Fundam. appl. Nematol., 1992, **15** (2), 141-147.

The comparative development of the cuticle of *Romanomermis culicivorax* in susceptible and resistant hosts

Nayna VYAS-PATEL

Department of Agriculture, University of Reading, Earley Gate, P.O. Box 236, Reading, RG6 2AT, UK.

Accepted for publication 26 December 1990.

Summary — The development of *Romanomermis culicivorax* cuticle in the resistant host *Toxorhyncites brevipalpis* was studied ultrastructurally and compared to that in the susceptible host *Aedes aegypti*. In susceptible hosts, reared at 26 °C, the parasitic stage of *R. culicivorax* was characterized by the formation and persistance of a surface coat over the epicuticle, 24-48 hours after host infection. The surface coat was lost 24-48 hours after exit from the host. In the resistant host, *T. brevipalpis*, the surface coat was invaded by capsular material. The defense response of *T. brevipalpis* manifested itself as an initial, strong, humoral reaction, the capsule of which was later surrounded by haemocytes. The changes in cuticular ultrastructure, leading to the observed one parasitic and one post-parasitic moults are described.

Résumé — ***Développement comparé de la cuticule de* Romanomermis culicivorax *chez les hôtes sensibles et résistants*** — Une étude comparative d'ultrastructure a porté sur le développement de la cuticule de *Romanomermis culicivorax* chez un hôte résistant, *Toxorhyncites brevipalpis*, et un hôte sensible, *Aedes aegypti*. Chez l'hôte sensible, élevé à 26 °C, le stade parasite de *R. culicivorax* est caractérisé par la formation et la persistance d'un enduit recouvrant l'épicuticule 24 à 48 h après l'infestation. Cet enduit superficiel disparaît 24 à 48 h après la sortie hors de l'hôte. Chez l'hôte résistant, *T. brevipalpis*, cet enduit est pénétré par des matières capsulaires. La réaction de défense de *T. brevipalpis* se traduit par une réponse initiale hormonale marquée, la capsule étant plus tard entourée par des hémocytes. Les modifications ultrastructurales de la cuticule conduisant à la mue parasitaire et à la mue post-parasitaire observées sont décrites.

Key-words : *Romanomermis*, cuticle, résistance, *Aedes*, *Toxorhyncites*.

The immune response to *Romanomermis culicivorax* has been described ultrastructurally from *Aedes triseriatus* and *Culex territans* (Poinar *et al.*, 1979). However, comparative observations — examining the parasite in susceptible hosts (when the host's immune response was either evaded or not elicited) had yet to be made. Such examinations should differentiate between cellular events following infection in a resistant as opposed to a susceptible host.

This study followed the changing ultrastructure during the general daily development of *R. culicivorax* in the susceptible host *Aedes aegypti* and compared it to the observations found in a known resistant host, *Toxorhyncites brevipalpis* (Vyas-Patel, 1988). Furthermore, as little information existed on the normal cuticular development of post-parasitic *R. culicivorax*, the ultrastructural observations were extended to post-parasitic *R. culicivorax*.

Materials and methods

Second instar *A. aegypti* larvae were infected with *R. culicivorax* (Louisiana strain) at a ratio of 1:5 host to nematode, for 6 hours and reared at 26 °C. Every 24 h, nematodes were dissected from hosts and prepared for observation under the light or transmission electron microscope. Similarly, post-parasites were also examined every 24 h after exit from the host, until they had completed a moult. Nematodes were mounted in lactophenol for light microscopy. For electron microscopy, nematodes were fixed in chilled 1.25 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C, for a few days, transferred to 1 % buffered osmium tetroxide (1 h at 4 °C), dehydrated and embedded in Spurr's resin. Sections were stained in uranyl acetate and lead citrate, and examined, using a Philips transmission electron microscope.

Second instar *T. brevipalpis* larvae were infected in two batches, one at the low 1:5 host to nematode ratio, the other at the high 1:20. At intervals of 5, 10, then every 24 hours after infection, for 6 days, nematodes were dissected from the hosts and processed for examination as described.

