Parasitol Res (2003) 90: 243–248
DOI 10.1007/s00436-003-0833-6

ORIGINAL PAPER

T. Matsuo · M. Sato · N. Inoue · N. Yokoyama
D. Taylor · K. Fujisaki

Morphological studies on the extracellular structure of the midgut of a tick, *Haemaphysalis longicornis* (Acari: Ixodidae)

Received: 30 September 2002 / Accepted: 11 December 2002 / Published online: 18 March 2003

Abstract In the present study, morphological observations on the extracellular structures found on the apical surface of the midgut epithelium, known as the peritrophic membrane (PM) or glycocalyx, are described in *Haemaphysalis longicornis* females and larvae. These structures have been hypothesized to provide protection to the microvilli of epithelial cells of the digestive tract. Our aim was to determine whether the extracellular structures are important in the digestion of the blood meal and/or as a protection against infection or injury. The PM was detectable in the midgut of engorged larvae by electron microscopy, but not in engorged females. However, a PM-like structure, stainable with toluidine blue, was observed in females by light microscopy. From the results of confocal laser scanning and electron microscopic observations with wheat germ agglutinin (WGA lectin) staining for chitin of the PM, however, the structure was clearly recognized. The structure in the female is likely to be PM because staining with WGA lectin in the presence of GlcNAc indicates the presence of chitin and various morphologies of PM have been reported in insects and ticks. These results show morphologically that different types of PM-like structure are formed in larvae and females of *H. longicornis*.

T. Matsuo · N. Inoue · N. Yokoyama · K. Fujisaki (✉)
National Research Center for Protozoan Diseases,
Obihiro University of Agriculture and Veterinary Medicine,
Obihiro 080-8555, Hokkaido, Japan
E-mail: fujisaki@obihiro.ac.jp
Tel.: +81-155-495646
Fax: +81-155-495643

M. Sato
Comparative Pathology Section,
Kyushu Research Station,
National Institute of Animal Health,
Kagoshima 891-0105, Japan

D. Taylor
Institute of Agriculture and Forestry,
University of Tsukuba, Tennohdai, Tsukuba,
Ibaraki 305-0003, Japan

Introduction

Two extracellular structures found on the apical surface of the midgut epithelium, the peritrophic membrane (PM) and the glycocalyx, have been proposed to provide protection for the microvilli of the digestive tract in many arthropods (Shen et al. 1999). The PM has been most extensively investigated in arthropods and is of particular interest in blood-sucking vectors (Lehane 1997; Shao et al. 2001). It is generally accepted in blood-sucking insects that the PM, which is characterized by chitin and peritrophin, is an extracellular sac derived from secretions of the epithelial cells of the midgut (Lehane 1997; Terra 2001). Moreover, a fluid (peritrophic gel) or PM film surrounding the food bolus has been described in most insects (Terra 2001).

PMs are classified according to their sites of synthesis: type 1 PM is expressed by the midgut epithelia of insects while type 2 is produced by specialized cells in the gut (Peters 1992). The PM apparently functions to protect the midgut epithelium from damage by hard food and is freely permeable to digestive enzymes and to the products of digestion (Rudzinska et al. 1982). It has also been reported that the PM is not freely permeable, but rather shows selective permeability to both digestive enzymes and digestion products (Terra 2001). Moreover, the significance of the PM as a barrier to the pathogenic invasion into the midgut epithelium by pathogens for which the arthropod is a vector has also been investigated (Laurence 1966; Rudzinska et al. 1982; Evans and Ellis 1983; Friedhoff 1987). In contrast, the glycocalyx was recently reported to be a protective structure for the midgut microvilli in malarial mosquitoes and is an integral part of the microvillar membrane, appearing as a fuzzy coat on the outer surface of the microvilli (Shen et al. 1999). The glycocalyx found in mosquitoes is rich in carbohydrates, as may be recognized by a variety of lectins. This structure is of potential importance because components of the glycocalyx may serve as receptors or attachment sites for the invasion of pathogens (Shen

et al. 1999). However, no experimental data are available on the molecular composition of the insect midgut glycocalyx.

