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THE MORPHOLOGY OF AUTOTOMY STRUCTURES IN THE SEA CUCUMBER *EUPENTACTA QUINQUESEMITA* BEFORE AND DURING EVISCERATION

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Summary

Evisceration in the dendrochirotid sea cucumber *Eupentacta quinquesemita* is a whole-body response involving a predictable series of events including muscle contraction and failure of three autotomy structures: (i) the introvert, the dexterous anterior extensible portion of the body wall, (ii) the tendon linking the pharyngeal retractor muscle to the longitudinal body wall muscle and (iii) the intestine–cloacal junction. The autotomy structures are histologically complex, consisting of muscle, nervous and connective tissue. Autotomy resulted from complete loss in the tensility of the connective tissue ground substance. Separation of the autotomy structures was facilitated by muscle contraction. The cell and tissue changes involved with autotomy were documented by microscopic examination of autotomising tissue. Change in the autotomy structures appears to initiate from the peritoneal side with delamination of the peritoneum followed by a wave of disruption as the connective tissue is infiltrated by coelomic fluid. Evisceration and autotomy in *E. quinquesemita* are neurally controlled, so particular attention was paid to the fate of neuronal elements. Neurosecretory-like processes containing large dense vesicles and axons were present in the connective tissue layers of the autotomy structures in association with extracellular matrix, muscles and neurons. These neuronal elements remained largely intact during autotomy and did not appear to be a source of factors that effect connective tissue change. They may, however, be involved in muscle activity. Holothuroid autotomy structures are completely or partially bathed in coelomic fluid, so there is potential for hormonal or neurosecretory activity using the coelomic fluid as a conduit. Connective tissue change during evisceration appears to be effected or mediated by an evisceration factor present in coelomic fluid that has a direct transmitter-like or neurosecretory-like mode of operation. The final outcome, expulsion of the viscera, is likely to result from a suite of factors that interact in a manner yet to be determined.

Key words: Holothuroidea, evisceration, mutable connective tissue, sea cucumber, *Eupentacta quinquesemita*.

Introduction

The possession of mutable connective tissues (MCT) that undergo rapid muscle-like, nerve-mediated changes in their mechanical properties is characteristic of the Echinodermata (Wilkie, 1984, 1996; Motokawa, 1984). There are two manifestations of this phenomenon; ‘catch’, in which the tissues exhibit reversible stiffening/softening properties, and ‘autotomy’, in which the tissues exhibit irreversible catastrophic softening leading to the loss of body parts. For the Holothuroidea, catch connective tissue in the body wall plays a major role in posture control and has been well-studied in aspidochirotids (Motokawa, 1984, 1987; Birenheide et al., 1998) and more recently in dendrochirotids (Trotter and Koob, 1995; Thurmond and Trotter, 1996; Trotter et al., 1996). Evisceration in holothuroids is a rather dramatic form of autotomy resulting in expulsion of the viscera (Smith and Greenberg, 1973; Byrne, 1985a; Dolmatov, 1996; Garciá-Arrarás et al., 1999). Aspidochirotids expel the digestive tract posteriorly through the anus, while dendrochirotids expel the digestive tract along with the tentacles and pharyngeal complex through rupture of the anterior body wall. Despite the notorious ability of holothuroids to dissolve their tissues and discard internal organs, the processes underlying these phenomena are not understood. Most descriptions of evisceration are based on laboratory studies (Emson and Wilkie, 1980), but this unusual behaviour does occur in nature. Evisceration in the dendrochirotid *Eupentacta quinquesemita* occurs seasonally, potentially as a means of discarding accumulated waste or parasite loads and as a defensive response to predatory sea stars (Byrne, 1985a,b, 1986a).

Evisceration in *E. quinquesemita* is effected by softening and rupture of three autotomy structures (Fig. 1): (i) the introvert, the dexterous anterior extendible portion of the body wall; (ii) the tendon (P-L tendon) linking the pharyngeal retractor muscle to the longitudinal body wall muscle and (iii) the intestine–cloacal junction (Byrne, 1982, 1985a,c,d). The introvert is a highly compliant structure and, in mechanical

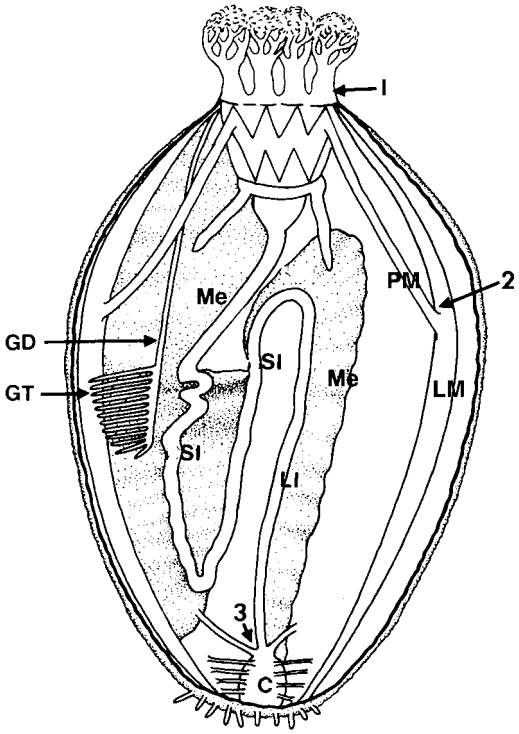

Fig. 1. Diagrammatic view of the autotomy structures of *Eupentacta quinquesemita*. I, introvert; 2, P-L tendon (linking the pharyngeal retractor muscle to the longitudinal body wall muscle); 3, intestine–cloacal junction; C, cloaca; GD, gonoduct, GT, gonad tubules, LM, longitudinal body wall muscle; LI, large intestine; Me, mesentery: PM, pharyngeal retractor muscle; SI, small intestine (from Byrne, 1985a, with permission).

tests, exhibited viscous creep behaviour deforming by 900 % of its original length before rupture (Byrne, 1985c). This structure does not, however, have a pre-existing mechanical weakness to account for its failure during autotomy (Byrne, 1985c). The P-L tendon forms a strong collagenous link between two major muscles and, thereby, anchors the oral complex (pharynx and buccal tentacles) to the body wall (Fig. 1). At its anterior end, the pharyngeal retractor muscle attaches to the calcareous ring, and its posterior end links with the longitudinal body wall muscle. Despite its small size, the tendon is mechanically strong. In breaking tests, the retractor muscle usually failed at its insertion into the ossicle and not at the tendon (Byrne, 1985c). Catastrophic autotomy-type changes can be elicited *in vitro* by manipulation of the ionic environment with rapid softening of introvert and tendon preparations in response to elevated [K^+] (Byrne, 1986b).

In the present study, the cell and tissue processes involved with autotomy in *E. quinquesemita* were investigated through microscopic examination of intact and autotomising tissues. Autotomy structures at various stages of breakdown were fixed instantly by coelomic perfusion. As is characteristic of echinoderms, connective tissue autotomy in *E. quinquesemita* appears to be under neural control (Byrne, 1986b), so particular attention was paid to the fate of neuronal elements. Neurosecretory-like processes containing large dense vesicles (LDVs) are abundant in the autotomy structures of *E. quinquesemita*, and their fate during autotomy was followed. In ophiuroid, crinoid and asteroid arm autotomy, these processes, termed juxtaligamental cells, are thought to be a source of agents that effect connective tissue change

