Characterization of Two Components of the Peripheral Nervous System of *Chelyosoma productum* and *Corella inflata*

by

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ABSTRACT

This study was undertaken to determine the characteristics of the dorsal strand complex in *Chelyosoma productum* and *Corella inflata*, and compare it to the 'cholinergic' visceral and branchial innervation.

Tunicates possess an extensive peripheral nervous system, one component of which has cell bodies in the neural ganglion and shows a strong cholinesterase activity. Previous researchers, however, describe another component: the dorsal strand plexus (dsp) that lies in close association with the dorsal strand. The dorsal strand is proposed to be involved in gonadogenesis and neuroblastogenesis, giving rise to the neurons of the plexus but the role of the plexus remains unclear.

The extent of the peripheral nervous system was mapped using histochemical and immunocytochemical techniques. A certain degree of overlap of innervation exists between the two components; however, the dsp is more localized around the dorsal strand and the reproductive tract. Immunocytochemistry, coupled with electron microscopy, reveals the GnRH-like peptide is localized in the dense core vesicles of the neurons and the neurites of the plexus. The dorsal strand plexus differs from the cholinergic system in having its cell bodies in the periphery, having no cholinesterase activity, its lack of reactivity with tubulin antiserum and its strong immunoreactivity with GnRH antiserum. The significance of these findings is discussed.

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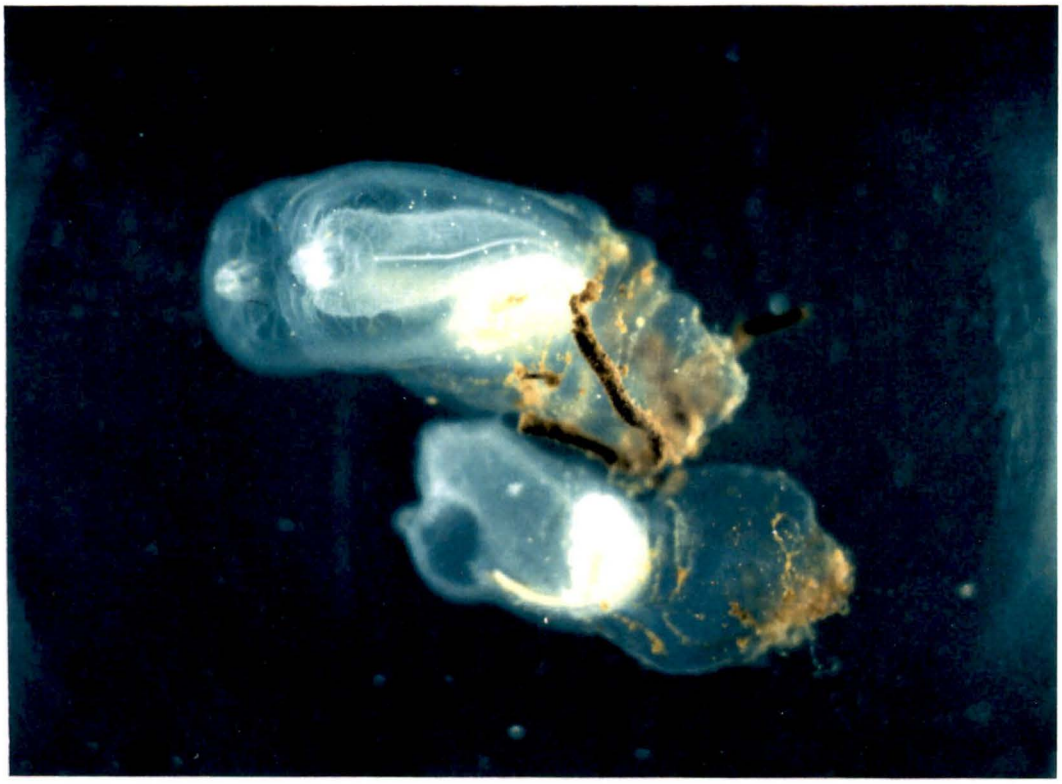

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DEDICATION

In memory of my father, Dr. Om Parkash, and my mother, Krishna Om Parkash.

INTRODUCTION

Tunicates are marine animals belonging to the subphylum Urochordata of the Phylum Chordata. The tadpole larva of these animals has attracted attention as it exhibits certain vertebrate characteristics: notochord, dorsal hollow nerve cord, pharyngeal gill slits and post-anal tail. However, this chordate organization is lost at metamorphosis in all tunicates except those belonging to the class Larvaceae. The presence of vertebrate characteristics during the larval stage suggests that tunicates are living representatives of ancestral animals belonging to a line of evolution that led from invertebrates to vertebrates.

Tunicates possess a rich cholinergic innervation of the branchial sac, mantle and muscles, as well as the viscera (Arkett et al., 1989; Florey, 1967). The cell bodies of the cholinergic nerves are present in the cerebral ganglion. There is evidence of the presence of yet another population of neurons (termed dorsal strand plexus) that shows a rich but localized distribution along the length of the dorsal strand of tunicates (Huus, 1924; Fedele, 1938; Millar, 1953). However, these neurons appear to be morphologically different from the cholinergic nerves; their cell bodies lie in the periphery, whereas the cell bodies of the cholinergic nerves are located in the cerebral ganglion.

The dorsal strand plexus is present along the entire length of the dorsal strand. Markman (1958) further traced these cells to the posterior proximity of the pericardium, to the oesophageal end of the endostyle and to the gonads in *Ciona*. Bone (1959) also observed this nerve plexus on the gonoducts in *Salpa*.

Sheath cells resembling ganglionic cells, present in close association with the dorsal strand itself, led earlier authors to propose that the strand was a part of

the peripheral nervous system (Kowalevsky, 1871; Julin, 1881; Metcalf, 1900; Lorleberg, 1907). Huus (1924), however, made a clear distinction between the dorsal strand and the sheath of cells around it. He proposed that the 'small nerve fibre' originates in the posterior part of the neural ganglion, in the root of the right siphonal nerve, whereas the dorsal strand originates from the neural gland. Huus further observed that, though the sheath lies in close proximity of the dorsal strand, there is no association between the two, except that they both accompany one another down the dorsal fold. Brien (1927 in Berrill, 1950) further confirmed the difference between the nerve sheath and the non-nervous nature of the dorsal strand. Although this nerve sheath has been termed 'small nerve fibre', 'raphael nerve', 'dorsal cord plexus', etc., to avoid confusion, it will be now referred to in the text as the dorsal strand plexus.

In 1980, Georges and Dubois found populations of cells in and around the neural ganglion of juvenile *Ciona* that are immunoreactive with mammalian anti-luteinizing hormone releasing hormone antiserum (LHRH, now termed GnRH). GnRH is present in all vertebrates, and acts as the master control hormone for reproduction. It usually occurs in two forms. One form is concerned with release of follicle stimulating-hormone and luteinizing hormone, while the other is thought to be a neurotransmitter or modulator in the brain and the dorsal root ganglia (Sherwood et al., 1993). Kelsall et al. (1990) found immunoreactive gonadotropin-releasing hormone in extracts of the neural complex of adult *Chelyosoma productum*, and reported tracts of GnRH-immunoreactive fibres and cell bodies in the brain and its roots. Furthermore, two forms of GnRH have been identified in *Chelyosoma*, Tunicate-I and Tunicate-II (Powell, 1995). The tunicate forms of GnRH are 60% identical to the mammalian form but have individual structures.

The presence of two 'subsets' of neurons and their projections raises several questions: (i) Are the peripheral cell bodies and their projections a part of the cholinergic system; or (ii) do these peripheral neurons constitute a completely separate component of the peripheral nervous system? Furthermore, (iii) are the GnRH-immunoreactive cells a part of the dorsal strand plexus?

This study attempts to characterize these two components of the peripheral nervous system: the visceral "cholinergic" system and the dorsal cord plexus. The hypothesis under consideration suggests that cells showing immunoreactivity to mammalian GnRH (Georges and Dubois, 1985; Kelsall et al., 1990) are a part of the dorsal strand plexus and involved in gonadogenesis and reproduction.

Two animals, *Chelyosoma productum* and *Corella inflata* were used for this study. Both the animals belong to class Ascidiacea, and represent the family *Corellidae*. Immunofluorescence techniques were used to map the extent of innervation by the dorsal strand plexus in both the animals and to compare its degree of overlap of innervation to that of the visceral nerve. The latter was studied using histochemical techniques. Using transmission electron microscopy (TEM), I have attempted to ascertain the extent of innervation of various structures in the animals. Also, following up on Irons' observations of dense core vesicles (dcvs) in the nerves innervating gonads of immature *Corella* (Irons, 1986), I observed similar vesicles in some axons lying in the vicinity of gonoducts of adult *Corella* and *Chelyosoma*. Using immunocytochemistry, I have attempted to determine whether the dcvs are GnRH-immunoreactive and contained in axons that constitute the dorsal strand plexus. These results will be compared to the innervation patterns of the visceral nerve observed using histochemical techniques.

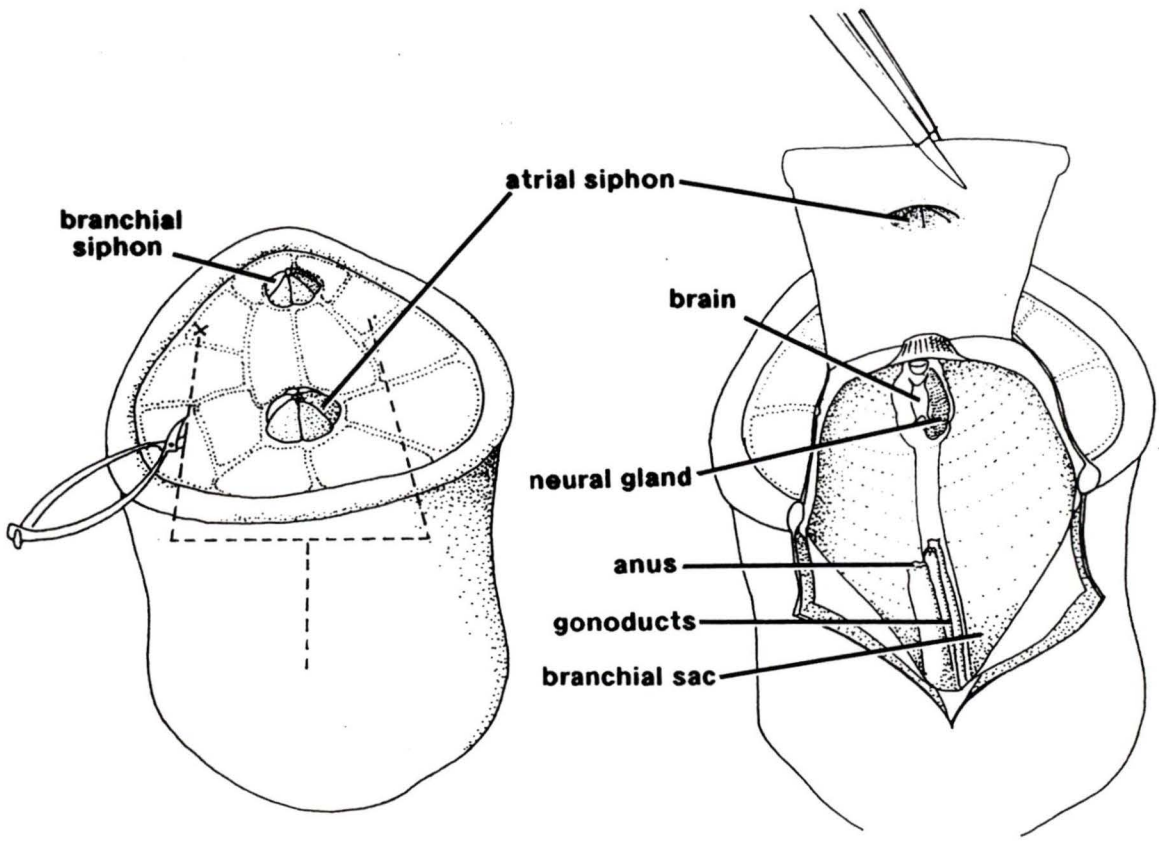
MATERIAL AND METHODS

(A) Tissue Preparation

Chelyosoma productum (Stimpson) and *Corella inflata* (Huntsman)(Plate I) for these investigations were collected off the Ogden Point breakwater, Fisherman's Wharf and Oak Bay Marina docks in Victoria, British Columbia, Canada. *Corella* can be found under floating docks, attached to kelp or other structures that remain permanently submerged. *Chelyosoma* was collected by divers using SCUBA. Animals were kept in a recirculating sea water system at 10° C and were used within two to three weeks of collection.

For the study of the nervous system of these animals, the dorsal fold region of the animal, which includes the neural ganglion, major posterior roots and the dorsal strand plexus, was dissected out. *Corella* possesses a transparent tunic that facilitates dissection of the animal. In these animals the tunic overlying the atrial pouch was cut and the tunic and the mantle covering the branchial basket subsequently removed. *Chelyosoma* possesses a leathery, opaque tunic and was dissected out in the following manner (Fig.1). Two parallel cuts were made on either side of the siphons, running from the atrial siphon to the branchial siphon. (Usually the atrial siphon can be distinguished from the branchial siphon as it lies further away from the lateral margin of the disc.) Following the initial cuts the disc margin on the atrial side was cut off. The disc was then lifted up and pulled out over the siphons, while the underlying adhering musculature was gently teased to free the disc. The neural ganglion and the neural gland lie immediately beneath the intersiphonal discs. Further dissection for both animals followed the same procedures. The overlying mantle was removed and the branchial basket was isolated by cutting the trabeculae that attach it to the mantle. The dorsal fold,

Figure 1. Drawings of anterio-dorsal view of *Chelyosoma* showing the anterior disc and the siphons. For dissecting out the neural complex and the dorsal fold region, two parallel cuts are made in the tunic on either side of the siphons, as shown in the figure. This is followed by cutting and lifting the anterior disc, carefully teasing away the attached tissue underneath. Approximate lengths of *Chelyosoma* and *Corella* used for this study ranged from 0.5cm to 4.0cm.


branchial siphon
atrial siphon
brain
neural gland
anus
gonoducts
branchial sac

including the ciliated funnel was cut out from the basket. This included the lengths of gonoducts and the rectum that are attached to the atrial surface of the dorsal lamina by a fold of the mantle.

The tissue was pinned out on Sylgard (Dow-Corning) lined Petri-dishes with fine cactus spines (*Opuntia: the spines are flexible and do not react with the chemicals*) and further trimmed down to limit the size of the tissue preparation. For visceral preparations, the tissue/organ under study was cleared of associated structures as much as possible while still attached to the animal.

(B) Structural Studies: Optical and Electron Microscopy

Whole mount preparations were examined live without staining by phase contrast and bright-field microscopy. For histological studies at the EM level, the dorsal fold region of the animal, including the ciliated funnel, neural ganglion, neural gland and associated tracts of the gonoducts was used. Tissue was fixed in 2.5% glutaraldehyde buffered with 0.4 M Millonig's Phosphate buffer at pH 7.3 for two hours. It was then rinsed in the same buffer for 15 minutes and post fixed in 1% osmium tetroxide in same buffer for an hour at 4°C. Tissue was briefly washed in distilled water and dehydrated using a graded ethanol series and propylene oxide. It was subsequently infiltrated overnight in 1:1 mixture of propylene oxide and Epon 812 (Luft, 1961) and then embedded in pure Epon and polymerised at 60 °C for 18 hours. Thick (1μm) and thin sections were cut on a Reichert OMU2 ultramicrotome with glass knives prepared on LKB 7810B Knifemaker. For light microscopy, thick sections were stained with Richardson's stain (Richardson et al.,1960). Thin sections were collected on 200 mesh copper grids, stained with 2% uranyl acetate in double distilled water, pH 4.5, for one hour. Grids were washed in double distilled water and further stained in a 0.2% aqueous solution of lead

citrate for eight minutes. Grids were examined using a Phillips EM 300 and a Hitachi H-7000.

