HISTOLOGICAL OBSERVATIONS ON *BORRELIA BURGDORFERI* GROWTH IN NATURALLY INFECTED FEMALE *IXODES RICINUS*

BY Z. ZHU *

BORRELIA BURGDORFERI
GROWTH
SPIROCHAETAL TRANSMISSION
IXODES RICINUS FEMALE
SYSTEMIC INFECTION
HISTOLOGY
SILVER STAIN
ULTRASTRUCTURE

SUMMARY: *Borrelia burgdorferi* in naturally infected, flat, blood-feeding and replete females was visualized by direct immunofluorescence assay, silver stain and electron microscopy. In the majority of flat females, borreliae were detected in the midgut only. During blood-feeding, midgut lumen infection was only present in females fed for 0.5 and 1 day on rabbits. All infected females fed for 2 days or more, including engorged ticks, contained bacteria within all tissues other than midgut lumen. These phenomena indicate that, if regurgitation can occur as a mode of *B. burgdorferi* transmission, it must probably takes place at the beginning of blood-feeding. The demonstration of numerous spirochaetes in the tissue of acini and ducts of salivary glands in all systemically infected blood-feeding females, provides further support for the salivary transmission of the bacterium. Although systemic borreliae could be found intracellularly within different tick tissues, they were predominantly situated in the extracellular site. Penetration of host cells by *B. burgdorferi* was often detected in the basal region of the hypodermis and midgut and ovarian epithelium. The results indicate that *B. burgdorferi* multiplies in various tick tissues during and after blood-feeding.

BORRELIA BURGDORFERI
CROISSANCE
TRANSMISSION DU SPIROCHETE
IXODES RICINUS FEMELLES
INFECTION SYSTEMIQUE
HISTOLOGIE
COLORATION ARGENTIQUE
ULTRASTRUCTURE

RÉSUMÉ : Chez des femelles infectées naturellement, à jeûn, prenant leur repas de sang, et gorgées, *Borrelia burgdorferi* a été visualisée par essai d'immunofluorescence, par coloration par l'argent, et en microscopie électronique. Chez la majorité des femelles à jeûn, les borrelies ont été détectées dans l'intestin moyen seulement. Pendant le repas de sang, l'infection de la lumière de l'intestin moyen a été présente seulement chez des femelles nourries pendant 0,5 et 1 jour sur des lapins. Toutes les femelles infectées nourries pendant 2 jours ou davantage, incluant des tiques gorgées, ont contenu des bactéries dans tous les tissus autres que la lumière de l'intestin moyen. Ces phénomènes indiquent que si la régurgitation peut produire un mode de transmission de *B. burgdorferi*, cela doit probablement prendre place au commencement du repas de sang. La démonstration de la présence de spirochètes nombreux dans les tissus des acini et des canaux des glandes salivaires de toutes les femelles prenant leur repas de sang systématiquement infectées, fournit un argument de plus à la transmission salivaire de la bactérie.

Bien que des borrélies systémiques aient pu se trouver intracellulairement dans différents tissus de la tique, elles se sont situées de façon prépondérante dans un site extracellulaire. La pénétration des cellules de l'hôte par *B. burgdorferi* a souvent été détectée dans la couche basale de l'hypodermis, et de l'épithélium de l'intestin moyen et de l'ovaire. Les résultats indiquent que *B. burgdorferi* se multiplie dans les différents tissus de la tique pendant et après le repas de sang.

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Introduction

The multiplication and migration of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae), the aetiological agent of Lyme borreliosis, are two important aspects of the development of the spirochaete in its ixodid tick vectors (Acari: Ixodidae). Morphological demonstrations of the agent in tick tissues have often been used to evaluate the behaviour of the bacterium (Benach *et al.*, 1987; Burgdorfer *et al.*, 1989; Zung *et al.*, 1989; Gern *et al.*, 1990). Although the salivary route of *B. burgdorferi* transmission has now been generally accepted, only mild systemic spirochaetal infections have been observed during blood-feeding (Benach *et al.*, 1987; Zung *et al.*, 1989; Gern *et al.*, 1990). On the other hand, whether *B. burgdorferi* can multiply during blood-feeding needs further confirmation (Piesman *et al.*, 1990), and whether the bacterium grows in other organs in addition to the midgut (Burgdorfer, 1984; Barbour & Hayes, 1986; Benach *et al.*, 1987; Zung *et al.*, 1989; Gern *et al.*, 1990; Spielman, 1992) remains uncertain. The aim of the present investigation is to further evaluate the development of *B. burgdorferi* in female *Ixodes ricinus* by visualizing spirochaetes in naturally infected flat, blood-feeding and replete female ticks. The present results have been partially presented at the First International Conference on Tick-Borne Pathogens at the Host-Vector Interface: an Agenda for Research, Saint Paul, Minnesota September (Zhu *et al.*, 1992*c*).

