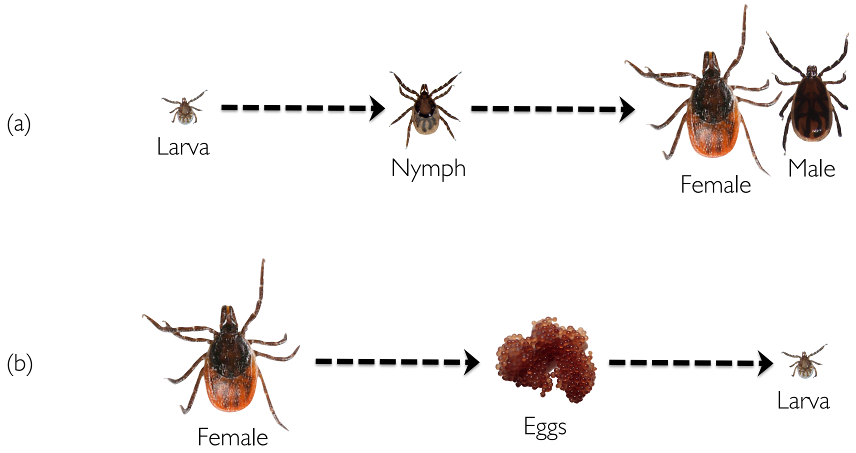

Fig. 2.8 Lyme disease spirochetes maintenance between different developmental stages (dashed arrows) in ticks of the *I. ricinus* complex by (a) transstadial transmission and (b) transovarial transmission (Anderson, 1991) (Pictures: J. K. Ty [http://www.astrophotophils.com/images/091012tick-d.jpg], S. Sollfors [http://www.eurospiders.com/Ixodes_ricinus.htm], N. Tonetti, modified).

Another way by which *Borrelia* spirochetes are maintained in tick lineage is transovarial transmission. The latter is the transmission of infectious agents from the female tick to its progeny (Fig. 2.8b). Although *Borrelia* bacteria are usually restricted to tick midgut (Gern et al., 1990; Lebet and Gern, 1994; Gern et al., 1996), they may be present in tick ovary (Burgdorfer et al., 1989; Bellet-Edimo et al., 2005), resulting in transmission of borreliae from the female tick to its offspring. As far as ticks of the *I. ricinus* complex are concerned, transovarial transmission of Lyme disease spirochetes has been reported in *I. ricinus* (Stanek et al., 1986; Bellet-Edimo, 2005), *I. pacificus* (Lane and Burgdorfer, 1987), *I. scapularis* (Magnarelli et al., 1987; Burgdorfer et al., 1988), *I. hexagonus* (Gern et al., 1991; Toutoungi and Gern, 1993), and *I. persulcatus* (Balashov et al., 1998). However, this phenomenon is rare as it appears that only 4.6 % of infected females in *I. ricinus* (Bellet-Edimo et al., 2005) and 6.3 % of infected females in *I. persulcatus* (Nefedova et al., 2004) transmit spirochetes to the next generation. Transovarial transmission of *B. burgdorferi* spirochetes may even be lower than described in the early literature (before 1995) (or inexistent) since reported *B. burgdorferi* infections might have been confused (due to unspecific detection methods) with infections with *B. miyamotoi*, a *Borrelia* species closely related to relapsing-fever spirochetes and described in 1995 (Funkunaga et al., 1995). A recent study has revealed this controversy and observed transovarial transmission of *B. miyamotoi* (at a rate of 0.9 to 2.4 %) but not of *B. burgdorferi* spirochetes from field-sampled *I. scapularis* females to larvae (Rollend et al., 2013). This seems to confirm what another study, in which field-collected unfed *I. ricinus* larvae harboured *B. miyamotoi* but not *B. burgdorferi* spirochetes (Richter et al., 2012), has suggested: that transovarial transmission might not occur at all in *B. burgdorferi*-infected ticks.

2.3.4 Genospecies

Speciation among *B. burgdorferi* s.l. is assumed to be a recent phenomenon since Le Fleche et al. (1997) have reported that divergence of the *rrs* gene sequences within different *Borrelia* representatives may not exceed 1 %. Assuming that *B. burgdorferi* has evolved clonally, a greater diversity of the isolates within a given genospecies should indicate a longer period available for evolution. The origin where speciation among *B. burgdorferi* genospecies has taken place is still under debate. Since the multiplicity of genospecies is much greater in Europe than in North America, it has been suggested that the *B. burgdorferi* complex originated in Europe. However, according to *ospC* typing, a closer relationship between European strains than North American ones exists, supporting the reverse conclusion, i.e. the introduction of *B. burgdorferi* to Europe from America (Foretz et al., 1997; Marti Ras et al., 1997). The latest published paper on that matter reaffirms that *B. burgdorferi* has originated from Europe rather than from North America, based on a multilocus sequence typing scheme comprising eight chromosomal housekeeping genes of *B. burgdorferi* (Margos et al., 2008).

To date, nineteen genospecies belonging to the *B. burgdorferi* complex have been described worldwide (all of which are transmitted by ticks belonging to the *I. ricinus* complex): *B. burgdorferi* sensu stricto (s.s.) (Baranton et al., 1992), *B. garinii* (Baranton et al., 1992), *B. afzelii* (Canica et al., 1993), *B. japonica* (Kawabata et al., 1993), *B. andersonii* (Marconi et al., 1995), *B. tanukii* (Fukunaga et al., 1996), *B. turdi* (Fukunaga et al., 1996), *B. lusitaniae* (Le Fleche et al., 1997), *B. valaisiana* (Wang et al., 1997), *B. bissettii* (Postic et al., 1998), *B. sinica* (Masuzawa et al., 2001), *B. spielmanii* (Richter et al., 2004), *B. californiensis* (Postic et al., 2007), *B. yangtze* (Chu et al., 2008), *B. carolinensis* (Rudenko et al., 2009a), *B. bavariensis* (Margos et al., 2009), *B. americana* (Rudenko et al., 2009b), *B. kurtenbachii* (Margos et al., 2010), and *B. finlandensis* (Casjens et al., 2011).

B. burgdorferi s.l. spirochetes have been detected on four continents: Europe, East Asia, North America, and North Africa (Table 2.3) (Bergström et al. 2002, Miyamoto and Masuzawa 2002). Their distribution is closely tied to that of the ticks of the *I. ricinus* complex (see sections 2.2.4 and 2.3.6) (Fig. 2.5). Some genospecies appear to be confined to certain areas, such as *B. andersonii* in northeastern USA, *B. bavariensis* in Central Europe, *B. finlandensis* in Finland, *B. japonica* in Japan, or *B. sinica* in China and Nepal, while other genospecies are widely distributed such as *B. burgdorferi* s.s., observed Europe, East Asia, North America and North Africa, or *B. garinii*, present in Europe, East Asia and North Africa (Table 2.3).

Another *Borrelia* species related to relapsing fever spirochetes, *B. miyamotoi*, has also been reported in ticks belonging to the *I. ricinus* complex. It was first described in *I. persulcatus* in Asia (Fukunaga et al., 1995), but it was later observed in *I. scapularis* in eastern North America (Scoles et al., 2001), *I. ricinus* in Europe (Fraenkel et al., 2002), and *I. pacificus* in western North America (Mun et al., 2006).

Table 2.3 Worldwide distribution of *B. burgdorferi* genospecies and relapsing fever-like *B. miyamotoi* (Scoles et al., 2001; Younsi et al., 2001; Barral et al., 2002; Bergström et al., 2002; Fraenkel et al., 2002; Miyamoto and Masuzawa, 2002; Piesman, 2002; Shih and Chao, 2002; Sarih et al., 2003; Masuzawa, 2004; Mun et al., 2006; Richter et al., 2006; Hulinska et al., 2007; Cotté et al., 2010; Gern et al., 2010; Kjelland et al., 2010; Hasle et al., 2011; Rudenko et al., 2011; Geller et al., 2012; Heylen et al., 2013; Norte et al., 2013).

Borrelia species	East Asia	Europe	North Africa	North America
B. afzelii	Northern and western China, Japan, Korea	Throughout	-	-
B. americana	-	-	-	Eastern and western USA
B. andersonii	-	-	-	Northeastern USA
B. bavariensis	-	Central Europe	-	-
B. bissettii	-	Czech Republic	-	Eastern and western USA
B. burgdorferi s.s.	Taiwan	Throughout	Morocco	Throughout
B. californiensis	-	-	-	Western USA
B. carolinensis	-	Western France	-	Southeastern USA
B. finlandensis	-	Finland	-	-
B. garinii	Northern and western China, Japan, Korea	Throughout	Tunisia, Morocco	-
B. japonica	Japan	-	-	-
B. kurtenbachii	-	Slovenia	-	Eastern USA, Canada
B. lusitaniae	-	Western, Central and eastern Europe	Tunisia, Morocco	-
B. miyamotoi	Japan	Central and northern Europe	-	Canada, eastern and western USA
B. sinica	China, Nepal	-	-	-
B. spielmanii	-	Central and eastern Europe	-	-
B. tanukii	Japan, Nepal	-	-	-
B. turdi	Japan	Norway, Portugal, Belgium	-	-
B. valaisiana	Southern China, Taiwan, Thailand, southern Korea, southern Japan	Throughout	-	-
B. yangtze	Southwestern China	-	-	-

2.3.5 Reservoir Hosts

Even though vectors of *B. burgdorferi* s.l. throughout the world feed on a large range of host species, only some of them may act as reservoir hosts for the pathogen. A reservoir host is a vertebrate host that participates significantly in the circulation of the spirochetes in nature by infecting ticks and by providing a medium where spirochetes multiply, disseminate and persist for a considerable amount of time (Gern et al., 1998). Hence, reservoir hosts increase the number of infected ticks in a particular area, exerting a positive ecological effect on the pathogen (Kahl et al., 2002). Such hosts do not appear to develop Lyme disease (Kurtenbach et al., 2002c).

Diversity of reservoir hosts of *B. burgdorferi* s.l. is important throughout the world but is greatest in Europe (Table 2.4). Reservoir hosts belong to three animal classes: mammals, birds and reptiles. Mammals constitute the majority of reservoir hosts of *B. burgdorferi* s.l., followed by birds, which are well represented. In contrast, reptiles constitute a small minority. Among mammals, rodents including mice, voles, rats and squirrels, predominate (Piesman 2002; Piesman and Gern, 2004). However, insectivores (shrews) and lagomorphs, and to a lesser extent carnivores may be reservoir hosts of *B. burgdorferi* s.l. (Gern et al., 1998; Piesman and Gern, 2004). Among birds, reservoir hosts are mainly passeriformes (Gern et al., 1998; Hanincova et al. 2003b). To date, lizards are the only reservoir hosts of *B. burgdorferi* s.l. among reptiles (Dsouli et al., 2006; Richter and Matuschka, 2006). There has been some controversy about the role ungulates play in the ecology of *B. burgdorferi* and its tick vector. Some authors have reported that cervids are reservoir hosts of the spirochetes (Anderson, 1989; Miyamoto and Masuzawa, 2002). However, it appears that cervids including elks *Alces alces* (Tälleklint and Jaenson, 1994), deer *Capreolus capreolus* (Jaenson and Tälleklint, 1992), *C. elaphus* (Gray et al., 1995), *Dama dama* (Gray et al., 1992) and *Odocoileus virginianus* (Telford III et al., 1988), and bovids including wild sheep *Ovis ammon* (Matuschka et al., 1993), do not infect feeding ticks with *B. burgdorferi* s.l., i.e. are non-reservoir hosts. Moreover, complement contained in cervid sera (i.e. in *C. elaphus* (Kurtenbach et al., 1998a) and *C. nippon* (Nelson et al., 2000) sera) is highly lytic to *B. burgdorferi*. Hence, a dilution in infection by *B. burgdorferi* s.l. might be observed in tick populations feeding on such hosts in nature. Nevertheless, ungulates are important hosts for immature and mature stages of ticks of the *I. ricinus* complex (see section 2.2.2.2). Even if such hosts do not appear to develop systemic infections (Telford III et al., 1988; Jaenson and Tälleklint, 1992; Matuschka et al., 1993; Kurtenbach et al., 1998a), they may play a key role in co-feeding transmission (see section 2.3.3) of *Borrelia* spirochetes in nature (Kimura et al., 1995; Ogden et al., 1997).

A parameter that complicates the determination of reservoir competence of a vertebrate host is the specificity between a particular host and the different *B. burgdorferi* s.l. genospecies. *B. afzelii* and *B. burgdorferi* s.s. are mainly associated with rodents, such as squirrels (*Sciurus* spp) (Craine et al., 1997; Humair and Gern, 1998), mice (*Apodemus* spp) and voles (*Myodes* spp) (Nakao et al., 1994; Humair et al., 1995; Kurtenbach et al., 1998b; Humair et al., 1999; Hanincova et al., 2003a; Morán Cadenas et al., 2007b). In addition, mice (*Apodemus* spp) have been found to be reservoir hosts of *B. bavariensis* (Hügli et al., 2002) and edible dormice (*Glis glis*) of *B. spielmanii* (Richter et al., 2004). In contrast, *B. garinii* and *B. valaisiana* are primarily associated with birds, like pheasants (*Phasianus colchicus*) (Kurtenbach et al., 1998b, 2002b) and thrushes (*Turdus* spp) (Nakao et al., 1994; Humair et al., 1998; Hanincova et al., 2003b; Comstedt et al., 2006). Lizards, which were thought to be non-reservoir hosts since sera of some North American species are borreliacidal (Lane and Quistad, 1998; Kuo et al., 2000; Ullmann et al., 2003), appear to be reservoir hosts of *B. lusitaniae* in North Africa (Dsouli et al., 2006) and in Europe (Majlathova et al., 2006; Richter and Matuschka, 2006; Amore et al., 2007).

Table 2.4 Reservoir hosts of *B. burgdorferi* s.l. in Asia (♣), Europe (♥), North Africa (♠), and North America (♦) (Anderson, 1989; Fish and Daniels, 1990; Barker et al., 1993; Stafford et al., 1995; Oliver, 1996; Craine et al., 1997; Gern et al., 1998; Wan et al., 1999; Gern and Humair, 2002; Miyamoto and Masuzawa, 2002; Piesman, 2002; Piesman and Gern, 2004; Estrada-Peña et al., 2005; Dsouli et al., 2006; Hanincova et al., 2006; Majlathova et al., 2006; Richter and Matuschka, 2006; Morán Cadenas et al., 2007b; Vourc'h et al., 2007; Salkeld et al., 2008; Gern, 2009; Scott et al., 2010a; Richter et al., 2011; Rydzewski et al., 2011).

Birds			
	Charadriiformes		*Alca torda* (♥), *Fratercula arctica* (♥)
	Galliformes		*Phasianus colchicus* (♥)
	Passeriformes		*Anthus trivialis* (♥), *Catharus fuscescens* (♦), *C. ustulatus* (♦), *Coccothrautes coccothrautes* (♥), *Emberiza spodocehala* (♣). *E. rustica* (♣), *Erithacus rubecula* (♥), *Fringilla coelebs* (♥), *Geothlypis trichas* (♦), *Luscinia luscinia* (♥), *L. svecica* (♥), *Melospiza melodia* (♦), *Parus major* (♥), *Phoenicurus phoenicurus* (♥), *Pipilo erythrophthalmus* (♦), *Psylloscopus collybita* (♥), *Quiscalus quiscula* (♦), *Sylvia atricapilla* (♥), *S. communis* (♥), *Thryothorus ludovicianus* (♦), *Troglodytes troglodytes* (♥), *Turdus chrysolaus* (♣), *T. merula* (♥), *T. migratorius* (♦), *T. pallidus* (♣), *T. philomelos* (♥), *Zonotrichia atricapilla* (♦)
Mammals			
	Artiodactyls		
		Boars	*Sus scrofa* (♥)
	Carnivores		
		Foxes	*Vulpes vulpes* (♥♣)
		Raccoons	*Procyon lotor* (♦)
		Skunks	*Mephitis mephitis* (♦)
	Insectivores		
		Hedgehogs	*Erinaceus europaeus* (♥)
		Shrews	*Blarina brevicauda* (♦), *Neomys fodiens* (♥), *Sorex araneus* (♥), *S. minutus* (♥), *S. unguiculatus* (♣)
	Lagomorphs		
		Hares	*Caprolagus sinensis* (♣), *Lepus europaeus* (♥), *L. timidus* (♥)
		Rabbits	*Oryctolagus cuniculus* (♥), *Sylvilagus floridanus* (♦)
	Marsupials		
		Opossums	*Didelphis virginiana* (♦)
	Rodents		
		Chipmunks	*Tamias sibiricus* (♥♣), *T. striatus* (♦), *T. ochrogenys* (♦)
		Dormice	*Eliomys quercinus* (♥), *Glis glis* (♥), *Muscardinus avellanarius* (♥)
		Groundhogs	*Marmota monax* (♦)
		Jumping Mice	*Napaeozapus insignis* (♦)
		Kangaroo Rats	*Dipodomys californicus* (♦)
		Mice	*Apodemus agrarius* (♥♣), *A. argenteus* (♣), *A. flavicollis* (♥), *A. speciosus ainu* (♣), *A. sylvaticus* (♥), *Peromyscus gossypinus* (♦), *P. leucopus* (♦), *P. maniculatus* (♦)
		Rats	*Rattus confucianus* (♣), *R. coxingi* (♣), *R. edwardsi* (♣), *R. fulvescens* (♣), *R. norvegicus* (♥♦♣), *R. rattus* (♥), *Sigmodon hispidus* (♦)
		Squirrels	*Sciurus carolinensis* (♥), *S. griseus* (♦), *S. vulgaris* (♥)
		Voles	*Eothenomys smithii* (♣), *Microtus agrestis* (♥), *M. ochrogaster* (♦), *M. pennsylvanicus* (♦), *Myodes glareolus* (♥), *M. rufocanus bedfordia* (♣), *M. rutilus mikado* (♣)
		Woodrats	*Neotoma floridana* (♦), *N. fuscipes* (♦)
Reptiles			
	Lizards		*Podarcis muralis* (♥), *Lacerta agilis* (♥), *Lacerta viridis* (♥), *Psammodromus algirus* (♠)

While antibodies appear to control the rate of transmission of Lyme disease spirochetes from natural host to tick (Kurtenbach et al., 1994), the complement system has been proven to be a major determinant of host specificity between *Borrelia* genospecies and their vertebrate hosts (Kurtenbach et al., 1998a, 2002a). Brade et al. (1992) first observed that different *B. burgdorferi* s.l. genospecies differed in sensitivity to human serum. More recent studies have shown that there is a clear pattern of resistance or sensitivity, which is consistent with patterns of transmissibility (Kurtenbach et al., 2002a). If borreliae are sensitive to complement system of a particular species they are lysed in the tick midgut when they encounter it, before transmission to the host occurs (Papatheodorou and Brossard, 1987; Kurtenbach et al., 2002a). Incompatible *Borrelia* genospecies do not survive the blood meal (Kurtenbach et al., 2002b). Hence, Kurtenbach et al. (1998a) has reported that bird sera are borreliacidal for *B. afzelii* – usually associated with rodents – but not for *B. garinii* or *B. valaisiana* – usually associated with birds. In contrast, rodent sera display the reverse pattern, destroying *B. garinii* and *B. valaisiana*, but not *B. afzelii*. Moreover, deer sera are borreliacidal for all the genospecies tested, suggesting the zooprophylactic role of cervids in *B. burgdorferi* ecology (Kurtenbach et al., 1998a).

To date, no reservoir host for *B. miyamotoi* in Europe has been described in the literature. However, suspicions that rodents play a role in the transmission of these relapsing-fever like spirochetes have emerged since *B. miyamotoi* is often associated with *B. afzelii* or *B. burgdorferi* s.s. spirochetes, i.e. rodents-associated genospecies (Gern et al., 2010). Moreover, infection transmission from *B. miyamotoi*-infected *I. scapularis* nymphs to *P. leucopus* mice and later recovery of the spirochetes from uninfected xenodiagnostic larvae (Scoles et al., 2001) seems to indicate that rodent are reservoir hosts for *B. miyamotoi* in North America. However, *B. miyamotoi* has been detected in 58 % of wild turkey tissues (*Meleagris gallopavo*) infested with *Amblyomma americanum*, suggesting that these spirochetes might also be linked to birds (Scott et al., 2010b).

2.3.6 *Ixodes* Vectors

Four tick species play major roles in Lyme disease epidemiology: *I. ricinus* is the main vector of Lyme spirochetes in Europe and North Africa, *I. persulcatus* in eastern Europe and Asia, *I. pacificus* in western North America, and *I. scapularis* in eastern North America (Stanek et al., 2012). However, other ticks of closely related species (19 spp.) play minor roles throughout the world: *I. affinis*, *I. angustus*, *I. columnae*, *I. dentatus*, *I. frontalis*, *I. granulatus*, *I. hexagonus*, *I. jellisoni*, *I. minor*, *I. muris*, *I. nipponensis*, *I. ovatus*, *I. pavlovskyi*, *I. sinensis*, *I. spinipalpis*, *I. tanuki*, *I. trianguliceps*, *I. turdus*, and *I. uriae* (Table 2.5). Ticks transmitting *B. burgdorferi* s.l. are hard ticks and belong to the genus *Ixodes* (Wall and Shearer, 2001). However, all *Ixodes* are not able to transmit *B. burgdorferi*. Spirochetes have also been detected in non-vector hard ticks belonging to other genera such as *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma* or *Rhipicephalus* (Mather and Mather, 1990; Eisen and Lane, 2002), soft ticks, mites and insects (Magnarelli and Anderson, 1988; Oliver, 1996; Gern and Humair, 2002; Kampen et al., 2004).

Ticks identified as vectors of *B. burgdorferi* s.l. belong either to exophilic or endophilic species. Exophilic ticks quest, as described in *I. ricinus* (see section 2.2.2.2) and tend to possess a range of host species, although some of them are more adapted to certain host species. In contrast, endophilic (or nidicolous) ticks remain secluded within their host's cave, burrow or nest throughout their life cycle. Hence, endophilic ticks tend to specialise in one or a few host species or families (Table 2.5) and come into contact with human beings less frequently than their exophilic counterparts (Sonenshine, 1991).

Table 2.5 *Ixodes* ticks transmitting *B. burgdorferi* s.l. spirochetes (and relapsing fever-like *B. miyamotoi*): their distribution, lifestyle, and transmitted *Borrelia* genospecies. The four tick species playing a major role in Lyme disease epidemiology are in grey shade. (Cotton and Watts, 1967; Burgdorfer et al., 1985; Burgdorfer and Gage, 1986; Telford III and Spielman, 1989; Gern et al., 1991; Brown and Lane, 1992; Marconi et al., 1995; Gern et al., 1997; Wan et al., 1998; Gylfe et al., 1999; Lane et al., 1999; Burkot et al., 2000; Dolan et al., 2000; Peavey et al., 2000; Wall and Shearer, 2001; Eisen and Lane, 2002; Korenberg et al., 2002; Eisen et al., 2003; Oliver et al., 2003; Sun et al., 2003; Masuzawa, 2004; Mun et al., 2006; Gern et al., 2010; Korenberg et al., 2010; Strube et al., 2010; Casjens et al., 2011; Hasle et al., 2011; Margos et al., 2011; Ogden et al., 2011; Stanek et al., 2012; Heylen et al., 2013).

Tick species	Geographical distribution	Lifestyle (preferred host)	Genospecies transmitted
Ixodes affinis	Southeastern North America	Exophilic	*B. bissettii*, *B. burgdorferi* s.s.
Ixodes angustus	Western North America	Endophilic (rodents)	*B. burgdorferi* s.s.
Ixodes columnae	Northeastern Asia	Endophilic (birds)	*B. valaisiana*
Ixodes dentatus	Eastern North America	Endophilic (rabbits)	*B. andersonii*
Ixodes frontalis	Europe, eastern Asia	Endophilic (birds)	*B. turdi*
Ixodes granulatus	Southeastern Asia	Exophilic	*B. valaisiana*, *B. yangtze*
Ixodes hexagonus	Throughout Europe, North Africa	Endophilic (hedgehogs)	*B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, *B. spielmanii*
Ixodes jellisoni	Western North America	Endophilic (kangaroo rats)	*B. californiensis*
Ixodes minor	Southeastern North America	Endophilic (woodrats)	*B. americana*, *B. bissettii*, *B. burgdorferi* s.s.
Ixodes muris	Northeastern North America	Endophilic (mice and birds)	*B. burgdorferi* s.s.
Ixodes nipponensis	Northeastern Asia	Exophilic	*B. afzelii, B. garinii, B. yangtze*
Ixodes ovatus	Northeastern Asia	Exophilic	*B. japonica, B. sinica*
Ixodes pacificus	Western North America	Exophilic	*B. americana*, *B. bissettii*, *B. burgdorferi* s.s., *B. californiensis, B. miyamotoi*
Ixodes pavlovskyi	Central Asia	Exophilic	*B. afzelii*, *B. garinii*
Ixodes persulcatus	Eastern Europe to eastern Asia	Exophilic	*B. afzelii, B. garinii*, *B. miyamotoi*
Ixodes ricinus	Throughout Europe, North Africa	Exophilic	*B. afzelii*, *B. bavariensis*, *B. bisettii*, *B. burgdorferi* s.s., *B. finlandensis*, *B. garinii*, *B. lusitaniae*, *B. miyamotoi*, *B. spielmanii*, *B. turdi*, *B. valaisiana*
Ixodes scapularis (also known as *I. dammini*)	Eastern North America	Exophilic	*B. bissettii*, *B. burgdorferi* s.s., *B. kurtenbachii*, *B. miyamotoi*
Ixodes sinensis	Southeastern Asia	Exophilic	*B. garinii*
Ixodes spinipalpis (also known as *I. neotomae*)	Western North America	Endophilic (woodrats and mice)	*B. bissettii*, *B. burgdorferi* s.s., *B. californiensis*
Ixodes tanuki	Northeastern Asia	Endophilic (raccoon dogs)	*B. tanukii*
Ixodes trianguliceps	Western Europe to Central Asia	Endophilic (voles)	*B. afzelii*, *B. garinii*
Ixodes turdus	Northeastern Asia	Endophilic (birds)	*B. turdi*
Ixodes uriae	Circumpolar region	Endophilic (seabirds)	*B. garinii*

2.3.7 Prevalence in Questing Ticks

B. burgdorferi s.l. prevalence varies greatly from one region to another. This variation is mainly due to two factors: tick habitat suitability and competent host availability. *B. burgdorferi* s.l. infection rate in *I. ricinus* nymphs and adults is higher in ticks collected from habitats that have complex vegetation compositions such as woodlands and the arboretum, while a lower spirochetal prevalence is observed in ticks sampled in habitats with reduced complexity of vegetation (Kirstein et al., 1997a). Furthermore, *B. burgdorferi* s.l. prevalence in questing *I. ricinus* varies with altitude. In fact, the density of *Borrelia*-infected nymphs and adults usually decreases with increasing altitude (Jouda et al., 2004b; Burri et al., 2007; Gern et al. 2008). However, the density of *Borrelia*-infected ticks may increase with increasing altitude under particular conditions, such as the north-facing slope on Chaumont Mountain, Switzerland (Morán Cadenas et al., 2007a).

In Europe, *B. burgdorferi* s.l. prevalence in questing *I. ricinus* ticks ranges from 5 to 45 % (Table 2.6). In Switzerland, infection prevalence range is similar to that of other countries in Central Europe. However, *Borrelia* prevalence varies greatly depending on the area. In fact, it reaches 26 % in Bern (Lommano et al., 2012b), 25 % in Fribourg (Lommano et al., 2012b), 24 % in Jura (Lommano et al., 2012b), 17 to 35 % in Neuchâtel (Casati et al., 2004; Morán Cadenas et al., 2007a; Lommano et al., 2012b), 11 to 15 % in Ticino (Jouda et al., 2003; Casati et al., 2004;), 20 to 35% in Valais (Casati et al., 2004; Burri et al., 2007), and 19 % in Vaud (Lommano et al., 2012b).

As far as genospecies are concerned, the most common ones in Europe are *B. garinii* and *B. afzelii*. More precisely, *B. garinii* tends to predominate in southwestern parts while *B. afzelii* tends to prevail in Central and northeastern parts of Europe (Hubalek and Halouzka, 1997; Rauter and Hartung, 2005). The prevalence of other genospecies, such as *B. burgdorferi* s.s., *B. spielmanii*, and *B. valaisiana*, varies widely but they are always less abundant (Hubalek and Halouzka, 1997; Rauter and Hartung, 2005). *B. lusitaniae* is commonly detected in south western countries such as Portugal, Spain and Italy but it is less often observed when going eastwards (Rauter and Hartung, 2005; Bertolotti et al., 2006; Toledo et al., 2009). In contrast, *B. bissettii* appears to be restricted to eastern Europe (Hulinska et al., 2007; Rudenko et al., 2008). The most common mixed infection is *B. garinii* and *B. valaisiana*, occurring more than twice as often than all other genospecies combinations (Rauter and Hartung, 2005).

Infection prevalence of *B. burgdorferi* s.l. does not only fluctuate geographically but also temporally, presumably due to factors involved in shaping *Borrelia* prevalence, such as host availability and climatic conditions that change over time (Morán Cadenas et al., 2007a). In addition, prevalence of *B. burgdorferi* s.l. differs among tick developmental stages. *B. burgdorferi* s.l. infection prevalence in *I. ricinus* adult ticks is higher than that in nymphs that is, in turn, higher than that in larvae (Hubalek and Halouzka, 1998; Kurtenbach et al., 1998b; Jouda et al., 2004a). However, it seems that there is usually no significant difference in *B. burgdorferi* infection prevalence between *I. ricinus* male and female ticks (Hubalek et al., 1994; Christova et al., 2001; Quessada et al., 2003; Cotté et al., 2010), probably because males and females take the same number of blood meals prior to reaching adulthood, i.e. one as larvae and one as nymphs.

To date, spirochete load in questing *I. ricinus* in Europe has been investigated in one single study. Hence, Rauter et al. (2002) have performed a real-time PCR targeting the borrelial *ospA* gene and have reported a median of 4,000 spirochetes per tick. However, it is important to note that Rauter et al. (2002) did not differentiate between tick developmental stages or *Borrelia* genospecies present in the infected ticks.

Table 2.6 Recent prevalences of *B. burgdorferi* s.l. spirochetes in questing *I. ricinus* nymphs (n) and adults (a) in Europe.

Country	Prevalence ✎	Reference
Austria	16 % (n), 25 % (a)	Blaschitz et al., 2008
Belgium	9 to 22 % (n), 33 % (a)	Misonne et al., 1998; Tack et al., 2012a
Bulgaria	10 % (n), 32 to 41 % (a)	Christova et al., 2001, 2003
Croatia	41 % (n), 47 % (a)	Rijpkema et al., 1996
Czech Republic	5 to 18 % (n), 6 to 27 (% a)	Derdakova et al., 2003; Daniel et al., 2009
Denmark	13 % (n), 4 % (a)	Skarphedinsson et al., 2007
England	3 to 6 % (n), 16 % (a)	Kurtenbach et al., 1998b; Vollmer et al., 2011
Estonia	15 % (a)	Mäkinen et al., 2003
Finland	4 % (n), 9 % (a)	Mäkinen et al., 2003
France	1 to 18 % (n), 10 to 25 % (a)	Ferquel et al., 2006; Cotté et al., 2010; Reis et al., 2011
Germany	9 to 30 % (n), 11 to 40 % (a)	Rauter et al., 2002; Hildebrandt et al., 2010; Bingsohn et al., 2013
Hungary	3 % (n & a)	Egyed et al., 2012
Ireland	12 to 18 % (n), 18 to 20 % (a)	Kirstein et al., 1997a, 1997b; Pichon et al., 2005
Italy	17 to 22 % (n), 19 to 40 % (a)	Bertolotti et al., 2006; Menardi et al., 2008; Nazzi et al., 2010
Latvia	11 to 20 % (n), 30 to 33 % (a)	Etti et al., 2003; Bormane et al., 2004; Vollmer et al., 2011
Lithuania	10 % (n), 14 % (a)	Paulauskas et al., 2008
Luxembourg	8 % (n), 15 % (a)	Reye et al., 2010
Netherlands	13 to 23 % (n), 8 % (n & a), 11 % (a)	Rijpkema et al., 1995; Wielinga et al., 2006; Gassner et al., 2011
Norway	6 to 11 % (n), 6 to 33 % (a)	Paulauskas et al., 2008; Soleng and Kjelland, 2013
Poland	6 to 7 % (n), 12 to 21 % (a)	Skotarczak et al., 2002; Sroka et al., 2009; Sytykiewucz et al., 2012
Portugal	75 % (a)	De Michelis et al., 2000
Romania	19 % (n), 15 % (a)	Coipan and Vladimirescu, 2011
Russia	34 % (a)	Masuzawa et al., 2008
Serbia	43 % (a)	Milutinovic et al., 2008
Slovakia	16 to 20 % (n), 42 % (a)	Hanincova et al., 2003a, 2003b
Spain	1 % (n), 13 to 25 % (a)	Barral et al., 2002; Toledo et al., 2009
Sweden	11 % (a)	Fraenkel et al., 2002
Turkey	20 (a)	Sen et al., 2011

✎ Although many methods (BSK-H medium cultivation, dark-field or phase-contrast microscopy, immunofluoresence assays, or PCR-based) may be used for detection of *B. burgdorferi* s.l., only results obtained through PCR-based methods are considered in the present section for consistency, since PCR-based detection is more sensitive, precise and subjective than all other methods (Rauter and Hartung, 2005).

In North America, Lyme disease spirochetes are primarily transmitted by two major tick vectors, *I. scapularis* and *I. pacificus*, which are located on the East coast and the West coast, respectively. In the northeastern United States, *B. burgdorferi* s.l. prevalence in questing *I. scapularis* nymphs ranges from 15 to 30 %, with a mean of 25 %, while it oscillates between 25 to 50 % in *I. scapularis* adults (Maupin et al., 1991; Piesman, 2002; Wang et al., 2003), reaching up to 64 % in New York state (Tokarz et al., 2010). Virtually all infections are due to *B. burgdorferi* s.s., which is the most common genospecies throughout North America (Piesman, 2002). In mid-western USA, *Borrelia* infection rate in questing *I. scapularis* nymphs and adults tends to be lower, fluctuating between 9 to 16 % and 31 to 37 %, respectively (Walker et al., 1994; Hamer et al., 2010). In the southwestern United States, *B. burgdorferi* s.l. prevalence is even lower than in the Midwest and, interestingly, questing *I. pacificus* nymphs display a higher infection rate than adults, infection reaching 14 % in nymphs and 4 to 6 % in adults (Clover and Lane, 1995; Holden et al., 2003).

Spirochete load in questing *I. scapularis* ticks has been investigated in two separate studies in the northeastern USA. Wang et al. (2003) have reported a range of 25 to 197,200 spirochetes in questing *I. scapularis* ticks, with a mean of 1,964 and 5,351 spirochetes per nymph and adult tick, respectively. Similarly, Ornstein and Barbour (2006) have observed a mean of 2,240 spirochetes per *I. scapularis* nymph, which may increase up to 223,900 spirochetes per tick after engorgement on uninfected mice.

In Asia, Lyme disease spirochetes are mainly transmitted by *I. persulcatus* ticks, although many other tick vectors are present (Table 2.5). In eastern Russia, 30 % of questing adults are infected by borreliae (Korenberg et al., 2010). In China, 37 to 40 % of questing adult ticks harbour *B. burgdorferi* s.l. whereas it oscillates around 14 % in nymphs (Takada et al., 2001; Cao et al., 2003). In Japan, *B. burgdorferi* s.l. prevalence ranges from 16 to 38 % in adults, while it fluctuates around 7 % in questing nymphs (Miyamoto et al., 1992; Nakao et al., 1992, 1996). Overall, the most common genospecies observed in questing *I. persulcatus* ticks in Asia is *B. garinii* (Takada et al., 2001; Korenberg et al., 2010; Liu et al., 2012).

In North Africa, although *I. ricinus* tick populations are sparse and restricted to selected humid areas, ticks are infected at rates that match or exceed those in Switzerland. Hence, 31 to 45 % and 13 to 33 % of questing *I. ricinus* adults and nymphs, respectively, harbour *B. burgdorferi* spirochetes in Tunisia (Zhioua et al., 1999; Younsi et al., 2001; Dsouli et al., 2006). In Morocco, 48 % of questing adults are infected by Lyme disease spirochetes (Sarih et al., 2003). However, unlike Switzerland, most of *B. burgdorferi* infections in North Africa are due to *B. lusitaniae* (Younsi et al., 2001; Sarih et al., 2003).

2.3.8 Lyme Disease Incidence and Genospecies Pathogenicity

Lyme disease, or Lyme borreliosis, is the most frequent tick-borne disease throughout the world, accounting for approximately 85,500 new patients annually (Hubalek, 2009). Among these 85,500 new cases, roughly 65,500 occur in Europe, 16,500 in North America, 3,500 in Asia, and 10 in North Africa. However, this number is probably an underestimate since many *B. burgdorferi* s.l. infections are suspected not to be reported or go undetected. The number of reported cases of Lyme borreliosis has increased from the early 1990s in many European countries (Lindgren and Jaenson, 2006) but recent surveys show that the overall prevalence of Lyme disease may be stabilising (Rizzoli et al., 2011). However, the geographical distribution of cases is expanding significantly (Lindgren and Jaenson, 2006; Rizzoli et al., 2011), presumably due to both an increased level of awareness in the general population and among medical personnel, and changes in vector abundance and distribution latitudinally and altitudinally (see section 2.2.4).

Lyme disease is a multistage and multisystem disease mainly affecting the skin, nervous system, heart, and joints (Stanek et al., 1996). Because of its extremely variable clinical manifestations, Lyme borreliosis has been described as the new "great imitator" of various human diseases (Pachner, 1989). It affects different body areas in varying degrees as it progresses. Three stages have been described: early localised (days to weeks after tick bite), early disseminated (weeks to months after tick bite), and late persistent infection (months to years after tick bite). The most frequent and characteristic clinical manifestation of Lyme disease is erythema migrans, which affects the skin in the form of an expanding red patch, often with central clearing, occurring within days after tick bite (Piesman and Gern, 2004; Nau et al., 2009). Within weeks after tick bite, *B. burgdorferi* s.l. spirochetes may spread in patients' blood or lymph throughout the body and affect various organs, causing neuroborreliosis (meningitis, facial palsy) (Steere et al., 1987; Nau et al., 2009), rheumatismal symptoms (Steere, 1989), or borrelial lymphocytoma (a bluish-red tumor-like skin swelling) (Stanek et al., 2002). Chronic symptoms including Lyme arthritis and acrodermatitis chronica atrophicans (a bluish-red discolouration and swollen skin on extremities) develop months to years after disease onset (Steere, 1989; Stanek et al., 2002). However, the disease itself does not necessarily develop in stages (Steere, 1989; Rosa et al., 2005).

To date, seven *B. burgdorferi* genospecies have been reported to be pathogenic to humans (Stanek and Reiter, 2011). *B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, and *B. bavariensis* (which used to be identified as *B. garinii* until recently; Margos et al., 2009) are pathogenic for humans and cause localised, disseminated and chronic manifestations of Lyme disease (Baranton et al., 1992; Margos et al., 2009). *B. spielmanii*, which has initially been observed in early skin disease only (Wang et al., 1999a; Richter et al., 2004; Fingerle et al., 2007b), has recently been shown to be pathogenic (Baranton and De Martino, 2009). *B. bissetti* and *B. valaisiana* have also been detected in a few cases of Lyme disease (Rijpkema et al., 1997; Fingerle et al., 2007b; Rudenko et al., 2008). The pathogenic potential of an eighth genospecies, *B. lusitaniae*, is strongly suspected (Zeidner et al., 2001; Collares-Pereira et al., 2004; De Carvalho et al., 2008).

B. miyamotoi, which is more closely related to relapsing fever spirochetes than to *B. burgdorferi* bacteria (Fukunaga et al., 1995), is harboured by hard ticks of the *I. ricinus* complex (Fukunaga et al., 1995; Scoles et al., 2001; Fraenkel et al., 2002; Mun et al., 2006), while relapsing fever spirochetes usually infect soft ticks of the genus *Ornithodoros* (Rebaudet and Parola, 2005). Interestingly, this *Borrelia* genospecies is pathogenic to humans and produces clinical manifestations that strictly belong to relapsing fever and Lyme disease. Hence, patients infected by *B. miyamotoi* may display relapsing fever episodes, which are very typical of tick-borne relapsing fever, but also an erythema migrans (Platonov et al., 2011; Krause et al., 2013), and meningoencephalitis (Gugliotta et al., 2013), both symptoms that may occur in Lyme disease patients.

3 MATERIAL AND METHODS

3 MATERIAL AND METHODS

This thesis is divided into five projects. Four of them deal with the influence of *B. burgdorferi* s.l. on *I. ricinus* (1) survival under hot and dry conditions, (2) attraction to humidity, (3) fat content, and (4) survival under cold conditions. These projects followed an identical methodology, which consisted of: tick sampling, maintenance and rehydration (see section 3.1), physiological or behavioural tests (see section 3.2), *Borrelia* detection (see section 3.3), and statistical analyses (see section 3.4). Only physiological or behavioural tests (see sections 3.2.1, 3.2.2, 3.2.3, and 3.2.4) differed between these four projects. The fifth project consisted of exploring and analysing statistically a large dataset on *Borrelia* genospecies in questing *I. ricinus* nymphs collected over three years.

3.1 Tick Sampling, Maintenance and Rehydration

3.1.1 Sampling

Tick sampling was performed during *I. ricinus* peak activity in spring and in autumn (see section 2.2.3.4) because we aimed at collecting as many ticks as possible over a short period of time. Ticks were collected over several successive days, when weather permitted it, i.e. if rain and strong wind were absent.

3.1.1.1 Study Area

Questing ticks were sampled in a mixed forest dominated by deciduous trees (Fig. 3.1) and located on the south-exposed face of Chaumont Mountain (47°00' to 47°02' N and 6°55' to 6°58' E), north of Neuchâtel, Switzerland (Appendix 9.1). Site altitude varied between 595 and 735 m above sea level. Ticks were collected on both sides of forest walking or biking trails.

Fig. 3.1 Study area located in a mixed forest on the south-exposed face of Chaumont Mountain, north of Neuchâtel, Switzerland (Pictures: C. Herrmann).

3.1.1.2 Method

Questing *I. ricinus* ticks were sampled using the flagging technique (Aeschlimann, 1972). This consisted of dragging a white terry flag (1m x 1m) aside over the low vegetation for several meters (Fig. 3.2). The flag was examined for ticks every 20 m or so. Ticks were collected with angled metal tweezers and kept in plastic tubes containing blades of grass (see section 3.1.2).

Fig. 3.2 White terry flag that was dragged over the vegetation, with angled metal tweezers and plastic tubes containing sampled ticks (Picture: C. Herrmann).

Tick sampling was performed during three successive years (2009, 2010 and 2011). Sampling was conducted only in spring in 2009, whereas it was conducted in spring and autumn in 2010 and 2011 (Table 3.1). In 2009, ticks were collected between 1st and 5th May. In 2010, ticks were sampled between 18th and 25th May, and between 10th and 13th September. In 2011, ticks were collected between 15th and 19th April, and between 23rd and 27th September. Overall, 19,655 *I. ricinus* ticks were sampled over three years.

Table 3.1 Detailed numbers of collected *I. ricinus* ticks during repeated sampling periods distributed over three successive years.

