Role of the surface coat of *Romanomermis culicivorax* in immune evasion

Muhammad M. SHAMSELDEAN [1,*], Edward G. PLATZER [2] and Randy GAUGLER [3]

[1] *Applied Center for Entomonematodes (ACE), Department of Agricultural Zoology and Nematology, Faculty of Agriculture, Cairo University, El-Gamaa St., Giza 12613, Egypt*
[2] *Department of Nematology, University of California, Riverside, CA 92521, USA*
[3] *Department of Entomology, Rutgers University, New Brunswick, NJ 08901, USA*

Summary – Interactions of the mermithid nematode *Romanomermis culicivorax* with the immune system of mosquito larvae were examined by scanning electron microscopy. The host immune system rapidly recognised invading parasites, as granulocytes and discharged granules were observed attached to parasitic nematodes within 5 min. Melanin deposition was infrequently observed. As a counter measure, the parasites secreted and shed an extracellular surface coat which aided immune evasion. During the first 4 days of infection, when parasite growth was limited, the coat served as a disposable, renewable barrier between parasite and host that was intermittently shed to cleanse the nematode of adhering host immune products. In the later infection phase the parasite grew rapidly and was beyond the effect of the depleted host immune response. The broad host range of *R. culicivorax* within culicines may be partly a function of the nonspecific defence it mounts against the host immune system. In summary, shedding of the surface coat is an adaptive counter response by *R. culicivorax* to the mosquito immune reaction to infection and provides a classic example of host-parasite coevolution.

Keywords – *Anopheles quadrimaculatus*, *Culex pipiens*, Culicidae, haemocytes, Mermithidae, mosquito, Nematoda, scanning electron microscopy.

The mermithid nematode *Romanomermis culicivorax* Ross & Smith is the most extensively studied of nematode species parasitising mosquitoes, an interest driven by the potential of mermithids for vector control. Mosquito-parasitic mermithids, particularly *R. culicivorax*, have been field tested successfully against an array of culicines (Platzer *et al*., 2005). Although eclipsed by the commercial development of *Bacillus thuringiensis* var. *israelensis* as a storage-stable, inexpensively produced, biological insecticide, mosquito mermithids still have a role to play where inoculative rather than inundative biological control is the objective.

The early interest in *R. culicovorax* resulted in part from their wide host range. Whereas aquatic mermithids tend to be highly specific insect endoparasites, often even species specific (*e.g*., *Empidomermis riouxi* for *Aedes detritus*) or genus (*e.g*., *Strelkovimermis peterseni* for *Anopheles*), *R. culcivorax* has a multi-genera host range in the culicines (Petersen, 1975). The immune response of nonpermissive mosquito larvae to *R. culicivorax* parasitism has been documented. A lethal encapsulation has been demonstrated in resistant hosts such as *Mansonia uniformis*, *Aedes triseriatus* and *Culex territans* (Petersen, 1975) larvae. Poinar *et al*. (1979), for example, reported that *C. territans* larvae responded to *R. culicivorax* juveniles by cellular encapsulation accompanied by pigment, presumably melanin, formation, resulting in the death and disintegration of the parasite. Thus, emphasis has rested on the host defensive response in overcoming mermithid parasitism, with less consideration of how the mermithid is able to counter the immune system in permissive hosts.

Our study assessed interactions between the host immune system and *R. culicivorax* in two permissive hosts: *Anopheles quadrimaculatus* and *Culex pipiens*. A SEM approach was used, coupled with a host fracture method,

---

* Corresponding author, e-mail: mshamseldean@hotmail.com
Address for correspondence: *49 Charles de Gaulle St., Shafik Building, Apt. #123, Giza, Egypt.*

to reveal events occurring at the site of nematode interaction with the host immune system.