To date, about 850 species of the suborder Ixodida have been reported (Keirans 1992) and it is thought that most of them form a PM in the midgut. However, the presence of a PM has only been reported in three species, *Ixodes scapularis* (= *Ixodes dammini*) (Rudzinska et al. 1982), *Ixodes ricinus* (Zhu et al. 1991) and *Ornithodoros moubata* (Grandjean 1984). In Japan, *Haemaphysalis longicornis* Neumann is known to be a vector of *Theileria orientalis* (= *Theileria sergenti*), a causative parasite of bovine piroplasmosis (Fujisaki et al. 1993a, 1993b; Kawazu et al. 1999), but PM has not been described in *Haemaphysalis*, the dominant genus tick in Asia. In 1991, Fujisaki et al. suggested the existence of a so-called midgut infection barrier (Hardy et al. 1983) in *H. longicornis* for the control of *T. orientalis*. The *Theileria* and *Babesia* parasites, which are transmitted by ticks, may penetrate protective structures like the PM. These parasites must recognize the surface of the midgut epithelium and then invade it much as malaria parasites do in the midgut of mosquitoes (Huber et al. 1991; Shen et al. 1999). In the present study, morphological observations on the PM in engorged *H. longicornis* females and larvae were carried out in order to clarify the presence of midgut extracellular structures and to understand their importance for digestion and protection against pathogen infection.

Materials and methods

Standard light and electron microscopy

H. longicornis females and larvae of the parthenogenetic Okayama race (Fujisaki et al. 1976) were allowed to complete feeding on the ears of rabbits (SPF; CLEA, Japan). The midgut of engorged females and larvae was removed, fixed with cold 3% glutaraldehyde in sodium cacodylate buffer (pH 7.4) overnight at 4°C, post-fixed with 1% OsO_4 in the same buffer for 2 h after washing thoroughly with the same buffer, dehydrated in an ethanol series and embedded in epon 812 resin (TAAB, UK). Semi-thin sections (approximately 1.5 μm thick) for light microscopy were cut on a Leica UCT ultramicrotome using a diamond knife, and stained with toluidine blue. Thin sections (approximately 80 nm thick) for electron microscopy were cut on the same microtome and doubly stained with uranyl acetate and lead citrate before examination in a Hitachi H-7500 electron microscope. Additionally, semi-thin sections, in which a structure strongly stained with toluidine blue was confirmed by light microscopy, were re-embedded and then observed with the electron microscope.

Confocal laser scanning microscopy using the wheat germ agglutinin (WGA lectin)-fluorescence method

The whole body of an engorged female with the dorsal integument removed was fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH 7.4) and embedded in OCT compound (Tissue-Tek, USA). Frozen sections (approximately 10 μm thick) were cut on a Leica CM 3050 cryostat and put on poly-L-lysine coated glass slides. These sections were incubated in 36 μg/ml biotin labeled WGA lectin (WAKO Chemical, Japan) (Allen et al. 1973; Peters and Latka 1986) with 0.5 M *N*-acetyl-D-glucosamine (GlcNAc) (Peters and Latka 1986; Rudin and Hecker 1989; Tellam and Eisemann 2000) for 2 h at room temperature and visualized by streptavidin labeled Texas Red-X fluorescence (Molecular Probes, USA) with a Leica TSC NT confocal laser scanning microscope. Sections treated with PBS, instead of biotin labeled WGA lectin, were used as negative controls.

Electron microscopy using the WGA lectin-gold method

Thin sections (approximately 80 nm thick) of the female midgut embedded in epon resin were cut on the same ultramicrotome, put on nickel grids and incubated in 10× diluted WGA lectin-gold conjugate (BBI International, UK) with and without 0.5 M GlcNAc overnight at 4°C (after Tellam and Eisemann 2000). The grids were then double stained with uranyl acetate and lead citrate before examination in the same electron microscope.