Results

CUTICULAR DEVELOPMENT OF *R. CULICIVORAX* IN THE SUSCEPTIBLE HOST *A. AEGYPTI*

Parasitic stage

Days 1-4 post-infection : Figure 1 A (day 1) shows an immature but rapidly developing stage of the nematode. The layers of the cuticle were not well defined, and in keeping with Platzer's observations, the thick and thin

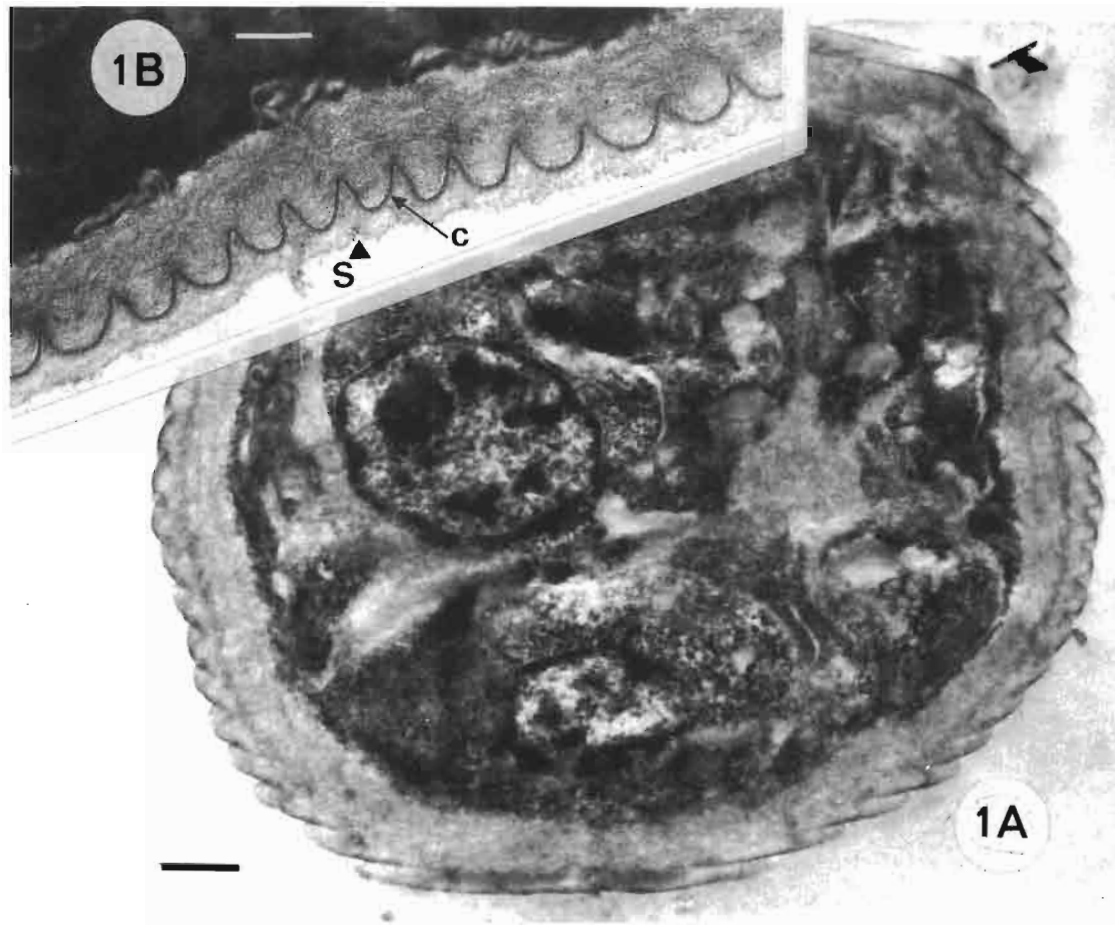

Fig. 1. Parasitic nematode stage from a susceptible host. A : 24 h post infection; surface coat on the cuticle is absent; B : 48 h post infection (S = surface coat, C = nematode epicuticle). *(Bars : A = 7 µm; B = 1.4 µm).*

striations of the cortical zone of pre-parasites were also absent in this study (Platzer & Platzer, 1985). A surface coat over the epicuticle was absent in all the parasitic stages examined on day one.