Materials and methods

EXPERIMENTAL INFECTIONS

Romanomermis culicivorax was maintained on an autogenous strain of *C. pipiens* according to the procedures of Platzer and Stirling (1978). Mosquito larvae were reared in tap water at 27°C on a 3:1 mixture of rabbit chow and brewer's yeast (*C. pipiens*) or a 2:1 mixture of dog biscuit and wheast (yeast grown on milk protein) (*A. quadrimaculatus*). Mosquitoes were infected by placing 100 second-instar larvae and 1000 preparasites (infective juveniles) together in 10 ml of deionised water in a Petri dish (10 × 35 mm), a procedure resulting in the desired 100% parasitism. After 10 min of host-parasite exposure at 24°C, larvae were recovered on a 60 mesh sieve (238 μm pore size), gently rinsed for 2 min with deionised water to remove adhering nematodes, and transferred to fresh deionised water at 27°C. Larvae were collected for fixation at the following intervals after the 10 min exposure: 5 min, 12, 24, 48, 72, 96, 120, 144 and 168 h. All of the 100 host larvae examined were parasitised.
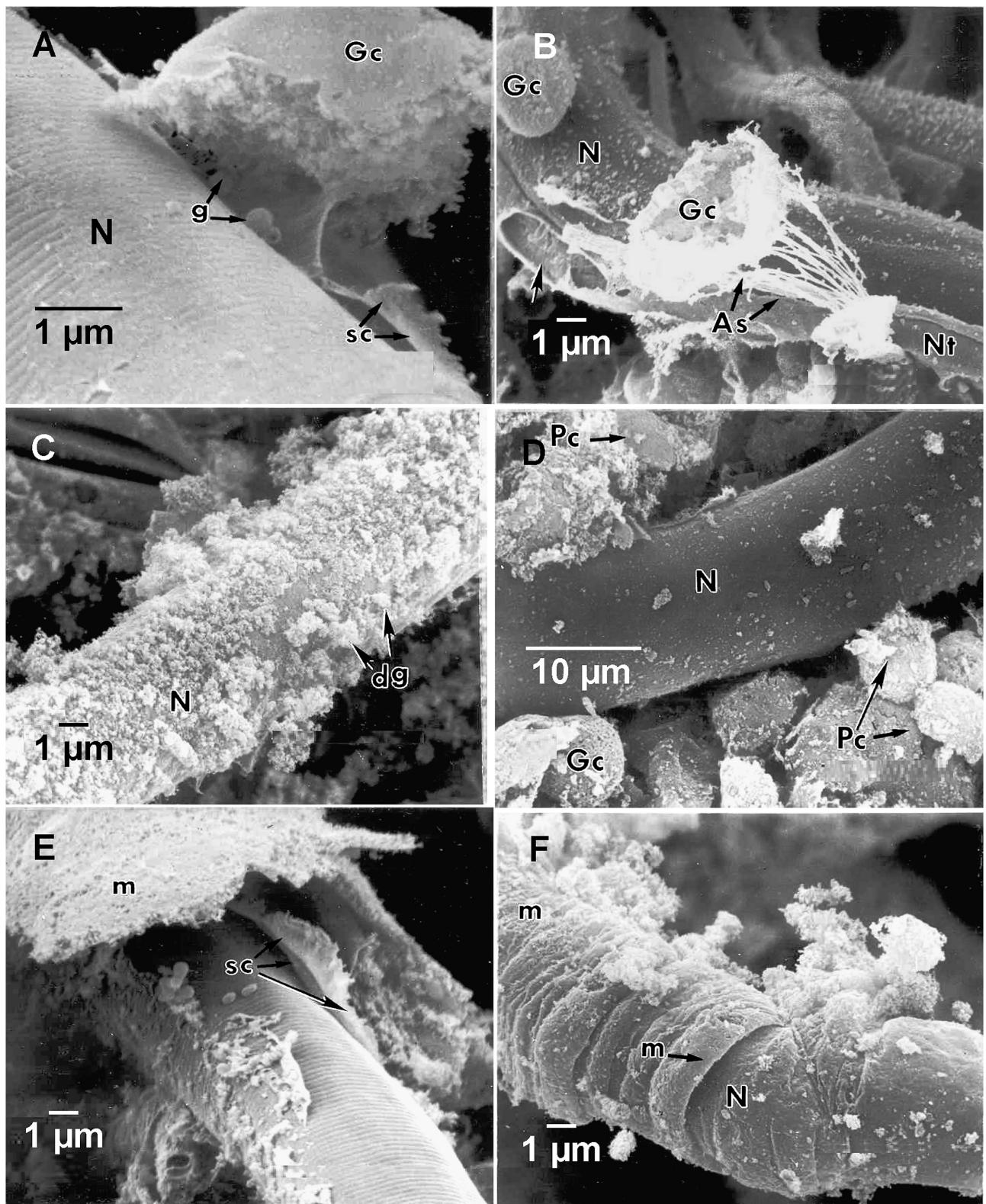
SEM PROCEDURES