(C) Cholinesterase Histochemistry

For cholinesterase histochemistry of the dorsal fold preparations, gonoducts, rectum and pericardium, the tissue was fixed in 10% buffered formalin, pH 7.4, for two hours at room temperature. After fixation, the tissue was rinsed in three changes of 0.1M sodium phosphate buffer, pH 7.4 over a period of a further two hours. This was followed by treatment with a solution of 0.5 mg of acetylthiocholine iodide dissolved in 6.5 mls of 0.1 M sodium phosphate buffer, pH 6.0. To this solution was added, in sequential order with shaking, 0.5 ml 0.1 M sodium citrate, 1 ml 30 mM copper sulphate, 1 ml distilled water and 1 ml 5 mM potassium ferricyanide [Karnovsky and Roots, (1964), as modified by Arkett et al. (1989)]. Incubations were carried out for a period of four to eight hours at room temperature. The reaction was stopped by a brief rinse in distilled water. The tissue was then dehydrated using a graded ethanol series, cleared in xylene and mounted in Histoclad. Weights were placed on the coverslip to flatten out the tissue while drying at 50°C.

(D) Immunolabelling: Optical and Electron microscope Techniques

Immunocytochemical studies of the dorsal strand plexus and the visceral roots were carried out at the light microscope level. Biotin/Strepavidin results were further processed for examination at the electron microscope level. Branchial sac preparations were pinned out on Sylgard lined Petri-dishes with the atrial side facing down. This permitted better penetration of the antibodies on the branchial

surface. Preparations were trimmed to limit the tissue to the dorsal lamina and the portion of the branchial basket in its immediate vicinity. Thicker wholemount preparations were stretched out to permit better viewing. The Petri dishes were lined with rolled moist Kimwipes to maintain a high ambient humidity level and prevent dehydration of the tissue.

(i) Light Microscopy:

The following fixatives were tried for immunocytochemistry: (i) Zamboni's fixative (Zamboni and DeMartino, 1967), pH 7.3, (ii) 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.3, (iii) 10% buffered formalin in 0.1 M sodium phosphate buffer, pH 7.4, and (iv) 4% paraformaldehyde in 0.2 M cacodylate buffer pH 7.2 containing 0.1% glutaraldehyde. Satisfactory preservation of antigenicity and general anatomy for light microscopy and electron microscopy resulted from use of 4% paraformaldehyde in 0.2 M cacodylate buffer containing 0.1% glutaraldehyde. Zamboni's fixative enhanced penetration of solutions and amplified peroxidase-DAB results. This is due to leakage of cell contents into the extracellular space. However, Zamboni fixed tissue showed poor preservation of cellular components under an electron microscope.

The tissue was fixed for two to three hours at room temperature. The tissue preparations were rinsed in four changes of 0.1 M Phosphate Buffered Saline (PBS) containing 0.3% Triton-X (PBS+Tx) for two hours. Following rinsing the tissue was incubated in blocking serum (10% Goat serum in PBS+Tx) for one hour. It was then directly incubated overnight in the primary antibody. The following primary antibodies were tried: Bla-4 and Bla-5 prepared in rabbit against lamprey GnRH, GF-6 prepared in rabbit against catfish GnRH, MGnRH prepared in rabbit against mammalian GnRH (*courtesy: Dr. N. Sherwood, UVic);* and,

U705-23 prepared in rabbit against mammalian LHRH (*courtesy: Gerry Kozlowski, University of Texas, Dallas, U.S.A.*); anti-tubulin (5-A6) (*courtesy: Dr. D.L. Brown, University of Ottawa, Canada)* (also see Crowther and Whittaker, 1992 for anti-tubulin studies on tunicates). The primary antiserum was used in a 1:100 dilution in PBS containing 10% goat serum and 0.3% Triton-X. Incubations were carried out at room temperature on an orbital shaker. Following incubation in the primary antiserum the tissue was rinsed in three to four changes of PBS+Tx, 30 minutes each, on a shaker. The preparation was then incubated overnight in the dark, with FITC conjugated goat anti-rabbit antibody (Sigma) in 1:100 dilution in PBS+Tx containing 10% goat serum. Incubations with U705-23 were carried out at 4°C. Controls were run using the same protocol, but (i) omitting the primary antibody, and (ii) using primary antibody that was preabsorbed with M-GnRH (*courtesy: Jean Rivier, Salk Institute, La Jolla, U.S.A.*). The wholemount was then thoroughly rinsed in 3-4 changes of PBS (30 minutes each), mounted in glycerol containing sodium gallate and observed using a Leica Aristoplan equipped with incident fluorescence optics and appropriate filters for flourescein.

For cryosectioning the anterior portion of the dorsal fold, including the neural ganglion, neural gland and the ciliated funnel and lower posterior portion of the dorsal fold along with the associated gonoducts and rectum was used. The tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.2, for two hours at room temperature. After fixation the tissue was rinsed in four to six changes of the same buffer over a period of three hours. The tissue was cryoprotected overnight in 30% sucrose in PBS and then mounted in OCT mounting media (Miles inc., U.S.A.) and frozen in a -86°C freezer. Cryosections, 10-15 μm thick, cryosections were cut at -27°C on a IEC cryostat model CTI and thaw-mounted on poly l-lysine coated slides. Nail polish was used

to make wells around the sections to prevent the solutions from running. All incubations were carried out in a moist chamber consisting of wide Petri dishes lined with PBS dampened filter paper, at 4°C.

The slides were rehydrated in PBS and then incubated in 10% goat serum for one hour. This was followed by incubation in the primary antiserum (U705-23 in 1:100 dilution in PBS containing 0.3% Triton X-100 and 10% goat serum) for two hours at room temperature or 24 hours at 4°C. Slides were rinsed in PBS, three changes 10 minutes each. The slides were subsequently incubated with biotinylated goat anti-rabbit antibody (Calbiochem. Biotin/Strepavidin Immunostaining Kit #568112) in 1:50 dilution in PBS, for one hour. After rinsing in PBS, three changes 10 minutes each, the slides were then incubated in strepavidin-peroxidase conjugate for another hour, followed by rinsing in PBS, four changes 10 minutes each. Reaction product was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Zymed Labs. Inc. Kit #: 00-02014) for five to ten minutes. Reaction was stopped by immersing the slides in distilled water. Slides were then dehydrated in graded ethanol series and mounted in histoclad. In control slides, the primary antiserum was omitted.

(ii) Electron Microscopy:

Tissue containing reaction products visualized using DAB was further processed for EM observations. The tissue was treated with 1% osmium tetroxide in PBS for 45 minutes. After a brief rinse in distilled water the tissue was dehydrated, using graded ethanol series and propylene oxide. After overnight infiltration in Epon 812 and propylene oxide in 1:1 ratio, the tissue was embedded in pure Epon and polymerised at 60°C for 24 hrs. Thick sections were used to determine the labelled areas and to further trim the block for thin sections.

Unstained thin sections and thin sections stained with 2% uranyl acetate in double distilled water, pH 7.5, were examined on a Hitachi H-7000 electron microscope. These sections were not stained with 0.2% aqueous solution of lead citrate, as lead citrate staining makes it difficult to differentiate the DAB label from other cellular inclusions.

(I) Anatomy of the Peripheral Nervous System

(A.1) The Cholinergic System-Branchial and Somatic Innervations: Previous Findings

(A.1a) Review of Anatomy of the Animals

This section reviews the general anatomy of *Chelyosoma* and *Corella*, and introduces the morphological similarities and differences that exist between the two animals.

The morphology of *Chelyosoma productum* Stimpson has been previously described in detail (Bancroft, 1898; Berrill, 1950). This ascidian is typically characterised by a dorsal, distinctly bounded, disc-like modification of the tunic, consisting of several immovable plates. Movable plates, usually six, surround each siphon. In addition, two central, or intersiphonal plates, lie between the atrial and the branchial siphons. These are commonly used landmarks for dissecting out the underlying neural complex. In appearance, the tunic of juveniles is translucent, but becomes brownish yellow in adults and develops a leathery consistency. The viscera are enclosed in a deep yellow mantle that attaches to the anterior disc by several muscles.

Corella inflata Huntsman is a solitary, ovo-viviparous ascidian of the family *Corellidae*. The tunic in these animals is noticeably transparent and lacks the leathery texture seen in the tunic of *Chelyosoma*. In addition, the body is elongated and more or less laterally compressed. *Corella inflata* also possess a definite pouch-like outgrowth of the atrial cavity towards the anterio-lateral atrial side, which serves as a brooding chamber for the developing tadpoles (Lambert, 1981).

Despite the external morphological differences, the internal anatomy of *Chelyosoma* and *Corella* are relatively similar (Berrill, 1950). The buccal siphon opens into a dilated pharynx, termed the branchial sac. Immediately posterior to the siphonal opening, the pharynx is lined by a row of peripharyngeal tentacles with a peripharyngeal band encircling the pharynx below these tentacles. This peripharyngeal band connects to the endostyle, which occupies the mid-lateral, branchial side of the branchial sac. The dorsal blood vessel lies on the mid-lateral surface on the atrial side of the branchial sac and extends from the roof of the pharynx to the level of the oesophagus. The walls of the branchial sac also possess several transverse and longitudinal blood vessels that are continuous with the dorsal blood vessel. At its anterior end, the dorsal vessel is dilated and encloses the neural ganglion and the neural gland.

The wall of the branchial sac is characterized by perforations called stigmata. Essentially, each stigma is lined by seven rows of ciliated cells, with the cilia projecting into the stigmatal opening. In *Corella* each stigma is curved and spirals towards the centre. Stigmata in *Chelyosoma* are comparatively smaller. The beating of the cilia lining the stigmata sets up a water current from the outside flowing through the branchial siphon into the branchial sac, and from within the sac to the atrial cavity. A moving sheet of mucous lining the inside of the branchial sac entraps minute organisms as the water is forced out of the sac through the stigmata. This sheet of mucous is continuously rolled up dorsally and ingested via the oesophagus.

On the mid-dorsal side of the branchial sac lies the dorsal lamina, a structure formed of series of languets that project into the branchial cavity. The dorsal lamina extends from below the anterior dilation of the dorsal blood vessel to

the opening of the oesophagus. The oesophagus leads into the stomach, which further opens into a looped gut.

The gonads are contained within the loop of the gut. Ascidians are monoecious and the ovaries and testes occupy overlapping territories. The gonoducts and the rectum collectively run anteriorly, adjacent to the atrial surface of the dorsal blood vessel, to the approximate mid-length of the branchial sac. Here they terminate into the respective gonopores and the anus.

The neural complex in these animals consists of the neural ganglion and the neural gland. In association with the neural gland is the ciliated funnel, which opens into the prebranchial part of the pharynx and connects with the gland by a duct. Various paired and unpaired nerve roots exit the brain and innervate the branchial sac and the viscera.

The innervation pattern of the branchial sac and the viscera by the peripheral nervous system has been studied by Arkett et al., (1989). The dorsal visceral root exits the brain posteriorly, running down the dorsal blood vessel and extending primary bundles into the branchial sac. The primary bundles further branch repeatedly within the blood vessels of the branchial sac. Single axons separate from these secondary and tertiary bundles and course though the branchial walls, terminating as synaptic boutons at the base of the ciliated cell clusters lining the individual stigma. Innervation of gonoducts, rectum, pericardium and gut by the visceral nerves has also been observed. It must be noted that the mantle innervation has not been well mapped, as the mantle is a thicker structure that is not readily penetrated by the reagents used in nerve staining.

Terminology of major roots

The nervous system of *Chelyosoma* and *Corella* consists of the neural ganglion and its projections into the viscera, and the dorsal strand plexus. Sensory cells with their cell bodies in the mantle epidermis have also been described in various tunicates (Bone and Mackie, 1982) but their distribution in Corellidae has not been explored. The neural ganglion itself lies within the anterior dilation of the dorsal blood sinus, midway between the atrial and the branchial siphons. Two major sets of roots exit it (Fig. 2). These can be designated as atrial (A) or branchial (B), depending on their orientation with regard to the two siphons. In both the animals, 'anterior' is towards the branchial siphon, 'posterior' towards the base of the animal, 'ventral' towards the endostyle and 'dorsal' towards the opposite side of the animal. In *Chelyosoma* two branchial medial roots (BM) extend anteriorly and diverge to either side of the ciliated funnel. These are designated as BM1 and BM2, the former being farthest from the neural gland. Two anterio-lateral smaller bundles, BL1 and BL2, extend from the neural ganglion to the mantle. Posteriorly, a set of three atrial median (AM) roots are present in both animals and are designated AM1, AM2 and AM3. Of these, AM3 extends down the dorsal blood vessel and bifurcates into two major bundles. These are jointly referred to as the visceral nerve roots.

(A.1b) Histochemical Studies

The ciliated cells of the ascidian branchial sac are innervated by branches of the visceral nerve. Cilia of these ciliated cells, lining the stigmata, are continuously beating, thus generating a current of water through the branchial sac. Sudden arrests of the rhythmic beating of these cilia have been observed in ascidians and is

a response to general vibrations, or foreign material entering the basket (Bone and Mackie, 1982). These ciliary arrests are accompanied by contractions of the siphons, branchial sac and the mantle, which generates squirting of water from the branchial siphon. In 1974, Mackie et al. found that an application of d-tubocurarine to ascidian branchial basket preparations blocks ciliary arrests. Arkett (1987) confirmed these findings and suggested acetylcholine (ACh) as a primary neurotransmitter at the neuro-ciliary synapses.

The presence of ACh has been reported in the neural ganglion (Florey, 1963). Researchers have also observed that muscles respond to application of exogenous ACh (Florey, 1967; Takahashi et al., 1973). Arkett et al. (1989) found a strong histochemical reaction for cholinesterase (an enzyme that breaks down acetylcholine at cholinergic synapses) in the neural ganglion and its projections. This technique made it possible to investigate the innervation pattern of the branchial basket in *Chelyosoma*. My own findings confirm and extend these results (section A.2a).

All these findings support the view that the branchial innervation is cholinergic; it will be referred to as such in the present account.

(A.2) The Cholinergic System: New Findings

(A.2a) Optical and Histochemical Results

Preparations of the neural ganglion and various regions of the body treated for cholinesterase show overall intense neural staining. Axons appear well defined with uniform staining and are characterized by varicosities along their entire length.

Cholinesterase-positive staining was observed in all peripheral projections of the neural ganglion. The anterior roots, BM1 and BM2, project anteriorly towards the branchial siphon. Root BM1 extends into the mantle to the anterior left side of the neural ganglion and runs around the branchial siphon in the vicinity of the peripharyngeal band. The main bundle branches repeatedly along its length and the branches and individual axons spread into the branchial mass (Fig. 3). Although there is a rich innervation of the prebranchial region of the sac and the siphon, no axons could be traced in the branchial tentacles. Some axons do extend up and around the ciliated funnel. In *Corella*, cholinesterase-positive axons are present in the vicinity of the orange spots (referred to as "ocelli" by some writers) that flank the lobes of the branchial siphon (Fig. 4). However, it is still unclear if these structures are innervated; individual axons certainly are present 'below' and 'above' the spots, but no synaptic boutons have been seen. Branches of the anterior roots also enter the mantle over the anterior portion of the branchial sac (Fig. 6). Axons ramify in a loose arrangement and innervate the mantle musculature, forming distinct motor end plates as described by Nevitt and Gilly (1986) (Fig. 7). Similar end plates also appear on the prebranchial muscles.

In both *Corella* and *Chelyosoma*, three major nerve bundles leave the ganglion from the posterio-dorsal side (i.e. on the side facing the atrial siphon) of

the brain. Of these, root AM3 bifurcates into two bundles that run posteriorly within the dorsal blood vessel. A band of muscle is also present, running parallel to the visceral bundles, and is innervated throughout its entire length by the cholinesterase-positive axons extending from the visceral bundles.