On day 2 (Fig. 1 B) the epicuticle was seen to be made up of two electron dense layers; pre-parasites have been reported to have three (Platzer & Platzer, 1985). On the surface of the epicuticle was a surface coat, the contours of which closely followed the epicuticle. The cuticular zones were not yet well formed.

By day 3 the zonation of the cuticle was evident as unformed layers. A surface coat was still present. On the 4th day post-infection, a moult appeared to be taking place in a number of the nematodes examined (Fig. 2 A). It was not clear which cuticle layers had been shed, but it seemed that only the outermost layer of the cuticle (probably the old epicuticle) was sloughed off into the surface coat. Four days post-infection, the epicuticle of the new cuticle consisted of two electron dense layers and was well enveloped by the surface coat. Numerous microvillar projections, which have previously been referred to as microvilli (Poinar & Hess, 1977; Platzer & Platzer, 1985) appeared to be extending from the hypodermis (Fig. 2 B).

Days 5-8 post-infection : In most cases, the surface coat appeared to be greatly reduced in thickness on day 5, presumably as a result of the previous moult. From day 5 onwards, the complex structure of the new cuticle layers became more apparent. The fibre layers were similar to those described by Lee (1970) for the mermithid *Mermis nigrescens*. The epicuticle was again only evident as two electron dense layers. Nematodes still parasitic on the 8th day post-infection, showed cuticular profiles similar to day 1 of the post-parasitic stage (Fig. 3 A).

Post-parasitic stage

Days 1-4 of the freeliving juvenile : The surface coating on the epicuticle (Fig. 3 A) was still present soon after the nematode's exit from the host. The interface be-