Methods for SEM were adapted from Eisenback (1985). Mosquito larvae were collected individually with a pipette, isolated on a 60 mesh sieve, rinsed with deionised water, and transferred to 1 ml of 0.1 M sodium cacodylate (pH 7.4) buffer. Specimens in buffer were added to perforated nylon vials and placed in individual wells of a 24 well tissue culture plate. One ml of 7% glutaraldehyde fixative in 0.1 M sodium cacodylate (pH 7.4) was added to the specimens to yield a final glutaraldehyde concentration of 3.5%. After holding at 5°C for 1 h, the larval

head and siphon were amputated and the torso transferred to fresh 3.5% glutaraldehyde in cacodylate buffer for an additional hour. Specimens were washed in cacodylate buffer and gradually dehydrated in a graded ethanol series (10% increments). Larvae were subsequently treated three times with 100% ethanol for 25 min each, then critical point-dried with carbon dioxide. Dried specimens were mounted on double-sided sticky tape applied to aluminum stubs. After mounting, a sharp insect pin was pressed to the surface of mosquito larvae to fracture the body and expose the nematodes as described by Shams-El-Din and Platzer (1987). Specimens were then sputter-coated with 20 nm gold/palladium alloy and examined with a Jeol JSM-35C SEM at 15 kV. Shape and external morphology were the criteria used to identify granulocytes, granules and plasmatocytes (Hall, 1983; Gupta, 1986). Observations for pigments indicative of melanin deposition were made by light microscopy prior to SEM preparations.

Results

The host immune responses displayed by the two species of mosquito larvae were similar, with both reacting quickly to *R. culicivorax* invasion. After 5 min, 83 (10 out of 12) and 90% (18 out of 20) of nematodes observed in the haemocoel of *A. quadrimaculatus* and *C. pipiens* larvae, respectively, were partially coated with host haemocytes or their products. Some granulocytes attached to the nematodes and were intact, but most lysed upon contact, releasing granules and cellular debris which adhered to the nematode surface (Fig. 1A). Other granulocytes ruptured before direct contact was made, releasing their contents directionally onto the nematodes (Fig. 1B), with adhesive strands linking these depleted granulocytes to their discharged cell products. There was no apparent preference site for haemocyte attachment as blood cells were observed at all points along the nematode body.

Fig. 1. *Scanning electron micrographs of mosquito larvae parasitised by* Romanomermis culicivorax. *A: Partially lysed granulocyte (Gc) of* Culex pipiens *larva discharging granules (g) onto the surface of* R. culicivorax *(N) at 5 min post-infection. Note the presence of the surface coat (sc); B: Depleted* Culex pipiens *granulocyte (Gc) with adhesive strands (As) attached to the tail (Nt) of* R. culicivorax *(N) at 12 h post-infection. Note the adhering granulocyte products on the nematode tail. An intact granulocyte (Gc) is attached to the body of the nematode. A shed tail cast of the surface coat is visible to the left (arrow); C: The surface of a parasitic stage of* R. culicivorax *covered with flocculent host material originating from degenerated granules (dg) of* Anopheles quadrimaculatus *larva; 12 h post-infection; D: Plasmatocyte (Pc) of* Culex pipiens *larva adhering to* R. culicivorax *at 48 h post-infection. Granulocytes (Gc) are also present. The nematode surface coat obscures the cuticular annulations usually visible in a second-stage juvenile of* R. culicivorax; *E: Melanin (m) from an* Anopheles quadrimaculatus *larva overlaying the nematode* R. culicivorax, *5 min post-infection. Shedding of the surface coat (sc); F: Melanin (M) from* Anopheles quadrimaculatus *larva encapsulating* R. culicivorax *(N) at 24 h post-infection. The melanin layer conforms to the annulations of the nematode body.*