Positive chemical reaction for cholinesterase in the primary bundles that enter the branchial sac and their branches reveals the richness of innervation of the organ. One pair of primary bundles (1°) separate from the visceral bundles in *Chelyosoma*, but in *Corella* several primary bundles extend into the branchial basket along the entire length of the visceral roots. Secondary bundles (2°) branch off from these 1° bundles at somewhat regular intervals and run perpendicular to them, extending towards the branchial siphon and the oesophagus respectively. The secondary bundles course through the blood sinuses of the basket and further separate into single axons. These axons run on the atrial side of the basket and extend down the coils of the stigmata (Fig. 5). Most axons bifurcate, or in some cases trifurcate, and these endings innervate the ciliated cell clusters that line the stigmatal opening. Axonal branches extend to adjacent cell clusters and terminate at their base in distinct synaptic boutons. A clear space, resembling a vesicle, is usually present in the centre of each bouton. This has also been observed in electron micrographs of the stigmatal region.

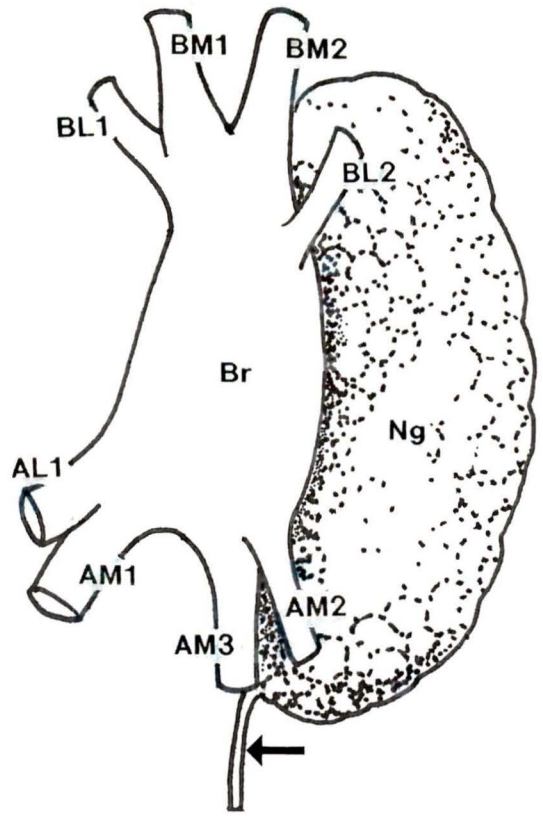
Although some colouration appears in the endostyle following the histochemical procedures, no axons are discernable in the structure. However, on its anterior surface, at the junction of the peripharyngeal band and the endostyle, axons have been traced in the walls of the endostyle .

Outside the branchial sac, a rich network of cholinesterase-positive neurites is seen over the surface of the oesophagus and the stomach. Innervation of the gut has both been reported by Arkett et al. (1989) and observed during my studies.

Figure 2. Diagram showing the dorsal view of the neural complex of *Chelyosoma* showing the various roots emerging from the ganglion and the dorsal strand (*arrow*). The organs are not represented in their true proportions. (*Br: neural ganglion; Ng: neural gland; AL: atrio-lateral; AM: atrio-medial; BL: Branchio-lateral; BM: Branchio-medial.*)

Figure 3. The neural ganglion in *Corella* along with its anterior projections after staining with cholinesterase. Root BL1 enters the mantle covering the prebranchial mass. The richness of innervation by the cholinergic nerves is evident. (*Br: neural ganglion; Bs: Branchial sac; AL: atrio-lateral; AM: atrio-medial; BL: Branchio-lateral; BM: Branchio-medial.*)

2


BM1
BM2
BL1
BL2
Br
Ng
AL1
AM1
AM2
AM3

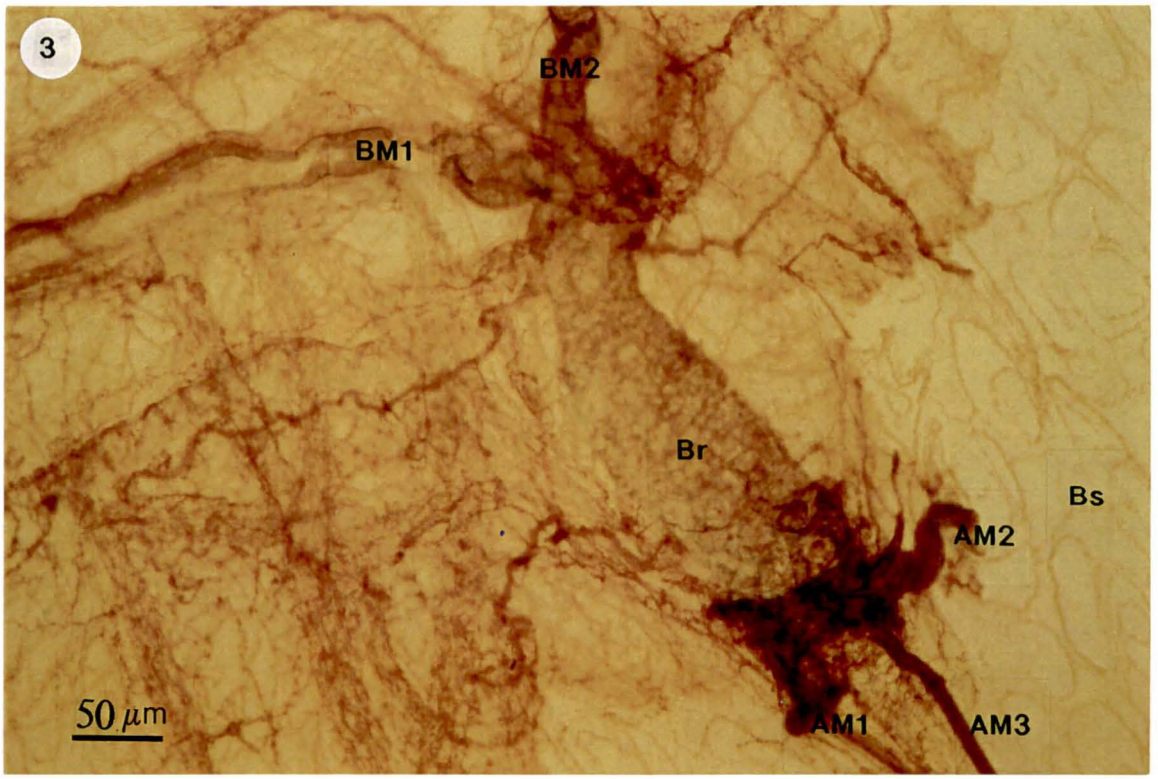
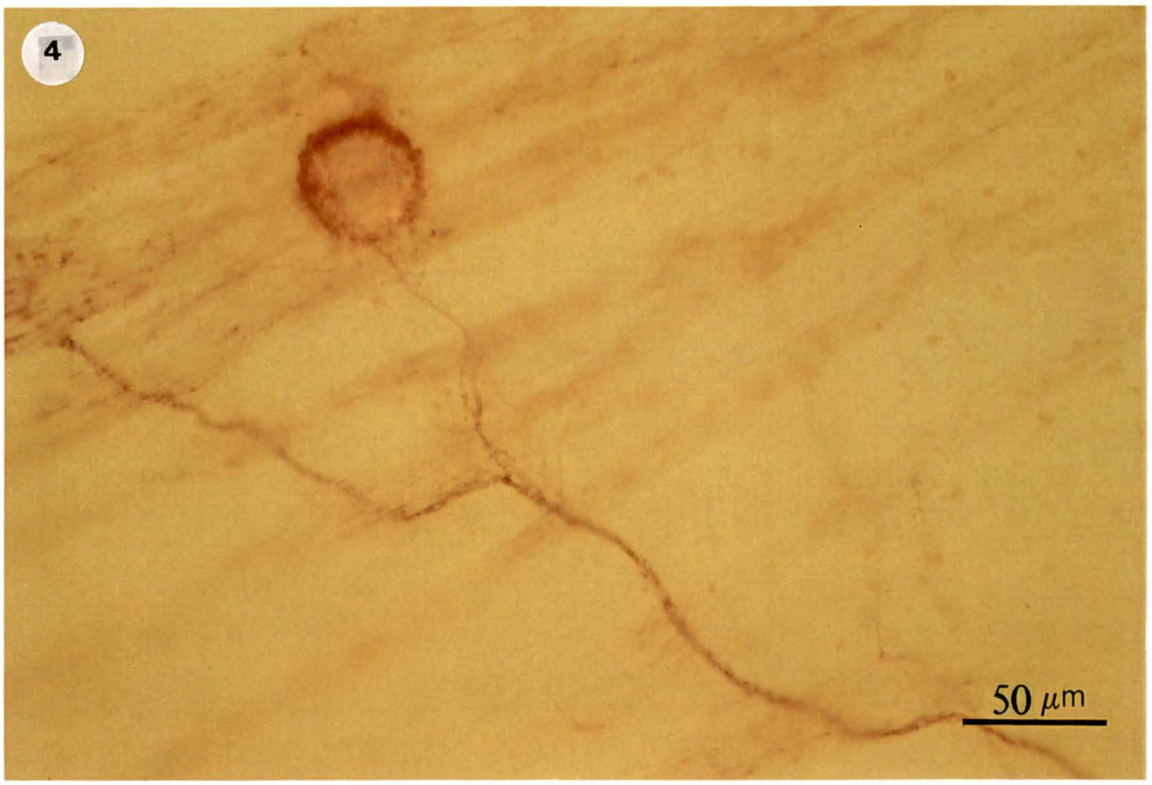
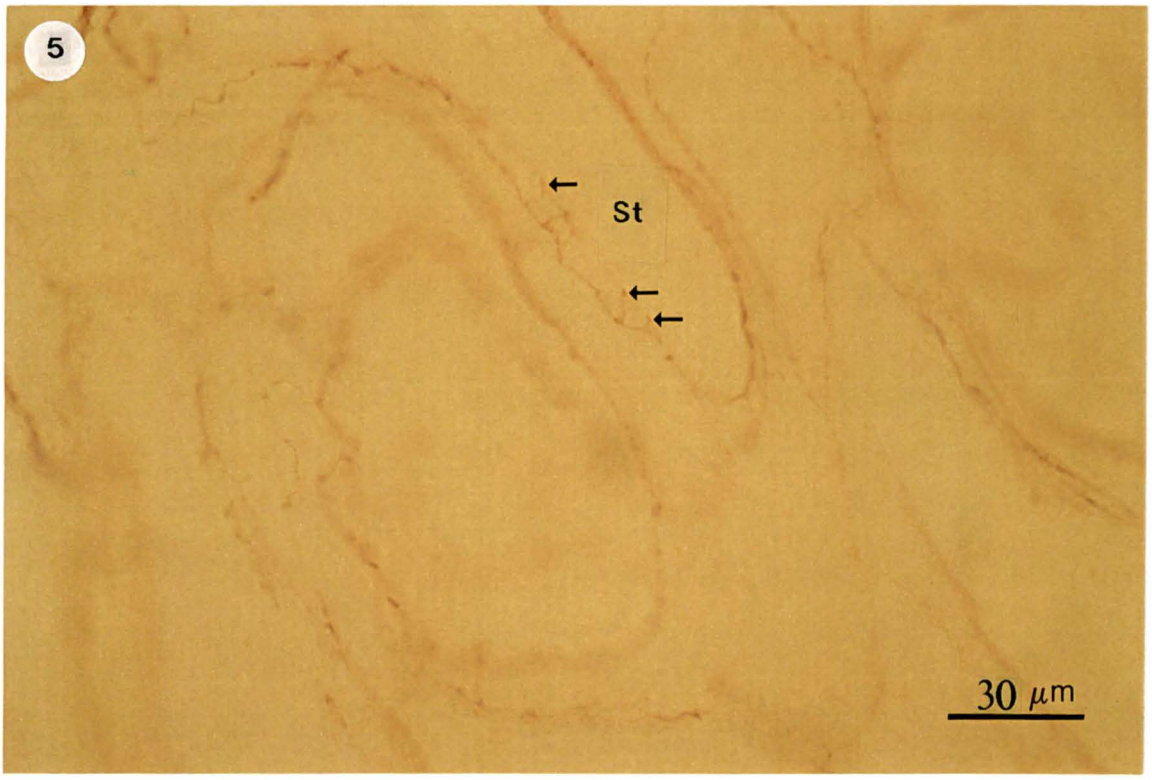

3
BM2
BM1
Br
Bs
AM2
AM1
AM3
50 μm

Figure 4. Cholinesterase-positive axons extend close to the pigmented spot in *Corella*. Although axons are seen in very close proximity to these spots, no contact between the two has been observed.

Figure 5. Innervation of the ciliated cell clusters in the branchial sac of *Corella*, visualised by staining for cholinesterase. Synaptic boutons (*arrows*) can be observed around the stigma (*St*).


4
50 μm


5
St
30 μm

Unfortunately, the gonads of a mature animal completely envelop the gut, making it difficult to clear the gut without damaging the overlying network of neurites. The rectum and associated gonoducts also are ensheathed in rich ramifications of the cholinergic axons. It appears that the axons run within the mantle sheath and not directly over the gonoducts. Examination of the rectum, on the other hand, shows the neurites spread over its surface.

It is difficult to obtain a consistent reaction for cholinesterase in *Chelyosoma*, as compared with staining in *Corella*. This is probably due to the thicker mantle and epithelial layers present in adult *Chelyosoma*. Incisions made into the wall of the dorsal blood sinus prior to fixation and/or after fixation aided penetration of the reagents. The branchial basket in *Corella* provided good results for cholinesterase histochemistry.

(A.2b) Immunocytochemical Results (anti-tubulin)

Incubation of the neural complex with anti-tubulin revealed a rich array of microtubular tracts within the ganglion and in its major roots (also see: Crowther and Whittaker, 1992). These tracts represent the cytoskeleton of individual neurites. In the ganglion itself, individual cell bodies are outlined. The cell bodies occupy a peripheral region (Figs. 8, 9). Although the neurons are scattered throughout the cortex of the ganglion, they are absent around the region of the roots. From each neuron at least one major process projects into the neuropil. Aggregates of the microtubular tracts of the neurites, aligned parallel to each other, exit as the respective roots. However, it is not possible to predict if neurons lying in the vicinity of a root contribute their processes to the root. The labelled microtubular tracts can be further traced as the primary, secondary and tertiary

bundles coursing through the branchial sac. Tracts are also discernible, even in the individual axons that extend to the base of the ciliated cell cluster.

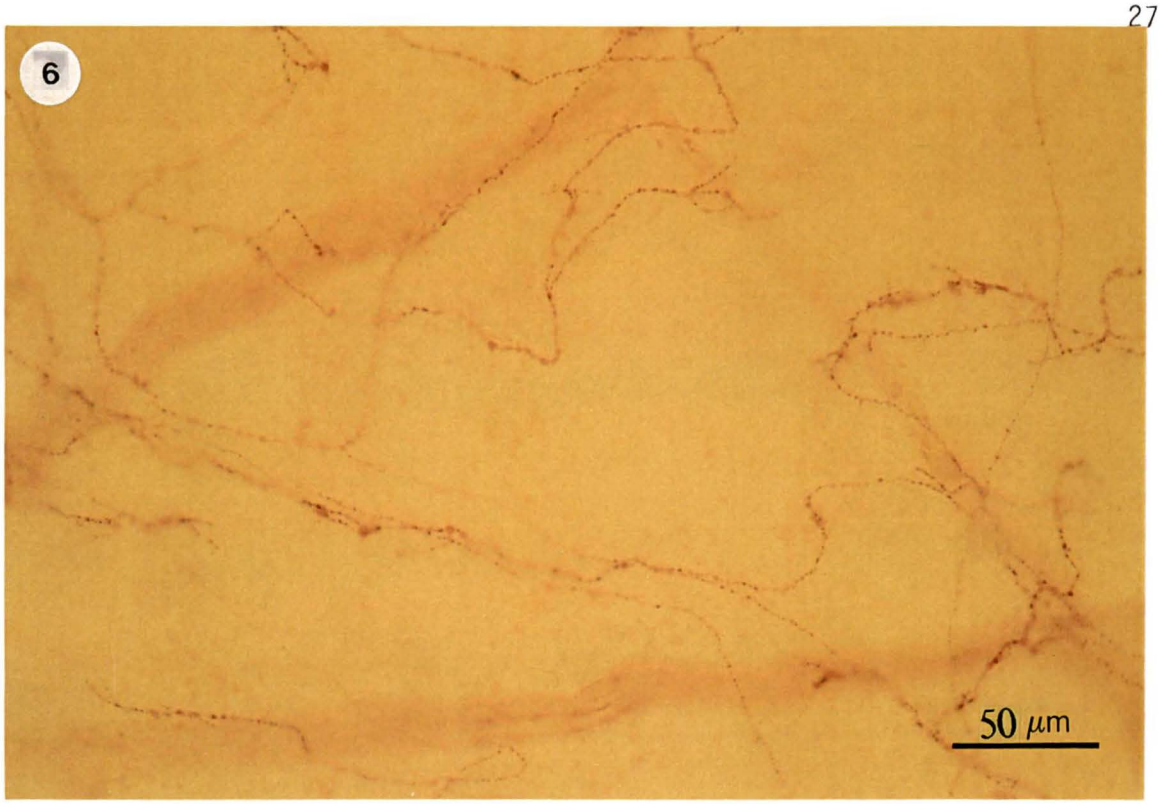
In the walls of the branchial sac much label is concentrated in the cilia of the stigmatal cell cells, but little is seen elsewhere. The gonoducts, especially the sperm duct, show a high degree of tubulin label in their ciliated inner wall. In the sperm duct the label is amplified by the presence of sperm, which are aligned to the longitudinal axis of the duct. Due to the presence of much labelling within the gonoducts, any axons lying over it are indiscernable. However, nerve bundles that run close to the gonoducts can be seen, by their labelled microtubular tracts. The background in this region lacks cells high in tubulin, thus providing a sharp contrast. This enables examination of the tubulin content in the cholinergic nerves and the GnRH-immunoreactive cell bodies and processes, and hence aids in distinguishing between the two.