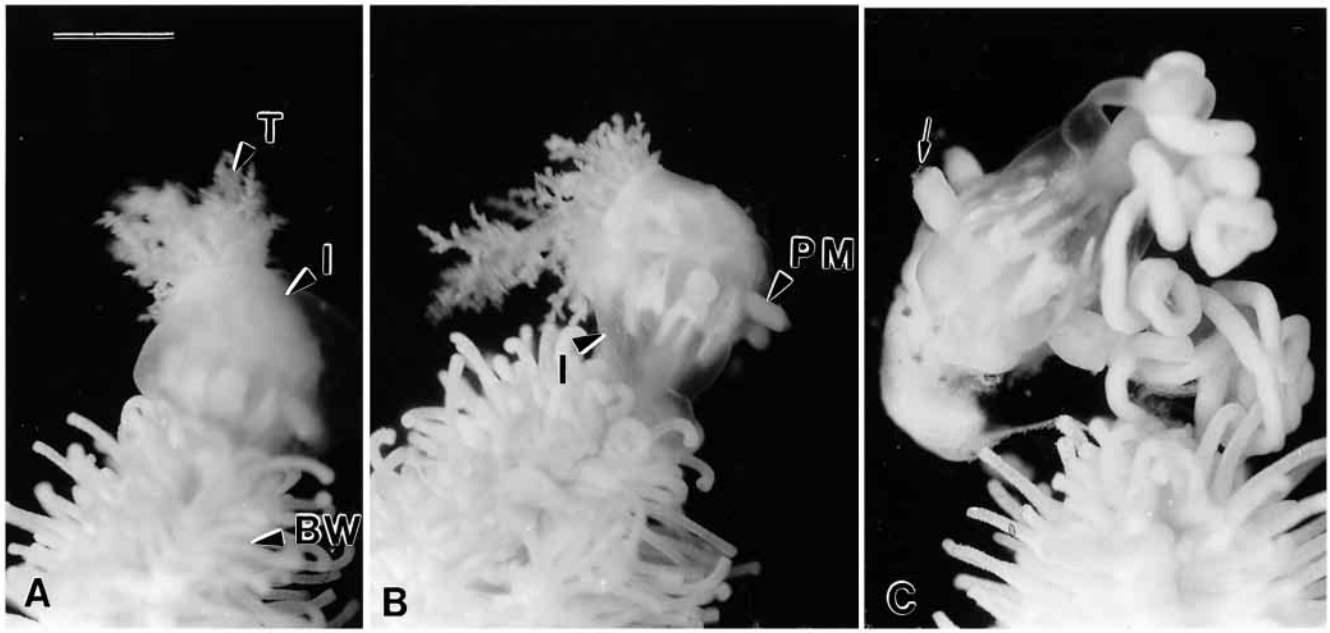

Fig. 2. Evisceration in *Eupentacta quinquesemita*. (A) At 3 min after the initial response to stimulus. The pharyngeal complex, freed from the body wall (BW) by tendon autotomy, is seen through the softening and expanding introvert (I). (B) At 4 min after the initial response. The coelomic fluid has been ejected through rupture of the introvert. (C) At 5 min after the initial response. The body is sealed off as the digestive tract is ejected. The only remaining connection between the introvert and the body wall is a viscid strand. The arrow in C points to the autotomised tendon. PM, pharyngeal retractor muscle; T, tentacles. Scale bar, 7.0 cm (from Byrne, 1985a, with permission).

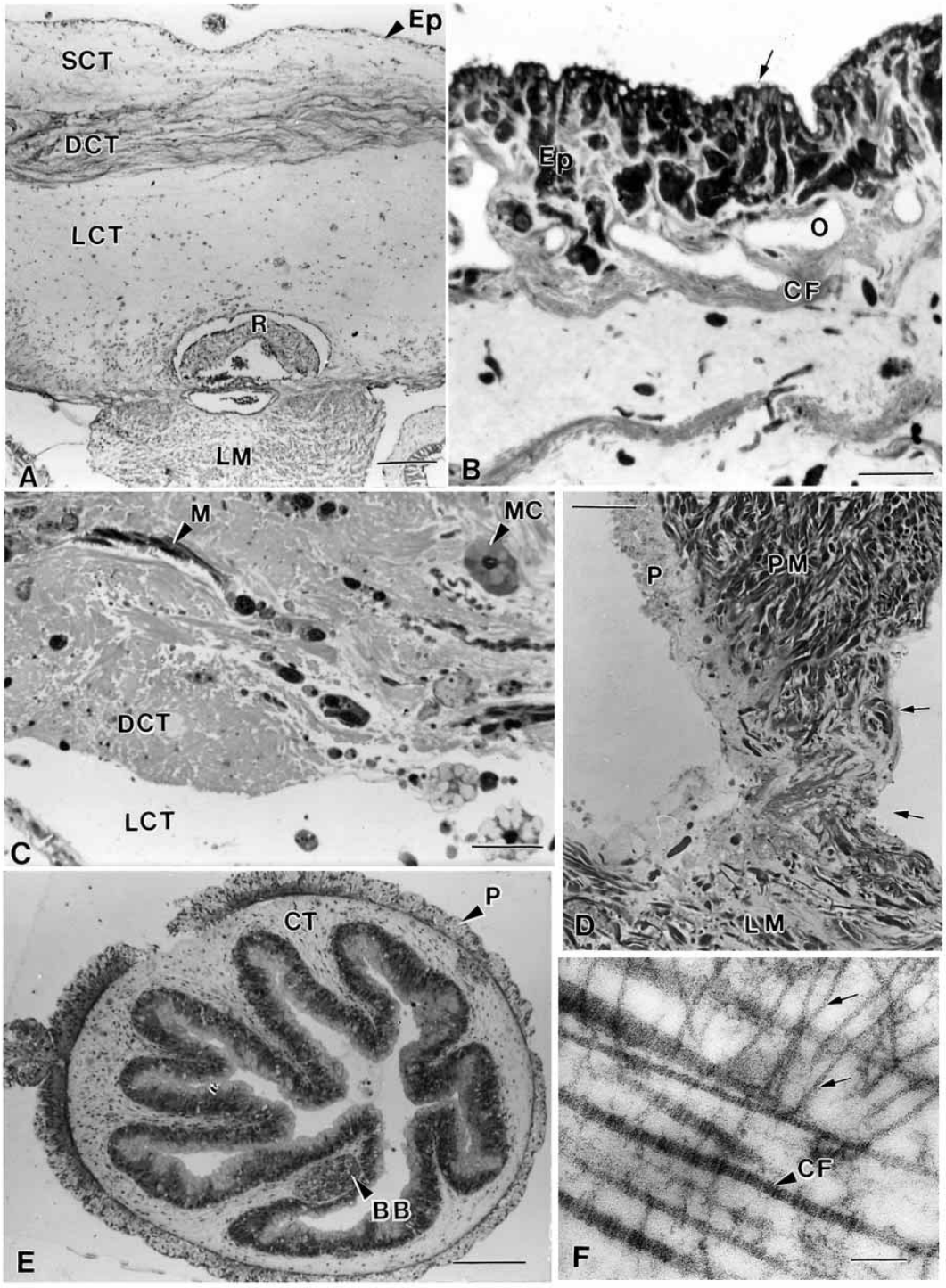

Fig. 3. Light micrographs (A–E) of autotomy structures in *Eupentacta quinquesemita*. (A) Paraffin section (7 μm thick) of the introvert showing epidermis (Ep) and dermal layers; subepidermal (SCT), dense (DCT) and loose (LCT) connective tissue. LM, longitudinal body wall muscle; R, radial nerve cord. (B,C) Plastic sections (0.5 μm thick) of introvert region showing epidermis (Ep), cuticle (arrow) and subepidermal connective tissue with ossicles (O) and associated collagen bundles (CF). (C) DCT–LCT interface showing abundance of collagen in the DCT and ground substance in the LCT. Muscle fibres (M) and morula cells (MC) are common. (D) Grazing longitudinal section of the pharyngeal retractor muscle (PM) and longitudinal body wall muscle (LM) and connecting P-L tendon (linking the pharyngeal retractor muscle to the longitudinal body wall muscle) (arrows). Collagen bundles (light tissue) and muscle fibres (darker tissue) pervade the tendon. (E) Intestine autotomy region comprising the inner digestive mucosa, connective tissue layer (CT) and muscle/peritoneal (P) layer. Brown body (BB) waste accumulations are common in the connective tissue. (F) Striated collagen fibrils (CF) and unstriated microfibrils (arrows) as seen here for the introvert are typical of the autotomy structures. Scale bars: A, 0.2 μm; B,C, 0.1 μm; D, 1.0 μm; E, 0.5 μm; F, 0.2 μm (E is taken from Byrne, 1985c, with permission).

(Cobb, 1985; Holland and Grimmer, 1981; Wilkie, 1979, 1988; Wilkie et al., 1990). The term juxtaligamental cells is not used here because in *E. quinquesemita* these processes are associated with muscle and with nervous and connective tissue. An evisceration factor that induces autotomy *in vitro* is present in the coelomic fluid expelled by eviscerating specimens (Byrne, 1986b), so the tissues were examined to assess the potential influence of this factor. An overview of previous research on autotomy in *E. quinquesemita* is presented, and our current understanding of evisceration in holothuroids is assessed.

Materials and methods

Eupentacta quinquesemita (Selenka) were collected using SCUBA near Victoria, British Columbia, Canada. In the laboratory, evisceration in this holothuroid is readily induced by injection (0.5 ml) of 0.45 mol l^{-1} KCl into the coelom and by electric shock (60 V) in 2.4 and 1.5 min respectively (Byrne, 1986a). For the present investigation, evisceration was induced by mechanical stimulation by holding specimens across their mid body region with forceps. With this method, evisceration takes up to 5 min to complete. Because of the predictable behavioural sequence that characterises evisceration in *E. quinquesemita* (Byrne, 1985a), the temporal pattern of change in the autotomy structures was examined through arrest of the process by injection of fixative into the coelom. Specimens at various stage of evisceration were perfused with 3 % glutaraldehyde in 0.2 mol l^{-1} cacodylate buffer (pH 7.4). Non-eviscerating specimens were also injected with fixative. Intact and autotomising tissues were processed for light and transmission electron microscopy. The autotomy structures were quickly dissected from injected specimens and placed in fresh fixative for 1 h at room temperature (20 °C). The tissues were post-fixed in 1 % OsO_4 in cacodylate for 1 h at 4 °C, decalcified in ascorbic acid (Dietrich and Fontaine, 1975), dehydrated and embedded in Epon resin. Thick sections (0.5 µm thick) were stained with Toluidine Blue, and paraffin-embedded sections were stained with haematoxylin and eosin. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips EM 300.