A — Gc, N, g, sc, 1 µm

B — Gc, N, Gc, As, Nt, 1 µm

C — N, dg, 1 µm

D — Pc, N, Gc, Pc, 10 µm

E — m, sc, 1 µm

F — m, m, N, 1 µm

As the discharged granules degenerated, a sticky layer of flocculant material surrounded the nematodes, covering the parasites entirely in a progressively thicker coating of host immune debris (Fig. 1C). Plasmatocytes also attacked the nematodes (Fig. 1D). Melanin deposition was infrequently observed and only in *A. quadrimaculatus* – one at 24 h and a second at 72 h of 73 parasites examined, or 2.74%. This was the only difference in host immune response or parasite counter response noted with the two host species. When present, melanin began to form within 5 min of parasite entry (Fig. 1E) and could completely enclose the nematode in a dense capsule within 24 h (Fig. 1F). Despite the host immune response, parasite growth proceeded normally as evidence of moulting (Fig. 2A) was noted at 72 and 98 h post-infection, as well as the appearance of pits (Fig. 2B) on the ventral nematode cuticle which are associated with development.

A nematode external envelope or surface coat, distinct from the epicuticle, was detected throughout the infection period including at the first observation after 5 min (Fig. 1E). The amorphous surface coat overlaid the entire nematode cuticle obscuring the annulations (Figs 1D, 2C). A loose attachment of the coat to the nematode cuticle was apparent from examining a discarded moulted cuticle (Fig. 2A). Sloughing-off or shedding of the surface coat (Figs 2D, E, F) was observed throughout parasitism including at the first observation (Fig. 1E). The surface coat was shed gradually as irregularly-sized and shaped fragments into the host haemocoel (Fig. 2C, D, E). At high magnification, texture and numerous perforations of varied dimension became apparent (Fig. 2F). Shedding of the surface coat was associated with continuous nematode forward migration in the host haemocoel.