Year	**Season**	**Field-collected ticks**		
		Nymphs	**Males**	**Females**
2009	Spring	700	650	600
2010	Spring	5675	1127	1075
	Autumn	1961	0	0
2011	Spring	5031	382	392
	Autumn	1940	61	61
TOTAL		15307	2220	2128

3.1.2 Maintenance and Rehydration

After sampling, field-collected ticks were brought back to the laboratory where they were maintained until they were tested in the physiological or behavioural tests. In the process, ticks rehydrated naturally since relative humidity (RH) was very high within the box and tubes in which they were maintained.

Plastic tubes containing freshly sampled ticks and blades of grass (Fig. 3.3) were held over water in a plastic box with a tight-fitting lid (98 % RH) placed within a cold chamber at 4°C in the dark (Crooks and Randolph, 2006) for a few days to a few months, until the experiment, in which ticks were intended to be used, started.

Fig. 3.3 Plastic tubes in which ticks were kept with blades of grass (Picture: C. Herrmann).

3.2 Physiological or Behavioural Tests

Physiological or behavioural tests dealt with survival under hot and dry conditions (see section 3.2.1), attraction to humidity (see section 3.2.2), energy reserves (see section 3.2.3) and survival under cold conditions (see section 3.2.4) in field-collected *I. ricinus* ticks. These tests were performed using living ticks. When tests were over, ticks were frozen and stored at -80°C until they were tested for *Borrelia* infection.

3.2.1 Hot and Dry Condition Survival Tests

Survival tests aimed at determining resistance, measured as survival rate, of *I. ricinus* ticks to unfavourable conditions of temperature (hot) and/or humidity (dry), and the effect of *Borrelia* infection on tick survival under these conditions. Tests were designed to study survival under extreme (but realistic) temperature and humidity (i.e. saturation deficit) values.

3.2.1.1 Fat Content Quantification

Before the start of survival tests, fat content was quantified in reduced samples (40 individuals) of the different tick developmental stages (nymphs, males, and females) to determine how much energy reserves they possessed.

Fat content was quantified as described in Randolph and Storey (1999). Ticks were incubated in an oven at 70°C for 24 h to remove water contained in their body. Out of the oven, they were immediately transferred and kept in a desiccator until subsequent weighing. Each nymph was weighed individually to the nearest 1 µg using a micro-balance (MX5, Mettler Toledo, Greifensee, Switzerland) kept in a quiet room. Nymphs were put in eppendorf tubes individually. Ticks were then immersed in three different changes of chloroform for 24 h each to remove fat from tick carcasses. Nymphs were re-dried in an oven at 70°C for 24 h, transferred into a desiccator and re-weighed to obtain fat-free dry mass. Fat content was calculated as described by Crooks and Randolph (2006).

Briefly, fat content was calculated by subtracting fat-free dry mass from initial dry mass. Randolph et al. (2002) observed that fat content was positively correlated with tick size. Fat content was therefore corrected for size using two different corrections to compare the results with other studies. The first correction was calculated by dividing fat content by fat-free dry mass (Crooks and Randolph, 2006). The second correction, fat index, was calculated by using the square root of fat content subsequently divided by fat-free dry mass (Randolph et al., 2002).

3.2.1.2 Saturated Salt Solutions

In the laboratory, hot and dry conditions were produced using incubators to control temperature, and saturated salt solutions to control relative humidity. Combinations of temperature and relative humidity values were expressed as saturation deficit values. Saturation deficit (SD) is a measure of the drying power of the atmosphere, depending on both temperature and relative humidity, and is defined mathematically as (Randolph and Storey, 1999):

$$SD = \left(1 - \frac{RH}{100}\right) \cdot \ 4.9463 \cdot \ e^{0.0621 \cdot \ T}$$

SD = saturation deficit (mmHg)
RH = relative humidity (%)
T = temperature (°C)
e = 2.71828

Some salts are known to produce varying relative humidities when they are used as saturated solutions at different temperatures (Winston and Bates, 1960). Saturated solutions were prepared by adding salt in boiling water until saturation was observed. Saturated solutions were cooled an instant and poured in testing tubes. Two temperatures, i.e. 12.5°C and 25°C, and four salts, i.e. potassium sulfate (K_2SO_4), sodium bromide (NaBr), magnesium chloride ($MgCl_2$), and lithium chloride (LiCl), were selected to produce five conditions ticks might experience in nature (Table 3.2).

Table 3.2 Detailed parameters of the thermo-hygrometric conditions used in survival tests, measured in the survival arenas (Appendix 9.2) (Winston and Bates, 1960).

Saturated salt solution	K_2SO_4	NaBr	LiCl	NaBr	$MgCl_2$
Temperature (°C)	25	12.5	12.5	25	25
Relative humidity (%)	89	61	13	51.5	32
Saturation deficit (mmHg)	2.57	4.19	9.35	11.33	15.89
Condition	**1**	**2**	**3**	**4**	**5**

3.2.1.3 Survival Arenas

Survival arenas were made of 5-ml plastic culture tubes (Milian, Meyrin, Switzerland) sealed together by silicone on their screw caps. Screw cap centres had previously been removed and replaced by polyamide mesh (500-µm grid, Sefar-Nitex, Sefar AG, Haiden, Switzerland). A piece of blotting paper was put in the culture tube that would later contain ticks.

3.2.1.4 Method

A total of 500 nymphs, 500 males, and 500 females sampled in spring 2009 were tested. Ticks were maintained and rehydrated for four weeks before the start of survival tests. Ticks (10 individuals of the same developmental stage) were placed in the upper culture tube of the survival arenas that stood vertically, while the lower culture tube was filled to its 2/3 with saturated salt solutions producing known RH at a given temperature (see section 3.2.1.2) (Fig. 3.4). Survival tests were conducted in temperature-controlled incubators in the dark. Survival of ticks under the various conditions was recorded after 48 h. Test duration was determined so that global survival would tend to be 50 % based on Perret (2002). When tests were over, ticks were frozen and stored at -80°C until they were analysed for *Borrelia* infection.

Fig. 3.4 Survival arenas used under hot and dry conditions, made of two 5-ml culture tubes sealed together and separated by polyamide mesh (Picture: C. Herrmann).

3.2.2 Humidity Attraction Tests

Humidity attraction tests aimed at determining how humidity attracted *I. ricinus* nymphs that had different hydration and energy levels, and the effect of *Borrelia* infection on tick attraction. Attraction was evaluated depending on tick movement towards or away from humidity.

3.2.2.1 Tick Conditioning

Nymphs were conditioned so that individuals possessed different hydration and energy levels. To obtain high-fat and fully hydrated ticks (group 1, 500 individuals), ticks were held over water in a box with a tight-fitting lid (98 % RH) in the dark within a cold chamber at 4°C for four weeks as described by Crooks and Randolph (2006). Group 2 (500 individuals) contained low-fat and moderately hydrated ticks. These ticks were maintained over water in a transparent plastic box with small holes allowing airflow (87% RH) at room temperature (~23°C). Ticks belonging to group 3 (500 individuals), which were low-fat and fully hydrated, were held over water in a transparent plastic box with a tight-fitting lid (98 % RH) at room temperature (~23°C). Group 2 and 3 ticks were exposed to natural daylight conditions in May–June for four weeks before being returned to the cold chamber until use (Crooks and Randolph, 2006).

3.2.2.2 Fat Content Quantification

Before the start of attraction tests, fat content was quantified in reduced samples (40 individuals) of the different tick conditioning groups to check whether tick conditioning had been efficient and determine how much energy reserves they possessed. Fat content quantification was performed as described in section 3.2.1.1.

3.2.2.3 Choice Arenas

Choice arenas were slightly modified from those described by Crooks and Randolph (2006). They were made of two transparent polystyrene boxes (19 cm x 9 cm x 9 cm) with tight-fitting lids, joined by a transparent polystyrene tunnel (3 cm diameter, 28 cm long) sealed into holes that had been cut in one side of each box (Fig. 3.5).

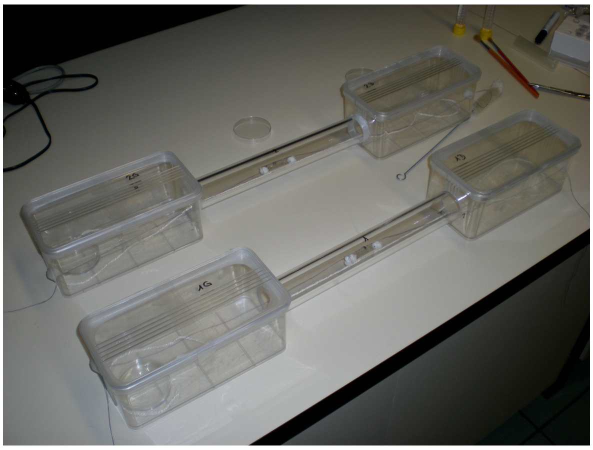

Fig. 3.5 Choice arenas made of two transparent boxes that were joined by a transparent tunnel. A small dish of water was placed in one box while the other remained empty (Picture: C. Herrmann).

Humidity was monitored using an EE07-PFT5 hygrometer (E+E Elektronik, Engerwitzdorf, Austria). The probe was positioned within different parts of the arena in order to study the establishment of a humidity gradient when a small dish of water was placed in one box whereas the other box remained empty (Appendix 9.3). A humidity gradient of 90 to 45 % RH was produced within 30 minutes. The humidity gradient did not evolve much over 3 h (end time of test, see section 3.2.2.4), only reaching 95 to 45 % RH. In the experimental room at ambient temperature (~23°C), this corresponded to SD values ranging from 1.10 mmHg to 10.98 mmHg (1 mmHg=133.3 Pa), as calculated by Randolph and Storey (1999).

3.2.2.4 Method

Each run consisted of a set of 20 nymphs placed into a 1 cm x 5 cm plastic culture tube (Milian, Meyrin, Switzerland) that was closed at both ends by pieces of cotton wool. Each piece of cotton wool was tied to a length of cotton thread. Experimental runs were conducted as described in Crooks and Randolph (2006). One tick-laden tube was positioned in the centre of the arena tunnel with the cotton threads passing through small holes cut into the box side that were closed by laboratory film (Parafilm M, Pechiney Plastic Packaging, Menasha, WI). The arena was left undisturbed for one hour with the lights on. The threads were gently pulled to remove the cotton wool from each end of the tube. The cotton wool pieces were left very close to the tube. Lights were left on for one hour and switched off for one additional hour since, according to Perret et al. (2003), darkness induces *I. ricinus* ticks to move. Ticks were collected two hours post-release and their position was recorded. Testing time was determined so that half of the ticks would walk out of the tube, based on Crooks and Randolph (2006). Humidity side and tick group were changed for every new experimental run. A total of 1500 nymphs sampled in spring 2010 were tested. When tests were over, ticks were frozen and stored at -80°C until they were analysed for *Borrelia* infection.

3.2.3 Energy Reserve Tests

Energy reserve tests aimed at determining how much energy *I. ricinus* ticks possessed, and the effect of *Borrelia* infection on tick energy reserves. Energy reserves were measured by quantifying fat content of nymphs that had been maintained for three months in the laboratory.

3.2.3.1 Method

Fat content quantification was performed as described in section 3.2.1.1. However, a total of 900 nymphs sampled in spring 2011 were tested in this project. In addition, an ultra-microbalance (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland) kept in a room with controlled temperature, atmospheric pressure and light intensity, and weighing samples to the nearest 0.1 µg, was used in these tests. When tests were over, ticks were frozen and stored at -80°C until they were analysed for *Borrelia* infection.

3.2.4 Cold Condition Survival Tests

Survival tests aimed at determining resistance, measured as survival rate, of *I. ricinus* nymphs to unfavourable conditions of temperature (cold), and the effect of *Borrelia* infection on tick survival under these conditions. Tests were designed to study tick survival under extreme temperature values. However, these values were chosen so that they were close to conditions ticks might experience in nature. These tests also aimed at determining whether spring and autumn *I. ricinus* nymphs differed in survival under cold conditions.

3.2.4.1 Fat Content Quantification

Before the start of survival tests, fat content was quantified in reduced samples (40 individuals) of spring and autumn collected nymphs to determine how much energy reserves they possessed. Fat content quantification was performed as described in section 3.2.1.1. However, an ultra-microbalance (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland) kept in a room with controlled temperature, atmospheric pressure and light intensity, and weighing samples to the nearest 0.1 µg was used in this project.

3.2.4.2 Survival Arenas

Survival arenas consisted of glass tubes (6 cm high, 1.5 cm in diameter) sealed by plastic caps with holes (Fig. 3.6). Tubes were kept over water in plastic boxes with tight-fitting lids (98 % RH). A piece of blotting paper was put in each survival arena that contained 50 nymphs.

Fig. 3.6 Survival arenas used under cold and extreme cold conditions, consisting of glass tubes sealed by plastic caps with holes (Picture: C. Herrmann).

3.2.4.3 Method

Survival tests were designed so that they recreated the winter conditions that *I. ricinus* ticks might experience in the field over short and long periods of time in Neuchâtel (located on the Swiss Plateau). Daily air temperature means of lowest and highest values of serial measures collected between 1864 and 2010 were obtained from MétéoSuisse. Data collected in Neuchâtel (47°00' N and 6°57' E, 485 m) from November to February were used to determine the temperature range. During the experiments, nymphs were kept in tubes (40 or 50 individuals per tube) at 98% RH when temperature was above 0°C.

Experiment 1 - Based on MétéoSuisse data, -10°C was chosen as a very cold temperature, with an exposure time of four days. To test whether the frequency of temperature variations had an effect on tick survival under very cold conditions (n = 1760), we maintained 440 spring and 440 autumn nymphs at -10°C (in a freezer) for 4 days (low frequency temperature variations, LF) while additional 440 spring and 440 autumn nymphs were exposed every day to temperature variations (18 h at -10°C, 2 h at 4°C (in a cold chamber), 2 h at 13°C (in a fridge), and 2 h at 4°C) for four days (high frequency temperature variations, HF).

Experiment 2 - Based on MétéoSuisse data and on results obtained in experiment 1, -5°C was chosen as a cold temperature. We tested two frequencies of temperature variations on tick survival. Low frequency temperature variations (LF) involved maintaining nymphs at -5°C (in a freezer) for 9 days followed by 24 h during which they experienced temperature variations (-5°C for 18 h, at 4°C for 2 h, at 13°C for 2 h, and at 4°C for 2 h). This 10-day cycle was repeated until the end of the experiment. High frequency temperature variations (HF) involved exposing nymphs to the same temperature variations but every day, rather than every ten days. In this experiment, 400 spring and 400 autumn nymphs were exposed to HF and additional 400 spring and 400 autumn nymphs were exposed to LF (n = 1600). For each treatment group the time to 50% mortality was determined using a subset of 100 spring and 100 autumn nymphs for each temperature variation frequency. The proportion of ticks surviving was assessed every day for the HF groups and every ten days for the LF groups. The point in the temperature cycle at which survival was assessed was the point after which nymphs had spent 2 h at 13°C after having spent 2h at 4°C. Survival was assessed on mobility of ticks. Any immobile ticks were exposed to human skin and breath at room temperature. If they did not move after a few minutes, they were considered dead. The time at which 50% of ticks within a treatment group had died was recorded, and the ticks from that group were harvested for laboratory analyses. This protocol is termed Experiment 2a in the following. The proportions of ticks surviving in the subset of ticks subject to daily or 10-daily monitoring continued to be assessed until all the ticks had died and in the following this protocol is termed Experiment 2b. All ticks submitted to survival tests at -5°C (experiments 2a and 2b) were frozen at -80°C until they were analysed for *Borrelia* infection.

3.3 *Borrelia* Detection

This part of the projects aimed to determine whether previously tested ticks were infected with *Borrelia* spirochetes using molecular techniques. Molecular analyses were performed on all ticks tested in physiological or behavioural tests (i.e. 5,500 individuals) except those used in survival tests under very cold conditions (Experiment 1). Moreover, an additional 4,860 ticks sampled in spring 2010 (2,250 nymphs, 900 males and 900 females), autumn 2010 (450 nymphs), spring 2011 (80 males and 80 females) and autumn 2011 (200 nymphs) were tested for *Borrelia*.

3.3.1 DNA Isolation

Isolation of tick DNA was achieved by using ammonium hydroxide (NH_4OH) as previously described (Guy and Stanek, 1991; Rijpkema et al., 1996) (Appendix 9.4). Ticks were boiled for 15 min at 100°C in 100 µl of 0.7 M NH_4OH and then cooled for one minute. They were boiled again for 15 to 20 min at 100°C in open tubes to evaporate the ammonia and cooled at room temperature. Negative controls were included during DNA isolation, which consisted of reagents without template DNA. Extracted DNA was stored at -20°C until further use.

3.3.2 Amplification and Quantification

Borrelia spirochetes were detected in ticks used in physiological or behavioural experiments by real-time (quantitative) PCR. Borrelial DNA was amplified and the number of copies was simultaneously quantified.

3.3.2.1 Quantification Standards

The strain NE1817 (*B. afzelii*) was used as quantification standard since Boschung (2006) showed that it was the most appropriate strain. This strain, which had been previously stored at -80°C, was cultured in BSK-H medium as previously described by Sinsky and Piesman (1989). Cultures were incubated in 5 ml tubes at 34°C for 14 days. They were regularly examined by dark-field microscopy. Some of the more abundant and more active cultures were transferred into new 15 ml tubes and fresh BSK-H medium was added. Tubes were incubated at 34°C for 7 to 10 days and regularly examined. This was repeated several times in order to have important volumes of culture. When important numbers of spirochetes had grown within the BSK-H medium (about 10^7 spirochetes per µl), the exact number of spirochetes per millimetre was determined using the Helber chamber. About 10 µl of culture medium was poured into the counting cell. Spirochetes were counted 3 times in 64 diagonally placed squares and the mean was calculated. The concentration of spirochetes per millimetre was calculated with the following formula:

$$\text{spirochetes/ml} = \frac{\text{spirochete number (mean in diagonal)}}{\text{64 (small square number)}} \cdot 2 \cdot 10^7$$

If the number of spirochetes present was close to 10^7 spirochetes per µl, the exact number was recorded and spirochete DNA was extracted from the culture medium according to Postic et al. (1994) (Appendix 9.5). Briefly, the culture was washed twice with phosphate-buffered saline/$MgCl_2$, and the pellet was resuspended in 30 µl of water and heated for 15 min at 100°C (Postic et al., 1994). *Borrelia* DNA stock was aliquoted at 10^5 spirochetes per µl and stored at -20°C. Serial dilutions were made from stored spirochete DNA in order to obtain five standard solutions with concentrations of *Borrelia* DNA of 10^1, 10^2, 10^3, 10^4, and 10^5 copies per µl. Standard solutions were analysed with a NanoDrop ND-1000 spectrophotometer (Witec, Littau, Switzerland) to check whether the concentration was correct.

3.3.2.2 Real-Time PCR

A real-time PCR amplifying a fragment of the flagellin gene (of 132 base pairs) described by Schwaiger et al. (2001) was used to detect and quantify *Borrelia* DNA in previously tested *I. ricinus* ticks (Appendix 9.6). Two primers, forward FlaF1A (AGCAAATTTAGGTGCTTTCCAA) and reverse FlaR1 (GCAATCATTGCCATTGCAGA), and a fluorogenic TaqMan® probe FlaProbe1 (TGCTACAACCTCATCTGTCATTGTAGCATCTTTTATTTG) were used. The fluorescent dyes at the 5' and the 3' ends of the probe were FAM (fluorescein) and TAMRA (tetramethylrhodamine), respectively.

The TaqMan® technology works as follows: as long as the probe is attached, or unattached, to template DNA and the DNA polymerase has not acted yet, the TAMRA fluorophore (quencher) reduces the fluorescence of the FAM fluorophore (reporter). During hybridisation, the probe and primers bind to their specific piece of template DNA. The Taq polymerase starts to add nucleotides to the single DNA strand, removing the probe and hydrolysing it in the process. This annihilates the quencher inhibiting effect on the reporter dye, which begins to emit fluorescence. The resulting fluorescence may thereby be detected and is proportional to the number of amplified DNA templates, allowing the simultaneous quantification of amplified DNA (Fig. 3.7).

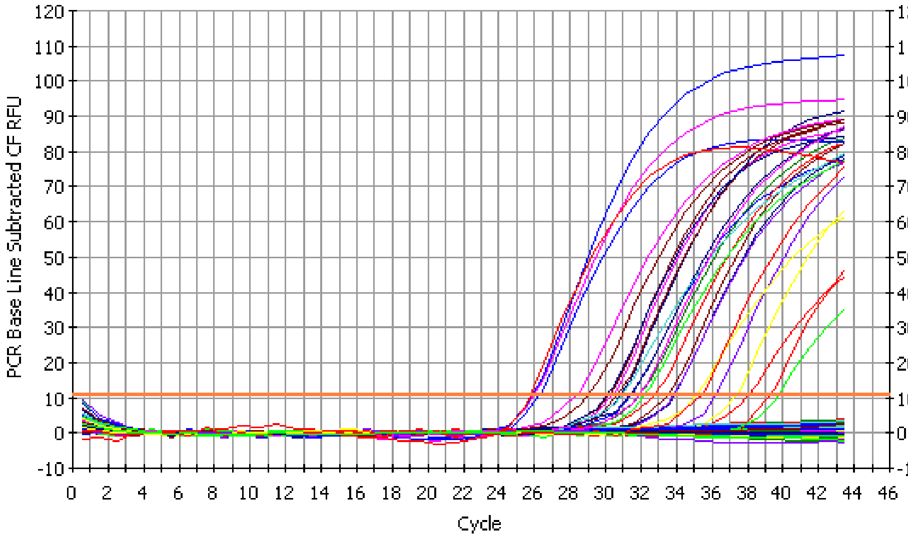

Fig. 3.7 Real-time PCR curves displaying the simultaneous amplification and quantification of DNA.

Instead of positive controls, quantification standards containing known DNA copies of the target gene were added to the PCR run so that fluorescence emitted by samples could be compared to that of standards. The number of DNA copies contained in samples could be determined using a regression line produced with standard curves.

The 50-µl real-time PCR mixture (Schwaiger et al., 2001) consisted of 10 µl of 5x buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl of 20 µM FlaF1A forward primer (Microsynth, Balgach, Switzerland), 1 µl of 20 µM FlaR1 reverse primer (Microsynth, Balgach, Switzerland), 1 µl of 10 µM FlaProbe1 probe (Microsynth, Balgach, Switzerland), 0.25 µl of HotStart Taq Polymerase (Kapa Biosystems, Woburn, MA), 20.75 µl of water and 10 µl of the extracted DNA. In each run, one extraction negative control (10 µl, see above), one PCR negative control (10 µl of water instead of 10 µl of the extracted DNA) and three replicates of five standards were included.

Following an incubation step at 95°C for 10 min, the samples were submitted to 45 repeated amplification cycles (95°C for 15 s, 60°C for 1 min) (Schwaiger et al., 2001) in an iCycler Optical Module (Bio-Rad, Reinach, Switzerland) using strip PCR tubes and flat caps (Scientific Specialties Inc, Lodi, CA).

3.3.3 Genospecies Determination

To identify *Borrelia* genospecies present in ticks, samples that were detected positive by real-time PCR were additionally analysed by touchdown PCR followed by reverse line blotting (see section 3.3.3.1 and 3.3.3.2).

3.3.3.1 Touchdown PCR

A touchdown PCR amplifying the variable spacer region between two repeated copies of the 23S and 5S ribosomal genes (of about 410 base pairs) (Fig. 3.8) described by Alekseev et al. (2001) was used (Appendix 9.7). The high variability of that region and the high stability of its flanking regions allowed a precise identification of species belonging to the *B. burgdorferi* s.l. complex (Postic et al., 1994) and of *B. miyamotoi* (Gern et al., 2010), a *Borrelia* species closely related to relapsing fever spirochetes. Two primers, forward 23S-Bor (TCAGGGTACTTAGATGGTTCACTT) and reverse B5S-Bor (GAGTTCGCGGGAGAGTAGGTTATT) were used. Biotin was added to the B5S-Bor primer 5' end. This primer modification was necessary to use it in reverse line blotting.

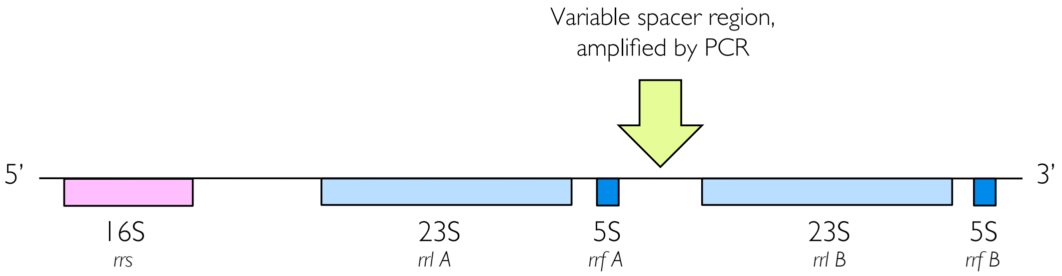

Fig. 3.8 Representation of the variable spacer region, located between gene 5S (*rrf A*) and gene 23S (*rrl B*), and its surrounding genes (Morán Cadenas, 2007, modified).

The 25-µl PCR mixture (Alekseev et al., 2001; Burri et al., 2007) consisted of 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM 23S-Bor forward primer (Microsynth, Balgach, Switzerland), 0.5 µl of 10 µM B5S-Bor reverse primer (Microsynth, Balgach, Switzerland), 0.125 µl of Taq Polymerase (Qiagen, Basel, Switzerland), 15.875 µl of water and 5 µl of the extracted DNA. Positive and negative controls were included in each PCR. In positive controls, isolates of *B. burgdorferi* s.s. (B31), *B. afzelii* (NE632), *B. garinii* (NE11), *B. lusitaniae* (PotiB1), or *B. valaisiana* (VS116), replaced DNA samples, whereas water substituted them in negative controls.

PCR amplifications were run in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) using a touchdown PCR program (Burri et al., 2007). Briefly, amplification started with an initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. During subsequent cycles, annealing was lowered by 1°C until it reached 52°C. Another 40 repeated amplification cycles (94°C for 20 s, 52°C for 30 s, 72°C for 30 s) followed the touchdown program. The PCR ended with a final extension at 72°C for 7 min.

3.3.3.2 Reverse Line Blot

The Reverse Line Blot (RLB) was used to identify *Borrelia* genospecies. This method consists of hybridising samples (previously amplified by PCR) with specific oligonucleotide probes covalently bound to a negatively charged Biodyne C (nylon) membrane. This phenomenon may then be visualised through the action of a peroxidase producing chemiluminescence, coupled to streptavidin interacting with the biotin contained in PCR products (Fig. 3.9) (Appendix 9.8, 9.9, and 9.10).

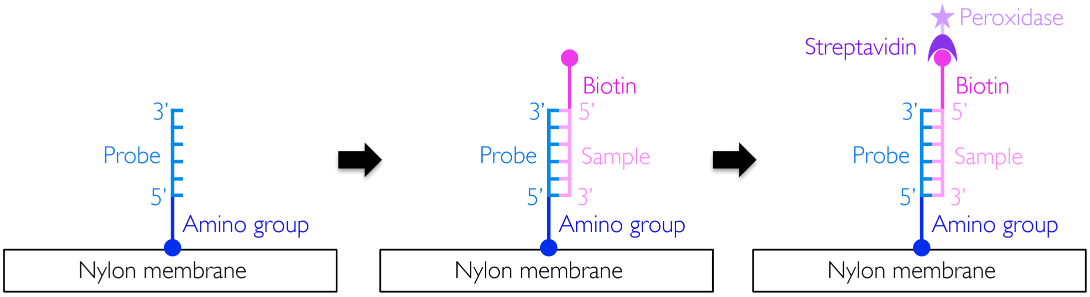

Fig. 3.9 Mechanism of Reverse Line Blotting: covalent coupling of probes to a nylon membrane, followed by hybridisation of DNA samples. The latter is visualised by chemiluminescence produced by a peroxidase linked to streptavidin, which interacts with biotin contained in DNA samples (Morán Cadenas, 2007, modified).

The first step of RLB was to activate the Biodyne C membrane (Pall Europe Ltd., Portsmouth, UK) by coupling covalently to it oligonucleotide probes of the target organism. Here, 17 oligonucleotide probes corresponding to different *Borrelia* genospecies were chosen: 3 generic probes, hybridising to all *B. burgdorferi* s.l. genospecies (SL1, SL2 and SL3) and 14 specific probes hybridising to one or two genospecies only (AF, BisNE, BisNE1, GA, GANE, GANE1, LusiNE, LusiNE1, LusiNE2, RFLNE, SpiNE, SpiNE1, SS, VSNE) (Table 3.3) (Rijpkema et al., 1995; Poupon et al., 2006; Gern et al., 2010). An amino group was added to the 5' end of all oligonucleotide probes so that they would bind covalently to the nylon membrane when applied on it.

Some oligonucleotide probes were diluted to 75 pmol/µl (AF, GA, GANE, LusiNE, SL1, SL2, SL3, SS, VSNE) and other to 100 pmol/µl (BisNE1, BisNE2, GANE1, LusiNE1, LusiNE2, RFLNE, SpiNE2, SpiNE3) in 150 µl of 500 mM $NaHCO_3$ pH 8.4. The Biodyne C membrane was cut at the appropriate size and rolled in a glass bottle. It was activated by a 10 min incubation in 10 ml of freshly prepared 16 % (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC) at room temperature in a rolling bottle. The membrane was rinsed with 10 ml of water and placed in a Miniblotter 45 (Immunetics, Cambridge, MA, USA). Residual water was removed from slots by aspiration. The slots of the Miniblotter, except for the first and the last one, were filled with 150 µl of diluted oligonucleotide mixture. The first and the last slots were used to mark the area where the oligonucleotide probes were applied by filling these with drawing pen ink diluted 1:100 in water. The Miniblotter was incubated for 1 min at room temperature. Oligonucleotide solutions were removed by aspiration and the membrane was inactivated by incubation in 100 ml of 100 mM NaOH for 10 min in a rolling bottle at room temperature. The membrane was rinsed with water and washed in 100 ml of 2x SSPE / 0.1 % SDS for 5 min at 60°C. At this point, the membrane was ready for use. If stored, the membrane was previously washed in 100 ml of 20 mM EDTA pH 8 for 15 min at room temperature, sealed in a plastic wrap and kept at 4°C until further use.

Table 3.3 Details on probes used to identify *Borrelia* genospecies in RLB (Rijpkema et al., 1995; Poupon et al., 2006; Gern et al., 2010). Generic probes are in bold whereas specific probes are in plain text.

Probe	Sequence (5'-3')	Target genospecies	Cross-hybridisation
SL1	**CTTTGACCATATTTTTATCTTCCA**	***B. burgdorferi*** **s.l.**	**-**
SL2	**CTTCCATCTCTATTTAGCCAATTT**	***B. burgdorferi*** **s.l.**	**-**
SL3	**TATTTTTATCTTCCATCTCTATTTT**	***B. burgdorferi*** **s.l.**	**-**
AF	AACATTTAAAAAATAAATTCAAGG	*B. afzelii*	-
BisNE1	AAACACTAACATTTAAAAAACAT	*B. bissettii*	-
BisNE2	AACTAACAAACATTTAAAAAACAT	*B. bissettii*	-
GA	AACATGAACATCTAAAAACATAAA	*B. garinii*	-
GANE	CAAAAACATAAATATCTAAAAACATAA	*B. garinii*	-
GANE1	AAAATCAATGTTTAAAGTATAAAAT	*B. garinii*	*B. burgdorferi* s.s.
LusiNE	TCAAGATTTGAAGTATAAAATAAAA	*B. lusitaniae*	*B. afzelii*
LusiNE1	CATTCAAAAAAATAAACATTTAAAAACAT	*B. lusitaniae*	*B. garinii*
LusiNE2	AAATCAAACATTCAAAAAAATAAAC	*B. lusitaniae*	-
RFLNE	CTATCCATTGATCAATGC	*B. miyamotoi*	-
SpiNE2	GAATGGTTTATTCAAATAACATA	*B. spielmanii*	-
SpiNE3	GAATAAGCCATTTAAATAACATA	*B. spielmanii*	-
SS	AACACCAATATTTAAAAAACATAA	*B. burgdorferi* s.s.	-
VSNE	TATATCTTTTGTTCAATCCATGT	*B. valaisiana*	-

The second step of RLB was to apply samples perpendicularly on the probes covalently hybridised to the nylon membrane. Ten µl of PCR products were diluted in 150 µl of 2x SSPE / 0.1 % SDS, heat-denatured for 10 min at 99°C, and cooled on ice immediately. The membrane was incubated in 100 ml of 2x SSPE / 0.1 % SDS for 5 min at room temperature. It was then placed on a support cushion (Immunetics, Cambridge, MA, USA) in the Miniblotter 45 with slots perpendicular to the line pattern of the applied nucleotides. Residual liquid was removed from the slots by aspiration. Slots were filled with 150 µl of denatured PCR products. An identical amount of 2x SSPE / 0.1 % SDS was applied to empty slots to prevent cross-flow. The Miniblotter 45 was incubated on a plane surface for 60 min at 42°C to maximize the hydridisation of denatured PCR products to specific oligonucleotide probes. PCR products were aspirated, and the membrane was washed twice in 100 ml of 2x SSPE / 0.5 % SDS for

10 min at 51°C in order to remove remaining unhybridised PCR products. The membrane was incubated with 2.5 µl of 1:4000 diluted peroxidase-labelled streptavidin conjugate (Roche, Basel, Switzerland) in 10 ml of 2x SSPE / 0.5 % SDS for 30 min in a rolling bottle at 42°C. It was then washed twice in 100 ml of 2x SSPE / 0.5 % SDS for 10 min at 42°C, and rinsed twice in 100 ml 2x SSPE for 5 min at room temperature to remove the excess of streptavidin conjugate that did not bind to the biotinated PCR products. The membrane was incubated for 1 min in 6 ml of enhanced chemiluminescence detection liquid (Amersham Biosciences Europe, Switzerland) (3 ml of each reagent), which produced chemiluminescence with the action of the peroxidase. An X-ray film (Hyperfilm, GE Healthcare, UK) was exposed to the membrane for 15 min to visualise the results.

The last step of RLB was to strip hybridised samples from the membrane so that the membrane could be reused. Hybridised PCR products were stripped from the membrane by two washes in 100 ml of 1 % SDS for 30 min at 80°C, followed by one wash in 100 ml of 20 mM EDTA pH 8 for 15 min at room temperature. The membrane was sealed in a plastic wrap and stored at 4°C until reuse.

3.3.3.3 Sequencing

Some samples that had been detected positive by real-time PCR could not be defined to genospecies level by RLB. These samples were purified using Wizard Plus Minipreps DNA Purification Systems (Promega, Madison, WI, USA) according to Promega instructions for use (Appendix 9.11) and sent for sequencing (one way only, primer 23S-Bor) to Microsynth (Balgach, Switzerland). Sequencing results were blasted on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find to what *Borrelia* genospecies corresponded the newly received sequence.

3.4 Statistical Analyses

Details on tests used for the different projects may be found in the results section. All statistics were calculated with R for Mac OS X (R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

4 RESULTS

4 RESULTS

4.1 Paper 1

Herrmann, C., and L. Gern. 2010. Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection. Journal of Medical Entomology 47: 1196-1204

4.2 Paper 2

Herrmann, C., and L. Gern. 2012. Do level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks? Parasitology 139: 330-337

4.3 Paper 3

Herrmann, C., M. J. Voordouw, and L. Gern. 2013. *Ixodes ricinus* ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves. International Journal for Parasitology 43: 477-483

4.4 Paper 4

Herrmann, C., and L. Gern. 2013. Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations. Ticks and Tick-Borne Diseases 4: 445-451

4.5 Paper 5

Herrmann, C., L. Gern, and M. J. Voordouw. Submitted. Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus.*

4.1 Paper 1

Survival of *Ixodes ricinus* (Acari: Ixodidae) under challenging conditions of temperature and humidity is influenced by *Borrelia burgdorferi* sensu lato infection. 2010. Journal of Medical Entomology 47: 1196-1204

Coralie Herrmann and Lise Gern

An unexpected result from burning vegetation to destroy *I. dammini* (later known as *I. scapularis*) habitat in a North American forest was observed by Mather et al. (1993) two decades ago. Instead of reducing *I. dammini* prevalence and thereby the risk for human infection with Lyme disease in the burnt area, the fire reduced the abundance of *I. dammini* nymphs by half but not the risk of encountering ticks infected with *B. burgdorferi*. In fact, the risk of being infected remained identical in the burnt and the unburnt area since *Borrelia* prevalence was twice as high in nymphs collected from the devastated section of the woodlot than the undamaged section. The higher death rate among the uninfected subpopulation of *I. dammini* remained unexplained. Less than a decade later, another intriguing phenomenon was observed in a forest above Neuchâtel, where *I. ricinus* density and infection with *B. burgdorferi* had been monitored for years. While *I. ricinus* density was exceptionally low in 1998 (a year with particularly high saturation deficit values in Switzerland) (Perret et al., 2000), unexpectedly high numbers of *Borrelia*-infected nymphs harboured high numbers of spirochetes compared to the previous and following years (Lise Gern, personal communication). These two unrelated observations suggested that *I. dammini* and *I. ricinus* ticks harbouring *B. burgdorferi* spirochetes might survive better under desiccating conditions in nature. In the context of climate change where tick habitat gets increasingly drier and hotter, it appeared crucial to determine if there was a difference in survival between *Borrelia*-infected and uninfected ticks. In this study, we therefore tested whether *I. ricinus* ticks infected with *Borrelia* survived differently from *Borrelia*-free ticks under hot and dry conditions produced in the laboratory.

Survival of *Ixodes ricinus* (Acari: Ixodidae) Under Challenging Conditions of Temperature and Humidity Is Influenced by *Borrelia burgdorferi* sensu lato Infection

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J. Med. Entomol. 47(6): 1196–1204 (2010); DOI: 10.1603/ME10111

ABSTRACT To determine whether *Borrelia burgdorferi* sensu lato (s.l.) influences tick survival under thermohygrometric stress, *Ixodes ricinus* (L.) (Acari: Ixodidae) questing ticks were tested under various relative humidities (13, 32, 51.5, 61, and 89% RH) at two different temperatures (12.5 and 25°C) and investigated for *Borrelia* infection. Survival rate of females was highest (77.6%), followed by males (51.6%), and nymphs (43.2%). The thermohygrometric factor that most importantly determined survival was saturation deficit (SD). As SD increased, tick survival rate decreased in all stages. Among the 1,500 ticks tested for *B. burgdorferi* s.l., 34.8% ($n = 522$) were infected. Adult infection rate (39.6%) was higher than that of nymphs (25.5%). Infection load in real-time polymerase chain reaction ranged from 1 to 1.2 million spirochetes per tick. *B. afzelii* (39.7%), *B. burgdorferi* sensu stricto (12.1%), *B. garinii* (37.9%), *B. myamotoi* (3.6%), and *B. valaisiana* (23.8%) were recorded. *B. garinii* infected significantly less nymphs than adults whereas *B. afzelii* displayed the opposite trend. Survival rate of nymphal and adult *I. ricinus* was significantly enhanced by infection by *B. burgdorferi* s.l. (χ^2: nymph, $P = 0.008$; adult, $P = 0.021$). In adults, a negative effect of infection on tick survival was observed when spirochete load overcame a threshold estimated at 160,000 spirochetes per tick but not in nymphs. Moreover, ticks infected by *B. afzelii* survived better than other ticks (infected by other genospecies or not). The results here indicate that infection by *B. burgdorferi* s.l., and more specifically infection by *B. afzelii*, confers survival advantages to *I. ricinus* under challenging thermohygrometric conditions.

KEY WORDS Lyme disease, *Ixodes ricinus*, climate change, tick survival, Switzerland

The hard-bodied tick *Ixodes ricinus* (L.) (Acari: Ixodidae) is the main vector of *Borrelia burgdorferi* sensu lato (s.l.), the etiological agent of the most common tick-borne disease in Europe, Lyme borreliosis. Ten *Borrelia* genospecies have been described in *I. ricinus* in Europe: *B. afzelii*, *B. bavariensis*, *B. bissettii*, *B. burgdorferi* sensu stricto (s.s.), *B. carolinensis*, *B. garinii*, *B. lusitaniae*, *B. spielmanii*, *B. valaisiana* as well as a *Borrelia* species related to relapsing fever spirochetes, *B. myamotoi* (Rauter and Hartung 2005, Margos et al. 2009, Gern et al. 2010, Cotté et al. 2010). Both *B. burgdorferi* s.l. and ticks of the *I. ricinus* complex have been thoroughly studied on their own, but knowledge on the influence of infection by *Borrelia* on ticks remains scarce.

The effect of infection by *B. burgdorferi* s.l. on the behavior of *Ixodes* ticks has been documented. Hence, it was observed that infection by *B. burgdorferi* s.l. in *I. persulcatus* and *I. ricinus* ticks reduced motor activity of both adult and immature infected individuals compared with uninfected ticks (Alekseev et al. 2000). Moreover, Lefcort and Durden (1996) reported that infection by *B. burgdorferi* s.l. influenced *I. scapularis* phototaxis, activity and questing height. Infected adults were less able to overcome physical obstacles, avoided vertical surfaces, were less active and quested at lower heights, whereas infected nymphs showed increased phototaxis and attraction to vertical surfaces. It also was observed that infection by *B. burgdorferi* s.l. increased questing activity in *Ixodes persulcatus* Schulze (Naumov 1999 cited by Uspensky et al. 2006) and host-finding efficacy in adult *I. ricinus* (Faulde and Robbins 2008).

In contrast, reports on the effect of parasites on the physiology of ticks are rare. Mather et al. (1993) observed a higher survival rate of *Ixodes scapularis* Say nymphs infected by *B. burgdorferi* s.l. than uninfected nymphs after habitat burning, suggesting that infection by *B. burgdorferi* s.l. makes ticks more resistant to heat and desiccation. Furthermore, in 1998 in Neuchâtel, Switzerland, a lower density of questing *I. ricinus* ticks was observed due to high desiccating climatic conditions (Perret et al. 2000) and an unexpectedly high percentage of nymphs harboring high numbers of *B. burgdorferi* s.l. was recorded compared with the previous and following

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0022-2585/10/1196–1204$04.00/0

years (L.G., unpublished data). In addition, Naumov (2003) (cited by Uspensky et al. 2006) noted that there was a trend toward an increase in the longevity of adult *I. ricinus* and *I. persulcatus* infected by *B. burgdorferi* s.l. spirochetes.

Similarly to other terrestrial arthropods, the biggest challenge for ticks is to maintain water balance while living in a relatively dry environment. This is even more crucial for *I. ricinus* survival because this tick is exceptionally sensitive to temperature and humidity compared with other ticks (Knülle and Rudolph 1982, Sonenshine 1991). Hence, combined with temperature, humidity is the most important limiting factor in *I. ricinus* survival and activity because the different developmental stages are known to display little resistance to desiccation (MacLeod 1935, Lees 1946, Aeschlimann 1972). In fact, relative humidity needs to be >70–80% to allow questing tick activity and survival (MacLeod 1935). More precisely, saturation deficit (SD), a measure of the drying power of the atmosphere depending on both temperature and relative humdity (Randolph and Storey 1999), limits duration of questing (Perret et al. 2003, 2004) and survival of *I. ricinus* in nature (Perret 2002, Perret et al. 2000, 2004; Burri et al. 2007).

