BORRELIA BURGDORFERI IN REPLETE NYMPHAL *IXODES RICINUS*: A LOCALIZATION STUDY USING LIGHT AND ELECTRON MICROSCOPY

BY Zhenqin ZHU *

BORRELIA BURGDORFERI
GEMMA
IXODES RICINUS
REPLETE NYMPH
LOCALIZATION
PERITROPHIC MEMBRANE
ELECTRON MICROSCOPY
SILVER-STAIN

SUMMARY: The ticks examined were in the early middle to middle pre-moulting period corresponding to the nymph-pharate adult transitional phase. Although *Borrelia burgdorferi* could be found intracellularly within Malpighian tubule, ovarian and syncytial muscular tissues, the spirochaete was predominantly located in the extracellular sites associated with different organs. A large number of borreliae were observed in the very narrow endoperitrophic space resulting from the peritrophic membrane atrophy in systemically infected nymphs at day 15 after a non-infectious blood meal. This observation indicates that some spirochaetes in the midgut lumen of unfed nymphs are enveloped within the endoperitrophic space once the peritrophic membrane occurs and/or condenses and survive there at least until ticks begin to enter pharate stage of the adult. The absence of *B. burgdorferi* from the ectoperitrophic space in systemically infected early middle to middle moulting nymphs suggests that spirochaetes persisting in this space may become systemic thereafter. The detection of borreliae only in the narrow endoperitrophic space of a male nymph at day 15 after repletion indicates that all pre-feeding midgut spirochaetes may be enclosed in this space when the peritrophic membrane appears and/or condenses, and the peritrophic membrane may become a barrier preventing spirochaetes from penetrating the midgut epithelium.

BORRELIA BURGDORFERI
GEMMA
IXODES RICINUS
NYMPHE REPUE
LOCALISATION
MEMBRANE PERITROPHIIQUE
MICROSCOPIE ELECTRONIQUE
COLORATION ARGENTIQUE

RÉSUMÉ: Les tiques examinées étaient en période de pré-mue précocement moyenne à moyenne, correspondant à la phase de transition adulte pharate-nymphe. Bien que *Borrelia burgdorferi* ait pu se trouver intracellulaire dans le tube de Malpighi et les tissus musculaires syncitiaux et ovariens, le spirochète s'est essentiellement localisé dans des sites extracellulaires associés à différents organes. Un grand nombre de borrelies, la plupart de formes atypiques, gemmae y compris, s'observèrent dans un espace endopéritrophique très étroit, provenant de l'atrophie de la membrane péritrophique, chez des nymphes systémiquement infestées 15 jours après un repas sanguin non infectieux. Cette observation montre que dans la lumière de l'intestin moyen de nymphes à jeûn quelques spirochètes s'enveloppent dans un espace endopéritrophique une fois formée et/ou condensée la membrane péritrophique et y survivent au moins jusqu'à ce que les tiques entrent en stade d'adulte pharate. L'absence, dans l'espace ectopéritrophique, de *B. burgdorferi* chez des nymphes infestées systémiquement, en mue précocement moyenne à moyenne, suggère que les spirochètes persistant dans cet espace doivent devenir ensuite systémique. La détection des borrelies seulement dans l'étroit espace endopéritrophique d'une nymphe mâle 15 jours après son repas montre que tous les spirochètes dans l'intestin moyen avant le repas de sang doivent s'enfermer dans cet espace lorsque apparaît et/ou se condense la membrane péritrophique, et la membrane péritrophique doit devenir une barrière prévenant la pénétration des spirochètes dans l'épithélium intestinal. L'origine des formes atypiques de *Borrelia burgdorferi* et leur rôle dans le cycle de vie du spirochète sont discutés.

* Department of Biology, East China Normal University, 3663 Zhong Shan Bei Lu, 200062 Shanghai, China.

Introduction

Following the discovery of the spirochaete *Borrelia burgdorferi* (Spirochaetales: Spirochaetacea), the causative agent of Lyme borreliosis, in *Ixodes dammini* in the United States (Burgdorfer *et al.*, 1982) and in *I. ricinus* collected in Switzerland (Burgdorfer *et al.*, 1983), many investigations have been concentrated on the development of the spirochaete in its ixodid tick vectors, mainly during blood-feeding. The studies dealing with the behaviour of the spirochaete in the immature stages of ixodid ticks are relatively few, especially in moulting ticks. *B. burgdorferi* has been demonstrated in all tissues, including haemolymph and salivary glands, of unfed and blood-feeding *I. dammini* nymphs (Zung *et al.*, 1989). Similar findings are lacking for engorged nymphs, though a rapid increase in spirochaete number in the period immediately after nymphal repletion and a rapid decrease as ticks moulted from nymphs to adults in *I. dammini* were reported (Piesman *et al.*, 1990). In addition, a rapid peritrophic membrane (PM) atrophic process was recently described in moulting nymphal *I. ricinus* during nymph-pharate adult transitional phase (Zhu *et al.*, 1993). It remains undetermined whether the atrophying PM has some influences on the development of midgut lumen spirochaetes, especially on the organisms enclosed within the endoperitrophic space when the PM occurs and condenses (Zung *et al.*, 1989; Zhu, 1995). The aim of the present investigation is to localize *B. burgdorferi* and evaluate its behaviour in replete *I. ricinus* nymphs. Part of the present results has been presented at the fifth International Conference on Lyme Borreliosis, Arlington, Virginia, USA (Zhu *et al.*, 1992*b*).

