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ORIGINAL PAPER

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Ultrastructure of the surface structures and electron immunogold labeling of peptide immunoreactivity in the nervous system of *Pseudothoracocotyla indica* (Polyopisthocotylea: Monogenea)

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Abstract Transmission electron microscope studies of the tegument of the tropical marine fish monogenean parasite *Pseudothoracocotyla indica* describe surface specialisations and detail the ultrastructure of the tegument and the haptor. The tegument consists of a syncytium, numerous electron-dense granules, electron-lucent vesicles and large multivesicular bodies. The posterior tegumental syncytium is infolded to form tegumental ridges that are present on both the ventral and dorsal surfaces. A thin coat of glycocalyx is present on the tegument surface. In contrast, the tegumental syncytium of the haptor is relatively thin, containing electron-dense granules and various-sized electron-lucent vesicles. Exocytosis of the electron-dense and electron-lucent vesicles apparently occurs in the syncytium of the haptor and general body surface. Tegumental damage was observed on the dorsal surface in the mid-body region and may possibly have been due to natural mechanical forces. The haptor consists of electron-dense clamp sclerites embedded within a matrix covered by the tegumental syncytium. The sclerites are connected to each other and to the basal lamina by radially oriented muscle fibres. The haptor is richly supplied with non-myelinated nerve axons. Both uniciliated and non-ciliated presumed sensory structures are present on the body surface and haptor. Uniciliated sensory structures were found mainly around the oral sucker. Groups of neurons and nerve processes containing neurosecretory vesicles were frequently observed in the vicinity of the clamps. Electron immunogold labeling studies demonstrated that neuropeptide F [NPF (*Moniezia expansa*)] immunoreactivity was confined to electron-dense-cored neurosecretory vesicles in nerve fibres from the posterior and haptor regions of the fluke.

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Introduction

Monogeneans are amongst the most common and abundant ectoparasites of fish, occurring mainly on the skin and gills, although a few genera may be found as endoparasites in the bloodstream, the urinary system, the body cavity and the gut of fish and other vertebrates. Within their natural hosts, monogeneans generally occur in relatively low numbers and present few, if any, host problems (Ramasamy et al. 1985; Ramasamy and Ramalingam 1989; Rohde 1993); however, in conditions where the hosts are maintained in culture, monogeneans have been shown to be pathogenic, resulting in economic loss (Paperna et al. 1984; Schmidt and Roberts 1989). Monogeneans are mainly ectoparasitic and as such they require an efficient mechanism of attachment to the host. This attachment is normally achieved via a well-developed, highly complex adhesive organ called a haptor. This specialised organ readily distinguishes the monogeneans from other groups of parasitic helminths. The haptor is posteriorly situated and consists of clamps and anchor hooks. The clamps vary in size, shape and number. The variation in the morphology and mode of action of the haptor can be related to the topography of the parasite's selected habitat. The morphology of the haptor, with respect to the host species, is of great importance and is probably a central factor in host and site specificity exhibited by the monogeneans.

Little information is available regarding the functions of the monogenean tegument as compared with that reported for the digenean tegument (Smyth and Halton 1983). Indeed, descriptions of the tegument are mostly restricted to scanning electron microscope (SEM) studies (Ramasamy and Hanna 1985, 1986a, b, 1989), with only a few transmission electron microscope studies (TEM) involving *Allodiscocotyla diacanthi* (Ramasamy et al. 1995), *Gotocotyla bivaginalis* (Ramasamy and Bhu-

vaneswari 1993), *Pricea multae* and *Vallisia indica* (Ramasamy et al. 1986, 1987) and *Diclidophora merlangi* (Halton 1979). The ultrastructural evidence available to date suggests that the monogenean tegument is morphologically similar to that of the digeneans and is thought to be secretory with an absorptive potential. An osmoregulatory function and a role in excretion has also been suggested, and since most monogeneans are ectoparasites and probably respire aerobically, the tegument may also have an important role in respiratory exchange (Lyons 1973).

Few immunocytochemistry (ICC) studies have been performed on monogeneans. Using ICC procedures on *D. merlangi*, Halton et al. (1990, 1994) have revealed that the clamps are richly innervated by peptidergic fibres derived from ganglionic masses in the haptor. Electron immunogold labeling studies demonstrated the presence of pancreatic polypeptide (PP), neuropeptide F (NPF) and FMRFamide immunoreactivity in dense-cored neurosecretory vesicles in the nervous system of *D. merlangi* (Halton et al. 1991; Brennan et al. 1993a).

Pseudothoracocotyla indica is a fish-gill parasite of the marine fish *Scomberomorus* species (mackerel). Rohde (1976, 1979) reviewed their site specificity, geographical distribution and morphology and showed that *P. indica* is site-specific, the common niché for these flukes being on the basal region of the outer gill filaments, mostly on the first gill and less frequently on the second gill, of both *S. commersoni* and *S. queenslandicus*.