In the context of climate change and with observations of increasing values and longer lasting periods of high saturation deficits, as for example in the Neuchâtel region (Perret et al. 2000, Morán Cadenas et al. 2007b), it seemed necessary to study whether infection by *B. burgdorferi* s.l. may influence *I. ricinus* survival under thermohygrometric stress conditions.

Materials and Methods

Sampling of Questing Ticks. The sampling site was a mixed forest (deciduous dominant) situated at 600 m above sea level (47° 00′N, 6° 57′E) on the south-facing slope of Chaumont Mountain (Neuchâtel, Switzerland). Host-seeking ticks were sampled by dragging a 1-m^2 terry flag over the low vegetation. The flag was examined for ticks every 10 m. Questing nymphs and adults were collected on two different days in May 2009. They were held over water (98% RH) at 4°C in the dark for rehydration for 4–6 wk (depending on testing day) as described in Crooks and Randolph (2006).

Fat Content Analysis. Fat content was quantified in field-collected and laboratory-reared ticks to take into account variable energy resources when assessing survival rate of *I. ricinus* under unfavorable thermohygrometric conditions. Samples of field-collected nymphs, males, and females were analyzed for their fat content as described in Randolph and Storey (1999) and as calculated in Crooks and Randolph (2006). As controls for nymphs, laboratory-reared nymphs from the colony at the University of Neuchâtel known to be free of spirochetal infection also were analyzed for their fat content. This colony is maintained at room temperature and natural light conditions.

Survival Tests. Survival arenas were made of 5-ml plastic culture tubes (Milian, Meyrin, Switzerland) sealed together by their screw caps with silicone. Screw cap centers had been removed previously and replaced by polyamide mesh (500-μm grid, Sefar-Nitex, Sefar AG, Haiden, Switzerland). Arenas stood vertically, with the top culture tube containing ticks and the bottom tube filled two thirds with saturated salt solutions (K_2SO_4, LiCl, $MgCl_2$, or NaBr), producing known relative humditities at a given temperature based on Winston and Bates (1960). They were adjusted after 48-h tests conducted under experimental conditions.

Tick survival was studied under five thermohygrometric conditions: 25°C, 89% RH; 12.5°C, 61% RH; 12.5°C, 13% RH; 25°C, 51.5% RH; and 25°C, 32% RH, corresponding to saturation deficits of 2.57, 4.19, 9.35, 11.33, and 15.89 mmHg (1 mmHg = 133.3 Pa), respectively, as calculated by Randolph and Storey (1999). Survival tests were conducted in the dark. Survival of ticks was recorded after 48 h. This duration was determined so that global survival would tend to be 50% based on Perret (2002). All ticks, dead or alive, were then stored at −80°C until analysis for *Borrelia* infection by real-time polymerase chain reaction (PCR) and reverse line blotting (RLB).

***Borrelia* Infection in Ticks.** Before DNA isolation, ticks were soaked in 70% ethanol and air-dried. Extraction of DNA from ticks was achieved using ammonium hydroxide as described previously (Guy and Stanek 1991, Rijpkema et al. 1996). Negative controls were included during DNA isolation, which consisted of reagents without template DNA.

A real-time PCR amplifying a fragment of the flagellin gene (Schwaiger et al. 2001) was used to detect and quantify *Borrelia* DNA in all field-collected ticks that were subjected to the survival tests. The strain *B. afzelii* NE1817 was used as quantification standard. Spirochete concentration in culture was evaluated using the Helber chamber. To extract DNA, the culture was washed twice with phosphate-buffered saline/$MgCl_2$, and the pellet was resuspended in 30 μl of water and heated for 15 min at 100°C (Postic et al. 1994). The *Borrelia* DNA stock was aliquoted at 10^5 spirochetes per μl and stored at −20°C. Serial dilutions were made from stored spirochete DNA to obtain six standard solutions with concentrations of *Borrelia* DNA ranging from 1 to 10^5 copies per μl.

The 50-μl real-time PCR mixture (Schwaiger et al. 2001) consisted of 5 μl of 10× $MgCl_2$ buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 1 μl of 20 μM FlaF1A forward primer, 1 μl of 20 μM FlaR1 reverse primer, 1 μl of 10 μM FlaProbe1 probe, 0.25 μl of HotStar *Taq* Polymerase (QIAGEN, Basel, Switzerland), 28.75 μl of water, and 10 μl of the extracted DNA. In each run, one extraction negative control (10 μl; see above), one PCR negative control (10 μl of water instead of 10 μl of the extracted DNA), and three series of the six standards were included.

After an incubation step at 95°C for 10 min, the samples were submitted to 45 repeated amplification cycles (95°C for 15 s, 60°C for 1 min) (Schwaiger et al. 2001) in an iCycler Optical Module (Bio-Rad Laboratories, Reinach, Switzerland) by using strip optical

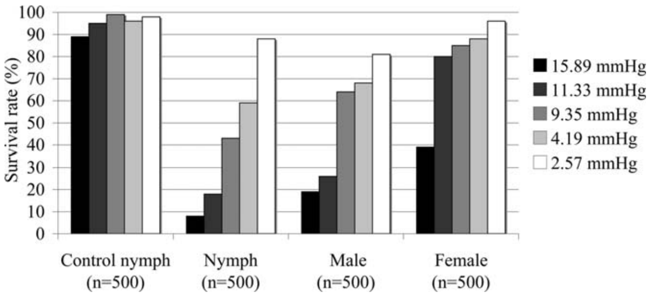

Fig. 1. *I. ricinus* survival rate after 48 h spent under thermohygrometric conditions corresponding to values of saturation deficit ranging from 2.57 to 15.89 mmHg.

tubes and caps Type F (Life Systems Design, Merenschwand, Switzerland).

PCR and RLB were used to identify the *Borrelia* species infecting the field-collected ticks. The variable spacer region between two repeated copies of the 23S and 5S ribosomal genes was amplified with primers 23S-Bor and B-5S-Bor (Alekseev et al. 2001). PCR amplifications were run in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touchdown PCR program (Burri et al. 2007). Positive and negative controls were included in each PCR. In positive controls, isolates of *B. afzelii* (NE632), *B. burgdorferi* s.s. (B31), and *B. valaisiana* (VS116) replaced DNA samples, whereas water was substituted in negative controls.

For *Borrelia* identification by RLB, PCR products were hybridized to 15 oligonucleotide probes (Rijpkema et al. 1995, Poupon et al. 2006, Gern et al. 2010) blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd., Portsmouth, United Kingdom) using a Miniblotter 45 (Immunetic, Cambridge, MA). Hybridization was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham Biosciences Europe, Basel, Switzerland) and by exposing the membrane to X-ray film (Hyperfilm, GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Statistical Analysis. All statistics were calculated with SPSS Statistics 17.0 for Macintosh (SPSS Inc, Chicago, IL). The relationships between fat content in field-collected nymphs and laboratory-reared nymphs between infection loads in field-collected nymphs, males and females, and between *Borrelia* genospecies and infection load were evaluated with the Mann–Whitney test. The χ^2 test on contingency table was used for assessing the influence of life stages on survival, infection, distribution of *Borrelia* genospecies, and distribution of infection load categories. Influence of the infection, infection load category, and *Borrelia* genospecies on survival was assessed by the χ^2 test on contingency table. Influence of the thermohygrometric factors and infection load on survival was estimated by binary logistic regressions.

Results

Fat Content Analysis. Field-collected nymphs contained less fat (8.4%) than field-collected males (16.2%; Mann–Whitney test, $P = 0$) or females (13.3%; Mann–Whitney test, $P = 0.004$). In contrast, field-collected males and females did not differ in lipid content. Laboratory-reared nymphs (control) (14.5%) contained more fat than field-collected nymphs (8.4%) (Mann–Whitney test, $P = 0$).

Survival Tests. Globally, there were differences in survival rates among tick life stages under all five tested conditions. Laboratory-reared nymphs were weakly affected by challenging thermohygrometric conditions showing a survival rate of 95.4% (477/500). Among field-collected ticks, females displayed the highest survival rate (77.6%; 388/500), followed by males (51.6%; 258/500) and nymphs (43.2%; 216/500).

Temperature and relative humidity had a significant impact on field-collected ticks (binary logistic regression, temperature: nymph, $P = 0$; male, $P = 0$; female, $P = 0$ and binary logistic regression, relative humidity: nymph, $P = 0$; male, $P = 0$; female, $P = 0$). In contrast, temperature had no significant influence and relative humidity a light effect on laboratory-reared nymphs (binary logistic regression, $P = 0.119$ and $P = 0.027$, respectively). More ticks survived when RH increased and temperature decreased.

SD strongly affected field-collected and laboratory-reared ticks (binary logistic regression: control, $P = 0.007$; nymph, $P = 0$; male, $P = 0$; female, $P = 0$) (Fig. 1). Nymphs seemed to suffer from SD at a rate that was proportional to SD increase, whereas in adults some kind of threshold (≈12 mmHg in males and 15 mmHg in females) was observed beyond which survival rate decreased rapidly.

***Borrelia* Infection in Ticks.** Among the 1,500 field-collected ticks that were tested for *B. burgdorferi* s.l. by real-time PCR, 34.8% ($n = 522$) revealed to be infected: 8.4% ($n = 126$) were nymphs, 13.2% ($n = 198$) males, and 13.2% ($n = 198$) females. Male (39.6%; 198/500) and female ticks (39.6%; 198/500) were more frequently infected than nymphs (25.5%; 126/500) (χ^2 test, $P = 0.001$). Globally, infection load in these ticks ranged from 1 to 1.2 million spirochetes per tick. Mean spirochete number was highest in females (52,400 spirochetes per tick), followed by males (47,600 spi-

Table 1. Distribution of *Borrelia* genospecies in field-collected *I. ricinus* ticks

Borrelia species[a]	Nymph[b]	Male[b]	Female[b]	Total[c]
af	71 (14.2)	52 (10.4)	45 (9)	168 (11.2)
ga	19 (3.8)	59 (11.8)	69 (13.8)	147 (9.8)
my	3 (0.6)			3 (0.2)
ss	6 (1.2)	13 (2.6)	18 (3.6)	37 (2.5)
va	11 (2.2)	34 (6.8)	33 (6.6)	78 (5.2)
One species	110 (22)	158 (31.6)	165 (33)	433 (28.9)
af & ga		3 (0.6)	1 (0.2)	4 (0.3)
af & my	2 (0.4)	4 (0.8)	4 (0.8)	10 (0.7)
af & ss	2 (0.4)	10 (2)	8 (1.6)	20 (1.3)
ga & my	1 (0.2)	2 (0.4)		3 (0.2)
ga & vs	8 (1.6)	15 (3)	17 (3.4)	40 (2.7)
ss & ga			1 (0.2)	1 (0.1)
ss & vs		1 (0.2)	1 (0.2)	2 (0.1)
Two species	13 (2.6)	35 (7)	32 (6.4)	80 (16)
af & ga & vs		2 (0.4)		2 (0.1)
af & my & ss		1 (0.2)	1 (0.2)	2 (0.1)
af & ss & vs		1 (0.2)		1 (0.1)
ga & my & vs	1 (0.2)			1 (0.1)
Three species	1 (0.2)	4 (0.8)	1 (0.2)	6 (0.4)

[a] af, *B. afzelii;* ga, *B. garinii;* my, *B. myamotoi;* ss, *B. burgdorferi* sensu stricto; va, *B. valaisiana.* Values in parentheses are percentages.

[b] $n = 500$.

[c] $n = 1{,}500$.

rochetes per tick) and nymphs (18,640 spirochetes per tick). However, the differences were not significant (Mann–Whitney test: nymph-male, $P = 0.632$; nymph-female, $P = 0.823$; male-female, $P = 0.560$).

To compare the infection loads between adults and nymphs, five infection load categories were arbitrarily chosen: very low (<180 spirochetes per tick), low (181–2,100 spirochetes per tick), medium (2,101–16,000 spirochetes per tick), high (16,001–160,000 spirochetes per tick), and very high (>160,000 spirochetes per tick). Infections in adults fell more often into the high and very high infection load categories (male: 26.8%, 53/198 and female: 29.3%, 58/198) than infections in nymphs (16.7%; 21/126). In fact, the distribution of infection categories was different between adults and nymphs but not between males and females (χ^2 test: nymph-male, $P = 0.003$; nymph-female, $P = 0.001$; male-female, $P = 0.928$).

Identification of *Borrelia* Species in Ticks. Identification of *Borrelia* genospecies by RLB was possible in 519/522 ticks. Globally, 84% (433/519) of these ticks were infected by one *Borrelia* species, 15% (80/519) by two species and 1% (6/519) by three species (Table 1). Mixed infections were more frequently observed in adults (male: 19.7%, 39/198; female: 16.7%, 33/198) than in nymphs (11.1%, 14/126).

Five *Borrelia* species—*B. afzelii, B. burgdorferi* s.s., *B. garinii, B. myamotoi,* and *B. valaisiana*—were identified by RLB (Table 1). Taking into account single and mixed infections, *B. afzelii* was the most frequent genospecies ($n = 207$; 39.7%), followed by *B. garinii* ($n = 198$; 37.9%), *B. valaisiana* ($n = 124$; 23.8%), whereas *B. burgdorferi* s.s. was less frequent ($n = 63$; 12.1%). The genospecies that was the least often detected was *B. myamotoi* ($n = 19$; 3.6%). *B. bissettii, B. lusitaniae,* and *B. spielmanii,* were not observed.

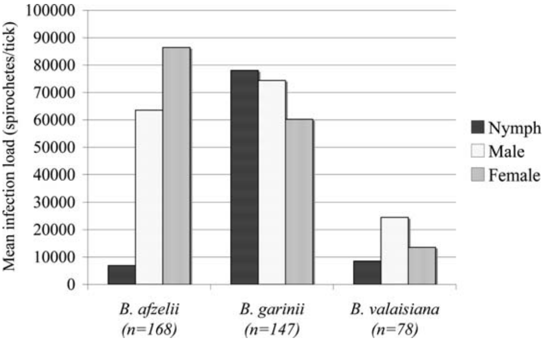

Fig. 2. Mean spirochete load (spirochetes per tick) in nymphal, male and female *I. ricinus* infected by *B. afzelii*, *B. garinii*, and *B. valaisiana*.

There was no difference in the distribution of *B. afzelii, B. garinii,* and *B. valaisiana* between males and females (χ^2 test: *B. garinii,* $P = 0.416$; *B. afzelii,* $P = 0.136$; *B. valaisiana,* $P = 0.819$). However, important dissimilarities between nymphs and adults were observed in the frequency of some *Borrelia* genospecies. In fact, *B. garinii* and *B. valaisiana* were more frequent in adults than in nymphs (χ^2 test: nymph-male, $P = 0$; nymph-female, $P = 0.001$; nymph-male, $P = 0.036$; and nymph-female, $P = 0.022$, respectively), whereas the contrary was observed for *B. afzelii* (χ^2 test: nymph-male, $P = 0$; nymph-female, $P = 0$).

Differences in the infection load were found between tick stages according to *Borrelia* species (Fig. 2). In nymphs, infections by *B. garinii* consisted of more spirochetes (78,000 spirochetes per tick) than infections by all other *Borrelia* species (7,680 spirochetes per tick) (Mann–Whitney test, $P = 0.001$), whereas this difference was less marked in males (74,400 and 42,800 spirochetes per tick, respectively; Mann–Whitney test, $P = 0.028$) and in females (60,400 and 52,400 spirochetes per tick, respectively; Mann–Whitney test, $P = 0.059$). In adults, infections by *B. valaisiana* showed less spirochetes (male: 24,520 spirochetes per tick; female: 13,560 spirochetes per tick) than infections by all other *Borrelia* species (male: 62,800 spirochetes per tick; female: 66,400 spirochetes per tick) (Mann–Whitney test: male, $P = 0.007$; female, $P = 0.005$). In nymphs, infections by *B. valaisiana* also displayed less spirochetes than all other *Borrelia* species, but the difference was not significant (8,520 and 20,880 spirochetes per tick, respectively) (Mann–Whitney test, $P = 0.864$). Finally, it seemed that nymphs infected by *B. afzelii* (6,880 spirochetes per tick) bore less spirochetes than nymphs infected by other genospecies (41,600 spirochetes per tick) (Mann–Whitney test, $P = 0.025$). Interestingly, the reverse trend was observed in adults, although the difference was not significant (male: 63,600 and 50,400 spirochetes per tick, respectively; female: 86,400 and 44,400 spirochetes per tick, respectively) (Mann–Whitney test: male, $P = 0.368$; female, $P = 0.176$).

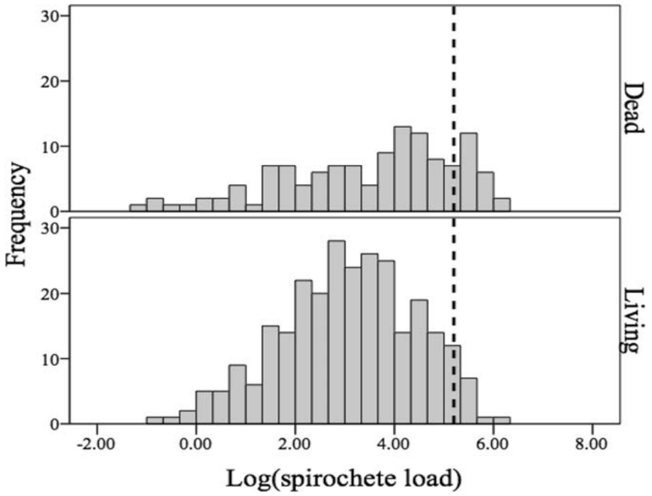

Fig. 3. Distribution of spirochete load (log[spirochetes per tick]) among dead and living *I. ricinus* adults. The dashed line lies at 160,000 spirochetes per tick.

Effect of *Borrelia* Infection on Tick Survival. Infection load had an effect on adult survival (binary logistic regression, $P = 0.031$) but had no effect on survival of nymphs (binary logistic regression, $P = 0.553$). In adults, survival rate increased as infection load decreased, i.e., a higher infection load negatively influenced adult survival. Specifically, it seemed that infections consisting of >160,000 spirochetes per tick had a negative impact on adult survival (Fig. 3). In nymphs, however, high spirochete numbers did not negatively influence survival (data not shown).

Based on the results of the survival tests, we considered that thermohygrometric conditions were challenging for ticks when the survival of ticks was below 70%. Because males and females survived well and were affected by thermohygrometric conditions similarly, both categories were merged together to evaluate the SD values corresponding to a survival rate <70%. These SD values were 4.19, 9.35, 11.33, and 15.89 mmHg in nymphs and 11.33 and 15.89 mmHg in adults. Under these thermohygrometric conditions, infection revealed to have a positive effect on survival of nymphs and adults: 42/98 infected nymphs (42.9%) and 62/123 infected adults (50.4%) survived, whereas only 86/302 uninfected nymphs (28.5%) and 95/251 uninfected adults (37.9%) survived (χ^2 test: nymph, $P = 0.008$; adult, $P = 0.021$).

The number of genospecies in the tick had no impact on tick survival (χ^2 test, $P = 0.589$). However, differences were observed between *Borrelia* genospecies. Hence, nymphs infected by *B. afzelii* survived better (44.1%; 26/59) than nymphs that were not infected or infected by other *Borrelia* genospecies (29.9%; 102/341) (χ^2 test, $P = 0.031$). The same trend was observed in adults where a higher survival rate (53.5%; 23/43) was observed in adults infected by *B. afzelii* than in adults that were not infected or infected by another genospecies (39.5%; 141/357) (χ^2 test, $P = 0.057$).

Discussion

Fat Content Analysis. In this study, we determined whether *B. burgdorferi* s.l. influenced *I. ricinus* survival under thermohygrometric stress. Fat content in *I. ricinus* was measured to take into account variable energy resources when assessing *I. ricinus* survival. It was observed that fat content in field-collected females was slightly higher than that in field-collected males and considerably higher than that in field-collected nymphs. These results are in line with what is expected because fat content is a source of energy derived from each bloodmeal (Uspensky 1995). Larvae, which ingest less blood than further developmental stages (Ogden et al. 1998), contain less fat than adults as they molt into nymphs. Likewise, nymphal males that take relatively less blood than nymphal females (Graf 1978, Mbow et al. 1994) molt into adult males with smaller energy resources than adult females.

Survival Tests. Globally, differences in survival were observed among *I. ricinus* life stages: females survived better (77.6%) than males (51.6%), which in turn survived better than nymphs (43.2%). Lower fat content in nymphs seemed to contribute to lower survival rate. Moreover, adults have a higher water storage capacity due to their size, and their lower surface-to-volume ratio probably allowed them to reduce water loss under dry conditions. Nevertheless the most striking difference in survival lay between laboratory-reared and field-collected nymphs. Although both belonged to the same life stage, the laboratory-reared nymphs survived extremely well (global survival rate, 95.4%) under any thermohygrometric condition, whereas the field-collected nymphs did not (global survival rate, 43.2%). This might be explained by the higher fat content, lack of any kind of stress of laboratory-reared nymphs, or a combination. Hence, they had full and ready-to-use stored energy resources when tests began.

Ixodes ticks are exceptionally sensitive to temperature and humidity compared with other ticks (Knülle and Rudolph 1982, Sonenshine 1991). More precisely, it has been observed that *I. ricinus* most important climatic feature for survival is a high degree of humidity and that increasing temperature limits survival (MacLeod 1935). Saturation deficit, defined as the drying power of the air (Randolph and Storey 1999), also has revealed to influence *I. ricinus* questing activity (Randolph and Storey 1999; Perret et al. 2000, 2003, 2004) and survival in nature (Perret et al. 2000, 2004; Perret 2002; Burri et al. 2007). Identical observations were made in the current study. Increasing relative humidity influenced positively *I. ricinus* survival, whereas increasing temperature affected it negatively. As a combination of the last two factors, SD had a stronger impact on *I. ricinus* than temperature and relative humidity alone. Hence, survival rate was inversely proportional to SD, i.e., more ticks survived as SD decreased.

The latter was true for all field-collected ticks, although it was more marked in nymphs. This matched fat content results: nymphs that had lower fat content than adults survived less well. Thus, it seems to indicate that available energy resources determine life span, which was described in Randolph and Storey (1999), and that more challenging thermohygrometric conditions increase fat usage. This also applies to laboratory-reared nymphs, although results are not as clear-cut as those of field-collected ticks. It may be due to a stress-free state (fully hydrated, high fat) of colony ticks, leading to a very low death rate (4.6%; 23/500) in the survival tests and less straightforward interpretations. However, these results clearly display how different laboratory-reared and field-collected ticks may be.

***Borrelia* Infection in Ticks.** Overall prevalence of infection by *Borrelia* (34.8%) was similar to that of another study conducted in the same area (32.7%) (Casati et al. 2004). Infection prevalence in adults was higher (39.6%) than in nymphs (25.5%), as often described in Europe (Rauter and Hartung 2005) and Switzerland (Casati et al. 2004, Jouda et al. 2004). Similarly, adult infections consisted more frequently of >160,000 spirochetes than those of nymphs. These differences in infection prevalence and infection load among stages are probably due to the number and size of bloodmeals taken by ticks in their earlier developmental stages (Ogden et al. 1998).

Genospecies prevalence was in line with what had been observed in the same area: *B. afzelii* (33.8%), *B. burgdorferi* s.s. (10.3%), *B. garinii* (32.1%), *B. myamotoi* (3.1%), and *B. valaisiana* (20.2%) (Casati et al. 2004, Jouda et al. 2004, Morán Cadenas et al. 2007b, Gern et al. 2010). The *Borrelia* genospecies were similarly distributed among males and females. However, *B. afzelii* prevailed in nymphs compared with adults, and *B. garinii* and *B. valaisiana* were more frequent in adults compared with nymphs. Different favored hosts for bloodmeal and specific host–parasite association may explain this discrepancy (Humair et al. 1995, 1998, 1999; Humair and Gern 1998; Kurtenbach et al. 1998; Morán Cadenas et al. 2007a).

High loads of spirochetes were usually observed in *B. garinii*-infected nymphs and adults, and low loads were observed in *B. valaisiana*-infected mature and immature ticks. *B. afzelii* was observed in low numbers in nymphs, whereas higher numbers of spirochetes were observed in adults. It has been demonstrated that transmission from *I. ricinus* to host differs among *Borrelia* genospecies (Crippa et al. 2002). More precisely, *B. afzelii* is transmitted to host earlier and more efficiently than *B. burgdorferi* s.s. The latter might be true in the opposite direction, i.e., from host to tick. Nymphs that feed for a longer time than larvae may have ingested more *B. afzelii* spirochetes resulting in adults harboring higher borreliae numbers than nymphs. Thus, early-transmitted spirochetes such as *B. afzelii* (Crippa et al. 2002) and probably also *B. garinii* according to the present results may be found in higher numbers in ticks than late-transmitted ones such as *B. burgdorferi* s.s. (Crippa et al. 2002) and supposedly *B. valaisiana.*

Infection load in questing *I. ricinus* in Europe has been investigated by Rauter et al. (2002), who conducted a real-time PCR targeting the *ospA* gene (compared with a fragment of the flagellin gene here). They reported a median of 4,000 spirochetes per tick, which is higher than the one observed in the current study (2,760 per nymph; 1,496 per male; 2,078 per female). However, Rauter et al. (2002) did not discriminate between life stages or between *Borrelia* genospecies (both factors have been shown to significantly influence infection load).

Effect of *Borrelia* Infection on Tick Survival. In view of the present results, the hypothesis that infection by *B. burgdorferi* s.l. enhances *I. ricinus* survival under thermohygrometric stress conditions is confirmed. However, the phenomenon does not apply in every situation and acts subtly. Its impact varies between life stages, as well as according to infection load. Hence, nymphal survival revealed to be highly positively influenced by infection by *Borrelia,* whatever the infection load. In contrast, adults seemed to be positively influenced by spirochete colonization up to a threshold of infection load (estimated at 160,000 spirochetes per tick), where an increasing number of borreliae became harmful and the positive effect was reversed to a negative effect. That this deleterious effect of spirochete "overdose" was observed in adults but not in nymphs is probably related to the extremely rare proportion of nymphs bearing >160,000 spirochetes per tick (1.6%; 2/126) compared with adults (8.6%; 34/396). Thus, a potentially negative effect of infection load could not be detected in nymphs due to poor occurrence of very high infection load. Very high *Borrelia* numbers might trigger *I. ricinus* immune response (Coleman et al. 1997, Johns et al. 2001, Sonenshine and Hynes 2008) requiring too much energy resources. That would, in turn, reduce energy allowance to other stress sources such as challenging thermohygrometric conditions thereby impairing vector resistance to heat and/or desiccation.

Although the number of different genospecies infecting the tick did not impact on tick survival, one particular genospecies, *B. afzelii,* revealed to be positively associated with *I. ricinus* survival, especially in nymphs. Therefore, ticks infected by *B. afzelii* had an advantage over uninfected ticks or those infected by other *Borrelia* genospecies when stressed by unfavorable thermohygrometric conditions. Interestingly, it has been observed during the past decade that infection rate by *B. afzelii* in *I. ricinus* has increased in the Neuchâtel region (L.G., unpublished data) where hot and dry climatic conditions have been shown to have a negative impact on questing tick densities (Perret et al. 2000, 2004; Morán Cadenas et al. 2007b).

Various hypotheses can be put forward to explain the higher survival rate of ticks infected by *Borrelia* observed in the current study. Although spirochetes in questing ticks are usually limited to the tick midgut, they may be disseminated in other tissues and organs producing a systemic infection in up to 11% of *I. ricinus*-infected nymphs (Burgdorfer et al. 1989, Lebet and Gern 1994). Hence, we can surmise that spirochetes in the midgut and those that are systemically distributed in the tick might be able to modify the physiology, metabolism, or both, of organs, such as those involved in bloodmeal digestion or water sorption/loss, resulting in optimized blood digestion increasing energy resources or optimized water sorption/retention enhancing water storage, respectively.

However, it is known that infection by *Borrelia* can change gene expression in ticks as observed in *I. scapularis.* It was shown that the expression of one gene was selectively increased in salivary glands of ticks infected by *B. burgdorferi* s.s. during blood feeding (Ramamoorthi et al. 2005). Therefore, it may be hypothesized that spirochetes, which are known to change their gene expression depending on temperature (Ojaimi et al. 2003), might be able to modify tick gene expression under varying temperature conditions as well, inactivating genes governing vector nonvital functions, slowing metabolism down, so that energy resources would essentially be devoted to resisting unfavorable thermohygrometric conditions while maintaining favorable living conditions for spirochetes.

It also has been observed that spirochetes are noninfectious (possibly evading tick immune response) when they are in unfed ticks (Crippa et al. 2002). Moreover it could be suggested that the turning off of infection factors by spirochetes in unfed ticks also might trigger tick metabolism to slow down, explaining the better survival of infected ticks under challenging thermohygrometric conditions.

More specifically, the positive effect of *B. afzelii* infection on survival of *I. ricinus* nymphs may be due, either to the fact that infections by *B. afzelii* consisted of low spirochete numbers and rarely reached a threshold beyond which spirochete load was harmful. Or, as mentioned before, it may be linked to *B. afzelii* being transmitted to the host earlier and more efficiently, compared with *B. burgdorferi* s.s., suggesting some influence on tick organ physiology (Crippa et al. 2002).

The molecular mechanisms at the tick–pathogen interface that are responsible for the higher survival rate of *I. ricinus* ticks infected by *Borrelia* spp. remain to be investigated and additional studies are expected in this subject. According to our results, infection by *B. burgdorferi* s.l. provides a survival advantage to *I. ricinus* ticks under challenging conditions of temperature and humidity. In fine, both organisms take advantages from each other, hence such a mutualism may have accompanied the entire evolution of *Borrelia* together with their tick vectors.

Acknowledgments

We thank Luca Gigandet, and Kangaji Kasongo for help in collecting ticks; Olivier Rais, Francisca Morán Cadenas, Caroline Burri, and Elena Lommano for precious technical help and advice; and Jason Grant for reviewing the English of the manuscript. Results obtained here are part of a project financed by the Swiss National Science Foundation (FN 320030_113936 and FN 310030_127064) to L.G. Some of the results are part of the Ph.D. dissertation of C.H.

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Received 26 April 2010; accepted 13 July 2010.

4.2 Paper 2

Do level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks? 2012. Parasitology 139: 330-337

Coralie Herrmann and Lise Gern

We observed in the first study that *I. ricinus* nymphs and adults harbouring *Borrelia* spirochetes survived better than uninfected ticks under hot and dry conditions (Herrmann and Gern, 2010). However, the mechanisms allowing infected ticks to survive better under desiccating conditions remained to be discovered. One hypothesis that might explain the previously observed phenomenon was that *Borrelia* spirochetes might modify the physiology and/or metabolism of organs involved in water sorption and/or loss resulting in enhanced water storage in infected ticks. Tick need for humidity might therefore be modified by *Borrelia* infection. To challenge this hypothesis, we tested in this study whether *I. ricinus* nymphs infected with *Borrelia* spirochetes walked differently from uninfected nymphs within a humidity gradient, i.e. were differently attracted to humidity under laboratory conditions. Moreover, we tested whether nymphs with different energy reserves and hydration status behaved differently within a humidity gradient.

Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks?

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(*Received 28 September 2011; revised 26 October 2011; accepted 26 October 2011; first published online 5 January 2012*)

SUMMARY

Ixodes ricinus horizontal movement within a humidity gradient and the influence of infection by *Borrelia burgdorferi* sensu lato (s.l.) on tick walking were investigated. Nymphs were placed within an arena containing a humidity gradient ranging from 45 to 95% relative humidity (RH). After 1 h of acclimation at 70% RH ticks were released so that they could either stay, or walk towards either the wet or the dry end. Their position was recorded 2 h post-release. Fat content was quantified and *Borrelia* infection was detected using real-time PCR and PCR followed by Reverse Line Blotting. Among the 1500 ticks tested, 29·85% were infected. More low-fat nymphs walked inside the arena than high-fat individuals. When nymphs walked, more low-fat ticks walked towards wetter than drier air, whereas more high-fat individuals walked towards drier than wetter air. Among high-fat nymphs, a lower proportion of *Borrelia*-infected ticks walked inside the arena compared to uninfected individuals, as though spirochetes manipulated their arthropod vector to stay. However, *Borrelia* infection had no effect on walking direction towards the dry or the wet end. Hence, it appears that *I. ricinus* nymphs walk horizontally over short distances within a humidity gradient depending on both energy resources and *Borrelia* infection.

Key words: *Borrelia burgdorferi* s.l., *Ixodes ricinus*, energy resource, humidity, tick movement, vector behaviour.

INTRODUCTION

In Europe the hard-bodied tick *Ixodes ricinus* (L.) (Acari: Ixodidae) is the main vector of *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of the most commonly reported tick-borne disease of the northern hemisphere, Lyme borreliosis. Eleven *Borrelia* genospecies have been found associated with this tick species in Europe: *B. afzelii, B. bavariensis, B. bissettii, B. burgdorferi* sensu stricto (s.s.), *B. carolinensis, B. finlandensis, B. garinii, B. lusitaniae, B. spielmanii, B. valaisiana*, and a *Borrelia* genospecies related to relapsing fever spirochetes, *B. miyamotoi* (Rauter and Hartung, 2005; Margos *et al.* 2009; Gern *et al.* 2010; Cotté *et al.* 2010; Casjens *et al.* 2011).

The biggest survival challenge for ticks is to maintain water balance whilst living in a relatively dry environment. This is even more essential to *I. ricinus* since this tick is extremely sensitive to temperature and humidity compared with other tick species (MacLeod, 1935; Lees, 1946; Aeschlimann, 1972; Knülle and Rudolph, 1982; Sonenshine, 1991). Hence, saturation deficit, a measure of the drying power of the atmosphere depending on both temperature and relative humidity (Randolph and Storey, 1999), limits *I. ricinus* duration of questing (Perret *et al.* 2003, 2004) and survival in nature (Perret, 2002; Perret *et al.* 2000, 2004; Burri *et al.* 2007; Morán Cadenas *et al.* 2007) and in laboratory settings (Herrmann and Gern, 2010).

Whilst questing on vegetation for their vertebrate hosts, *I. ricinus* ticks increase water loss so that they must return periodically to moister surroundings, such as the litter layer. There, they can extract water vapour from the atmosphere above a certain humidity level (Knülle and Rudolph, 1982). Hence, ticks are considered to move primarily in the vertical rather than the horizontal plane in order to seek the two resources that are necessary to their survival, i.e. water vapour and/or hosts (Goddard, 1993). However, Perret *et al.* (2003) observed that nymphs walked great distances, up to 9·56 m in a laboratory setting. In these experiments, ticks were constrained within vertical channels and, although it was difficult to evaluate the part that would represent horizontal movements, Perret *et al.* (2003) assumed that some of the displacements could represent horizontal movements. Later, Crooks and Randolph (2006) showed that nymphs with high-energy resources walked horizontally but over short distances only. Furthermore, they also reported that ticks that were slightly dehydrated were more likely to walk towards fully saturated than drier air.

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Parasitology (2012), **139**, 330–337.
doi:10.1017/S0031182011002095

Recently, we observed that field-collected *I. ricinus* ticks infected by *B. burgdorferi* s.l. survived better under unfavourable conditions of temperature and humidity (saturation deficit), suggesting that infected ticks might be affected differently by surrounding humidity (Herrmann and Gern, 2010). In that context, it was interesting to determine whether field-collected *I. ricinus* nymphs of varying hydration status and level of energy reserves displayed a different walking activity and direction within a humidity gradient in the horizontal plane when infected by *B. burgdorferi* s.l. spirochetes.

MATERIALS AND METHODS

Tick collection and maintenance

The sampling site was a mixed forest (deciduous dominant) situated at 600 m above sea level (47°00′N and 6°57′E) on the south-facing slope of Chaumont Mountain (Neuchâtel, Switzerland) (Herrmann and Gern, 2010). Host-seeking *I. ricinus* nymphs were sampled by flagging the low vegetation using a 1-m^2 terry flag on 3 consecutive days in May 2010. In the laboratory, collected nymphs were divided into 3 groups exposed to different conditions. To obtain high-fat and fully hydrated ticks (H&F, Group 1), ticks were held over water in a box with a tight-fitting lid (98% relative humidity; RH) in the dark within a cold chamber at 4 °C for at least 4 weeks as described by Crooks and Randolph (2006). Group 2 contained low-fat and moderately hydrated (L&M) ticks. These ticks were maintained over water in a transparent plastic box with small holes allowing airflow (87% RH) at room temperature (~23 °C); while group 3 ticks, low-fat and fully hydrated (L&F), were held over water in a transparent plastic box with a tight-fitting lid (98% RH) at room temperature (~23 °C). L&M and L&F ticks were exposed to the natural daylight conditions in May–June for 4 weeks before being returned to the cold chamber until use (Crooks and Randolph, 2006).

Fat content analysis

Fat content was quantified in order to verify whether fat content of high-fat and low-fat groups of ticks differed. Samples of 40 nymphs from each group (H&F, L&F, and L&M) were analysed for their fat content as described by Randolph and Storey (1999) and as calculated by Crooks and Randolph (2006). Briefly, ticks were dried at 70 °C for 24 h, weighed individually to the nearest 1 μg, washed in 3 changes of chloroform for 24 h each, re-dried at 70 °C for 24 h and re-weighed using a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland).

Attraction tests

Choice arenas were slightly modified from those described by Crooks and Randolph (2006). They were made of 2 transparent polystyrene boxes (19 cm × 9 cm × 9 cm) with tight-fitting lids, joined by a transparent polystyrene tunnel (3 cm diameter, 28 cm long) sealed into holes cut in one side of each box. Humidity was monitored using an EE07-PFT5 hygrometer (E+E Elektronik, Engerwitzdorf, Austria) with a probe within different parts of the arena. A small dish of water was placed in one box whereas the other box remained empty, producing a humidity gradient of 90–45% RH within 30 min. The humidity gradient did not evolve much after 3 h (end time of test, see details below), only reaching 95–45% RH. In the experimental room at ambient temperature (~23 °C), this corresponds to saturation deficits (SD) ranging from 1·10 mmHg to 10·98 mmHg (1 mmHg = 133·3 Pa), as calculated by Randolph and Storey (1999).

Each replicate consisted of a set of 20 nymphs placed into a 1 cm × 5 cm plastic culture tube (Milian, Meyrin, Switzerland) that was closed at both ends by pieces of cotton wool. Each piece of cotton wool was tied to a length of cotton thread. Experimental runs were conducted as described by Crooks and Randolph (2006). Briefly, 1 tick-laden tube was positioned in the centre of the arena tunnel with the cotton threads passing out through small holes cut into the box side that were closed by laboratory film (Parafilm M, Pechiney Plastic Packaging, Menasha, WI, USA). The arena was left undisturbed for 1 h with the lights on. Then the threads were gently pulled to remove the cotton wool from each end of the tube. The cotton wool pieces were left very close to the tube. Lights were left on for 1 h and switched off for 1 additional hour since, according to Perret *et al.* (2003), darkness induces *I. ricinus* ticks to move. Ticks were collected 2 h post-release and their position was recorded. Testing time was determined so that half of the ticks would walk out of the tube, based on the protocol of Crooks and Randolph (2006). Twenty-five replicates were performed for each tick group (H&F, L&F, and L&M). Humidity side and tick group were changed for every new experimental run.

Borrelia *infection in ticks*

Borrelia infection in ticks was detected using real-time PCR. Before DNA isolation, ticks were soaked in 70% ethanol and air-dried. Extraction of DNA from ticks was achieved using ammonium hydroxide as previously described (Guy and Stanek, 1991; Rijpkema *et al.* 1996; Herrmann and Gern, 2010). Negative controls were included during DNA isolation, which consisted of reagents without template DNA.

A real-time PCR amplifying a fragment of the flagellin gene (Schwaiger *et al*. 2001; Herrmann and Gern, 2010) was used to detect and quantify *Borrelia* DNA in all field-collected ticks that were subjected to the humidity gradient tests. The strain *B. afzelii* NE1817 was used as quantification standard. Spirochete concentration in culture was evaluated using the Helber chamber. To extract DNA, the culture was washed twice with phosphate-buffered saline/$MgCl_2$, and the pellet was resuspended in 30 μl of water and heated for 15 min at 100 °C (Postic *et al*. 1994). The *Borrelia* DNA stock was aliquoted at 10^5 spirochetes per μl and stored at −20 °C. Serial dilutions were made from stored spirochete DNA in order to obtain 5 standard solutions with concentrations of *Borrelia* DNA ranging from 10^2 to 10^5 copies per μl.

The 50 μl real-time PCR mixture (Schwaiger *et al*. 2001) consisted of 10 μl of 5 × buffer, 5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 1 μl of 20 μM FlaF1A forward primer, 1 μl of 20 μM FlaR1 reverse primer, 1 μl of 10 μM FlaProbe1 probe, 0·25 μl of HotStart Taq Polymerase (Kapa Biosystems, Woburn, MA, USA), 20·75 μl of water and 10 μl of the extracted DNA. In each run, 1 extraction negative control (10 μl, see above), 1 PCR negative control (10 μl of water instead of 10 μl of the extracted DNA) and 3 series of the 5 standards were included.

Following an incubation step at 95 °C for 10 min, the samples were submitted to 45 repeated amplification cycles (95 °C for 15 s, 60 °C for 1 min) (Schwaiger *et al*. 2001) in an iCycler Optical Module (Bio-Rad, Reinach, Switzerland) using strip PCR tubes and flat caps (Scientific Specialties Inc, Lodi, CA, USA).

PCR and RLB were used to identify the *Borrelia* species in the field-collected ticks that were detected positive by real-time PCR as described in Herrmann and Gern (2010). The variable spacer region between 2 repeated copies of the 23S and 5S ribosomal genes was amplified with primers 23S-Bor and B-5S-Bor (Alekseev *et al*. 2001). PCR amplifications were run in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touch-down PCR program (Burri *et al*. 2007; Herrmann and Gern, 2010). Positive and negative controls were included in each PCR. In positive controls, isolates of *B. valaisiana* (VS116), *B. lusitaniae* (PotiB1), *B. burgdorferi* s.s. (B31) or *B. garinii* (NE11) replaced DNA samples, whereas water substituted them in negative controls.

For *Borrelia* identification by RLB, PCR products were hybridized to 15 oligonucleotide probes (Rijpkema *et al*. 1995; Poupon *et al*. 2006; Gern *et al*. 2010) blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd, Portsmouth, UK) using a Miniblotter 45 (Immunetic, Cambridge, MA, USA). Hybridization was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham Biosciences Europe, Switzerland) and by exposing the membrane to X-ray film (Hyperfilm, GE Healthcare, UK).

Statistical analysis

The Mann-Whitney test was performed in order to determine whether there was a difference in fat content between tick groups, and whether spirochete load differed among *Borrelia* genospecies. Simple χ^2 tests were used to compare walking activity, direction of movement between tick groups. All statistics were calculated with R for Mac OS X (R Development Core Team, 2011).

