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A PRELIMINARY OBSERVATION ON THE PERITROPHIC MEMBRANE ATROPHY IN ENGORGED NYMPHAL *IXODES RICINUS* (ACARI: IXODIDAE) USING LIGHT AND ELECTRON MICROSCOPES

by

ZHENQIN ZHU, LISE GERN AND ANDRÉ AESCHLIMANN

WITH 4 FIGURES

INTRODUCTION

A peritrophic membrane (PM) has been described in the midgut lumen of all three developmental stages of *Ixodes dammini* and *I. ricinus* (RUDZINSKA *et al.* 1982; ZHU *et al.* 1991). It is a transient branched tube-like structure produced after blood-feeding stimulation and covers the whole midgut contents. The occurrence time of the PM in *I. dammini* and *I. ricinus* (RUDZINSKA *et al.* 1982; ZHU *et al.* 1991), its morphological changes in *I. ricinus* during and after blood-feeding (ZHU *et al.* 1991) and its time of disappearance in molting larval *I. dammini* (RUDZINSKA *et al.* 1982) have been investigated. However, the atrophy and/or disintegration of the PM in replete ticks have never been reported before. As vectors of diverse diseases, *I. dammini* and *I. ricinus* transmit numerous pathogenic microorganisms to man and animals. Findings involving the atrophy and/or disintegration of the PM may shed some light on the evaluation of the behaviour of the microorganisms contained within endoperitrophic space, including *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae), the causative agent of Lyme borreliosis, which had been demonstrated in the endoperitrophic space in the midgut lumen of blood-feeding *I. dammini* and *I. ricinus* nymphs (ZUNG *et al.* 1989; N. LEBET unpublished data). The purpose of this study was to observe the morphological process of the PM atrophy in replete nymphal *I. ricinus.*

MATERIALS AND METHODS

Nymphal *I. ricinus* were collected by flagging vegetation in a forest near Neuchâtel, Switzerland in May, 1990. Collected ticks were fed on uninfected New Zealand white rabbits (GRAF 1978). Replete nymphs were kept

at 20-22°C and in a saturated humidity condition. Alternate periods of 16 hours of light and 8 of darkness were maintained. Ticks were sampled at day 15 (n = 15) and 21 (n = 9) after repletion and longitudinally halved in phosphate buffered saline (pH 7.35). Halved ticks were fixed in freshly prepared Karnovsky's fixative (KARNOVSKY 1965) at 4°C overnight and processed for transmission electron microscopy (TEM) (AGBEDE *et al.* 1986; ZHU *et al.* 1991). Spurr's (Electron Microscopy Sciences Fort Washington, PA)-embedded halved ticks were sectioned transversally. Both semi-thin and ultra-thin sections were made with an ultra microtome (Reichert-Jung Ultracut E, Reichert-Jung Optische Werke Ag Austria). Semi-thin sections were stained with toluidine blue and observed with an Olympus Vanox-S (OLYMPUS OPTICAL CO., LTD) optical microscope (OM). Ultra-thin sections were first stained with uranyl acetate and then with lead citrate and examined with a Philips EM 201 electron microscope. For the control of the molting duration, replete nymphs (n = 26) were also maintained until they molted to adults.

RESULTS

The molting period of the examined nymphs

All nymphs kept as controls molted to adults at day 36-43 after repletion. Thus, the nymphs examined at day 15 and 21 after repletion were in the early middle to middle pre-molting period. OM and TEM observations showed that in nymphs examined at day 15 after repletion, the detachment of the hypodermis from the old cuticle had taken place, in varying degrees, at the anterior part of the ticks. In nymphs examined at day 21 after repletion, the hypodermis had completely separated from the old cuticle. Thus, the apolysis (JENKIN and HINTON 1966) of the examined nymphs had begun or finished and the ticks were in the nymph-pharate adult transition phase.