Results

Behavioural events

Evisceration in *Eupentacta quinquesemita* followed a predictable a series of behavioural events (Fig. 2). In response to the evisceration stimulus, the anterior body region rapidly shortened as a result of a strong contraction of the pharyngeal retractor muscle. This often created a short-lived mid-body constriction in the body wall where the tendons attached to the longitudinal body wall muscle (Fig. 1). The tendons lost their mechanical strength within a few seconds of retractor muscle contraction. Rupture of the tendon severed the connection between the oral complex and the body wall (Figs 1, 2A,B). Tendon autotomy was a two-step process involving rapid muscle contraction followed by softening and rupture of the tendon. Contraction of the retractor muscle and longitudinal body wall muscle facilitated rapid separation of these muscles after tensility changes had occurred within the tendon. An intact tendon was only seen in a few specimens preserved in the process of autotomy. In some individuals, two or three of the five tendons were still intact, indicating that tendon rupture was not simultaneous. Autotomy of the intestine–cloacal junction (Fig. 1) occurred simultaneously with or shortly after P-L tendon autotomy. Some specimens fixed in the process of evisceration had 2–3 intact tendons and a detached intestine, while others autotomised all the tendons before intestinal autotomy. Mechanical separation of the intestine may be effected by tension generated by contraction of the intestine and cloacal muscles. Contraction of the gut muscles following autotomy caused the detached end of the intestine to roll back on itself.

After internal autotomy, strong contraction of the body wall muscles propelled the viscera and coelomic fluid anteriorly into the introvert (Fig. 2A). The introvert changed from a firm opaque structure to one that was thin, soft and translucent as it filled with coelomic fluid and autotomised organs (Fig. 2A,B). As it distended, the introvert enlarged like a balloon and eventually ruptured, expelling the oral complex and digestive tract (Fig. 2B,C). Final rupture of the introvert and expulsion of the viscera took up to 5 min. After autotomy, the introvert was reduced to a soft mucous-like mass. On the surface, the epidermis and the dermal layer containing the ossicles delaminated from the underlying tissue and could be removed with ease. Ossicles embedded in this tissue became visible to the eye. After shunting viscera and coelomic fluid into the introvert, muscle contractions sealed off the anterior end of the body (Fig. 2C).

Structure of intact introvert

The introvert provides a flexible support for the tentacles (Fig. 1), and its compliant properties were reflected in its high content of proteoglycan-dominated ground substance

Fig. 4. Transmission electron micrographs of intact introvert. (A) Dense connective tissue layer with collagen bundles (CF), muscle fibres (M) and large dense vesicle (LDV)-containing processes. (B,C) Axon bundle in dense connective tissue layer containing clear (CV), small dense (SV) and dense-cored (DV) vesicles and a glial-like cell (Gl) surrounded by basal lamina. (D,E) LDV-containing neurosecretory-like processes with axial microtubules (MT) and adjacent muscle (M), sarcoplasmic process (S), collagen fibrils (CF) and microfibrils (MF). (F) Loose connective tissue (LCT) with morula cells (MC), collagen fibrils and nerves (arrows) embedded in a gel-like ground substance and adjacent peritoneum (P), body wall muscles (M) and nerve plexus (NP). (G) Processes in the LCT with LDVs differing in size and shape. (H) Axo-axonal synapse in the nerve plexus. The varicosity on the lower axon contains dense-cored (DV) and clear (CV) vesicles. Ax, axon. Scale bars: A, 2.0 µm; B,C,E, 0.5 µm; D,G, 1.0 µm; F, 0.3 µm; H, 0.45 µm (G is taken from Byrne 1985c, with permission).

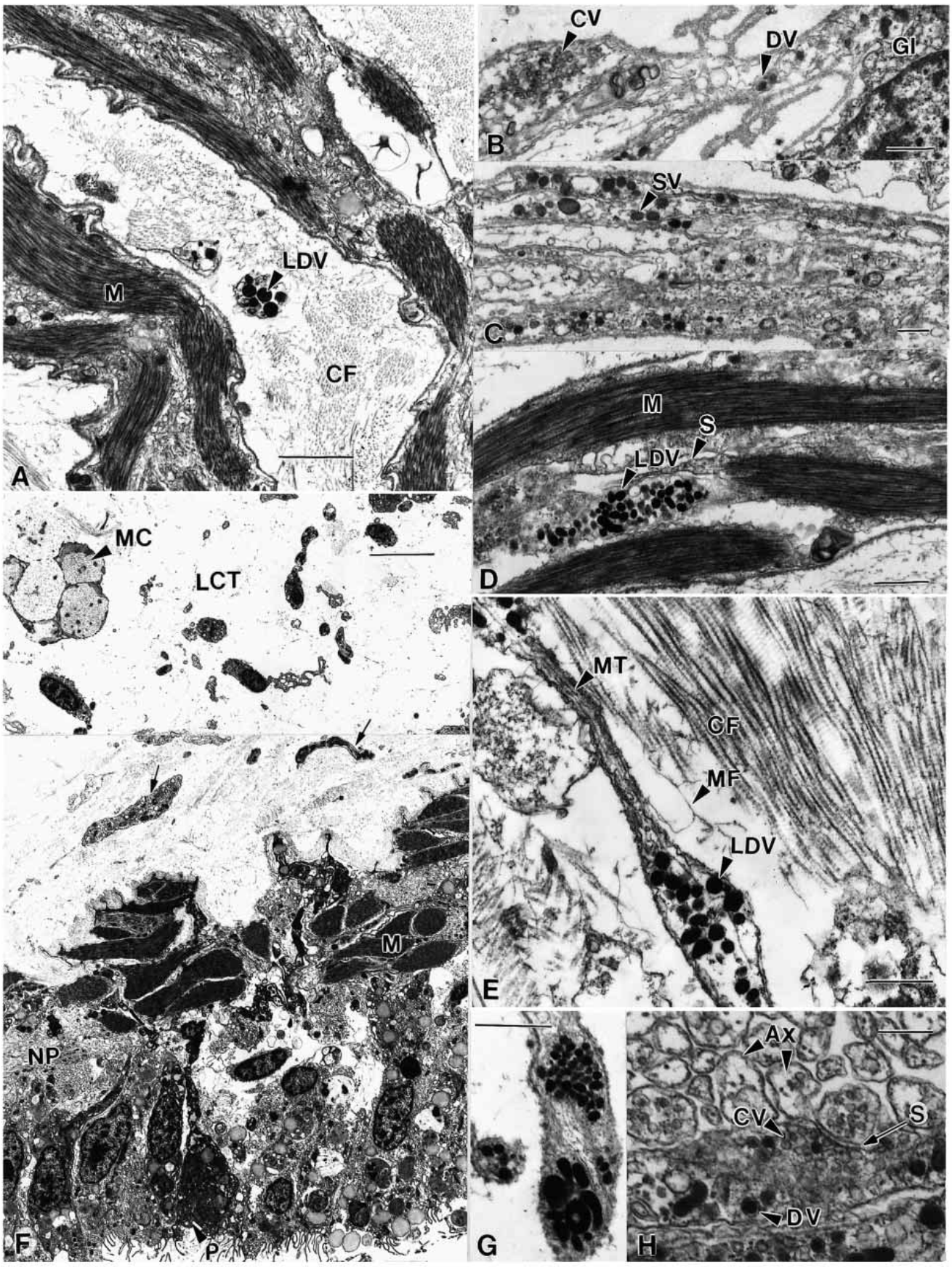
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Fig. 4

(Fig. 3A–C). Muscle fibres were dispersed throughout the dermis, occupying 1–4 % of the cross-sectional area (Figs 3C, 4A,D). The introvert is made up of the epidermis overlain by a well-developed cuticle (90–130 μm thick), the dermal connective tissue layer and the inner body wall muscle/peritoneal layer (Fig. 3A–C). The epidermis, one to several cells deep, projected into the underlying dermis (Fig. 3B). These cells were joined by desmosome/septate junction complexes, and their basal region rested on the basal lamina separating the epidermis and dermis. Numerous hemidesmosomes formed cuticle/epidermis/dermis junction complexes, linking the superficial regions of the integument.