RESULTS

Fat content analysis

The fat content of the low-fat moderately hydrated (L&M) ticks (4·15 ± 3·98 μg) was not statistically different from that of the low-fat fully hydrated (L&F) ticks (4·28 ± 3·38 μg) (Mann-Whitney test; $P = 0{\cdot}51$). In contrast, the fat content of high-fat fully hydrated (H&F) ticks (9·25 ± 6·64 μg) was significantly higher than that of the L&M and L&F ticks (Mann-Whitney test; H&F-L&M $P = 0$, H&F-L&F $P = 0{\cdot}0001$). According to Crooks and Randolph (2006), fat content is positively correlated with tick size, measured by tick fat-free (reduced) dry mass. Therefore a correction for size was calculated by dividing fat content by tick reduced dry mass (Crooks and Randolph, 2006). After correction for size, fat content of the H&F ticks (0·137 ± 0·099) remained significantly higher than that of the L&M (0·073 ± 0·076) and L&F (0·074 ± 0·059) ticks (Mann-Whitney test; H&F-L&M $P = 0{\cdot}0003$, H&F-L&F $P = 0{\cdot}001$) and it did not differ between the L&M and L&F ticks (Mann-Whitney test; $P = 0{\cdot}78$).

Borrelia *infection in ticks*

Among the 1500 questing nymphs that were tested for *B. burgdorferi* s.l. by real-time PCR, 29·85% ($n = 448$) were infected. Globally, infection load in questing nymphs ranged from 2 to 896 000 spirochetes per tick. Mean spirochete number was 33 971 spirochetes per nymph while median spirochete number was 4300.

Identification of *Borrelia* genospecies by RLB was possible in 413/448 nymphs. Nymphs were mainly infected by 1 *Borrelia* genospecies (86·4%) (Table 1). Six *Borrelia* genospecies were identified: *B. afzelii, B. bavariensis, B. burgdorferi* s.s, *B. garinii, B. miyamotoi*, and *B. valaisiana* (Table 1). *B. bissettii, B. lusitaniae*, and *B. spielmanii* were not observed.

Spirochete load varied between *Borrelia* genospecies (Table 1). Infections by *B. bavariensis*

Table 1. Distribution of *Borrelia* genospecies and mean spirochete number in questing *Ixodes ricinus* nymphs in Neuchâtel, Switzerland

Borrelia genospecies[a]	Infected ticks[b]	Mean spirochete number[c]
af	165 (40·0%)	15 531
bav	24 (5·8%)	89 419
ga	86 (20·8%)	69 660
miy	2 (0·5%)	na
ss	18 (4·4%)	8985
vs	62 (15·0%)	12 981
Infection by 1 species	357 (86·4%)	32 678
af & *ga*	4 (1·0%)	na
af & *miy*	3 (0·7%)	na
af & *ss*	2 (0·5%)	na
af & *vs*	2 (0·5%)	na
bav & *vs*	4 (1·0%)	na
ga & *miy*	2 (0·5%)	na
ga & *vs*	32 (7·7%)	87 706
Infection by 2 species	52 (12·6%)	64 345
af & *ga* & *vs*	1 (0·2%)	na
ga & *miy* & *vs*	3 (0·7%)	na
Infection by 3 species	4 (1·0%)	na

[a] af, *B. afzelii*; bav, *B. bavariensis*; ga, *B. garinii*; miy, *B. miyamotoi*; ss, *B. burgdorferi* sensu stricto; *vs*, *B. valaisiana*.

[b] n = 413.

[c] Mean spirochete number was not calculated when frequency was below 10.

and by *B. garinii* consisted of significantly more spirochetes per tick (89 419 and 69 660, respectively) than infections by *B. afzelii* (15 531) (Mann-Whitney test; $P = 0$ and $P = 0{\cdot}008$, respectively), by *B. valaisiana* (12 981) (Mann-Whitney test; $P = 0$ and $P = 0{\cdot}005$, respectively), and *B. burgdorferi* s.s. (8985) (Mann-Whitney test; $P = 0{\cdot}001$ and $P = 0$, respectively). Infections by *B. miyamotoi* ($n = 2$) were excluded from the statistical analyses due to their low frequency.

Walking activity

Within 2 h, a significantly higher number of low-fat ticks walked out of the tube than stayed inside (χ^2 test; L&M: $P = 0$; L&F: $P = 0$). Hence, 65% (323/500) L&M and 60% (298/500) L&F nymphs crawled out of the tube. In contrast, high-fat ticks had a higher tendency to stay inside the introduction tube (54%, 270/500) than walk out (χ^2 test; $P = 0{\cdot}07$). Thus, more low-fat nymphs (L&M: 323/500 and L&F: 298/500) walked out of the tube than high-fat (H&F: 230/500) individuals (Fig. 1). The difference was highly significant for moderately hydrated ticks (χ^2 test; $P = 0$) and for fully hydrated ticks (χ^2 test; $P = 0$). Among low-fat individuals, there was no difference in walking activity between moderately (323/500 out of tube) and fully hydrated ticks (298/500 out of tube) (χ^2 test; $P = 0{\cdot}12$).

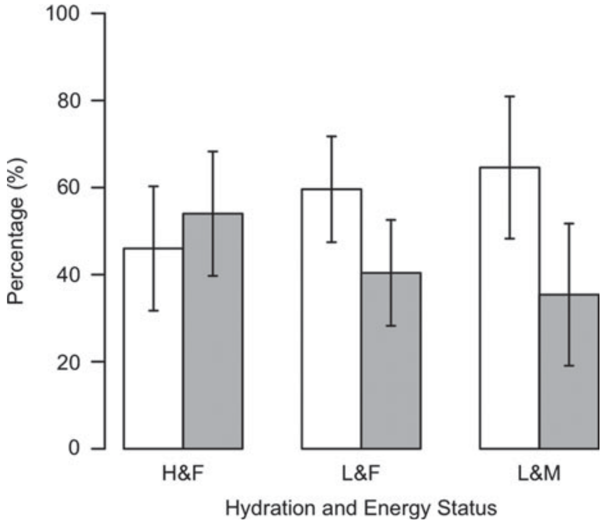

Fig. 1. The percentage of high-fat fully hydrated (H&F), low-fat fully hydrated (L&F), and low-fat moderately hydrated (L&M) *Ixodes ricinus* ticks that stayed inside (light grey bars) or walked (white bars) out of the introduction tube after 2 h within a humidity gradient.

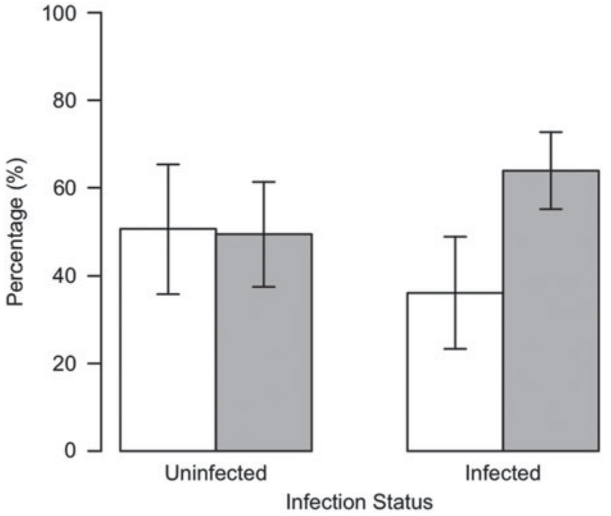

Fig. 2. The effect of *Borrelia* infection on walking activity of high-fat fully hydrated *Ixodes ricinus* nymphs after 2 h within a humidity gradient. Light grey bars represent ticks that stayed within the introduction tube while white bars represent those that walked out.

Among low-fat (L&M and L&F) ticks, walking activity was not different between uninfected and *Borrelia*-infected individuals. In fact, among L&M ticks 64% (222/347) uninfected and 66% (101/153) infected individuals crawled out of the tube (χ^2 test; $P = 0{\cdot}74$), and among L&F nymphs 61% (221/363) uninfected and 56% (77/137) infected ticks walked out of the tube (χ^2 test; $P = 0{\cdot}4$). In contrast, among H&F individuals, significantly more uninfected ticks (51%, 173/342) walked out of the tube than infected ticks (36%, 57/158) (χ^2 test; $P = 0{\cdot}003$) (Fig. 2). Hence, H&F infected ticks rather stayed inside the introduction tube (64%, 101/158) than walked out of it (36%, 57/158).

Direction of movement (dry versus wet end of the arena)

To evaluate direction of movement, 3 categories of ticks were considered: (1) those that reached the empty box, i.e. moved to the dry end, (2) those that reached the box containing the dish of water, i.e. moved to the wet end, and (3) those that stayed in the tunnel, i.e. did not move. Although some ticks might have moved 1–2 cm inside the tunnel and have been categorized as 'did not move', such cases were rare (less than 1%). There was a clear distinction between ticks that moved to the boxes or stayed close to the plastic culture tube. A higher proportion of low-fat individuals moved towards the wet than the dry end of the arena (χ^2 test; L&M: $P=0$; L&F: $P=0$), whereas a lower proportion of high-fat nymphs (H&F) walked towards the wet than the dry end of the arena (χ^2 test; $P=0{\cdot}01$) (Fig. 3). Hence, 42% (210/500) L&M and 38% (191/500) L&F ticks moved towards the wet end, and 23% (113/500) L&M and 21% (107/500) L&F nymphs walked to the dry end of the arena. In contrast, 19% (96/500) H&F ticks walked towards the wet end and 27% (134/500) H&F nymphs moved towards the dry end of the arena. The difference in walking direction between groups of ticks with different fat contents was statistically significant (χ^2 test; L&M-H&F: $P=0$, L&F-H&F: $P=0$). However, tick hydration status (only tested among low-fat ticks) (moderate, L&M: 87% RH, or fully, L&F: 98% RH) had no effect on the direction of movement (χ^2 test; $P=0{\cdot}88$). Similarly, *Borrelia*-infection had no significant effect on walking direction towards the dry or the wet end of the arena (χ^2 test; H&F: $P=0{\cdot}09$, L&F: $P=0{\cdot}16$, L&M: $P=0{\cdot}29$).

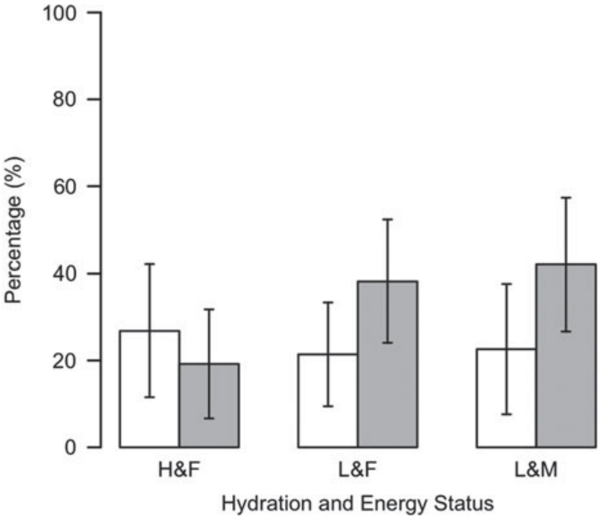

Fig. 3. The percentage of high-fat fully hydrated (H&F), low-fat fully hydrated (L&F), and low-fat moderately hydrated (L&M) *Ixodes ricinus* ticks that walked towards the wet (light grey bars) or the dry end (white bars) of the arena after 2 h.

DISCUSSION

Fat content analysis

Fat content is a source of energy derived from each bloodmeal (Uspensky, 1995). Here it was quantified in order to determine available energy reserves in *I. ricinus* nymphs among the different groups (high-fat and low-fat). Nymphs treated to conserve their fat did indeed contain higher lipid content (9·3 µg) than those treated to lose some of their fat (4·2–4·3 µg). However, it appeared that low-fat nymphs from the present study had a fat content that was in between that of high-fat and low-fat nymphs (4·7 µg and 3·9 µg, respectively) from the work by Crooks and Randolph (2006). In fact, nymphs collected in the spring in Neuchâtel did not only have a higher mean fat content (9·3 µg, this study; 7·12 µg; Herrmann and Gern, 2010) but also a higher fat range (1–25 µg, this study; 1–29 µg; Herrmann and Gern, 2010) than those sampled in southern England all over the year (0·2–15 µg; Randolph *et al.* 2002; 1·6–18·2 µg; Randolph and Storey, 1999). Moreover, when fat content of spring ticks from Neuchâtel was corrected for size and converted into fat index (Randolph *et al.* 2002) (mean = 0·035), it was strikingly higher than that of spring ticks from southern England (approximately 0·015), and even slightly higher than that of fall ticks from southern England (approximately 0·032). This suggests that *I. ricinus* ticks from central Europe and southern England are more distinct than previously thought. Hence, they seem to distinguish themselves not only by different host-seeking behaviour and activity as suggested by Kurtenbach *et al.* (2006), i.e. in southern England, ticks might quest higher in the vegetation and host-seeking activity is present in spring-summer while in central Europe questing activity is present in spring and occasionally in autumn, but also by overall fat content.

Borrelia *infection in ticks*

Prevalence of infection by *Borrelia* in *I. ricinus* nymphs (29·85%) was similar to that reported by Casati *et al.* (2004) (29%) and slightly higher than that observed by Herrmann and Gern (2010) (25·5%). All these studies were conducted in the same suburban forest of Neuchâtel, Switzerland. Such slight fluctuations of *B. burgdorferi* s.l. prevalence over the years have already been observed in a nearby area on Chaumont Mountain (Jouda *et al.* 2004; Morán Cadenas *et al.* 2007).

Borrelia genospecies frequency was in line with what was previously observed in this area: the most abundant species was *B. afzelii* (42·9%), followed by *B. garinii* (31%), *B. valaisiana* (25·4%), *B. burgdorferi* s.s. (5·6%), while *B. miyamotoi* (2·9%) was the least common one (Casati *et al*. 2004; Jouda *et al*. 2004; Morán Cadenas *et al*. 2007; Gern *et al*. 2010; Herrmann and Gern, 2010). *B. bavariensis* (6·8%), formerly *B. garinii* OspA serotype 4, a newly described genospecies (Margos *et al*. 2009), is reported for the first time in the area, although it was already described in Switzerland (Staatswald) (Hu *et al*. 2001; Huegli *et al*. 2002; Pérez *et al*. *manuscript submitted*).

The spirochete load in questing ticks observed in the present study reached a mean of 33 971 and a median of 4300 spirochetes per tick, showing a higher load of spirochetes than that reported in a previous study conducted in the same area (mean = 18 640; median = 2760 spirochetes per tick) (Herrmann and Gern, 2010). Since *Borrelia* genospecies influence infection load (Herrmann and Gern, 2010), these differences in spirochete load may be due to different genospecies distribution in the two studies. For example, infections by *B. garinii*, which consisted of high spirochete numbers (78 000 per tick), represented 23·4% of infections in the study by Herrmann and Gern (2010), while infection by *B. garinii* and by *B. bavariensis* (previously included in infections by *B. garinii*) (69 660 and 89 419, respectively) accounted for 37·8% of infections in this study.

Influence of Borrelia *infection on walking activity*

To date, numerous vector-borne parasites have been shown to alter phenotypic traits of their arthropod vectors, most likely in a way that enhances their probability of transmission (Hurd, 2003; Lefèvre and Thomas, 2008). For example, *Trypanosoma cruzi* increases biting rate in bug *Mepraia spinolai* (Botto-Mahan *et al*. 2006), *Plasmodium mexicanum* alters temperature preference in sand fly *Lutzomyia vexator* (Fiahlo and Schall, 1995), *P. falciparum* alters immune response in mosquito *Anopheles gambiae* (Lambrechts *et al*. 2007), *Babesia microti* increases lifespan in the tick *I. trianguliceps* (Randolph, 1991), or *B. burgdorferi* increases survival in *I. ricinus* under hot and dry conditions (Herrmann and Gern, 2010). Another example of behavioural/physiological alteration in the arthropod vector due to parasite presence is brought to light in the present study. Although walking activity was similar between low-fat uninfected and *Borrelia*-infected nymphs, it seems that among high-fat ticks, *Borrelia* infection influenced *I. ricinus* walking activity. Thus, more high-fat uninfected nymphs walked out of the introduction tube than high-fat *Borrelia*-infected individuals, which rather stayed inside the tube. Higher locomotion activity in uninfected ticks than in individuals infected by *B. burgdorferi* s.l. has been previously reported. In fact, Alekseev *et al*. (2000) observed that infection by *B. burgdorferi* s.l. in *I. persulcatus* and *I. ricinus* ticks suppressed walking activity of both adult and immature individuals compared to uninfected individuals. Hence, *B. burgdorferi* s.l. spirochetes seem to reduce tick motor activity in high-fat individuals (ticks used by Alekseev *et al*. in 2000 were field-collected and stored, similarly to high-fat individuals in this study), apparently manipulating ticks to behave in a beneficial way to them. It may be hypothesized that *Borrelia* spirochetes only affect the behaviour of ticks with high-energy reserves because they may have access to their host vector energy resources without threatening to make the tick run out of energy and die, which would be a disaster for the spirochetes. Thus, energy-using borreliae may manipulate the tick to reduce its motor activity when the arthropod is placed in favourable surroundings for the spirochete, for example when the tick is on the vegetation, increasing the tick chances to find a passing host, in turn allowing the spread of *Borrelia* spirochetes to a new host. In contrast, spirochetes in ticks with reduced energy reserves may not have access to their vector energy resources, forcing them to reduce their metabolism and preventing the spirochetes from manipulating tick behaviour in any way.

Influence of energy reserves and hydration status on walking activity

A 2-h period was chosen as testing time based on Crooks and Randolph (2006) so that half of the ticks would walk out of the tube. The estimate was quite correct since 46% of the high-fat and 62% of the low-fat ticks moved out of the tube during that period.

Interestingly, high-fat nymphs walked less than low-fat ones in the present study while the opposite phenomenon (about 70% and 40%, respectively) was observed by Crooks and Randolph (2006). Differing levels of energy reserves between the nymphs in the two studies seem to be one of the factors explaining this differential walking behaviour. The proportion of low-fat nymphs that walked (62%), which proved not to be affected by *Borrelia* infection, was in between that of high-fat (70%) and low-fat (40%) individuals that walked in the study by Crooks and Randolph (2006). Similarly, fat content in low-fat ticks in our study (4·2–4·3 μg) was in between that of high-fat (4·7 μg) and low-fat (3·9 μg) ticks, in Crooks and Randolph (2006). Thus, low-fat nymphs in our study did not correspond to either low-fat or high-fat individuals in the work by Crooks and Randolph (2006). They had more energy reserves than the first and less than the second, and behaved as such, i.e. walked more than low-fat ticks and less than high-fat ticks.

Another factor that may explain the difference between the two studies is *Borrelia* infection. Although Crooks and Randolph (2006) did not provide information about *Borrelia* infection, it may be considered that infection prevalence was much lower among the nymphs they tested. In fact, tested ticks were collected in southern England where infection prevalence is below 12·4% (Rauter and Hartung, 2005; Vollmer *et al*. 2011) whereas infection prevalence reached 30% in the present study. Hence, this difference in infection prevalence may explain the lower proportion of high-fat nymphs walking outside the introduction tube (since *Borrelia* reduced walking activity of high-fat individuals) in the present study (36% of infected ticks moved versus 51% of uninfected ticks).

Hydration status (only tested among low-fat nymphs) had no significant effect on tick walking activity. However, it appeared that moderately hydrated individuals had a higher tendency to walk than fully hydrated ones. This is in line with what Lees (1948) described. He reported that dehydrated ticks were intensely active in dry air (about 70% RH in this study) while hydrated individuals were not. This seems to indicate that ticks with higher deficiencies in water reserves are more prone to walk, probably in order to seek for what they lack.

Direction of movement (dry versus wet end of the arena)

Hydration status, which only differed among low-fat individuals, appeared not to influence direction of movement. This may probably be explained by the fact that their hydration status was very similar (they were maintained at 98% and 87% RH, respectively). Moderately hydrated ticks would probably have needed to be maintained under drier conditions in order to be differentiated from fully hydrated individuals.

The level of energy reserves played a role in the direction of movement of *I. ricinus* nymphs within a humidity gradient. Low-fat individuals (fully and moderately hydrated) tended to walk up the humidity gradient, i.e. towards the area saturated in water vapour, whereas high-fat individuals tended to move down the humidity gradient, i.e. towards an area containing less water vapour than where they were released. Walking of high-fat fully hydrated ticks towards the dry end of the arena is in line with what has been described by Lees (1948). He reported that nymphal and female *I. ricinus* ticks that had been previously exposed to saturated air avoided the wet side (95% RH) of the arena, preferring to walk towards the dry side (34% RH). On the contrary, ticks that were kept under unsaturated air conditions moved towards the opposite direction. This behaviour was observed on the horizontal and the vertical plane (Lees, 1948). It appears in the present study that when *I. ricinus* nymphs are treated so that they become low-fat, they behave similarly to ticks exposed to unsaturated air in the study of Lees (1948), suggesting that not only state of hydration but also the level of energy reserves plays a role in triggering the locomotion response.

These results seem to indicate that *I. ricinus* nymphs that are not in immediate need for energy or water move towards unfavourable dry surroundings, supposedly representing in nature the walk up the vegetation to quest for a host where humidity decreases. In contrast, ticks with lower energy reserves, despite high water reserves, tend to walk towards a wet comfortable zone where they remain fully hydrated, such as the litter layer in the field. Thus, it is as though nymphs with sufficient energy reserves may take the risk to wander randomly outside their comfort zone in order to find a potential host, while leaner counterparts need to be cautious about their energy-costly movements and move towards favourable (wet) conditions before waiting until a positive stimulus (such as host odour) trigger them to walk again.

Nymphs infected by *Borrelia* spirochetes did not walk preferentially towards the wet or the dry end within a humidity gradient. This seems to indicate that borreliae do not affect the need of *I. ricinus* nymphs for moist conditions as might have been expected because of higher survival of ticks infected by *B. burgdorferi* s.l. under hot and dry conditions (Herrmann and Gern, 2010).

In conclusion, the results described here point out that *I. ricinus* nymphs with lower energy reserves are more likely to walk horizontally than their counterparts with higher energy reserves. When *I. ricinus* nymphs walk, those with lower energy reserves are more likely to move towards fully saturated air than drier air, while those with higher energy reserves are more likely to move to the opposite direction. Moreover, it seems that *I. ricinus* immature ticks that are infected by *Borrelia* spirochetes are less likely to move on the horizontal plane than uninfected specimens, suggesting a manipulation by spirochetes in ticks with sufficient available energy reserves. Thus, it appears that *I. ricinus* nymphs will crawl horizontally over short distances within a humidity gradient depending both on their energy reserves and on the presence of *B. burgdorferi* spirochetes.

ACKNOWLEDGEMENTS

We would like to thank C. Dupasquier and O. Rais for their help in collecting ticks, as well as P. Guerin for providing useful material. This study is part of the Ph.D. thesis of C. Herrmann and has been supported by the Swiss National Science Foundation grant (FN 310030_127064).

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4.3 Paper 3

Ixodes ricinus **ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves. 2013. International Journal for Parasitology 43: 477-483**

Coralie Herrmann, Maarten J. Voordouw and Lise Gern

We showed in the two previous studies that (1) *I. ricinus* ticks harbouring *Borrelia* spirochetes survived better than uninfected ticks under hot and dry conditions (Herrmann and Gern, 2010) and that (2) *I. ricinus* nymphs infected with *Borrelia* spirochetes had a lower need to move towards an environment favourable for maintaining water balance than uninfected nymphs (Herrmann and Gern, 2012). Hence, *I. ricinus* ticks harbouring *Borrelia* spirochetes seemed to display an increased tolerance to desiccating conditions than uninfected ticks. Since we know that in nature *I. ricinus* ticks go up and down the vegetation to rehydrate more often when desiccation increases (Perret et al., 2000) and doing so they consume more energy, we surmised that the observed higher tolerance to desiccation in *Borrelia*-infected ticks could be due to higher energy reserves in the latter. In this study, we therefore quantified fat content in field-sampled nymphs to determine if *Borrelia*-infected and uninfected nymphs had different energy reserves.

Reprinted from International Journal for Parasitology, 43, C. Herrmann, M. J. Voordouw and L. Gern, Ixodes ricinus *ticks infected with the causative agent of Lyme disease,* Borrelia burgdorferi *sensu lato, have higher energy reserves, 477-483, copyright (2013), with permission from Elsevier*

International Journal for Parasitology 43 (2013) 477–483

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Ixodes ricinus ticks infected with the causative agent of Lyme disease, *Borrelia burgdorferi* sensu lato, have higher energy reserves

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ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form 19 December 2012
Accepted 22 December 2012
Available online 13 February 2013

Keywords:
Borrelia burgdorferi s.l
Ixodes ricinus
Energy reserves
Fat content
Lyme disease
Tick body size

ABSTRACT

Ticks use their energy reserves to maintain their water balance, search for hosts and transmit tick-borne pathogens. However, the influence of tick-borne pathogens on the energy reserves of the tick vector has not been well studied. The relationship between *Borrelia burgdorferi* sensu lato (s.l.) infection status and fat content in questing *Ixodes ricinus* nymphs was examined. Nymphs were sampled from the field. Their body mass and fat content were measured, and their *Borrelia* genospecies infection status (using reverse line blot analysis), and spirochete load (using quantitative PCR) were analysed. Of the 900 nymphs tested, 21.2% were infected with a variety of *Borrelia* genospecies. *Borrelia*-infected nymphs had 12.1% higher fat content than uninfected ticks after correcting for body size. For the subset of *Borrelia*-infected nymphs, no relationship was found between spirochete load and fat content and bioenergetics calculations suggest that *Borrelia* spirochetes consume a negligible fraction of the tick energy reserves. While the mechanism that causes the association between *Borrelia* infection and higher fat content in *I. ricinus* nymphs remains unknown, the present study complements our previous findings that *Borrelia*-infected nymphs had higher survival times under desiccating conditions and walked less within a humidity gradient.

1. Introduction

Lyme borreliosis is the most common vector-borne disease in the northern hemisphere, accounting for approximately 85,000 new cases in Europe (Lindgren and Jaenson, 2006) and 20,000 in the United States each year (Bacon et al., 2008). The causative agents of this disease are spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex, which are transmitted to humans by hard-bodied ticks (Acari:Ixodidae) during blood feeding. In Europe, *Ixodes ricinus* is the most important tick species and to date at least 10 *B. burgdorferi* s.l. genospecies have been associated with this vector: *B. burgdorferi* sensu stricto (s.s.), *Borrelia afzelii*, *Borrelia garinii*, *Borrelia bissettii*, *Borrelia lusitaniae*, *Borrelia spielmanii*, *Borrelia valaisiana* (Rauter and Hartung, 2005; Gern et al., 2010), *Borrelia bavariensis* (Margos et al., 2009), *Borrelia carolinensis* (Cotté et al., 2010), *Borrelia finlandensis* (Casjens et al., 2011), as well as a genospecies related to relapsing fever, *Borrelia miyamotoi* (Fraenkel et al., 2002).

In haematophagous arthropods, fat content is a source of energy derived from the blood meal (Lehane, 1991). Although the blood meal primarily consists of proteins (up to 95%), the products obtained during digestion are largely converted to, and stored as, fat (lipids) (Lehane, 1991). In ticks, lipids are stored in epithelial cells of the midgut and in the fat body, which is a diffuse organ of highly dispersed strands of cells (primarily trophocytes) adhering to the branches of the tracheal system and other internal organs (Sonenshine, 1991). *Ixodes ricinus* ticks use their fat supplies to quest for hosts on vegetation and to maintain their water balance. Ticks are highly sensitive to desiccation (Lees, 1946) and have to move from their questing position to the underlying leaf litter where they take up atmospheric water (Lees and Milne, 1951; Knülle and Rudolph, 1982; Needham and Teel, 1991; Kahl and Alidousti, 1997). In *I. ricinus*, the rate of lipid consumption has been shown to increase under unfavourable conditions of temperature (Van Es et al., 1998) and humidity (Randolph and Storey, 1999). As ticks have no other energy sources, their body fat content declines between blood meals (Steele and Randolph, 1985). Thus tick fat content reflects both the history of the tick and the energy reserves remaining for future use (Randolph and Storey, 1999; Randolph et al., 2002).

Borrelia spirochetes influence both the survival and behaviour of their tick vector. Naumov (2003) reported that *I. ricinus* and *Ixodes persulcatus* ticks infected with *B. burgdorferi* lived slightly longer than uninfected individuals. More recently, we reported that field-collected *I. ricinus* ticks infected with *B. burgdorferi* s.l. survived better than uninfected individuals when exposed to unfavourable conditions of temperature and humidity (high saturation deficit) (Herrmann and Gern, 2010). With respect to tick

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http://dx.doi.org/10.1016/j.ijpara.2012.12.010

behaviour, a number of studies have shown that *Borrelia*-infected ticks move less than uninfected individuals. Alekseev et al. (2000) noticed that the locomotor activity in *Borrelia*-infected *I. ricinus* and *I. persulcatus* ticks was reduced compared with uninfected individuals. Lefcort and Durden (1996) reported that *Borrelia*-infected adult *Ixodes scapularis* were less active than uninfected ticks. We observed that *I. ricinus* nymphs infected with *B. burgdorferi* s.l. moved less within a humidity gradient than uninfected individuals (Herrmann and Gern, 2012). These observations led us to hypothesise that *Borrelia* spirochetes are associated with high energy reserves (measured as fat content) in *I. ricinus*. The purpose of this experiment was to test our hypothesis.

2. Material and methods

2.1. Pilot study

A pilot study was performed to determine whether it was possible to quantify fat content in field-collected *I. ricinus* ticks, followed by *Borrelia* detection. We wanted to make sure that the different steps used to remove fat from ticks (such as exposure to high temperature and 3-day chloroform immersion, described in Section 2.3) did not interfere with the efficiency of *Borrelia* detection and genospecies identification in tick carcasses (described in Sections 2.4 and 2.5). The pilot study was conducted on 80 nymphs collected in May ($n = 40$) and September ($n = 40$) 2010, and maintained as described in Section 2.2.

2.2. Tick collection and maintenance

The sampling site was a mixed forest (dominated by deciduous trees) situated 600 m above sea level on the south-facing slope of Chaumont Mountain, Neuchâtel, Switzerland (47°00′ N, 6°57′ E). Nine hundred questing *I. ricinus* nymphs were sampled by flagging low vegetation using a 1 m^2 cotton towelling flag on April 15th 2011. In the laboratory, ticks were kept over water in a box with a tight-fitting lid (98% relative humidity; RH) and placed within a cold chamber at 4 °C in the dark for 3 months as described in Crooks and Randolph (2006).

2.3. Fat content quantification

The fat content of each of 900 nymphs was measured as described in Randolph and Storey (1999). Ticks were incubated in an oven at 70 °C for 24 h to remove the water from their body. Following incubation, the dried ticks were immediately transferred to a desiccator until subsequent weighing (initial dry mass). Each nymph was individually weighed to the nearest 0.1 μg using an ultra-microbalance (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland) kept in a room with controlled temperature, atmospheric pressure and light intensity. To remove the fat content, each nymph was immersed in chloroform for 24 h and this step was repeated twice. Nymphs were re-dried in an oven at 70 °C for 24 h, transferred to a desiccator and re-weighed to obtain the fat-free dry mass (hereafter referred to as "body size") and thereby the fat content (Crooks and Randolph, 2006). Tick carcasses were stored at −80 °C for *Borrelia* detection and genospecies identification.

Randolph et al. (2002) observed that fat content was positively correlated with tick body size. Fat content was therefore corrected for body size using the two different methods described by Randolph et al. (2002) and Crooks and Randolph (2006) to facilitate comparisons between our study and the published literature. The simpler correction is achieved by dividing fat content by body size (Crooks and Randolph, 2006), whereas using the square root of fat content as the numerator is necessary to achieve a more complete correction for body size (referred to as the 'fat index' by Randolph et al., 2002).

2.4. Borrelia detection and quantification using quantitative PCR

DNA was extracted from tick carcasses using ammonium hydroxide as previously described (Herrmann and Gern, 2010, 2012). Briefly, ticks (each one in its own Eppendorf tube) were boiled for 15 min at 100 °C in 100 μl of 0.7 M NH_4OH and then cooled for a few minutes. The ticks were boiled again for 15–20 min at 100 °C in the open tubes to evaporate the ammonia and cooled at room temperature. Negative controls were included during DNA extraction, which consisted of reagents without template DNA.

A quantitative PCR amplifying a fragment of the flagellin gene (Schwaiger et al., 2001) was used to detect and quantify *Borrelia* DNA in each field-collected tick that had been subjected to fat content analysis. The strain *B. afzelii* NE1817 was used as the quantification standard as follows. Spirochete concentration in culture was estimated using a Helber chamber. To extract spirochete DNA, the culture was washed twice with PBS/$MgCl_2$, the pellet was resuspended in 30 μl of distilled water and heated for 15 min at 100 °C (Postic et al., 1994). The *Borrelia* DNA stock was aliquoted at 10^5 spirochetes per μl and stored at −20 °C. Serial dilutions were made from stored spirochete DNA in order to obtain five standard solutions with concentrations of *Borrelia* DNA ranging from 10 to 10^5 copies per μl.

The 50-μl quantitative PCR mixture (Schwaiger et al., 2001; Herrmann and Gern, 2010, 2012) consisted of 10 μl of 5× buffer, 5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 1 μl of 20 μM FlaF1A forward primer, 1 μl of 20 μM FlaR1 reverse primer, 1 μl of 10 μM FlaProbe1 probe, 0.25 μl of HotStart Taq Polymerase (Kapa Biosystems, Woburn, MA, USA), 20.75 μl of water and 10 μl of the extracted DNA. In each run, one extraction negative control (10 μl, see above), one PCR negative control (10 μl of water instead of 10 μl of the extracted DNA) and three series of the five standards were included. Following an incubation step at 95 °C for 10 min, the samples were submitted to 45 repeated amplification cycles (95 °C for 15 s, 60 °C for 1 min) (Schwaiger et al., 2001) in an iCycler Optical Module (Bio-Rad, Reinach, Switzerland) using strip PCR tubes and flat caps (Scientific Specialties Inc, Lodi, CA, USA).

2.5. Borrelia genospecies identification

PCR and Reverse Line Blotting (RLB) were used to identify the *Borrelia* genospecies in the ticks that were positive for *Borrelia* DNA in the quantitative PCR as described in Herrmann and Gern (2010, 2012). The variable spacer region between two repeated copies of the 23S and 5S ribosomal genes was amplified (Alekseev et al., 2001).

The 25-μl touchdown PCR mixture (Herrmann and Gern, 2010, 2012) consisted of 2.5 μl of 10× buffer, 0.5 μl of 10 mM dNTPs, 0.5 μl of 10 μM 23S-Bor forward primer, 0.5 μl of 10 μM B5S-Bor reverse primer, 0.125 μl of Taq Polymerase (Qiagen, Basel, Switzerland), 15.875 μl of water and 5 μl of the extracted DNA. Positive and negative controls were included in each PCR run. Positive controls consisted of isolates of *B. afzelii* (NE632), *B. lusitaniae* (PotiB1), *B. burgdorferi* s.s. (B31) or *B. garinii* (NE11) and negative controls contained water. PCR amplifications were run in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touchdown PCR program (Burri et al., 2007). The amplification started with an initial denaturation at 94 °C for 3 min, followed by denaturation at 94 °C for 20 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. During the subsequent cycles, the annealing was lowered by 1 °C until it reached 52 °C. Another 40 repeated amplification cycles (94 °C for 20 s, 52 °C for 30 s, 72 °C for 30 s) followed the touchdown program. The PCR ended with a final extension at 72 °C for 7 min.

For *Borrelia* identification by RLB, PCR products were hybridised to three generic oligonucleotide probes (SL1, SL2, SL3) and 14 specific oligonucleotide probes (SS, GA, GANE, AF, VSNE, LusiNE, RFLNE, SpiNE, SpiNE1, LusiNE1, LusiNE2, GANE1, BisNE, BisNE1) (Poupon et al., 2006; Gern et al., 2010) blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd, Portsmouth, UK) using a Miniblotter 45 (Immunetic, Cambridge, MA, USA). Hybridization was visualised by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham Biosciences Europe, Switzerland) and by exposing the membrane to X-ray film (Hyperfilm, GE Healthcare, UK).

2.6. Statistical analysis

2.6.1. Pilot study

2.6.1.1. Effect of season on the body size-fat content relationship. An independent two-sample *t*-test was used to test whether there was a difference in body size between spring and autumn nymphs. Analysis of co-variance (ANCOVA) was used to test the following three hypotheses: (i) whether there was a relationship between body size and fat content, (ii) whether fat content for a given body size changed with season, and (iii) whether the slope of the body size-fat content relationship was the same between spring and autumn nymphs. As tick body size and fat content are related to each other via a power relationship, where fat content = a (body size)^b (a and b being two constants), both variables were log-transformed to control the variances and to linearise the relationship, where log (fat content) = log(a) + b $*$ log(body size).

2.6.2. Main study

2.6.2.1. Effect of Borrelia genospecies on spirochete load in infected ticks. The effect of genospecies on spirochete load was analysed for the subset of ticks that were infected with at least one of the four most common *Borrelia* genospecies ($n = 183$ ticks). Infections by *B. bavariensis* ($n = 5$) and *B. miyamotoi* ($n = 6$) were excluded from the statistical analyses due to their low prevalence. Parasite burden variables such as spirochete load are almost never normally distributed and are best modelled using generalised linear models with a negative binomial distribution (Wilson et al., 1996; Wilson and Grenfell, 1997). The glm.nb() function in R was therefore used to test whether a particular genospecies alone or in combination with another genospecies (interaction) had a positive or negative effect on *Borrelia* spirochete number. The glm.nb() function was also used to test whether there was a relationship between spirochete load and body size or fat content.

2.6.2.2. Effect of Borrelia infection status on the body size-fat content relationship. An independent two-sample *t*-test was used to test whether there was a difference in body size between *Borrelia*-infected and uninfected nymphs. ANCOVA was used to test the following three hypotheses: (i) whether there was a linear relationship between body size and fat content (after log-transforming these two variables), (ii) whether *Borrelia* infection status influenced tick fat content for a given body size, and (iii) whether the slope of the body size-fat content relationship was the same between *Borrelia*-infected and uninfected ticks. For the subset of nymphs ($n = 183$) infected with the four most common *Borrelia* genospecies (i.e. *B. afzelii*, *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana*), ANCOVA was also used to test whether *Borrelia* genospecies influenced the slope or the intercept of the body size-fat content relationship.

All statistics were calculated with R for Mac OS X (R Development Core Team, 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org). All means are reported with their standard error. The *P* value was considered significant when below 0.05.

3. Results

3.1. Pilot study

Tick body size of questing *I. ricinus* nymphs was slightly higher in the spring (72.9 ± 16.5 μg; $n = 40$) than the autumn sample (67.9 ± 14.7 μg; $n = 40$) but this difference was not significant ($t = 1.509$, df = 78, $P = 0.135$). The ANCOVA found no significant interaction between season and tick body size ($F_{1,76} = 2.052$, $P = 0.156$) and there was no significant relationship between body size and fat content ($F_{1,77} = 1.618$, $P = 0.207$). However, the mean fat content of autumn nymphs (13.2 ± 10.7 μg; range = 2–47 μg) was 1.7 times greater than that of the spring nymphs (7.8 ± 7.1 μg; range = 1–31 μg) and this difference was statistically significant ($F_{1,77} = 6.452$, $P = 0.013$). The estimates of fat content divided by body size and the fat index following Randolph et al., 2002 are shown in Table 1.

With respect to *Borrelia* infection, 25.0% (10/40) of the spring nymphs and 27.5% (11/40) of the autumn nymphs were infected. The mean and median spirochete numbers were higher in spring than in autumn (Table 2). Identification of *Borrelia* genospecies was successful in nine of 10 spring nymphs and 10 of 11 autumn nymphs. Spring nymphs were infected with *B. afzelii* ($n = 7$), *B. valaisiana* ($n = 1$) and unidentified *B. burgdorferi* s.l. ($n = 1$). Autumn individuals were infected with *B. garinii* ($n = 3$), *B. afzelii* ($n = 1$), *B.*

Table 1

Fat content of *Ixodes ricinus* nymphs in the present study and in previously published studies conducted in Switzerland and the United Kingdom. For each study we report three fat content variables (i) absolute fat content (measured in μg), (ii) fat content corrected for size (following Crooks and Randolph, 2006), and (iii) the fat index (following Randolph et al., 2002). For each of the three fat content variables we report the mean, SD and median. *n* refers to the number of nymphs in the study. Cells with data missing are left blank.

Study	Season and year	*n*	Absolute fat content		Fat content corrected for size		Fat index	
			Mean (±SD)	Median	Mean (±SD)	Median	Mean (±SD)	Median
Pilot study	Spring 2010	40	7.8 (±7.1)	5.5	0.118 (±0.119)	0.077	0.037 (±0.021)	0.032
	Autumn 2010	40	13.2 (±10.7)	9.5	0.205 (±0.171)	0.162	0.052 (±0.028)	0.047
Main study	Spring 2011	900	7.8 (±4.3)	7.1	0.119 (±0.069)	0.106	0.041 (±0.013)	0.039
Herrmann and Gern (2010)	Spring 2009	40	7.1 (±6.8)	5	0.100 (±0.103)	0.071	0.034 (±0.018)	0.033
Herrmann and Gern (2012)	Spring 2010	40	9.3 (±6.6)	8	0.137 (±0.099)	0.115	0.043 (±0.018)	0.039
Steele and Randolph (1985)	Spring 1982		∼6.0					
Randolph et al. (2002)	Spring 1998	36					∼0.015	
	Autumn 1998	15					∼0.032	
Crooks and Randolph (2006)	Spring 2004	40	4.7 (±2.7)					

Table 2
Borrelia prevalence, mean and median spirochete number in questing *Ixodes ricinus* nymphs sampled during three different years in Neuchâtel, Switzerland.

Study	Season and year	Number of tested ticks	Prevalence of *Borrelia*-infected ticks	Spirochete number per tick	
				Mean	Median
Pilot study	Spring 2010	40	10 (25.0%)	41,475	2,410
	Autumn 2010	40	11 (27.5%)	14,752	1,390
Main study	Spring 2011	900	191 (21.2%)	15,556	3,410
Herrmann and Gern (2010)	Spring 2009	500	126 (25.5%)	18,638	2,760
Herrmann and Gern (2012)	Spring 2010	1500	448 (29.9%)	33,971	4,300

valaisiana ($n = 1$), and mixed infections of *B. garinii* and *B. valaisiana* ($n = 4$), and *B. afzelii* and *B. miyamotoi* ($n = 1$).

3.2. Main study

3.2.1. Fat content quantification

Mean and median tick body size of questing *I. ricinus* nymphs sampled in spring 2011 were 66.4 ± 13.2 µg and 66 µg, respectively ($n = 900$; range = 23.6–128.4 µg). Mean fat content in these nymphs was 7.8 ± 4.3 µg while median fat content was 7.1 µg (range = 1–29 µg). The estimates of fat content corrected for body size and the fat index following Randolph et al. (2002) are shown in Table 1.

3.2.2. Borrelia infection in ticks

Of the 900 questing *I. ricinus* nymphs, 21.2% ($n = 191$) were infected with *B. burgdorferi* s.l. Identification of *Borrelia* genospecies was possible in 188 of 191 infected nymphs. Ticks were primarily infected by one *Borrelia* genospecies (89.9%, 169/188). Infections by two *Borrelia* genospecies were considerably less frequent (10.1%, 19/188) while no tick was infected by three or more genospecies. Among single infections, *B. afzelii* was the most common (64.5%, 109/169), followed by *B. valaisiana* (13.6%, 23/169), *B. garinii* (11.8%, 20/169), *B. burgdorferi* s.s. (7.1%, 12/169) and *B. bavariensis* (3.0%, 5/169). The 19 mixed infections consisted of three types: *B. afzelii* and *B. miyamotoi* (31.6%, 6/19), *B. afzelii* and *B. burgdorferi* s.s. (36.8%, 7/19), and *B. garinii* and *B. valaisiana* (31.6%, 6/19). The genospecies *B. bissettii*, *B. lusitaniae* and *B. spielmanii* were not detected.