The most recently documented research on *P. indica* was that of Ramasamy and Hanna (1985) detailing the surface topography of the body, the haptor and the clamps using SEM. They also examined the attitude and effects of reattachment of *P. indica* on the body surface of co-habiting *P. multae* and observed that reattachment of *P. indica* did occur under in vitro conditions, suggesting that it has the potential for reattachment if displaced from its primary position.

In the present study on *P. indica*, TEM was used to investigate and describe the structure and function of the surface tegument and associated sensory structures and the organs of attachment. Electron immunogold labeling (IgG) was employed to demonstrate the occurrence, distribution and localization of neuropeptides within the nervous system.

Materials and methods

Adult specimens of *Pseudothoracocotyla indica* were obtained from the gills of locally caught *Scomberomorus commersoni* (Spanish mackerel) landed at Marina beach, Madras, India, by local fishermen. The flukes were washed thoroughly in natural seawater to remove any fish-gill mucus prior to fixation. For general ultrastructural observations, standard TEM procedures were carried out following those described by Ramasamy et al. (1995) for marine flatworms.

For the sub-cellular localization of neuropeptide immunoreactivity (IR), flukes were fixed in 2% double-distilled glutaraldehyde (DDG) in 0.1 *M* cacodylate buffer (pH 7.2) containing 3% sucrose and 0.5% NaCl at 4°C for 1 h. Fixed specimens were then buffer-washed, dehydrated through graded ethanols and infiltrated and embedded in Epon resin at 60°C for 48 h. Sections (60–70 nm in thickness) were cut on a Reichert Ultracut E ultramicrotome and collected on bare 200-mesh nickel grids. After drying, the sections were rinsed with 20 m*M* TRIS/HCl buffer (pH 8.2) containing 0.1% bovine serum albumin (BSA) and Tween 20 (1:40 dilution) and were incubated first with normal goat serum for 30 min at room temperature (to block non-specific proteins) and then with the primary antibody NPF 792(3) diluted to 1:5,000, 1:10,000 and 1:20,000 with TRIS/BSA for 18 h at room temperature. Sections were subsequently washed in TRIS/BSA and transferred to a 25-µl droplet of 10-nm-size gold-conjugated goat anti-rabbit IgG for 1 h at room temperature. The sections were lightly fixed with 2% DDG, washed in buffer and rinsed in distilled water. Finally, sections were double-stained with uranyl acetate (8 min) and lead citrate (6 min) and examined in a JEOL 100CX transmission electron microscope at 100 kV. Controls consisted of (1) use of non-immune rabbit serum in place of the primary antiserum, (2) omission of the primary antiserum and (3) pre-absorption of the primary antiserum with NPF standard (200–500 ng/100 µl distilled antiserum).

Results

General morphology

The general morphology of *Pseudothoracocotyla indica* Unnithan (1956) is illustrated in Fig. 1.

Transmission electron microscopy

Ultrastructure of the tegument

Ultrastructural examination of the dorsal and ventral surface teguments revealed the tegumental syncytium to be delimited by apical and basal plasma membranes (Figs. 2–4). Beneath the apical plasma membrane, a uniformly fibrous electron-dense terminal web, 0.03–0.05 µm in thickness, was present and the underlying tegumental syncytium consisted of a dense, finely granular matrix containing numerous electron-dense secretory granules, larger electron-lucid vesicles, multivesicular bodies, granular endoplasmic reticulum and occasional mitochondria (Figs. 3, 4). The basal plasma membrane was infolded to form numerous finger-like projections. These infoldings were found throughout the entire syncytium, occurring most frequently on the dorsal surface in the anterior region of the fluke. Situated beneath the basal lamina of the tegument and attached to it via electron-dense hemidesmosomes were both circular and longitudinal muscle bundles (Figs. 2–6). These muscle fibres were non-striated, each being delimited by a sarcolemma, and were separated from one another by interstitial material (Figs. 2, 4–6). In the posterior region of the worm the tegumental syncytium was characterised by deep infoldings of the apical plasma membrane that formed tegumental ridges. These tegumental ridges were present on both the dorsal and ventral surfaces, although no apparent difference between them could be observed. On the ventral surface the tegumental ridges had pointed