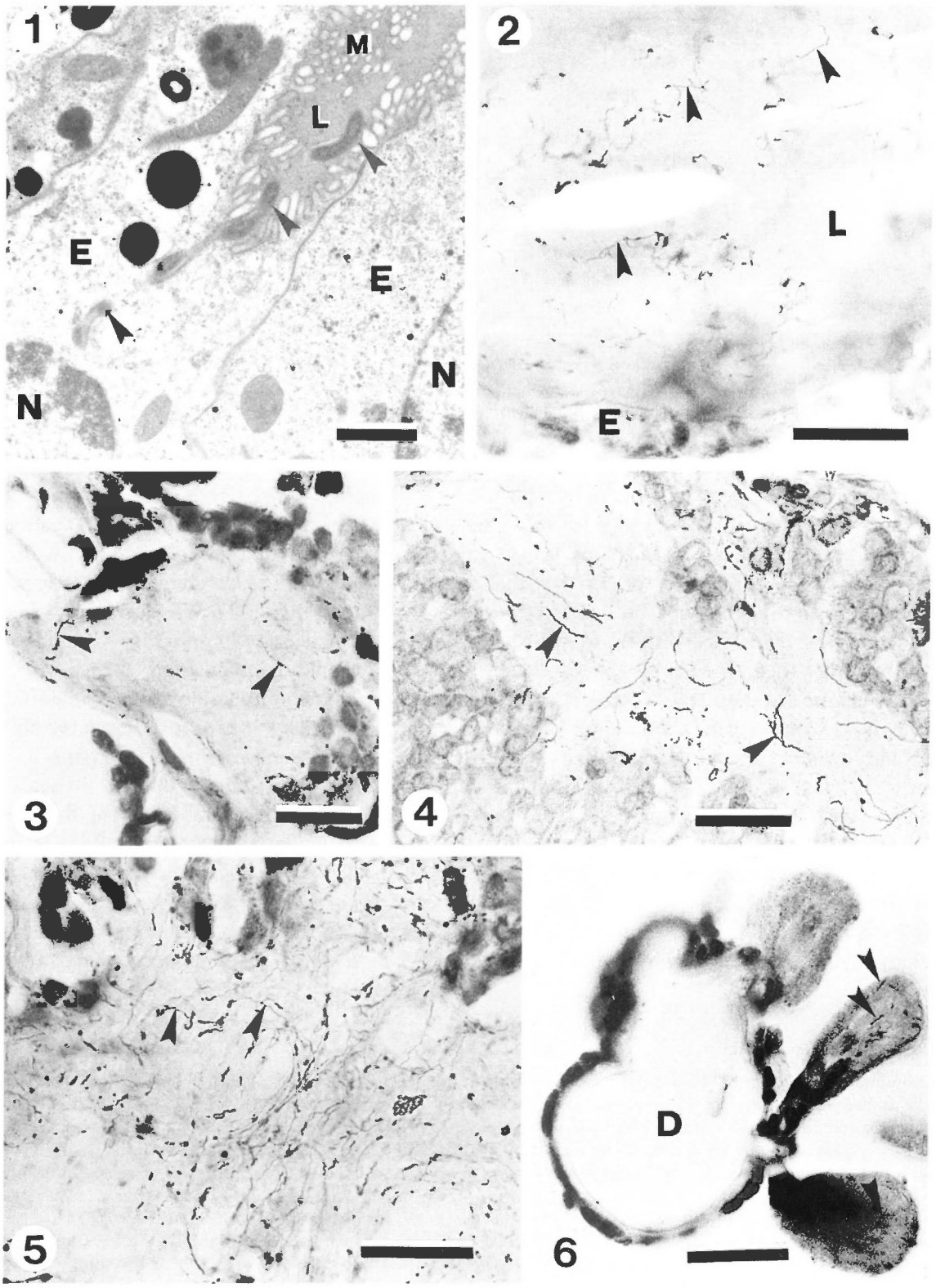
Materials and methods

Starved adult *I. ricinus* were collected by flagging vegetation in a forest near Neuchâtel, Switzerland. Blood-feeding of ticks was conducted on the ears of uninfected New Zealand rabbits (Graf, 1978). Replete ticks were held at 20–22°C and saturated humidity. Ticks were sampled at different intervals after placement on rabbits or after engorgement. *B. burgdorferi* in flat, blood-feeding and replete females was visualized simultaneously by direct immunofluorescence assay (Gern *et al.*, 1990; Gern, *et al.*, 1991), histological silver stain (van Orden & Greer, 1971; Gern *et al.*, 1990) and transmission electron microscopy (TEM). For histology, halved ticks or pieces of tick organs were fixed in freshly prepared phosphate buffered 4% formaldehyde (pH=7.4) at 4°C overnight. Tick tissues were then embedded in paraffin, sectioned at a thickness of 8 µm and stained using Dieterle spirochaete stain (van Orden & Greer, 1971; Gern *et al.*, 1990). Halved ticks or tick tissues were also fixed in freshly prepared Karnovsky's fixative (Karnovsky, 1965) at 4°C overnight and further treated for TEM observation as previously described (Zhu *et al.*, 1991; Zhu *et al.*, 1992*a*).

Results

Six out of 27 unfed females proved to be infected with *B. burgdorferi.* Of these, 5 had borreliae in the midgut only, and 1 harboured spirochaetes in both the midgut and synganglion (Table 1). In this systemically infected tick, a spirochaete was observed to be just penetrating the apical plasma membrane of the midgut epithelium. Half of the spirochaete was embedded in an epithelial cell and the rest was suspended in the midgut lumen (Fig. 1). In the synganglion, spirochaetes were limited in number and most of them were intracellular. Spirochaetes with multiple protoplasmic cylinders were sometimes observed in the midgut lumen.

Fig. 1: A *B. burgdorferi* spirochaete (arrowheads) is just penetrating the apical plasma membrane of the midgut epithelium of a starved female with gut and synganglion infection only. Note that the bacterium is in a position across the apical plasma membrane of an epithelial cell (E). L, midgut lumen; M, microvilli of the midgut epithelial cell; N, nucleus of epithelial cells. Electron micrograph. Scale bar, 1 µm. Fig. 2: *B. burgdorferi* (arrowheads) diffusely distributed in the midgut lumen (L) of a female fed for 1 day. E, midgut epithelium. Light micrograph, Dieterle silver stain. Scale bar, 20 µm. Fig. 3: *B. burgdorferi* (arrowheads) in the synganglion of a female fed for 2 days. Light micrograph, Dieterle silver stain. Scale bar, 20 µm. Fig. 4: *B. burgdorferi* (arrowheads) in the synganglion of a female fed for 5 days. Light micrograph, Dieterle silver stain. Scale bar, 20 µm. Fig. 5: *B. burgdorferi* (arrowheads) in the synganglion of a female at day 15 after repletion. Light micrograph, Dieterle silver stain. Scale bar, 20 µm. Fig. 6: *B. burgdorferi* (arrowheads) in the agranular acini of salivary glands of a female fed for 2 days. D, duct of the salivary glands. Light micrograph, Dieterle silver stain. Scale bar, 20 µm.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Day(s) after placement/ repletion	No of examined ticks	No of infected ticks	No of systemically infected ticks	Remarks on *Borrelia burgdorferi* infection
0	27	6	1	Mild systemic infection[A] present in one female with *B. burgdorferi* in midgut lumen and synganglion only
0.5	16	1	0	Mild to moderate midgut lumen infection
1	16	1	0	Moderate[B] to massive[C] midgut lumen infection
2	12	1	1	Mild systemic infection
4	12	0	0	
5	21	2	2	Massive systemic infection
6	9	0	0	
6/0.5	11	1	1	Moderate to massive systemic infection
7/1	4	0	0	
7/7	10	0	0	
6/15	12	1	1	Massive systemic infection
Total No	150	13	6	Infected ticks/examined ticks: 8.7% (13/150); Systemically infected ticks/infected ticks: 46.1% (6/13)

[A] Spirochaetes sparcely distributed at 6 × 100 magnification; [B] Spirochaetes densely distributed, but can be counted at 6 × 100 magnification; [C] Spirochaetes too densely distributed to be counted at 6 × 100 magnification.