The dermis formed the bulk of the introvert and consisted of an outer subepidermal layer, a middle dense connective tissue layer (DCT) and an inner loose (LCT) connective tissue layer (Fig. 3A–C). The superficial dermis was largely ground substance with a few bundles of small-diameter (40–60 nm) collagen fibrils (Fig. 3B). Unstriated fibrils (7–20 nm in diameter) were scattered through the superficial dermis. The latter are common in the connective tissue of all the autotomy structures of *E. quinquesemita* (Fig. 3F) and are likely to be homologous to the fibrillin-containing microfibrils described from the body wall of another dendrochirotid species (Thurmond and Trotter, 1996). The ossicles formed a skeletal layer just below the epidermis (Fig. 3B). The DCT (0.1–0.5 mm thick) formed a collagenous (20–160 nm diameter fibrils) stratum around the circumference of the introvert (Figs 3A,C, 4A,E). In this tissue layer, the collagen fibrils were either organised into small bundles (collagen fibres) or distributed as single fibrils. The collagen fibres were not orientated in a particular pattern with respect to the body axis. The collagen fibrils in the introvert and in the other autotomy structures had a longitudinal periodicity (60–67 nm) typical of Type I collagen (Fig. 3F). Unstriated microfibrils were attached to, and crossed between, adjacent collagen fibrils (Figs 3F, 4E). Bundles of muscle fibres were scattered through the DCT and, because of fixation-induced contraction, their sarcolemma and associated basal lamina had an undulating profile (Figs 3C, 4A). The muscle fibres gave rise to terminal sarcoplasmic processes devoid of myofilaments and containing axial microtubules (Fig. 4D). These 'muscle tails' are characteristic of echinoderm muscles (Cobb and Begbie, 1994) and in *E. quinquesemita* were often associated with neuronal or neurosecretory-like processes (Fig. 4D). Mitochondria and small subsarcolemmal cisternae were located in the peripheral sarcoplasm of muscle fibres in the introvert and other autotomy structures.

Bundles of axons surrounded by a basal lamina and often ensheathed by a glial-like cell were scattered through the DCT (Fig. 4B,C). Some of these nerves were adjacent to muscle bundles and others were surrounded by connective tissue. The axons contained several types of vesicles including, clear (70–80 nm diameter), dense-cored (80–140 nm diameter) and small dense (Fig. 4B,C) vesicles. The latter were round (130 nm diameter) to ellipsoidal (200–250 nm×120–150 nm). Axial microtubules and mitochondria were characteristic of the axons. Neurosecretory-like processes filled with large electron-dense vesicles (LDVs) and surrounded by basal lamina were scattered through the connective tissue and were also associated with muscle fibres (Fig. 4A,D,E). These processes contained axial microtubules and were similar to those of the juxtaligamental cells, which are considered to be characteristic of echinoderm connective tissues (Wilkie, 1996). The vesicles varied in shape from round (150–300 nm in diameter) to ellipsoidal or elongate (180–550 nm×125–220 nm). Some processes contained only round vesicles, while others contained only elongate ones (Fig. 4A,E,G). The diverse array of vesicle profiles in most processes precluded categorisation into different types. It is likely that the variable profiles observed represent different types of section (cross, longitudinal and tangential) through what may be elongate spindle-shaped granules. Other cells encountered in the DCT and elsewhere in the integument included phagocytes and morula cells (Figs 3C, 4F). The latter are large migratory cells, characteristic of holothuroids, that appear to function in the maintenance of connective tissues as a source of ground-substance material (Byrne, 1986c).

Internally, the LCT was an electron-lucent layer containing scattered cells suspended in what appeared to be a gel-like medium (Figs 3A,C, 4F,G). This tissue layer was predominantly proteoglycan-dominated ground substance with numerous unstriated microfibrils (7–12 nm in diameter) and a few collagen fibrils (40–60 nm in diameter) embedded in it. Muscle bundles similar to those seen in the DCT were present, as were nerve and neurosecretory-like processes (Fig. 4G). Morula cells, phagocytes and other migratory cells were abundant in the LCT (Figs 3C, 4F). The peritoneum and associated circular and longitudinal body wall muscles and nerve plexus formed the innermost tissue layer of the introvert (Figs 3A, 4F). The nerve plexus, positioned at the base of the peritoneum adjacent to the body wall muscles (Fig. 4F), consisted of bundles of axons with the range of vesicle types listed above. This is a major nervous structure, and axo-axonal synapses were occasionally observed (Fig. 4H). Body wall muscle fibres gave rise to sarcoplasmic processes that mingled with axons.

Autotomising introvert

During evisceration, the introvert exhibited viscid flow as a result of breakdown of the ground substance (Fig. 2). This loss in connective tissue tensility resulted in interfibrillar slippage. Despite this disruption, the collagen fibrils remained intact in structure (Fig. 5A–C). Disruption of the peritoneum was one of the first changes during autotomy (Fig. 5D). The peritoneal cells, nerve plexus and muscle cells peeled away from the introvert and dissociated into the coelom (Fig. 5D,F). Most of the axons and their vesicles appeared intact, although a few swollen axons were observed (Fig. 5F). The LCT was infiltrated by coelomic fluid, as marked by the presence of haemocytes and amoebocytes in tissue sections (Fig. 5H). This tissue layer changed from the gel-like state in intact introvert

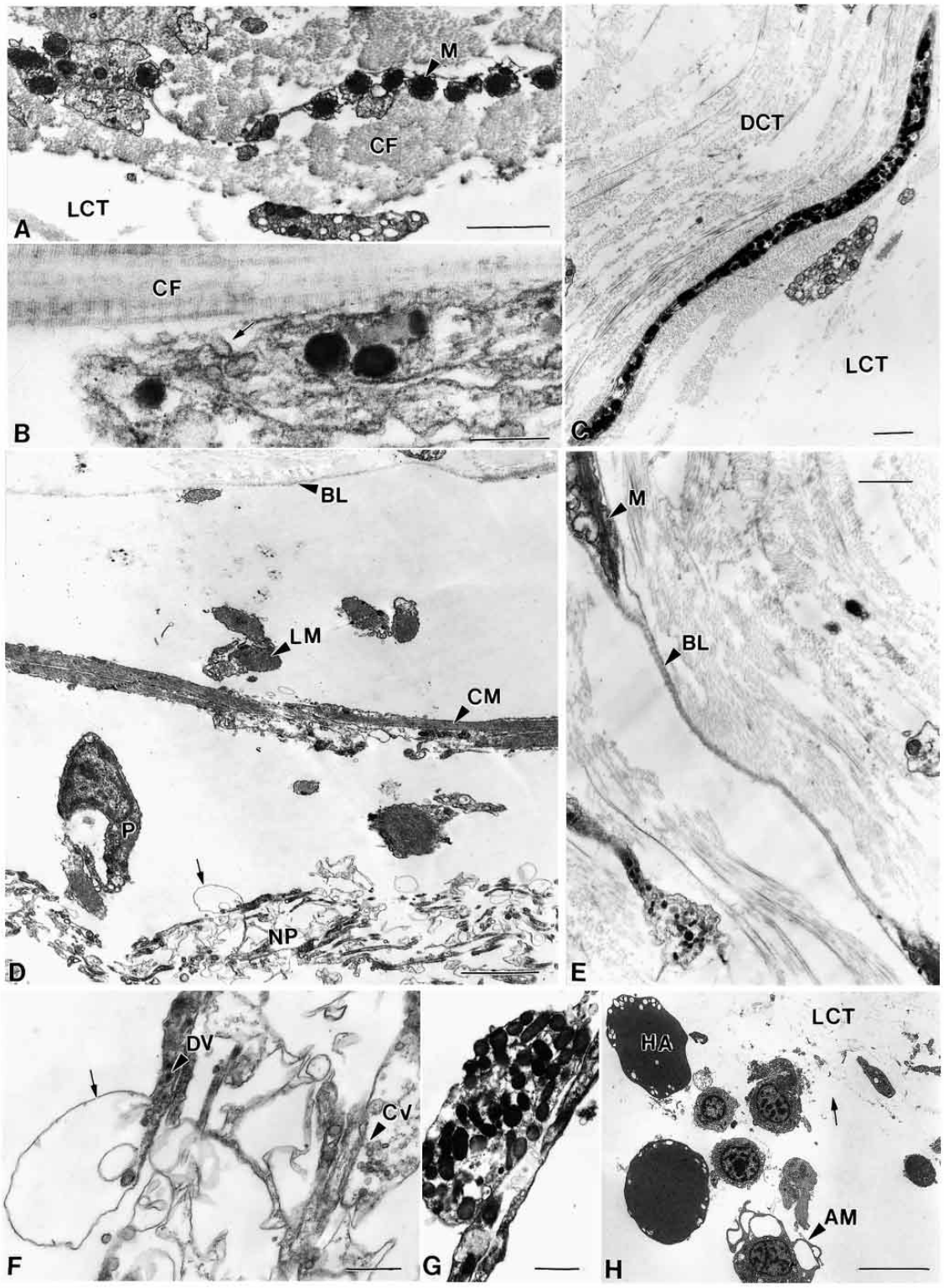

Fig. 5. Transmission electron micrographs of autotomising introvert. (A–C,E) Collagen fibrils (CF) remained intact as the introvert extends. The muscle fibres (M), their basal lamina (BL) and large dense vesicle-containing processes extended in the direction of distension. The LDV remained intact. Only one exocytotic-like profile (arrow in B) was seen. LCT, loose connective tissue; DCT, dense connective tissue. (D) The peritoneum (P), longitudinal (LM) and circular (CM) muscle fibres and nerve plexus (NP) detached from the loose connective tissue (LCT) layer and dispersed into the coelom. Some axons were swollen (arrow). (F) Detail of swollen axon (arrow) with dense-cored (DV) and clear (CV) vesicles intact. (G) LDV-containing process in the coelom. (H) Coelomic fluid infiltrates (arrow) the LCT as indicated by the presence of amoebocytes (AM) and haemocytes (HA). Scale bars: A, 1.5 μm; B, 0.2 μm; C,E,G, 1.0 μm; D, 3.0 μm. F, 0.5 μm, H, 8.0 μm (C is taken from Byrne 1985c, with permission).

to a fluid state. Coincident with this change, the components of the LCT became disorganised and fell out of position. The axons, neurosecretory-like processes, cells and fibrils embedded in the LCT floated away into the coelom (Fig. 5H). Processes filled with intact LDV and axons containing small vesicles were among the dissociated cells (Fig. 5G).