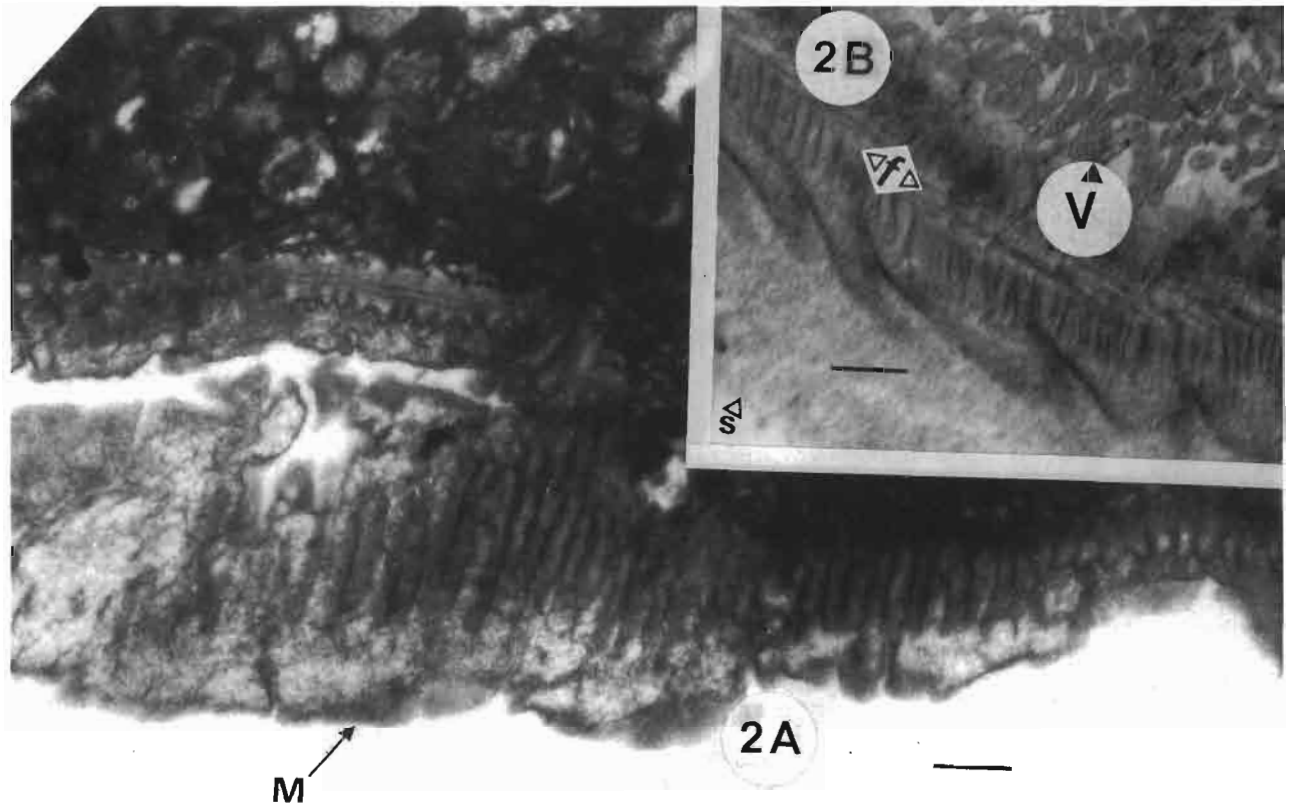

Fig. 2. Parasitic nematode stage from a susceptible host, 96 h post infection. A : Moult (M), note the remains of the epicuticle within the surface coat; B = Microviller extensions (V); fibre layer (f). *(Bars : A = 3.5 µm; B = 10.5 µm).*

tween the hypodermis and the cuticle was the region where the new cuticle was later formed.

By day 2, all of the post-parasites examined had lost their surface coat. The onset of cuticle separation from the hypodermis was evident from the 4th day in most cases.

Days 5-7 of the freeliving juvenile : The old cuticle continued to separate from the nematode. Numerous large, dense, nuclei were evident along all of the hypodermal layer from day 5 (Fig. 3 B). On day 6, the old cuticle was well separated (and is off the edge of Fig. 4); the new cuticle continued to form. In Figure 4, day 6, material appeared to be passing into the newly formed cuticle and could be contributing to formation of the new cuticle. By day 10, most of the nematodes examined were entirely free of the old cuticle and adults.

Light microscopy observations : The parasitic stages of the nematode generally lost their stylet (which is not replaced in adults), between the 4th and 5th days of infection, in keeping with the observations of Gordon *et al.* (1974) and the present EM study. As stylets are cast during moulting, a moult was deemed to have taken place. The onset of moulting in post-parasites, as indicated by the detachment of the anterior cuticular head capsule, was seen in most cases on day 7. The majority of post-parasites had wriggled free of the posterior part of the detached cuticle by days 10-12.

Ultrastructure of melanization in *T. brevipalpis*

Low host infections

A swift (24-48 h), strong, host response occurred, which allowed for little nematode growth, and quite often gave rise to entirely spherical capsules. Solubility tests (Pearce, 1960) on the capsule were positive for melanins. The deposition of electron dense material extended into the cuticular annulations and adhered firmly to the nematode's epicuticular layer, until nematode hypertrophy occurred. If a surface coat had formed