Results

Standard morphological observation

The PM-like structure was detectable in the midgut of engorged *H. longicornis* larvae by standard electron microscopy (Fig. 1). It was observed on the apical surface of the midgut epithelium, attached closely to the epithelial cells. PM also appeared to fill up the space between the microvilli, so the ectoperitrophic space found in other arthropods was not formed. Since the electron density of the PM was higher than that of the

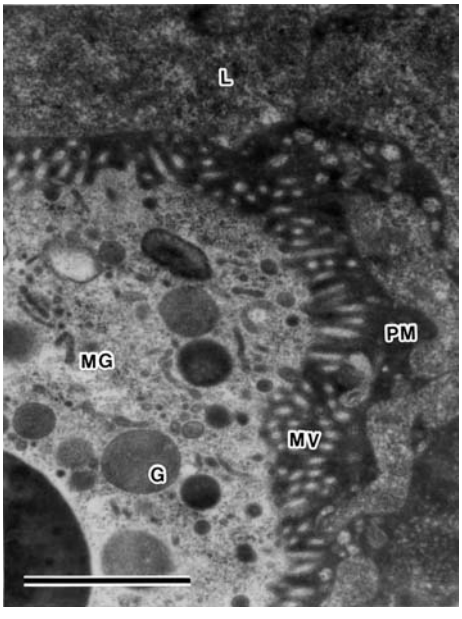


Fig. 1 Electron micrograph showing the peritrophic membrane (*PM*)-like structure on the apical surface of the midgut epithelium (*MG*) in engorged *Haemaphysalis longicornis* larvae. The electron-density of the PM is higher than that of the contents in the midgut lumen (*L*). Note that the PM closely attaches to the epithelium and envelopes the microvilli (*MV*). *G* Granule in the epithelial cell. *Bar* = 2 μm

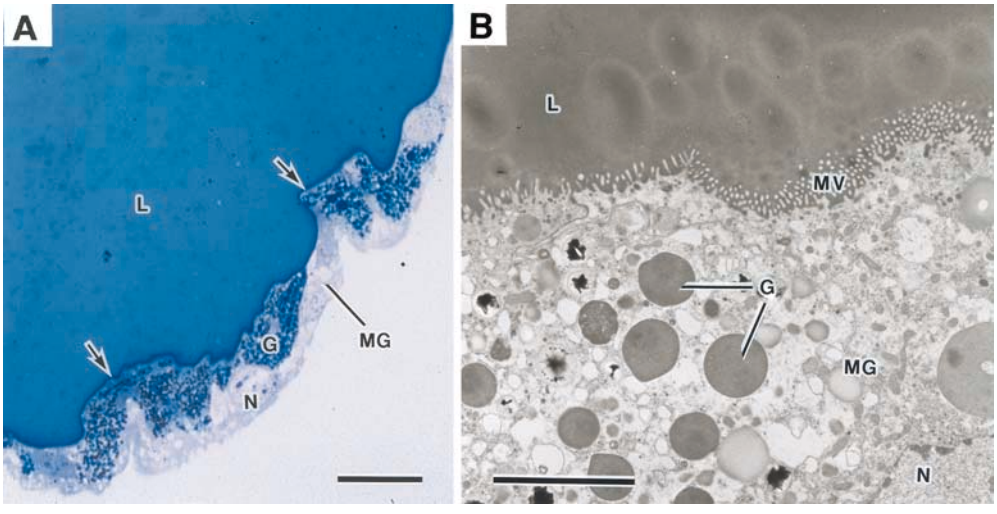
gut lumen contents, an uneven single layer boundary with a thickness of about 0.3–2.5 μm was apparent.