3.2.3. Effect of Borrelia genospecies on spirochete load in infected ticks

The mean spirochete load in *Borrelia*-infected nymphs was 15,556 spirochetes per nymph ($n = 191$ nymphs) while the median spirochete load was 3,410 spirochetes (range = 1–496,000 spirochetes; Table 2). The mean spirochete load of *B. garinii* infections (22,442) was higher than *B. afzelii* (11,144), *B. burgdorferi* s.s. (9,886) and *B. valaisiana* (7,116) infections. However, the generalised linear model (GLM) analysis found no effect of any of the genospecies or their two-way interactions on the spirochete load inside the tick ($\chi^2 = 5.93$, df = 5, $P = 0.313$; Fig. 1). Similarly, the GLM found no effect of body size or fat content on the spirochete load inside the tick ($\chi^2 = 3.78$, df = 2, $P = 0.151$). For the GLM analyses, the residual deviances were similar to the residual degrees of freedom, suggesting that the negative binomial error function was a reasonable fit for the spirochete load data.

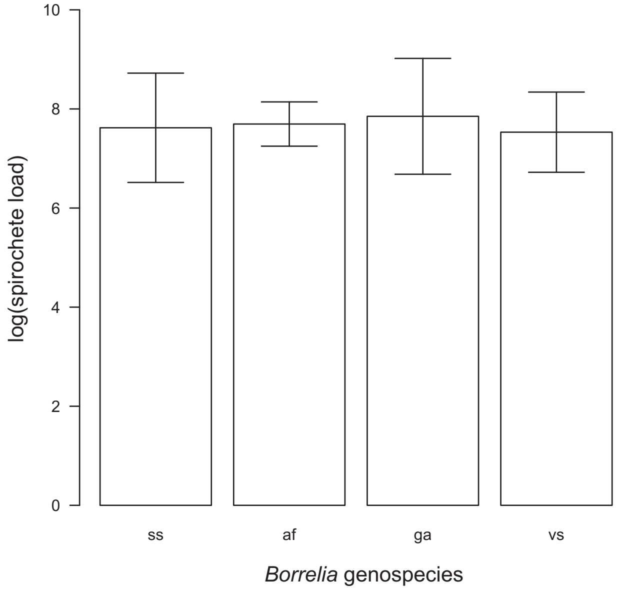

Fig. 1. Log-transformed spirochete loads for the four most common *Borrelia* genospecies: *Borrelia afzelii* (af), *Borrelia burgdorferi* sensu stricto (ss), *Borrelia garinii* (ga) and *Borrelia valaisiana* (vs) found in *Ixodes ricinus* nymphs sampled in Neuchâtel, Switzerland. Shown are the means and the 95% confidence intervals.

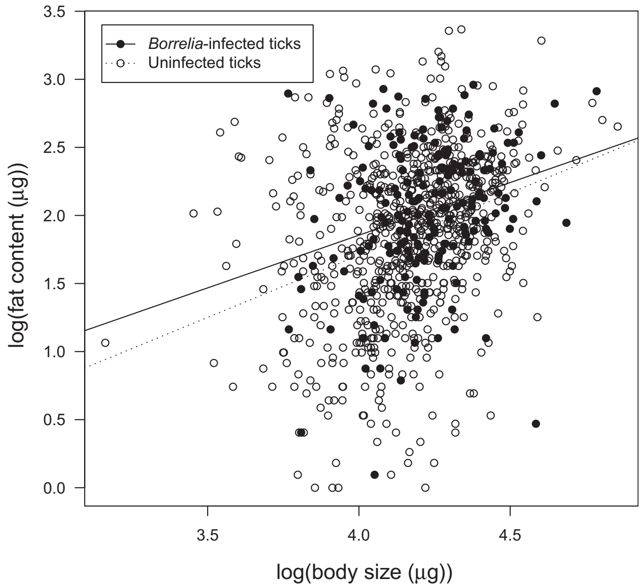

Fig. 2. The relationship between fat content and tick body size for uninfected and *Borrelia*-infected *Ixodes ricinus* nymphs.

3.2.4. Effect of Borrelia infection on the body size – fat content relationship

Borrelia-infected nymphs had a larger body size (69.3 ± 12.3 µg; $n = 191$) than uninfected individuals (65.7 ± 13.3 µg; $n = 709$) and the difference was highly significant ($t = 3.639$, df = 898, $P < 0.001$). The ANCOVA found no significant interaction between *Borrelia* infection status and tick body size ($F_{1,896} = 0.303$, $P = 0.582$), indicating that the slopes of the fat content versus tick body size regressions were the same between infected and uninfected ticks (Fig. 2). Larger nymphs had more fat as there was a positive and statistically significant relationship between body size and fat content ($F_{1,897} = 89.979$, $P < 0.001$). The main effect of infection status was also statistically significant ($F_{1,897} = 6.265$, $P = 0.012$), indicating that infected ticks had a higher fat content than uninfected ticks after controlling for body size. For an average body size of 66.4 μg, the mean fat content of a *Borrelia*-infected tick (7.40 μg) was 12.1% higher than that of an uninfected tick (6.60 μg). The lines of best fit are as follows: uninfected ticks: log (fat content) = −1.854 + 0.891 ∗ log (body size); infected ticks: log (fat content) = −1.738 + 0.891 ∗ log (body size).

There was no effect of *Borrelia* genospecies on the slope ($F_{3,156} = 1.176$, $P = 0.321$) or intercept ($F_{3,159} = 0.978$, $P = 0.405$) of the body size-fat content relationship in the subset of ticks infected with the four most common *Borrelia* genospecies ($n = 183$). Thus for a given body size, infected ticks had a similar fat content regardless of the *Borrelia* genospecies they harboured. As before, there was a significant and positive relationship between body size and fat content ($F_{1,159} = 11.062$, $P = 0.001$).

4. Discussion

Parasites of arthropods can affect the resource levels of their hosts. For example, in a triatomine-trypanosomatid system, *Rhodnius prolixus* bugs infected by the parasite *Trypanosoma rangeli* show a twofold increase in their amount of lipids compared with uninfected individuals (Ferreira et al., 2010). In this study, we found that for a given body size, *Borrelia*-infected *I. ricinus* nymphs had a higher fat content than uninfected ticks. Similarly, Gassner (2010) showed that infected nymphs had a higher fat content than uninfected nymphs, but that study was restricted to *I. ricinus* nymphs infected by *B. afzelii*. Here, ticks were infected by various *Borrelia* genospecies (*B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. bavariensis* and by *B. miyamotoi*, a relapsing fever like spirochete), but for a given body size, infected ticks had a similar fat content regardless of the *Borrelia* genospecies they harboured.

The higher fat content in *Borrelia*-infected nymphs may be caused by a variety of processes operating on tick-host interactions (blood feeding), on tick physiology (digestion, and moulting), and/or tick behaviour. The presence of *Borrelia* spirochetes in the vertebrate host might modify the quantity or quality of the larval blood meal, resulting in higher fat content in the nymph. Previous work on *Babesia microti* found that this tick-borne parasite increased the blood meal size and thus the engorged weight in immature *Ixodes trianguliceps* (Randolph, 1991) and *I. scapularis* ticks (Hu et al., 1997). One potential explanation for this phenomenon is that the *B. microti* parasites suppress the acquired immune response of rodents against ticks. Previous studies have shown that rodents can develop an acquired immunity to ticks, which reduces the quality of the tick blood meal (Oberem, 1984; Dizij and Kurtenbach, 1995) and that *B. microti* suppresses the rodent immune system (Purvis, 1977; Gray and Phillips, 1983). Thus, ticks feeding on *Babesia*-infected, immunosuppressed mice may obtain larger blood meals (resulting in higher fat content) than ticks feeding on uninfected mice. Although it is not known whether a similar mechanism operates at the interface between *Borrelia*, *I. ricinus* and the reservoir host, there is evidence that *Borrelia* suppresses the immune response in vertebrate hosts (Giambartolomei et al., 1998; Rupprecht et al., 2008), which presumably would also help blood feeding of ticks. Additionally, *Borrelia* might modify the quality of the blood meal in the vertebrate host (e.g. by increasing the glucose concentration) thereby increasing tick fat content. However, this phenomenon has never been reported in the literature.

Alternatively, *Borrelia* spirochetes might alter tick gene expression in such a way that fat storage is increased. A recent study found that entomopathogenic fungal parasites changed the lipid profiles in cattle ticks (Angelo et al., 2010) suggesting that parasites can change the expression of genes related to fat metabolism and fat levels in ticks. Similar phenomena might take place via mechanisms related to the digestion and moulting process in *Borrelia*-infected ticks, resulting in increased fat content in nymphs harbouring spirochetes. The *4E-BP* gene involved in lipid storage (Kume et al., 2012) or the *ATG* genes involved in autophagy (Umemiya-Shirafuji et al., 2010) in ticks might be potential targets for spirochete manipulation.

Finally, *Borrelia* spirochetes might alter behaviour of ticks in a way that conserves fat reserves. Fat reserves can be metabolised to provide energy as well as water since the latter is a by-product of fat metabolism. *Ixodes ricinus* uses this energy for questing activity and maintenance of water balance under desiccating environmental conditions (Van Es et al., 1998; Randolph and Storey, 1999). A number of studies have shown that *Borrelia*-infected nymphs move less than uninfected individuals (Lefcort and Durden, 1996; Alekseev et al., 2000; Herrmann and Gern, 2012). Therefore, we expect *Borrelia*-infected, low-activity ticks to have higher fat levels than their uninfected, high-activity counterparts. In conformity with this hypothesis, *Borrelia* induces low activity levels in the tick, thereby conserving the tick fat reserves. In addition, energy is better conserved by infected ticks since their larger body size enhances water retention. Indeed, since surface to volume ratio is smaller in larger individuals, larger ticks lose proportionally less water than smaller ticks. Our study thus suggests that *Borrelia*-infected ticks can spend more time questing, thereby increasing their chances of finding a host compared with uninfected ticks, which have to return more frequently to the leaf litter to rehydrate. There is the potential for positive feedback mechanisms where larger, *Borrelia*-infected ticks do not suffer the costs of rehydration as often as smaller, uninfected ticks, which allows them to further conserve their fat supplies.

The present study also suggests that *Borrelia*-infected ticks should be more tolerant to desiccation than uninfected ticks, which is consistent with the results from our previous studies: (i) that *Borrelia*-infected ticks survived better under desiccating conditions (Herrmann and Gern, 2010) and (ii) that *Borrelia*-infected ticks were less likely to move to an environment that was favourable for maintaining water balance (Herrmann and Gern, 2012). Our results may also explain observations by Naumov (2003) who reported longer survival in *I. ricinus* and *I. persulcatus* ticks infected with *B. burgdorferi* compared with uninfected individuals. Ticks with a higher fat content relative to body size have larger energy reserves and therefore a higher likelihood of survival.

Parasites consume host energy resources. In *Borrelia*-infected larval ticks, the spirochete load increases dramatically following the blood meal (Gern et al., 1990; Piesman et al., 1990; De Silva and Fikrig, 1995) presumably by consuming the tick energy reserves. However, no significant relationship between tick energy reserves and spirochete load was found, suggesting that the spirochete load represents a negligible energetic cost for the tick. To check this hypothesis, we calculated how much energy it takes to grow one spirochete using tick glucose under anaerobic conditions. *Borrelia burgdorferi* uses glycolysis to meet its energetic demands for maintenance and growth (Von Lackum and Stevenson, 2005) as the genome lacks enzymes for both the citric acid cycle and oxidative phosphorylation (Fraser et al., 1997). A large cylindrically shaped spirochete (length: 30 μm, diameter: 0.3 μm) has a volume of 2.121 μm^3 and a dry mass of $5.089 * 10^{-13}$ g (cells have a density of 1.2 g/cm^3 of which 80% is water). Under anaerobic conditions, it takes $5.089 * 10^{-12}$ g of glucose to grow a single spirochete, corresponding to $8.143 * 10^{-11}$ kJ of energy. The average tick has a fat content of 7.8 μg, which corresponds to $2.886 * 10^{-4}$ kJ of energy. Thus the energy requirements to grow the median population of 3,410 spirochetes using glucose under anaerobic conditions corresponds to 0.10% of the total fat reserves of the nymphal tick. These bioenergetics calculations suggest that the median spirochete population consumes a very small portion of the tick energy budget and provide a potential explanation for the lack of a negative relationship between tick energy reserves and spirochete load.

The present study assumes that *Borrelia* infection is causal in influencing variation in tick body size and fat content. However, the alternative hypothesis that tick body size could influence the probability that a tick becomes infected cannot be excluded. For example, larger ticks might take larger blood meals, which are more likely to contain spirochetes. However, this explanation is unlikely as previous studies have shown that most of the variation in host-to-tick transmission rates is caused by differences among host individuals (Gern et al., 1994) and, in particular, by differences in spirochete load inside the host tissue (Raberg, 2012). In addition, the possibility that natural selection in the field might have caused differences in relative fat content between *Borrelia*-infected and uninfected ticks prior to sampling cannot be excluded. For example, if *Borrelia* spirochetes exert some sort of survival cost on ticks, only those individuals with a high fat content (relative to body mass) survived to be included in the sample. This explanation seems unlikely since our bioenergetic calculations suggest that the median spirochete population consumes a negligible portion of the tick energy budget. Sampling bias could also produce the observed results if flagging is more likely to capture *Borrelia*-infected ticks with high fat content than low fat content but no such fat content-related sampling bias exists for uninfected ticks. Future studies should include manipulative experiments to test whether ticks feeding on *Borrelia*-infected hosts are larger and have higher fat content than ticks feeding on control hosts.

The pilot study showed that the protocol for fat content quantification did not interfere with the subsequent protocol for *Borrelia* detection and genospecies identification. The mean and median spirochete loads in individuals collected in spring and autumn (Table 2) were similar to the levels observed in an earlier study (unpublished data; spring: mean = 45,850, median = 3,390 spirochetes per tick; autumn: mean = 16,231, median = 2,275 spirochetes per tick) where nymphs were not tested for fat content prior to the *Borrelia* infection analysis. *Borrelia* prevalences in the pilot study (spring: 25%; autumn: 17.5%) were similar to the unpublished study (spring: 26.4%, 595/2250; autumn: 26.7%, 120/450) and the prevalences of the *Borrelia* genospecies were similar to two previously published studies in the same area (Herrmann and Gern, 2010, 2012). Finally, the fat content of the spring nymphs was similar to that previously described in Switzerland (Table 1). The similarity between the pilot study and the literature confirmed our belief that the *Borrelia* quantification and identification results of the main study were not biassed by the upstream fat content protocol.

To date, studies quantifying fat content in *I. ricinus* have been conducted in Wales (Steele and Randolph, 1985), England (Randolph and Storey, 1999; Randolph et al., 2002; Crooks and Randolph, 2006), and Switzerland (Herrmann and Gern, 2010, 2012). In Switzerland, the fat content of spring nymphs was 1.5 to 2.0 times higher than in the United Kingdom (UK) (Table 1). The *Borrelia* infection rate of nymphs in Switzerland (>21.2%) was much higher than that in the UK (<12.4% according to Vollmer et al., 2011). Thus one potential explanation is that Swiss larvae are more likely to feed on *Borrelia*-infected hosts and thus moult into *Borrelia*-infected nymphs with higher fat content. Ticks in Switzerland and the UK also may have evolved different body sizes and/or fat content levels in response to the very different climatic conditions in these two countries with UK ticks experiencing much higher rainfall (Gray, 1998). Randolph and Storey (1999) observed that ticks under dry conditions consume their fat resources twice as fast as those under wet conditions, which might explain why Swiss ticks have evolved to have more fat than their UK counterparts.

In the pilot study, tick body size was slightly (although not significantly) higher in the spring than the autumn sample. This result is consistent with a previous study showing that larger ticks become active earlier in the spring while ticks questing in autumn reflect the full size range (Randolph et al., 2002). Fat content in autumn nymphs was 1.4 to 1.9 times higher than spring nymphs. This phenomenon has been reported previously in southern England (Randolph et al., 2002). The autumn nymphs have higher fat content because they obtained their larval meal that same summer whereas the spring nymphs obtained their larval blood meal the previous summer and consumed their fat stores during the winter months.

We found a strong positive association between *Borrelia* infection status and body size corrected for fat content. We suggest that *Borrelia* infection increases tick fat content through mechanisms operating in the host (e.g. *Borrelia*-induced immune suppression of anti-tick immunity) or the tick (e.g. *Borrelia*-induced manipulation of tick fat metabolism). Higher fat content allows *Borrelia*-infected ticks to better maintain their water balance under desiccating conditions. This explanation accounts for our previous observations that *Borrelia*-infected ticks are more likely to survive desiccating conditions than uninfected ticks and that the former will quest for a longer period without having to move to an environment that is favourable for maintaining water balance (Herrmann and Gern, 2010, 2012). Additional studies are needed and expected on this topic so as to understand the multiple ways in which *Borrelia* and *Ixodes* have evolved to benefit from their close and long-lasting association.

Acknowledgements

We would like to thank M. Pluess, A. Schmid and O. Rais for their help in collecting ticks, as well as P. Fuchs and the Swiss Federal Office of Metrology (METAS) for access to useful material such as the ultra-microbalance. Also thanks to Gerrit Voordouw for help with the bioenergetics calculations. This study is part of the Ph.D. thesis of C. Herrmann and has been partly supported by the Swiss National Science Foundation grant (FN 310030_127064) and the Canton de Neuchâtel, Switzerland.

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4.4 Paper 4

Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations. 2013. Ticks and Tick-Borne Diseases 4: 445-451

Coralie Herrmann and Lise Gern

In the context of climate change where winter conditions are changing in the main distribution area of *I. ricinus* in Switzerland, i.e. decrease of snowfall frequency and quantity (North et al., 2007), reduction of day-to-day temperature variability due to loss of coldest extremes in winter (Rebetez, 2001), it appeared interesting to determine what climatic factors influenced *I. ricinus* survival under winter conditions. Moreover, the fact that (1) *Anaplasma phagocytophilum* increases *I. scapularis* nymph resistance to cold by inducing the expression of a gene coding for antifreeze proteins (Neelakanta et al., 2010) and that we observed that (2) *Borrelia*-infected *I. ricinus* nymphs possess higher energy reserves than uninfected nymphs in our third study (Herrmann et al., 2013) incited us to test whether *Borrelia* infection influenced *I. ricinus* survival under winter conditions. In this study, we tested how tick age (spring- versus autumn-sampled), frequency of temperature variations (high versus low), cold severity and *Borrelia*-infection affected survival under cold conditions in *I. ricinus* nymphs under laboratory conditions.

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Ticks and Tick-borne Diseases 4 (2013) 445–451

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Survival of *Ixodes ricinus* (Acari: Ixodidae) nymphs under cold conditions is negatively influenced by frequent temperature variations

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ARTICLE INFO

Article history:
Received 4 February 2013
Received in revised form 30 April 2013
Accepted 2 May 2013
Available online 10 July 2013

Keywords:
Borrelia burgdorferi s.l.
Ixodes ricinus
Survival
Cold conditions
Lyme borreliosis
Winter

ABSTRACT

In this study, we tested the survival of *Ixodes ricinus* under cold conditions in the laboratory. We investigated how the frequency of temperature variations (from −5 °C or −10 °C to 13 °C), and infection with *Borrelia burgdorferi* sensu lato (s.l.) influenced survival of questing nymphs collected in spring and autumn 2011. In experiment 1, survival of 1760 nymphs was tested at −10 °C over a short period of time to simulate very cold winter conditions. In experiment 2, survival of 1600 nymphs was tested under cold condition (−5 °C) over a long period of time to simulate common winter conditions. Ticks used for survival tests at −5 °C were screened for *Borrelia* by quantitative PCR, and genospecies identification was achieved by reverse line blotting. Tick age and frequency of temperature variations had a highly significant effect on *I. ricinus* survival while *Borrelia* infection was marginally significant. Hence, survival rate was higher in younger (autumn) than older (spring) nymphs and in nymphs exposed to low rather than high-frequency temperature variations. *Borrelia*-infected ticks tended to survive better than their uninfected counterparts. These findings suggest that in nature (i) frequent temperature changes in winter threaten tick survival more importantly than very low temperatures, (ii) older (spring) ticks are less resistant to cold than younger (autumn) individuals, and (iii) *Borrelia* infection plays a marginal role in *I. ricinus* survival during winter conditions.

Introduction

The hard-bodied tick *Ixodes ricinus* (L.) (Acari: Ixodidae) is the vector of a variety of pathogens which have both medical and veterinary relevance, the most prevalent being *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of Lyme borreliosis. This tick, which is present in Europe, shows a seasonal activity. In spring, questing activity starts when the daily maximum temperature has reached 7 °C for around one week (MacLeod, 1936; Perret et al., 2000). In autumn, when temperatures fall below the temperature threshold of questing activity and day-lengths are shortened, ticks reduce their activity. Unfed ticks enter behavioural diapause (Belozerov, 1982) or more precisely winter quiescence (Dautel et al., 2008), which is a phase of inactivity that occurs when climatic conditions become harsh and stops as soon as favourable conditions are back. During this time, *I. ricinus* ticks remain in the leaf litter or in the upper layers of the soil (up to 5–7 cm deep) where temperatures are milder than on the vegetation (Dusbábek et al., 1971; Daniel et al., 1972; Gigon, 1985). Ticks belonging to the *I. ricinus* complex can resist temperatures falling far below 0 °C for short periods, as shown in nymphs of *I. persulcatus* and *I. nipponensis* (Fujimoto, 1994), *I. scapularis* (Burks et al., 1996; Vandyk et al., 1996; Neelakanta et al., 2010), and *I. ricinus* (MacLeod, 1935; Gigon, 1985; Dautel and Knülle, 1997). Increased winter temperatures due to global warming and climate change are affecting *I. ricinus* European populations, in particular, allowing ticks to colonize regions at higher latitudes (Lindgren et al., 2000; Jore et al., 2011; Jaenson et al., 2012), higher altitudes (Morán Cadenas et al., 2007; Jore et al., 2011), and allowing them to extend their winter activity (Dautel et al., 2008).

In this study, we evaluated in the laboratory how cold conditions and the frequency of exposure to cold temperatures may influence the survival of resident *I. ricinus* populations under winter conditions. In addition, it was recently reported that *Anaplasma phagocytophilum* increases *I. scapularis* nymph resistance to cold by inducing the expression of a gene coding for antifreeze proteins (Neelakanta et al., 2010). Consequently, we investigated whether *Borrelia* spirochaetes, known to be associated with a higher fat content in *I. ricinus* (Herrmann et al., 2013), may as well influence tick survival under cold conditions.

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http://dx.doi.org/10.1016/j.ttbdis.2013.05.002

Materials and methods

Tick collection and maintenance

The sampling site was a mixed (deciduous dominant) forest at 600 m above sea level in Neuchâtel on the Swiss Plateau, Switzerland (47°00′ N and 6°57′ E). Host-seeking *I. ricinus* nymphs were sampled by flagging low vegetation during several consecutive days in April 2011 (spring ticks) and September 2011 (autumn ticks). Spring ticks were maintained in tubes over water in a box with a tight-fitting lid (98% relative humidity; RH) in the dark within a cold chamber at 4 °C, as described in Crooks and Randolph (2006), for 5 months until autumn sampling was performed. Before the start of the survival tests, nymphs collected in spring (spring nymphs) and in autumn (autumn nymphs) were acclimated at 13 °C for a week, followed by another week at 4 °C and 98% RH.

Fat content quantification

Fat content was measured before the survival tests in a sample of 40 spring and 40 autumn nymphs to quantify the energy reserves as described by Herrmann et al. (2013). Briefly, ticks were dried at 70 °C for 24 h, weighed individually to the nearest 0.1 μg (UMT 5 Comparator, Mettler Toledo, Greifensee, Switzerland) (initial dry mass), washed in 3 changes of chloroform for 24 h each, redried at 70 °C for 24 h and reweighed (fat-free dry mass). Fat content was calculated according to Crooks and Randolph (2006), i.e. by subtracting fat-free dry mass (hereinafter referred to as body size, according to Randolph et al., 2002) from initial dry mass.

Survival tests

Survival tests were designed so that they recreated the winter conditions that ticks might experience in the field over short and long periods of time in Neuchâtel (located on the Swiss Plateau). Daily air temperature means of lowest and highest values of serial measures collected from 1864 to 2010 were obtained from MétéoSuisse. Data collected in Neuchâtel (47°00′ N and 6°57′ E, 485 m) from November to February were used to determine the temperature range. During the experiments, nymphs were kept in tubes (40 or 50 individuals per tube) at 98% RH when temperature was above 0 °C.

Experiment 1: survival under very cold conditions

Based on MétéoSuisse data, −10 °C was chosen as a very cold temperature, with an exposure time of 4 days. To test whether the frequency of temperature variations had an effect on tick survival under very cold conditions ($n = 1760$), we maintained 440 spring and 440 autumn nymphs at −10 °C (in a freezer) for 4 days (low-frequency temperature variations, LF) while additional 440 spring and 440 autumn nymphs were exposed every day to temperature variations (18 h at −10 °C, 2 h at 4 °C (in a cold chamber), 2 h at 13 °C (in a fridge), and 2 h at 4 °C) for 4 days (high-frequency temperature variations, HF).

Experiment 2: survival under cold conditions

Based on MétéoSuisse data and on results obtained in experiment 1, −5 °C was chosen as a cold temperature. We tested 2 frequencies of temperature variations on tick survival. Low-frequency temperature variations (LF) involved maintaining nymphs at −5 °C (in a freezer) for 9 days followed by 24 h during which they experienced temperature variations (−5 °C for 18 h, at 4 °C for 2 h, at 13 °C for 2 h, and at 4 °C for 2 h). This 10-day cycle was repeated until the end of the experiment. High-frequency temperature variations (HF) involved exposing nymphs to the same temperature variations, but every day, rather than every 10 days. In this experiment, 400 spring and 400 autumn nymphs were exposed to HF and an additional 400 spring and 400 autumn nymphs were exposed to LF ($n = 1600$). For each treatment group, the time to 50% mortality was determined using a subset of 100 spring and 100 autumn nymphs for each temperature variation frequency. The proportion of ticks surviving was assessed every day for the HF groups and every 10 days for the LF groups. The point in the temperature cycle at which survival was assessed was the point after which nymphs had spent 2 h at 13 °C after having spent 2 h at 4 °C. Survival was assessed on the mobility of ticks. Any immobile ticks were exposed to human skin and breath at room temperature. If they did not move after a few minutes, they were considered dead. The time at which 50% of ticks within a treatment group had died was recorded, and the ticks from that group were harvested for laboratory analyses. This protocol is termed experiment 2a in the following. The proportions of ticks surviving in the subset of ticks subject to daily or 10-daily monitoring continued to be assessed until all the ticks had died, and in the following this protocol is termed experiment 2b. All ticks submitted to survival tests at −5 °C (experiments 2a and 2b) were frozen at −80 °C until they were analyzed for *Borrelia* infection.

Borrelia detection and quantification by real-time PCR

DNA was extracted from ticks using ammonium hydroxide as previously described (Herrmann and Gern, 2010, 2012; Herrmann et al., 2013). A real-time PCR amplifying a fragment of the flagellin gene (Schwaiger et al., 2001) was used to detect and quantify *Borrelia* DNA in nymphs. *B. afzelii* NE1817 was used as quantification standard. Spirochaete concentration in culture was evaluated using the Helber chamber. DNA was extracted by heating spirochaetes for 15 min at 100 °C (Postic et al., 1994). Serial dilutions were made from stored spirochaete DNA in order to obtain 5 standard solutions with concentrations of *Borrelia* DNA ranging from 10 to 10^5 copies per μl (Herrmann and Gern, 2012).

The 50-μl real-time PCR mixture (Herrmann and Gern, 2010, 2012) consisted of 10 μl of 5 × buffer, 5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 1 μl of 20 μM FlaF1A forward primer, 1 μl of 20 μM FlaR1 reverse primer, 1 μl of 10 μM FlaProbe1 probe, 0.25 μl of HotStart Taq Polymerase (Kapa Biosystems, Woburn, MA), 20.75 μl of water and 10 μl of the extracted DNA. In each run, one extraction negative control (10 μl of extraction reagents without template DNA), one PCR negative control (10 μl of water), and 3 series of the 5 standards were included. Following an incubation step at 95 °C for 10 min, the samples were submitted to 45 repeated amplification cycles (95 °C for 15 s, 60 °C for 1 min) (Schwaiger et al., 2001) in an iCycler Optical Module (Bio-Rad, Reinach, Switzerland) using strip PCR tubes and flat caps (Scientific Specialties Inc., Lodi, CA).

Borrelia genospecies identification by PCR and RLB

PCR followed by RLB was used to identify the *Borrelia* genospecies in ticks that were detected positive by real-time PCR as described in Herrmann and Gern (2010, 2012) and in Herrmann et al. (2013). The variable spacer region between 2 repeated copies of the 23S and 5S ribosomal genes was amplified with primers 23S-Bor and B-5S-Bor (Alekseev et al., 2001) in a Tgradient Thermocycler 96 (Whatman Biometra, Göttingen, Germany) by using a touchdown PCR program (Burri et al., 2007). Positive [*B. afzelii* (NE632), *B. lusitaniae* (PotiB1), *B. burgdorferi* s.s. (B31), *B. valaisiana* (VS116), or *B. garinii* (N11)] and negative (water) controls were included in each PCR.

RLB analysis was performed using 15 oligonucleotide probes (Rijpkema et al., 1995; Poupon et al., 2006; Gern et al., 2010) blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd., Portsmouth, UK) held in a Miniblotter 45 (Immunetic, Cambridge, MA). Hybridization was visualized by incubating the membrane with enhanced chemiluminescence detection liquid (Amersham Biosciences Europe, Switzerland) and by exposing the membrane to X-ray film (Hyperfilm, GE Healthcare, UK).

Statistical analysis

Body size and fat content: we used an independent two-sample *t*-test to test whether there was a difference in body size between spring ($n = 40$) and autumn nymphs ($n = 40$). We used an ANCOVA to test 3 hypotheses: (1) whether there was a relationship between body size and fat content (a measure of tick energy reserves), (2) whether tick fat content for a given body size varied between spring and autumn nymphs, and (3) whether the slope of the body size-fat content relationship was the same between spring and autumn nymphs. We log-transformed the variables tick body size and fat content to control the variances and to linearize their relationship.

We analyzed the effect of *Borrelia* genospecies on spirochaete load in ticks that were infected with *B. afzelii*, *B. garinii*, or *B. valaisiana* ($n = 242$ nymphs). Infections by *B. burgdorferi* s.s., *B. bavariensis*, *B. miyamotoi*, and mixed infections were excluded from the statistical analyses due to their low frequency (Table 1). We used a two-way ANOVA to test whether sampling season and the presence of *B. afzelii*, *B. garinii*, or *B. valaisiana* in single infections influenced spirochaete load in nymphs. Since spirochaete load data were not normally distributed, we used Spearman's rank correlation to test whether spirochaete load among infected ticks varied over time during the survival tests.

Experiment 1, survival under very cold conditions (−10 °C): Simple chi-square tests were used to compare survival rate between spring and autumn nymphs and between high- and low-frequency temperature variations.

Experiment 2a, survival under cold conditions (−5 °C): Mean daily survival rate was calculated for spring and autumn nymphs exposed to HF and LF. Daily survival rate was calculated in each tube according to the following formula:

$$D_{50\%}\sqrt{\frac{\mathrm{liv}_{50\%}}{\mathrm{tot}}}$$

where $D_{50\%}$ is the number of days until 50% mortality was reached, $\mathrm{liv}_{50\%}$ is the number of living nymphs in the tube when 50% mortality was reached, and tot is the total number of nymphs in the tube. We used a paired *t*-test to test whether there was a difference in survival between *Borrelia*-infected ($n = 278$) and uninfected ticks ($n = 922$) without taking into account whether nymphs had been collected in spring or autumn, or exposed to HF or LF. The paired *t*-test compared the survival rate of infected nymphs to that of uninfected nymphs in the 24 tubes used in this experiment ($n = 24 \times 50$ nymphs $= 1200$). We then used an ANOVA to test whether survival ratio (= logarithm of the survival rate of infected nymphs divided by the survival rate of uninfected individuals) in the 24 tubes was different between spring and autumn nymphs and between nymphs exposed to high and low frequencies of temperature variation.

Experiment 2b, survival under cold conditions (−5 °C): We used GLMs with a Gamma error function to test whether sampling season, frequency of temperature variations, *Borrelia* infection, or the interactions between sampling season and *Borrelia* infection, and between frequency of temperature variations and *Borrelia* infection had an effect on the hazard rate, i.e. the daily probability of dying, of *I. ricinus* nymphs.

All statistics were calculated with R for Mac OS X (R Development Core Team, 2012).

Results

Body size and fat content in ticks collected in spring and autumn

Mean body size of questing *I. ricinus* was slightly higher in the autumn ($62.3 \pm 12.0\,\mu$g; $n = 40$) than in the spring nymphs ($62.0 \pm 12.0\,\mu$g; $n = 40$), but this difference was not significant ($t = -0.101$, df = 78, $p = 0.920$). The ANCOVA found no significant interaction between sampling season and tick body size ($F_{1,76} = 0.015$, $p = 0.903$). However, there was a significant relationship between body size and fat content ($F_{1,77} = 4.628$, $p = 0.035$). In addition, the mean fat content was 1.6 times higher in the autumn nymphs ($7.4\,\mu\text{g} \pm 3.5\,\mu\text{g}$) than in the spring nymphs ($4.6\,\mu\text{g} \pm 3.9\,\mu\text{g}$) ($F_{1,77} = 17.730$, $p < 0.001$).

Borrelia infection in ticks

B. burgdorferi s.l. was more frequently detected in spring (23.4%, 187/800) than in autumn ticks (20.0%, 160/800), but not significantly ($\chi^2 = 0.192$, df = 1, $p = 0.661$). Six *Borrelia* species were identified (Table 1). Both spring and autumn ticks were primarily infected by one *Borrelia* genospecies (87.9% and 83.0%, respectively). Infections by 2 *Borrelia* genospecies were less frequent (12.1% in spring and 14.3% in autumn ticks) while infections by 3 genospecies were only observed in autumn ticks (2.7%).

Spirochaete load among infected ticks

The mean spirochaete load in infected nymphs was 6584 and 5696 spirochaetes per nymph in spring ($n = 187$) and autumn samples ($n = 160$), respectively, while the median spirochaete load was 1060 and 536 spirochaetes per nymph, respectively. In spring nymphs, the mean spirochaete load of *B. afzelii* infections was higher than that of *B. garinii* and *B. valaisiana* infections (Table 1). In autumn nymphs, the mean spirochaete load of *B. garinii* infections was higher than that of *B. afzelii* and *B. valaisiana* infections. The ANOVA found no significant interaction between sampling season and the presence of *B. afzelii*, *B. garinii*, or *B. valaisiana* (df = 2, LR stat = 2.998, $p = 0.223$). Similarly, no difference in the spirochaete load was found between spring and autumn nymphs (df = 1, LR stat = 0.022, $p = 0.882$) or between ticks infected by these 3 *Borrelia* species (df = 2, LR stat = 3.921, $p = 0.141$).

Experiment 1: survival under very cold conditions

Nymphs submitted to low-frequency temperature variations survived better (spring ticks: 12.7%, 56/440; autumn ticks: 89.6%, 394/440) than those exposed to high-frequency temperature variations (spring ticks: 6.8%, 30/440; autumn ticks: 85.0%, 374/440) ($\chi^2 = 8.71$, df = 1, $p = 0.003$ spring ticks; $\chi^2 = 4.09$, df = 1, $p = 0.043$ autumn ticks) independently of the sampling season (Fig. 1). Moreover, autumn ticks survived significantly better at −10 °C (87.3% survival rate, 768/880) than spring individuals (9.8%, 86/880) ($\chi^2 = 1058.02$, df = 1, $p < 0.001$) independently of the frequency of temperature variations.

Experiment 2: survival under cold conditions

Experiment 2a: 50% survival in spring nymphs exposed to high-frequency temperature variations (HF) and low-frequency temperature variations (LF) was reached after 3 days (survival rate 57.3%, $n = 300$) and 5 days (survival rate 47.5%, $n = 300$), respectively. In autumn nymphs exposed to HF and LF, 50% survival

Table 1
Distribution of *Borrelia* genospecies and mean spirochaete load in questing *I. ricinus* nymphs collected in Neuchâtel, Switzerland, in spring and autumn 2011.

Borrelia genospecies[a]	Spring ($n = 800$ ticks)		Autumn ($n = 800$ ticks)	
	Infected ticks ($n = 173$)[b]	Mean spirochaete load[c]	Infected ticks ($n = 147$)[b]	Mean spirochaete load[c]
af	80 (46.2%)	8361	47 (32.0%)	5663
bav	8 (4.6%)	na	7 (4.8%)	na
ga	30 (17.3%)	5242	22 (15.0%)	9324
miy	0 (0.0%)	na	0 (0.0%)	na
ss	8 (4.6%)	na	9 (6.1%)	na
vs	26 (15.0%)	3156	37 (25.2%)	4719
Infection by one species	152 (87.9%)	7286	122 (83.0%)	6156
af & *bav*	1 (0.6%)	na	0 (0.0%)	na
af & *ga*	1 (0.6%)	na	1 (0.7%)	na
af & *miy*	2 (1.2%)	na	1 (0.7%)	na
af & *ss*	5 (2.9%)	na	4 (2.7%)	na
af & *vs*	1 (0.6%)	na	6 (4.1%)	na
bav & *vs*	1 (0.6%)	na	1 (0.7%)	na
ga & *ss*	2 (1.2%)	na	0 (0.0%)	na
ga & *vs*	8 (4.6%)	na	8 (5.4%)	na
Infection by 2 species	21 (12.1%)	7198	21 (14.3%)	3516
af & *ga* & *vs*	0 (0.0%)	na	3 (2.0%)	na
af & *miy* & *vs*	0 (0.0%)	na	1 (0.7%)	na
Infection by 3 species	0 (0.0%)	na	4 (2.7%)	na

na, not answered.

[a] af, *B. afzelii*; bav, *B. bavariensis*; ga, *B. garinii*; miy, *B. miyamotoi*; ss, *B. burgdorferi sensu stricto*; *vs*, *B. valaisiana*.

[b] *Borrelia* species identification by RLB in 173/187 infected spring and 147/160 infected autumn nymphs.

[c] Mean spirochaete number was not calculated when frequency was below 10.

was reached after 12 days (survival rate 48.8%, $n = 300$) and 24 days (survival rate 48.5%, $n = 300$), respectively. Mean daily survival rate was greater in autumn nymphs exposed to LF (0.96), followed by autumn nymphs exposed to HF (0.93), and spring nymphs exposed to LF (0.86). Mean daily survival rate was lowest in spring nymphs exposed to HF (0.84). No difference in survival was observed between nymphs infected by *Borrelia* ($n = 302$) and uninfected individuals ($n = 998$) ($t = 0.680$, df = 23, $p = 0.504$). Moreover, the ANOVA found no significant effect of sampling season (df = 1, $F = 0.0101$, $p = 0.921$) or frequency of temperature variations (df = 1, $F = 0.0439$, $p = 0.836$) on survival ratio of *Borrelia*-infected versus uninfected nymphs.

Experiment 2b: spring nymphs submitted to LF ($n = 100$) were all dead when their survival was assessed for the first time, i.e. 10 days after the start of the experiment and were therefore not used in the GLM. Mortality reached 100% after 7 days in spring nymphs ($n = 100$) exposed to HF and after 42 days and 80 days in autumn nymphs exposed to HF ($n = 100$) and LF ($n = 100$), respectively (Fig. 2). The GLM found that frequency of temperature variations and sampling season had both a significant effect on *I. ricinus* survival time (df = 1, deviance = 46.161, $p < 0.001$ and df = 1, deviance = 92.752, $p < 0.001$, respectively). In fact, the hazard rate, i.e. the daily probability of dying, of spring nymphs (0.293) was more than 4 times that of autumn nymphs (0.072), while the hazard rate of nymphs exposed to HF (0.072) was more than twice that of nymphs exposed to LF (0.028). In contrast, the interaction between frequency of temperature variations and *Borrelia* infection and the interaction between sampling season and *Borrelia* infection had no significant effect on survival time (df = 1, deviance = 0.0003, $p = 0.972$; df = 1, deviance = 0.0218, $p = 0.759$, respectively). The main effect of *Borrelia* infection was marginally significant on tick survival time (df = 1, deviance = 0.7111, $p = 0.077$) so that the

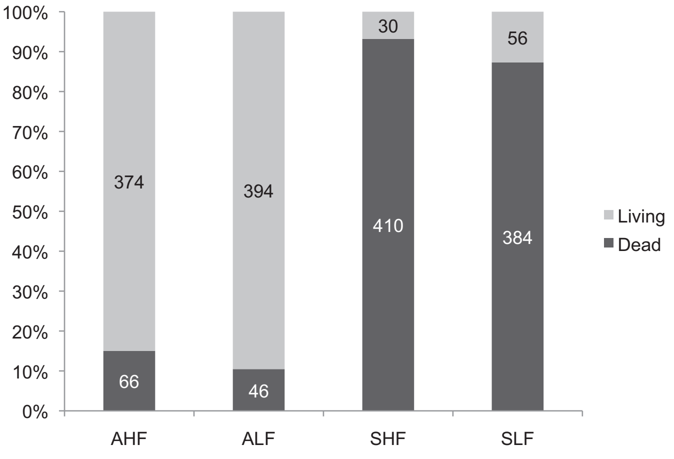

Fig. 1. Survival rate under very cold conditions (−10 °C) in autumn and spring nymphs exposed to high- and low-frequency temperature variations after 4 days. AHF and ALF: autumn nymphs exposed to high- and low-frequency temperature variations ($n = 440$ and $n = 440$), respectively. SHF and SLF: spring nymphs exposed to high- and low-frequency temperature variations ($n = 440$ and $n = 440$), respectively. Dead ticks are represented in dark grey while living ticks are represented in light grey.

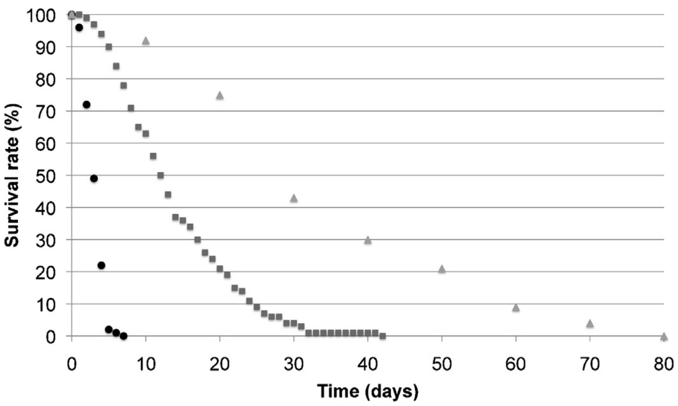

Fig. 2. Survival rate under cold conditions (−5 °C) in spring nymphs exposed to high-frequency temperature variations ($n = 100$, black circles), autumn nymphs exposed to high-frequency temperature variations ($n = 100$, dark grey squares), and autumn nymphs exposed to low-frequency temperature variations ($n = 100$, light grey triangles) over time (experiment 2b).