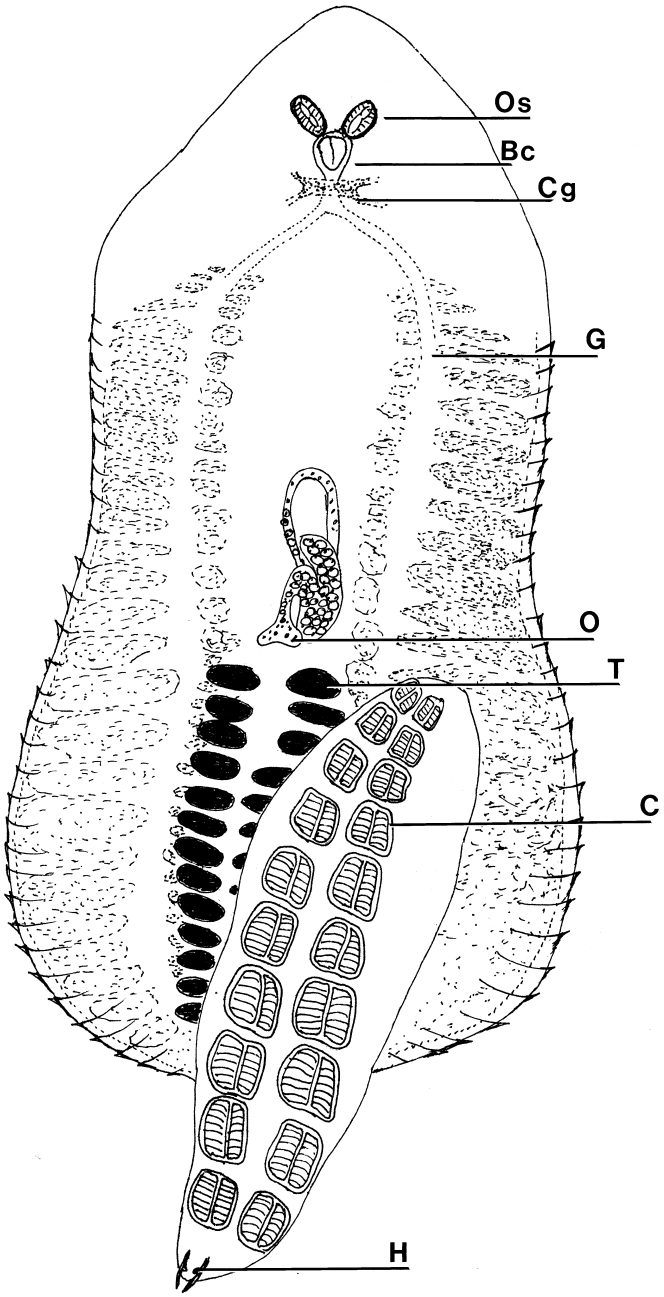

Fig. 1 Diagrammatic illustration of the general morphological features of *Pseudothoracocotyla indica* (*Os* Oral sucker, *Bc* buccal cavity, *Cg* cerebral ganglia and commissure, *G* gut caecum, *O* ovary, *T* testes, *C* clamps on haptor, *H* hooks on haptor)

tips and deep infoldings were observed (Fig. 2). The ridges were formed mainly by an increase in the outer anucleate zone of the syncytium. However, on the dorsal surface the ridges had rounded tips and infoldings were observed in both the apical and basal plasma membranes (Fig. 3). The dorsal ridges consisted mainly of a nucleated zone covered by an anucleate zone, which, although thicker at the ridge tips, was much thinner than the ridges on the ventral surface. Tegumental changes were observed throughout the length of the fluke. The syncytium of the mid-body region contained both electron-dense secretory granules and, large electron-lucid vesicles as well as occasional multivesicular bodies. These secretory granules and vesicles were also present in the tegumental cells situated beneath the basal lamina and tegumental musculature and were connected to the syncytial layer via trabeculae (Fig. 6).

Ultrastructure of the haptor

The clamp and rib-sclerites of the haptor region were electron-dense structures embedded within a dense sclerite matrix and invested by a relatively thin tegumental syncytium (Figs. 7, 8). The sclerites of each clamp were connected to each other and to the basal lamina by radially oriented muscle fibres. The tightly arranged myofibrils were not separated by interstitial tissue, although tight junctions were present between the sarcolemma of adjacent fibres. In certain regions, sclerites that protruded from the edge of the clamp were covered only by the apical plasma membrane of the tegument, which occasionally appeared to be broken in some areas, although this damage was not believed to be a fixation artifact as it was limited to a small region.

The tegumental syncytium of the haptor was similar to that described for the main body of the fluke, containing numerous electron-dense granules, various-sized electron-lucent vesicles, mitochondria, and occasional ribosomes. Some of these secretory vesicles exhibited the processes of exocytosis, a process apparently prevalent both in the syncytium of the clamps and in the general body tegument. The haptor was richly supplied with nerve fibres that presumably extended into the clamp muscles. The nerve axons were apparently non-myelinated and contained numerous electron-dense and electron-lucent neurosecretory granules and mitochondria. Associated with the nerve fibres entering the clamps were groups of neurons present in the haptor (Fig. 10). The cell bodies of the neurones contained neurosecretory granules that resembled those observed in the axon, whereas other neurones appeared to contain smaller granules (Fig. 12).