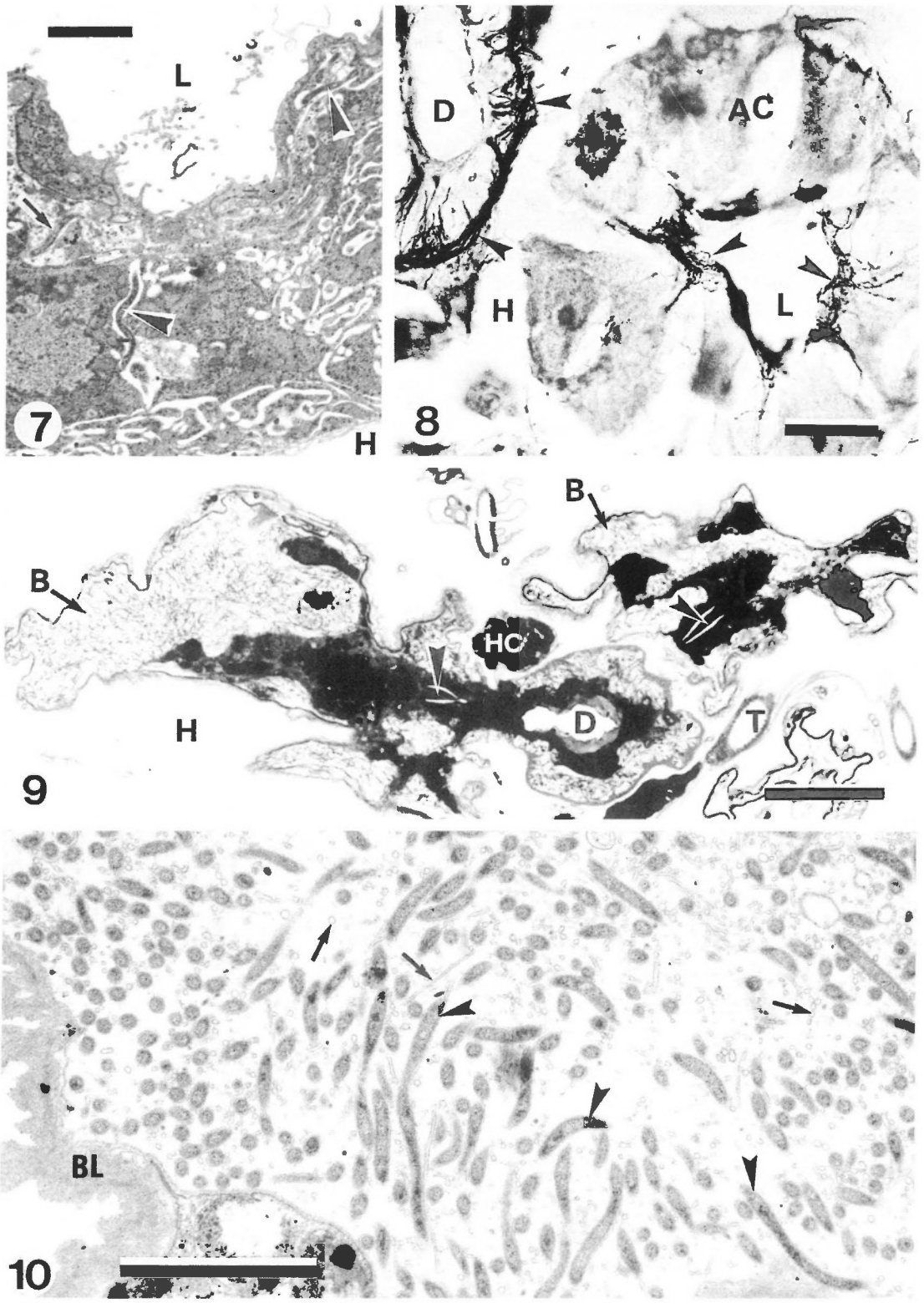
TABLE 1: General results of histological observations on *B. burgdorferi* growth in naturally infected female *I. ricinus*.

During blood-feeding, the midgut lumen infection was only observed in two females fed for 0.5 (1/16) and 1 day (1/16) (Table 1). They had no spirochaetes in other organs. In the female fed for 0.5 day, the infection was mild to moderate and the majority of the spirochaetes were associated with the apical surface of the midgut wall. In the female fed for 1 day, the infection was moderate to massive and borreliae were diffusely distributed in the midgut lumen (FIG. 2). No peritrophic membrane was present in the female fed for 0.5 day, but a definite peritrophic membrane had occurred in the female fed for 1 day. In the latter female spirochaetes were found in both ecto- and endoperitrophic spaces. The majority of the spirochaetes were located in the large endoperitrophic space. Some spirochaetes showed an atypical form with multiple cylinders and/or a blebbed outer cell membrane.

In engorging females fed for 2 days or more and in replete females, all infected ticks (5/91) had numerous borreliae within all tissues, including tissues of ducts and different types of acini of the salivary glands (Table 1 and Figs. 3–16). However, spirochaetes were very rare in the hemolymph (Fig. 16) and never observed in the midgut lumen. Spirochaetes were restricted to the narrow peripheral region of the midgut wall and their density was not particularly higher than that of the spirochaetes in other organs.