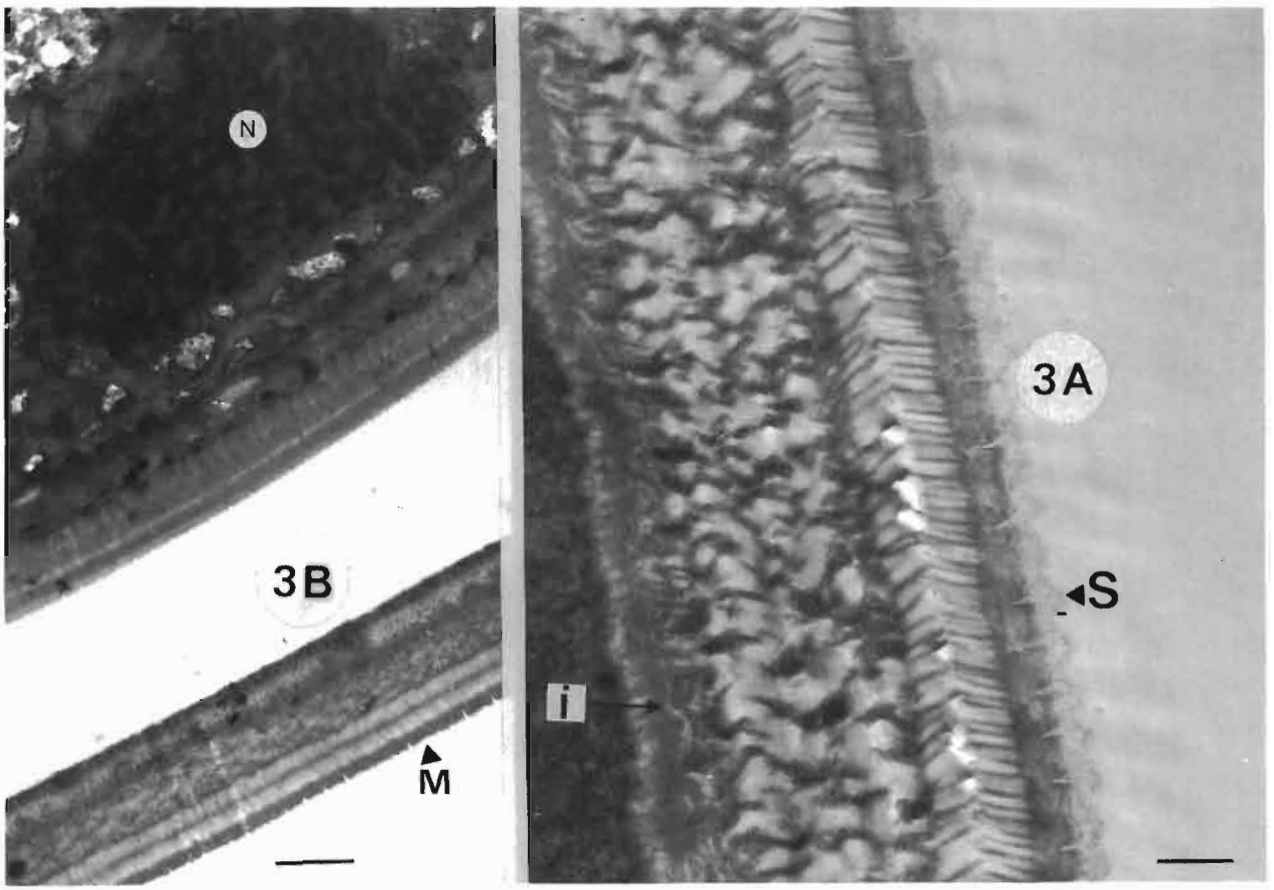

Fig. 3. A : Freeliving nematode stage, 24 h post emergence. (S = surface coat; I = interface where new cuticle forms); B : Freeliving nematode stage, 120 h post emergence. (N = nucleus in the hypodermal layer; M = moulted cuticle). *(Bars : A = 7 µm; B = 2.1 µm).*

prior to the onset of electron dense material deposition, it could not be seen. A grey area, of presumably less deposit of electron dense material, always accompanied the initial, very dark, capsular layer in contact with the nematode cuticle (Fig. 5 A). In many cases, this grey area was in contact with host haemocytes.

High host infections

With a higher parasite burden, the host response was just as swift as in low infections of hosts, but appeared less intense per nematode at the same point in time, presumably as the finite host response was spread over a higher parasite burden. A number of nematodes were longer, probably because they were able to absorb nutrients and grow. The surface coat was clearly visible on day 4 in some nematodes where the deposition of capsular material had yet to commence. In other cases, where the surface coat had formed, the electron dense material was seen to have penetrated through the surface coat to adhere to the nematode epicuticle. The edge of the surface coat is visible within the light deposit of electron dense material in Figure 5 B.

Discussion

The comparison of the development of *R. culicivorax* in susceptible and resistant hosts gave rise to some useful observations, the most interesting being the invasion of the surface coat by the electron dense material, formed in the resistant host *T. brevipalpis*. In susceptible hosts, the surface coat persisted until the exit of the nematode from the host; also, during the parasitic moult, it was present on newly formed cuticle, as well as the old, cast, epicuticle. This meticulous preservation of the surface coat, during the development of *R. culicivorax* in susceptible hosts, and its invasion in resistant hosts, gives rise to many queries on the role of the surface coat in nematode defense.