Ultrastructure of sensory endings

Two types of presumed sensory receptors were observed, the more common dome-shaped, putative sensory structures lacking a cilium and the less numerous structures displaying a single cilium (Figs. 8, 9). The uniciliated sensory structures, which occurred mostly in the oral sucker region, consisted of a nerve bulb attached to the apical tegumental membrane by a ring desmosome. The nerve bulb was connected to the peripheral nervous system by a nerve axon passing through the syncytical cytoplasm and underlying tissues. The cytoplasm of the nerve bulb was finely granular and contained spherical or granular electron-dense vesicles and microtubules. A sin-

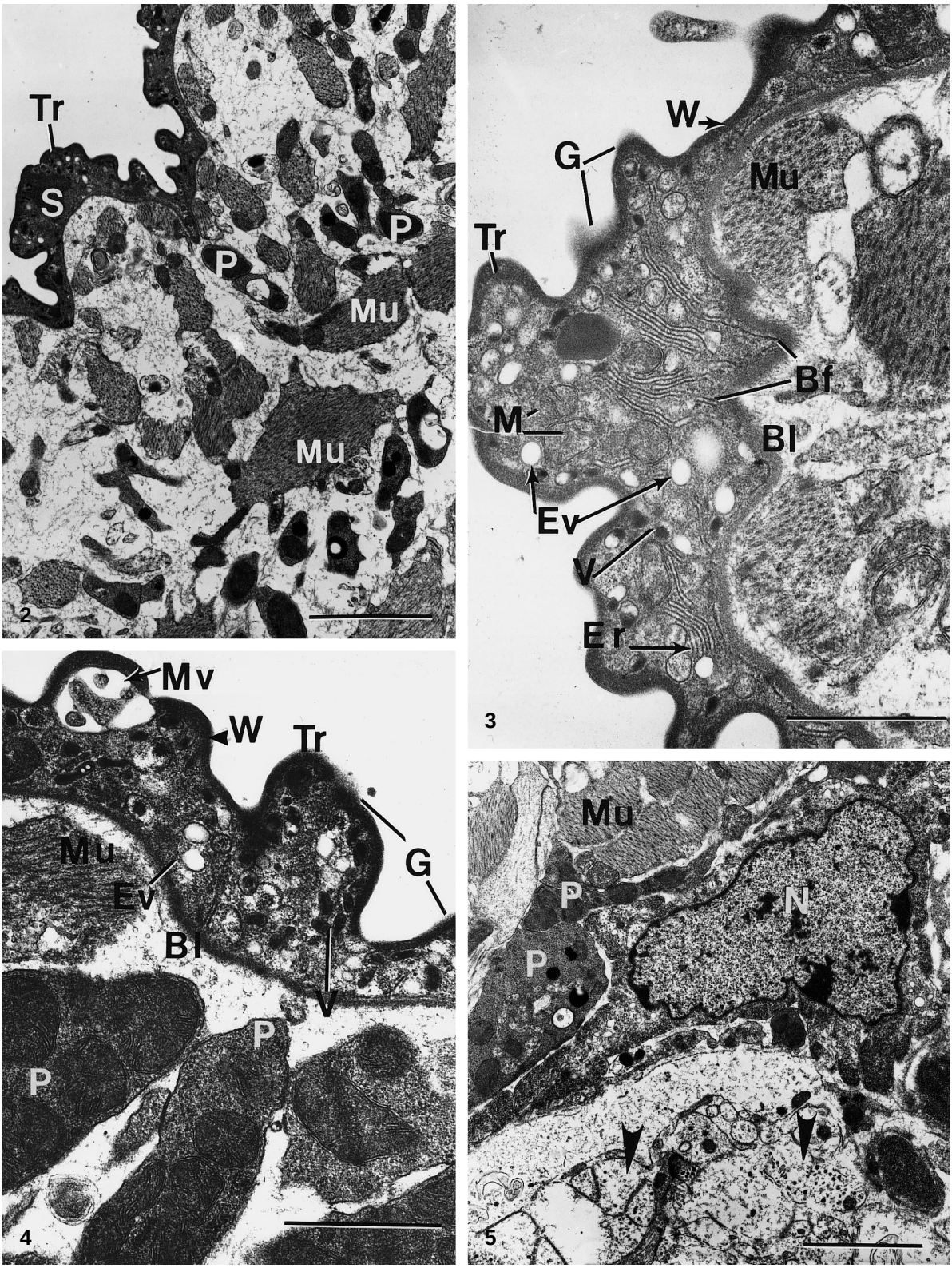

Fig. 2 *P. indica*: low-power electron micrograph of the tegument of the midventral surface of the body. The tegumental syncytium (*S*) is characterized by infoldings of the apical plasma membrane that give rise to pointed tegumental ridges (*Tr*). (*Mu* Muscles, *P* parenchymal cell extension). Scale bar = 2 µm. **Fig. 3** *P. indica*: transmission electron micrograph of a portion of the dorsal tegumental syncytium. The apical plasma membrane is infolded, forming rounded tegumental ridges (*Tr*). (*W* Electron-dense web, *M* mitochondria, *Er* granular endoplasmic reticulum, *V* electron-dense vesicles, *Ev* electron-lucent vesicles, *Bf* basal infold, *Bl* basal lamina, *G* glycocalyx, *Mu* muscles) Scale bar = 1 µm. **Fig. 4** Section of tegument showing the fibrous electron-dense web beneath the apical plasma membrane (*W*). A multivesicular body (*Mv*) and electron-dense (*V*) and electron-lucent (*Ev*) vesicles are present in the tegumental syncytium. (*Bl* Basal lamina, *G* glycocalyx, *Mu* muscles, *P* parenchymal cell extensions containing numerous mitochondria and glycogen, *Tr* tegumental ridge) Scale bar = 1 µm. **Fig. 5** Section through a portion of a tegumental cell (*N* Nucleus, *P* parenchymal cell extension, *Mu* muscle). Note the neurosecretory vesicles (*arrows*). Scale bar = 2 µm