The level of infection in tick tissues obviously increased with the prolongation of the time period after attachment or repletion (Table 1 and Figs. 3–12). All systemically infected females fed for 5 days or more, or examined after repletion, showed a massive systemic infection in all tissues (Table 1 and Figs. 4–5, 8–12). Indeed, spirochaetes were countless in the hypodermis, synganglion (Fig. 4), salivary glands

FIG. 7: Ultra-thin section of a thin-walled granular acinus from a female fed for 2 days, showing *B. burgdorferi* in the extracellular spaces formed by the invagination of the basal plasma membrane of acinus cells (arrowheads) and intracellularly in an acinus cell (arrow). H, hemocoel; L, lumen of acinus. Electron micrograph. Scale bar, 2 µm. FIG. 8: *B. burgdorferi* (arrowheads) in the wall of a thick-walled granular acinus (AC) and a duct (D) of the salivary glands of a female fed for 5 days. Countless spirochaetes are located along the apical narrow region of the acinus wall, whereas spirochaetes are mainly situated in the peripheral region of the duct. L, lumen of acinus; H, hemocoel. Light micrograph, Dieterle silver stain. Scale bar, 20 µm. FIG. 9: Semi-thin section of atrophied salivary glands from a female at day 15 after repletion, displaying that the almost unstained wide peripheral region of the salivary glands is full of *B. burgdorferi* (B). Note two atrophied acini with acinus valves (arrowheads). D, salivary gland duct; H, hemocoel; HC, hemocyte; T, trachea. Light micrograph, Toluidine blue. Scale bar, 20 µm. FIG. 10: Enlarged view of portion of the spirochaete-containing spaces shown in Fig. 9, demonstrating densely distributed *B. burgdorferi* (arrowheads) in association with countless tubular, vesicular or pearl-like structures (arrows). BL, basal lamina. Electron micrograph. Scale bar, 2 µm.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(Fig. 8) and genital organs (Figs. 11–12) of the females fed for 5 days and in all organs (Figs. 5, 9–10) of the female at day 15 after repletion.

TEM observations showed that, in systemically infected females, although spirochaetes could be found intracellularly within different tick tissues (Figs. 7, 14), the agent of Lyme borreliosis was predominantly located in the extracellular sites, such as the spaces between the basal lamina and the basal plasma membrane of host cells, the interspaces between adjacent cells, the spaces between the basal plasma membrane infoldings and the extracellular spaces in the connective tissues associated with various tick organs (Figs. 7, 9–11, 13–16).

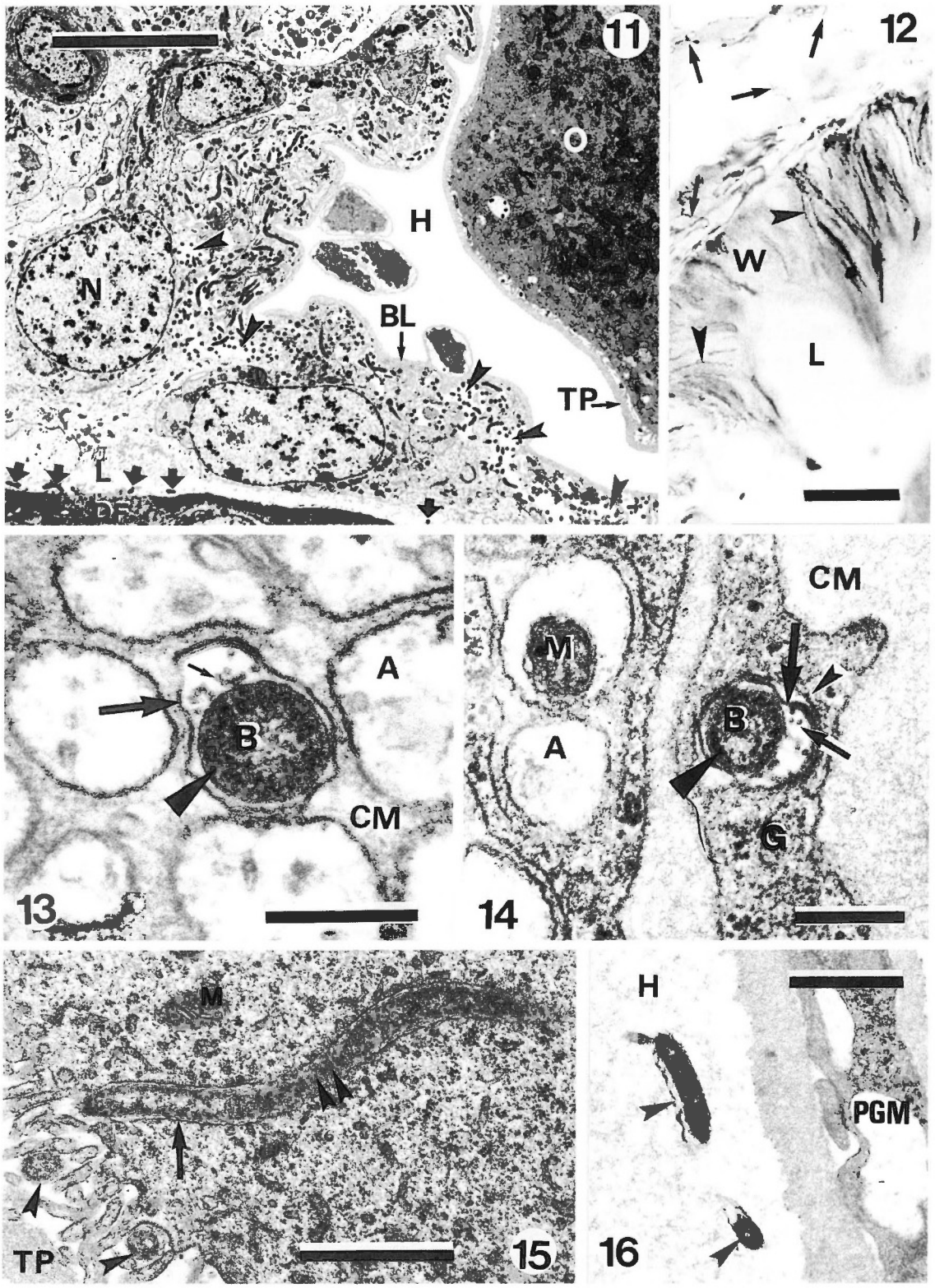
Penetration of host cells by the spirochaete was often observed in the basal region of the hypodermis, midgut and ovarian epithelium in females fed for 5 days. Spirochaetes were also found penetrating oocytes in these ticks (Fig. 15). The second envelope of the intracellular portion of the penetrating spirochaete was very prominent, and it obviously linked up with the plasma membrane of the host cells (Fig. 15).

In a female at day 15 after repletion, a large quantity of borreliae were found in the ovarian lumen, in the extracellular spaces of the muscular connective tissue and in the large invaginations of the basal plasma membrane of midgut epithelial cells. They were accompanied with countless tubular, vesicular or pearl-like structures, presumably derived from the outer cell envelope of spirochaetes (BURGDORFER & HAYES, 1990). A similar phenomenon was also present in the atrophied salivary glands (Fig. 10). The electron-lucent spaces in the peripheral region of the acini and ducts of salivary glands were very wide and full of *B. burgdorferi* and the accompanying structures (Fig. 10).