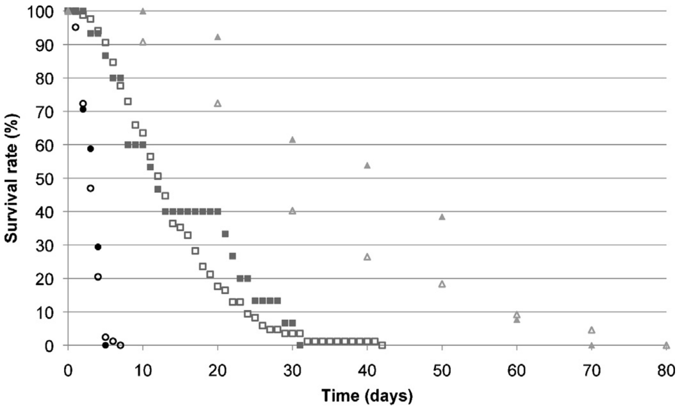

Fig. 3. Survival rate under cold conditions (−5 °C) in *Borrelia*-infected individuals (filled markers) versus uninfected individuals (empty markers) over time (experiment 2b). Spring nymphs exposed to high-frequency temperature variations (black circles), autumn nymphs exposed to high-frequency temperature variations (dark grey squares), and autumn nymphs exposed to low-frequency temperature variations (light grey triangles) are treated separately.

hazard rate of *Borrelia*-infected nymphs (0.066) was 1.1 times lower than that of uninfected nymphs (0.072) (Fig. 3). Fig. 3 shows that infected spring nymphs exposed to HF displayed a 10% higher survival rate on days 3 and 4, and autumn nymphs exposed to HF 22% on days 14–20. It also shows that infected autumn nymphs exposed to LF displayed a higher survival rate until day 50. At other points in time, *Borrelia*-infected and uninfected nymphs had similar survival rates. When the last infected nymph died, few uninfected ticks were still alive (2 spring nymphs exposed to HF, 3 and 4 autumn nymphs exposed to HF and LF, respectively). Among infected ticks ($n = 45$) spirochaete load was negatively correlated to the number of days that ticks survived ($S = 22248.27$, rho = −0.466, $p = 0.001$), i.e. the longer nymphs survived, the lower the spirochaete load they harboured (Fig. 4).

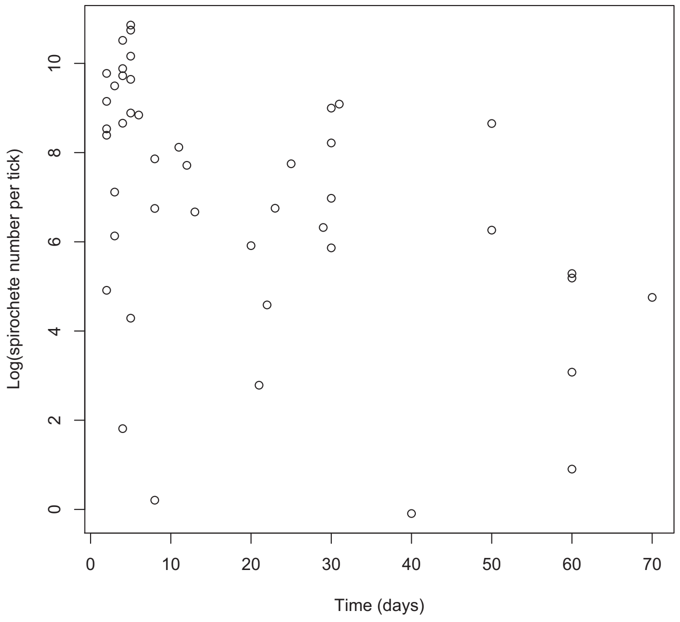

Fig. 4. Log-transformed spirochaete load depending on time (measured in days) that infected (spring and autumn) nymphs ($n = 45$) survived under cold conditions (−5 °C).

Discussion

Fat content in ticks collected in spring and autumn

Autumn nymphs possessed close to twice the fat content of spring individuals (representing tick energy reserves according to Randolph et al., 2002). It is likely that the lower fat content in spring nymphs was due to the fact that these ticks were older and belonged to a different cohort. Nymphs collected in spring had emerged the previous autumn and survived cold winter conditions, while nymphs collected in autumn had newly emerged. Moreover, a sampling bias might have occurred in spring. In April, we might have collected smaller ticks, because larger ticks had already been picked up by hosts, since it is known that larger ticks start questing earlier in the spring (Randolph et al., 2002).

The spring (4.6 μg) and autumn (7.4 μg) nymphs sampled in 2011 which were used in this study possessed lower mean fat content than nymphs collected during the same spring season (7.8 μg) and nymphs collected in autumn 2010 (13.2 μg) (Herrmann et al., 2013). One explanation for the latter differences is that fat content is known to decrease over time (Steele and Randolph, 1985; Herrmann and Gern, 2012). Since we maintained spring nymphs at 4 °C for 6 months versus 3 months in the study by Herrmann et al. (2013), the difference between spring ticks was possibly related to the difference in maintenance time. In addition, fat content is consumed faster when climate conditions are unfavourable, i.e. warmer and drier (Van Es et al., 1998; Randolph and Storey, 1999). Hence, nymphs collected in autumn 2011 probably consumed more fat to maintain their water balance than nymphs collected in autumn 2010 (Herrmann et al., 2013) since 2011 was warmer and drier than 2010 (MétéoSuisse).

Survival under cold and very cold conditions

Milder climate (especially during winter) due to climate change and global warming has allowed tick species such as *I. persulcatus*, *I. ricinus*, and *I. scapularis* to colonize regions at higher latitudes (Dennis et al., 1998; Lindgren et al., 2000; Yasyukevich et al., 2009; Jore et al., 2011; Jaenson et al., 2012; Leighton et al., 2012) and higher altitudes (Morán Cadenas et al., 2007; Jore et al., 2011) and has allowed *I. ricinus* to quest during winter months (Dautel et al., 2008). In such context, we focused on the influence of cold conditions on the survival of resident *I. ricinus* populations during winter. Brunner et al. (2012) reported that cold-related overwintering mortality did not impact greatly on *I. scapularis* populations. Similarly, we observed that *I. ricinus* ticks resisted constant cold conditions rather well. However, experiencing frequent temperature variations was more detrimental to *I. ricinus* survival than being maintained under constant cold temperature. Nymphs survived better when they stayed at −5 °C or −10 °C for a few days without temperature changes than when they experienced frequent temperature variations. These results are consistent with Gigon (1985), although he used more extreme conditions (temperature variations from −4.5 °C to 21 °C, −10.5 °C to 21 °C, or −36 °C to 21 °C) and smaller samples ($n = 100$). Lower survival rates in ticks exposed to frequent temperature variations (from quiescence to questing activity temperatures, i.e. temperatures below and over 7 °C) were likely due to the adaptations (costing energy) these variations generated. Moreover, when the temperature reaches an activity threshold (i.e. above 7 °C), tick metabolism increases. Energy reserves were probably depleted faster in ticks exposed to high-frequency temperature variations, resulting in shorter survival time. The fact that variations of temperature are more detrimental to ticks than constant temperature conditions is interesting in the context of climate change. During the past decades, a decrease in snowfall frequency and quantity was reported on the

Swiss Plateau (main distribution area of *I. ricinus* and tick collection site) (North et al., 2007). This may have a negative impact on tick population, since overwintering ticks in this area are most likely no longer protected by snow cover and may be more exposed to severe variation in temperatures. However, it was shown that day-to-day temperature variability decreased during the last century in Neuchâtel (tick collection site), mainly due to a loss of extremely low temperatures, particularly in winter (Rebetez, 2001). Hence, according to our results, we expect overwintering *I. ricinus* ticks to benefit from the observed reduced snow cover associated with a reduction of temperature variability, resulting in lower tick mortality during winters with these conditions. Such a trend may even increase in the future due to global warming. However, if reduced snow cover was associated with important temperature fluctuations during a particular winter, we would expect *I. ricinus* populations to be more severely impaired, resulting in higher tick mortality during that winter.

I. ricinus nymphs sampled in autumn survived better at −5 °C and −10 °C than individuals sampled in the spring of the same year. Autumn nymphs possessed close to twice more fat content than spring individuals. The lower fat content in spring nymphs was likely due to the fact that these ticks were older and belonged to a different cohort, as mentioned above. The combination of these differences likely explains why younger autumn nymphs resisted longer under cold conditions than older spring nymphs.

In experiment 2a, no effect of *Borrelia* infection on nymph survival was observed. In contrast, in experiment 2b, nymphs infected with *Borrelia* spirochaetes tended to survive slightly better (marginally significant) than uninfected individuals. As it has recently been reported that nymphs infected with *B. burgdorferi* s.l. have a higher fat content (12% more after correction for body size) than uninfected ticks (Herrmann et al., 2013), we expected infected ticks to survive better. When the survival rate was closely examined in experiment 2b (Fig. 3), it appeared, in fact, that at some time points, *Borrelia*-infected nymphs survived better. However, the last surviving nymphs were uninfected independently of sampling season (i.e. tick age) or frequency of temperature variations. The latter probably occurred because uninfected ticks were more numerous than infected nymphs, resulting in higher numbers of uninfected ticks with a high fat content (due to a normal distribution of fat content, unpublished data). Such ticks would therefore survive longer than infected nymphs. The fact that no difference could be observed in experiment 2a might be because the point in time at which higher survival in infected nymphs occurred had not been reached or had already been passed when survival was assessed. However, further investigations are needed to confirm such a phenomenon.

Borrelia infection in ticks

Prevalence of *Borrelia* infection in spring (23.4%) and autumn nymphs (20.0%) was consistent with previous reports from Neuchâtel varying from 17.4% to 29.8% between 1999 and 2011 (Jouda et al., 2004; Morán Cadenas et al., 2007; Gern et al., 2010; Herrmann and Gern, 2010, 2012; Herrmann et al., 2013). *Borrelia* genospecies distribution was similar to that previously reported in the same area, *B. afzelii*, *B. garinii*, and *B. valaisiana* being the most common genospecies (Herrmann and Gern, 2010, 2012; Herrmann et al., 2013).

The load of *Borrelia* spirochaetes per nymph (spring mean = 6584; autumn mean = 6604) was lower than previously reported in the area (mean = 18,638, Herrmann and Gern, 2010; mean = 33,971, Herrmann and Gern, 2012; mean = 15,556, Herrmann et al., 2013). Although spirochaete numbers might fluctuate in questing *I. ricinus* ticks over time, spirochaete numbers observed in the present study were particularly low. We observed that spirochaete load and the number of days that nymphs survived were negatively correlated, suggesting that spirochaete number was reduced in nymphs during maintenance under cold conditions. Interestingly, a significant decrease of spirochaete numbers was also observed in *I. scapularis* adults that overwintered in nature (Sharon et al., 1992). Explanations may be that important resources for spirochaete survival were depleted in ticks when the temperature was reduced, resulting in reduced numbers of spirochaetes, and/or that spirochaetes, which are primarily located in tick midgut (Lebet and Gern, 1994), were gradually digested by cold-exposed nymphs and used as additional energy resources.

Conclusion

We observed that spring *I. ricinus* nymphs possessing less fat content than their autumn counterparts were less resistant to cold, most likely due to their lower energy reserves. Moreover, the presence of *Borrelia* spirochaetes in nymphs was associated with a slightly better survival, presumably because infected nymphs have higher fat contents than their uninfected counterparts (Herrmann et al., 2013). Last but not least, the frequency of temperature variations between temperatures below and above activity thresholds was the most important factor impairing *I. ricinus* nymphal survival under winter conditions. We might expect low survival of *I. ricinus* populations during winters with high temperature fluctuations and those associated with reduced snow cover (protecting ticks from extreme cold temperature variations) on the Swiss Plateau (North et al., 2007). However, since a reduction in the day-to-day temperature variability was observed during the past century in Neuchâtel, mainly due to less extreme cold temperatures in winter (Rebetez, 2001), ticks are likely to be less frequently exposed to temperature variations in the future. Under such conditions, we might expect a better survival of *I. ricinus* populations during winter in the Neuchâtel area.

Acknowledgments

We would like to thank C. Holzer, J. Santoli, M. Pluess, and O. Rais for their help in collecting ticks, and D. Varidel from the Physics Institute of University of Neuchâtel for providing useful material. We would like to thank M.J. Voordouw for his help in statistics, and Jonathan Hamley for improving the English language of the manuscript. Finally, we would like to thank the two anonymous reviewers, who helped improve significantly the quality of the manuscript. This study is part of the Ph.D. thesis of C. Herrmann and has been supported by the Swiss National Science Foundation (grant FN 310030_127064).

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4.5 Paper 5

Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. Submitted

Coralie Herrmann, Lise Gern and Maarten J. Voordouw

Over 7000 *I. ricinus* nymphs were collected in the same sampling area over three years in both spring and autumn. They were tested in survival or behavioural tests and subsequently analysed for *Borrelia* infection using the same detection methods. The value of this data set was that it was large enough to test the significance of a number of rare double infections between *Borrelia* genospecies, especially those involving the more recently described and rare *Borrelia* genospecies occurring in the Neuchâtel area, such as *B. bavariensis* and *B. miyamotoi*, which had not been previously possible. In addition, the fact that spirochete load was quantified allowed us to determine how genospecies interacted to shape spirochete load in ticks infected by more than one genospecies. In this study, we thus analysed statistically previously produced data to identify which *Borrelia* genospecies were positively or negatively associated, and to determine whether spirochete load was increased or reduced depending on *Borrelia* genospecies co-infecting *I. ricinus* nymphs.

TITLE: Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*

RUNNING TITLE: *Borrelia* genospecies interactions in ticks

JOURNAL SECTION: Microbial Ecology

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Summary

Mixed infections have important consequences for the ecology and evolution of host-parasite interactions. In vector-borne diseases, interactions between pathogens occur in both the vertebrate host and the arthropod vector. Spirochete bacteria belonging to the *Borrelia burgdorferi* sensu lato (s. l.) genospecies complex are transmitted by *Ixodes* ticks and cause Lyme borreliosis in humans. In Europe, there is a high diversity of *Borrelia* pathogens and the main tick vector, *Ixodes ricinus*, is often infected with multiple *Borrelia* genospecies. In the present study, we characterized the pairwise interactions between five *B. burgdorferi* s.l. genospecies in a large data set of *I. ricinus* ticks collected from the same field site in Switzerland. We measured two types of pairwise interactions: (1) co-occurrence, whether double infections occurred more or less often than expected and (2) spirochete load additivity, whether the total spirochete load in double infections was greater or less than the sum of the single infections. Mixed infections of *Borrelia* genospecies specialized on different vertebrate reservoir hosts occurred less frequently than expected (negative co-occurrence) and had joint spirochete loads that were lower than the additive expectation (inhibition). By contrast, mixed infections of genospecies that share the same reservoir hosts were more common than expected (positive co-occurrence) and had joint spirochete loads that were similar or greater than the additive expectation (facilitation). Our study suggests that the vertebrate host plays an important role in structuring the community of *B. burgdorferi* s.l. genospecies inside the tick vector.

Introduction

Most hosts are infected with multiple parasite species or parasite strains (42, 49, 50). Interactions among parasite taxa infecting the same host can take a variety of forms (54). Competition for limited host resources can result in the elimination of the less competitive parasite; for example, *Wolbachia* bacteria prevent dengue viruses, chikungunya viruses, and malaria parasites from infecting *Aedes aegypti* mosquitoes (37). Alternatively, the presence of one pathogen may facilitate opportunistic infection by another pathogen; for example, HIV and fungal/bacterial infections (9, 10, 16), intestinal helminth and malaria infections (38), or influenza and pneumococcal infections (35). Multiple infections involving genetically distinct clones of the same parasite species are of particular interest because their interactions are important in shaping the evolution of parasite virulence and disease severity (11, 34, 40), as shown in malaria (2, 8) and *Pasteuria* infections (4). Characterizing the interactions among parasite taxa in multiply infected hosts represents a major challenge for understanding the epidemiology of infectious diseases. In the case of vector-borne diseases, this task is further complicated because the parasites interact in both the arthropod vector and the vertebrate host.

The *Borrelia burgdorferi* sensu lato (s.l.) complex is a group of tick-borne spirochete bacteria that cause Lyme borreliosis, the most common tick-borne disease in the Northern Hemisphere (43). In Europe there are at least ten different *B. burgdorferi* s.l. genospecies (5, 7, 32, 47). All of these spirochetes are vectored by the hard-bodied tick *Ixodes ricinus* and maintained in a variety of vertebrate reservoir hosts (mostly birds and small mammals) (13). Previous field surveys have repeatedly shown that questing *I. ricinus* ticks often carry multiple spirochete infections (12, 47), providing ample opportunity for interactions among *Borrelia* genospecies. While many studies report the incidence of single and multiple *Borrelia* infections in *I. ricinus* (reviewed in (47)), to our knowledge only one study (29) has tested whether the incidence of multiple infections deviates from the random expectation. To better understand patterns of co-occurrence and abundance of these tick-borne pathogens it is crucial that we compare their observed distributions against the random or neutral expectation (6, 15).

The specificity of *Borrelia* genospecies for their vertebrate reservoir hosts plays a key role in shaping the ecology of mixed *Borrelia* infections in both the host and the tick vector. Previous work has shown that *B. burgdorferi* sensu stricto (s.s.), *B. afzelii* and *B. bavariensis* are specific for rodents (22, 24, 25, 30) whereas other genospecies such as *B. garinii* and *B. valaisiana* are specific for birds (26, 30). This host specificity appears to be mediated by the complement system of the vertebrate host (28, 31). The vertebrate complement system thus reduces the probability of encounter between rodent- and bird-adapted *Borrelia* genospecies. When encounters between mal-adapted co-infection partners do occur, the vertebrate immune system likely plays a key role in shaping the joint spirochete load in the tick vector. Spirochete growth rates may also influence the outcome of the mixed infection inside the tick vector. For example, the total spirochete population first expands following the larval blood meal and then declines during the larval pre-moulting period (44), providing further opportunities for competitive interactions among *Borrelia* genospecies.

The purpose of this paper was to characterize the pattern of interactions among *B. burgdorferi* s.l. genospecies in *I. ricinus* ticks. We sampled ticks from the same sampling site in Switzerland multiple times over a three-year period, and used this dataset (7400 nymphal ticks) to test whether double infections occurred more or less often than the random expectation. We limited our study to genospecies interactions in questing *I. ricinus* nymphs because this stage is responsible for infecting the next generation of reservoir hosts and is therefore the most important from an epidemiological point of view. We predicted that mixed infections of rodent- and bird-adapted *Borrelia* genospecies (e.g. *B. afzelii-B. garinii*, *B. afzelii-B. valaisiana*) in *I. ricinus* nymphs would be relatively rare compared to the random expectation. We also predicted that the joint spirochete load of such mixed infections would be lower than the additive expectation because we expected the host complement system to suppress the density of the mal-adapted co-infection partner. We predicted that mixed infections of *B. burgdorferi* s.l. genospecies that use the same vertebrate reservoir hosts (e.g. *B. garinii-B. valaisiana*) would occur more often than expected by chance. For these mixed infections we did not have a clear prediction for the joint spirochete load. However, as the tick vector presumably sets some upper limit on the spirochete load, we expect negative interactions between spirochete loads to be the norm. Detecting positive and negative patterns of species co-occurrence is an important first step toward understanding species interactions in general, and competitive and facilitative interactions in particular. Although such comparative data cannot decipher the underlying causal mechanisms, they are critical for generating new hypotheses that can further be tested experimentally.

Materials and Methods

Meta-analysis of Borrelia *infection data from the same population of* I. ricinus *ticks*

The present study is a meta-analysis of seven independent sampling occasions that were conducted by Coralie Herrmann over the course of her PhD thesis (17-20). The ticks collected on these sampling occasions were used in studies to quantify fat content in ticks and to test how temperature, humidity and *Borrelia* infection influenced the physiology, behavior, and survival of *I. ricinus* nymphs (Table 1). Over a period of three years (2009 to 2011), 7400 questing *I. ricinus* nymphs were collected from the same sampling site in Switzerland and all these ticks were processed in the same way with respect to quantification of spirochete load and *Borrelia* genospecies identification. This data set therefore presented a unique opportunity to test how *B. burgdorferi* s.l. genospecies interact in the tick vector. After pooling the data from the sampling occasions, the large sample size gave us sufficient statistical power to test whether the frequency and spirochete load of mixed infections was different from the random expectation.

Tick collection, spirochete load and Borrelia *genospecies identification*

The sampling site was a mixed forest situated 600 m above sea level on the south-facing slope of Chaumont Mountain, Neuchâtel, Switzerland (47°00’ N, 6°57’ E). Field sampling of questing ticks and subsequent molecular methods have been described in detail elsewhere (17, 19, 20). Briefly, our molecular protocol consisted of two successive steps: (1) qPCR to identify *Borrelia*-infected nymphs and to estimate the spirochete load and (2) reverse line blot (RLB) of the *Borrelia*-infected nymphs to identify the *Borrelia* genospecies. Thus the sensitivity of our protocol depended on the ability of the qPCR to identify the *Borrelia*-infected ticks whereas the ability to discriminate among the various *Borrelia* genospecies depended on the RLB. As the qPCR protocol estimated the total spirochete load, we do not have separate spirochete loads for each genospecies in the case of doubly infected nymphs.

We used the qPCR protocol from Schwaiger et al. (52), which targets the flagellin gene. For the RLB, we used the primers of Alekseev et al. (1) to amplify the variable spacer region between 2 repeated copies of the 23S and 5S ribosomal genes. The RLB protocol contains three general probes for *B. burgdorferi* s.l. in addition to specific probes that allow us to detect each of the five *B. burgdorferi* s.l. genospecies present at our study site: *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. valaisiana*, and *B. bavariensis* (12). Our RLB protocol also contains a probe that allows us to detect the relapsing fever-like spirochete *B. miyamotoi* (12). However, in the present paper, we only consider the pairwise interactions between *Borrelia* genospecies belonging to the *B. burgdorferi* s.l. complex.

Statistical Methods

Positive and negative co-occurrence of B. burgdorferi *s.l. genospecies*

We focused on interactions between pairs of *B. burgdorferi* s.l. genospecies because higher-order interactions (i.e. triple infections) were exceedingly rare. Our study area contained five different *B. burgdorferi* s.l. genospecies (*B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. valaisiana*, and *B. bavariensis*) resulting in ten possible genospecies pairs. We used log-linear analysis to characterize the co-occurrence patterns between pairs of *B. burgdorferi* s.l. genospecies in the tick vector. For each genospecies pair, the data consists of the counts of four groups of ticks: (1) uninfected, (2) infected with genospecies A, (3) infected with genospecies B, (4) and infected with genospecies A and B. A log-linear analysis essentially consists of modeling these count data as a function of genospecies A (presence/absence), genospecies B (presence/absence), and the genospecies A: genospecies B interaction using a generalized linear model with a Poisson error distribution. The sign (positive or negative) and statistical significance of the interaction term test whether the two genospecies co-occurred more or less often than expected by chance. The advantage of log-linear analyses over chi-square tests is that the sign and magnitude of the interaction term provide a quantitative estimate of the genospecies co-occurrence pattern.

We used a two-step approach to hypothesis testing. In the first step, we used three-way log-linear analysis to test whether we were justified in pooling the data from the seven sampling occasions for each of the ten *B. burgdorferi* s.l. genospecies pairs. Specifically, we tested whether the genospecies co-occurrence pattern was the same among the seven sampling occasions by evaluating the three-way interaction term for sampling occasion, genospecies A, and genospecies B. In the second step, we used two-way log-linear analysis to test the co-occurrence pattern for each of the ten genospecies pairs depending on the results of the first step. If the three-way interaction (sampling occasion: genospecies A: genospecies B) was not statistically significant, we pooled the data from the different sampling occasions and estimated the global two-way interaction. If the three-way interaction was statistically significant, we did not pool the data and analyzed the two-way interaction separately for each of the seven sampling occasions. We used the glm() function in R with a Poisson error distribution to run the log-linear analyses. All statistics were calculated with R for Mac OS X (45).

Spirochete load in doubly infected ticks (inhibition and facilitation)

We analyzed the subset of *B. burgdorferi* s.l. infected ticks (n = 1696 nymphs = 1731 *Borrelia*-infected nymphs - 35 *B. miyamotoi* infected-nymphs in Table 2) to test whether the spirochete load of mixed infections deviated from the neutral (additive) expectation. For this analysis, we pooled the results of the seven sampling occasions to maximize our sample size and statistical power. The naive or null hypothesis of additivity assumes that the expected spirochete load in a doubly infected tick ($\overline{X}_{A \cap B}$) is simply the sum of the mean spirochete loads of genospecies A ($\overline{X}_A$) and genospecies B ($\overline{X}_B$) in singly infected ticks (i.e. $\overline{X}_{A \cap B} = \overline{X}_A + \overline{X}_B$). The two alternative hypotheses are inhibition and facilitation where the spirochete load of doubly infected ticks is less than ($\overline{X}_{A\cap B} < \overline{X}_A + \overline{X}_B$) or greater than ($\overline{X}_{A\cap B} > \overline{X}_A + \overline{X}_B$) the additive expectation.

For each of the ten *B. burgdorferi* s.l. genospecies pairs, we calculated the observed mean spirochete load ($\overline{X}_{A\cap B}$; Table 2) for the sample of co-infected ticks (n_{AB}; Table 2). To generate the null distribution of additivity, we randomly sampled (with replacement) n_{AB} pairs of spirochete loads from the set of singly infected ticks for each genospecies in the pair. We summed each randomly sampled pair of spirochete loads to form the sample of joint spirochete loads then calculated the expected mean spirochete load. We repeated this random sampling protocol 100,000 times to create a null distribution with sufficient precision for calculating p-values. Thus for each genospecies pair, the mean and variance of the null distribution were $\overline{X}_A + \overline{X}_B$ and $\sigma_A^2 + \sigma_B^2$, respectively. We used the 2.5th and the 97.5th percentiles from the null distribution as the 95% confidence limits (CL) of the mean expected spirochete load. To facilitate comparison among genospecies pairs, we divided the mean expected spirochete load and the 95% CL by the corresponding observed mean spirochete load. Genospecies pairs where the 95% CL of the ratio (R = expected mean/observed mean) overlap 1.0 exhibit additivity. Genospecies pairs where the 95% CL of the ratio are above or below 1.0 exhibit inhibition and facilitation, respectively.

Results

Prevalence of Borrelia *genospecies and spirochete loads*

Among the 7400 questing nymphs sampled, there were 1520 single, 211 double, and ten triple infections. The triple infections were excluded from our analyses. *B. afzelii* was the most common genospecies, representing 50.4% of single and double infections (872/1731; Table 2), followed by *B. garinii* (24.0% = 415/1731), and *B. valaisiana* (22.8% = 395/1731). *B. burgdorferi* s.s. (7.0% = 121/1731), *B. bavariensis* (5.4% = 94/1731), and the relapsing fever-like spirochete *B. miyamotoi* (2.0% = 35/1731) were less common. Among nymphs infected with a single *Borrelia* genospecies, the rank order of median spirochete load per tick was as follows: *B. bavariensis* (23050), *B. garinii* (5080), *B. burgdorferi* s.s. (3410), *B. afzelii* (3140), *B. valaisiana* (1830), and *B. miyamotoi* (1160).

Positive and Negative Co-occurrence of B. burgdorferi *s.l. genospecies*

The three-way log-linear analysis showed that the co-occurrence between *B. burgdorferi* s.l. genospecies was the same across the seven sampling occasions for nine of the ten genospecies pairs (Table 3). This result means that we were justified in pooling the data of the seven sampling occasions and interpreting the global two-way interaction for these genospecies pairs. Of the nine genospecies pairs where pooling was justified, three showed positive co-occurrence and six showed negative co-occurrence (Table 4). Two of the three positive co-occurrences (*B. afzelii-B. burgdorferi* s.s, and *B. garinii-B. valaisiana*) and three of the six negative co-occurrences (*B. afzelii-B. bavariensis*, *B. afzelii-B. garinii*, and *B. bavariensis-B. garinii*) were statistically significant. Pooling was not justified for the *B. afzelii-B. valaisiana* genospecies pair (df = 6, deviance = 20.232, p-value = 0.0025; Table 3) and examination of the seven sampling occasions found six cases of negative co-occurrence (four statistically significant) and one case of positive co-occurrence (not statistically significant) (data not shown).

Spirochete load in doubly infected ticks (inhibition and facilitation)

We used simulations to describe the spirochete load of nymphal ticks infected with two *B. burgdorferi* s.l. genospecies. Specifically, for each genospecies pair we set out to test whether the joint spirochete load observed in doubly infected ticks was unusual relative to a null distribution. We generated the null distribution by randomly sampling and then summing the spirochete loads of the singly infected ticks belonging to each member of the genospecies pair. We calculated a null distribution for eight of the ten genospecies pairs (Figure 1). Two genospecies pairs were excluded (*B. bavariensis-B. burgdorferi* s.s. and *B. bavariensis-B. garinii*) because they had no doubly infected ticks. Of the eight remaining genospecies pairs, two pairs (*B. afzelii-B. garinii*, *B. afzelii-B. valaisiana*) showed inhibition (95% CL of the ratio > 1.0), one pair (*B. afzelii-B. burgdorferi* s.s.) showed facilitation (95% CL of the ratio < 1.0), and the remaining five pairs showed additivity (Figure 1; Table 5). After Bonferonni-correction, three of the eight spirochete load interactions remained statistically significant (Table 5). Spirochete loads in nymphs with double infections of *B. afzelii-B. garinii* or *B. afzelii-B. valaisiana* were six to nineteen times lower than the additive expectation. In contrast, spirochete loads of double infections of *B. afzelii-B. burgdorferi* s.s. were four times greater than the additive expectation.

Relationship between co-occurrence and spirochete load inhibition in doubly infected ticks

B. burgdorferi s.l. genospecies pairs fell into two broad categories when jointly considering the two types of interactions measured in this study (Figure 2). Genospecies pairs that had negative co-occurrence had combined spirochete loads that were lower than expected (stronger inhibition) whereas genospecies pairs with positive co-occurrence had spirochete loads that met or surpassed the additive expectation (weaker inhibition or facilitation). Thus broadly speaking, *B. burgdorferi* s.l. genospecies pairs either fell in the negative co-occurrence/inhibition or the positive co-occurrence/facilitation quadrant (Figure 2). Across the set of eight genospecies pairs, there was a significant negative correlation between the two types of interactions (Pearson r = -0.851, t = -3.97, df = 6, p-value = 0.0074).

Discussion

To our knowledge, this is the first study to test the frequency and spirochete load of mixed *B. burgdorferi* s.l. infections in the epidemiologically relevant nymphal stage of *I. ricinus* against the background null hypotheses of random species co-occurrence patterns and additive spirochete loads in double infections. The value of our statistical approach was that it allowed us to quantify the degree of co-occurrence and the degree of spirochete load inhibition between co-infection partners. Plotting these measures of co-occurrence and spirochete load inhibition revealed broad patterns of interactions between *B. burgdorferi* s.l. genospecies pairs (Figure 2). Common co-infections have higher-than-expected joint spirochete loads inside the nymphal tick (Figure 2). *B. burgdorferi* s.l. genospecies that share the same vertebrate reservoir hosts (*B. afzelii-B. burgdorferi* s.s. and *B. garinii-B. valaisiana*) frequently occur together and exhibit weak inhibition and even facilitation with respect to the spirochete load inside the nymphal tick. Conversely, *B. burgdorferi* s.l. genospecies pairs that are specialized on different vertebrate reservoir hosts (*B. afzelii-B. garinii*, *B. afzelii-B. valaisiana*, *B. garinii-B. burgdorferi* s.s., *B. burgdorferi* s.s.-*B. valaisiana*) rarely occur together and exhibit strong inhibition with respect to spirochete load. This negative association between occurrence and spirochete load inhibition is likely driven by the vertebrate immune system. Specifically, the complement system, which is present in the tick midgut (41), is capable of lysing *B. burgdorferi* s.l. spirochetes that are not adapted to that particular vertebrate host (28, 31). In cases where the complement system fails to eliminate the mal-adapted co-infection partner, the spirochete load of the latter would likely be much reduced resulting in the observed pattern of inhibition in the nymphal tick. For example, when the same larva takes multiple blood meals from different vertebrate hosts after interrupted attachments (23, 36), the complement system of the second host could reduce the spirochete load of the *Borrelia* pathogen from the first host. Thus the vertebrate immune system is the most likely explanation as to why co-infections involving rodent- and bird-adapted *B. burgdorferi* s.l. genospecies exhibit lower-than-expected spirochete loads. Our results for the more common genospecies associations correspond to what has previously been reported in the literature (29, 43, 47). Rauter and Hartung (47) reported that *B. garinii-B. valaisiana* was the most common mixed infection of *I. ricinus* ticks but they did not test whether mixed infections occurred more or less often than the random expectation. Kurtenbach et al. (29) used a simple Chi-square test to show that *B. garinii-B. valaisiana* and *B. afzelii-B. garinii* mixed infections were more and less common than the random expectation in a variety of European countries. However, their study focused on adult ticks, which mostly feed on incompetent hosts such as deer and therefore do not contribute significantly to the epidemiology of Lyme borreliosis.

The specificity of rodent and avian-adapted *Borrelia* genospecies for their respective reservoir hosts is not perfect. Our study found that double infections between rodent and avian-adapted *Borrelia* genospecies do occur (Table 2) and we suggest four possible mechanisms. First, some *B. burgdorferi* s.l. strains might be generalists that are capable of infecting both rodent and bird reservoir hosts. For example, a recent field study on the Siberian chipmunk found one double infection with *B. afzelii* and *B. garinii* (33). Second, larvae may acquire a double infection via a combination of systemic and co-feeding transmission (21). Systemic transmission refers to the standard mode where ticks acquire spirochetes that have established a widespread and long-term infection in the host. Co-feeding transmission refers to the process where hosts are not systemically infected but instead function as a “temporary bridge” (46) that facilitates transmission between infected and uninfected ticks feeding in close proximity to each other (14). Third, double infections may result from a combination of vertical (trans-ovarial) and horizontal (blood meal) transmission of *Borrelia* genospecies, although the former is believed to be rare or nonexistent in *Ixodes* ticks (3, 39, 51). Fourth, larval ticks taking multiple blood meals from different hosts (interrupted blood meals) (23) could also produce double infections of rodent- and bird-specific genospecies (36). These four mechanisms illustrate the diversity of transmission pathways that can produce ticks doubly infected with rodent- and bird-adapted *Borrelia* genospecies. These double infections are of interest because they connect the avian and rodent Lyme borreliosis systems and provide opportunities for genetic exchange between *Borrelia* genospecies.

Importantly, while the prevalence of *B. burgdorferi* s.l. genospecies often fluctuates through time and space, the nature of the pairwise interaction appeared to be robust. For six of ten genospecies pairs, the nature of the pairwise interaction was always in the same direction for each of the seven sampling occasions (Table 4), despite the fact that the questing nymphs had been collected in different years, different seasons, and exposed to different abiotic conditions prior to *B. burgdorferi* s.l. genospecies identification (Table 1). Similarly, the fact that the three-way interaction was not statistically significant for nine of the ten genospecies pairs (Table 3) indicates that the different experimental conditions of the seven sampling occasions did not bias the co-occurrence patterns of the *B. burgdorferi* s.l. genospecies. Thus an important aspect of this study is our demonstration that the genospecies associations appear to be relatively constant over time at our site.

Most of the double infections had spirochete loads that were considerably lower than the additive expectation, suggesting that genospecies compete for limited resources in the tick (i.e. for seven of the eight genospecies pairs in Table 5, the average ratio of the mean expected spirochete load to the mean observed spirochete load is greater than one suggesting inhibition). The two statistically significant inhibition interactions include the *B. afzelii-B. garinii* and the *B. afzelii-B. valaisiana* genospecies pairs. As previously discussed, this inhibition was likely caused by the host complement system suppressing the spirochete load of the mal-adapted co-infection partner. By contrast, the only statistically significant facilitation interaction involved two genospecies, *B. afzelii-B. burgdorferi* s.s., that are both adapted to rodent reservoir hosts (24, 25, 27, 30). One possible explanation for facilitation is that one genospecies suppresses the host immune system and thereby enhances the infection and transmission success of its co-infection partner.

Our approach of using qPCR to detect *Borrelia* infections followed by RLB to determine the community of *B. burgdorferi* s.l. genospecies had its advantages and disadvantages. Advantages of our approach include low cost, relative simplicity, and time efficiency. Our qPCR protocol gives reliable estimates of the spirochete load because the median spirochete load in the present study (3200 spirochetes/tick across all *Borrelia* genospecies) was similar to another study (4000 spirochetes/tick) on *B. burgdorferi* s.l. infections in *I. ricinus* ticks (48). Our RLB protocol gives reliable identification of the *B. burgdorferi* s.l. genospecies and *B. miyamotoi* because there were only three out of 1731 *Borrelia*-infected ticks (as detected by qPCR) where the PCR product failed to hybridize with a genospecies-specific probe (subsequent sequencing revealed two *B. afzelii* and one *B. garinii* infection). The reverse situation where the RLB protocol identifies infections in ticks but the qPCR fails to detect them may also occur (unpublished data). One major advantage of RLB is that the technique allows identification of mixed infections in samples. A disadvantage of our approach using qPCR is that we could not estimate separate spirochete loads for each partner in the mixed infections. Thus in the case of inhibition or facilitation, we do not know which of the two *B. burgdorferi* s.l. genospecies reduced or increased their spirochete load in the mixed infection relative to the single infection. Future studies should use next-generation sequencing approaches that can identify all possible *B. burgdorferi* s.l. genospecies and estimate genospecies-specific spirochete loads. In addition, experimental infection experiments would greatly clarify the underlying mechanisms of the pairwise interactions observed in the present study.

In addition to *B. burgdorferi* s.l. genospecies, the RLB identified a low prevalence of the relapsing fever-like spirochete, *B. miyamotoi,* (2.0% = 35/1731; Table 2) in our *I. ricinus* population. The identity of this relapsing fever-like spirochete has been confirmed with DNA sequencing of the flagellin gene and the 16S rDNA genes of samples hybridizing with the *B. miyamotoi* RLB probe (12). Screening ticks for *Borrelia* infection using only the RLB protocol (i.e. no upstream qPCR identification of infected ticks) found that between 0.5% (7/1324) (unpublished data) and 5.3% (5/94) (12) of nymphs collected in the same forest were infected with *B. miyamotoi* showing that the prevalences of *B. miyamotoi* are similar regardless of the protocol. In the present study, double infections with *B. miyamotoi* and rodent-specialized genospecies such as *B. afzelii* (n = 20; Table 2) were more common than double infections with bird-specialized genospecies such as *B. garinii* (n = 3; Table 2). A recent study conducted on field-captured rodents in Switzerland (C. Burri, O. Schumann, C. Schumann, and L. Gern, submitted for publication) showed that rodents are relevant reservoir hosts for *B.*

miyamotoi and *B. afzelii,* which explains the high number of double infections involving these two genospecies. Alternatively, the co-occurrence of these two genospecies in nymphs may be due to a combination of transovarial transmission of *B. miyamotoi* (53) and horizontal transmission of *B. afzelii*.

In conclusion, the present study found that the co-occurrence and joint spirochete load of rodent- and bird-adapted *B. burgdorferi* s.l. genospecies in *I. ricinus* nymphs were both lower than the neutral expectation. This observation is consistent with the theory that the vertebrate immune system plays an important role in structuring the *B. burgdorferi* s.l. genospecies community in the *I. ricinus* tick vector. Conversely, *B. burgdorferi* s.l. genospecies that are specialized on the same reservoir host co-occurred more often than expected from chance and their joint spirochete loads followed or exceeded the additive expectation suggesting facilitation. Future experimental infection experiments will further elucidate the mechanisms shaping the community ecology of *B. burgdorferi* s.l. genospecies inside the tick vector.

Acknowledgements

This study is part of the Ph.D. thesis of C. Herrmann and has been supported by the Swiss National Science Foundation grant (FN 320030_113936 and FN 310030_127064) and the Canton de Neuchâtel, Switzerland. Thanks to Blake Matthews and two anonymous reviewers for comments on this manuscript.

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Tables

Table 1. Description of the seven studies (A to G), which provided the data on *Borrelia* genospecies infections in *I. ricinus* nymphs.

Study	Season of collection	Year of collection	Sample size	Treatment	Publication
A	Spring	2009	500	Survival under hot conditions	(19)
B	Spring	2010	1500	Humidity Attraction	(17)
C	Spring	2010	2250	Survival under hot conditions	unpublished
D	Autumn	2010	450	Survival under cold conditions	unpublished
E	Spring	2011	900	Fat content quantification	(20)
F	Spring	2011	800	Survival under cold conditions	(18)
G	Autumn	2011	1000	Survival under cold conditions	(18)

Table 2. Number of single and double infections (upper right, unshaded area) and the corresponding mean spirochete load (lower left, shaded area) for the six *Borrelia* genospecies (on the diagonal) and the 15 genospecies pairs (off the diagonal) in questing *I. ricinus* nymphs (n = 7400) sampled in Neuchâtel, Switzerland. Some genospecies associations did not occur (n = 0) and so the mean spirochete load was not available (NA).

Genospecies[a]	af	bav	ga	miy	ss	vs
af	788 12277	1	13	20	35	15
bav	6040	92 84841	0	1	0	10
ga	9574	NA	290 41895	3	3	106
miy	19778	45	33888	8 259	3	0
ss	85034	NA	4987	7251	79 9697	1
vs	1084	47967	36970	NA	732	263 7969

[a] af = *B. afzelii*; bav = *B. bavariensis*; ga = *B. garinii*; miy = *B. miyamotoi*; ss = *B. burgdorferi* sensu stricto; vs = *B. valaisiana*

Table 3. Three-way log-linear analyses testing whether the pairwise co-occurrence between *Borrelia* genospecies differed among the seven sampling occasions for each of the ten genospecies pairs. Shown are the degrees of freedom, the deviance and the P-value testing whether the three-way sampling occasion:genospecies A:genospecies B interaction was statistically significant. After Bonferonni-correction, $\alpha/n = 0.05/10 = 0.005$ (where n = the number of pairwise comparisons) only one of the ten genospecies pairs (af-vs) had a significant three-way interaction.

Genospecies A[a]	Genopecies B[a]	Degrees of freedom	Deviance	P-value	Significance[b]
af	bav	6	4.938	0.5518	
af	ga	6	5.486	0.4832	
af	ss	6	9.125	0.1667	
af	vs	6	20.232	0.0025	*
bav	ga	6	< 0.001	1.0000	
bav	ss	6	< 0.001	1.0000	
bav	vs	6	1.849	0.9330	
ga	ss	6	9.444	0.1501	
ga	vs	6	7.750	0.2570	
ss	vs	6	2.847	0.8278	

[a] af = *B. afzelii*; bav = *B. bavariensis*; ga = *B. garinii*; ss = *B. burgdorferi* sensu stricto; vs = *B. valaisiana*

[b] Level of significance after Bonferroni correction: low significance (*) p-value = 0.05/10 = 0.005; intermediate significance p-value = 0.01/10 = 0.001; high significance (***) p-value = 0.001/10 = 0.0001.