The distribution and morphology of the PM in nymphs examined at day 15 after repletion

A PM was present in the midgut lumen of each of the 15 nymphs examined at day 15 after repletion. The development of the PM was not synchronous among the examined nymphs. Based on the appearance and the distribution of the PM, 3 situations (I-III) were recognized:

I): In two of the 15 nymphs, a thick, winding and multi-layered PM similar to that previously described in molting nymphs sampled at day 14 after repletion (ZHU *et al.* 1991), was seen throughout the midgut lumen. That is to say, the PM was still situated quite close to the midgut epithelial cell surface and the endoperitrophic space remained very wide. Its electron density was obviously lower than that of the endo- and ectoperitrophic spaces.

II): In 3 nymphs, a PM was detected in the lumen of the stomach and in the basal parts of the midgut diverticula (fig. 1). A considerable length of the terminal diverticular lumen was free of the PM. Disintegration was not

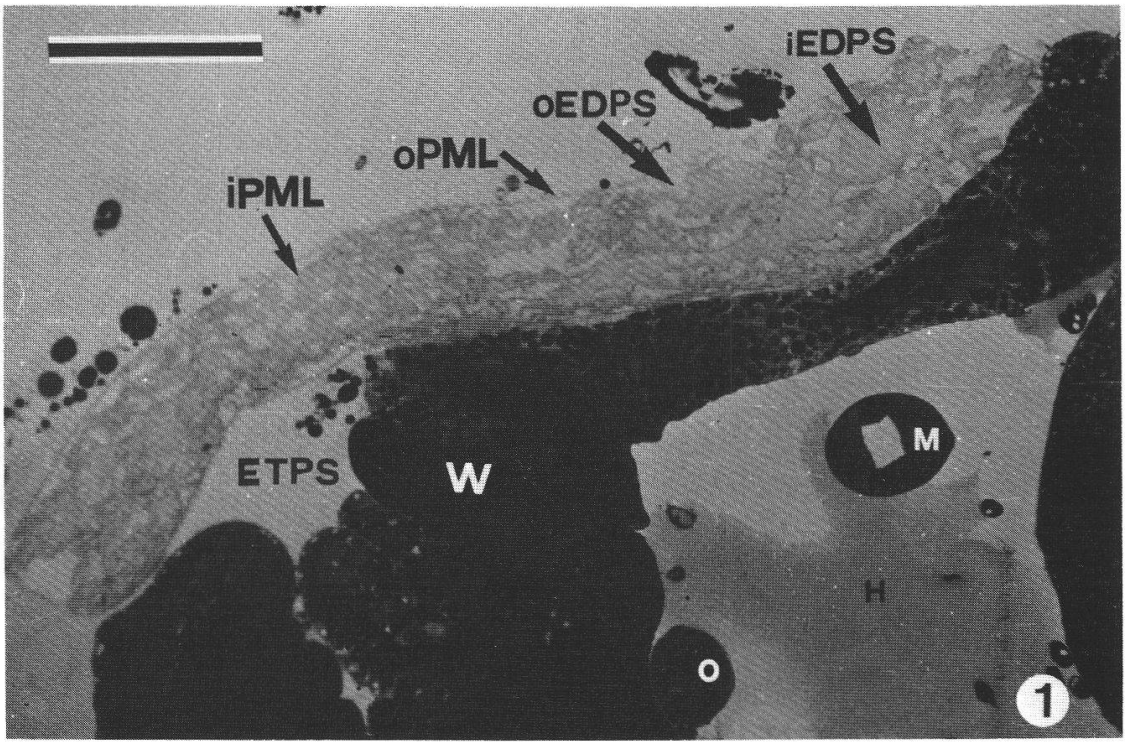

Fig. 1. Peritrophic membrane in the stomach lumen of a nymph examined at day 15 after repletion (the second situation). The peritrophic membrane consists of two layers: an almost non-rippled outer layer (oPML) and a greatly folded inner one (iPML). The inner endoperitrophic space (iEDPS) enclosed by the iPML has largely decreased. The folds of the iPML is still distinguishable and remain un-disintegrated. The outer endoperitrophic spaces (oEDPS) between the oPML and the iPML can still be recognized. Note that half of the stomach wall had been removed during dissection. ETPS ectoperitrophic space; H hemocoel; M Malpighian tubule; O ovarian primordium; W wall of the stomach. Optical micrograph, Toluidine blue, Bar = 100 μm.

found at the distal part of the diverticular branches of the PM. The terminal midgut diverticula were devoid of a histologically visible lumen which was completely closed with the enlarged epithelial cells (TILL 1961).