Materials and methods

Nymphal *I. ricinus* were collected by flagging vegetation in a forest near Neuchâtel, Switzerland. Collected ticks were fed on uninfected New Zealand white rabbits. Replete nymphs were kept at 20–22°C and saturated humidity, and sampled at day 15 (n=18) and 21 (n=12) after repletion. Each tick was then dissected in phosphate buffered saline (pH 7.4) and processed simultaneously by direct immunofluorescence assay (DIFA) for the immunological identification of *B. burgdorferi* infection, and by histological and transmission electron microscopic techniques (TEM) for the localization of the spirochaete within tick tissues. For DIFA, air-dried slides with organ smears of the ticks were fixed in acetone, incubated with fluorescein isothiocyanate conjugated antibodies to *B. burgdorferi* (Peacock *et al.*, 1971; Gern *et al.*, 1990, 1991) and were examined for spirochaetes by fluorescence microscopy. For histology, longitudinally halved ticks or pieces of tick organs were fixed in cold phosphate buffered 4% formaldehyde, pH 7.4, at 4°C overnight. The paraffin embedded tissues were sectioned at a thickness of 8 µm and stained using Dieterle spirochaete stain (Van Oden & Greer, 1977; Gern *et al.*, 1990). Longitudinally 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 according to Agbede *et al.* (1986) and Zhu *et al.* (1991, 1992, 1993).

Results

B. burgdorferi infection was demonstrated by DIFA, silver-stain and TEM, in 3 of 18 nymphs examined at day 15 and in one of 12 nymphs examined at day 21 after repletion. The examined ticks were in the early middle to middle pre-moulting period (Zhu *et al.*, 1993). The nymphs examined at day 15 after repletion were just in the course of apolysis and the ticks examined at day 21 after repletion had begun their pharate stage of the adult (Zhu *et al.*, 1993). The sexual differentiation of the examined ticks were very distinct (Zhu *et al.*, 1992). Among the infected ticks, only one examined at day 15 after repletion was found to be a male.

B. burgdorferi *in nymphs at day 15 after repletion*

Two female nymphs examined at day 15 after repletion had a systemic infection with *B. burgdorferi* in every organ. Spirochetal distribution and abundance in various organs were similar in these two nymphs.