In the infected ovaries, spirochaetes were more abundant in the more developed ventral portion (Fig. 11). Many large electron lucent extracellular spaces, in which a large number of spirochaetes were demonstrated, were observed along the basal region of the ventral portion of the ovarian wall in systemically infected females fed for 5 days (Fig. 11). Similar spaces were never present in the dorsal folds of the ovaries. In a systemically infected ovipositing female examined at day 15 after repletion, oocytes harbouring a large number of borreliae but devoid of microvilli and egg-shell deposition in the space between the plasma membrane and the *tunica propria* membrane, were often observed.

In one of two systemically infected females fed for 5 days, all three pairs of nerves (optical, cheliceral and pedipalpal nerves) harboured *B. burgdorferi* and up to 17 spirochaetes could be found in a transverse section of a nerve. Most spirochaetes were located extracellularly among or between axons and were embedded in the matrix of the connective tissue, with their long axes parallel to those of the adjacent axons (Fig. 13). Intracellular spirochaetes were limited in number and only detected in the cytoplasmic processes of glial cells (Fig. 14).

FIG. 11: Electron micrograph of the ventral ovarian area opposite the dorsal fold (DF) of the ovary from a female fed for 5 days. In the ovarian wall, spirochaetes (arrowheads) are mostly located along the basal region of the organ and many large electron lucent extracellular spaces (arrowheads) are filled with spirochaetes. Almost every oocyte (O) in this part of the ovary is infected with numerous spirochaetes in the spaces between the tunica propria (TP) and the oocytoplasm (O). A few spirochaetes (bold arrows) are present in the ovarian lumen (L). BL, basal lamina; H, hemocoel; N, nucleus. Scale bar, 10 μm. FIG. 12: Transversal section of the wall of the cervical part of the vagina (W) from a female fed for 5 days, displaying countless *B. burgdorferi* (arrowheads) radially within the wall epithelium and numerous spirochaetes (arrows) within the surrounding muscular-connective tissue. L, lumen of the cervical part of the vagina. Light micrograph, Dieterle silver stain. Scale bar, 20 μm. FIG. 13: Electron micrograph of nerve tissue from a female at day 5 after placement, showing a *B. burgdorferi* spirochaete (B) extracellularly located among axons (A) and embedded in the connective tissue matrix (CM). Note the loose outer cell envelope (big arrow), the flagella (small arrow) in the periplasmic space between the outer cell envelope and the inner cytoplasmic cylinder (arrowhead). Scale bar, 0.2 μm. FIG. 14: Electron micrograph of nerve tissue from a female at day 5 after placement, demonstrating a *B. burgdorferi* spirochaete (B) in a cytoplasmic process of a glial cell (G). The spirochaete has a second envelope of host cell origin (small arrowhead). Note the flagella (small arrow) in the periplasmic space between the outer cell envelope (big arrow) and the inner cytoplasmic cylinder (big arrowhead). A, axons; CM, connective tissue matrix; M, mitochondrion in an axon. Scale bar, 0.2 μm. FIG. 15: Electron micrograph of the peripheral region of an oocyte from a female at day 5 after placement. Note that a spirochaete (double arrowheads) is just penetrating into the oocyte. The second envelope (arrow) covering the penetrating spirochaete is very prominent and appears to be fused with the plasma membrane of the oocyte. Also note two spirochaetes (single arrowheads) in the space between the tunica propria (TP) and the plasma membrane of the oocyte. M, mitochondrion. Scale bar, 1 μm. FIG. 16: *B. burgdorferi* (arrowheads) suspended in the hemolymph (H) of a female at 0.5 day after repletion. PGM, periganglionic membrane. Electron micrograph. Scale bar, 1 μm.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DISCUSSION

The present investigation confirms that *B. burgdorferi* is principally an extracellular spirochaete in its tick vector (BARBOUR & HAYES, 1986; ZHU *et al.*, 1992*b*), though it can also be found intracellularly in tissues of blood-feeding and replete ticks. In the present study, the location of the spirochaetes within synganglion tissue in replete and blood-feeding females was observed only by light microscopy. Thus, we do not know the ultrastructural location of the spirochaetes in these ticks. However, it has been found that most of the spirochaetes within the synganglion tissue of a field-collected unfed *I. ricinus* female were intracellular (ZHU, 1995). Whether synganglion harbours more intracellular spirochaetes than other organs needs further observation.

We observed that of 6 naturally infected unfed females, one (16.7%) had a systemic infection with spirochaetes in midgut and synganglion only. This result is in agreement with the previous observation that systemic spirochaetes become restricted to the synganglion when nymphs molt to adults (BURGDORFER, 1984).

Of 7 blood-feeding and replete females, 5 (71.4%) showed a systemic infection with borreliae within all tissues. These results clearly show an increased rate of systemic infection and an evidently enlarged range of infected tick tissues. Thus, the gut penetration of the spirochaete does occur but most probably during early blood-feeding, since midgut lumen spirochaetes were not observed in females fed for 2 days or more and in replete females.

Our previous study revealed that during slow phase of blood-feeding of artificially infected females the majority of the spirochaetes persisted in the midgut lumen (GERN *et al.*, 1990). This observation confirms the results reported by BENACH *et al.* (1987). However, the present study showed that spirochaetes disappeared from the midgut lumen of field-collected infected females fed for 2 to 5 days or fed until engorgement. The real cause for this phenomenon is unknown. At present, it can be suggested that this phenomenon may be attributed to the difference of the spirochaetal behaviours between the laboratory-cultured and the field-collected strains.

Spirochaetes in a same isolate may show different morphology and serological reactions (HOVIND-HOUGEN, 1984; HOVIND-HOUGEN *et al.*, 1986; STIERNSTEDT *et al.*, 1985). Thus, the spirochaetes in the isolate used for artificial infection in the previous study may consist of a small and a large population different from each other in their behaviour, as suggested by BENACH *et al.* (1987). Spirochaetes in the small population may have succeeded in penetrating the midgut wall and become systemic during blood-feeding, while the majority of the spirochaetes representing the large population persist in the gut lumen (GERN *et al.*, 1990). Spirochaetes of the large population may have lost their ability to disseminate systemically during serial *in vitro* cultures, since culture process can cause variations of the outer surface protein A (OspA) and B (OspB) (BISSET & HILL, 1987). Such variations may change the matching relationship between surface proteins of spirochaetes and the surface receptor of tick midgut epithelial cells (MILLER & LEHANE, 1993) and, therefore, inhibit the midgut penetration of spirochaetes. The midgut spirochaetes in field-collected infected females may have become systemic after 1 to 2 days' blood-feeding.