During introvert expansion, the epidermis spread out, causing rupture of intercellular junctions. The subepidermal connective tissue took on a hydrated appearance as it was infiltrated by sea water. Subsequently, the epidermis and subepidermal layer delaminated from the underlying tissue. Changes in the DCT layer were evident when normal and autotomising tissues were compared. This layer decreased in diameter to 0.01–0.1 mm as collagen fibres and individual collagen fibrils slid across and away from each other. The collagen fibrils became uniformly distributed with their long axis aligned along the direction of distension (Fig. 5A,C). This displacement of collagen fibrils continued for several minutes and finally resulted in complete fibril disarray. The muscle cells stretched in the direction of elongation (Fig. 5A,E). They became highly attenuated and very thin in cross section, reduced to only a few bundles of thick filaments. Tears appeared in their basal lamina, and they eventually ruptured. Similarly, the long axis of the axons and neurosecretory-like processes became aligned along the direction of distension (Fig. 5C). In the advanced stages of autotomy, rupture of the neurilemma was evident, but the vesicular contents of the axons appeared intact. Intact LDVs were prominent in autotomised tissues. A concerted search was undertaken for evidence of changes in dermal neuronal elements. Only one exocytotic-like profile was encountered (Fig. 5B).

Intact tendon

The P-L tendon formed a compact layer of collagen fibrils (30–40 nm in diameter) and unstriated microfibrils (10–15 nm in diameter) surrounding and infiltrating through the central region containing the tapered ends of the pharyngeal retractor muscle fibres (Figs 3D, 6A). The collagen fibrils were organised into short fibres that had a predominantly longitudinal orientation. Axons containing clear and dense vesicles and neurosecretory-like processes were abundant in the connective tissue (Fig. 6B–E). The retractor muscle fibres were arranged in bundles surrounded by a basal lamina (Fig. 6A,C), and adjacent myocytes were occasionally joined by desmosomes. Sarcoplasmic processes intermingled with axons in the middle of the bundles (Fig. 6C) and the two were often difficult to tell apart. The axons contained clear (90–100 nm in diameter), dense-cored (90–120 nm in diameter) and small dense (90–120 nm in diameter) vesicles (Fig. 6E). Processes containing LDVs were also often associated with muscle fibres (Fig. 6C). Although axons and LDV-containing processes were closely associated with the sarcolemma of adjacent myocytes, synaptic specialisations were not observed. Other cells in the tendon included phagocytes and secretory fibroblast-like cells (Fig. 6A). On its outer surface, the tendon was covered by the coelomic peritoneum (Figs 3D, 6A).

Fig. 6. Transmission electron micrographs of intact tendon. (A) The tendon is made up of a compact layer of collagen bundles (CF) surrounding a central region containing pharyngeal retractor muscle fibres (M) and overlain by the peritoneum (P). The peritoneal cells contain abundant lipid droplets (L) and extend microvilli (MV) into the coelom. Fibroblast-like amoebocytes (arrowheads) with prominent rough endoplasmic reticulum (R) were common. (B) Neurosecretory-like cell body (NC) vesicles and large dense vesicle (LDV)-containing process in the connective tissue adjacent to muscle fibres (M). (C,D) LDV-containing processes associated with myocytes (My) and collagen fibrils (CF). The LDV-containing processes vary in profile from small round (arrowhead) to elongate (arrow). S, sarcoplasmic process. (E) Axons in connective tissue containing a variety of small (SV) and large dense (LDV) vesicles. Scale bars: A, 2.0 μm; B–D, 1.0 μm; E, 0.5 μm (A and D are taken from Byrne, 1982, with permission; C is taken from Byrne 1985c, with permission).

Autotomising tendon

During autotomy, the P-L tendon changes from a compact collagenous structure to one in disarray caused by a decrease in tensility of the interfibrillar matrix (Fig. 7A). Comparison of similar fields of normal and autotomised tendon shows the fibril disarray resulting from autotomy (Figs 6A, 7A). Myocytes were disrupted, and the peritoneum peeled away from the connective tissue layer (Fig. 7A,B). Autotomised muscle fibres were no longer organised into bundles, and their contractile apparatus was no longer evident because of disintegration of the myofilaments (Fig. 7B). Sarcolemmal disruption was evident, and the contents of some muscle cells dispersed.

Axons and neurosecretory-like processes were present in the tendon through autotomy (Fig. 7A–D). The neurosecretory-like processes remained surprisingly intact, although some vesicular loss through mechanical damage was expected (Fig. 7B). In the advanced stages of breakdown, the presence of intact LDVs and axonal vesicles contrasted with the poor condition of associated muscle fibres. Axons were dispersed irregularly throughout autotomising tendons and, although they showed some membrane irregularities, their vesicles appeared largely unchanged (Fig. 7C). Despite extensive tissue disruption, many axons and LDV-containing processes maintained a close association with adjacent myocytes, suggesting that these couplings may represent neuromuscular specialisations (Fig. 7D).

Intact intestine

In cross section, the intestine–cloacal junction was typical of the holothuroid intestine, with the inner digestive mucosa, a central connective tissue layer and the outer longitudinal and inner circular muscle layers, enteric nerve plexus and peritoneum (Fig. 3E). The nerve plexus, positioned alongside the muscle cells, consisted of axons with a variety of dense and clear vesicle inclusions (Fig. 8A). Processes containing LDVs