A structure strongly stained with toluidine blue was observed on the apical surface of the midgut epithelium in female *H. longicornis* by light microscopy (Fig. 2a). However, a PM like structure found in larvae was not observed by standard electron microscopy (Fig. 2b). The semi-thin sections in which a structure strongly stained with toluidine blue was confirmed by light microscopy were embedded in epon resin again, and thin sections were cut and re-examined with an electron microscope. Figure 2a and b shows the same region of the midgut, but we could not confirm any structures on the apical surface of the midgut epithelium using only electron microscopy.

Light and electron microscopy with WGA lectin

Frozen sections of the midgut of engorged *H. longicornis* females were incubated with WGA lectin in order to detect glycoproteins, especially chitin of the PM. WGA lectin reacted with the apical surface of the midgut epithelium, the site strongly stained with toluidine blue, and also the basement membrane (Fig. 3a). These reactions were not observed in negative control sections without WGA lectin (Fig. 3b). Furthermore, in electron microscopy with WGA lectin-gold conjugate, WGA lectin clearly reacted with an area away from the apical surface of the midgut epithelium (Fig. 4a). This area was 1.5–3.0 μm in thickness and formed an ectoperitrophic space. However, the structure was indistinguishable from the gut lumen contents without lectin staining, showing that the morphology of this structure in engorged females is obviously different from that of larvae. The structure was PM-like and contained chitin. Non-specific binding to cell organelles of the midgut epithelium was observed when the grids were treated only with the WGA-gold conjugate lacking GlcNAc (Fig. 4b).

Fig. 2A, B Light and electron micrographs showing the midgut of engorged *H. longicornis* females. **A** Light micrograph of a cross section of the midgut. *Arrows* indicate a layer strongly stained with toluidine blue on the apical surface of the midgut. **B** Electron micrograph of the midgut at just the same region as the light micrograph. The layer found in the light micrograph is not apparent on the surface of the epithelium in the lumen (*L*). *G* Granule, *MG* midgut epithelial cell, *MV* microvillus, *N* nucleus. *Bar* **A** 30 μm, **B** 5 μm



Discussion

Extracellular structures like PM and glycocalyx were originally investigated as structures protecting the gut from ingested food (Peters 1992). However, it has been shown recently that these structures also have a protective function from ingested parasites like protozoa. The PM reported in various arthropods apparently functions to protect the midgut epithelium from damage afflicted by hard food such as crystallized blood, and is freely permeable to digestive enzymes and to the products of digestion. There is also discussion as to the significance of the PM as a barrier to the invasion into the midgut epithelium of pathogens for which the arthropod is a vector. Many histochemical and biochemical techniques indicate the presence of chitin, which is characteristic of the PM, although these methods are often adversely influenced by the presence of highly glycosylated proteins, a principal component of the matrix (Tellam and Eisemann 2000; Shao et al. 2001).

The extracellular structure of the midgut in *H. longicornis* females was morphologically different from the PM found as an electron-dense structure in larvae, but was strongly stained with toluidine blue and reacted with WGA lectin. These results indicate that different types of extracellular structure are formed in larval and female *H. longicornis*. Chitin appeared to be present because the structure in *H. longicornis* females was recognized by WGA lectin in the presence of GlcNAc. Various morphologies of the PM have been reported in insects and ticks, and it is widely accepted that the PM is charac-