The resistance of *T. brevipalpis* to *R. culicivorax* was

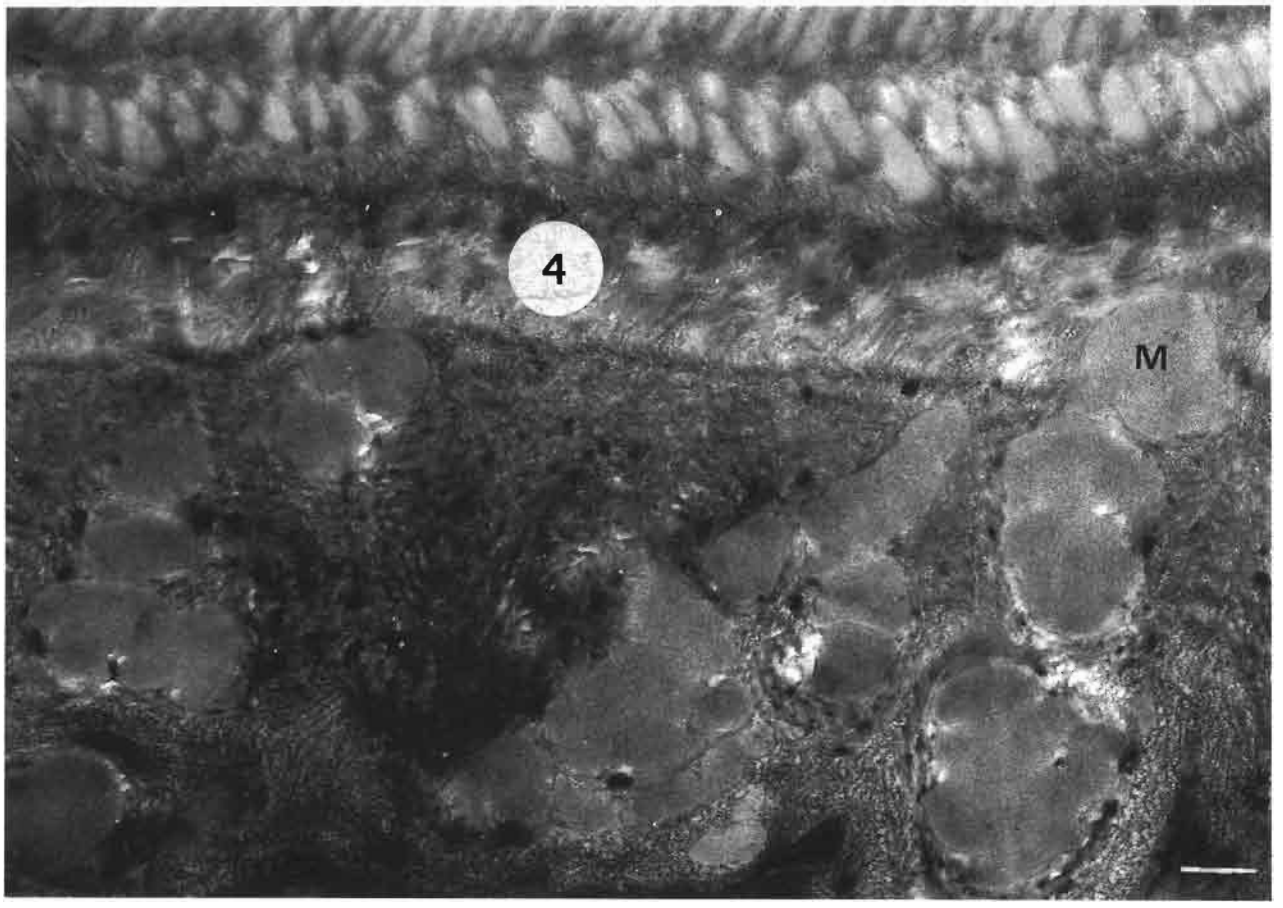

Fig. 4. Freeliving stage, 144 h post emergence (M = cellular material passing into the cuticular layer). *(Bar = 7 μm).*

seen as an initial, strong, humoral response, as indicated by the deposition of very dark, then grey, electron dense material (melanin), which was later surrounded by haemocytes. Such a defense response has been reported from adult mosquitoes to the filarial nematode parasite *Brugia pahangi* (Chen & Laurence, 1985). So far, a humoral response (without haemocyte adherence to the electron dense material) has been reported from *Aedes triseriatus*, and a cellular response from *Culex territans* (Poinar *et al.*, 1979). In the case of cellular encapsulation, the micrographs presented in the report by Poinar *et al.* (1979), illustrated that the surface coat (referred to as the homogenous deposit), remained intact, and was not invaded by the cellular capsule. It would seem that in the case of cellular encapsulation in *C. territans*, haemocytes merely surround (not invade) the surface coat of *R. culicivorax*, without any direct contact with its epicuticle.