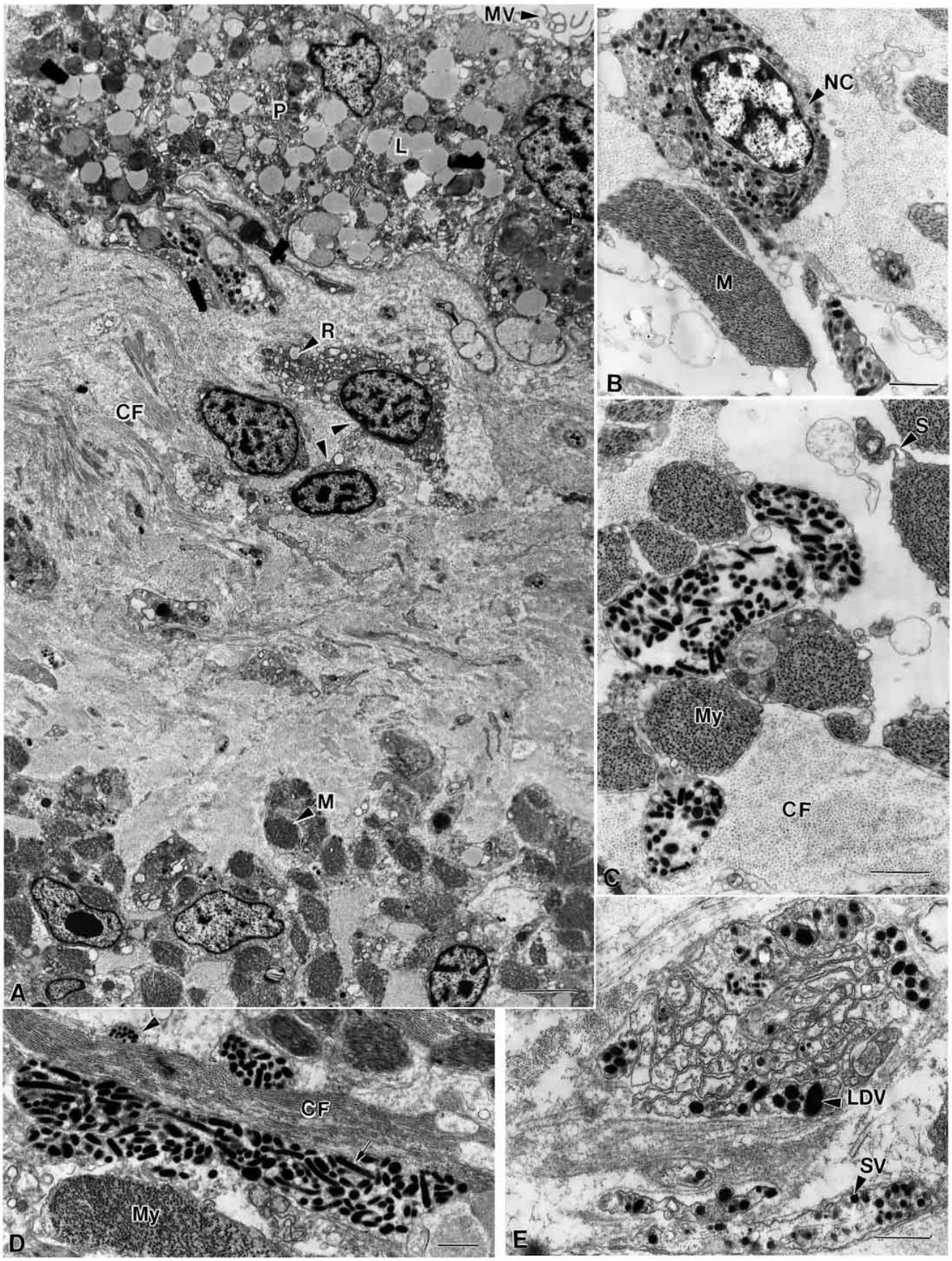

Fig. 6

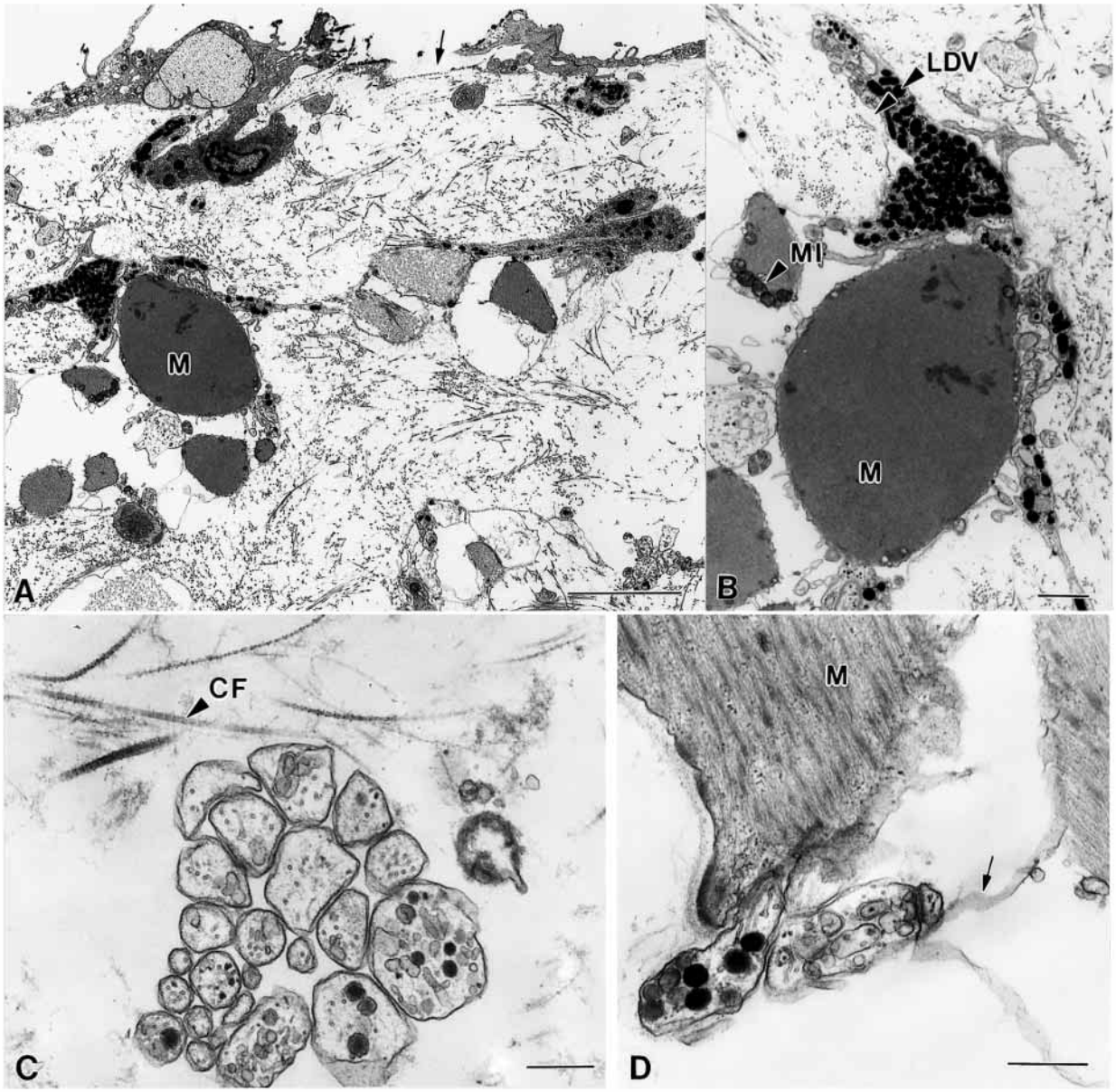

Fig. 7. Transmission electron micrographs of autotomised (A,B) and autotomising (C,D) tendon. (A,B) Collagen fibrils (compare with Fig. 6A) are in disarray and muscle fibres (M) lack a well-defined contractile apparatus. The connective tissue is bathed in coelomic fluid as a result of rupture of the peritoneum (arrow). The large dense vesicles (LDV) remain intact. MI, mitochondria. (C) During autotomy, some axons appeared somewhat swollen but still contained vesicles. CF, collagen fibrils. (D) Sarcoplasmic processes become swollen (arrow), but the LDV-containing processes remained firmly associated with the sarcolemma. Scale bars: A, 2.0 μm; B, 1.0 μm; C,D, 0.5 μm (A and B are taken from Byrne 1985c, with permission).

were present in the plexus among muscle cells and extended into the connective tissue (Fig. 8A). Sarcoplasmic processes mingled with the axons. The connective tissue layer was largely ground substance containing an abundance of unstriated microfibrils (4–10 nm in diameter) and a few collagen (20–40 nm in diameter) fibrils. The latter were most common along the epithelial basal laminae, where they were organised into small collagen fibres. Axons were rarely encountered in the connective tissue, but LDV-containing processes were common (Fig. 8A,B). Morula cells, phagocytes and other amoeboid cells were abundant. The intestine–cloacal junction had no specific morphological features distinguishing it from the adjacent intestinal region.

Autotomised intestine

The peritoneum, muscle layer and nerve plexus detached from the connective tissue layer during autotomy (Fig. 8C). Cells from these layers dissociated and dispersed into the coelom. As a result, the connective tissue layer was immersed in coelomic fluid. Internally, the mucosa remained intact. Similar fields of intact and autotomised intestine show the disorganised appearance of the connective tissue as the ground