The PM was found to have an important influence on the behaviour of the enclosed microorganisms and may function as a membranous barrier preventing spirochaetes contained in the endoperitrophic space from gut penetration (ZHU, GERN & AESCHLIMANN, 1993; author's unpublished data). Having failed to demonstrate the systemic infection in nymphal *I. dammini* ticks fed for 0–96h, HAYES & BURGDORFER (1992) suggested that the PM may act as a limiting barrier to migration of spirochaetes from the midgut to other tissues. Thus, another explanation for the phenomenon mentioned above could be that only those bacteria persisting in the ectoperitrophic space when the PM occurs and/or condenses (ZHU *et al.*, 1991, 1993, 1995) could pass through the gut wall and invade other tick organs. These spirochaetes may

represent the small population. The PM may act as a barrier preventing the spirochaetes enclosed within the endoperitorphic space from migrating from midgut to other tissues. The spirochaetes in the endoperitrophic space may represent the large population. This hypothesis explains well the spirochaetal behaviour observed in the artificially infected blood-feeding females (BENACH *et al.*, 1987; GERN *et al.*, 1990). However, how can one explain the phenomenon that countless spirochaetes were present in the midgut lumen in females at 1 day after tick attachment, when a definite PM had occurred, but spirochaetes could no longer be demonstrated in the lumen in females fed for 2 days or more and in replete females? The answer may be that, in these females, while the spirochaetes situated in the ectoperitrophic space could have penetrated through the midgut and become systemic, the spirochaetes present in the endoperitrophic space could have died off because of presently unknown factors in the gut lumen of blood-feeding ticks. These factors may be present or absent depending on the tick strains and/or the vertebrate hosts on which ticks feed.

The regurgitation, which appears to occur in *Ornithodoros moubata* (HESSE, 1983; CONNAT, 1991) and *Amblyomma americanum* (BROWN, 1988), may also play a role in the spirochaetal transmission and it may occur in both artificially and naturally infected females. However, one can think that only proportion of the spirochaetes in the midgut could be injected into host skin. In addition, the experimentally infected ticks may harbour many more spirochaetes. This could be an additional factor explaining why spirochaetes remain numerous in the gut lumen of artificially infected females.

Further investigation is needed to clarify the complex behaviour of midgut spirochaetes.

Findings so far available show that the midgut infection of *B. burgdorferi* in unfed infected nymphal and female *I. dammini* and *I. ricinus* is present without exception (BARBOUR & HAYES, 1986; BENACH *et al.*, 1987; BURGDORFER *et al.*, 1988; ZUNG *et al.*, 1989). The midgut of the blood-feeding ticks was described to be a site for *B. burgdorferi* multiplication (BURGDORFER, 1984; BARBOUR & HAYES, 1986; BENACH *et al.*, 1987; BURGDORFER *et al.*, 1982; BURGDORFER *et al.*, 1988; BURGDORFER, 1989*a*, 1989*b*; ZUNG *et al.*, 1989; BURGDORFER & HAYES, 1990; FRIEDHOFF, 1990; GERN *et al.*, 1990), and the hemolymph a medium *via* which *B. burgdorferi* reaches and infects various tick organs contained in the hemocoel (BENACH *et al.*, 1987; RIBEIRO *et al.*, 1987; BURGDORFER *et al.*, 1982, 1988; BURGDORFER, 1989*a*, 1989*b*; ZUNG *et al.*, 1989; GERN *et al.*, 1990). We found that in the midgut of all systemically infected blood-feeding females fed for 2 to 5 days or fed until engorgement, borreliae were restricted to the narrow basal region of the midgut wall and that the level of spirochaetal infection in this region was not particularly higher than in other organs. We also remarked that the hemocoel harboured only a few spirochaetes or was devoid of the bacterium and that spirochaete abundance in various tick tissues increased greatly during and after blood-feeding. Thus, it appears that, during the early blood-feeding period, the spirochaetes in the midgut lumen, especially those persisting in the ectoperitrophic space, either could have penetrated into or passed through the midgut wall and become systemic or they could have been injected into their vertebrate hosts by tick regurgitation (HESSE, 1983; BROWN, 1988; CONNAT, 1991). Alternatively, both situations may have occurred. The newly-disseminated bacteria may then multiply, mainly extracellularly, within various tick tissues, although the mechanism leading to the systemic infection still remains unclear. On the other hand, a mild systemic infection of *B. burgdorferi* can occur in a small percentage of unfed ticks (BURGDORFER *et al.*, 1983; BURGDORFER *et al.*, 1988; BURGDORFER, 1989*b*; ZUNG *et al.*, 1989). These pre-feeding systemic spirochaetes may also multiply within various tick tissues after the start of blood-feeding. The previous observation that the hemolymph infection of *B. burgdorferi* is rare (BURGDORFER, 1989*b*; BURGDORFER & HAYES, 1990) or very mild if present (BENACH *et al.*, 1987; BURGDORFER, 1989*b*; GERN *et al.*, 1990) strongly supports our suggestions of systemic *B. burgdorferi* multiplication. If spirochaetal multiplication takes place only or mainly in the midgut, the spirochaetal migration *via* hemolymph must be much more frequent and the spirochaetes in the hemocoel must be much more numerous to induce such a massive systemic infection as shown in our present study.

Tubular, vesicular or pearl-like structures were reported to be associated with *B. burgdorferi* during the extensive growth period in the ovarian lumen and were considered to result from outer cell envelope blebbing (BURGDORFER & HAYES, 1990). In the present investigation, in a female examined at day 15 after repletion, a similar phenomenon was found not only in the ovarian lumen but also in the extracellular sites associated with midgut wall, muscular-connective tissues, and particularly in the acini and ducts of salivary glands. These new findings provide further evidences for the multiplication of systemic spirochaetes after repletion.

PIESMAN *et al.* (1987) found that the longer the time period between tick attachment and examination, the greater the probability of infection of experimental hosts by spirochaetes. The present investigation revealed that systemic infection appeared at day 2 after tick attachment and that all systemically infected females had numerous spirochaetes in the acinus and duct tissue of salivary glands but no spirochaetes in the midgut lumen. Furthermore, we also found that spirochaetal number in salivary glands increased greatly during blood-feeding. These findings lend strong support to the suggestion of a salivary route of spirochaetal transmission. In addition, the presence of countless *B. burgdorferi* in all tick organs during blood-feeding, indicates that the transmission of the bacterium to its vertebrate hosts may also occur through the wound by crushing the fixed ticks or *via* the remnants of the tick mouthparts in host skin.