(A.2c) Summary of Results

The cholinergic system comprises the neural ganglion and its peripheral projections. All major roots from the ganglion show the cholinesterase reaction. Anterior to the ganglion, the branchial siphon and its musculature is innervated. Some stain was visible in the branchial tentacles, but no axons could be discerned. In *Corella* the orange pigmented spots that flank the upper rim of the branchial siphon show presence of cholinergic axons in their immediate vicinity, but no clear innervation of these structures has been observed. Cholinergic axons also extend over the ciliated funnel. Branches of the anterior roots and the lateral roots project upwards into the mantle. The mantle is traversed by several cholinergic axons, that form distinct terminal boutons on the muscles of the mantle. No neuronal cell bodies were seen in the mantle.

Figure 6. Cholinergic axons in the atrial side mantle of *Corella*. Muscles can be seen below the plane of focus.

Figure 7. A cholinergic axon innervates a muscle in the mantle (*arrow*), forming a distinct end plate.


6
50 μm

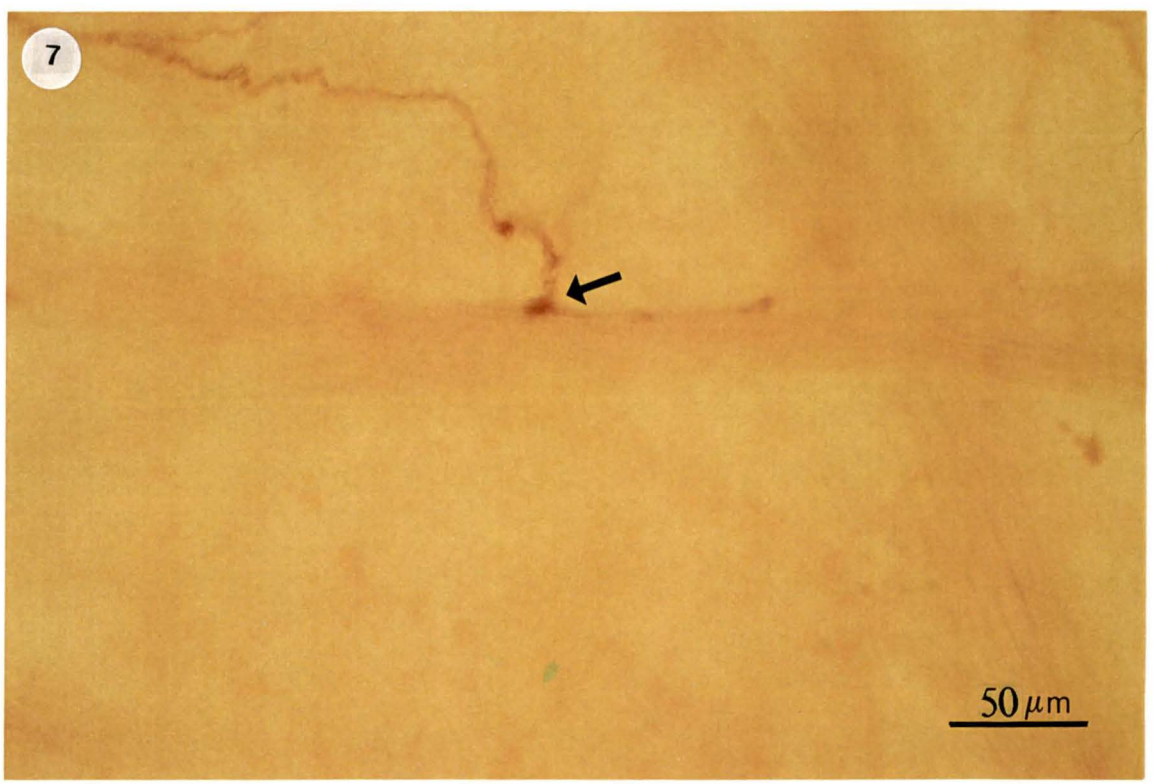
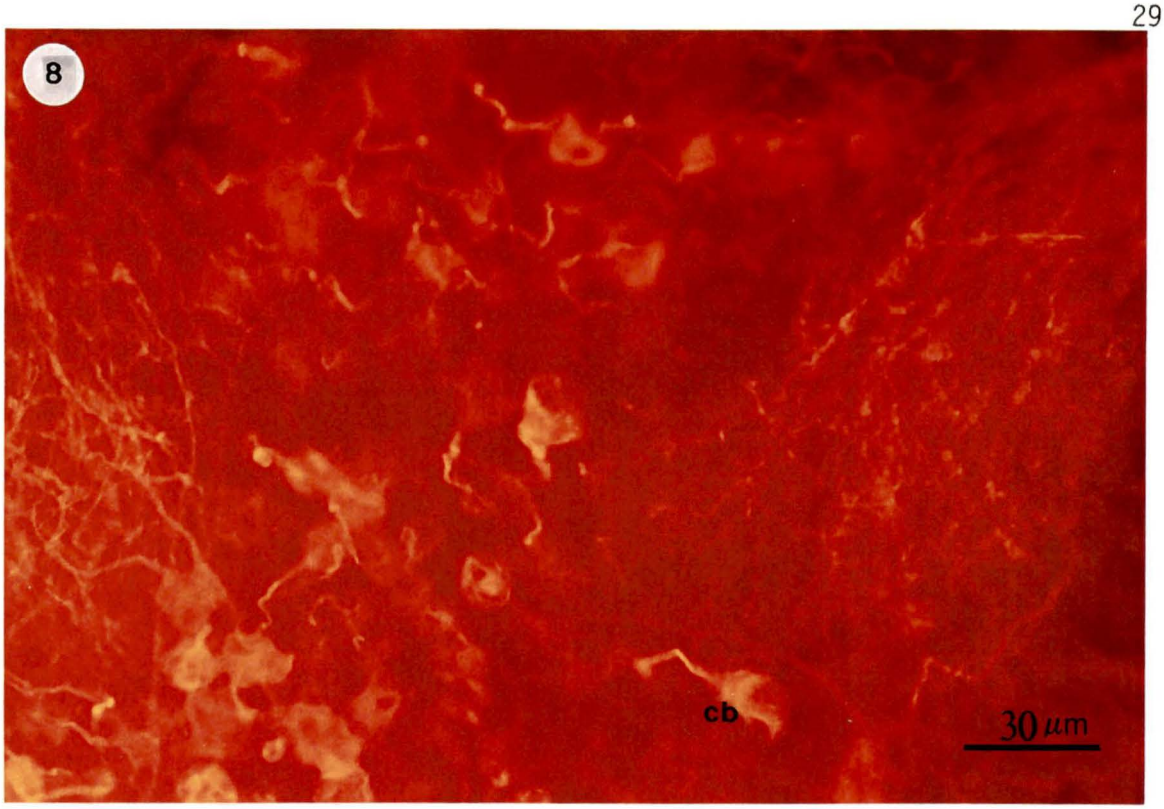
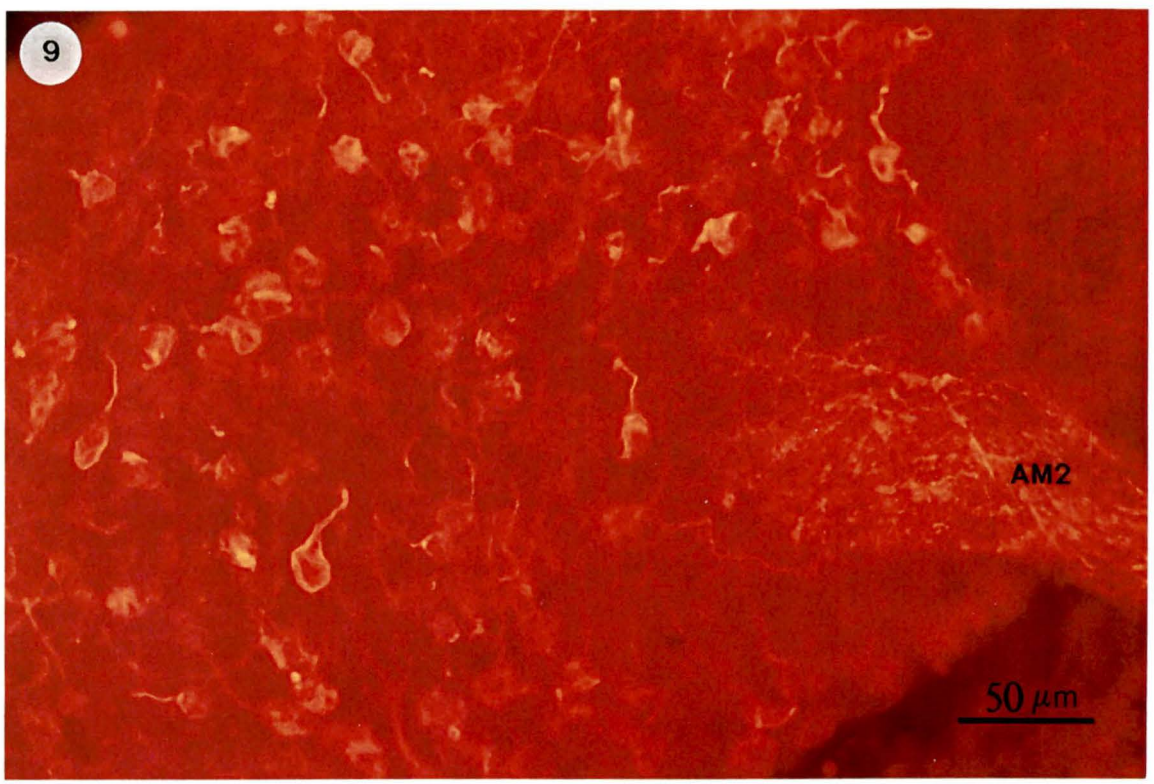

7
50 μm

Figure 8. Cell bodies (cb) and axonal tracts in the neural ganglion of *Chelyosoma* revealed using anti-tubulin.

Figure 9. Lower magnification of the region seen in figure 8. reveals the axonal tract labelled using anti-tubulin collectively exit the neural ganglion of *Chelyosoma*. The root is atrio-medial 2 (AM2).


8
cb
30 μm


9
AM2
50 μm

The posterior roots, AM1 and AM2, diverge from the posterior end of the neural ganglion to the lateral regions of the branchial basket and the overlying mantle. Root AM3 runs posteriorly in the dorsal vessel and splits into two bundles. Primary bundles branch off the visceral roots and extend perpendicularly into the branchial sac. The primary bundles branch into secondary bundles, that further branch and innervate the entire branchial basket. Individual axons extend to the ciliated cell clusters that line the stigmata. These axons terminate at the base of the cell clusters as distinct boutons, although an axon may sometimes further branch and innervate adjacent cell clusters.

The viscera are also innervated by the cholinergic system. The stomach, intestine and the rectum show ramification of tiny nerves all over their surface. The gonoducts are also innervated, but most of the nerves appear to run in the mantle sheath that surrounds the rectum and the gonoducts.

(B). Dorsal Strand Plexus /GnRH-Immunoreactive System

(B.1). Previous Findings:

(B.1a) Anatomical Studies

Several investigators in the last 100 years have made reference to a loose sheath of cells lying in close association with the dorsal strand (Huus, 1924; Fedele, 1938; Millar, 1953; Markman, 1958; Bone, 1959; Mackie et al. 1974). This section presents a review of the existing information regarding this plexus.

Fedele (1938) describes the dorsal strand plexus (dsp) cells associated with the dorsal strand as a bilayer of multipolar fusiform cells forming a plexus along the length of the strand. Other workers reiterated Fedele's findings, describing the cells of the plexus as being usually unipolar, bipolar, or multipolar with both short and long processes (Millar, 1953; Markman, 1958; Mackie et al., 1974; Irons, 1986). As stated earlier, the dorsal strand plexus is present along the entire length of the dorsal strand. Markman (1958) mapped the dorsal stand plexus originating around the right posterior brain root (the visceral nerve or AM3) in *Ciona*. He further traced it to the posterior proximity of the pericardium, to the oesophageal end of the endostyle and to the gonads in *Ciona*. Bone (1959) also observed this nerve plexus on the gonoducts in *Ciona*. Posterior aggregation of the dsp cells around the portion of the dorsal strand lying over the ovary in *Ciona* have been compared to a visceral ganglion by Millar (1953). Bone (1959) considered these dsp cells comparable to nerve cells in salp plexus. He describes cells resembling fibroblasts, but with extremely long processes, upon the viscera of salps. Bone found these cells morphologically similar to the ones he observed in the gonoduct plexus (or the dorsal strand plexus) of *Ciona*.

The fibrous processes of these cells extend anteriorly and posteriorly along the dorsal strand, interconnecting the cells of the dorsal strand plexus. However, Millar found it difficult to establish the relationship between unipolar cells and the fibres of other cells connecting to them. It seems that the fibres from other cells terminate near the cell body of the unipolar cell. Furthermore, fibres of the dsp cells also extend into the visceral nerves and cannot be distinguished from the fibres of the visceral nerve. Mackie et al. (1974) confirmed Millar's findings with their own phase contrast observations in *Corella*. They found that, while unipolar and multipolar ganglionic cells of the dorsal strand plexus appear to be intimately associated with the visceral nerve, they seem to lie outside the visceral nerve bundles. Mackie et al. postulate that the dorsal strand plexus fibres could enter the branchial sac along with the branches of the visceral nerves, but, they note that the shortness of the sheath cell processes make this theory unlikely. In *Dendrodoa* however dorsal strand plexus fibres penetrate the branchial sac (Mackie, 1995).

Sheath cells resembling ganglionic cells, present in close association with the dorsal strand itself, led earlier authors to propose that the strand was a part of the peripheral nervous system (Kowalevsky, 1871; Julin, 1881; Metcalf, 1900; Lorleberg, 1907). Huus (1924), however, made a clear distinction between the dorsal strand and the sheath of cells and their processes that are present around it. He proposed that this small nerve fibre (the dorsal strand plexus) has its origin in the posterior part of the neural ganglion, or in the root of the right siphonal nerve, whereas the strand originates from the neural gland. He considered this nerve sheath homologous to Julin's (1892) "cordon nerveux visceral" or "nerve visceral" in *Dendrodoa glossularia*, to Metcalf's (1900) "rapheal nerve", and to Lorleberg's (1907) "dorsal nerve" in *Perophora listeri*. Huus further observed that, though the

sheath lies in close proximity of the dorsal strand, there is no association between the two except that they both run down the dorsal fold. Brien (1927, cited by Berrill) further confirmed the difference between the nerve sheath and the non-nervous nature of the dorsal strand. Although this nerve sheath has been termed as "small nerve fibre", "rapheal nerve", "dorsal cord plexus", etc., to avoid confusion, it will be now referred to in the following text as the dorsal strand plexus.