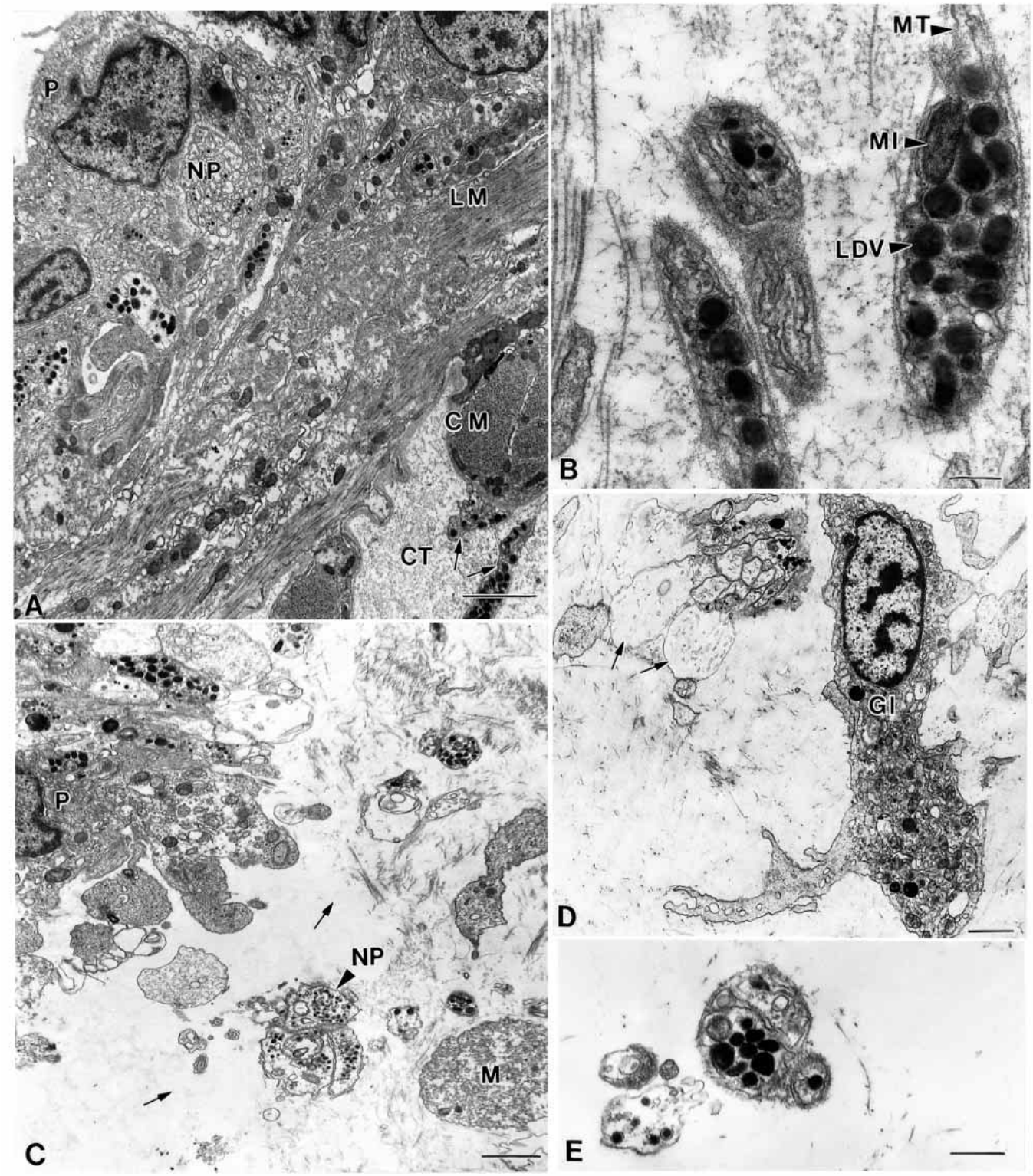

Fig. 8. Transmission electron micrographs of intact (A,B) and autotomised (C–E) intestine. (A) The peritoneal (P) layer has an extensive nerve plexus (NP) associated with the longitudinal (LM) and circular (CM) muscles giving rise to large-dense-vesicle (LDV)-containing processes (arrows) that extend through the connective tissue (CT). (B) The LDV-containing processes also have axial microtubules (MT) and mitochondria (MI). (C) In autotomised intestine, the peritoneum detaches and the nerve plexus (NP) fragments. The muscle fibres (M) disintegrate. Coelomic fluid infiltrates (arrows) the connective tissue. (D) The glial-like (Gl) cells separate from associated axons. Some axons appear intact, while others are swollen (arrows). (E) Large dense vesicles in the connective tissue remain intact. Scale bars: A, 2.0 μm; B, 0.25 μm; C,D, 1.0 μm; E, 0.4 μm (B is taken from Byrne 1985c, with permission).

substance lost its structural integrity (Fig. 8A,C). The ground substance changed from a granular matrix to one with a 'hydrated-dissolved' appearance similar to that seen in autotomised introvert LCT. The collagen fibrils remained intact. Some axons from the dissociated nerve plexus and neurosecretory-like processes from the connective tissue layer had a swollen appearance, and there was some disruption of the basal laminae around nerves (Fig. 8D). For the most part, however, the vesicular content of the axons and the LDVs appeared intact (Fig. 8C,E).

Discussion

The loss of tensility in the autotomy structures of *E. quinquesemita* is effected by a catastrophic decrease in connective tissue tensility. The collagen and microfibrillar network in the autotomy structures is characteristic of echinoderm connective tissues (Thurmond and Trotter, 1996; Wilkie, 1996). During evisceration, these fibrillar elements remained intact, but they and the cells associated with them dispersed in an apparently 'dissolving-hydrating' ground substance. These phenomena are characteristic of echinoderm autotomy (Motokawa, 1984; Wilkie, 1984, 1996). In addition to changes in the connective tissue, evisceration involved intrinsic muscle activity, including pharyngeal retractor muscle and longitudinal body wall muscle contraction. Whole-body muscle contraction accelerated the failure of the introvert and ejection of the viscera. Similar muscular activity occurs during evisceration in other holothuroids (Dolmatov, 1996). Intrinsic muscle contraction and disruption are also characteristic of asteroid arm autotomy (Wilkie et al., 1990, 1995). Autotomy in crinoids and ophiuroids, in contrast, involves only connective tissue (Holland and Grimmer, 1981; Wilkie, 1988, 1996). Although ophiuroid intervertebrate muscles detach from the skeleton during autotomy, this change is effected by dissolution of tendon connective tissue, not muscle tissue (Wilkie and Emson, 1987). In holothuroids, the final result of evisceration is extensive tissue and organ rupture, leading to loss of a large proportion of organism biomass. Holothuroid evisceration appears to be the most energetically costly form of autotomy in echinoderms. Asteroid autotomy also involves extensive tissue disruption and loss of energetically important organs (Wilkie et al., 1990; Wilkie et al., 1995; Mazzone, 1999).

Only a small proportion of holothuroids eviscerate, and the rationale underlying this behaviour has been speculated upon for some time (Emson and Wilkie, 1980). It is assumed to be defensive, although data to support this supposition are scarce. *E. quinquesemita* readily eviscerates in response to the presence of predatory sea stars, and this behaviour may also be used to expel enteric parasites (Byrne, 1985a,b). Seasonal evisceration in *E. quinquesemita* may be a mechanism to discard a waste-laden digestive tract (Byrne, 1986a). Newly regenerated intestines are yellow and, as digestion proceeds, they become progressively pigmented as brown body waste deposits (Fig. 3E) accumulate in the connective tissue.

In contrast to the homogeneous collagenous arm autotomy ligaments of ophiuroids and crinoids (Holland and Grimmer, 1981; Wilkie, 1988), the autotomy structures in *E. quinquesemita* are histologically complex, comprising several tissue types (nervous, muscle and connective). Asteroid autotomy structures also consist of several tissue types (Wilkie et al., 1990, 1995). The introvert is dominated by ground substance which, together with its muscle component, contributes to its dexterity. The abundance of ground substance is an important autotomy specialisation facilitating distension of the introvert during evisceration. The DCT is likely to have 'catch' properties that assist in the maintenance of tentacle posture during feeding. In its histological organisation into subepidermal, dense and loose connective tissue layers, the introvert is similar to the body wall of *E. quinquesemita* and other holothuroids (Menton and Eisen, 1970). In contrast to the body wall, however, there is a higher content of ground substance, and muscle fibres are present. The origin of these muscles is not known; they could not be traced to the inner body wall musculature.

The collagenous component of the P-L tendon is integral to its mechanical functions as a tendon and as an autotomy structure. In its intact state, the tendon plays a conventional role in forming a strong connection between two major muscles. During autotomy, contraction of the pharyngeal retractor and longitudinal body wall muscles provides mechanical tension to accelerate separation of these muscles after tensility changes have occurred in the tendon. Tendon autotomy can be mimicked *in vitro* through treatment with elevated $[K^+]$ (Byrne, 1985c). This treatment elicited strong retractor muscle contraction and softening of the tendon. Autotomy can occur in the absence of muscle contraction, as demonstrated by treatment of anaesthetised P-L tendon preparations with high $[K^+]$ (Byrne, 1985c). Strong contraction of *in vitro* preparations in response to acetylcholine was not followed by autotomy (Byrne, 1986b).

The intestine is typical of the holothuroid digestive tract, with the central connective tissue layer providing structural support and the muscle layer playing a major role in gut contraction (Rosati, 1968; García-Arrarás et al., 2001). Unlike the introvert and tendon, the intestine–cloacal junction did not differ from adjacent intestinal regions, and there were no obvious morphological specialisations for autotomy. There is, however, a sharp anatomical and histological change between it and the cloaca. The visceral autotomy plane may be a thin ring of dehiscence. Detachment of the gut is likely to be assisted by differential contraction of the gut and cloacal muscles.

Neurosecretory-like processes containing LDVs are common in echinoderm MCT and are thought to be involved in the control of mutability as a source of agents that effect connective tissue change (Motokawa, 1984; Wilkie, 1979, 1984, 1996). Despite extensive breakdown of the autotomy structures of *E. quinquesemita*, the neurosecretory-like processes remained largely intact, dissociating into the coelom with no apparent change in vesicle contents. This suggests that

LDVs are not a local source of agents that effect connective tissue change. Axons in the connective tissue also appeared to remain intact. The presence of swollen axons in autotomised tissues probably resulted from mechanical rupture. There was little or no evidence of change in any vesicular inclusions. It has been suggested that the exocytotic profiles and the reduced electron density of LDVs in juxtaligamental processes in the autotomising dermis of asteroids and autotomised ligaments of crinoids and ophiuroids are part of a secretion mechanism whereby release of vesicle contents leads to destabilisation of the extracellular matrix (Holland and Grimmer, 1981; Wilkie and Emson, 1987; Wilkie et al., 1990). Interestingly, some categories of neurosecretory-like granules remained intact through asteroid arm autotomy (Wilkie et al., 1990).