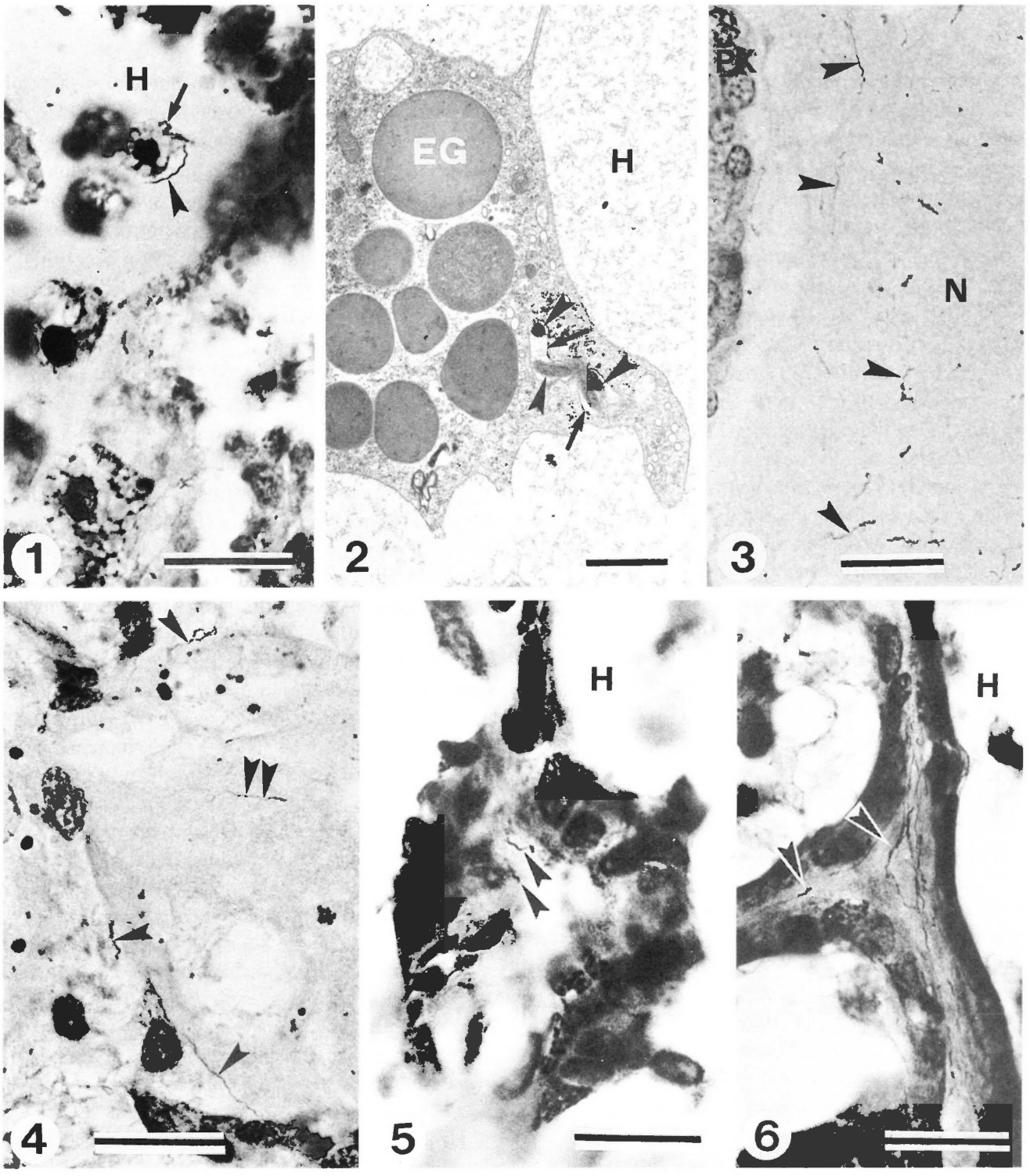

FIG. 1. *B. burgdorferi* (arrowhead) adhering to a hemocyte (arrow) in the haemolymph of a female nymph examined at day 15 after repletion. H hemocoel. Light micrograph, Dieterle stain. Bar = 20 µm. — FIG. 2. *B. burgdorferi* (arrowheads) within phagocytic vacuoles (arrows) of a type II granulocyte. EG electron-dense granules; H hemocoel. Transmission electron micrograph. Bar = 1 µm. — FIG. 3. *B. burgdorferi* (arrowheads) within the synganglion tissue of a nymph examined at day 15 after repletion. N neuropile region; PK perikaryo-region of neurons. Light micrograph, Dieterle stain. Bar = 20 µm. — FIG. 4. *B. burgdorferi* within (double arrowheads) and along the margin (arrowheads) of a weakly silver-stained large surviving agranular acinus of salivary glands (Balashov, 1968), measuring about 85–105 µm in diameter, in a female nymph examined at day 15 after repletion. Light micrograph, Dieterle stain. Bar = 20 µm. — FIG. 5. *B. burgdorferi* (arrowheads) in a heavily silver-stained small acinus of salivary glands, measuring about 45–65 µm in diameter, in a female nymph examined at day 15 after repletion. H hemocoel. Light micrograph, Dieterle stain. Bar = 20 µm. — FIG. 6. *B. burgdorferi* (arrowheads) in the lumen of a duct of the salivary glands from a nymph examined at day 15 after repletion. Note the duct is strongly silver-stained. H hemocoel. Light micrograph, Dieterle stain. Bar = 20 µm.

They were limited in number in haemolymph (Figs. 1–2), tracheae, fat body and nephrocytes, but quite numerous within other tissues (Figs. 3–12). The heaviest infection was found in the muscular tissue, in the greatly narrowed endoperitrophic space of the midgut lumen as described by ZHU *et al.* (1993) (Figs. 7–10), and in the epithelium of Malpighian tubules (Figs. 11–12).

Although many *B. burgdorferi* could be found intracellularly within Malpighian tubules (Fig. 11–12) and seldom in the phagocytic vacuoles of type II granulocytes (Fig. 2), the bacterium was principally located in the extracellular sites associated with various organs, such as the spaces between the basal lamina and the basal plasma membrane of host cells, the large vacuoles in the basal region of hypodermis, the extracellular spaces in the connective tissues, the intercellular spaces between ovarian cells as previously described by authors (ZHU *et al.*, 1992), the lumens of the acini and ducts of salivary glands (Figs. 4–6), and the endoperitrophic spaces of midgut lumen (ZHU *et al.*, 1993) (Figs. 7–10). Most spirochaetes in the syncytial muscular tissue were situated in the electron-lucent sarcoplasmic matrix. The ultrastructural location of *B. burgdorferi* in the synganglion has not been investigated.

Although spirochaetes were relatively few in the midgut wall and almost exclusively confined to the extracellular site along the very narrow basal margin of the organ, a large number of *B. burgdorferi* was present in the midgut lumen (Figs. 7–10). They were enclosed in the endoperitrophic space (Figs. 7–10) narrowed by the peritrophic membrane (PM) atrophy (ZHU *et al.*, 1993); whereas they were not seen in the ectoperitrophic space. Some spirochaetes still showed a typical *B. burgdorferi* appearance, but most of them had changed their morphology (Figs. 7–10). A large number of gemmae were present (Figs. 7–10). They consisted of a highly blebbed outer cell envelope and a coiled or folded inner protoplasmic cylinder as described by BURGDORFER & HAYES (1989). In the ultra-thin sections, the coiled cylinder often appeared as several separated cylinders adhering to the outer cell envelope. Huge protoplasmic cylinders as those reported by BURGDORFER & HAYES (1989) were sometimes observed within gemmae. They measured about 0.4–0.7 μm in diameter and showed a lower electron density than other ones. Some protoplasmic cylinders within gemmae were surrounded with 2 or 3 membranous layers. No electron-dense chromatin bodies were observed within gemmae, despite an extensive TEM examination. Giant spirochaetes with a huge protoplasmic cylinder measuring approximately 0.35–0.50 μm in diameter, were occasionally found in the narrow endoperitrophic space. Some un-gemmated spirochaetes occurring in this space possessed a protoplasmic cylinder surrounded with 2 or 3 membranous layers (Figs. 7, 9–10). Many small electron-dense granules and electron-lucent to moderately dense vesicles or blebs were seen in association with these atypical forms of the spirochaete (Figs. 7–10). They measured approximately 0.05–0.15 μm in diameter, and were much smaller than a typical protoplasmic cylinder of the spirochaete.

Giant spirochaetes with similar appearance to those found in the migut lumen also occurred in the wall of the Malpighian tubules (Figs. 11–12). They were often observed by TEM and located either in the membranous vacuoles in the cytoplasm of epithelial cells or in the extracellular spaces between the basal membrane infoldings. They had a dimension of approximately 0.6–0.8 μm. Their protoplasmic cylinders measured about 0.4–0.6 μm in diameter. The flagella were loosely associated with the protoplasmic cylinder and diffusely suspended in the highly enlarged periplasmic space between the outer envelope and the inner protoplasmic cylinder.

The infected male nymph was found to harbour a large quantity of borreliae in the narrowed endoperitrophic space, but not in other organs. The morphology of these spirochaetes was similar to that of the bacteria found in the endoperitrophic space of the two systemically infected female nymphs.