In histological sections, the PM was found to consist of two morphologically different layers: a nearly non-rippled outer PM layer, and a highly rippled and greatly folded inner one (fig. 1). The occurrence of the outer PM layer had led to the presence of a space between the inner and outer PM layers. For the convenience of description, this space is tentatively named the outer endoperitrophic space and the space enclosed by the inner PM layer the inner endoperitrophic space (fig. 1).

The inner endoperitrophic space enveloped by the highly rippled and folded inner PM layer had largely decreased in volume but was still recognizable in histological sections (fig. 1). The folds of the inner PM layer remained still distinguishable, and showed an un-disintegrated appearance (fig. 1). Both the outer and the inner PM layer were found to be multilayered by TEM. The electron density of both the ectoperitrophic space and the outer endoperitrophic space was identical, but it was obviously lower than that of the inner endoperitrophic space. The outer PM layer and the

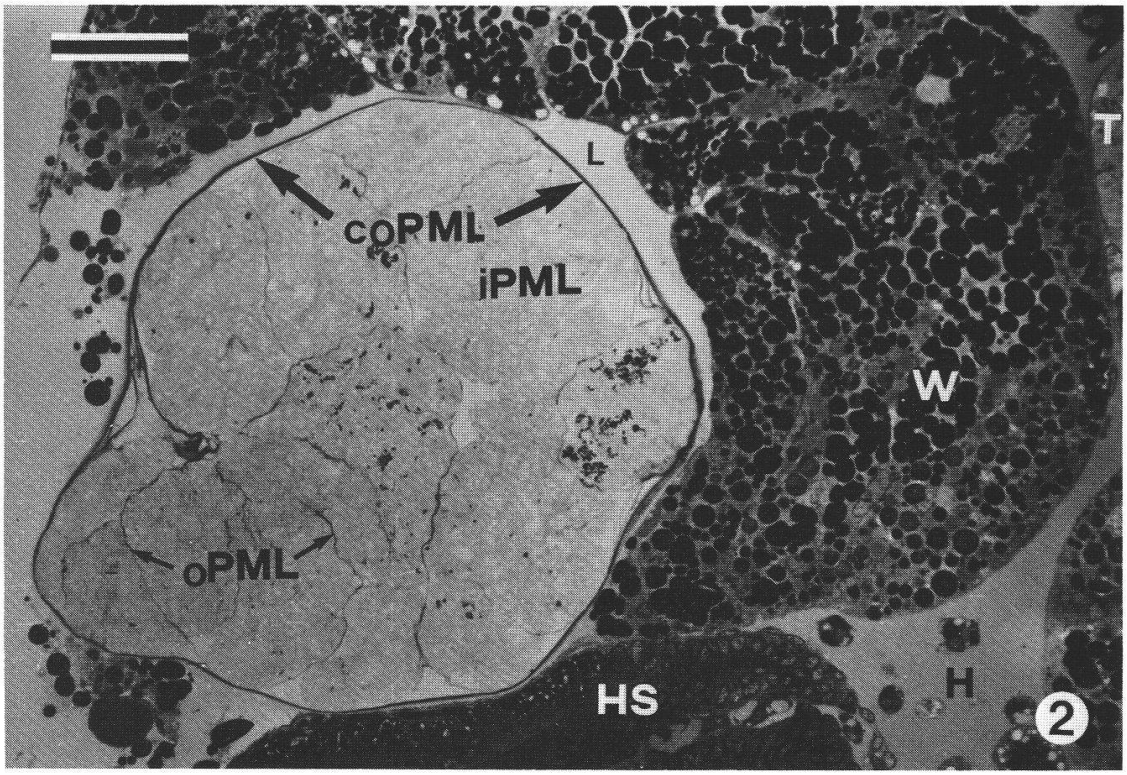

Fig. 2. Semi-thin section crossing the anterior part of a nymph examined at day 15 after repletion (the third situation), showing a large peritrophic membrane mass in the stomach lumen. The large mass consists of several small masses and is enclosed within a common outer peritrophic membrane layer (coPML). Each small mass itself is surrounded with a relatively thin outer peritrophic membrane layer (oPML). The inner peritrophic membrane layer (iPML) is extremely folded. Note that half of the gut wall had been removed during dissection. H hemocoel; HS hypodermal sac of the genital organ; L lumen of the stomach; T testicular primordium; W wall of the stomach. Optical micrograph, Toluidine blue, Bar = 50 µm.

folded inner PM layer showed a higher electron density than the inner endoperitrophic space, and particularly than the outer endoperitrophic space and the ectoperitrophic space.

III): In the remaining 10 ticks, a PM was only found in the stomach lumen (figs. 2-3). In the transversal histological sections, it appeared to be a large mass consisting of a surrounding common outer PM layer and several small PM masses (fig. 2). Each of these small masses was enclosed with a relatively thin outer PM layer (fig. 2). In these small masses, the inner PM layer was so greatly folded and so densely distributed that the spaces enclosed by inner PM layer (belonging to the inner endoperitrophic space) and those between the outer and the inner PM layers (belonging to the outer endoperitrophic space) were hard to be recognized in the histological sections (fig. 2). However, by TEM, they could still be distinguished from each other and the inner endoperitrophic space was represented by numerous very narrow spaces between the folds of the inner PM layer (fig. 3). The electron density of these spaces was obviously lower than that of the greatly folded inner PM layer and particularly than that of the outer PM