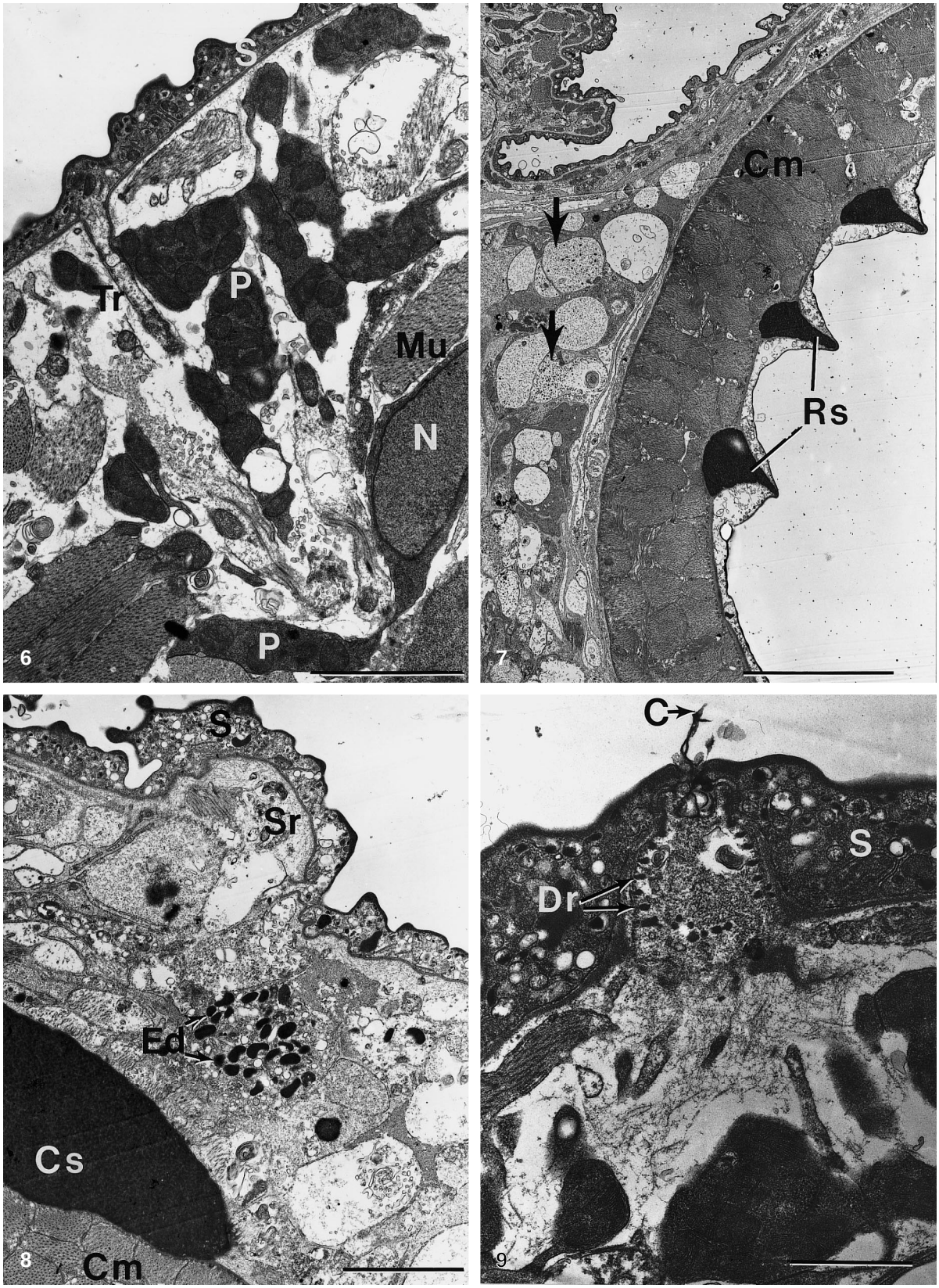

Fig. 6 Tegument showing the trabeculae (*Tr*), syncytium (*S*), muscles (*Mu*), and parenchymal cell nucleus (*N*). Note the numerous mitochondria within the parenchymal cell extensions (*P*). Scale bar = 2 µm. **Fig. 7** Section through a portion of a clamp sclerite, showing the electron-dense rib-sclerites (*Rs*), clamp muscles (*Cm*), and nerve processes containing numerous neurosecretory vesicles (*arrows*). Note the syncytium covering the rib-sclerites. Scale bar = 5 µm. **Fig. 8** Section through a portion of a clamp sclerite, showing the fibrous, electron-dense clamp sclerite (*Cs*) and a non-ciliated presumed sensory receptor (*Sr*). Note the clamp muscles (*Cm*), the electron-dense secretory vesicles (*Ed*) and the syncytium (*S*). Scale bar = 2 µm. **Fig. 9** Section through a unciliated sensory receptor (*C* Cilium, *S* syncytium, *Dr* ring desmosomes). Scale bar = 1 µm