The *R. culicivorax* surface coat was invariably recognised and attacked by the mosquito immune system. Shedding of the surface coat, however, repeatedly removed adhering haem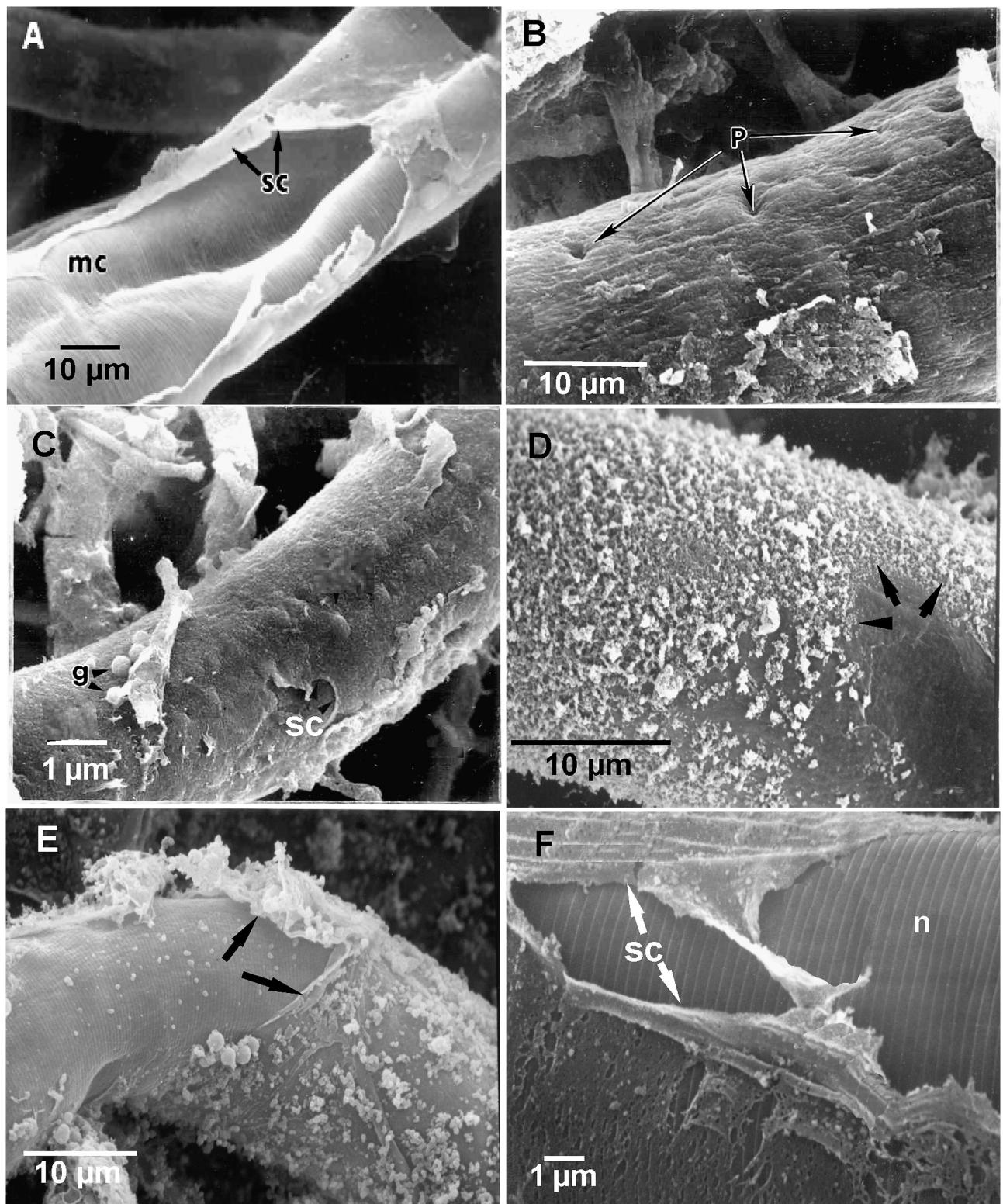ocytes, granules, and other host immune products, effectively cleaning the nematode of host immune products. This point is effectively illustrated where nematodes otherwise covered with attached host immune products have shed small flakes (Fig. 2D) or large areas (Fig. 2E) of the surface coat, exposing underlying epicuticles virtually free of immune particulates. Although coat shedding freed parasites of host products, immune attacks continued, resulting in a recurrent cycle of attachment and shedding. Throughout the first 4-5 days of the study, parasites could be found in parallel that were free of host materials, with various thicknesses of encapsulation or in the process of surface coat shedding. Parasites where encapsulation was advanced, especially those with substantial melanin deposition (Fig. 1F), were unable to shed the attacking cells and were eventually killed.

Minor nematode growth was observed during the first 3-4 days of parasitism, but growth proceeded swiftly after the moult to the third-stage juvenile. As growth progressed, the nematode lost the distinct cuticular annulations of the previous stage and displayed an increasingly smooth cuticle surface. An immune response continued to be elicited, but was greatly diminished so that in late infections the nematodes were free of adhering host immune debris or only traces were found. In contrast to the constant movement and migration of the second-stage juvenile, third-stage juveniles soon became nearly sedentary until host emergence on day 7 or 8. The surface coat was discernable throughout the parasitic phase.

Discussion

The response of the mosquito immune system to mermithid parasitism appears unremarkable, proceeding in a predictable sequence of events. What is remarkable is that this immune sequence is repeatedly interrupted and resumed in a persistent cycle of host response-parasite counter response.