These descriptions of the dorsal strand plexus, based on anatomical observations, point to some consistent conclusions: (i) the dorsal strand plexus occupies an overlapping domain with the dorsal strand but retains its individual identity; (ii) the plexus is visible at the base of the AM3 root, or the visceral root, and extends posteriorly to the gonads; (iii) elements of the plexus consist of unipolar, bipolar and multipolar cells interconnected by their processes; (iv) based on their similarities with ganglionic cells, it is probable that these cells are nervous in nature and may be immature (Fedele, 1938; Mackie, 1995); (v) some processes of these cells associate with the visceral nerve bundles, but seem to lie only on the exterior of the visceral bundles (Mackie et al., 1974); and, (vi) it is not clear if processes of the plexus cells enter the branchial sac, though it is likely that they may do so along with the visceral nerve branches.

(B.1b) Endocrinological and Immunocytochemical Studies

In 1881, Julin suggested a homology between the neural gland of the ascidians and the pituitary gland of the vertebrates. Huus (1924) believed the neural gland is involved with the development and the reproductive process of the gonads. He suggested that the gland is a homologue of the pituitary and its secretions controlling gamete release. The pituitary-like affinity of the neural gland

has been supported further by findings of many cells containing immunoreactive substances, localized in the neural gland, using antisera raised against some mammalian hormones and neuropeptides: Prolactin (Pestarino, 1984a); Secretin (Pestarino, 1984b); ß-endorphin (Pestarino, 1985a); ACTH (Georges and Dubois, 1985; Pestarino, 1985b); Cholecystokinin (CCK) (Pestarino, 1985c); Melanotropin and Corticotropin (Pestarino, 1988a); Bombesin (Pestarino, 1988b); Glucagon (Pestarino, 1990); and Insulin, (Neil et al., 1986).

In 1937, Hogg reported that injections of an extract of the neural complex of *Polycarpa obtecta* Traustedt in mice caused an increase in the size and number of follicles in the ovaries of the animals. Carlisle (1950) reported similar results, using neural complex extracts from *Ciona* and *Phallusia mammillata* in mice. He also found that injections of these extracts into male frogs caused sperm discharge. However, Benazzi (1939) and Dodd and Dodd (1966) obtained negative results when they injected similar extracts into mice. Dodd and Dodd (1966) hypothesized that a possible reason for this lack of response was a seasonal variation, but they also noted that Carlisle's experiments involved extracts from the neural complex of sexually mature ascidians. Hence, this could imply that the ascidians were not in a reproductive phase and thus not producing secretions responsible for sperm discharge in frogs in Carlisle's experiments.

In 1950, Carlisle first reported gonadotropin activity in the neural gland extracts of ascidians. In 1951, he further tested the role of the neural complex in the control of gamete release, observing that injections of mammalian chorionic gonadotropin into *Ciona* and *Phallusia* stimulated gamete discharge after an approximately 20 hour delay. In animals where the heart was removed and the blood drained, the response could be elicited only by injections into the neural region. Sectioning the visceral nerves abolished this response. Carlisle further

found that when the nerves to the gonads, probably the visceral nerves, are left intact while the others nerves are severed, gametes release occurs. Ablation of the neural gland still yields a positive response, as long as the neural ganglion is left intact. Gametes are released even when chorionic gonadotropin is replaced by highly concentrated extracts of ascidian neural gland, or if the animals are fed with gametes from other animals of their own species. Based on his results, Carlisle concluded that extrinsic stimulation caused hormone secretion from the neural gland. He suggested that this hormone further excites the neural ganglion, which in turn stimulates the release of gametes via the visceral nerve. These results suggests that the neural gland is mandatory for gamete release in ascidians.

However, in 1966, Hisaw et al. found that ablation of the entire neural complex in *Chelyosoma* caused no dysfunction in gonadal activity of the animals. A year later, Bouchard-Madrelle (1967) reported that ablation of the neural complex in *Ciona* resulted in a decrease in the number of germinal sites in the ovary and in the number of mature follicles. Their results were further supported by Sengel and Keiny's (1962, 1963a, 1963b) experiments involving in vitro culture of ascidian gonads. They found that maturation of oocytes in the cultured gonads of *Molgula manhattensis* continued only in the presence of explanted neural complex. Controls using tissue other than the explanted neural complex yielded negative results.

In 1980, Georges and Dubois found a population of cells in the neural ganglion of juvenile *Ciona* that are immunoreactive with antisera against mammalian anti-luteinizing hormone releasing hormone (LHRH, now termed GnRH). In very young animals two to three cells were observed between the posterior root area of the ganglion. Sections of more mature animals revealed five to six cells in the same location. In adults a group of five to ten immunoreactive

cells are present in the space between the neural ganglion and the neural gland. These cells do not exhibit the radial orientation seen in the neurons within the ganglion but, instead, are longitudinally arranged along the duct of the ciliated funnel that connects to the neural gland. A second group of immunoreactive cells lies posterior to the ganglion, along a small nerve following the dorsal vessel and the genital ducts. These cells are bipolar and bound by short processes. This population of cells forms a continuous line from the posterior part of the ganglion to the posterior part of the gonopores and apparently continue to the gonads. The entire cytoplasm of these cells is fluorescent and the nucleus is small and located to a side of the cell. It is interesting to note that these cells are also immunoreactive with anti-GnRH throughout the year.

Kelsall et al. (1990) found similar labelling within the ganglion and its roots using antiserum against lamprey GnRH. Though the authors were unable to discern cell bodies in their preparations, they reported staining of nerve fibres in the neural ganglion and in at least one anterior and one posterior root. Both Georges and Dubois (1980) and Kelsall et al. (1990) report no labelling for GnRH-like immunoreactivity in the neural gland, even though their area of study involved the entire neural complex. This sharply contrasts with previous ideas of the neural gland being responsible for some sort of gonadotropic control of the gonads.

Ascidians periodically squirt water from their siphons, which is a result of contraction of the body of the animal and subsequently the contraction of the branchial sac. Ruppert (1990) reports that the ascidian neural gland acts as a blood volume regulatory organ, restoring the volume of water lost from the blood system during squirting. He suggests that the elevated pressure in the blood vessels of the branchial sac due to compression could result in the ultrafiltration of the blood into the lumen of the branchial sac and into the atrium (from whence it is expelled

during squirting). Ruppert further suggests that the neural gland is not a nervous structure, as his investigations revealed a lack of innervation in the neural gland of *Ascidia interuppta*. Furthermore, he concludes that the absence of ultrastructural evidence supporting the previous immunocytochemical studies of the gland suggest the gland is not an endocrine structure. However, some authors continue to regard the neural gland as a pituitary homologue (e.g. Gorbman, A., 1995.).

In conclusion, then, the role of the neural gland in the reproductive biology of ascidians remains uncertain. For the purpose of this study, however, it will be of interest to establish if GnRH-immunoreactive cells and fibres are present in the neural gland. This would define the extent of the dorsal strand plexus and the possible significance of the neural gland in reproductive biology of these animals.

(B.2). Dorsal Strand Plexus: New Findings

(B.2a) Optical and Immunocytochemical(GnRH) Results

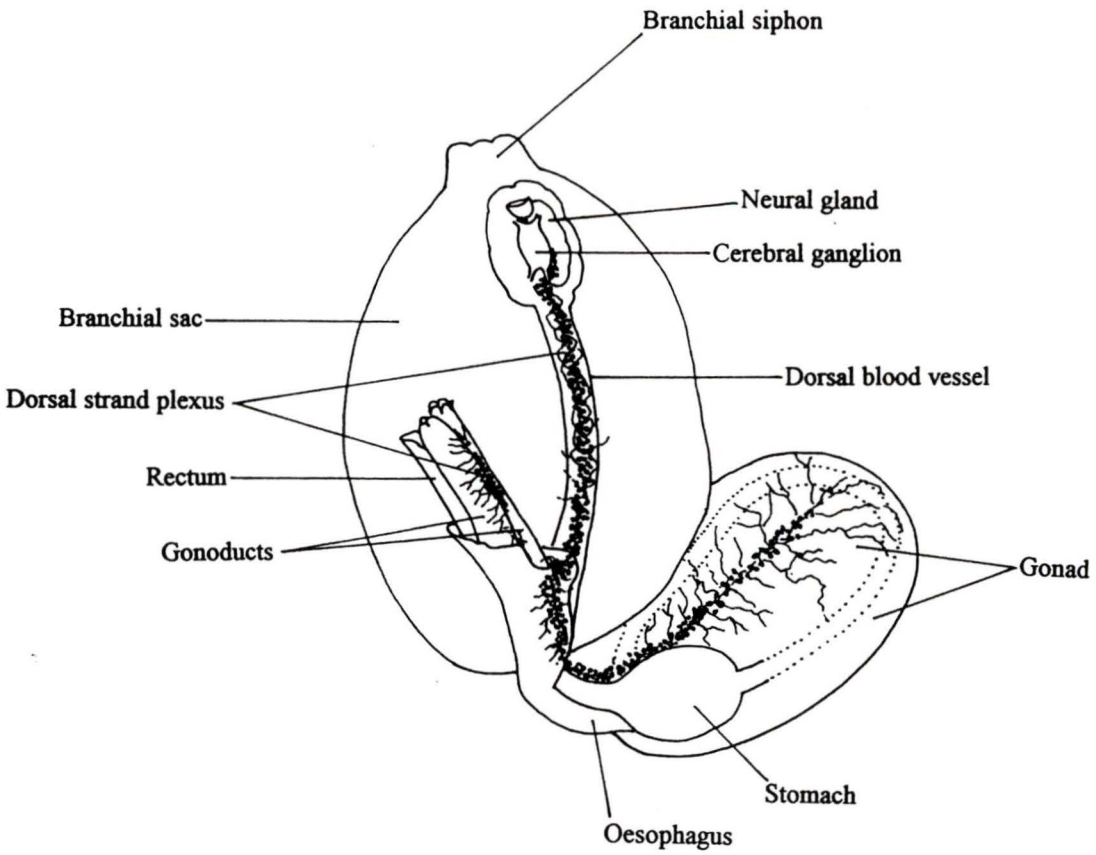
This study confirms the presence of a distinct rich plexus of neurons and their processes in the dorsal fold of *Chelyosoma* and *Corella*. Furthermore, the elements of the plexus exhibit a strong GnRH-like immunoreactivity and are extensively distributed within the body of the animals.

The dorsal strand plexus was visualised using Nomarski optics and phase contrast optics. FITC conjugated secondary antibodies and Strepavidin/Biotin complex visualized with DAB were used in conjunction with various anti-GnRH sera to further establish the neurosecretory map of the plexus.

The dorsal strand plexus consists of bipolar and multipolar neurons that vary in shape, from spherical to ovoid, and their processes. A prominent rounded nucleus is visible in the neurons. In both *Chelyosoma* and *Corella* the cell bodies of the dorsal strand plexus are concentrated in the dorsal fold region of the branchial basket. This aggregation of GnRH immunoreactive cell bodies and their neurites extends from the anterior-most region of the dorsal blood vessel, immediately below the neural ganglion (Fig.10) to the posterior end of the dorsal fold at the level of the oesophagus. From here the plexus extends posteriorly over the gonoducts and the gonads (Fig. 10). Within the dorsal blood vessel, the plexus runs along the dorsal strand, around which the cell bodies and their fibres seem to be arranged preferentially (Figs. 11, 12, 15). However, in *Corella* the cell bodies are further apart and the neurites are more loosely arranged than in *Chelyosoma* (Fig. 13).

In both animals the plexus is conspicuous at the posterior end of the neural ganglion. It lies around the AM3 root, which extends down the dorsal fold as the

Figure 10. Diagrammatic representation of the extent of the dorsal strand plexus mapped in an adult *Chelyosoma*. The animal has been dissected open to display the organs. The tunic and mantle are not shown in the diagram. Organs are also not shown in their correct orientation or true proportion.


Branchial siphon
Neural gland
Cerebral ganglion
Branchial sac
Dorsal blood vessel
Dorsal strand plexus
Rectum
Gonoducts
Gonad
Stomach
Oesophagus

visceral root (Fig. 13). Here, the neurons of the plexus and their processes form a compact sheath over the visceral root. Some fibres can be seen extending into the superficial layer of the ganglion itself. However, as the tissue is quite dense in whole mounts, it has not been possible to follow these fibres further into the ganglion.

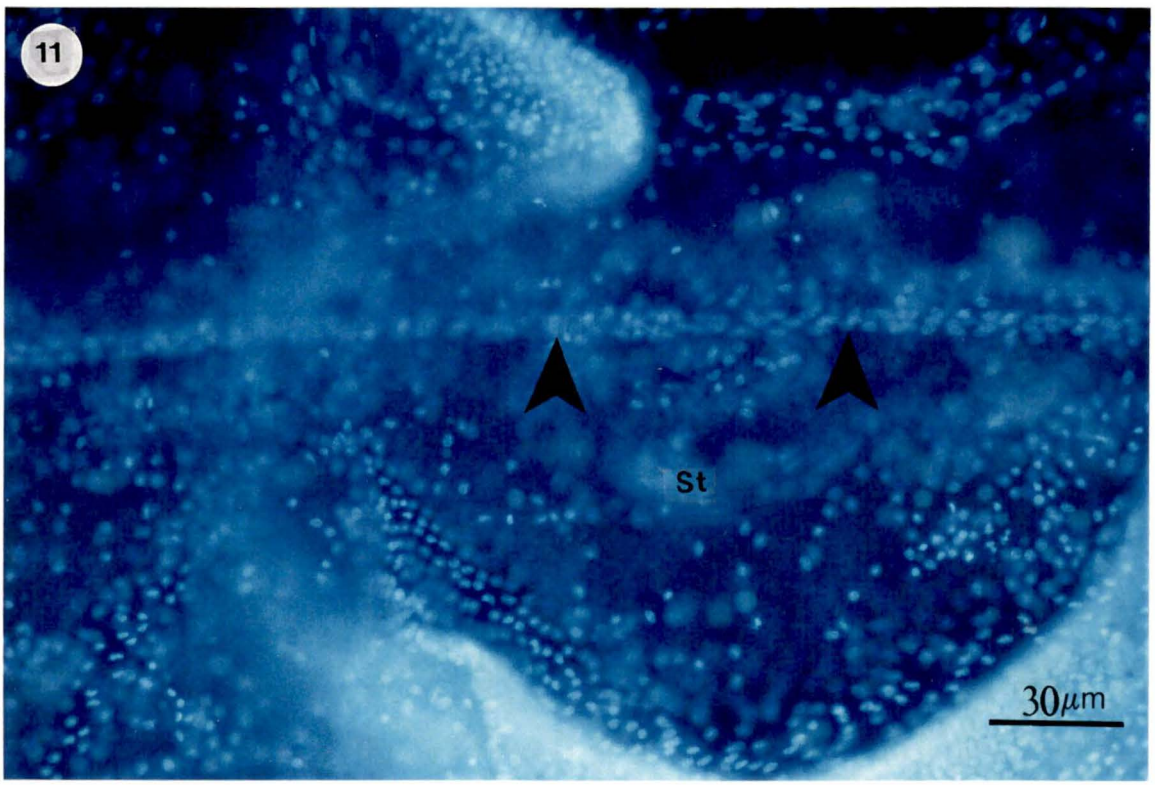
As the visceral root, along with the ensheathing plexus, extends posteriorly, the main body of the plexus shifts and comes to lie between the two visceral nerve bundles (Fig. 14). It is possible that this shift reflects the anterior-most association of the plexus and the dorsal strand. The cell bodies of the dorsal strand plexus are now scattered around the dorsal strand; the neurites are spread out within the lumen of the dorsal blood vessel. Though some neurites terminate on other neurons, it is unclear where the others extend to or terminate. A few GnRH-immunoreactive axons can be traced entering the branchial basket through some transverse blood vessels in juvenile animals (Fig. 16).