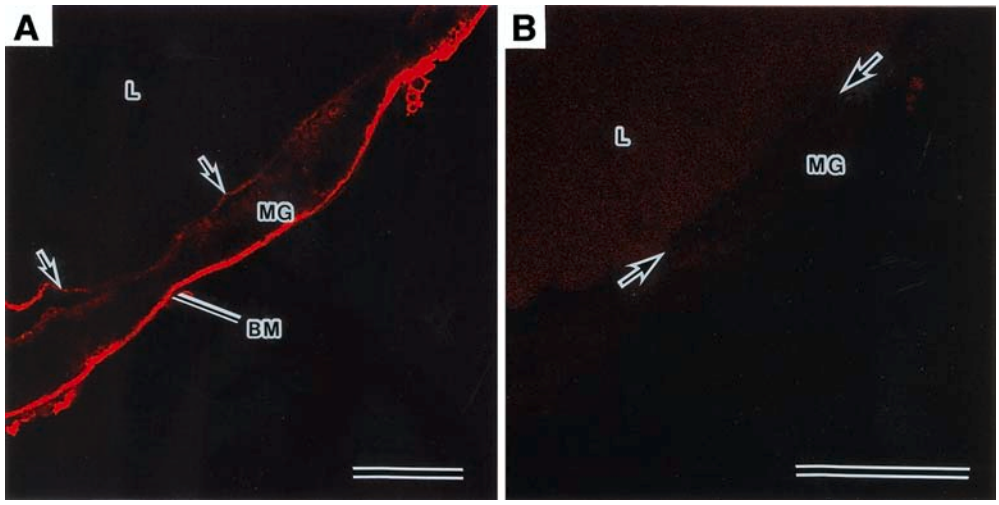


Fig. 3A, B Confocal laser scanning micrographs showing the midgut of engorged *H. longicornis* females stained with WGA lectin and visualized with Texas Red-X fluorescence. **A** The apical surface of the epithelium, where the same region was stained with toluidine blue, is recognized with WGA lectin (*arrows*). The small apical part of the cells and basement membrane (*BM*) of the epithelial cells also reacted. **B** Negative control without WGA lectin. *Arrows* indicate the boundary between the epithelium and the lumen (*L*). *MG*, midgut epithelial cell. *Bars* 30 μm