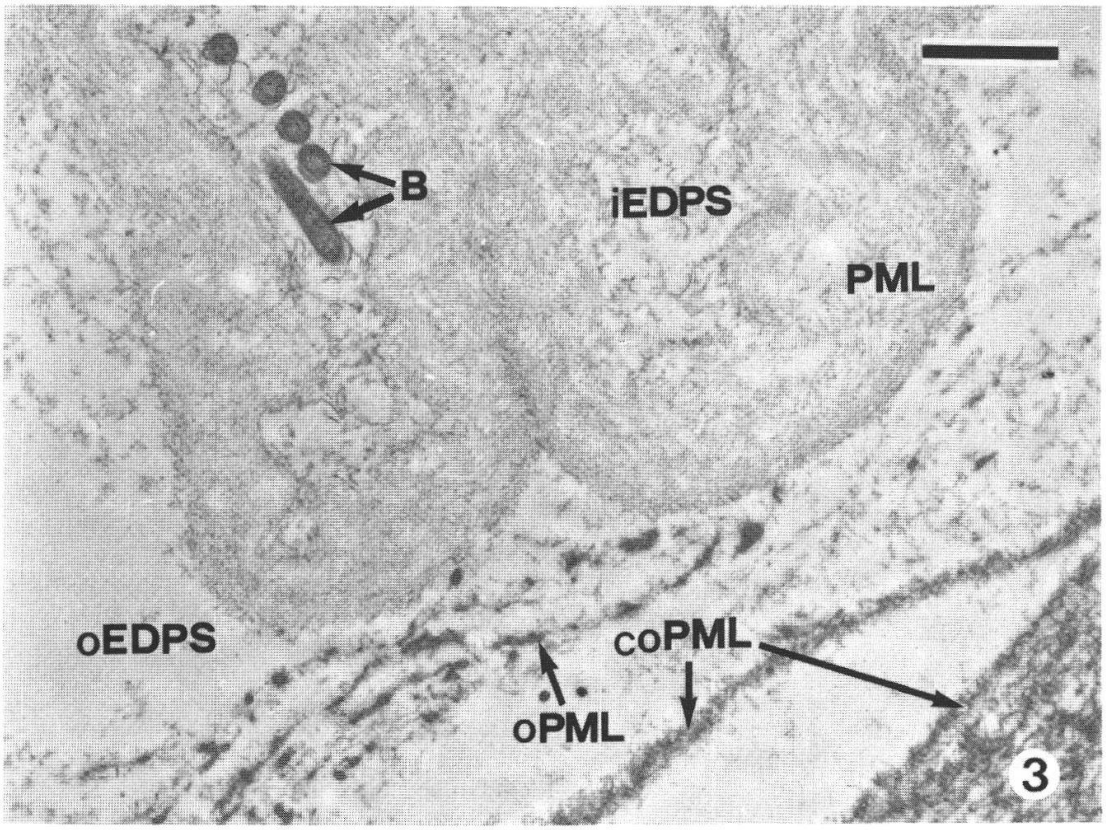

Fig. 3. Ultra-thin section showing the greatly folded inner peritrophic membrane layer (iPML), and the multi-layered outer (oPML) and common peritrophic membrane (coPML) layers in a nymph examined at day 15 after repletion (the third situation). Note that several *B. burgdorferi* are situated in a narrow inner endoperitrophic space (iEDPS). The iEDPS shows a lower electron density than the iPML, oPML and coPML. B *B. burgdorferi*. Transmission electron micrograph, Bar = 1 μm.

layer and the common outer PM layer (fig. 3). TEM observation also showed that the common outer PM layer, the outer PM layer and the inner PM layer had a multi-layered appearance (fig. 3). Although the PM had completely changed its appearance, it seemed to be still un-disintegrated (figs. 2-3).

The distribution and morphology of the PM in nymphs examined at day 21 after repletion

The development of the PM was also not synchronous among the 9 nymphs examined at day 21 after repletion. According to the presence or absence of a PM, two additional situations (IV-V) were distinguished:

IV): In 3 of the 9 nymphs, the lumen of the stomach was still quite large, though most part of the diverticula lumen was not detectable in the semi-thin sections by OM. A disintegrating PM mass was observed in the stomach lumen (fig. 4). The commom outer PM layer was still very prominent, but the enclosed contents appeared to be homogeneous, that is to say, the con-