Only one moult was observed during the EM study in the parasitic stage of susceptible hosts. The fate of the old, cast cuticle, together with the surface coating material remains unclear. It appeared not to be resorbed by the nematode and it seemed that the susceptible host showed no visible response to its presence in the haemocoel. As one moult alone occurs during the post-parasitic stage (Vyas-Patel, 1989) and one in the egg (Poinar & Otieno, 1974), it would seem that *R. culicivorax* undergoes three moults only during its life cycle.

The composition of the amorphous surface coat needs to be analysed, especially whether it possesses any antigenic properties (Lumsden, 1975). If the presence or absence of the surface coat could be confirmed from the ultrastructural study of nematodes grown *in vitro* (Castillo *et al.*, 1982; Giblin & Platzer, 1987), it would indicate whether this coating is the sole product or a derivative of the nematode, the host, or both.

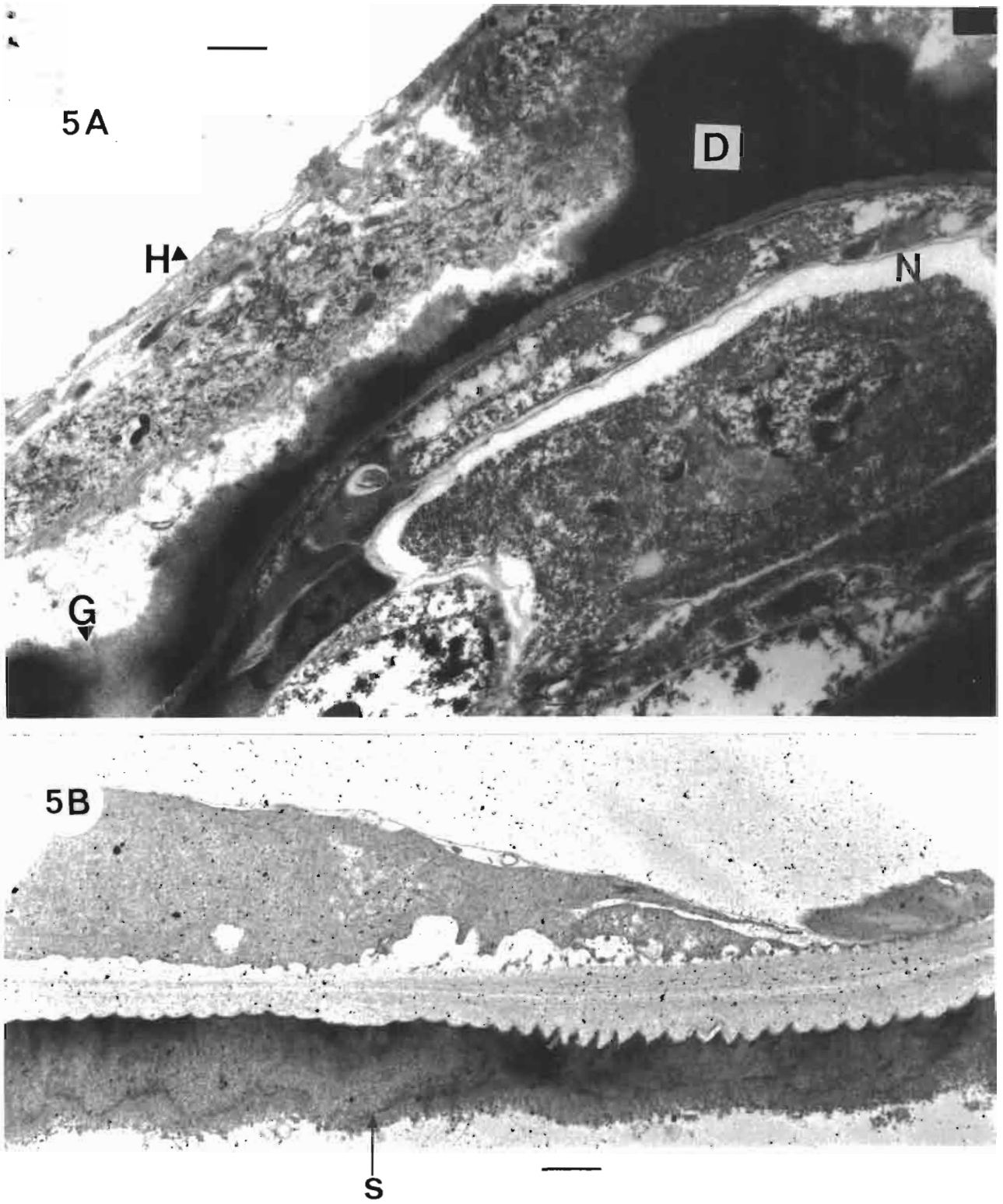

Fig. 5. Nematode from a resistant host, infected at low parasite densities. A : Nematode (N); very dark electron dense layer (D); grey area, of less electron dense material (G); haemocyte (H); B : Surface coat, visible through the electron dense layer (S). *(Bars : A = 3.5 μm; B = 7 μm).*

Acknowledgments

The support of Dr. N. G. M. Hague (University of Reading), and the invaluable help and advice of Prof. R. E. Sinden, Dr. A. F. F. Evans, and Mr. R. Hartley (Imperial College of Science & Technology); and the counsel of Prof. D. L. Lee (University of Leeds) are acknowledged.

References

CASTILLO, J. M., PING CHIN & ROBERTS, D. W. (1982). Growth and development of *Romanomermis culicivorax* in vitro. *J. Nematol.*, 14 : 476-485.