B. burgdorferi *in a nymph at day 21 after repletion*

The infected female nymph at day 21 after repletion contained borreliae in every organ. In the midgut lumen, where the PM had disappeared, no spirochaetes were detected by TEM. The spirochaete number in hypodermis, midgut wall, synganglion, Malpighian tubule epithelium, salivary glands and muscular tissue in this nymph was obviously lower than that in the nymphs examined at day 15 after repletion. In

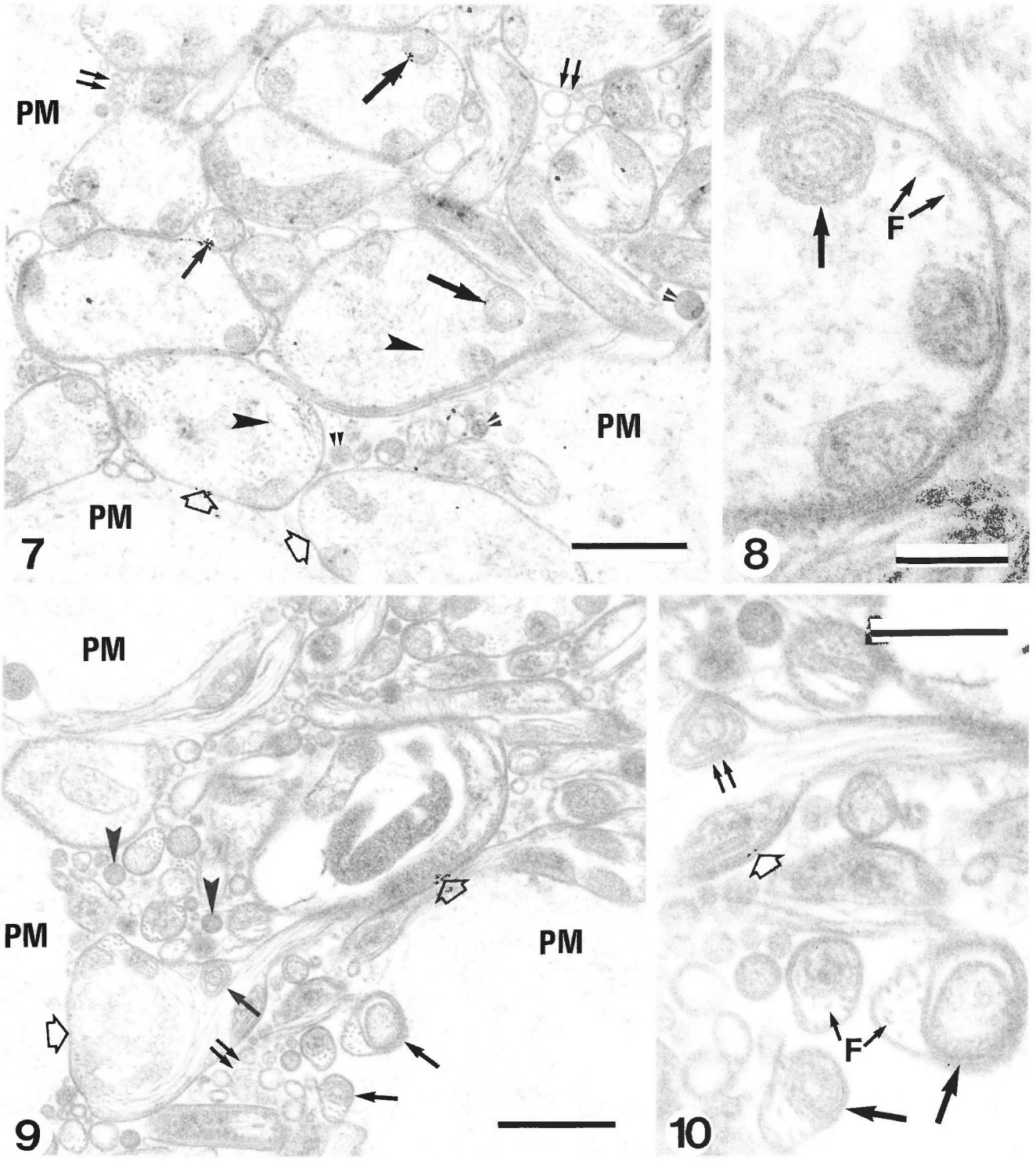

FIG. 7. *B. burgdorferi* imprisoned in the greatly narrowed endoperitrophic space of a nymph examined at day 15 after repletion. Note the densely packed gemmae (open arrows), electron-dense small granules (small arrowheads), electron-lucent to moderately dense vesicles or blebs (double small arrows) and a spirochaete having a protoplasmic cylinder surrounded with three membranous layers (small arrow). Within the gemmae, the flagella are suspended in the gemma contents (big arrowheads) and some protoplasmic cylinders are enclosed with three membranous layers (big arrows). PM peritrophic membrane. Transmission electron micrograph. Bar = 0.5 μm. — FIG. 8. Enlarged view of one cytoplasmic cylinder with three membranous layers (arrow) in a gemma shown in Fig. 7. F flagella. Transmission electron micrograph. Bar = 0.125 μm. — FIG. 9. *B. burgdorferi* imprisoned in the greatly narrowed endoperitrophic space of a nymph examined at day 15 after repletion. Note gemmae (open arrows), electron-dense small granules (arrowheads), electron-lucent to moderately dense vesicles or blebs (double arrows), and cytoplasmic cylinders possessing two or three membranous layers in a gemma and in two un-gemmated spirochaetes (arrows). Within the gemma(e) (open arrows), the coiled protoplasmic cylinder(s) can clearly be seen and part of the cylinder(s) shows a high electron density. The peritrophic membrane (PM) shown in this figure was not well exposed due to the excessive electron contrast between the PM and the enclosed spirochaetes. Transmission electron micrograph. Bar = 0.5 μm. — FIG. 10. Enlarged view of part of Fig. 9, demonstrating that one cytoplasmic cylinder (double arrows) in a gemma (open arrow) is enclosed with two membranous layers and two spirochaetes (arrows) have a cytoplasmic cylinder with two or three membranous layers. F flagella. Transmission electron micrograph. Bar = 0.25 μm.