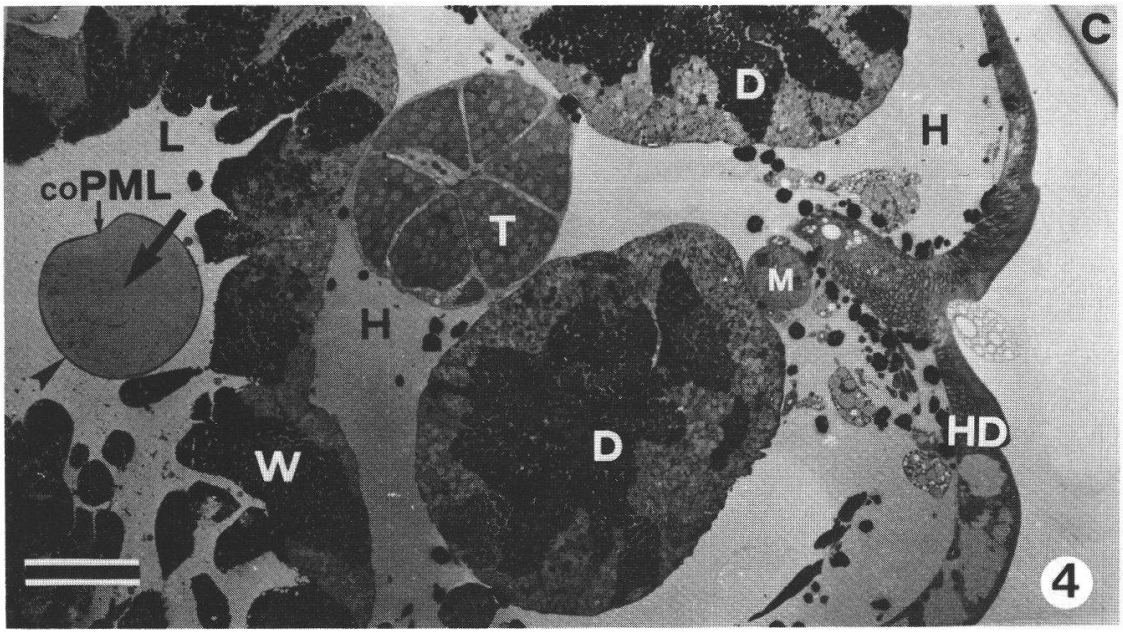

Fig. 4. Semi-thin section traversing the anterior part of a nymph examined at day 21 after repletion (the fourth situation), demonstrating a disintegrating peritrophic membrane mass (arrowhead) in the stomach lumen. Note that the common outer peritrophic membrane layer (coPML) is still prominent but the enclosed content shows a homogeneous appearance (big arrow). The diverticula (D) lack a visible lumen and the hypodermis (HD) has totally separated from the old cuticle (C). H hemocoel; L lumen of the stomach; M Malpighan tubule; T testicular primordium; W wall of the stomach. Optical micrograph, Toluidine blue, Bar = 100 μm.

tained small PM masses present in the nymphs belonging to the third situation, were no longer distinguishable (fig. 4).

V): In the remaining 6 ticks, most part of the midgut lumen was completely enclosed with the enlarged epithelial cells (TILL 1961; BALASHOV 1968). TEM observation showed no PM in these ticks.

DISCUSSION

The present study reveals that, under our experimental conditions, during the early middle to middle pre-molting period, the PM of nymphal *I. ricinus* undergoes a rapid atrophy process, though this phenomenon is not synchronous among the ticks examined. This period corresponds to the transition phase from nymph into pharate adult. The temperature and the daytime have an important influence on the molting duration of the nymphal *I. ricinus* (KAHL *et al.* 1990). The molting duration is variable among nymphal *I. dammini* under the same laboratory conditions (PIESMAN *et al.* 1990). A PM was reported in a nymphal *I. ricinus* examined at day 30 after repletion, though it had become thick, winding and multi-layered (ZHU *et al.* 1991). Moreover, in our present investigation the PM of most molting nymphs examined at day 21 after repletion has completely disappeared. This developmental delay may be mainly due to the relatively low temperature