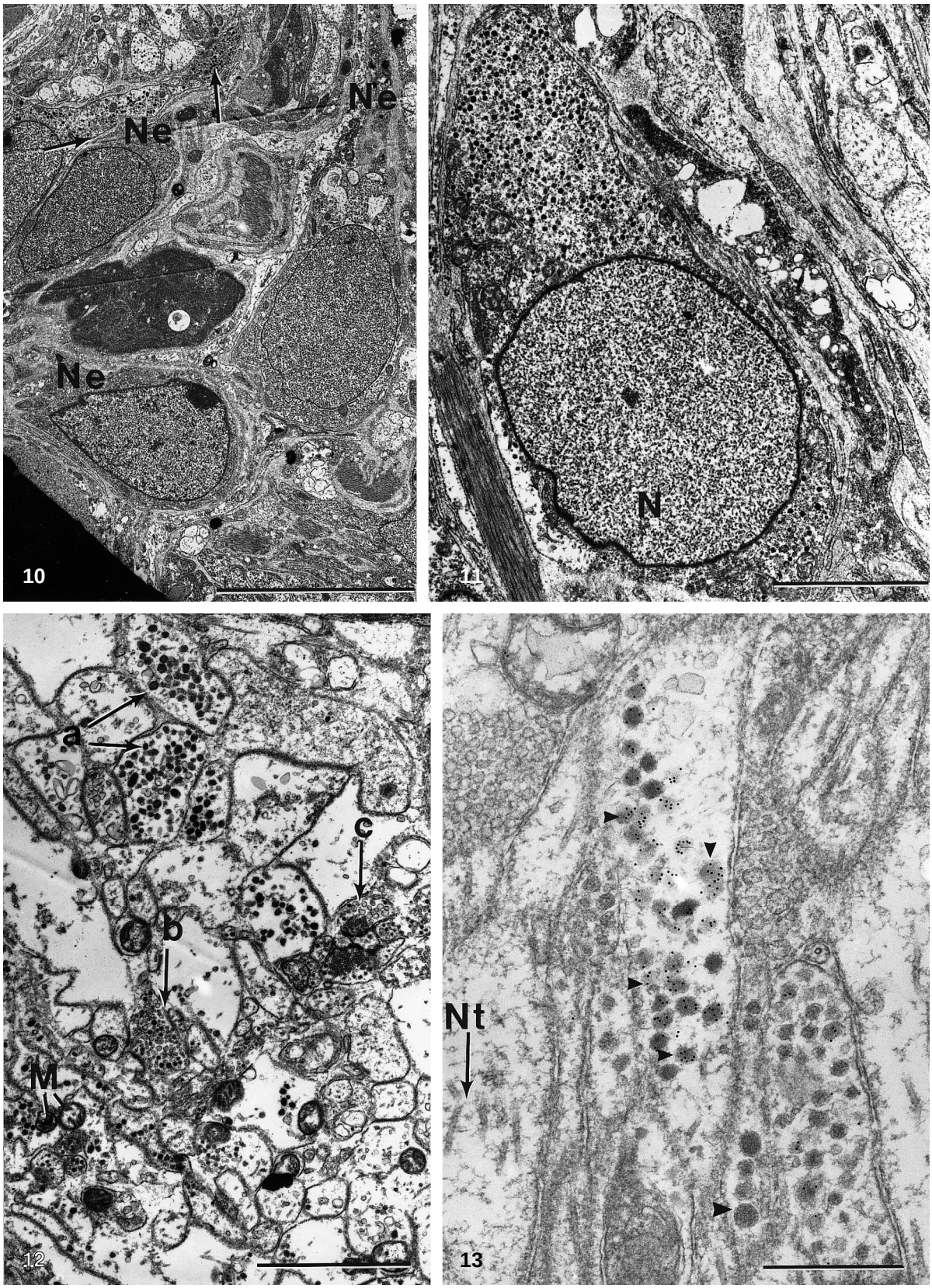

Fig. 10 A group of neurones (*Ne*) in the haptor region. Numerous dense-cored neurosecretory vesicles are present in the axon (*arrows*). Scale bar = 5 μm. **Fig. 11** High magnification of a neurone containing numerous dense-cored neurosecretory vesicles. (*N* Nucleus). Scale bar = 2 μm. **Fig. 12** Portion of a nerve axon from the posterior central nervous system (CNS) showing three kinds of neurosecretory vesicles: the larger dense-cored vesicles (*a*), the smaller dense-cored vesicles (*b*) and the electron-lucent vesicles (*c*). (*M* Mitochondria). Scale bar = 2 μm. **Fig. 13** Dense-cored vesicles in a nerve axon from the CNS immunolabeled with gold probes (10-nm size; *small arrowheads*), demonstrating NPF immunoreactivity. A separate population of non-reactive neurovesicles is also present (*large arrowhead*). (*Nt* neurotable). Scale bar = 0.5 μm

gle cilium was anchored in the nerve bulb by a basal body. The non-ciliated structures were present over the entire body surface and were more abundant in the haptor region. They appeared similar in structure to the uniciliated sensory structures but lacked the cilium and the ring desmosome.

Immunogold electron microscopy

Sampling of the ultrastructure of the nerve fibres from the posterior and haptor regions revealed three morphologically distinct types of membrane-bound vesicle: small lucent vesicles (average diameter, 40–50 nm), small dense-cored vesicles (60–70 nm), and large dense-cored vesicles (100–110 nm; Figs. 10–12). The small lucent vesicles were found either as a single type in some nerve axons or occasionally co-localized with the small dense-cored vesicles. The two forms of dense-cored vesicle were never observed together in the same nerve axon (Fig. 12). This was also true for the nerve-cell bodies, which contained only one of the two types of dense vesicle (Fig. 11).

Immunogold labeling revealed immunoreactivity (IR) to NPF associated only with the contents of the large dense-cored vesicles, hence identifying peptide-immunoreactive nerve cells (Fig. 13). The labeling was specific as indicated by the number of gold probes concentrated over the matrix of the vesicles; no labeling was seen in the other vesicle types or in any of the adjacent tissues. NPF immunoreactivity was blocked completely following pre-incubation of the sections with NPF standard, and incubations of the sections when the primary antiserum was omitted or substituted by non-immune serum were at all times negative.

Discussion

The body of *Pseudothoracocotyla indica*, like many platyhelminths, is extensively contractile, and much of the folding observed in the tegument may have been due to contractions of the underlying circular and longitudinal muscles during fixation of the specimen. Similar annular corrugations have been observed in *Diclidophora merlangi* (Halton 1979). The parallel array of tegumental ridges that are found throughout most of the tegumental surface appear to be independent of the fixed state of contraction and generally serve to amplify the free surface area of the