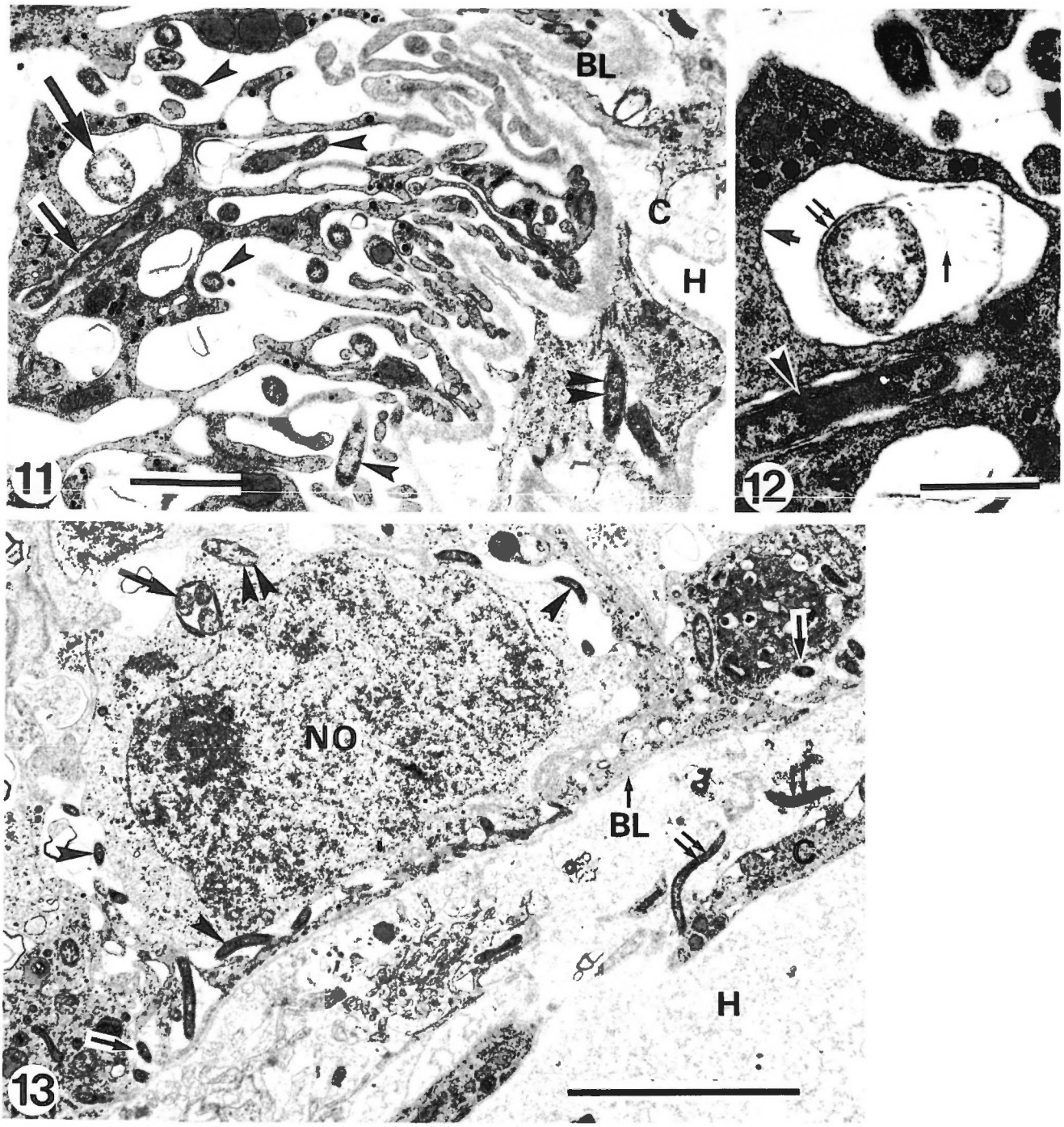

FIG. 11. Ultra-thin section of the Malpighian tubule tissue from a female nymph examined at day 15 after repletion, showing numerous *B. burgdorferi* (arrowheads) in the epithelium. Note that most spirochaetes (arrowheads) are situated in the electron-lucent spaces between the basal plasma membrane infoldings of the epithelial cells. A giant spirochaete (large arrow) is in a membranous vacuole of the host cell, and an intracellular spirochaete (small arrow) is just adjacent to the giant spirochaete. *B. burgdorferi* (double arrowheads) is also seen within the muscular-connective tissue surrounding the tubule. BL basal lamina; H hemocoel. Transmission electron micrograph. Bar = 1 μm. — FIG. 12. Enlarged view of the giant *B. burgdorferi* (double small arrows) shown in Fig. 11. Note about 16 flagella (small arrow) in the wide space between the outer cell envelope and the inner huge cytoplasmic cylinder. The big arrow indicates the host cell vacuole harbouring the giant spirochaete. An intracellular spirochaete (arrowhead) is just adjacent to the giant spirochaete. Transmission electron micrograph. Bar = 0.5 μm. — FIG. 13. *B. burgdorferi* (arrowheads) within the ovarian tissue of a nymph examined at day 21 after repletion. Note that spirochaetes can be seen in the intercellular spaces between ovarian cells (arrowheads), in the spaces between the basal lamina and the basal membrane of ovarian cells (small arrows), and in the extracellular spaces of the connective tissue (double small arrows) surrounding the ovary. Also note that single (double arrowheads) or grouped rickettsia-like microorganisms (large arrow) are located in the cytoplasm of the ovarian cells. BL basal lamina; C connective tissue; H hemocoel; NO nucleus of the primordial ovarian cell. Transmission electron micrograph. Bar = 4 μm.

fact, in the Malpighian tubule epithelium, only a few bacteria were present and giant spirochaetes were never detected. In contrast, the ovarian borreliae (Fig. 13) were much more abundant than those in the female nymphs examined at day 15 after repletion, despite the fact that the primordial ovary of this nymph was obviously more developed and had greatly enlarged. The heaviest spirochetal infection was present in the primordial ovary (Fig. 13). Some intracellular spirochaetes could be seen in the peripheral region of the primordial ovary. Within other tissues, the location of the bacterium was similar to that described in the female nymphs at day 15 after repletion.