(18-20°C) (ZHU *et al.* 1991) and a relatively short-day condition (alternate periods of 10.5 hours of light and 13.5 of darkness) applied to the experiments but not mentioned in the resultant publication (ZHU *et al.* 1991). In addition, ticks used in the previous study were from a laboratory strain, while nymphs for the present experiments were captured in nature. This may also be responsible for the different molting durations.

The authors' previous study has demonstrated that after repletion the PM in nymphal *I. ricinus* becomes thicker and thicker, increasingly winding and multi-layered (ZHU *et al.* 1991). Simultaneously, the distance between the PM and the midgut epithelium increases continuously (ZHU *et al.* 1991). The present investigations show that these morphological changes of the PM are greatly accelerated during early middle to middle pre-molting period. At the same time, the PM disappears gradually from the terminal parts of the midgut diverticula towards stomach. Thus, it appears that the PM atrophy begins soon after repletion, but only during early middle to middle pre-molting period, when nymphs begin to enter their pharate stage of adult, does a rapid atrophy process of the PM occur. The fate of the PM in diapausing nymphs (KAHL *et al.* 1990) deserves to be investigated.

The observation that in the majority of the nymphs examined at day 15 after repletion, the PM was only present in the stomach lumen and had become a large mass (figs. 2-3) suggests that the PM might be able to contract itself towards stomach during its rapid atrophy.