Fig. 2. *Scanning electron micrographs of mosquito larvae parasitised by* Romanomermis culicivorax. *A: Moulted cuticle (MC) of* R. culicivorax *with remnants of surface coat (sc) from* Anopheles quadrimaculatus *larva; B: The ventral surface of* R. culicivorax *in* Culex pipiens *larva showing the appearance of pits (P) on the ventral cuticle at 72 h post-infection; C:* R. culicivorax *in* A. quadrimaculatus *larva at 72 h post-penetration with surface coat (sc) enveloping the nematode. A small flake of surface coat has been shed (arrowheads). Adhering host immune material includes discharged granules (g); D:* R. culicivorax *from an* A. quadrimaculatus *larva at 98 h post-infection showing adhering host immune debris. A small patch of the surface coat has been shed (coat edges indicated by arrows), clearing immune products away to show the underlying nematode epicuticle; E:* R. culicivorax *from an* A. quadrimaculatus *larva at 24 h post-infection showing adhering host immune debris. A large fragment of the surface coat has been shed (coat edges indicated by arrows), clearing immune products away to show the underlying nematode epicuticle; F:* Romanomermis culicivorax *(n) from a* C. pipiens *larva at 12 h post-infection showing partial shedding of the surface coat (sc) layer. Numerous perforations in the surface coat are apparent.*

The *R. culicivorax* surface coat provides a means of overcoming the mosquito immune defences. Shedding of the surface coat cleans the parasite of adhering immune products. Thus, the *R. culicivorax* surface coat serves as a disposable but renewable physical barrier between parasite and host similar to that described for the zooparasitic nematode *Trichinella spiralis* (Modha *et al*., 1999). The discarded sheds, coupled with *R. culicvorax* migration away from the sheds, presumably also diverts and inactivates additional mosquito haemocytes and so are analogous to the flares military aircraft discharge to divert attacking missiles. Secretion and shedding may explain slow nematode growth during the first 4 days of parasitism (Gordon *et al*., 1974); parasite resources are diverted to surface coat production. Energy costs associated with migration, as well as some interference with transcuticular uptake of nutrients resulting from the surface coat barrier, could be contributing factors. In the final phase of infection when parasite growth surges, the limited number of blood cells mosquitoes possess (Hall, 1983) may be reduced from days of unrelenting demand, explaining the dearth of attacking haemocytes during late infection. The reduced host immune response permits parasite resources to be redeployed from coat production to growth. The parasite grows rapidly thereafter (Gordon *et al*., 1974), increasing hundreds of times in size as development progresses, and is quickly beyond the effect of the depleted host immune response.

Although it has been suggested that the observed envelope is derived from the host (Poinar *et al*., 1979), our results indicate that this layer is a nematode secretion. In non-infected mosquitoes, granulocytes and plasmatocytes were intact and no evidence of attachment, lysis, or interaction with other host tissues was observed. In infected mosquitoes, these cells attached or degranulated on the secretion layer in a clear response to foreign materials. Studies with immunofluorescent techniques provide further evidence for the nematode origin of the secretion or surface coat in *R. culicivorax* (Platzer, 1989; Platzer *et al*., 1992). Prior to infection, binding of concanavalin A was restricted to the mouth, amphids, and head of infective juveniles. By contrast, binding was intense over the entire surface of juveniles recovered from the haemocoel of *C. pipiens* within 15 min post-infection. Exposure of infective juveniles to isosmotic saline to simulate entrance into the host haemocoel resulted in an equivalent secretion of concanavalin A glycoprotein. In unpublished studies (E.G. Platzer) with polyclonal antibodies produced in mice immunised with infective

juveniles, the binding pattern of mouse antibodies as detected with fluorescent labelled anti-mouse antibodies was equivalent to that of concanavalin A; *i.e*., there was some binding to the mouth, amphids, and head of infective juveniles, whilst binding post-infection was extensive over the entire surface of the juveniles. Thus, the lectin and nematode-specific antibody binding bolster our SEM observations of a surface coat originating from the juvenile nematodes.

The spores of some nematophagous fungi attach exclusively to sensory openings and the vulva of *Caenorhabditis elegans* (Jansson, 1994), suggesting the surface coat renders the nematode biochemically invisible to sensory or adhesive products of parasites. In our study, the surface coat was immediately recognised by the immune system with attachment of haemocytes occurring at all points along the *R. culicivorax* body, demonstrating that the surface coat is recognised as an immunogen.