Discussion

The present investigations show that, in replete nymphal *I. ricinus*, *B. burgdorferi* is principally located in the extracellular sites associated with different tick organs, although many spirochaetes can be found intracellularly within Malpighian tubule and ovarian cells and in the syncytial muscular tissue in systemically infected ticks. This confirms that *B. burgdorferi* is predominantly an extracellular spirochaete in its tick vector (Barbour & Hayes, 1986). In the present study, the ultrastructural location of the bacterium within the synganglion tissue has not been investigated. However, most spirochaetes detected in the synganglion of a starved naturally infected female were seen to be intracellular (Zhu, 1995). Whether more spirochaetes within synganglion tissue are intracellular than in other organs waits the results of the investigations in progress.

Findings so far available show that the PM is impenetrable for enveloped microorganisms once it condenses and remains structurally intact (Stohler, 1961; Mehlhorn, 1988; Miller & Lehane, 1993), except *Babesia microti* in *I. dammini* and *Plasmodium gallinaceum* in *Aedes aegypti*. The former is capable of traversing a mature and intact PM due to its arrowhead organelle (Rudzinska *et al.*, 1982); while the latter can penetrate even an abnormally thickened PM probably by means of a chitinase (Miller & Lehane, 1993). A midgut lumen infection of *B. burgdorferi* seems to be always present in infected unfed nymphs and females of both *I. dammini* and *I. ricinus* (Benach *et al.*, 1987; Burgdorfer *et al.*, 1982, 1988; Zung *et al.*, 1989; Zhu *et al.*, 1992*a*). During blood-feeding, numerous spirochaetes were present in both the endo- and ectoperitrophic spaces of midgut lumen in nymphal *I. dammini* (Zung *et al.*, 1989). The present investigations demonstrated a great number of *B. burgdorferi* in the midgut lumen, but only in the greatly narrowed endoperitrophic space in replete nymphal *I. ricinus* examined at day 15 after a non-infectious blood meal. These findings suggest that pre-feeding spirochaetes can persist in both the endo- and ectoperitrophic spaces when the PM occurs, and that the spirochaetes present in the ectoperitrophic space during blood-feeding could become systemic during and/or after blood-feeding. In addition, these findings also indicate that spirochaetes can persist in the endoperitrophic space of replete nymphs and can be concentrated in this space when the PM atrophies. The presence of borreliae exclusively in the narrow endoperitrophic space of a male nymph examined at day 15 after repletion suggests that all pre-feeding midgut lumen spirochaetes may be enclosed in this space once the PM occurs and condenses, and the PM may act as a real barrier preventing *B. burgdorferi* from penetrating the midgut epithelium.

Although *Spirochaeta duttoni* was reported to be able to form gemmae in the gut epithelium of its vector *Ornithodoros moubata* after being ingested (Dutton & Todd, 1907) and empty gemmae of *B. burgdorferi* were described in the ovarian lumen of a female *I. dammini* (Burgdorfer & Hayes, 1989), our present histological and ultrastructural investigations demonstrated a large number of gemmae of the spirochaete only in the greatly narrowed endoperitrophic space in replete nymphal *I. ricinus*. Gemma-like borreliae with multiple protoplasmic cylinders were found in the midgut lumen of starved *I. dammini* nymphs (Zung *et al.*, 1989) and starved and blood-feeding *I. ricinus* nymphs (Lebet *et al.*, personal communication; Zhu, 1995). Therefore, gemmae of *B. burgdorferi* might occur in the midgut lumen not only of moulting ticks but also of starved and blood-feeding nymphs. These gemmae may be then concentrated in the narrowed endoperitrophic space due to the shrinkage of the PM during atrophy, and a high concentration of gemmae may occur, as is shown in

the present study. If this is the case, the atrophying PM may play a role in gathering gemmae and other forms of *B. burgdorferi*. Another explanation is that, with the exhaustion of the ingested food (BALASHOV, 1968) and the rapid reduction of the endoperitrophic space, the conditions in this space may become no longer suitable for spirochaetes to develop and the concentration of the spirochaetes becomes too high in many small endoperitrophic spaces. Adverse changes in the spirochaete's environment might strongly induce gemma formation (PILLOT *et al.*, 1964). Thus, the majority of the gemmae found in the narrowed endoperitrophic spaces in the midgut of the moulting nymphs might occur during the atrophy of the PM.