The gonoducts and the rectum run along the atrial side of the dorsal fold, with the anus and the gonopores situated around the mid-length of the branchial sac (Fig. 19). In *Chelyosoma* the dorsal strand plexus crosses over from the dorsal fold, posterior to the level of the gonopores and the anus, and comes to lie in close proximity to the gonoducts (Fig. 18, 24). From this point the plexus continues posteriorly in association with the gonoduct (Figs. 19, 20, 21). However, several axons extend into the dorsal fold and form a parallel network that extends down the remaining length of the dorsal blood vessel, to the level of the oesophagus. The dorsal strand plexus seems to occupy a more intermediate position in *Corella*, lying between the dorsal fold and the gonoducts.

In *Chelyosoma* there appears to be an aggregation of GnRH-positive axons at the cross-over zone of the plexus. Numerous axons run across the surface of the

Figure 11. The dorsal strand (*arrow-heads*) in *Corella* visualised using Hoechst. (*St: stigma.*)

Figure 12. The dorsal strand plexus in the same location as in figure 11 visualised using U705-23 (anti-LHRH) and FITC secondary antibody. The plexus surrounds the dorsal strand in the anterior region of the dorsal fold in *Corella* and *Chelyosoma*. A neuron of the dorsal strand plexus is visible (*arrow*). (*St: stigma.*)


11
St
30μm

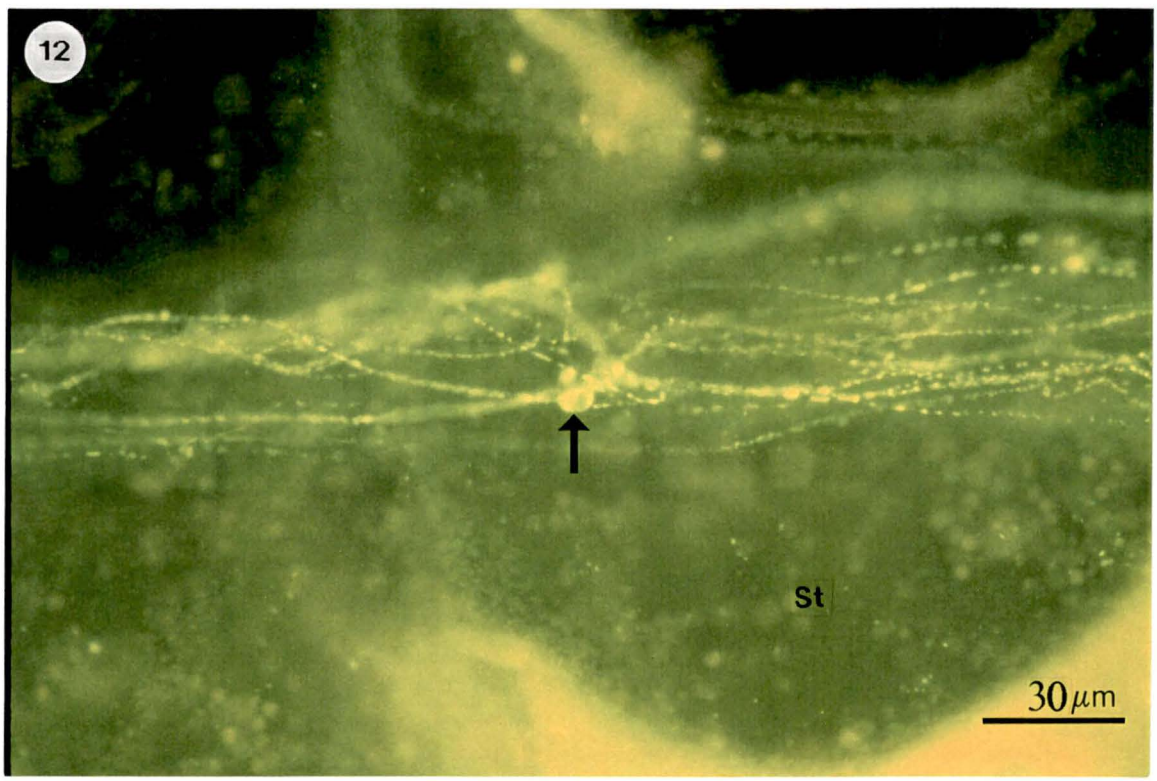
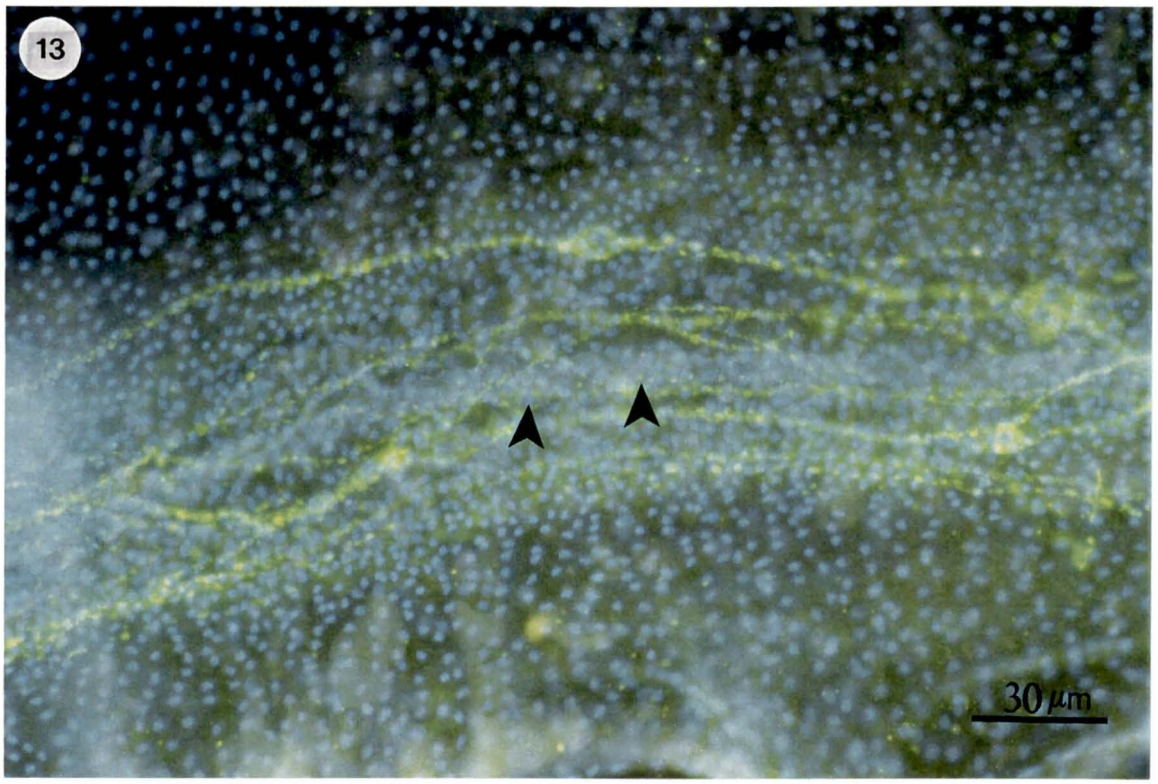

12
St
30μm

Figure 13. In *Corella* a looser arrangement of the cell bodies and axons of the dorsal strand plexus around the dorsal strand (*arrow-heads*) is present. *(Bla-4, FITC)*.

Figure 14. The elements of the dorsal strand plexus (*arrow-heads*) are concentrated around the root AM3 of the neural ganglion on *Chelyosoma*, ensheathing its origin from the ganglion. There is a short overlap between the two structures, immediately posterior to the ganglion. (*Wholemount;Br: neural ganglion; Ng: neural gland; AM: atrio-medial; Bs: Branchial sac*). *(U705-23, FITC.)*


13
30 μm

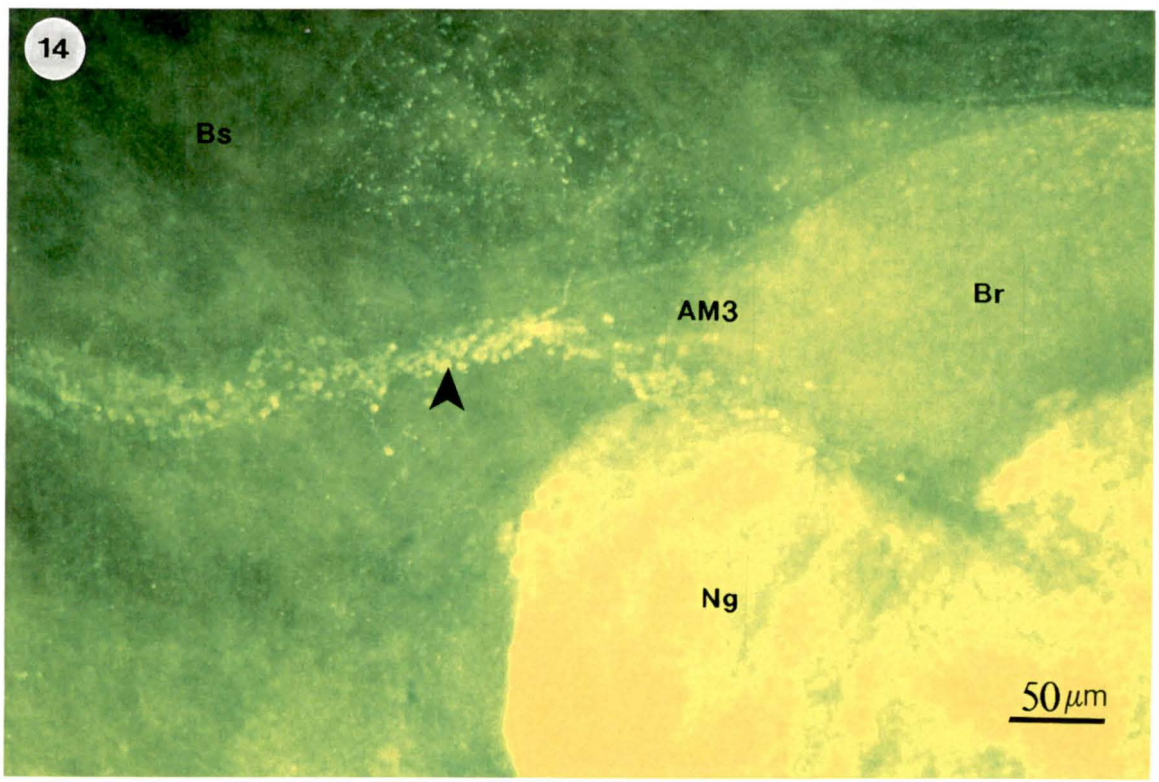
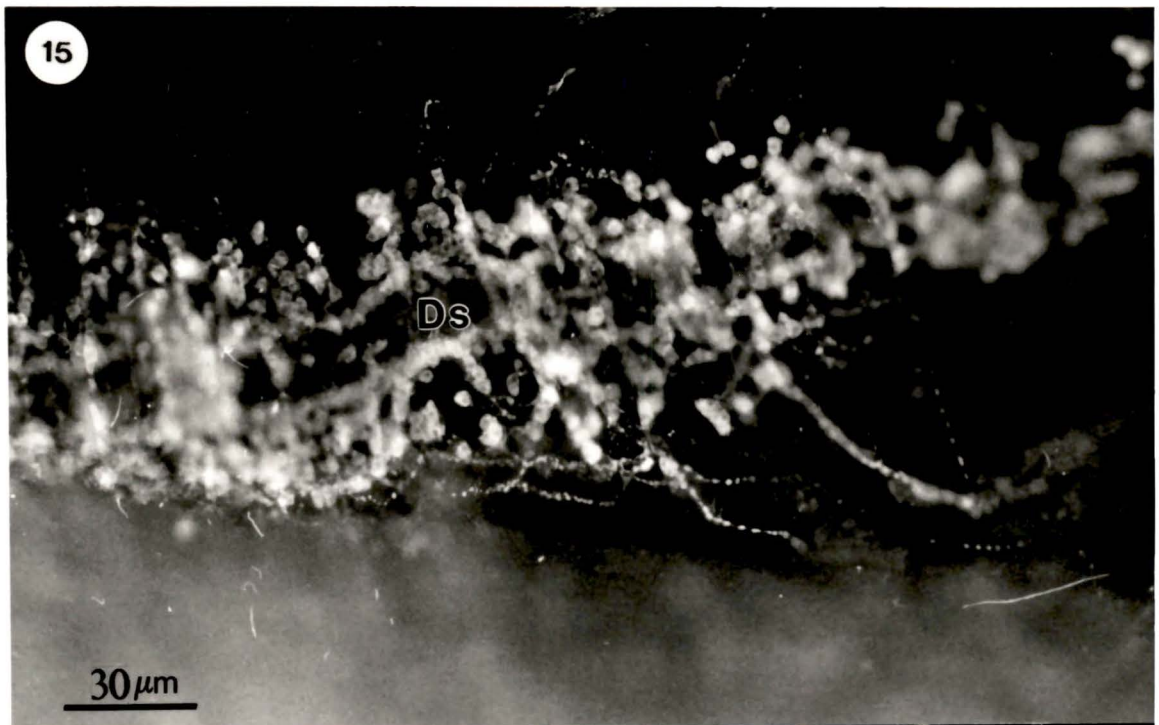
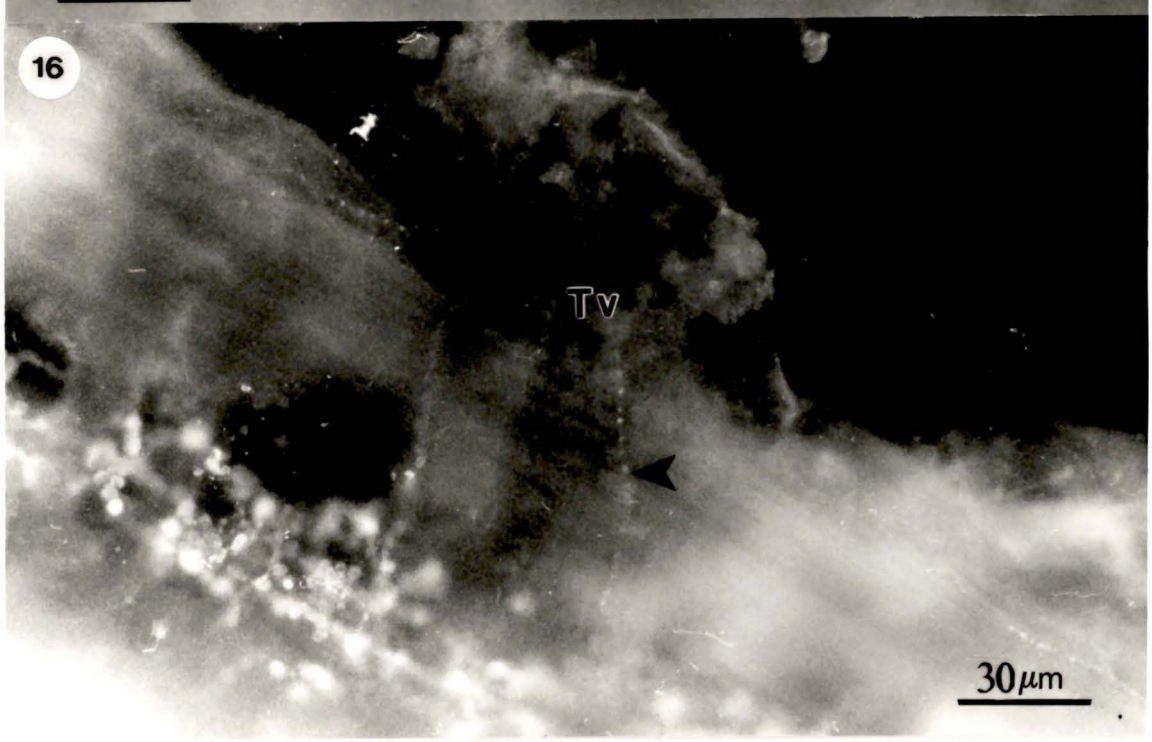