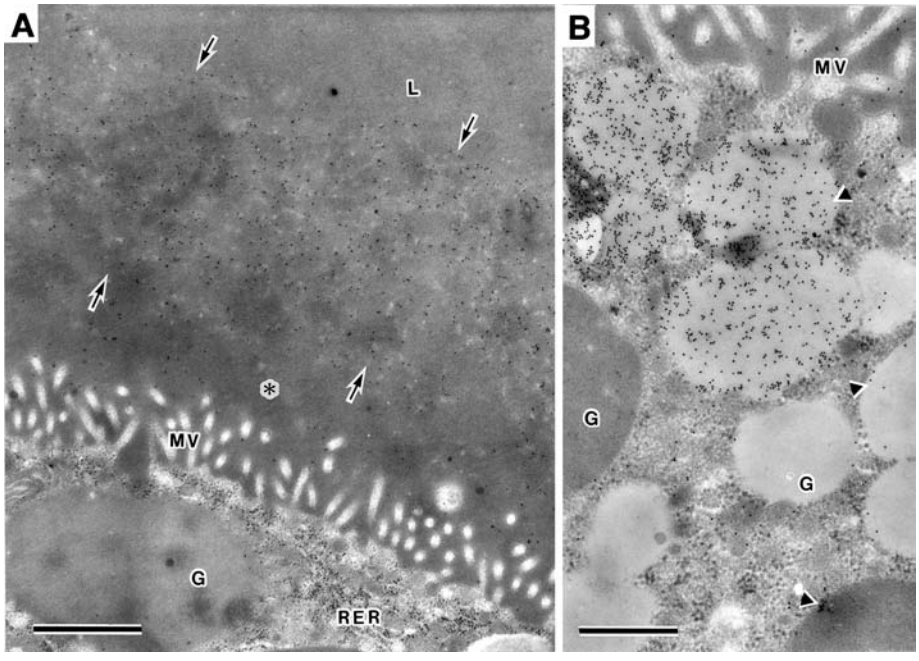


Fig. 4A, B Electron micrographs showing the midgut of adult *H. longicornis* treated with WGA lectin-gold conjugate. **A** Section incubated with WGA lectin and *N*-acetyl-D-glucosamine (GlcNAc). Gold particles occur densely in a limited area of the lumen (*L*) between *arrows facing each other* and sparsely between the epithelium and the stained area (*asterisk*: ectoperitrophic space). No gold particle is observed inside the area (*upper right region*). **B** Section incubated only with WGA lectin. WGA lectin appears to bind non-specific sites of whole area, or different types of granules (*arrowheads*) and cytoplasmic matrix, in epithelial cells without GlcNAc. *MG* Midgut epithelial cell, *MV* microvillus, *G* granule. *Bar***A** 1 μmand **B** 0.5 μm

terized by the presence of chitin (Peters 1992; Lehane 1997). WGA lectin binds to GlcNAc-containing oligosaccharide attached to glycoproteins but has a much greater affinity for the linear oligosaccharides of GlcNAc such as that present in chitin (Allen et al. 1973; Peters and Latka 1986). However, simply examining WGA lectin localization in a tissue cannot differentiate between the lectin binding to chitin or glycoproteins. It has been suggested that WGA lectin can be selectively displaced from oligosaccharides attached to glycoproteins, but not chitin, using high concentrations of GlcNAc (0.2–0.5 M) (Peters and Latka 1986; Rudin and Hecker 1989). This supports our observations of a non-specific reaction to cell organelles in sections incubated only in WGA lectin without GlcNAc, and a specific reaction to the extracellular structures on sections incubated in WGA lectin with GlcNAc. Furthermore, it is known that glycocalyx is the fuzzy coat of the microvilli (Shen et al. 1999). Therefore, the structures in *H. longicornis* females and larvae are not like glycocalyx morphologically, since in females an ectoperitrophic space was found and in larvae the structure was electron-dense and clear. While, the electron density of the PM was lower than that of the lumen contents, an ectoperitrophic space was observed and some layers were observed in the PM of *I. ricinus* (Zhu et al. 1991). However, the layers were not identified in either engorged *H. longicornis* females or larvae. In addition, the structure in females may be similar to "peritrophic gel" (Terra 2001) because it was morphologically fuzzy, but chitin was detected by WGA lectin staining. However, the structure in females is likely to be PM. Further

morphological studies are needed to clarify whether the structure in females is another type of PM or some other structure like the peritrophic gel and glycocalyx.

It is also generally known that ookinetes of *Plasmodium* transmitted by mosquitoes penetrate the PM and invade the epithelia by chitinase. Many studies on mosquito-malaria parasite interactions and the role of ookinete chitinase in the penetration of the PM have been reported (Huber et al. 1991; Billingsley and Rudin 1992; Vinetz et al. 2000). If most vector ticks of *Theirelia* and *Babesia* parasites have a PM in their gut, chitinase may have developed in protozoa transmitted by ticks, allowing them to invade the midgut epithelium. However, there are only a few descriptions on tick midgut-protozoa interactions; penetration of the peritrophic membrane (Rudzinska et al. 1982), invasion into the midgut epithelium (Potgieter et al. 1976; Rudzinska et al. 1983; Agbede et al. 1986) and protozoan development in the tick gut (Potgieter and Els 1977; Zapf and Schein 1994; Higuchi et al. 1999). The coating of glycoproteins has been reported as another extracellular structure of the midgut (Shen et al. 1999). However, microorganisms ingested into the vector's digestive tract must recognize and adhere to the extracellular structures or the apical surface of the gut in order to infect or penetrate the gut epithelium (Yuda et al. 1999; Zieler et al. 1999). It is important that the coating matrix serves as an adhesive site for invasion into the midgut epithelium. The glycocalyx has been reported to be a protective structure of the midgut in malarial mosquitoes (Shen et al. 1999). It is of potential importance because its components may serve as receptors or attachment sites for invading parasites such as *Plasmodium* (Shen et al. 1999).