Gemma forms of spirochaetes were considered either to be involved in spirochetal reproduction (DUTTON & TODD, 1907; HINDLE, 1911; HAMP, 1950; DELAMATER *et al.*, 1951), or to be simply degenerative products (KLEINE & ECKARD, 1913; WITTROCK, 1913; FENG & CHANG, 1936; BURGDORFER, 1951; KLEINE & KRAUSE, 1983). The chromatin bodies within gemmae of *Spirochaeta duttoni* were considered to be the primordia of the new spirochetal generation (DUTTON & TODD, 1907). Recently, BURGDORFER & HAYES (1989) found genetic material in the form of linear and circular plasmids in gemmae and blebs of *in vitro* cultured *B. burgdorferi*. Nevertheless, they observed no evidence that chromatin bodies produce a new generation of spirochaetes. They suggested that these gemmae or blebs may play a role in the storage and/or in the exchange of genetic material. In our present investigations, we failed to find chromatin bodies within gemmae, despite an extensive TEM observations. The huge protoplasmic cylinders were present either as one of several cylinders in a section of a gemma, or as one single cylinder of a giant spirochaete present in the endoperitrophic space or Malpighian tubule epithelium. The protoplasmic cylinders surrounded by two or three membranous layers were present only in the endoperitrophic space and have never been reported before. They occurred either as one or several cylinders in a gemma section or as one only cylinder of an un-gemmated spirochaete. The function of these unusual spirochaetes and the relationship between these atypical forms remains obscure.

Recently, spherical bodies similar in appearance to the gemmae observed in our present investigations were reported in *in vitro* cultured human oral spirochaete strains of *Treponema denticola* (WALF *et al.*, 1993). The close resemblance of the atypical spirochaetes observed in these two different spirochaete species belonging to two deeply branching groups (PASTER *et al.*, 1991) provides additional evidence that gemmae or spherical bodies may represent a form of spirochaetes generally occurring under certain *in vitro* or *in vivo* environmental conditions (BURGDORFER & HAYES, 1989). WALF *et al.* (1993) thought the spherical bodies of *Treponema denticola* to be the surviving form of the spirochaete. Our present study showed that in the systemically infected nymph at day 21 after repletion, any form of *B. burgdorferi* was not detected in the midgut lumen, in which the PM had disappeared. Presumably spirochaetes occur in the endoperitrophic space of this systemically infected tick before the disappearance of the PM, as was found in all three infected nymphs at day 15 after repletion. The gemmae and other forms of the bacterium may have disintegrated and disappeared together with the atrophied PM, or the organisms that survived the PM disintegration may have passed through the midgut epithelium and entered various tick tissues. Alternatively, the surviving bacteria were too few to be detected in the lumen by TEM. These questions need further investigation.

The present investigation showed that spirochaetes in various organs, except in the primordial ovary, were much more abundant in nymphs at day 15 after repletion than at day 21 after repletion. The difference of the spirochaete number in midgut lumens and Malpighian tubules between these ticks was particularly great. Indeed, borreliae had completely disappeared from the midgut lumen of the nymph at day 21 after repletion. In contrast, spirochaetes were much more abundant in the ovarian primordium of the 21-day nymph even though the organ had considerably enlarged (ZHU *et al.*, 1992). Because the number of the infected ticks in the present investigation was limited, it is uncertain whether *Borrelia burgdorferi* growth in the ovarian primordia of replete nymphs is delayed.

The number of spirochaetes in haemolymph was very limited. This confirms previous observations

(BENACH *et al.*, 1987; BURGDORFER, 1989; GERN *et al.*, 1990). The detection of spirochaetes in the phagocytic vacuoles within the type II granulocytes suggests that phagocytosis may be one of the factors limiting the spirochaete number in the haemolymph. However, it appears that the phagocytosis by haemocytes can not destroy all haemolymph spirochaetes and prevent the bacteria from invading the organs contained in the haemocoel.

A 10-fold decrease in the spirochaete number was observed as *I. dammini* nymphs moulted to adults (PIESMAN *et al.*, 1990). Nevertheless, the nymph-adult transition of *B. burgdorferi* does occur both in nature and in the laboratory (BURGDORFER *et al.*, 1983; 1985; 1988; MONIN *et al.*, 1989; GERN *et al.*, 1990). In fact, in naturally infected unfed adults, 3.9% of *I. dammini* (BURGDORFER *et al.*, 1988), 5.4% of *I. ricinus* (BURGDORFER *et al.*, 1983) and 32% of *I. pacificus* (BURGDORFER *et al.*, 1985) were found to be systemically infected. The detection of numerous spirochaetes within various organs of moulting nymphs, especially in synganglion and primordial ovary suggests that, the systemic infection of unfed adults may be realized by inheriting spirochaetes from their corresponding moulting nymphal organs (BURGDORFER *et al.*, 1988). In moulting ticks, ectodermal derivatives and certain muscle groups undergo histolysis, and salivary acini are completely replaced, but most tick organs change gradually throughout the tick's life-span (BALASHOV, 1968, 1979; OBENCHAIN & GALUN, 1982; HOOGSTRAAL, 1985). This characteristic of tick moulting is in favour of above hypothesis.

ACKNOWLEDGEMENTS

This study was financed by the Swiss National Science Foundation (No. 3.975.87), the Laboratory of Diagnostic Parasitology, Institute of Zoology, University of Neuchâtel, Switzerland and the Laboratory of Animal Biology, Centre of Biology and Ecology, University of Perpignan, France.

REFERENCES

AGBEDE (R. I. S.), KEMP (D. H.), & HOYTE (H. M.), 1986. — Secretory and digest cells of female *Boophilus microplus*: Invasion and development of *Babesia bovis*; Light and electron microscopic studies. — In: SAUER, J. R. & HAIR, J. A. (eds.) Morphology, Physiology, and Behavioral Biology of Ticks. Ellis Horwood, England, pp 457-471.

BALASHOV (Yu. S.), 1968. — Bloodsucking ticks (Ixodoidea)—vectors of diseases of man and animals. — Nauka Publishers, Lenningrad. [In English: Translation 500 (T500), Medical Zoology Department, United States Naval Medical Research Unit Number Three, Cairo, Egypt, pp. 376].

BALASHOV (Yu. S.), 1979. — An atlas of ixodid tick ultrastructure. — Nauka Publishers, Lenningrad. [In English (1983) Entomol. Soc. Am., Washington, D. C. pp 1-289].