Table 4. Two-way log-linear analyses testing whether the pairwise associations between *B. burgdorferi* s.l. genospecies are positive or negative when the seven independent sampling occasions are combined into one single data set. Shown are the deviance, the P-value, the estimate and standard error (SE) of the two-way interaction term describing the pairwise associations between *B. burgdorferi* s.l. genospecies. The 'co-occurrence type' column indicates whether the genospecies pair exhibits positive co-occurrence (two-way interaction is positive) or negative co-occurrence (two-way interaction is negative). The 'by study' column indicates the number of studies where the sign of the two-way interaction was in the same direction (positive or negative) as the overall estimate ('estimate' column). The three-way log-linear analysis (Table 3) found a significant effect of study on the pairwise association for the *B. afzelii*-*B. valaisiana* genospecies pair (shaded grey).

Genospecies A[a]	Genospecies B[a]	Deviance	P-value	Significance[b]	Interaction term		Co-occurrence type	By study[c]
					Estimate	SE		
af	bav	19.390	0.00001	***	-2.656	1.006	Negative	7 (100%)
af	ga	43.425	< 0.00001	***	-1.483	0.285	Negative	7 (100%)
af	ss	26.730	< 0.00001	***	1.158	0.205	Positive	6 (86%)
af	vs	31.567	< 0.00001	***	-1.238	0.266	Negative	6 (86%)
bav	ga	13.254	0.0003	**	-24.78	55500	Negative	7 (100%)
bav	ss	3.314	0.0687	NS	-22.640	38540	Negative	7 (100%)
bav	vs	2.776	0.0957	NS	0.607	0.338	Positive	5 (71%)
ga	ss	2.601	0.1068	NS	-0.829	0.588	Negative	5 (71%)
ga	vs	198.560	< 0.00001	***	2.049	0.129	Positive	7 (100%)
ss	vs	7.395	0.0065	NS	-1.917	1.006	Negative	7 (100%)

[a] af = *B. afzelii*; bav = *B. bavariensis*; ga = *B. garinii*; ss = *B. burgdorferi* sensu stricto; vs = *B. valaisiana*

[b] Level of significance after Bonferroni correction: no significance (NS) p-value > 0.05/10 = 0.005; low significance (*) p-value = 0.05/10 = 0.005; intermediate significance p-value = 0.01/10 = 0.001; high significance (***) p-value = 0.001/10 = 0.0001.

[c] Number of times the co-occurrence type was observed out of the seven studies (%).

Table 5. Results from the randomization protocol testing the null hypothesis of additivity of *B. burgdorferi* s.l. spirochete load. Above the diagonal: the ratio of the mean expected spirochete load to the mean observed spirochete load; the 95% confidence limits are in parentheses (the 2.5th and the 97.5th quantiles from the null distribution of expected spirochete loads divided by the observed spirochete load). Ratios with 95% confidence limits that overlap 1 indicate additivity, ratios with 95% confidence limits less than 1.0 indicate facilitation, and ratios with 95% confidence limits greater than 1.0 indicate inhibition. Below the diagonal: proportion of randomized values that were more extreme than the observed mean spirochete load multiplied by 2.0 (two-tailed test). The randomization protocol was not possible for two genospecies pairs and these are described as not available (NA). For the remaining eight genospecies pairs, we used a Bonferroni-corrected α level of 0.05/8 = 0.00625 to determine the statistical significance (shown in bold text).

Genospecies[a]	af	bav	ga	ss	vs
af		16.137 (0.08-82.59)	**5.668** **(1.71-12.47)**	**0.259** **(0.15-0.40)**	**18.732** **(7.39-36.25)**
bav	0.2428		NA	NA	1.938 (0.50-4.92)
ga	**0.0040**	NA		10.350 (0.55-42.63)	1.349 (0.88-1.90)
ss	**< 0.0002**	NA	0.1206		24.117 (0.10-134.43)
vs	**< 0.0002**	0.4444	0.1660	0.2512	

[a] af = *B. afzelii*; bav = *B. bavariensis*; ga = *B. garinii*; ss = *B. burgdorferi* sensu stricto; vs = *B. valaisiana*

Figures

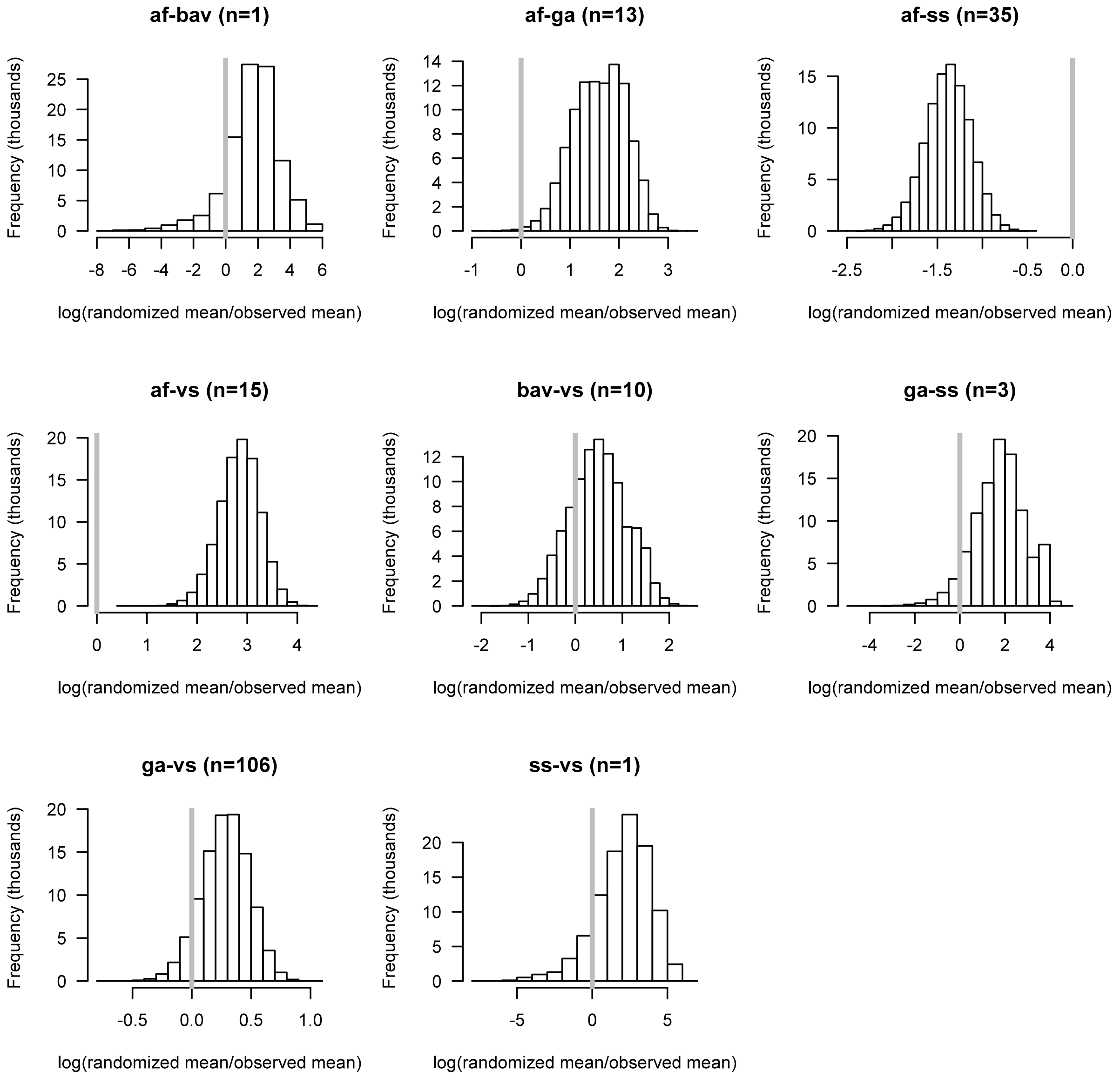

Figure 1. Distribution of the expected *B. burgdorferi* s.l. spirochete load under the null hypothesis of additivity for 8 genospecies pairs. To facilitate visualization of the null distribution, expected values were divided by the observed value and subsequently log-transformed. Applying this transformation sets the observed value to zero (vertical gray line). Null distributions centred on zero indicate additivity. Null distributions centred on positive values (right-shifted) indicate inhibition among genospecies pairs. Null distributions centred on negative values (left-shifted) indicate facilitation. Abbreviations: af = *B. afzelii*; bav = *B. bavariensis*; ga = *B. garinii*; ss = *B. burgdorferi* sensu stricto; vs = *B. valaisiana*.

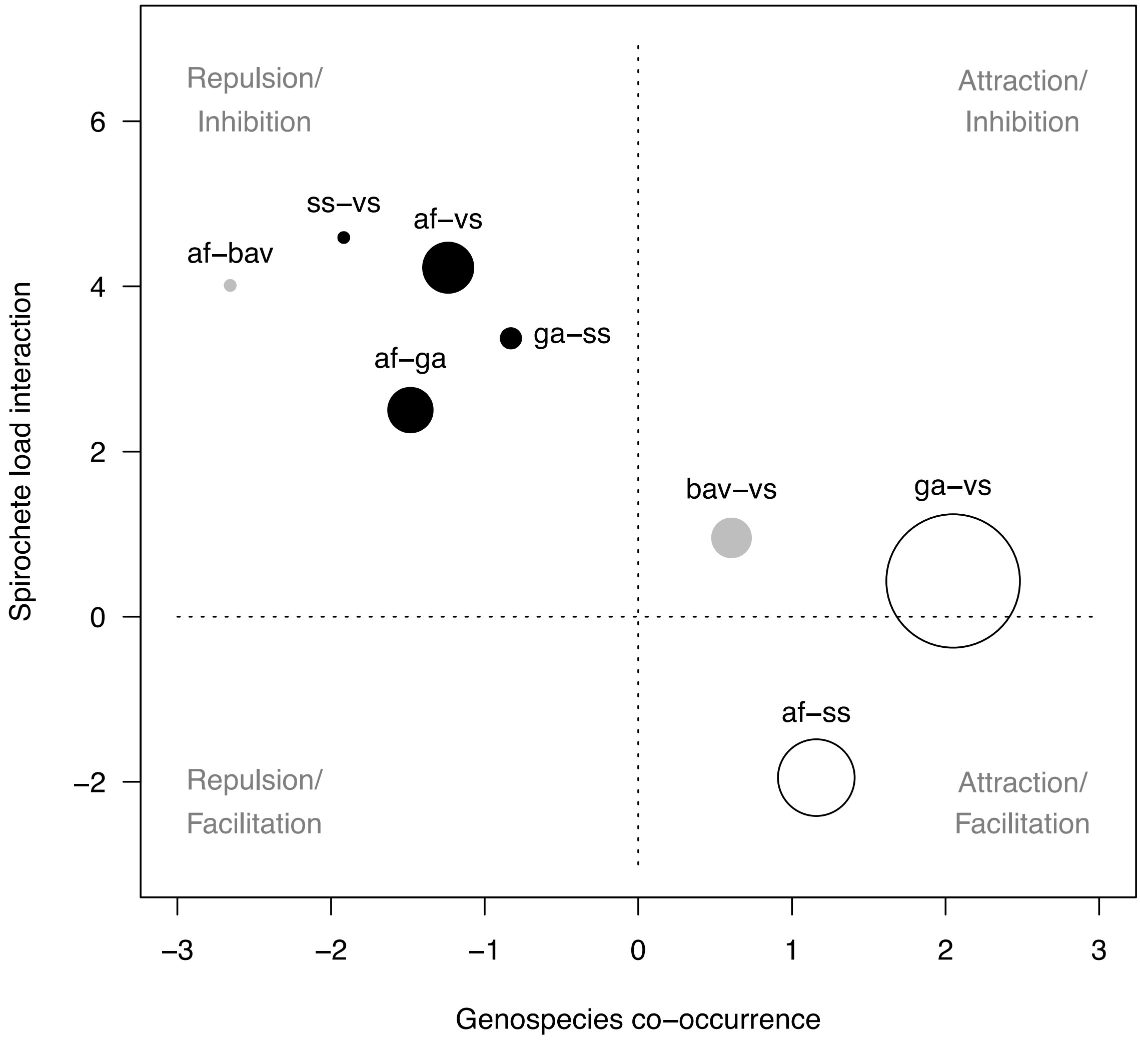

Figure 2. Relationship between our two measures of interaction for the 8 pairs of *B. burgdorferi* s.l. genospecies. Genospecies co-occurrence refers to whether double infections occurred more or less often than expected (positive or negative). The spirochete load interaction refers to whether the total spirochete load in the doubly infected ticks was greater or less than the sum of the single infections (facilitation or inhibition). The circle size refers to the sample size of double infections for each genospecies pair (Table 2). Solid black dots and open dots refer to genospecies pairs for which we had 'a priori' predictions of negative and positive co-occurrence respectively. Solid grey dots refer to genospecies pairs for which we had no 'a priori' predictions for co-occurrence. The dotted lines (Y = 0 and X = 0) refer to the null hypotheses of independence (i.e. frequency of double infections equals the product of the frequency of the single infections) and additivity (i.e. spirochete load of double infection equals the sum of the single infections). To facilitate the graphing of the spirochete interaction, we log-transformed the ratios in Table 5 (negative values indicate ratios < 1 = facilitation whereas positive values indicate ratios > 1 = inhibition). Abbreviations: af =*B. afzelii*; bav = *B. bavariensis*; ga = *B. garinii*; ss = *B. burgdorferi* sensu stricto; vs = *B. valaisiana*.

Supporting Information

Additional supporting information may be found in the online version of this article.

Supporting Information_Figure S1.pdf shows the log2-transformed spirochete loads for each of the six *Borrelia* genospecies. Each distribution contains the spirochete loads for the single and double infections of that particular genospecies.

Supporting Information_Table S1.xlsx shows the prevalences of the single and double infections for each of the six *Borrelia* genospecies for each of the seven sampling occasions (A-G).

5 DISCUSSION

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5.1 Experimental Procedures

Molecular techniques

Common laboratory techniques such as real-time PCR (also known as quantitative PCR) (Schwaiger et al., 2001) and touchdown PCR (Alekseev et al., 2001; Burri et al., 2007) followed by reverse line blot (RLB) (Poupon et al., 2006; Gern et al., 2010) have allowed us to answer a great variety of questions. Using these two techniques, we have been able to quantify the number of *Borrelia* spirochetes in ticks on the one hand and to identify *Borrelia* spirochetes to the species level on the other hand. The advantage of using both techniques subsequently was to target two different genetic regions of *B. burgdorferi* s.l. (the real-time PCR amplifying the flagellin gene and the touchdown PCR amplifying the variable spacer region between two repeated copies of the 23S and 5S ribosomal genes). The second technique confirmed or infirmed the results obtained with the first one, which reduced risks to get false positives (by contamination for instance). Moreover, screening for positive samples by real-time PCR first allowed us to save time, as only a reduced number of samples were tested by the second time-consuming touchdown PCR followed by RLB technique. A limitation of the real-time PCR technique used in this thesis is that spirochete number was quantified as a whole and was not differentiated for each genospecies involved in multiple infections. Nevertheless, it would be very useful to be able to quantify spirochete number for each genospecies involved in multiple infections individually. An attempt to develop such a technique has been made by Rauter et al. (2002): a real-time PCR (targeting the *OspA* gene) allowing the differentiation of three *Borrelia* genospecies (namely *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*) by their melting temperature, determined by the melting curve of the amplified product after thermal cycling. However, this real-time PCR has several flaws: its specificity is only 86 % (misclassifications occur) and *B. valaisiana* cannot be distinguished from *B. afzelii* due to sequence similarity (Rauter et al., 2002). Moreover, this technique cannot quantify spirochete number of each genospecies involved in multiple infections individually, similarly to the real-time PCR targeting the flagellin gene. In order to understand better the interactions between *Borrelia* genospecies in the tick, the development of a multiplex real-time PCR targeting *B. burgdorferi* s.l. should be considered, similarly to the recently developed multiplex real-time PCR detecting relapsing fever spirochetes (Elbir et al., 2013).

Physiological and behavioural tests

Physiological and behavioural tests have allowed us to test survival under hot and dry conditions, attraction to humidity, and survival under cold conditions, and to quantify energy reserves in field-collected *I. ricinus* ticks. These tests were designed so that conditions ticks experienced in the laboratory were close to those they experience in nature. Field-collected *I. ricinus* ticks were used in all tests performed during this thesis. In order to perform tests as standardised as possible, tick sampling was conducted so that collected *I. ricinus* ticks were as physiologically identical as possible. Ticks tested in one experiment were therefore sampled in the same region during several consecutive days. However, ticks are never identical (in terms of size, water content, fat content, etc.) in nature since they are likely to have experienced different conditions. Testing laboratory-reared ticks, which are more physiologically alike than field-collected individuals, in similar physiological and behavioural tests might therefore be interesting. However, tests using laboratory-reared ticks would not reflect exactly what happens in nature since laboratory-reared ticks behave differently from their wild counterparts (Olivier Rais, personal communication).

Hot and dry condition survival tests – The experimental design of these tests was based on similar experiments previously conducted by Perret (2002), who used combinations of five RHs (11 to 98 % RH) and four temperatures (14 to 34°C) producing twenty different SD conditions ranging from 0.25 to 36.4 mmHg, and tested male and female *I. ricinus* survival over ten days. We reduced the number of testing conditions and used temperature and RH conditions (89 % RH at 25°C, 61 % RH at 12.5°C, 13 % RH at 12.5°C, 51.5 % at 25°C, and 32 % RH at 25°C), corresponding to SD values ranging from 2.57 to 15.89 mmHg, that *I. ricinus* ticks might experience in the forest in Neuchâtel during late spring or summer days (although 13 % RH is extreme). Nymphs were included in these tests because ticks belonging to this stage play a major role in infecting vertebrate hosts and are therefore important from an epidemiological point of view. Testing time was reduced compared to that used by Perret (2002) since his results indicated that 50 % of the tested ticks had already died 48 h after the start of the experiment. As we aimed at stopping the tests at 50 % mortality in order to compare the number of *Borrelia*-infected individuals among dead and living ticks, we set testing time at 48 h. In both the experiment conducted by Perret (2002) and ours, conditions that ticks experienced over ten days and 48 h, respectively, do not reflect accurately natural conditions since climate conditions vary importantly over 24 h in nature (temperature decreases significantly overnight for instance). In nature, ticks usually experience stressful conditions such as those used in these tests over a few hours only, during daylight hours. Thus, such conditions represent an accumulation of short stressful periods over 48 h. This thereby allowed us to test the combined effects of a couple of stressful days over 48 h, i.e. a relatively short period of time. It would be interesting to test whether similar results are observed when test conditions reflect more accurately natural conditions. We therefore recommend future research to investigate *I. ricinus* survival in the laboratory under conditions that represent more precisely what happens in nature: desiccating conditions during the day stressing importantly tested ticks and moister conditions during the night allowing tested ticks to be momentarily relieved of the desiccation burden. Moreover, it may be interesting to assess tick mortality regularly (as described by Perret, 2002, for example) rather than at one time point only, as performed in the present thesis after 48 h, to obtain more precise information about *I. ricinus* survival under desiccating conditions over time. However, doing so would require removing dead ticks from survival arenas at every mortality assessment (to be able to compare survival between *Borrelia*-infected and uninfected individuals as infection is only detected afterwards), which might be complicated without disturbing experimental conditions significantly.

Humidity attraction tests – In these tests, *I. ricinus* nymphs were allowed to walk horizontally within a humidity gradient, which aimed at illustrating how ticks would move depending on humidity level in nature. The experimental design of these tests was based on the experimental design described by Crooks and Randolph (2006) since it was appropriate to study *I. ricinus* walking activity in the horizontal plane. Testing time, i.e. one hour with the lights on and one hour with the lights off (the onset of darkness triggers *I. ricinus* ticks to walk; Perret et al., 2003), was adapted from the results obtained by Crooks and Randolph (2006), as the authors observed that the majority of ticks had already walked and chosen the dry or the wet side two hours post-release. The humidity gradient produced within the choice arenas (45 to 95 % RH) reflected quite accurately what ticks might experience in nature. However, such humidity gradient is usually observed in the vertical plane, humidity gradient decreasing from the litter layer to the top of vegetation. To mimic more accurately conditions that ticks experience in nature, such tests should be conducted in the vertical plane, the humidity gradient increasing from the top to the bottom of the choice arenas. Vertical channels such as those described by Perret et al. (2003) might be used for such experiments. Alternatively, a vertical apparatus within which a humidity gradient could be created such as that described by Lees (1948) might also be used. In such apparatus, Lees (1948) has observed that unfed *I. ricinus* nymphs that have been exposed to fully saturated air before the start of the experiment initially avoid the wet side and therefore settle in the dry side but six days later, all the ticks are found on the wet side. Hence, it might also be interesting to increase testing time to study more precisely how ticks move within a humidity gradient over time. Under such conditions, it would be necessary to tag each tick individually to be able to compare movements between *Borrelia*-infected and uninfected ticks as infection is only detected afterwards.

Cold condition survival tests – Although studies such as that conducted by Dautel and Knülle (1997) exposing *I. ricinus* ticks to extreme cold temperatures over the short term documents *I. ricinus* resistance to cold temperatures (i.e. 50 % of *I. ricinus* nymphs survive a constant -10°C exposure for one month), they do not reflect accurately what happens under natural conditions. The experimental design of our tests was based on similar experiments previously performed by Gigon (1985), who studied survival in *I. ricinus* larvae, nymphs and females in both unfed and engorged state under cold conditions corresponding to three low temperatures (-4.5, -10.5, and -36°C) and four frequencies at which ticks were exposed to temperature variations (1, 4, 16, or 32 days). We reduced the number of testing conditions and used two low temperatures (-5°C and -10°C only, since -36°C was too extreme) and two frequencies of temperature variations (1 and 10 days) to create more accurately conditions that *I. ricinus* ticks might experience in the forest in Neuchâtel during winter days. Moreover, we adapted the range and the duration of temperature variations over 24 h to make it more realistic (reproducing more accurately temperature variations occurring during a winter day): temperature variation over 24 h consisted of 2 h at 4°C (representing late morning), 2 h at 13°C (midday), 2 h at 4°C (early afternoon) and 18 h at either -5°C or -10°C (night) in our tests compared to 6 h at 4°C, 6 h at 21°C and 12 h at -4.5, -10.5, or -36°C in the tests conducted by Gigon (1985). In contrast to Gigon (1985), who used first generation laboratory reared-ticks, we used field-collected ticks since laboratory-reared ticks do not experience stressful conditions occurring in the field and are therefore physiologically different from field-sampled individuals. Only nymphs were included in our tests because they are important from an epidemiological point of view. Although testing conditions used in our tests were more adapted than those used by Gigon (1985), they might also be more extreme than natural conditions. While such low temperatures may be regularly measured at ground level in the sampling area, they usually do not occur for as many consecutive days as they did in the tests we conducted. Test conditions might therefore have stressed ticks more than natural conditions. Moreover, temperature variations (-5 to 13°C or -10 to 13°C) that ticks experienced in these tests were probably more important than those occurring in nature, which might have accentuated tick stress further. Hence, although test conditions created in the laboratory were close to natural conditions, less extreme cold temperatures (-2°C for example) and smaller temperature variations (such as -2° to 5°C) might reflect more accurately natural winter conditions. The part of the tests that could be most significantly improved is the frequency of mortality assessment. Mortality was assessed regularly in a reduced sample of ticks while it was assessed at one time point (when survival was estimated to be 50 %) in the other ticks. We recommend that future tests assess tick mortality regularly in all ticks (as described by Gigon, 1985), as it would give more useful information about *I. ricinus* survival under cold conditions. In our tests, this was only performed in a reduced sample of ticks due to the difficulty of removing dead ticks from the survival arenas (to be able to compare survival between *Borrelia*-infected and uninfected individuals as infection is only detected afterwards) without disturbing experimental conditions significantly.

Energy reserve tests – To our knowledge, the method used to quantify fat content (representing energy reserves) in *I. ricinus* ticks in our study was first described by Steele and Randolph (1985). Later, *I. ricinus* fat content has been repeatedly quantified using this method in studies conducted in the United Kingdom (Randolph and Storey, 1999; Randolph et al., 2002; Crooks and Randolph, 2006). This method uses the properties of chloroform, which dissolves and extracts lipids contained in the tick. However, we cannot exclude that some lipids that constitute tick cuticle (or more precisely, the outer epicuticle, which is rich in various lipids) (Hackman and Filshie, 1982) might be dissolved in the process. Hence, we might have quantified lipids contained inside the tick and lipids constituting tick epicuticle using this method. Our results cannot determine whether observed higher fat content in *Borrelia*-infected ticks represented, in fact, higher lipid content within the tick (i.e. higher energy reserves) and/or denser epicuticle (i.e. decreasing water loss). Moreover, this method does not allow determining what types of lipids are quantified. One might imagine that some particular types of lipids are present in higher quantity in *Borrelia*-infected versus uninfected ticks. It might therefore be interesting to determine what types of lipids are present in *I. ricinus* ticks and if some of them are present in different quantities in ticks harbouring spirochetes and in uninfected ticks.

5.2 General Discussion

The tick *I. ricinus* has two basic and interconnected needs that govern its entire behaviour and life cycle: feeding blood on a vertebrate host and maintaining its water balance. *I. ricinus* challenge is to manage to find the right balance in order to fulfil these needs, which are often not in agreement.

I. ricinus, like other haematophagous arthropods, needs blood meals (one at each developmental stage) from which it acquires its energy. *I. ricinus* is a generalist tick that feeds on more than 300 different vertebrate species ranging from mammals (including rodents, insectivores, lagomorphs, carnivores and artiodactyls) to reptiles (such as lizards) and birds (mostly passerines) (Anderson, 1991). It finds its vertebrate host while questing, i.e. waiting on the vegetation with its first pair of legs outstretched in the air. When a vertebrate host brushes the spot where the tick is waiting, the arthropod immediately grabs the passing hosts, climbs onto it, and ultimately takes a blood meal. While feeding, the tick may acquire pathogens such as Lyme disease spirochetes, if the vertebrate host is a reservoir host for *B. burgdorferi* s.l. and is infected (Gern et al., 1998), or by co-feeding if other ticks feeding closely harbour *Borrelia* spirochetes (Gern and Rais, 1996; Randolph et al., 1996). In Switzerland, at least seven Lyme disease genospecies, namely *B. afzelii*, *B. bavariensis*, *B. burgdorferi* s.s., *B. garinii*, *B. lusitaniae*, *B. spielmanii*, and *B. valaisiana* (Jouda et al., 2004b, Gern et al., 2010, Lommano et al., 2012b), and *B. miyamotoi*, which is related to relapsing fever spirochetes (Gern et al., 2010), have been detected in *I. ricinus* ticks.

Specificity between reservoir host and *B. burgdorferi* genospecies has been widely studied: *B. afzelii*, *B. bavariensis*, *B. burgdorferi* s.s., and *B. spielmanii* are associated with rodents (Humair et al., 1995; Craine et al., 1997; Humair and Gern, 1998; Kurtenbach et al., 1998b; Humair et al., 1999; Hu et al., 2001; Hügli et al., 2002; Hanincova et al., 2003a; Richter et al., 2004; Morán Cadenas et al., 2007b), *B. garinii* and *B. valaisiana* with birds (Humair et al., 1998; Kurtenbach et al., 1998b; Hanincova et al., 2003b; Comstedt et al., 2006), and *B. lusitaniae* with lizards (Dsouli et al., 2006; Majlathova et al., 2006; Richter and Matuschka, 2006). Co-infections with *Borrelia* genospecies sharing the same reservoir hosts (in particular *B. garinii-B. valaisiana* co-infections) have been more frequently reported than co-infections involving genospecies using different reservoir hosts in *I. ricinus* ticks in Europe (Rauter and Hartung, 2005) and in Swizterland (Morán Cadenas et al., 2007a). However, only one study (Kurtenbach et al., 2001) tested statistically whether *Borrelia* genospecies were independently distributed in questing *I. ricinus*. The authors reported that *B. afzelii-B. garinii* co-infections were less frequent than expected, while *B. garinii-B. valaisiana* co-infections were more frequent than expected, suggesting a mutually exclusive colonisation of individual ticks by *B. garinii* and *B. valaisiana* on the one hand and by *B. afzelii* on the other hand. Due to the very low prevalence of co-infections (3 % to 3.5 %; Morán Cadenas et al., 2007a; Gern et al., 2010), statistical tests on mixed infections are hardly performed, in particular when genospecies that occur rarely are involved. The large data set (7400 nymphs) used in our study (paper 5, Herrmann et al., submitted) allowed us to test the occurrence frequency of double infections (between six *Borrelia* genospecies, namely *B. afzelii*, *B. bavariensis*, *B. burgdorferi* s.s., *B. garinii*, *B. miyamotoi*, and *B. valaisiana*), even that of rare genospecies. Moreover, we quantified spirochete load in ticks co-infected with two *Borrelia* genospecies, which represented competition level between genospecies (higher spirochete load than expected, i.e. facilitation, and lower spirochete load than expected, i.e. inhibition). Our findings demonstrate statistically a more frequent co-occurrence than expected (positive covariance) between *B. afzelii-B. burgdorferi* s.s. and *B. garinii-B. valaisiana*, and a less frequent co-occurrence than expected (negative covariance) between *B. afzelii-B. garinii* and *B. afzelii-B. valaisiana*, presumably due to specificity between reservoir host and *Borrelia* genospecies. Moreover, they show novel and rare associations between genospecies, namely the positive covariance between *B. afzelii-B. miyamotoi* and the negative covariance between *B. afzelii-B. bavariensis*. Both associations are of particular interest since they give us additional information on *Borrelia* genospecies and their reservoir hosts. Hence, the positive covariance between *B. afzelii* and *B. miyamotoi* (whose reservoir host in Europe is currently unknown) suggests that the two genospecies share the same rodent reservoir hosts. In contrast, the negative covariance between *B. afzelii* and *B. bavariensis*, which both use *Apodemus* mice as reservoirs (Kurtenbach et al., 1998b; Humair et al., 1995, 1999; Hügli et al., 2002; Hanincova et al., 2003a), suggests exclusion between the genospecies, presumably competing for the same habitat and/or resources inside the vertebrate host. In nymphs co-infected with two genospecies using different reservoir hosts such as *B. afzelii-B. garinii* and *B. afzelii-B. valaisiana*, exceptionally low spirochete numbers were observed. This probably shows that the specificity between reservoir host and *Borrelia* genospecies is not exclusive but influences the number of spirochetes in the tick. Host complement system presumably reduces the spirochete number of the non-adapted *Borrelia* genospecies in the tick (Kurtenbach et al., 1998a, 2002a, 2002b). By contrast, unexpectedly high spirochete numbers were observed in nymphs harbouring *B. afzelii* and *B. burgdorferi* s.s. spirochetes, which are known to share the same rodent hosts (Humair and Gern, 1998; Humair et al., 1999). This seems to indicate that one or both genospecies facilitate each other's colonisation of the tick vector by suppressing vertebrate host and tick immune system. Overall, this study shows that *Borrelia* genospecies interactions fall into two broad categories: negative covariance associated with inhibition or positive covariance associated with facilitation, and that the host immune system plays a role in shaping both the frequency of double infections and the spirochete load in co-infected *I. ricinus*. Moreover, it highlights how interactions between the different actors of this host-vector-parasite system shape Lyme disease ecology, evolution, and epidemiology.

I. ricinus, like other terrestrial arthropods, needs to maintain its water balance in an environment whose relative humidity (RH) is often below its critical equilibrium humidity. Critical equilibrium humidity (situated between 86 to 96 % RH according to Lees (1946) and between 80 to 85 % RH according to Kahl (1989) in *I. ricinus*) is the humidity threshold below which ambient RH causes the tick to continuously lose water and above which passive diffusion and active uptake of atmospheric water causes the tick to gain water (Knülle and Wharton, 1964; Needham and Teel, 1986). When RH is below critical equilibrium humidity, the tick needs to minimise water loss and maximise active uptake of water vapour from the atmosphere (Knülle and Rudolph, 1982). *I. ricinus* loses water through the integument of its general body surface when it is exposed to humidity conditions below its critical equilibrium humidity, a phenomenon called transpiratory loss (Knülle and Rudolph, 1982). This occurs when *I. ricinus* is questing on the vegetation. In fact, *I. ricinus* adults may lose up to 10 % of their original body mass in one day when they are exposed to dehydrating conditions below their critical equilibrium humidity such as those they experience on grass tips while questing (Lees, 1946). Nymph and adult *I. ricinus* cannot tolerate water loss higher than 15 to 20 % of their original body mass (Kahl and Alidousti, 1997). In addition, *I. ricinus* loses water in the course of respiratory exchange via the tracheal system, a phenomenon named respiratory loss (Knülle and Rudolph, 1982). This occurs during *I. ricinus* mobility since respiration increases when ticks move (Rudolph and Knülle, 1979; Pugh et al., 1988; Lighton et al., 1993). Such water loss is particularly important in ticks that move up the vegetation to find a questing spot and later move down the vegetation to reach a moister environment. Water loss due to transpiration and respiration is compensated by acquiring water from the surrounding environment. *I. ricinus* does not drink liquid water (Lees, 1946; Kahl and Alidousti, 1997; Kröber and Guerin, 1999), but rather extracts water vapour from the atmosphere whose humidity level exceeds tick critical equilibrium humidity (Lees, 1946, 1948; Knülle and Rudolph, 1982). In nature, the tick achieves this by returning periodically to moist surroundings such as the litter layer (Lees, 1946; Lees and Milne, 1951), as shown by dehydrated *I. ricinus* nymphs moving preferentially towards fully saturated air under laboratory conditions (Lees, 1948; Crooks and Randolph, 2006). *I. ricinus* absorbs water vapour by active sorption (O'Donnell and Machin, 1988; Needham and Teel, 1991) faster when atmospheric humidity reaches 85 to 90 % (Lees, 1946).

I. ricinus is known to display little resistance to desiccation (MacLeod, 1935; Lees, 1946; Aeschlimann, 1972). RH needs to be above 70 to 80 % to allow *I. ricinus* questing activity and survival (MacLeod, 1935). Moreover, as decreasing humidity (and increasing water loss) is often associated with increasing temperature in nature, temperature plays a considerable role in *I. ricinus* activity (MacLeod, 1935; Needham and Teel, 1986). Hence, *I. ricinus* is active and negatively geotropic (i.e. climbs up to vegetation to quest) when temperature ranges between 7 and 24°C (MacLeod, 1935; Perret et al., 2000). In contrast, the tick reduces its activity and is positively geotropic (i.e. returns to the litter layer to rehydrate) when temperature reaches 30°C (MacLeod, 1935). Saturation deficit (SD), a measure of the drying power of the atmosphere depending on both temperature and RH (Randolph and Storey, 1999), also influences *I. ricinus*. SD values that are lower than 5 mmHg are favourable for *I. ricinus* questing activity and development, but the tick is already positively geotropic when SD reaches 4.4 mmHg (corresponding to 80 % RH at 24°C) (MacLeod, 1935; Perret et al., 2003; Burri et al., 2007). Increased SD reduces *I. ricinus* questing activity (Burri et al., 2007; Morán Cadenas et al., 2007a; Gern et al., 2008; Knap et al., 2009; Tagliapietra et al., 2011), duration of questing (Perret et al., 2003, 2004), and survival in nature (Perret, 2002; Perret et al., 2000, 2004) and under laboratory conditions (Perret, 2002). *I. ricinus* larvae are less tolerant of desiccation and therefore die earlier than nymphs when kept under desiccating conditions (MacLeod, 1935). In turn, *I. ricinus* nymphs are more importantly affected by desiccating conditions (therefore suffering a higher death rate) than adults, as shown by the partial restoration of questing adult population (but not of questing nymph population) after each drought event in nature (Perret et al., 2004). The lower resistance to desiccation observed in *I. ricinus* immature than adult stages (MacLeod, 1935; Lees, 1946; Perret et al., 2004) is suspected to be mainly due to the bigger size of adults, so that the latter have a higher water content and a lower surface area to volume ratio (causing a proportionally lower water loss) than the former (Randolph and Storey, 1999).

I. ricinus questing activity is therefore greatly influenced by weather conditions. Hence, during winter, when weather conditions are unfavourable, unfed *I. ricinus* ticks usually do not quest and are in the leaf litter or in the upper layers of the soil where temperatures are milder than on the vegetation (Dusbabek et al., 1971; Daniel et al., 1972; Gigon, 1985; Daniel et al., 1976, 1977). There, unfed ticks are in a state of inactivity, classically called behavioural diapause (Belozerov, 1982). However, depending on how Belozerov's terminology of diapause (i.e. diapause is exclusively triggered by changes in photoperiodicity) is interpreted, it may be argued whether the inactive state observed in overwintering ticks should be called a behavioural diapause or a facultative quiescence (Gray, 1991; Randolph et al., 2002), since the interaction between photoperiodicity and temperature appears to determine whether *I. ricinus* ticks are active or not during winter (Randolph et al., 2002; Dautel et al., 2008; Gray, 2008). Anyhow, behavioural diapause should not be confused with developmental diapause, which also occurs during winter months. Developmental diapause is a physiological state during which metabolic processes (and therefore development) are slowed down in engorged ticks, which is triggered by changes in photoperiodicity (Belozerov, 1982). Indeed, metabolism processes in diapausing (engorged) ticks do not accelerate when temperature increases during winter but remain constant until photoperiodicity changes in spring.

In Switzerland, questing ticks may be observed as early as mid-February to early March (Aeschlimann, 1972; Morán Cadenas et al., 2007a). In fact, questing *I. ricinus* ticks are active when the daily maximal temperature has reached 7°C over 5 days (Perret et al., 2000). Adults and nymphs usually emerge first, followed by larvae (Aeschlimann, 1972). Tick questing density increases progressively as weather conditions get warmer (Perret et al., 2000) until peak density is recorded in spring, usually between April and June (Aeschlimann, 1972; Morán Cadenas et al., 2007a). Questing density then decreases gradually due to increasing numbers of ticks on vertebrate hosts, and to increasingly drier climate conditions, so that *I. ricinus* ticks rarely quest during summer, except at higher altitudes where climate is milder (Burri et al., 2007; Morán Cadenas et al., 2007a). In autumn, when favourable conditions of temperature and humidity are back, a second activity peak may be observed (Aeschlimann, 1972; Morán Cadenas et al., 2007a). However, the autumn activity peak is of lower intensity than the one observed in spring and is absent if weather conditions are unfavourable (Aeschlimann, 1972; Perret et al., 2000, 2004; Morán Cadenas et al., 2007a). The last questing *I. ricinus* ticks are usually sampled in October to early November (Aeschlimann, 1972; Morán Cadenas et al., 2007a) as ticks return progressively to an inactive state during winter (Belozerov, 1982; Dautel et al., 2008).

As previously mentioned, *I. ricinus* has two basic needs that determine its actions during its whole life: to have a blood meal on a vertebrate host and to maintain its water balance. To fulfil these needs, the tick consumes energy when it climbs up the vegetation to quest and when it periodically moves down the vegetation to rehydrate in the litter layer. *I. ricinus* needs to move down the vegetation to rehydrate more often when weather conditions get drier (Randolph and Storey, 1999; Perret et al., 2003), consuming even more energy in the process. In *I. ricinus*, energy obtained from the blood meal is digested intra-cellularly within the midgut and gut epithelium (Akov, 1982; Franta et al., 2010). In ticks, part of the blood meal is converted and stored as fat (lipids) in epithelial cells of the midgut (Tarnowski and Coons, 1989; Umemiya-Shirafuji et al., 2010) and in the fat body (Sonenshine, 1991). As *I. ricinus* has no other energy sources, its body fat content declines over time between blood meals (Steele and Randolph, 1985; Randolph and Storey, 1999; Randolph et al., 2002). In nature, newly moulted *I. ricinus* ticks (i.e. with high fat contents) usually start questing in autumn (Randolph et al., 2002). Thereafter, tick fat contents decline steadily until ticks with very low fat content disappear completely from the questing population (usually the following autumn) (Randolph et al., 2002). In addition, the rate of lipid consumption in *I. ricinus* increases under unfavourable conditions of humidity (i.e. low humidity) (Randolph and Storey, 1999) and temperature (i.e. high temperature) (Van Es et al., 1998).

Due to *I. ricinus* high sensitivity to temperature and humidity (MacLeod, 1935; Lees, 1946; Aeschlimann, 1972; Knülle and Rudolph, 1982; Sonenshine, 1991), climate change affects this tick species considerably. This is illustrated by the distribution shifts towards higher latitudes in the northern distributions of *I. ricinus* in Sweden (Lindgren et al., 2000; Jaenson et al., 2012) and Norway (Jore et al., 2011) and towards higher altitudes in the Czech Republic (Daniel et al., 2003; Materna et al., 2005, 2008), Switzerland (Morán Cadenas et al., 2007a) and Norway (Jore et al., 2011). Such phenomenon is due to the fact that climate conditions and habitats at higher latitudes and altitudes become suitable for this tick. Higher temperatures in spring and autumn extend the vegetation period and the season during which *I. ricinus* develops, allowing vegetation communities (and therefore mammals on which *I. ricinus* feeds) and tick populations to colonise and establish at higher latitudes (Jaenson and Lindgren, 2011) and altitudes (Danielova et al., 2006). In addition, climate change affects *I. ricinus* populations in areas where the tick is already established, notably influencing its seasonal activity (Gray, 2008). And *I. ricinus* seems to gradually extend its seasonal activity as it uses milder winter periods for re-emerging and questing (Dautel et al., 2008; Gray, 2008).

In Switzerland, like in other Central European countries, winter conditions are changing in areas where *I. ricinus* populations have been established for centuries due to global warming. Globally, winter temperatures in Switzerland are 1 to 1.5°C higher today than they were in the 1950s (Beniston, 2004). Moreover, a reduction of day-to-day temperature variability due to the loss of coldest extremes, particularly in winter (Rebetez, 2001), and a decrease in snowfall frequency and quantity (North et al., 2007) have been observed during the last century on the Swiss plateau, which is the main distribution area of *I. ricinus*. Although it has been shown that *I. ricinus* nymphs may survive -10°C for one month under laboratory conditions (Dautel and Knülle, 1997), the impact of changing winter conditions due to climate change on *I. ricinus* populations remained unclear. In such context, we attempted to determine how low temperature (-5°C and -10°C) and the frequency of temperature variations would influence *I. ricinus* survival under cold conditions. Our findings (paper 4, Herrmann and Gern, 2013) demonstrate that younger *I. ricinus* nymphs (sampled in autumn) survive better than older nymphs (sampled in spring), presumably due to higher energy reserves in the former. As ticks collected in spring have usually spent one winter in an unfed state (Randolph et al., 2002), this observation suggests that unfed *I. ricinus* may not survive two successive winters without a blood meal. If the nymph does not find a host within a year from its emergence, it is likely to die during its second winter. Furthermore, this study shows that *I. ricinus* nymphs die earlier when they are exposed to temperature variations at high frequency than at low frequency. The higher death rate is probably due to higher energetic costs associated with the metabolic adaptations that temperature variations generate when experienced at high frequency. Such findings are of particular interest since overwintering *I. ricinus* populations are likely to be more exposed to winter temperature variations in the future due to the observed reduction of snow cover in the main distribution area of *I. ricinus* in Switzerland (North et al., 2007) and the predicted decrease in number of precipitation events in the future, as described in the Alps (Beniston et al., 2011) or in Europe (Beniston, 2009), reducing snow cover protection. *I. ricinus* populations are expected to benefit from the reduced day-to-day temperature variability due to the loss of coldest extremes, particularly in winter, observed in their distribution area (Rebetez, 2001), resulting in lower tick mortality during winter. However, if reduced snow cover happened to be associated to important temperature fluctuations during a particular winter, *I. ricinus* populations would be expected to be more severely impaired, resulting in unexpectedly high tick mortality during such winter.