The LDVs in *E. quinquesemita* exhibited considerable morphological variability in size and shape and were associated with a range of tissue types (muscle, connective, nervous). It is likely that the LDV-containing processes include several cell populations with varying neurosecretory-type physiological functions. Those in the dermal region may be homologous to the granulated processes seen in the holothuroid body wall, which are thought to be involved in connective tissue catch. Several attempts have been made to categorise echinoderm neurosecretory-like cells on the basis of vesicle size, shape, histochemistry and potential MCT-stiffening and MCT-softening activity (Wilkie, 1984; Wilkie et al., 1990; Trotter and Koob, 1995; Welsh et al., 1995; Wilkie, 1996), but their variable profile makes this intractable. The association of LDV-containing processes with muscle and nervous tissue in *E. quinquesemita* and the presence of axial microtubules support the contention that they may be neuronal in origin. Synaptic connections between axons and juxtaligamental cells have been demonstrated in ophiuroid MCTs (Cobb, 1985; Cobb and Begbie, 1994). In contrast to *E. quinquesemita*, LDV-containing processes are associated only with autotomy connective tissue in ophiuroids, crinoids and asteroids and are the only morphologically detectable source of agents that may effect connective tissue change (Holland and Grimmer, 1981; Wilkie, 1979; Wilkie and Emson, 1987; Wilkie et al., 1990).

The neurosecretory-like processes of *E. quinquesemita* associated with muscle fibres could possibly have a neuromuscular function. In autotomised tendons, these processes maintained a tight adhesion to the sarcolemma of the myocytes despite extensive muscle disruption. This suggests that these associations may be neuromuscular specialisations, as suggested for similar profiles in other echinoderms (Cobb, 1978; Sugi et al., 1982). Coordinated neuromuscular activity is an essential component of evisceration, the control of which is probably different from that effecting connective tissue change and may involve the LDV-containing processes.

Pharmacological evidence suggests that evisceration and autotomy in *E. quinquesemita* are neurally mediated (Byrne, 1985c, 1986b) but, on the basis of morphological evidence, local neural elements do not appear to be a source of factors that effect connective tissue change. An evisceration factor(s) that induces autotomy in intact *E. quinquesemita* and in isolated preparations is present in eviscerated coelomic fluid (Byrne, 1986b). This factor was obtained from a number of holothuroids, including species that do not eviscerate (Byrne, 1986b). In all cases, high levels of activity were observed with peritoneal extracts. As suggested by Smith and Greenberg (1973), the evisceration factor may be a multi-functional molecule in holothuroids. Size fractionation of peritoneal extracts from *E. quinquesemita* indicated that the active fraction had a molecular mass approximating that of a peptide of 30–50 amino acid residues (M. Byrne, unpublished results), similar to the evisceration factor of *Thyone briareus* (Smith and Greenberg, 1973). This factor is likely to be a small peptide, potentially a neuropeptide. Several small peptides and proteins that influence the catch properties of the holothuroid body wall have recently been characterised (Trotter et al., 1996; Birenheide et al., 1998; Koob et al., 1999). The autotomy-promoting factor isolated from sea stars also has a peptide component (Mladenov et al., 1989). On the basis of changes in granule structure during catch and autotomy, several studies suggest that these active factors reside in the LDVs (Trotter et al., 1996; Wilkie et al., 1995; Birenheide et al., 1998). There was little or no evidence for this, however, in *E. quinquesemita*. Considering that the peritoneum was a major source of evisceration factor in *E. quinquesemita*, disruption of this layer and its associated nerve plexus during evisceration may result in the release of factors that modulate or enhance autotomy. If the evisceration factor and other factors that influence connective tissue mutability are neuropeptides, then detecting their presence and activity in tissue sections may require the generation of specific antibodies for use in immunocytochemistry. At the light microscopic level, the sea star neuropeptide GFNSALMHamide has been localised to neuronal cell bodies and varicosities (Moss et al., 1997; Byrne et al., 1999). The subcellular location has not, however, been determined ultrastructurally for any echinoderm neuropeptide.

Peritoneal dissociation occurred in all the autotomy structures, but it is not clear whether this was effected by physical rupture or by chemical factors. Regardless of the cause, loss of the peritoneum appeared to enhance the dissolution-hydration of the connective tissues by hydration or by infiltration of coelomic agents. By shunting coelomic fluid anteriorly, the general peritoneal lining of the body is not exposed to potentially damaging coelomic factors.

The presence of an endogenous evisceration factor in discarded coelomic fluid suggests neurosecretory or hormonal control of evisceration (Smith and Greenberg, 1973; Byrne, 1986a). Similarly, the presence of an autotomy-promoting factor in asteroids (Mladenov et al., 1989) suggests that arm autotomy may also be mediated by a coelomic factor. In contrast to the autotomy ligaments of ophiuroids and crinoids, holothuroid and asteroid autotomy structures are completely or partially bathed in coelomic fluid. As a result, there is potential for hormonal or neurosecretory activity using the perivisceral coelom as a conduit in these echinoderms. Holothuroid cells

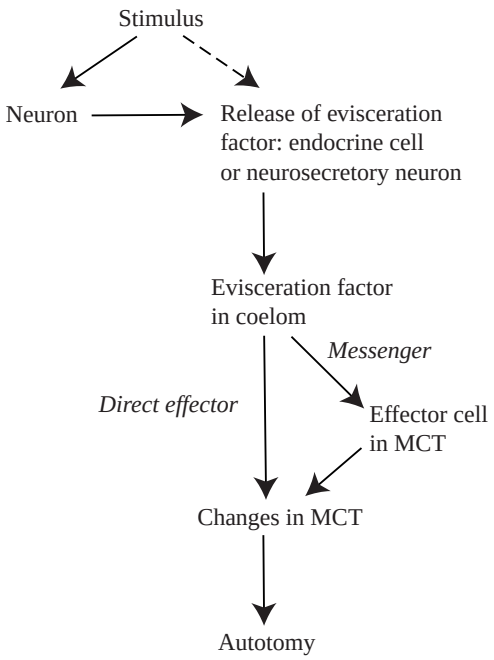

Fig. 9. Hypothetical sequence of events leading to autotomy in *Eupentacta quinquesemita* (taken from Byrne, 1986b, with permission). MCT, mutable connective tissue.

involved in autotomy, located at a distance from the MCT, may influence connective tissue breakdown and perhaps control muscle activity through release of factors into the coelom. As illustrated in Fig. 9, the evisceration stimulus may induce activity in upstream neurons or may act directly on evisceration-factor-producing cells. Once in the coelom, the evisceration factor may act as a direct effector of connective tissue change or it may act as a messenger substance that activates effector cells (Fig. 9). Alternatively, the presence of active factors in the coelom may be a result of evisceration, a stimulatory product released from damaged tissues. Coelomic fluid evidently plays a multifunctional role – as a source of hydrostatic pressure to effect introvert expansion and as a source of agents that effect connective tissue change and muscular responses.

Although the presence of evisceration factor(s) in dendrochirotids has been known for some time (Smith and Greenberg, 1973; Byrne, 1986a), their chemical nature is not known. We do not know whether the active component in tissue extracts is one or several molecules. Filling this gap in our knowledge is essential to understanding holothuroid autotomy, as is determination of the mechanism by which these factors effect connective tissue dissolution. Construction of antibody probes and application of immunocytochemistry to elucidate the origin of these factors will help to determine whether they reside in neurosecretory-like processes, as suggested for the peptides involved in body wall catch (Birenheide et al., 1998), or whether they are located outside the connective tissue, as suggested here.

On the basis of connective tissue specialisations, autotomy in echinoderms is without parallel in other phyla. Compared with other echinoderms, the processes underlying autotomy appear to be more complex in holothuroids. With the histological complexity of their autotomy structures, their elaborate behavioural response and their large coelom, it is not surprising that autotomy should differ in the Holothuroidea. There are, however, intriguing similarities between asteroid arm autotomy and holothuroid evisceration that warrant investigation. Like other echinoderms, autotomy in *E. quinquesemita* is controlled by an unconventional relationship between the nervous system and extracellular tissue, but the mechanism/triggers at the neuronal level are not known. There is strong evidence for the involvement of active factors distributed through the coelom that have a direct transmitter-like or neurosecretory-like mode of operation. The final outcome, expulsion of the viscera, is likely to result from a suite of factors that interact in a manner yet to be determined.

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