BARBOUR (A. G.), & HAYES (S. F.), 1986. — Biology of *Borrelia* species. — Microbiol. 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. Infectious Diseases, **155**: 1300-1306.

BURGDORFER (W.), 1951. — Analyse des Infektionsverlaufes bei *Ornithodoros moubata* und der natürlichen Uebertragung von *Spirochaeta duttoni*. — Acta Tropica, **8**: 196-262.

BURGDORFER (W.), 1989. — 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.), PÉTER (O.) & AESCHLIMANN (A.), 1983. — Erythema chronicum migrans—a tickborne spirochetosis. — Acta Tropica, **40**: 79-83.

BURGDORFER (W.) & HAYES (S. F.), 1989. — Vector-spirochaete relationships in louse-borne and tick-borne borreliosis with emphasis on Lyme disease. — Advances in Disease Vector Research, **6**: 127-150. Springer-Verlag New York Inc.

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.

BURGDORFER (W.), LANE (R. S.), BARBOUR (A. G.), GRESBRINK (R. A.) & ANDERSON (J. R.), 1985. — The western black-legged tick, *Ixodes pacificus*: A vector of *Borrelia burgdorferi*. — Amer. J. trop. Med. Hygiene, **34**: 925-93.

DELAMATER (E. D.), NEWCOMER (V. D.), HAANES (M.) & WIGGALL (R. H.), 1951. — Studies on the life cycle of spirochetes. VIII. Summary and comparison of observation on various organisms. — J. Investigative Dermatol., **16**: 231-256.

DUTTON (J. E.) & TODD (J. L.), 1907. — A note on the morphology of *Spirochaeta duttoni*. — Lancet, **2**: 1523-152.

FENG (L. C.) & CHANG (H. L.), 1936. — Studies on the development of *Spirochaeta duttoni* in *Ornithodoros moubata*. — Chinese med. J., **50**: 1158-1190.

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

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

HAMP (E. G.), 1950. — Morphologic, characteristics of smaller oral treponemes and *Borrelia vincenti* as revealed by smear, darkfield and electron microscopic technics. — J. Am. dental Assoc., **40**: 1-11.

HINDLE (E.), 1911. — On the life-cycle of *Spirochaeta gallinarum*. — Parasitology, **4**: 463-477.

HOOGSTRAAL (H.), 1985. — Argasid and nuttalliellid ticks as parasites and vectors. — In: J. R. BAKER & R. MULLER (Eds), Advances in Parasitology, Vol. 24, London: Academic Press. pp 136-238.

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

KLEINE (F. K.) & ECKARD (B.), 1913. — Uber die Lokalisation der Spirochaeten in der Rückfallfieberzecke (*O. moubata*). — Zeitschrift für Hygiene und Infektionskrankheiten, **74**: 389-394.

KLEINE (F. K.) & KRAUSE (M.), 1983. — Zur Kritik angeblicher Entwicklungsformen von Rückfallfieberspirochaeten in der Zecke (*Ornithodoros moubata*). — Archiv für Schiffs und Tropenhygiene, **36**: 190-191.

MEHLHORN (H.), 1988. — Peritrophic membranes. In: H. MEHLHORN (Ed.) Parasitology in Focus, Facts and Trends, Springer-Verlag, 1988, pp. 219-221.

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

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

OBENCHAIN (F. D.) & GALUN (R.), 1982. — Physiology of Ticks. — In: Current Themes in Tropical Science, Vol 1, Oxford: Pergamon.

PASTER (B. J.), DEWHIRST (F. E.), WEISBURG (W. G.), TORDOFF (L. A.), FRASER(G. J.), HESPELL (R. B.), STANTON (T. B.), ZABLEN (L.), MANDELCO (L.) & WOESE (C. R.), 1991. — Phylogenetic analysis of the spirochetes. — J. Bacteriol., **173**: 6101-6109.

PEACOCK (M.), BURGDORFER (W.) & ORMSBEE (R. A.), 1971. — Rapid fluorescent-antibody conjugation procedure. — Infection and Immunity, **3**: 335-337.

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

PILLOT (J.), DUPOUEY (P.) & RYTER (A.), 1964. — La signification des formes atypiques et la notion de cycle évolutif chez les spirochètes. — Ann. Instit. Pasteur, **107**: 484-502.

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.

STOHLER (H.), 1961. — The peritrophic membrane of bloodsucking Diptera in relation to their role as vectors of blood parasites. — Acta Tropica, **18**: 263-266.

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

WALF (V.), LANGE (R.) & WECKE (J.), 1993. — Development of quasi-multicellular bodies of *Treponema denticola*. — Arch. Microbiol., **160**: 206-213.

WITTROCK (O.), 1913. — Beitrag zur Biologie der Spirochaeta des Rückfallfiebers. — Zeitschrift für Hygiene, **74**: 55-60.

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

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

ZHU (Z.), GERN (L.) & AESCHLIMANN (A.), 1992*a*. — *Borrelia burgdorferi* in naturally infected female *Ixodes ricinus*. — Proceedings & abstracts, p. 85, D4-4, First International Conference on Tick-Borne Pathogens at the Host-Vector Interface: an Agenda for Research, Saint Paul, Minnesota 15-18 September 1992.

ZHU (Z.), GERN (L.), AESCHLIMANN (A.), 1992b. — Localization of *Borrelia burgdorferi* in molting *Ixodes ricinus* (Acari: Ixodidae) nymphs. — Abstract, V International Conference on Lyme Borreliosis, May 30-June 2, 1992, Arlington, Virginia, U. S. A.

ZHU (Z.), GERN (L.) & AESCHLIMANN (A.),1993. — A preliminary observation on the peritrophic membrane atro-

phy in engorged *Ixodes ricinus* (Acari: Ixodidae) nymphs. — 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.