Systemic infection in starved adult ticks was suggested to be inherited from the preceding systemically infected nymphal stage (BURGDORFER *et al.*, 1988). However, systemic infection in starved nymphs was speculated to result from gut penetration of spirochaetes (ZUNG *et al.*, 1989). In the present study, the detection of apical plasma membrane penetration by *B. burgdorferi* in the midgut epithelium of a flat female with spirochaetes in midgut and synganglion suggests that midgut penetration of the spirochaete may occur in starved ticks that lack a peritrophic membrane (RUDZINSKA *et al.*, 1982; ZHU *et al.*, 1991), though the direction of the spirochaetal migration remains unknown.

Host cell penetration by *B. burgdorferi* was often found in the basal region of the hypodermis, and midgut and ovarian epithelium in females examined at day 5 after placement on the laboratory host. The demonstration of the real conjunction of the second envelope, which covers the intracellular part of the penetrating spirochaetes, with the plasma membrane of host cells (Fig. 15) strongly confirms the host cell origin of this envelope (ZUNG *et al.*, 1989).

Although the genus *Borrelia* is thought to develop as symbiotes in ticks, especially in Argasidae (HOOGSTRAAL, 1979; 1985), the pathogenic effect of *B. burgdorferi* on the developing oocytes of a spent, naturally infected *I. dammini* female was observed by BURGDORFER *et al.* (1988). They found that numerous spirochaetes in the spaces between oocytes and tunica propria membranes could inhibit the vitelline process, the egg shell deposition and the formation of the microvilli of the oocytes. In our present study, a similar phenomenon was found in the ovary of a systemically infected ovipositing *I. ricinus* female examined at day 15 after repletion. In addition, we also observed that, in the ventral portion of the ovarian wall of systemically infected females examined at day 5 after placement, there were many large electron lucent extracellular spaces along the peripheral region of the wall, in which a large number of spirochaetes were demonstrated. Similar spaces were not observed in the dorsal fold of the ovary and have never been described in ticks before, as far as we are aware. Thus, these large spaces may be the lysed zones of the ovarian cells. In addition, whether numerous *B. burgdorferi* within different organs, particularly within the synganglion and nerves, may have some effects on the physiological activities including behaviour of the infected ticks, deserves further investigations.

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REFERENCES

BARBOUR (A. G.) & HAYES (S. F.), 1986. — Biology of *Borrelia* species. — Microb. Rev., **50**: 381-400.

BENACH (J. L.), COLEMAN (J. L.), SKINNER (R. A.) & BOSLER (E. M.) 1987. — Adult *Ixodes dammini* on rabbits: A hypothesis for the development and transmission of *Borrelia burgdorferi*. — J. infect. Diseases, **155**: 1300-1306.

BISSET (M. L.) & HILL (W.), 1987. — Characterization of *Borrelia burgdorferi* strains isolated from *Ixodes pacificus* ticks in California. — J. clin. Microbiol., **25**: 2296-2301.

BROWN (S. L.), 1988. — Evidence for regurgitation by *Amblyomma americanum*. — Vet. Parasitol., **28**: 335-342.

BURGDORFER (W.), 1984. — Discovery of the Lyme disease spirochete and its relation to tick vectors. — Yale J. Biol. Medicine, **57**: 609-612.

BURGDORFER (W.), 1989*a*. — *Borrelia burgdorferi*: its relationship to tick vectors. — In: STANEK, G., KRISTOFERITSH, W., PLETSCHETTE, M., BARBOUR, A. G. & FLAMM, H. (Eds) Lyme Borreliosis II. Zentralblatt f. Bakteriol., Suppl. **18**: 8-13.

BURGDORFER (W.), 1989*b*. — Vector/host relationships of the Lyme disease spirochete, *Borrelia burgdorferi*. — Rheumatic Disease Clinics N. Amer., **15**: 775-787.

BURGDORFER (W.), BARBOUR (A. G.), HAYES (S. F.), BENACH (J. L.), GRUNWALDT (E.) & DAVIS (J. P.), 1982. — Lyme disease, a tick-borne spirochetosis? — Science, **216**: 1317-1319.

BURGDORFER (W.), BARBOUR (A. G.), HAYES (S. F.), PETER (O.), & AESCHLIMANN (A.) 1983. — Erythema chronicum migrans — a tickborne spirochetosis. — Acta Tropica, **40**: 79-83.

BURGDORFER (W.) & HAYES (S. F.), 1990. — Vector-spirochete relationships in louse-borne and tick-borne borrelioses, with emphasis on Lyme disease. — In: HARRIS, K. F. (Ed.) Advances in Disease Vector Research, **6**: 127-150. Springer-Verlag, New York.

BURGDORFER (W.), HAYES (S. F.), & BENACH (J. L.), 1988. — Development of *Borrelia burgdorferi* in Ixodid tick vectors. — Ann. N. Y. Acad. Sci., **539**: 172-179.

CONNAT (J-L.), 1991. — Demonstration of regurgitation of gut content during blood meals of the tick *Ornithodoros moubata*, possible role in the transmission of pathogenic agents. — Parasitol. Res., **77**: 452-454.

FRIEDHOFF (K. T.), 1990. — Interaction between parasite and tick vector. — Int. J. Parasitol., **20**: 525-535.

GERN (L.), TOUTOUNGI (L. N.), HU (C. M.) & AESCHLIMANN (A.) 1991. — *Ixodes (Pholeoixodes) hexagonus*, an efficient vector of *Borrelia burgdorferi* in the laboratory. — Medical vet. Entomol., **5**: 431-435.

GERN (L.), ZHU (Z.) & AESCHLIMANN (A.) 1990. — Development of *Borrelia burgdorferi* in *Ixodes ricinus* females during blood feeding. — Ann. Parasitol. Hum. Comp., **56**: 89-93.

GRAF (J. F.), 1978. — Copulation, nutrition et ponte chez *Ixodes ricinus* L. (Ixodoidea: Ixodidae) 1re partie. — Bull. Soc. ent Suisse, **51**: 89-87.