Encapsulation kills invading nematodes by blocking the transfer of oxygen and nutrients (Salt, 1970). Thus, the surface coat must be permeable, even in the early stages of parasitism, while concurrently fulfilling its key function of anti-immune barrier. The perforations we observed are sufficient in size and number to facilitate gas exchange and the uptake of large molecular weight nutrients, yet are small enough not to interfere with cleansing the cuticle of immune particulates. In addition, the perforations assist in tearing and, therefore, shedding of the fragile envelope much as the perforations in disposable towels ease tearing and removal. It is unclear whether the coat is synthesised with the pores or whether pores are opened soon after by enzymatic or other action.

Surface coats have been described from free-living, plant-parasitic and animal-parasitic nematodes (Blaxter *et al*., 1992). Ours is the first report from an insect-parasitic taxon. Poinar *et al*. (1979) visualised a "thin homogenous deposit surrounding the cuticle" of *R. culicivorax* (see Fig. 7 in Poinar *et al*., 1979) but noted a "similarity" to host tissue. Shamseldean and Platzer (1989) correctly identified an "outer coat" in parasitic stage *R. culicivorax* but did not comment further. Wang and Gaugler (1999) extracted presumed surface coat proteins from *Steinernema glaseri* that suppressed melanotic encapsulation in scarabaeid hosts, but the surface coat was not visualised or suggested to serve as a physical barrier against immunocytes. By contrast, the present study indicates the surface coat acts in *R. culicivorax* not by suppression of the host humoral defences but by immune evasion. Further evidence is provided by Hall *et al*. (1975) in showing that

Steinernema carpocapsae-infective juveniles were encapsulated with melanin by *Aedes aegypti* larvae previously infected with *R. culicivorax*. Thus, *R. culicivorax* parasitism did not provide co-protection for a second, non-adapted parasite species, demonstrating that *R. culicivorax* does not provide a general immunosuppression but rather that the coat is inert and acts at the cuticle surface of individual nematodes.

Mosquitoes are capable of mounting strong humoral and cellular immune responses against foreign organisms. Yet over 90 species of mosquitoes in 13 genera are susceptible to infection with *R. culicivorax* (Petersen, 1985; Peng *et al*., 1992), reflecting a relative non-specificity for mosquito hosts. Only a few species are able to inhibit the development of *R. culicivorax* by encapsulation (Petersen, 1985). The non-specific host range of *R. culcivorax* may be a function of the simple non-specific defence it mounts against the host immune system. The resistance of some mosquito larvae is likely to be based in part on non-immune defences; for example, *Aedes triseriatus* resistance to *R. culicivorax* is based on physical or behavioural as well as physiological factors (Petersen, 1975). In general, relatively inactive species are highly susceptible, whereas species displaying a high degree of physical activity show low levels of parasitism. Further investigations will be required to separate the comparative impact of behavioural, physical, and physiological resistance in permissive and nonpermissive hosts.

Shedding of the surface coat is an adaptive counter-response by *R. culicivorax* to the mosquito immune response to infection and provides a classic example of host-parasite coevolution. Additional studies are needed to determine whether the resulting immune evasion is an anti-immune strategy deployed as a general feature throughout the Mermithidae or whether host-specific mermithids may offer more complex systems that include immunosuppression. Consider, for example, *Empidomeris* sp. parasitisng the adult snowpool mosquito, *Aedes stimulans* (Gaugler *et al*., 1981). Penetrating juveniles are encapsulated by larval hosts unless they migrate immediately to the brain, a refugia from haemocytes. When the host pupates, the parasite moves back into the abdomen but no longer elicits an immune response. This suggests *Empidomeris* changes rather than sheds its coat by acquiring host antigenic epitopes that permit molecular mimicry. Thus the mermithid surface coat may play a determinate role in one of the most important of all biological control parameters: breadth of host range.

References

BLAXTER, M.L., PAGE, A.P., RUDIN, W. & MAIZELS, R.M. (1992). Nematode surface coats: actively evading immunity. *Parasitology Today* 8, 243-247.