Only 3 of the 9 nymphs examined at day 21 after repletion possessed a PM, and the PM was restricted in the stomach lumen and displayed a disintegrating appearance. Furthermore, disintegrating PM was not found in any other ticks. These results suggest that the PM in repleted *I. ricinus* nymphs might finally disintegrate and disappear in the stomach lumen at the beginning of their pharate adult phase. However, the possibility that the atrophying PM disintegrates and disappears gradually from the terminal ends of its diverticular branches towards the stomach lumen should not be excluded, since the real extremity of the diverticular PM branches in the nymphs belonging to the second situation was difficult to be detected. Further investigation is needed to elucidate the concrete morphological and biochemical process of the PM disintegration.

We observed that in the nymphs pertaining to the second situation, the electron density of the outer endoperitrophic space was identical to that of the ectoperitrophic space, but lower than that of the inner endoperitrophic space. In addition, the outer PM layer was found to be nearly non-rippled, while the enclosed inner PM layer was greatly folded. These findings suggest that the outer PM layer may be secreted during the rapid shrinkage and folding of the inner PM layer which represents the PM produced before rapid atrophy. The presence of a thick common outer PM layer surrounding the whole large PM mass in the nymphs belonging to the third situation, provides an additional evidence for the secretion of the PM materials even after the PM had become restricted in the stomach lumen. The multi-layered appearance of both the outer PM layer and the common outer PM layer (figs. 2-3) implicates that these secretions might be intermittent.

We have found a large number of *B. burgdorferi,* most of which are atypical forms including gemmae, imprisoned in the greatly reduced inner endoperitrophic space in systemically infected molting nymphal *I. ricinus* examined at day 15 after repletion (the third situation) (fig. 3) (ZHU *et al.* 1992). The relationship between the atrophy and disintegration of the PM and the behaviour of *B. burgdorferi* enveloped in the endoperitrophic space will be reported in another paper.

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Summary

Although the peritrophic membrane atrophy in nymphal *I. ricinus* begins soon after tick repletion, only during early middle to middle pre-molting period, corresponding to the nymph-pharate adult transition phase, does the peritrophic membrane undergo a rapid atrophy process. When this rapid process begins, the tube-like peritrophic membrane becomes more infolded and shrinks rapidly and the endoperitrophic space rapidly decreases in volume. At the same time, the peritrophic membrane disappears gradually from the terminal parts of the diverticular lumen towards the stomach. In the majority of the nymphs examined at day 15 after repletion, it is only found in the stomach lumen and appears to be a large mass packed with membranous folds. The endoperitrophic space in these ticks is enormously reduced and is represented by numerous narrow spaces between the peritrophic membrane folds. The peritrophic membrane has completely disappeared in most ticks examined at day 21 after repletion.
Key-words: Peritrophic membrane atrophy, Molting nymphs, *Ixodes ricinus*, *Borrelia burgdorferi*, Light and electron microscopy.

Résumé

Bien que l'atrophie de la membrane péritrophique débute peu après le repas sanguin chez les nymphes d'*I. ricinus*, ce n'est que pendant la période précédant directement la mue conduisant au stade adulte que la membrane péritrophique subit une rapide atrophie. Celle-ci se manifeste par un rétrécissement et un plissement de la membrane péritrophique et l'espace endopéritrophique diminue de volume. En même temps, la membrane péritrophique disparaît graduellement des extrémités des diverticules intestinaux pour se concentrer dans l'estomac. Dans la majorité des nymphes examinées 15 jours après le repas sanguin, elle n'est visible que dans la lumière de l'estomac où elle apparaît comme une large masse remplie par les plis membranaires. L'espace endopéritrophique chez ces tiques est alors énormément réduit et se limite aux petits espaces situés entre les plis de la membrane péritrophique. Chez la plupart des tiques examinées 21 jours après le repas sanguin, la membrane péritrophique a complètement disparu.

Mots-clés: Atrophie de la membrane péritrophique, Nymphes en train de muer, *Ixodes ricinus*, *Borrelia burgdorferi*, Microscope optique et électronique.

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