worm. The tegumental ridges have previously been observed at both the light level and the SEM level with various degrees of contraction throughout the surface of the fluke. Rohde (1976) noted that the constrictions were usually coarsely serrated posteriorly and that fine constrictions were generally smooth anteriorly. Ramasamy and Hanna (1985) have suggested that the tegumental serrations may be involved in maintaining the fluke in its preferred orientation on the gills, similar to that of *Vallisiopis australis* (Young 1968), *Vallisia indica* (Ramasamy et al. 1987) and *Allodiscocotyla diacanthi* (Ramasamy et al. 1995).

The tegumental syncytium of *P. indica* is similar in structure to that described for the majority of monogeneans and digeneans (Smyth and Halton 1983). The monogenean tegument is a secretory epithelium containing secretory granules and vesicles transported from the underlying secretory cells in the nucleated zone. Presently, little is known of their chemical composition or function. In many monogenean and digenean parasites these secretory inclusions may contribute, by exocytosis, to the maintenance of a glycocalyx, or to repair of the tegumental syncytium or may possibly be involved in the provision of a protective mucus layer over the apical surface membrane to reduce mechanical, immunological, ionic and osmotic damage to the tegumental surface (Smyth and Halton 1983; Ramasamy et al. 1987). Schmahl and Mehlhorn (1985) demonstrated that vacuolization and subsequent exocytosis of syncytial materials helped the flukes to overcome mucus secretions of the gill and fluctating water currents, laden with particulate matter, in their gill habitat. The role of the glycocalyx in digeneans has been well documented (Threadgold 1976; Hanna 1980; Dunn et al. 1987a, b); however, in monogeneans the glycocalyx is not as well developed, suggesting that the transtegumental transport of nutrients is secondary to ingestion via the gut.

This study has demonstrated the occurrence of tegumental damage, possibly induced by mechanical forces in parts of the clamps. However, damage is not apparent throughout the general body surface of *P. indica* but instead is limited to discrete regions of the haptor, indicating that it is not a fixation artifact. It may result from (1) the withdrawal and extension of the clamps and attachment and detachment actions (Ramasamy and Hanna 1989) or (2) exposure of the parasite haptor to excess gill mucus or immunologically active secretions. By contrast, the rest of the body of the parasite is continuously washed by ventilation water currents and is free of tegumental damage. Rohde (1975, 1980) suggested that the ectoparasitic life-style of monogeneans was responsible for continuous sloughing off of the external syncytium in response to host reactions. The tegumental damage incurred may possibly have unknown adaptive values; besides releasing molecules that maintain and repair the glycocalyx, apical plasma membrane and syncytium, it may enhance the survival of the flukes in a mechanically stressful environment (Ramasamy and Bhuvaneswari 1993).