HAYES (S. F.), BURGDORFER (W.), 1992. — Peritrophic membrane: a potential barrier for *Borrelia burgdorferi* to establish generalized infection in feeding *Ixodes dammini* nymphs. — In: Proc. & Abstr. First Int. Conf. Tick Borne Pathogens at the Host-Vector Interface: An Agenda for Research, Saint Paul, Minnesota, September 15-18, 1992, p. B3-11.

HESSE (G.), 1983. — Evidence of regurgitation by *Ornithodoros moubata* (Ixodoidea: Argasidae) using radioactive tracer. — Zentralblatt f. Bakteriol., Mikrobiol. Hygiene, **256**: 267-268.

HOOGSTRAAL (H.), 1979. — Ticks and spirochetes. — Acta Tropica, **36**: 133-136.

HOOGSTRAAL (H.), 1985. — Argasids and nuttalliellid ticks as parasites and vectors. — Adv. Parasitology, **24**: 135-238.

HOVIND-HOUGEN (K.), 1984. — Ultrastructure of spirochetes isolated from *Ixodes ricinus* and *Ixodes dammini*. — Yale J. Biol. Medicine, **57**: 543-548.

HOVIND-HOUGEN (K.), ASBRINK (E.), STIERNSTEDT (G.), STEERE (A. C.) & HOVMARK (A.), 1986. — Ultrastructural differences among spirochetes isolated from patients with Lyme disease and related disorders, and from *Ixodes ricinus*. — In: STANEK G., FLAMM H., BARBOUR A. G., BURGDORFER W. (Eds), Lyme Borreliosis. Zentralblatt für Bakteriologie Hygiene, A263: 103-111.

KARNOVSKY (M. J.), 1965. — A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. — Cell Biol, **27**: 137-139.

MONIN (R.), GERN (L.) & AESCHLIMANN (A.), 1989. — A study of the different modes of transmission of *Borrelia burgdorferi* by *Ixodes ricinus*. — In: STANEK G., KRISTOFERITSCH W., PLETSCHETTE M., BARBOUR A. G. & FLAMM H. (Eds), Lyme Borreliosis II. Zentralblatt f. Bakteriol., Suppl. **18**: 14-20.

MILLER (N.) & LEHANE (M. J.), 1993. — Peritrophic membranes, cell surface molecules and parasite tropisms within arthropod vectors. — Parasitol. Today, **9**: 45-50.

PIESMAN (J.), MATHER (T. N.), SINSKY (R. J.) & SPIELMAN (A.), 1987. — Duration of tick attachment and *Borrelia burgdorferi* transmission. — J. clin. Microbiol., **25**: 557-558.

PIESMAN (J.), OLIVER (J. R.), & SINSKY (R. J.), 1990. — Growth kinetic of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). — Amer. J. trop. Medicine Hygine, **42**: 352-357.

RIBEIRO (J. M. C.), MATHER (T. N.), PIESMAN (J.) & SPIELMAN (A.), 1987. — Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae). — J. med. Entomol., **24**: 201-205.

RUDZINSKA (M. A.), SPIELMAN (A.), LEWENGRUB (S.), PIESMAN (J.) & KARAKASHIAN (S.), 1982. — Penetration of the peritrophic membrane of the tick by *Babesia microti.* — Cell Tissue Res., **221**: 471-481.

SPIELMAN (A.), 1992. — Development of Lyme disease spirochetes in vector ticks.— Proc. & Abstr. First Int. Conf. Tick-Borne Pathogens at the Host-Vector Interface: An Agenda for Research, Saint Paul, Minnesota, U.S.A., 15–18 Sept. 1992, p. 47-53.

STIERNSTEDT (G.), BARBOUR (A. G.), HOVIND-HOUGEN (K.), HANSSON (O.), SVEMUNGSSON (B.), JÖRBECK (H.), SKÖDENBERG (B.), 1985. — *Borrelia* strains isolated from *Ixodes ricinus* in Sweden—studies on their ultrastructure, and reactivities with antibodies of patients with spirochetal meningitis. — In: STIERNSTEDT G. (Ed.), Tick-borne *Borrelia* infection in Sweden, Vol II. Kongl Carolinska Medico Chirurgiska Inst., Stockholm, pp. 1-13.

VAN ORDEN (A. E.) & GREER (P. W.), 1977. — Modification of Dieterle spirochete stain. — J. Histol., **1**: 51-53.

ZHU (Z.), 1995. — Contribution à l'étude de la transmission de la borreliosis de Lyme en Europe. — Thèse de doctorat, Univ. Perpignan.

ZHU (Z.), GERN (L.) & AESCHLIMANN (A.), 1991. — The peritrophic membrane of *Ixodes ricinus.* — Parasitol. Res., **77**: 635-641.

ZHU (Z.), AESCHLIMANN (A.) & GERN (L.), 1992*a*. — Rickettsia-like microorganisms in the ovarian primordia of molting *Ixodes ricinus* (Acari: Ixodidae) larvae and nymphs. — Ann. Parasitol. Hum. Com., **67**: 99-110.

ZHU (Z.), GERN (L.) & AESCHLIMANN (A.) 1992*b*. — Localization of *Borrelia burgdorferi* in replete *Ixodes ricinus* nymphs. — Prog. & Abstr., A50, 292, Vth Int. Conf. Lyme Borreliosis, Arlington, Virgina, U. S. A., May 30–June 2, 1992.

ZHU (Z.), GERN (L.) & AESCHLIMANN (A.), 1992*c*. — *Borrelia burgdorferi* in naturally infected female *Ixodes ricinus*. — Proc. & Abstr., D4-4, First Int. Conf. Tick-Borne Pathogens at the Host-Vector Interface: An Agenda for Research, Saint Paul, Minnesota 15–18 Sept. 1992, p. 85.

ZHU (Z.), GERN (L.) & AESCHLIMANN (A.), 1993. — A preliminary observation on the peritrophic membrane atrophy in engorged nymphal *Ixodes ricinus* (Acari: Ixodidae) using light and electron microscopy. — Bull. Soc. Neuchâteloise Sci. nat., **116** (2): 15-23.

ZUNG (J. L.), LEWENGRUB (S.), RUDZINSKA (M. A.), SPIELMAN (A.), TELFORD (S. R.) & PIESMAN (J.) 1989. — The fine structural evidence for the penetration of the Lyme disease spirochete *Borrelia burgdorferi* through the gut and salivary tissues of *Ixodes dammini.* — Can. J. Zool., **67**: 1737-1748.