CHEN, C. C. & LAURENCE, B. R. (1985). An ultrastructural study on the encapsulation of microfilaria of *Brugia pahangi* in the haemocoel of *Anopheles quadrimaculatus*. *Int. J. Parasitol.*, 15 : 421-428.

LEE, D. L. (1970). The ultrastructure of the cuticle of adult female *Mermis nigrescens* (Nematoda). *J. Zool.*, 161 : 513-518.

LUMSDEN, R. D. (1975). Surface ultrastructure and cytochemistry of parasitic helminths. *Exp. Parasit.*, 37 : 267-339.

GIBLIN, R. M. & PLATZER, E. G. (1987). Evaluation of culture media for axenic growth of *Romanomermis culicivorax*. *Revue Nématol.*, 10 : 67-74.

GORDON, R., BAILEY, C. H. & BARBER, J. M. (1974). Parasitic development of the mermithid nematode *Reesimermis nielseni* in the larval mosquito *Aedes aegypti*. *Can. J. Zool.*, 52 : 1293-1302.

PEARCE, A. G. E. (1960). *Histochemistry, theoretical and applied*. Boston, USA, Little & Brown, 998 p.

PLATZER, A. & PLATZER, E. G. (1985). Permeability of the body wall of *Romanomermis culicivorax* to lanthium. *J. Nematol.*, 17 : 261-269.

POINAR, G. O., Jr. & HESS, R. (1977). *Romanomermis culicivorax* : morphological evidence of transcuticular uptake. *Exp. Parasit.*, 42 : 27-33.

POINAR, G. O., Jr., HESS, R. T. & PETERSEN, J. J. (1979). Immune responses of mosquitoes against *Romanomermis culicivorax* (Mermithidae : Nematoda). *J. Nematol.*, 11 : 110-115.

POINAR, G. O., Jr. & OTIENO, W. A. (1974). Evidence of four moults in the Mermithidae. *Nematologica*, 20 : 370.

VYAS-PATEL, N. (1988). Parasitism of Kenyan mosquito larvae (Diptera : Culicidae) by *Romanomermis culicivorax* (Nematoda : Mermithidae). *J. Nematol.*, 20 : 96-101.

VYAS-PATEL, N. (1989). Moulting in the post-parasitic stage of *Romanomermis culicivorax*. *J. Invert. Path.*, 53 : 134-136.