Climate changes may be observed during cold months, but they are usually more pronounced during warm months. For example, while overall mean temperature has increased by 1.5°C in 50 years in Switzerland, it is expected to rise by 2°C in winter and 3°C in summer by 2050 (North et al., 2007). As increasing temperature is usually associated with decreasing humidity (resulting in high SD values) in nature and *I. ricinus* is highly sensitive to desiccation, global warming stresses established tick populations most importantly in spring (questing activity peak) and summer, and to a lesser extent in autumn. As a matter of fact, *I. ricinus* questing density is low throughout the year when SD is high during spring and summer (Perret et al., 2000). Interestingly, an intriguing phenomenon was observed during such a year (i.e. 1998) in Switzerland. While *I. ricinus* questing density was exceptionally low (Perret et al., 2000), unexpectedly high numbers of nymphs harboured *Borrelia* spirochetes in high numbers during that year: 83.3 % of questing *I. ricinus* nymphs were heavily infected with borreliae in 1998, compared to 60 %

during the previous and following years (L. Gern, personal communication). This observation seemed to suggest that *I. ricinus* ticks infected with *B. burgdorferi* s.l. might perceive and/or tolerate desiccating conditions differently from uninfected individuals.

The idea that a parasite can modify the phenotype of its host by changing its perception of the environment and/or behaviour may be hard to believe. However, increasing examples of such phenomena prove that it is, in fact, well established and documented in hundreds of distinct host-parasite associations in all major phyla of living organisms (Moore, 2002; Poulin, 2010). While most of known cases involve only subtle modifications in host phenotypes, some are doubtlessly spectacular. Among the most exceptional host manipulations are the five following examples: (1) The trematode *Dicrocoelium dendriticum* causes its ant intermediate host to climb to the top of glass blades in the evening and stay there waiting for its sheep definitive host to eat the infected ant while grazing during the night (Carney, 1969). (2) Another trematode, *Leucochloridium* spp., changes the size, shape and colouration of the tentacles of its snail intermediate host and causes them to pulsate in response to light, so that its bird definitive host confuses the colourful tentacles with caterpillars and eats them (Lewis, 1974). (3) The nematode *Gasteromermis* spp. that needs water to release its eggs, when infecting a male mayfly host, feminises its host so that the male mayfly behaves like a female mayfly, returning to a stream to oviposit, although only the nematode lays eggs (Vance, 1996). (4) The protozoan *Toxoplasma gondii* causes its rat intermediate host to be attracted by cat odour, so that its cat definitive host is more likely to eat the infected rat (Berdoy et al., 2000). (5) Another nematode, *Myrmeconema neotropicum*, turns the gaster (i.e. posterior abdominal portion) of its ant intermediate host bright red and causes the ant to walk with their gaster in a conspicuous elevated position, so that its frugivorous bird definitive host confuses the gasters with berries and eats the infected ant (Yanoviak et al., 2008).

As shown in these examples, modifications of host phenotype by parasites aim at enhancing parasite transmission to the next host, and thereby completing the parasite life cycle. Although the preceding examples do not involve vectors and their parasites, numerous vector-borne parasites have been shown as well to modify phenotypic traits of their arthropod vectors so that their probability of transmission to vertebrate hosts is enhanced (Hurd, 2003; Lefèvre and Thomas, 2008). The best-known examples involve pathogens transmitted by blood-sucking insects taking short blood meals on multiple hosts such as mosquitoes, sand flies, or tsetse flies. In such cases, manipulation usually consists of modifying vector behaviour so that the number of bitten hosts (and thereby infected hosts) is increased using strategies such as increased biting rates, shortened blood meals, increased lifespan, etc. (Moore, 1993; Hurd, 2003; Rogers and Bates, 2007; Lefèvre and Thomas, 2008). Unlike haematophagous insects, ticks feed for days (Gern, 2009) and transmission of tick-borne parasites often needs some time to occur (i.e. several hours), as shown in *B. burgdorferi* s.l. transmission by *I. ricinus* (Kahl et al., 1998; Crippa et al., 2002). In contrast to insect-borne parasites, tick-borne parasites cannot increase the number of encountered hosts by their vectors during their life cycle. As a result, tick-borne parasites need to use different strategies to enhance their transmission to vertebrate hosts (Table 5.1). Examples show that bacteria and viruses that infect ticks belonging to the *I. ricinus* complex may modify their vector questing activity (Alekseev and Dubinina, 1994; Alekseev, 1996; Lefcort and Durden, 1996; Naumov, 1999; Alekseev and Dubinina, 2000; Alekseev et al., 2000; Perret, 2002; Belova et al., 2012; Romashchenko et al., 2012), perception of stimuli (Alekseev et al., 1993; Alekseev, 1996; Lefcort and Durden, 1996; Meiners et al., 2011; Vollandt et al., 2011) and survival (Randolph, 1991; Hu et al., 1997; Naumov, 2003; Gassner, 2010; Neelakanta et al., 2010).

Table 5.1 Phenotypic traits modified by tick-borne pathogens (bacteria and viruses) in tick vectors belonging to the *I. ricinus* complex.

Modified trait	Vector	Pathogen	Detailed effect	Reference
Questing activity	*I. persulcatus* adults	*B. burgdorferi* s.l.	Walk shorter distances	Alekseev and Dubinina (1994), Alekseev (1996)
	I. persulcatus adults	TBE virus	Walk faster, reach higher questing height, more tolerant of desiccation	Alekseev (1996)
	I. scapularis nymphs	*B. burgdorferi* s.s.	Walk longer distances, reach higher questing height, attracted by vertical surfaces	Lefcort and Durden (1996)
	I. scapularis adults	*B. burgdorferi* s.s.	Walk shorter distances, reach lower questing height, avoid vertical surfaces	Lefcort and Durden (1996)
	I. persulcatus nymphs and adults	*B. burgdorferi* s.l.	Questing activity is increased but inhibited more importantly by temperature	Naumov (1999)
	I. persulcatus nymphs and adults	*B. burgdorferi* s.l.	Questing triggered by higher temperature and lower relative humidity	Alekseev and Dubinina (2000)
	I. ricinus larvae, nymphs and adults	*B. burgdorferi* s.l.	Walk shorter distances	Alekseev et al. (2000)
	I. ricinus nymphs	*B. burgdorferi* s.l.	Interrupt quiescence more often, walk longer distances under desiccating conditions	Perret (2002)
	I. ricinus adults	TBE virus	Walk faster, more tolerant to tick-repellent	Belova et al. (2012)
	I. persulcatus adults	*B. burgdorferi* s.l.	Reach higher questing height, walk slower	Romashchenko et al. (2012)
Perception of stimuli	*I. ricinus* and *I. persulcatus* ticks	*B. burgdorferi* s.l.	React differently to plant odours	Alekseev et al. (1993), Alekseev (1996)
	I. scapularis nymphs	*B. burgdorferi* s.s.	Increased phototaxis	Lefcort and Durden (1996)
	I. ricinus nymphs and adults	*B. afzelii*	Respond more efficiently to odours	Meiners et al. (2011)
	I. ricinus nymphs and adults	TBE virus	Attracted only by odours of competent hosts	Vollandt et al. (2011)
Survival	*I. trianguliceps* larvae	*B. microti*	Increased blood meal size, increased moulting rate from larvae to nymphs	Randolph (1991)
	I. scapularis nymphs	*B. microti*	Increased blood meal size	Hu et al. (1997)
	I. ricinus adults	*B. burgdorferi* s.l.	Prolonged survival	Naumov (2003)
	I. persulcatus adults	*B. burgdorferi* s.l.	Prolonged survival	Naumov (2003)
	I. ricinus nymphs	*B. afzelii*	Higher energy reserves	Gassner (2010)
	I. scapularis nymphs	*A. phagocytophilum*	Increased survival under cold conditions	Neelakanta et al. (2010)

With that in mind, the important number of *I. ricinus* nymphs infected with high numbers of *Borrelia* spirochetes detected during 1998, a year with high death rate in *I. ricinus* tick populations due to desiccating conditions in Neuchâtel (Perret et al., 2000), seemed to indicate that *Borrelia* infection might play a role in survival of *I. ricinus* ticks under desiccating conditions. In such context, we undertook to test whether *I. ricinus* nymphs and adults infected with *B. burgdorferi* s.l. survived differently from uninfected individuals under hot and dry conditions. Our study (paper 1, Herrmann and Gern, 2010) shows that *I. ricinus* nymphs and adults harbouring *Borrelia* spirochetes survive better than their uninfected counterparts under desiccating conditions. In this case, pathogen transmission chances are increased by enhancing survival of the infected tick vector under unfavourable conditions, giving the tick more time and opportunities to find a vertebrate host (and thereby transmit the spirochetes). Although this study does not reveal the mechanisms leading to the observed phenomenon, it has allowed us to formulate the hypothesis that *Borrelia* spirochetes that are in the midgut and/or those that disseminate and produce a systemic infection in the tick (Burgdorfer et al., 1989; Lebet and Gern, 1994) might change the physiology and/or metabolism of organs involved in water sorption, storage, or loss. This might result in enhanced water storage in infected ticks, and *I. ricinus* need for humidity might therefore be modified by *Borrelia* infection. On the other hand, our findings might be due to modification of tick behaviour by spirochetes, so that infected ticks take more risks by staying immobile under desiccating conditions, although *I. ricinus* ticks are known to move importantly under desiccating conditions (Lees, 1948). Such risky behaviour might have been beneficial to infected ticks since they spared energy reserves while uninfected ticks depleted theirs trying to find a moister environment in the survival arenas without success, resulting in higher death rate in more active uninfected ticks.

Even though it has been shown that *I. ricinus* nymphs infected with *Borrelia* walk longer and more often in the vertical plane under very desiccating conditions (9.3 mmHg) (Perrett, 2002) and that dehydrated *I. ricinus* ticks move preferentially towards a moister environment in both the horizontal (Crooks and Randolph, 2006) and the vertical plane (Lees, 1948), the influence of *Borrelia* infection on *I. ricinus* movement within a humidity gradient in the horizontal plane remained unknown. That is why we tested whether *I. ricinus* nymphs harbouring *Borrelia* spirochetes were attracted to humidity differently from uninfected individuals by observing if infected nymphs walked differently within a humidity gradient. Our findings (paper 2, Herrmann and Gern, 2012) are similar to observations by Alekseev et al. (2000), i.e. *Borrelia*-infected nymphs walk shorter distances (on an inclined plane) than uninfected individuals. In addition, they show that *I. ricinus* nymphs harbouring *Borrelia* spirochetes have a lower need to move towards an environment that is favourable for maintaining water balance than uninfected nymphs. Indeed, infected individuals stayed immobile in an environment (70 to 75 % RH, corresponding to SD values of 6.2 to 5.2 mmHg at room temperature, ~23°C), which they were expected to leave since *I. ricinus* ticks move to a moister environment when SD reaches 4.4 mmHg (corresponding to 80 % RH at 24°C) in nature (MacLeod, 1935). In this case, vector manipulation consists of increasing the tick chances to find a vertebrate host (and thereby transmit the pathogen) by increased questing time, i.e. by allowing the tick to quest for a longer time under desiccating conditions before returning to a moister environment such as the litter layer to rehydrate.

According to our above-mentioned observations, *Borrelia*-infected ticks are more tolerant to desiccating conditions (better survival and increased questing time under these conditions) than uninfected ticks. However, the mechanisms leading to this phenomenon remained to be discovered. Since increasing desiccation forces *I. ricinus* ticks to consume more energy by moving down the vegetation to rehydrate more frequently in nature (Randolph and Storey, 1999; Perret et al., 2000), we hypothesised that the observed higher tolerance to desiccation in *I. ricinus* harbouring *Borrelia* spirochetes than in uninfected ticks might be due to higher energy reserves in the latter than the former. That is why we intended determining if energy reserves, measured by quantifying fat content, differed between *Borrelia*-infected and uninfected *I. ricinus* ticks. Our study (paper 3, Herrmann et al., 2013) reveals that *I. ricinus* nymphs harbouring *Borrelia* spirochetes possess higher fat content, and therefore more energy reserves, than their uninfected counterparts. Moreover, bioenergetics calculations show that *Borrelia* spirochetes consume a negligible fraction of the tick energy reserves to grow. However, the study does not allow us to comprehend how higher energy reserves in ticks and *Borrelia* infection happen to be associated, as mechanisms causing this phenomenon are presumably multiple and interconnected. Hypotheses may nevertheless be formulated. *Borrelia* spirochetes in the vertebrate host might suppress host immune response resulting in increased blood meal size in the tick, as shown in *I. trianguliceps* (Randolph, 1991) and *I. scapularis* ticks (Hu et al., 1997) feeding on *B. microti*-infected rodents. The increased blood meal size might be subsequently converted into more fat content in the tick. Alternatively, *Borrelia* spirochetes might influence their vector directly by changing the expression of genes involved in fat storage in ticks (such as the 4E-BP genes described by Kume et al., 2012) so that fat storage is enhanced during/after the blood meal. Although causes leading to such phenomenon remain unknown, we may imagine what consequences it may have. In this case of manipulation, the pathogen increases its transmission chances by increasing its vector energy reserves, thereby extending its vector life span, a phenomenon similarly reported by Naumov (2003) in *Borrelia*-infected *I. ricinus* and *I. persulcatus* adults under laboratory conditions. Higher energy reserves in *Borrelia*-infected nymphs means longer time for questing. Since infected nymphs can move up and down the vegetation more often before their fat reserves are depleted, they survive longer and questing time is increased. In turn, increased questing time implicates that nymphs harbouring *B. burgdorferi* spirochetes have increased chances to find hosts, and thereby increased chances to transmit spirochetes. In short, this situation illustrates how a pathogen modifies the phenotypic traits of its tick vector to enhance its transmission to another host: by increasing tick survival under desiccating conditions, tolerance to desiccation and energy reserves, eventually leading to increased opportunities for its vector to find a host.

The idea that *B. burgdorferi* s.l. infection increases *I. ricinus* opportunities to find a vertebrate host due to modified questing activity is particularly interesting. Faulde and Robbins (2008) have reported that host-finding efficacy is increased in female *I. ricinus* ticks harbouring *B. burgdorferi* spirochetes. These authors observed that *Borrelia* infection prevalence was higher in unfed ticks collected from clothing of volunteers than directly from vegetation in the same forest in Germany. In the context of climate change, which is expected to favour *Borrelia*-infected *I. ricinus* ticks according to our findings, we surmise that the risk for human population to encounter ticks harbouring *B. burgdorferi* spirochetes, and therefore to be bitten by such individuals, might increase in the future. As Lyme disease is to date the most frequent tick-borne disease throughout the world, accounting for approximately 85,500 new human cases annually, among which roughly 65,500 occur in Europe (Hubalek, 2009), we might expect this disease to become a greater public health concern if climate changes get more pronounced and *Borrelia*-infected ticks are more likely to find hosts under such conditions. However, such suppositions might reveal inaccurate in the future since Lyme disease epidemiology does not depend on the sole role of *I. ricinus* but rather on multiple parameters, notably *Borrelia* reservoir host populations, which might be negatively influenced by climate change.

6 CONCLUSION

6 CONCLUSION

In the context of climate change and global warming, the observations (1) that *I. dammini* density was reduced while *Borrelia*-infected *I. dammini* prevalence was increased after the burning of a forest area (Mather et al., 1993) and (2) that unexpectedly high numbers of *Borrelia*-infected nymphs harboured high numbers of spirochetes during a particularly desiccating year (resulting in high tick mortality) (Perret et al., 2000; Lise Gern, personal communication), suggesting that *I. dammini* and *I. ricinus* ticks harbouring *B. burgdorferi* spirochetes might survive better under desiccating conditions in nature, have stressed the importance of studying the biology of *Borrelia*-infected and uninfected ticks under different climate conditions.

This PhD thesis has answered several questions that arose from such observations. First, our results confirm that *I. ricinus* survival under hot and dry conditions is influenced by *B. burgdorferi* s.l. infection: *I. ricinus* nymphs and adults harbouring spirochetes survive better under desiccating conditions than uninfected individuals. Second, our findings show that *I. ricinus* behaviour within a humidity gradient is influenced by *B. burgdorferi* s.l. infection: *Borrelia*-infected *I. ricinus* nymphs walk less within a humidity gradient, i.e. show a lower need to move to an environment that is favourable for maintaining water balance, than uninfected individuals. Third, our results demonstrate that *I. ricinus* level of energy reserves is influenced by *B. burgdorferi* s.l. infection: *I. ricinus* nymphs harbouring spirochetes possess higher energy reserves than uninfected ticks. Consequences of these findings are the following: higher energy reserves in *Borrelia*-infected nymphs result in longer time for questing (due to longer survival and increased number of movements up and down the vegetation before energy reserves are depleted) in infected ticks, in turn leading to increased chances for infected ticks to find hosts and thereby to transmit *Borrelia* spirochetes. The latter is thought to be a manipulating strategy of *B. burgdorferi* spirochetes to enhance their transmission to other hosts by modifying phenotypic traits of their tick vector.

We have described how higher energy reserves in *Borrelia*-infected *I. ricinus* ticks may allow infected ticks to be more tolerant under desiccating conditions. However, higher tolerance to desiccation in *Borrelia*-infected ticks might also be induced by more efficient maintenance of water balance. As *Borrelia* spirochetes may disseminate and produce a systemic infection in the tick (Burgdorfer et al., 1989; Lebet and Gern, 1994), dispersed spirochetes, as well as spirochetes present in the midgut, might modify the physiology and/or metabolism of organs involved in water sorption or loss resulting in enhanced water storage in infected *I. ricinus* ticks. More efficient maintenance of water balance would help *Borrelia*-infected ticks spare energy reserves by lowering their need to move frequently to a moister environment to rehydrate under desiccating conditions, thereby increasing the difference in fat content between infected and uninfected ticks. Future research is expected to study the capacity of *Borrelia*-infected versus uninfected *I. ricinus* ticks to absorb and/or retain water and the physiology of organs involved in these phenomena.

Furthermore, our results suggest that *Borrelia*-infected *I. ricinus* ticks may be more likely to find a vertebrate host than their uninfected counterparts because their questing behaviour is optimised, presumably due to spirochete manipulation of their tick vector. However, having a blood meal is one step away from having increased chances to find a vertebrate host (although the latter is probably the hardest part). Since spirochete ultimate goal is to enhance their transmission to the host, it might be interesting to investigate whether infected ticks that have increased opportunities to find a vertebrate host due to modified questing activity are also more aggressive towards the host, i.e. whether they bite the host and start feeding more rapidly, than uninfected ticks.

As phenotypic traits are (at least partly) influenced by mechanisms working at the genetic level, determining what genes of the tick are targeted by spirochetes or which spirochetal genes are involved in manipulating the tick might be very useful to understand the precise mechanisms causing *I. ricinus* ticks harbouring *B. burgdorferi* s.l. to be so different from *Borrelia*-free individuals.

Besides, our results indicate that *I. ricinus* survival under cold conditions is likely to be influenced by *B. burgdorferi* s.l. infection: *Borrelia*-infected *I. ricinus* nymphs survive slightly better under cold conditions than uninfected individuals. More importantly, our observations show that increased frequency of temperature variations from negative to positive temperatures influences negatively *I. ricinus* survival during winter, and that *I. ricinus* ticks that have experienced a first winter season in an unfed state are unlikely to survive a second winter season without a blood meal.

Our findings confirm that *Borrelia* genospecies that are specialised for the same reservoir hosts frequently occur together in *I. ricinus* nymphs, while *Borrelia* genospecies that are specialised for different reservoir hosts rarely co-occur. Moreover, infections involving *Borrelia* genospecies frequently co-occurring consisted of high spirochete numbers, whereas infections involving *Borrelia* genospecies rarely co-occurring consisted of low spirochete numbers. These results suggest that the vertebrate host immune system plays a role in shaping both the frequency of infections involving two *Borrelia* genospecies and the spirochete load inside the tick harbouring two *Borrelia* genospecies. As specificity between reservoir hosts and *Borrelia* genospecies seems to be prominent in shaping *Borrelia* infection, we can surmise that *Borrelia* genospecies specialised for different vertebrates may have developed different manipulating strategies of their *Ixodes* vector to enhance their transmission to specific hosts. In fact, it would be more beneficial for bird-adapted genospecies such as *B. garinii* and *B. valaisiana* if their vector fed on a bird host, while rodent-adapted genospecies such as *B. afzelii*, *B. bavariensis* and *B. burgdorferi* s.s. would benefit more if their vector fed on a rodent host. That is why we can imagine that *I. ricinus* ticks infected with different genospecies may, for instance, be attracted differently by odours produced by bird and rodent hosts. Alternatively, *I. ricinus* ticks infected with different genospecies may tolerate desiccating conditions differently and therefore quest at higher heights. For example, low questing height in ticks infected with rodent-adapted spirochetes would optimise chances to make contact with rodents, as the latter live close to the ground. In contrast, higher questing height in ticks infected with bird-adapted spirochetes would enhance chances to make contact with hosts that live further away from the ground, such as birds. Such examples illustrate how each *Borrelia* genospecies may influence its vector differently depending on its specific needs in terms of transmission. Future investigations should therefore consider testing such aspects of the biology of infected *I. ricinus*.

In fine, although the underlying mechanisms leading to the phenomena described throughout these pages remain to be understood, this thesis contributes significantly to the acknowledgement of the influence of *B. burgdorferi* s.l. spirochetes on phenotypic traits of their tick vector *I. ricinus*, optimising their transmission to the next host in the context of climate change and global warming.

7 REFERENCES

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8 ACKNOWLEDGEMENTS

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J'aimerais remercier chaleureusement tous les anciens et actuels membres du groupe de parasitologie de l'université de Neuchâtel car cette aventure n'aurait pas été la même sans vous. Ma thèse n'a pas seulement été riche sur le plan scientifique mais aussi social. Vous me manquez déjà.

Mes plus sincères remerciements vont à:

Lise Gern, qui a dirigé cette thèse, qui m'a constamment motivée avec des commentaires positifs et sensés, avec qui j'ai eu des discussions interminables et excitantes sur la science mais aussi sur la vie en général, qui a contribué énormément à ma réussite, et qui a toujours été disponible quand il le fallait

Bruno Betschart, qui m'a engagée en tant qu'assistante pour enseigner les travaux pratiques

Jakob Koella, qui m'a soulagée de la charge d'enseigner les travaux pratiques pendant mon dernier semestre

Maarten Voordouw, qui a été mon expert externe lors de mon examen de mi-thèse, qui a conçu les analyses statistiques de la plupart de mes articles, qui a contribué de manière importante à la rédaction de nos publications communes et qui fait partie de mon jury de thèse

Sarah Randolph and Jean-Luc Perret, qui font partie de mon jury de thèse

Peter-Alan Diehl and Michel Brossard, qui m'ont donné de précieux conseils, notamment sur la littérature scientifique

Patrick Guerin, qui m'a prêté généreusement du matériel appartenant à son groupe

Lorenza Béati, qui m'a initiée à la génétique des populations et qui fait partie de mon jury de thèse

Jean-Pierre Duvoisin, qui m'a aidée à construire des arènes pour mes tiques

Daniel Varidel, qui m'a permis d'utiliser un incubateur appartenant à l'institut de physique et qui l'a surveillé attentivement durant les tests que j'ai menés

Peter Fuchs, qui m'a cordialement invitée à utiliser une microbalance unique en son genre à l'office fédéral de Métrologie METAS, Wabern

Radu Slobodeanu, qui a amélioré la qualité des tests statistiques utilisés pour analyser mes données

Olivier Rais, plus communément connu sous le pseudonyme Pitou, qui m'a répétitivement aidée à récolter des tiques à Neuchâtel, qui m'a initiée à différentes techniques utilisées dans le laboratoire, qui a toujours réagi au quart de tour lorsque je l'ai sollicité, et avec qui j'ai passé de nombreuses heures à refaire le monde dans le train

Francisca Morán Cadenas, ancien membre du groupe Lyme, qui m'a permis d'utiliser ses précieux échantillons pour mon projet de génétique des populations

Caroline Burri-Cordonnier et Elena Lommano, anciennes doctorantes dans le groupe Lyme, avec qui j'ai passé des conférences scientifiques sérieuses mais aussi délirantes, et qui m'ont enseigné certaines techniques de laboratoire

Nicolas Tonetti et Léonore Lovis, anciens assistants-doctorants, qui m'ont souvent donné de précieux conseils et m'ont montré comment enseigner les travaux pratiques de manière stimulante

Mirko Berggoetz, membre du groupe Lyme avec qui je partage mon bureau, qui a suivi les cours du programme doctoral avec moi, et avec qui j'ai eu de longues et passionnantes conversations

Marion Quartier, ancienne doctorante dans le groupe de parasitologie, qui m'a beaucoup aidée durant mon projet de génétique des populations

Jonas Durand et Maxime Jacquet, actuels doctorants dans le groupe de parasitologie, avec qui j'ai passé de sympathiques soirées et avec qui j'ai souvent joué au pingpong

Liselore Roelfstra, Barbara Molnar, Nikolaus Huber, membres du groupe de parasitologie, avec qui j'ai eu des discussions intéressantes au laboratoire, à la pause de midi et à nos fameux apéros

Jonathan Hamley et Antoine Barreaux, membre du groupe de parasitologie, avec qui j'ai enseigné les travaux pratiques durant ma dernière année

Christèle Dupasquier, ancienne étudiante en master dans le groupe Lyme, Christopher Holzer, Mégane Pluess, Angélique Schmid et Jeanne Santoli, anciens apprentis en biologie dans le groupe de parasitologie, qui m'ont aidée à récolter des milliers de tiques

Thomas Kröber, Michèle Vlimant, Martine Bourquin, Charles Chappuis, Jérôme Frei, Alexandre Gurba, Caroline Joris et Sébastien Kessler, membres du groupe de parasitologie, qui ont partagé leurs connaissances concernant le fonctionnement du matériel, la physiologie des parasites et les statistiques avec moi

Nathalie Veyrat, Kangaji Kasongo, Sabrina Joye, Séverine Monnier, Luce Bertaiola et tous mes amis, Lucia Garcia, Maude Sunier, Virginie Wyss, Mélody Schmid, Daniel Ston, Olivier et Coraline Schumann, Pierre Lehmann, Manel Frötscher, Mathieu Blaser et tous les autres anciens ou actuels membres du groupe de parasitologie, qui ont apporté leur entrain dans le laboratoire mais aussi en dehors

Toute la famille Stornetta, dont les membres ont toujours été intéressés d'en connaître plus sur le monde des tiques

Ma famille fantastique, Félix, Dina, Olivia, Fiona, et Alexis Herrmann, dont les membres n'ont pas eu peur de venir récolter des tiques avec moi et qui m'ont toujours encouragée durant ma thèse

Brian Gertsch, qui a créé des figures pour cette thèse, qui m'a montré le chemin vers l'excellence académique, et qui a m'a toujours encouragée à me dépasser pour donner le meilleur de moi-même

MERCI, MERCI, MERCI à vous tous et à ceux que j'aurais malheureusement oubliés pour avoir contribué d'une manière ou d'une autre à l'aboutissement de cette thèse.

9 APPENDIX

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9.1 Sampling Area

In Switzerland, Neuchâtel is located in the western part of the country, flanked by the Jura mountain range to the north and lake Neuchâtel to the south (Fig. 9.1). The sampling area was located on the south-exposed face of Chaumont Mountain, north of Neuchâtel, Switzerland (Fig. 9.2).

Fig. 9.1 Map of Switzerland, with the sampling area (Neuchâtel) highlighted by a black star in the western part of the country (http://www.mappi.net/images/map/country-switzerland/carte_suisse_villes_aeroports.jpeg, modified).

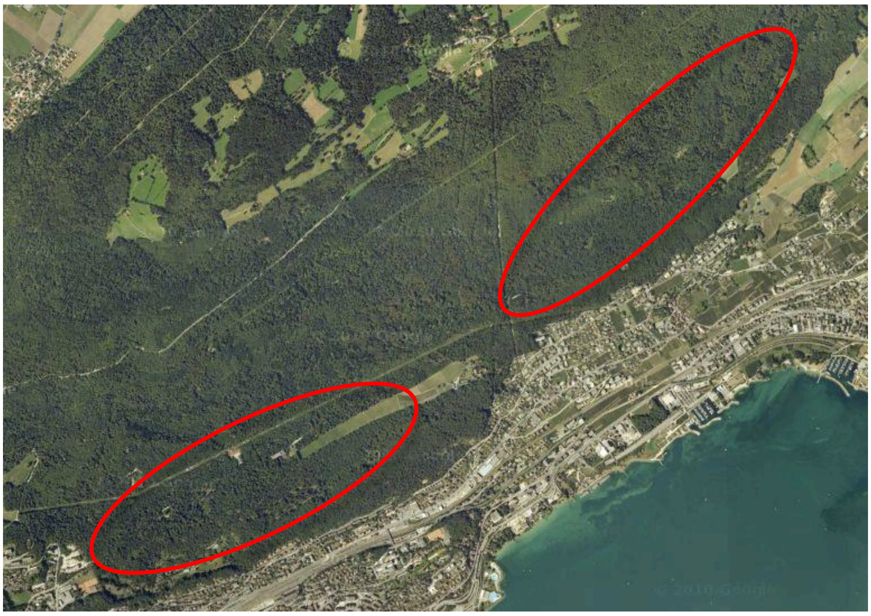

Fig. 9.2 Map of the Neuchâtel area, with the sampling area highlighted by red circles (http://www.mapplus.ch/?size=1, modified).

9.2 Relative Humidity Produced by Saturated Salt Solutions in Survival Arenas

Salts to be used for the saturated solutions were selected depending on the relative humidity (RH) that they were known to produce according to Winston and Bates (1960). Potassium sulfate, sodium bromide, magnesium chloride, and lithium chloride were chosen because they produced RHs quite distinct and also because their RHs appeared not to vary too widely when temperature increased and decreased slightly. The relative humidity produced by the selected saturated salt solutions in survival arenas was recorded over 48 h in order to check whether it was in line with what was expected (Fig. 9.3). This was done using a Vaisala hygrometer (HMI41 indicator, HMP42 probe, Helsinki, Finland).

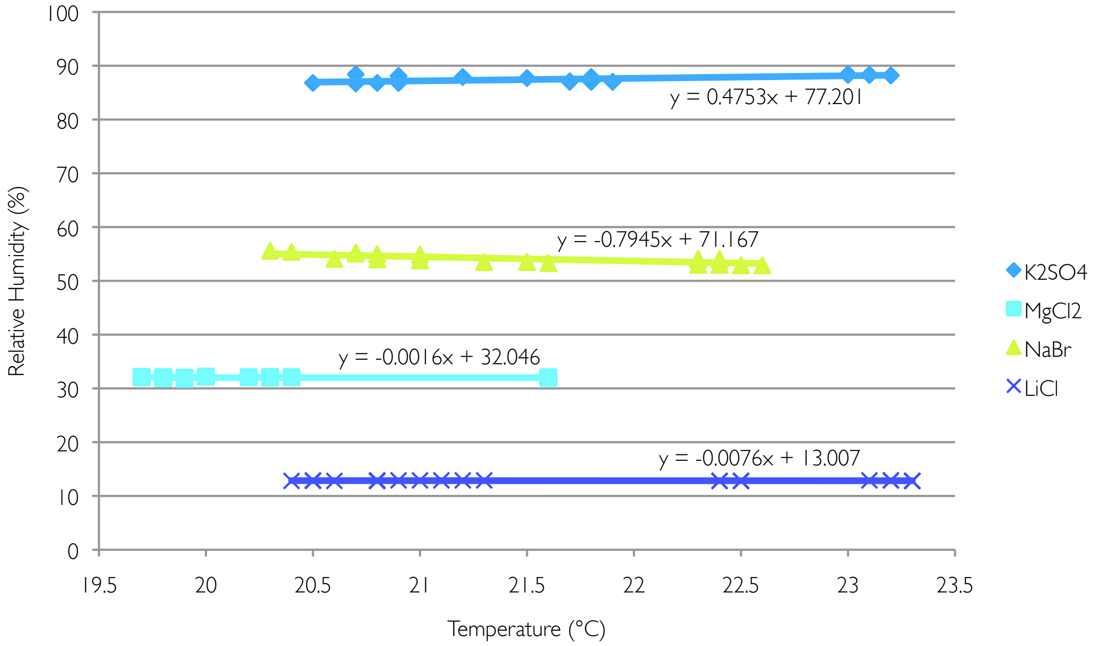

Fig. 9.3 Measurement of relative humidity variation produced by saturated salt solutions inside survival arenas depending on temperature, recorded at room temperature over 48 h.

Using the regression line obtained by plotting the measurements described above, the relative humidity (RH) and saturation deficit (SD) produced by the different saturated salt solutions in the survival arenas could be determined (Table 3.1).

9.3 Relative Humidity Produced by a Dish of Water in Choice Arenas

The experimental setup was tested before starting the experimental runs. Relative humidity variation was recorded at four different spots inside choice arenas: dry side, i.e. box without the dish of water, wet side, i.e. box with the dish of water, within the tunnel on the dry side, and within the tunnel on the wet side. Humidity was monitored using an EE07-PFT5 hygrometer (E+E Elektronik, Engerwitzdorf, Austria). Each recording was performed during 3 h (time of one experimental run) and was repeated twice at each spot (Fig. 9.4).

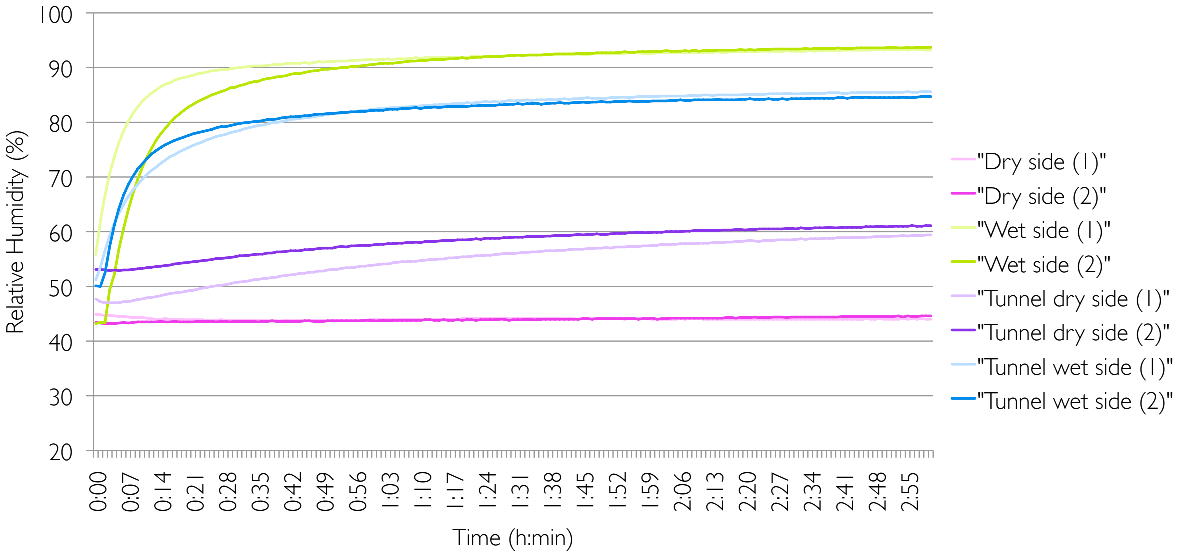

Fig. 9.4 Measurement of relative humidity produced by a dish of water in choice arenas over 3 h. Measurements were repeated twice in four sections of the arena (dry side, i.e. box without the dish of water, wet side, i.e. box with the dish of water, within the tunnel on the dry side, and within the tunnel on the wet side).

9.4 DNA Isolation from Ticks by Ammonium Hydroxide

- Wash ticks in ethanol
- Prepare fresh 0.7 M ammonium hydroxide (100 µl per tick plus 100 µl per negative control)
- Place each tick in its own 1.5 ml tube
- Distribute 100 µl 0.7 M ammonium hydroxide per tube
- Centrifuge the tubes until each tick is permanently immersed in ammonium hydroxide
- Incubate closed tubes at 100°C for 15 min
- Let tubes cool down at room temperature for a few minutes
- Centrifuge briefly
- Open tubes and incubate at 100°C for 15 to 20 min until a volume of 40 µl is left
- Let tubes cool at room temperature for a few minutes and store at -20°C

9.5 DNA Isolation from *Borrelia* Culture

- Pour 1 ml of *Borrelia* culture per 1.5 ml tube
- Centrifuge 15 min at 10'000 rpm
- Remove supernatant
- Resuspend pellet in 1 ml of phosphate-buffered saline/$MgCl_2$
- Centrifuge 15 min at 10'000 rpm
- Repeat last 3 steps once
- Resuspend pellet in 30 µl of water
- Incubate at 100°C for 15 min
- Let tubes cool at room temperature for a few minutes
- Stock at -20°C

9.6 *Borrelia* Amplification and Quantification by Real-Time PCR

- 50-µl mix (for 1 sample)

KAPA buffer 5x	10 µl
$MgCl_2$ (25 mM)	5 µl
dNTPs (10 mM)	1 µl
Forward primer FlaF1A (20 µM)	1 µl
Reverse primer FlaR1 (20 µM)	1 µl
Probe Flaprobe1 (10 µM)	1 µl
KAPA HotStart Taq polymerase (5 U/µl)	0.25 µl
H_2O	20.75 µl
DNA	10 µl

- Program

Initial Activation	95°C	10 min	
Denaturation	95°C	15 s	45 cycles
Annealing & Extension	60°C	1 min	

9.7 *Borrelia* Amplification by Touchdown PCR for Reverse Line Blot

- 25-µl mix (for 1 sample)

QIAGEN buffer 10x	2.5 µl
dNTPs (10 mM)	0.5 µl
Forward primer 23S Bor (10 µM)	0.5 µl
Reverse primer B5S Bor (10 µM)	0.5 µl
QIAGEN Taq polymerase (5 U/µl)	0.125 µl
H_2O	15.875 µl
DNA	5 µl

- Program

Initial Denaturation	94°C	3 min	
Denaturation I	94°C	20 s	-1°C per cycle, 9 cycles
Annealing I	60°C	30 s	
Extension I	72°C	30 s	
Denaturation II	94°C	20 s	40 cycles
Annealing II	52°C	30 s	
Extension II	72°C	30 s	
Final Extension	72°C	7 min	

9.8 Reverse Line Blot: Covalent Coupling of Probes to the Membrane

- Dilute oligonucleotide probes to 75 pmol/µl (SL1, SL2, SL3, GA, GANE, AF, VSNE, LusiNE) or to 100 pmol/µl (RFLNE, SpiNE2, SpiNE3, LusiNE1, LusiNE2, GANE1, BisNE1, BisNE2) in 150 µl of 500 mM $NaHCO_3$ pH 8.4
- Cut nylon membrane at the appropriate size (cushion size)
- Roll membrane in a rolling bottle
- Incubate membrane in 10 ml of freshly prepared 16 % (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC) in a rolling bottle for 10 min at room temperature
- Rinse membrane with 10 ml of water and place it in a miniblotter
- Remove residual water from slots by aspiration
- Fill first slot with drawing pen ink diluted 1:100 in water
- Fill following slots with 150 µl of diluted oligonucleotide mixture
- Fill last slot with drawing pen ink diluted 1:100 in water to mark the end of the area where probes were applied
- Incubate for 1 min at room temperature
- Remove all liquid from slots by aspiration
- Inactivate membrane with 100 ml of 100 mM NaOH for 10 min at room temperature in a rolling bottle
- Rinse membrane with water
- Wash membrane by shaking it in 100 ml of 2x SSPE / 0.1 % SDS for 5 min at 60°C
- If membrane needs to be stored, wash membrane by shaking it in 100 ml of 20 mM EDTA pH8 for 15 min at room temperature, seal it in plastic wrap and store it at 4°C

9.9 Reverse Line Blot: Hybridisation with PCR Products and Detection

- Dilute 10 µl of PCR products in 150 µl of 2x SSPE / 0.1 % SDS
- Denature PCR products for 10 min at 99°C and cool on ice immediately
- Shake membrane in 100 ml of 2x SSPE / 0.1 % SDS for 5 min at room temperature
- Place membrane on a support cushion in miniblotter with slots perpendicular to the line pattern of applied nucleotides
- Remove residual liquid from slots by aspiration
- Fill slots with 150 µl of denatured PCR products
- Fill empty slots with 2x SSPE / 0.1 % SDS
- Incubate miniblotter on a plane surface for 60 min at 42°C
- Remove PCR products by aspiration
- Wash membrane twice by shaking it in 100 ml of 2x SSPE / 0.5 % SDS for 10 min each at 51°C
- Incubate membrane with 2.5 µl of 1:4000 diluted peroxidase-labelled streptavidin conjugate in 10 ml of 2x SSPE / 0.5 % SDS for 30 min at 42°C in a rolling bottle
- Wash membrane twice by shaking it in 100 ml of 2x SSPE / 0.5 % SDS for 10 min each at 42°C
- Wash membrane twice by shaking it in 100 ml of 2x SSPE for 5 min each at room temperature
- Incubate membrane in 6 ml of ECL detection liquid on an overhead sheet (3 ml of each) for 1 min
- Transfer membrane a clean overhead sheet an cover it with another one

- Expose membrane to an X-ray film for 15 min

9.10 Reverse Line Blot: Stripping of the Membrane and Storage

- Wash membrane twice by shaking it in 100 ml of 1 % SDS for 30 min each at 80°C in order to strip hybridised PCR products
- Rinse membrane by shaking it in 100 ml of 20 mM EDTA pH 8 for 15 min each at room temperature
- Seal membrane in a plastic wrap and store at 4°C

9.11 Purification for Sequencing

Sequencing was done according to Promega (Madison, WI, USA) instructions for use. The only change applied to protocol was the elution in 25 µl Nuclease-Free Water instead of 50 µl Nuclease-Free Water.

- Add an equal volume of Membrane Binding Solution to the PCR amplification (15 µl)
- Insert SV Minicolumn into Collection Tube
- Transfer prepared PCR product to the Minicolumn assembly
- Incubate at room temperature for 1 min
- Centrifuge at 16'000 x g for 1 min
- Discard flowthrough and reinsert Minicolumn into Collection Tube
- Add 700 µl Membrane Wash Solution (ethanol added)
- Centrifuge at 16'000 x g for 1 min
- Discard flowthrough and reinsert Minicolumn into Collection Tube
- Repeat last 3 steps but with 500 µl Membrane Wash Solution
- Centrifuge at 16'000 x g for 5 min
- Empty Collection Tube and recentrifuge column assembly for 1 min with microcentrifuge lid open (or off) to allow evaporation of any residual ethanol
- Carefully transfer Minicolumn to a clean 1.5 ml tube
- Add 25 µl Nuclease-Free Water to the Minicolumn
- Incubate at room temperature for 1 min
- Centrifuge at 16'000 x g for 1 min
- Discard Minicolumn and store DNA in 1.5 ml tube at 4°C