14
Bs
AM3
Br
Ng
50 μm

Figure 15. The cell bodies and their neurites of the dorsal strand plexus exhibit a scaffolding-like arrangement around the dorsal strand (*Ds*) in an adult *Chelyosoma*. *(U705-23, FITC).*

Figure 16. In juvenile *Chelyosoma* a few axons (*arrow-heads*) extend from the dorsal strand plexus into the transverse vessels (*Tv*) of the branchial basket. In both juvenile and adult animals some GnRH-positive fibres are present in the branchial sac, lying close to its atrial surface. However, no GnRH immunoreactive cell bodies are found within the sac. *(U705-23, FITC).*


15
Ds
30μm


16
Tv
30μm

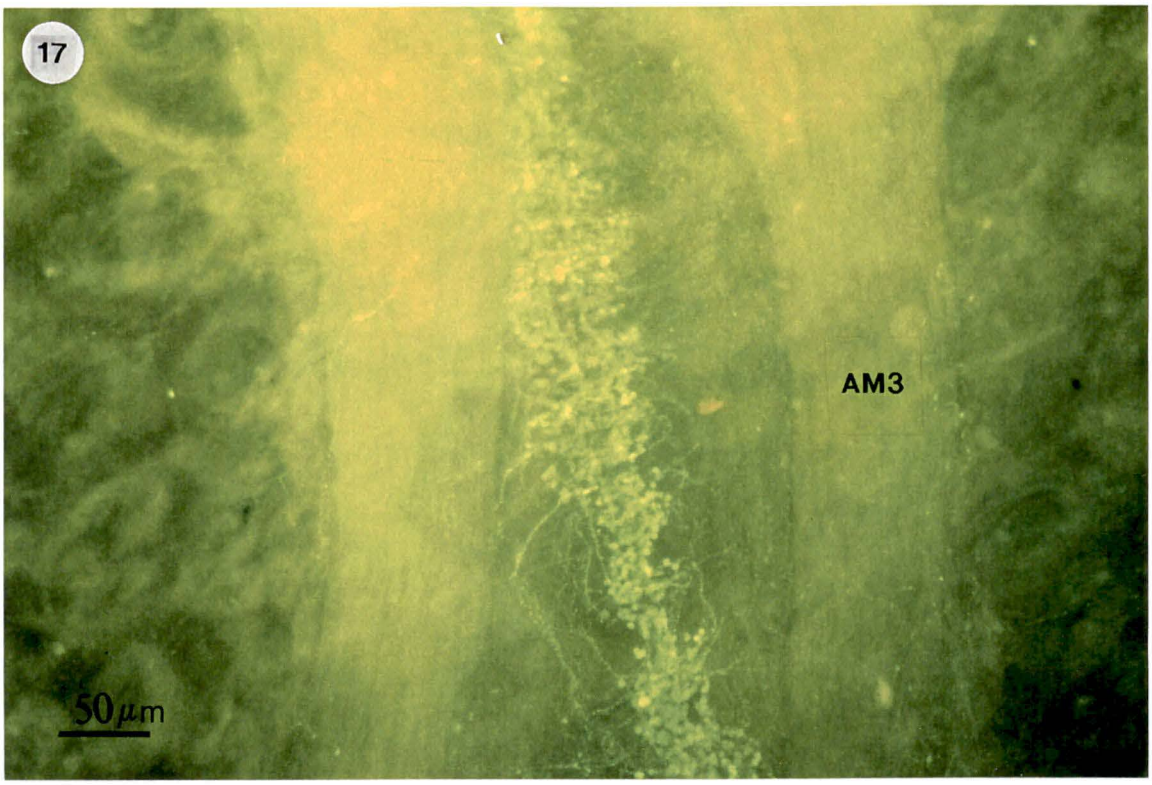
rectum and the gonopores, in a circular arrangement, following the sphincter muscles that are present around the anus and the gonopores (Fig. 25). Along the gonoduct itself, the cell bodies and axons of the dorsal strand plexus exhibit a more compact spatial arrangement, in comparison to that observed within the dorsal blood vessel. However, several axons traverse the width of the sperm duct and ramify over its surface (Figs. 21, 26). No cell body of the dorsal strand plexus has been observed over the gonoducts.

Various regions of the branchial basket were examined for extensions of the dorsal strand plexus. These studies revealed a few scattered GnRH-positive axons lying close to the atrial surface in the blood vessels of the branchial sac. No cell body was seen in the branchial basket itself, although there is a slight increase in the number of GnRH-positive axons in the portion of the branchial basket adjacent to the gonads. Fluorescent label accumulates in the folds of the endostyle during immunocytochemical studies, though no cell body or axons could be seen. The pericardium and the heart itself were also devoid of innervation by the dorsal strand plexus. In addition, it should be noted that one or two GnRH-positive axons have been observed in the layer of epithelium between the branchial sac and the gonads, but these results are not very consistent. Finally, no cell bodies or axons appeared in the mantle.

In juvenile *Chelyosoma*, where the gonads have not yet reached the level of morphological maturity exhibited by adult animals, the dorsal strand plexus extends over the stomach and across the gonads. In adult *Corella* the plexus can be traced extending posteriorly in the dorsal fold to the oesophageal opening and moving out along with the oesophagus. In the body cavity the plexus loops under the oesophagus, following the gut and the gonoducts, and then takes up a median position over the gonads where it extends to the middle of the intestinal loop.

Figure 17. As the dorsal strand plexus extends down the dorsal fold it occupies a more median position between the two visceral (AM3) bundles, as is evident in this lower magnification photograph of the field in figure 15. (*Chelyosoma, U705-23, FITC.)*

Figure 18. Posterior to the gonopores, the dorsal strand plexus sharply bends and comes to lie in close proximity of the gonoducts in *Chelyosoma*. (*Bs: Branchial sac; R: rectum; S: sperm duct.*) *(U705-23, FITC).*


17
AM3
50μm

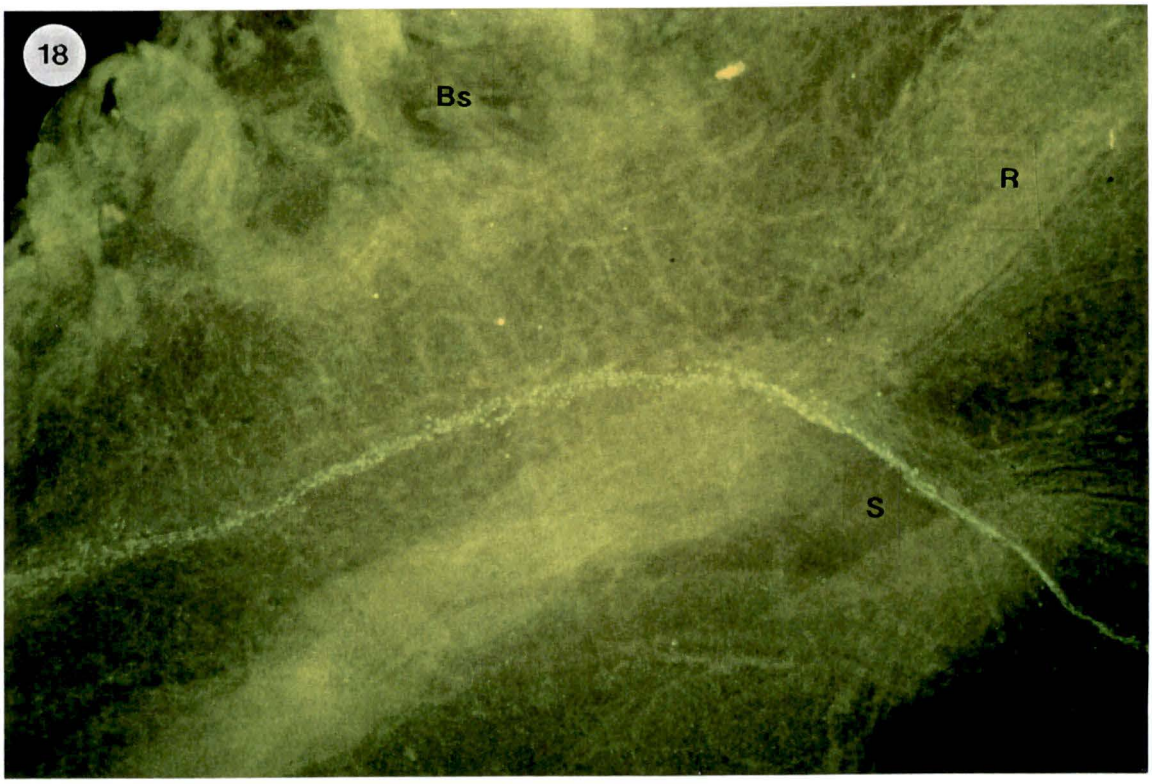

18
Bs
R
S

Figure 19. Diagrammatic representation of the dorsal strand plexus *(shown in red stippling)* in the dorsal fold region of *Corella*. The relative positions of the sperm duct, the oviduct and the rectum in relation to the dorsal fold are also shown; the organs are not represented in their true proportions.

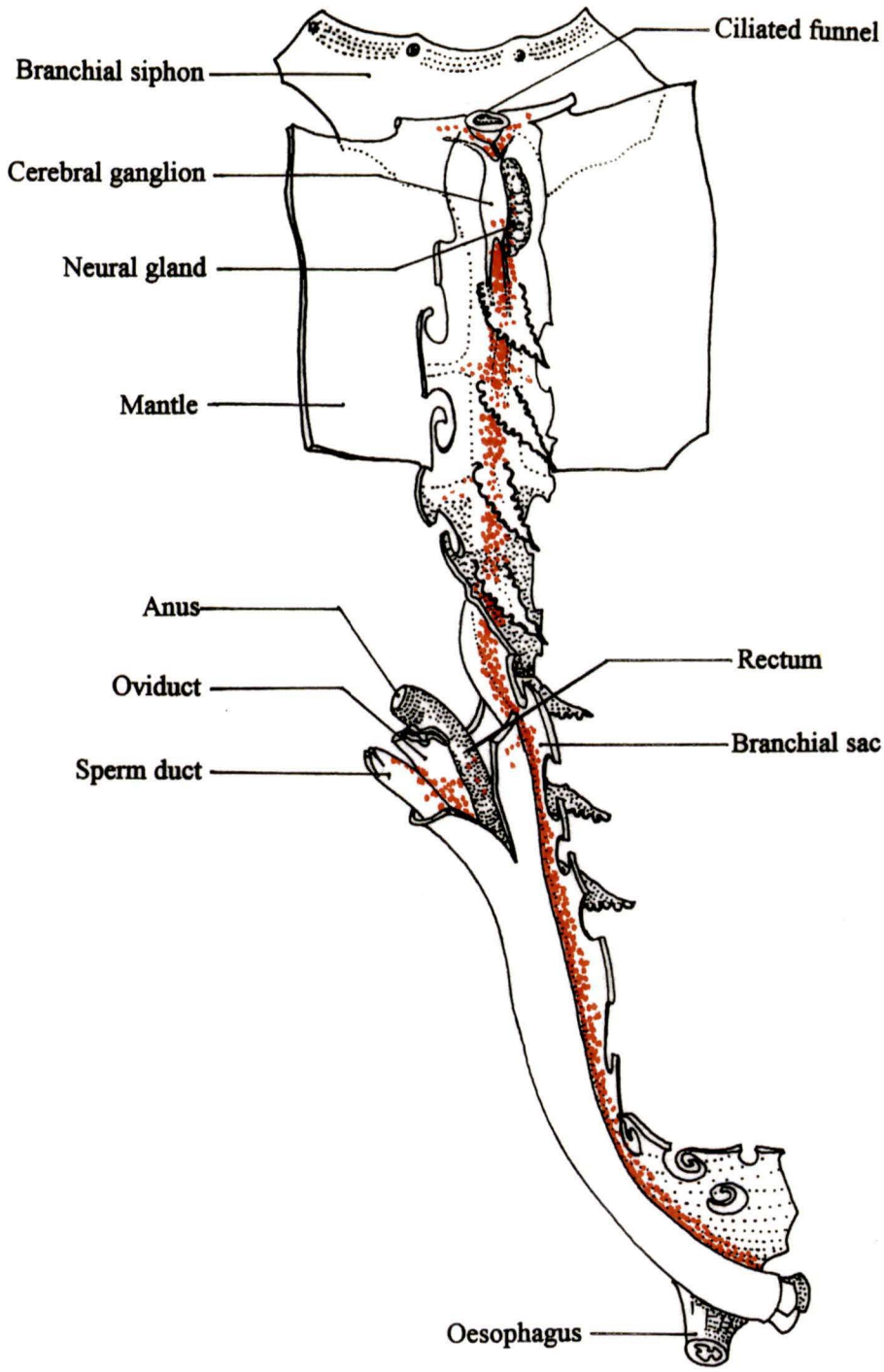
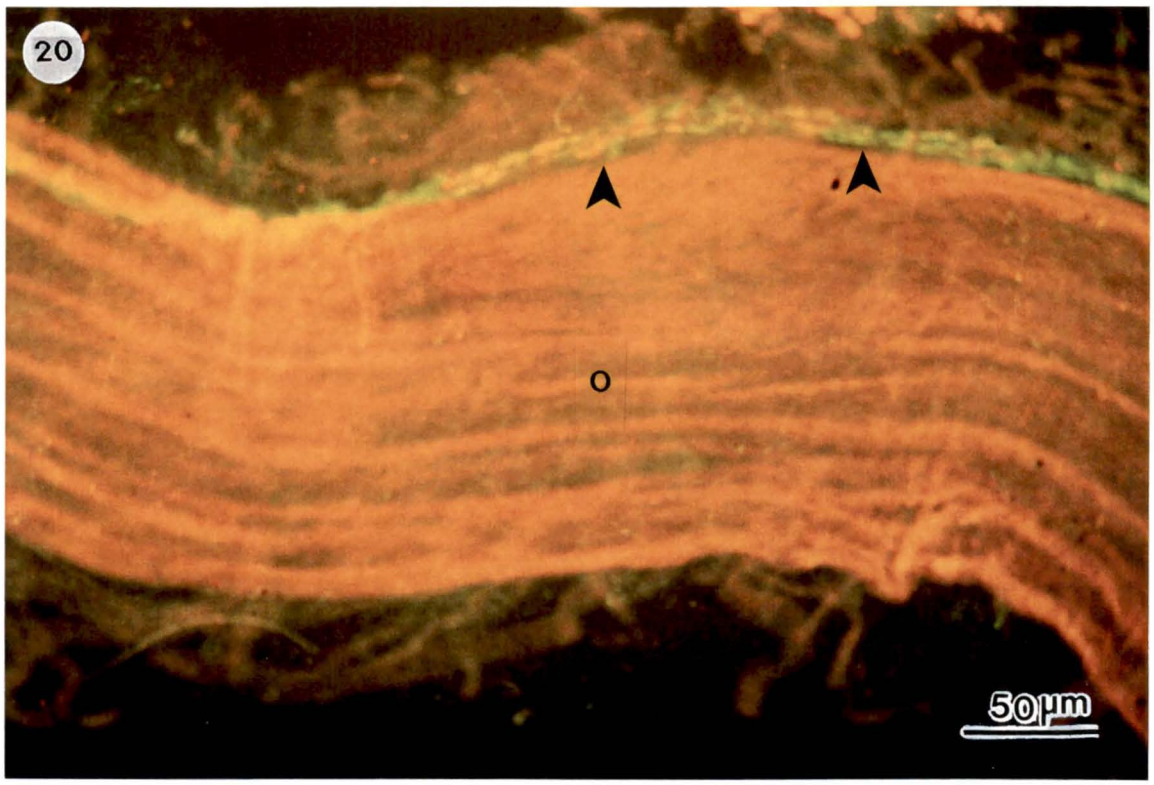

Ciliated funnel
Branchial siphon
Cerebral ganglion
Neural gland
Mantle
Anus
Rectum
Oviduct
Branchial sac
Sperm duct
Oesophagus

Figure 20. The dorsal strand plexus (*arrow-head*) in close association with the oviduct (O) in *Corella*. Double labelling with *U705-23* and anti-tubulin reveals the tubulin contents of the wall of the oviduct. The dorsal strand plexus, running along its side noticeably lacks label for tubulin.