EISENBACK, J.D. (1985). Techniques for preparing nematodes for scanning electron microscopy. In: Sasser, J.N. & Carter, C.C. (Eds). *An advanced treatise on* Melodoigyne, *Vol. II: Methodology*. Raleigh, NC, USA, North Carolina State University Graphics and USAID, pp. 79-105.

GAUGLER, R., WRAIGHT, S. & MOLLOY, D. (1981). Bionomics of a mermithid parasitizing snow-pool *Aedes* spp. mosquitoes. *Canadian Journal of Zoology* 62, 670-674.

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

GUPTA, A.P. (1986). Arthropod immunocytes: identification, structure, functions, and analogies to the functions of vertebrate B- and T-lymphocytes. In: Gupta, A.P. (Ed.). *Hemocytic and humoral immunity in arthropods.* New York, USA, John Wiley & Sons, pp. 3-60.

HALL, D.W. (1983). Mosquito hemocytes: a review. *Developmental and Comparative Immunology* 7, 1-12.

HALL, D.W., ANDREADIS, T.G., FLANAGAN, T.R. & KACZOR, W.J. (1975). Melanotic encapsulation of the nematode *Neoaplectana carpocapsae* by *Aedes aegypti* larvae concurrently parasitized by the nematode *Reesimermis nielseni*. *Journal of Invertebrate Pathology* 26, 269-270.

JANSSON, H.-B. (1994). Adhesion of conidia of *Drechmeria coniospora* to *Caenorhabditis elegans* wild type and mutants. *Journal of Nematology* 26, 430-435.

MODHA, J., ROBERTS, M.C., ROBERTSON, W.M., SWEETMAN, G., POWELL, K.A., KENNEDY, M.W. & KUSEL, J.R. (1999). The surface coat of infective larvae of *Trichinella spiralis*. *Parasitology* 118, 509-522.

PENG, Y., PLATZER, E.G., SONG, J. & PELOQUIN, J. (1992). Susceptibility of thirty-one species of mosquitoes to *Romanomermis yunanensis*. *Journal of West China University of Medical Sciences* 23, 412-415.

PETERSEN, J.J. (1975). Penetration and development of the mermithid nematode *Reesimermis nielseni* in eighteen species of mosquitoes. *Journal of Nematology* 7, 207-210.

PETERSEN, J.J. (1985). Nematodes as biological control agents: part I. Mermithidae. *Advances in Parasitology* 24, 307-346.

PLATZER, E.G. (1989). Lectin binding on juveniles of *Romanomermis* sp. *Journal of Nematology* 21, 580. [Abstr.]

PLATZER, E.G. & STIRLING, A.M. (1978). Improved rearing procedures for *Romanomermis culicivorax*. *Proceedings of the California Mosquito and Vector Control Association* 46, 87-88.

PLATZER, E.G., RYAN, E.G. & SHIH, J.J.M. (1992). Host invasion in an entomopathogenic nematode. *American Zoologist* 32, 127A. [Abstr.]

PLATZER, E.G., MULLENS, B. & SHAMSELDEAN, M.M. (2005). Mermithid nematodes. In: Grewal, P., Ehlers, R. & Shapiro-Ilan, D. (Eds). *Nematodes as biological control agents.* Wallingford, UK, CABI Publishing, pp. 411-418.

POINAR JR, G.O., HESS, R.T. & PETERSEN, J.J. (1979). Immune responses of mosquitoes against *Romanomermis culicivorax* (Mermithidae: Nematoda). *Journal of Nematology* 11, 110-116.

SALT, G. (1970). *The cellular defence reactions of insects.* Cambridge, UK, Cambridge University Press, 118 pp.

SHAMS-EL-DIN, M.M. & PLATZER, E.G. (1987). A simple technique for the study of mermithid cuticle by scanning electron microscopy. *Journal of Nematology* 19, 557. [Abstr.]

SHAMSELDEAN, M.M. & PLATZER, E.G. (1989). *Romanomermis culicivorax*: penetration of larval mosquitoes. *Journal of Invertebrate Pathology* 54, 191-199.

WANG, Y. & GAUGLER, R. (1999). *Steinernema glaseri* surface coat protein suppresses the immune response of Japanese beetle larvae. *Biological Control* 14, 45-50.