The present study showed that there are various structures in a vector tick, which may play an important role against microorganisms in the tick midgut. It is interesting that differences are found not only between different species but also between developmental stages of the same species. It is difficult to explain the difference between females and larvae found in the present study. However, *H. longicornis* females oviposit only once after feeding and then die. Thus, the significance of protection against hard food and parasites may be lower than in larvae, which are the first developmental stage. As mentioned above, some extracellular structures play reciprocal roles against parasites as a protective structure or recognition and adherence sites. The structure reported here in female *H. longicornis* is the PM and its structure and function in *H. longicornis* should be investigated in more detailed experiments, such as molecular biological methods, genetic experiments, infection experiments, because the midgut of vectors is the first contact site with a parasite. Thus, an understanding of the interaction at this first barrier is very important, as are studies on other immune systems in vectors.

Acknowledgements This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. This study was supported by a grant from The 21st Century COE Program (A-1) from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan.

References

Agbede RIS, Kemp DH, Hoyfe HMD (1986) *Babesia bovis* infection of secretory cells in the gut of the vector tick *Boophilus microplus*. Int J Parasitol 16:109–114

Allen K, Neuberger A, Sharon N (1973) The purification, composition and specificity of wheatgerm agglutinin. Biochem J 131:155–162

Billingsley PF, Rudin W (1992) The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of *Plasmodium* species. J Parasitol 78:430–440

Evans DA, Ellis DS (1983) Recent observations on the behaviour of certain trypanosomes within their insect hosts. Adv Parasitol 22:1–42

Friedhoff KT (1987) Interaction between parasite and vector. Int J Parasitol 17:587–595

Fujisaki K, Kitaoka S, Morii T (1976) Comparative observations of Japanese ixodid ticks under laboratory conditions. Nat Inst Anim Health Q (Jpn) 16:122–128

Fujisaki K, Kamio T, Kawazu S (1991) *Theileria sergenti* cannot be regarded as the same species as *T. bufferi* and *T. orientalis* because of its transmissibility only by *Kaiseriana* ticks. In: Dusbabeck F, Bukva V (eds) Modern acarology, vol 1. Academia, Prague, pp. 233–237

Fujisaki K, Kamio T, Kawazu S (1993a) Development of *Theileria sergenti* in vector ticks, *Haemaphysalis longicornis*, during blood sucking. Ann Trop Med Parasitol 87:95–97

Fujisaki K, Kamio T, Kawazu S (1993b) *Theileria sergenti*: transformation of zygotes into kinetes in vector ticks, *Haemaphysalis* (*Kaiseriana*) *longicornis*, and *H.* (*K.*) *mageshimaensis*. J Vet Med Sci 55:849–851

Grandjean O (1984) Blood digestion in *Ornithodoros moubata* Murry sensu stricto Walton (Ixodidea: Argasidae) female. I. Biochemical changes in the midgut lumen and ultrastructure of the midgut cell, related to intracellular digestion. Acarologia 25:147–165

Hardy JL, Houk EJ, Kramer LD, Reeves W (1983) Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu Rev Entomol 28:229–262

Higuchi S, Izumitani M, Hoshi H, Kawamura S, Yasuda Y (1999) Development of *Babesia gibsoni* in the midgut of larval tick, *Rhipicephalus sanguineus*. J Vet Med Sci 61: 689–691

Huber M, Cabib E, Miller LH (1991) Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. Proc Nat Acad Sci U S A 88:2807–2810

Kawazu S, Kamio T, Kakuda T, Terada Y, Sugimoto C, Fujisaki K (1999) Phylogenetic relationships of the benign *Theileria* species in cattle and Asian buffalo based on the major piroplasm surface protein (p33/34) gene sequences. Int J Parasitol 29:613–618