Infoldings of the basal plasma membrane give rise to numerous finger-like projections, suggesting tegumental involvment in nutrient uptake, excretion or osmoregulation. The absence of pino- and phagocytotic vesicles from the tegument suggests osmoregulation as the most likely possibility, similar to that postulated for *Fasciola hepatica* by Threadgold and Brennan (1978). *P. indica*, like many digeneans, has tegumental mitochondria, which would allow an active transport system to operate, and although microvilli are absent from its tegument, its

surface area is increased by ramifying tegumental folds similar to those observed in *Polystoma integerrimum* (Bresciani 1973), *Polystomoides malayi* and *P. renschi* (Rohde 1973).

In the nucleate zone of the tegumental syncytium of *P. indica*, parenchymal cells and fibrous interstitial materials fill the space around the tegumental cells. The parenchyma is well developed as a potential transport system, providing possible cell-to-cell communication via gap junctions. The interstitial fibres and matrix support the tegumental and parenchymal cells in addition to providing a framework for muscle attachment.

The clamp sclerites of *P. indica* are ultrastructurally similar to those described for other monogenean polyopisthocotyleans (Shaw 1979; Ramasamy et al. 1986, 1987, 1995). They may possibly arise through the process of protein synthesis in specialised areas of the tegument, unlike the spines of digeneans, which arise from cuticularisations (Ramasamy et al. 1986, 1995). The occurrence of neurones and nerve processes in and around the clamps of *P. indica* indicate a functional role in neuromuscular activity. Neurones and nerve axons together with the radial muscles of the clamp are important in coordinating adhesion to the gill filament. The clamp muscles appear to have ultrastructural differences as compared with the muscles present in the body wall, possibly reflecting functional differences in these two regions. The absence of an interstitial space between the sarcolemma of adjacent clamp-muscle bundles may help improve intermuscular communication and co-ordination (Ramasamy et al. 1986).

The ciliated and non-ciliated sensory structures present in the tegumental syncytium of *P. indica* are similar in structure to those described for other monogeneans (Smyth and Halton 1983; Ramasamy et al. 1986, 1987; Ramasamy and Bhuvaneswari 1993). They have been ascribed a variety of functions based largely on their distribution and ultrastructure, but as no behavioural or electrophysiological information yet exists, their exact functions remain uncertain. The uniciliated sensory endings present around the oral apertures are possibly involved in feeding and the selection of feeding sites, whereas at other sites on the body surface they may function as rheo-, tango- or chemoreceptors, aiding the fluke in its orientation with respect to the direction and flow of water currents and gill architecture. The non-ciliated sensory structures, which are present over the entire body surface and are more abundant in the haptor region, may act as mechano-receptors involved in host contact or contact with other flukes. However, although the sensory papillae thus far described from the monogeneans may have a sensory function, neurophysiological evidence for such a function is presently lacking.

Immunoelectron microscopy was used to demonstrate the sub-cellular distribution of neuropeptide F (NPF, *Moniezia expansa*) immunoreactivity in the clamp region of *P. indica*. Electron microscopic examination of the associated nerve cords clearly identified two types of axon, which contained inclusions of neurotubules and mitochondria but were distinguished by populations of different-sized neurosecretory vesicles. Only the large-sized, electron-dense vesicles were immunoreactive, and in all cases the immunogold labeling was confined to the contents of these vesicles. ICC studies have now shown that regulatory peptides form a substantial element of the platyhelminth nervous system (Halton et al. 1994). ICC studies on the nervous system of *D. merlangi* (Maule et al. 1992) showed that, similar to the situation in many other parasitic platyhelminths examined, the most intense immunoreactivity was obtained using antisera to NPF. Indeed, considering the peptides that have been localized in flatworms, the most predominant is the native neuropeptide NPF, which is structurally analogous to the vertebrate neuropeptide NPY superfamily (Halton et al. 1994). NPF is perhaps the most abundant neuropeptide identified in cestodes and trematodes (Halton et al. 1991; Brennan et al. 1993a, b).

In the present study, NPF immunoreactivity was demonstrated in dense-cored vesicles of the nerve cords that supply the clamps of the haptor of *P. indica*. In a separate study, Brennan et al. (1993a) demonstrated neuropeptide immunoreactivity in dense-cored vesicles of nerve axons to the peduncles and clamps of the monogenean *D. merlangi*. Although the specific action of NPF is unknown, its widespread occurrence in the nervous system of cestodes and trematodes suggests a fundamental role in nerve-muscle functioning.

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