Keirans JE (1992) Systematics of the Ixodida (Argasidae, Ixodidae, Nuttalliellidae): an overview and some problems. In: Fivaz BH, Petney TN, Horak IG (eds) Tick vector biology: medical and veterinary aspects. Springer, Berlin Heidelberg New York, pp. 1–21

Laurence BR (1966) Intake and migration of the microfilariae of *Onchocerca volvulus* (Leuckart) in *Simulium damnosum* Theobald. J Helminthol 40:337–342

Lehane MT (1997) Peritrophic membrane structure and function. Annu Rev Entomol 42:525–550

Lehane MT, Allingham PG, Weglicki P (1996) Composition of the peritrophic matrix of the tsetse fly, *Glossina morsitans morsitans*. Cell Tissue Res 283:375–384

Peters W (1992) Zoophysiology, vol 30, Peritrophic membranes. Springer, New York Berlin Heidelberg.

Peters W, Latka I (1986) Electron microscopic localisation of chitin using colloidal gold labeled with wheat germ agglutinin. Histochemistry 84:155–160

Potgieter FT, Els HJ (1977) Light and electron microscopic observations on the development of *Babesia bigemina* in larvae, nymphae and non-replete females of *Boophilus decoloratus*. Onderstepoort J Vet Res 44:213–232

Potgieter FT, Els HJ, van Vuuren S (1976) The fine structure of merozoites of *Babesia bovis* in the gut epithelium of *Boophilus microplus*. Onderstepoort J Vet Res 43:1–10

Rudin W, Hecker H (1989) Lectin-binding sites in the midgut of mosquitoes *Anopheles stephensi* (Liston) and *Aedes aegypti* L. (Diptera: Cullicidae). Parasitol Res 75:268–279

Rudzinska MA, Spielman A, Lewengrub S, Piesman J (1982) Penetration of the peritrophic membrane of the tick by *Babesia microti*. Cell Tissue Res 221:471–481

Rudzinska MA, Lewengrub S, Spielman A, Piesman J (1983) Invasion of *Babesia microti* into epithelial cells of the tick gut. J Protozool 30:338–346

Shao L, Devenport M, Iacobs-Lorena M (2001) The peritrophic matrix of hematophagous insects. Arch Insect Biochem Physiol 47:119–125

Shen Z, Dimopoulos G, Kafatos FC, Jacobs-Lorena M (1999) A cell surface mucin specifically expressed the midgut of the malaria mosquito *Anopheles gambiae*. Proc Nat Acad Sci U S A 96:5610–5615

Tellam RL, Eisemann C (2000) Chitin is only a minor component of the peritrophic matrix from larvae of *Lucilia cuprina*. Insect Biochem Mol Biol 30:1189–1201

Terra WR (2001) The origin and functions of the insect peritrophic membrane and peritrophic gel. Arch Insect Biochem Physiol 47(2): 47–61

Vinetz JM, Valenzuela JG, Specht CA, Aravind L, Langer RC, Ribeiro JMC, Kaslow DC (2000) Chitinases of the avian malaria parasite *Plasmodium gallinaceum*, a class of enzymes necessary for parasite invasion of the mosquito midgut. J Biol Chem 275:10331–10341

Yuda M, Sawai T, Chinzei Y (1999) Structure and expression of an adhesive protein-like molecular of mosquito invasive-stage malaria parasite. J Exp Med 189:1947–1952

Zapf F, Schein E (1994) The development of *Babesia* (*Theileria*) *equi* (Laveran, 1901) in the gut and the haemolymph of the vector ticks, *Hyalomma* species. Parasitol Res 80:297–302

Zhu Z, Gern, L, Aeschlimann A (1991) The peritrophic membrane of *Ixodes ricinus*. Parasitol Res 77:635–641

Zieler H, Nawrocki JP, Shahabuddin M (1999) *Plasmodium gallinaceum* ookinetes adhere specifically to the midgut epithelium of *Aedes aegypti* by interaction with a carbohydrate ligand. J Exp Biol 202:485–495