Figure 21. The cell bodies of the dorsal strand plexus can be seen in close proximity to the sperm duct (S). *(Chelyosoma, U705-23, FITC).*


20
O
50µm

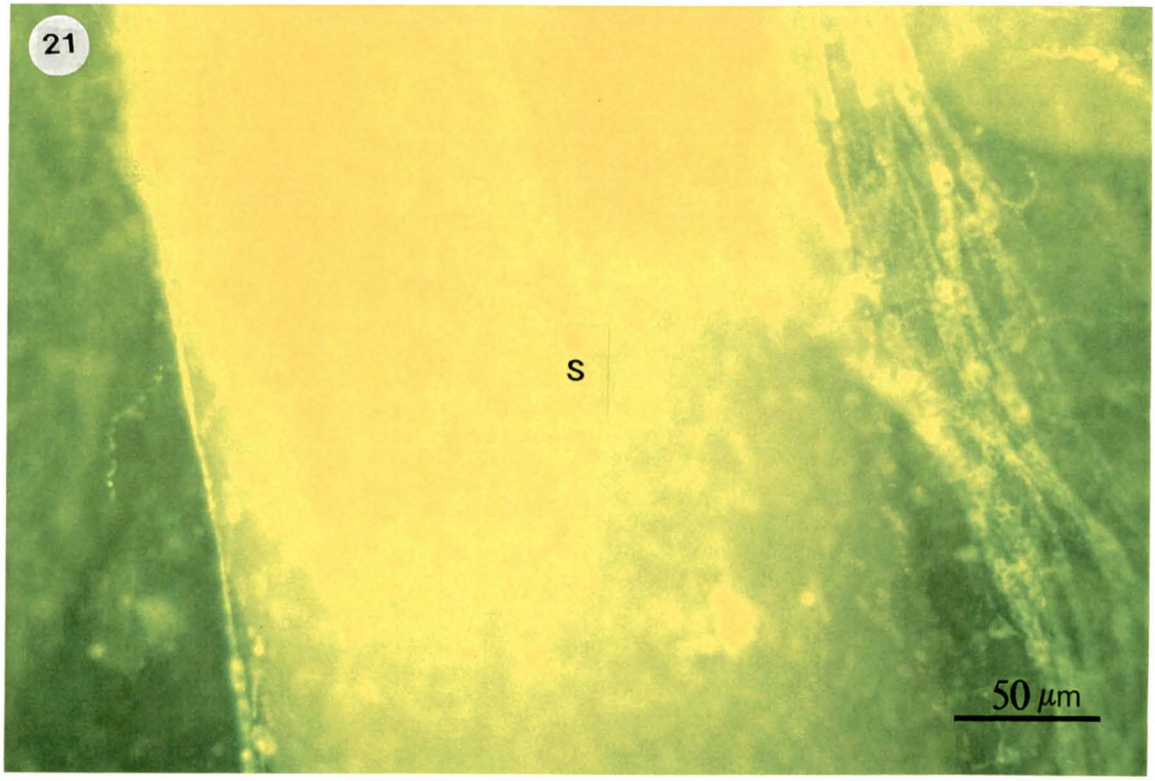
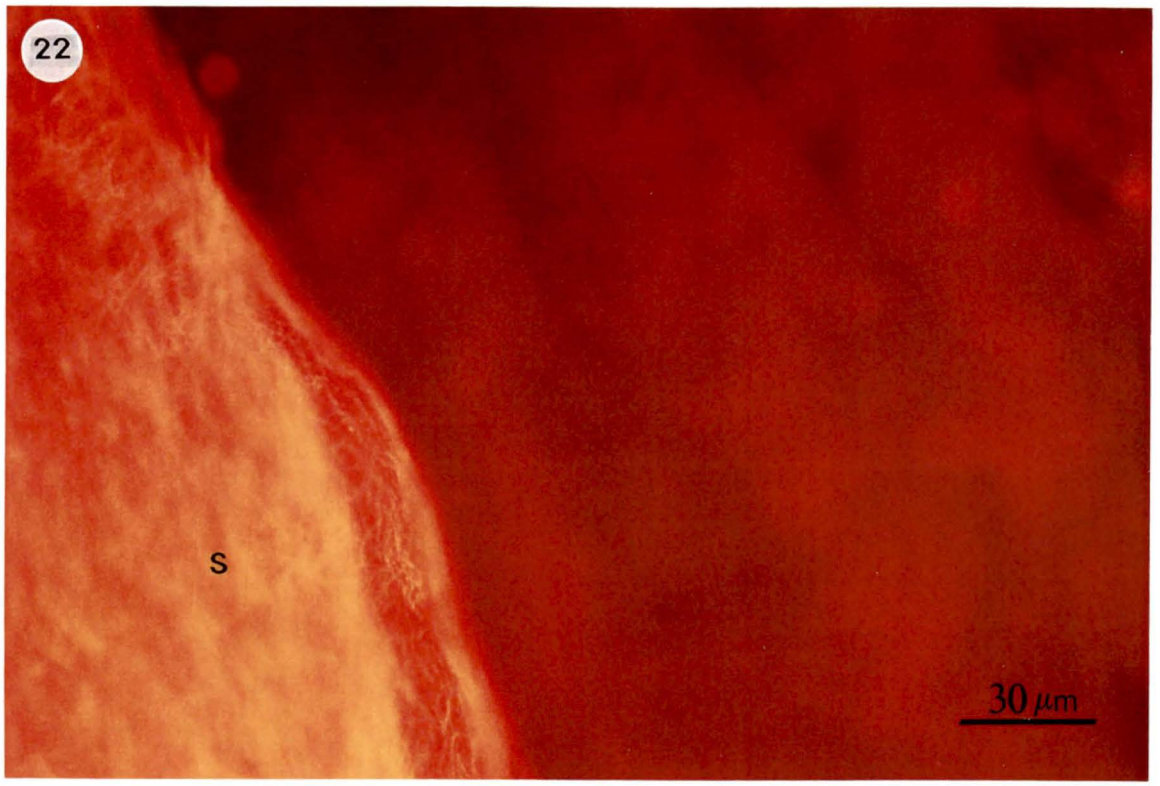

21
S
50 µm

Figure 22. Microtubular tracts visualized in the sperm duct (*S*) of *Chelyosoma* using anti-tubulin and Rhodamine conjugated secondary antibody. The labelled fibre-like structures in the peripheral region of the sperm duct are sperm tails.

Figure 23. Double incubations with anti-tubulin and U705-23 (anti-LHRH) reveal the ramifications of the GnRH-positive axons over the surface of the sperm duct (*S*) in *Chelyosoma*. Figures 22 and 23 show overlapping area of the sperm duct.


22
S
30 μm

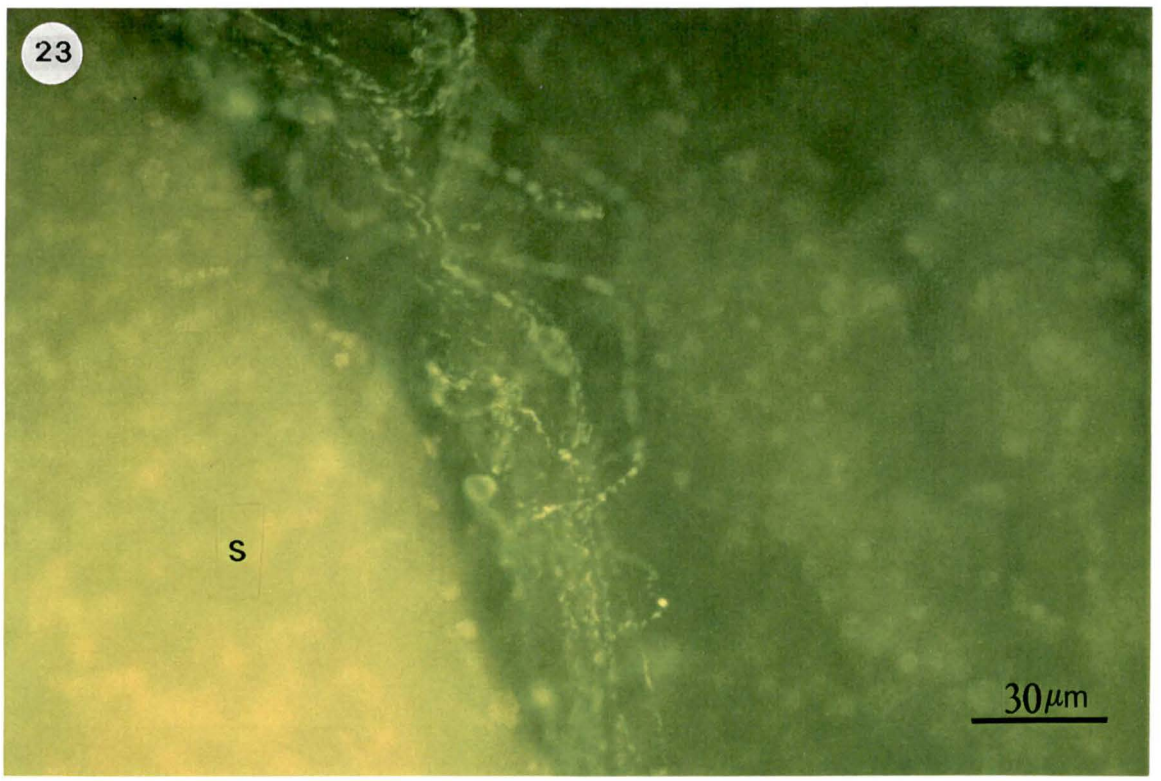

23
S
30μm

Figure 24. This thick section stained with Richardson's stain reveals the location of the DAB labelled GnRH-immunoreactive cell bodies and axons (*arrows*) in relation to the rectum (*R*), oviduct (*O*) and the sperm duct (*S*) in *Chelyosoma*.

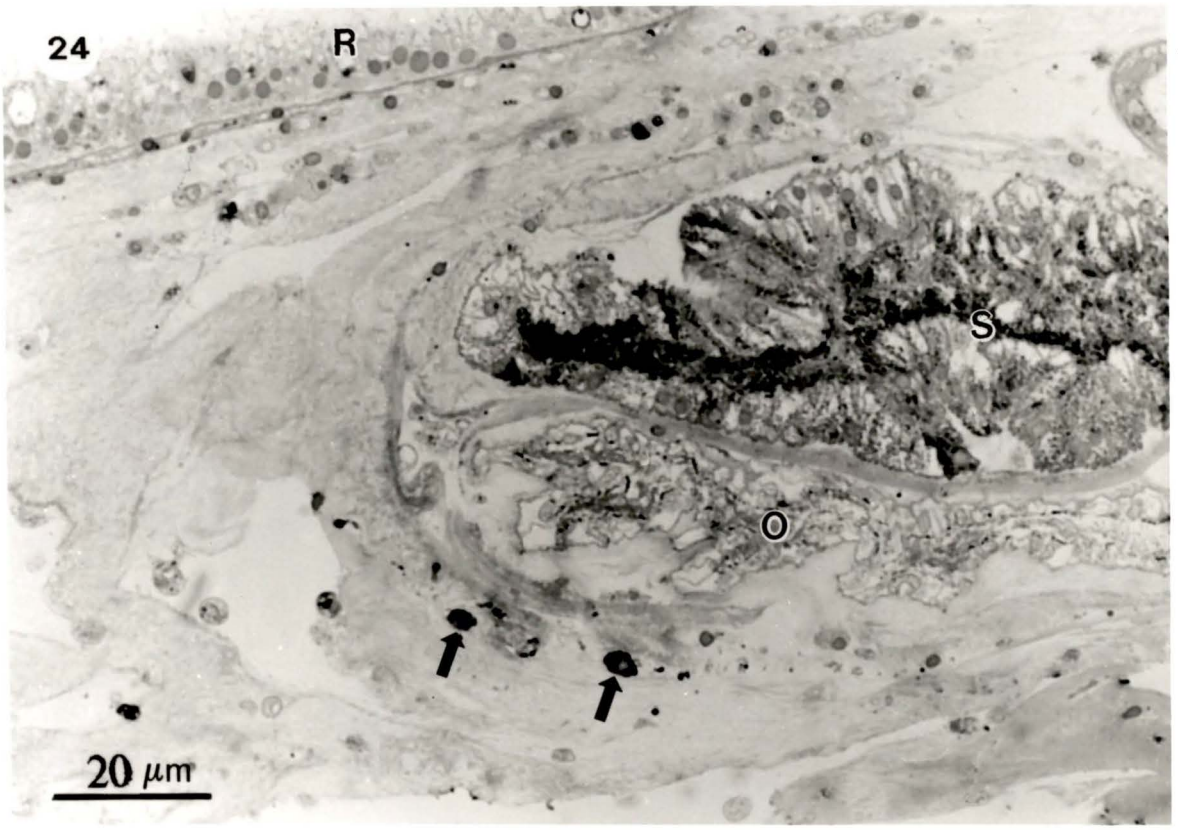
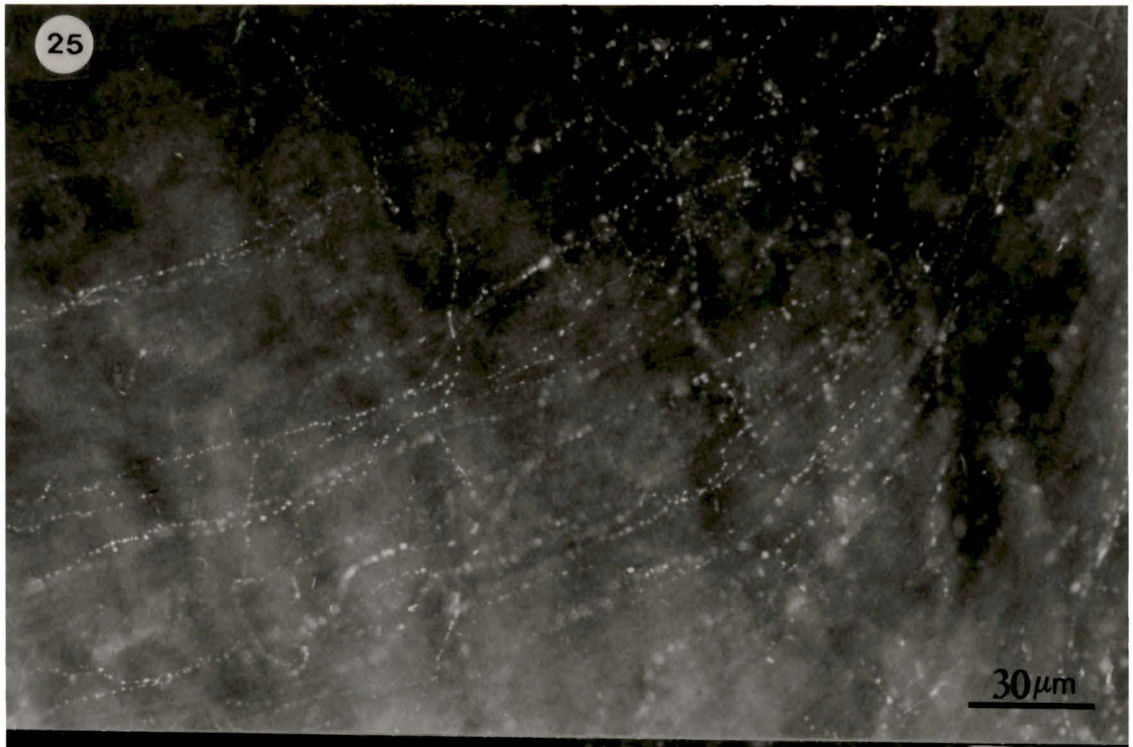
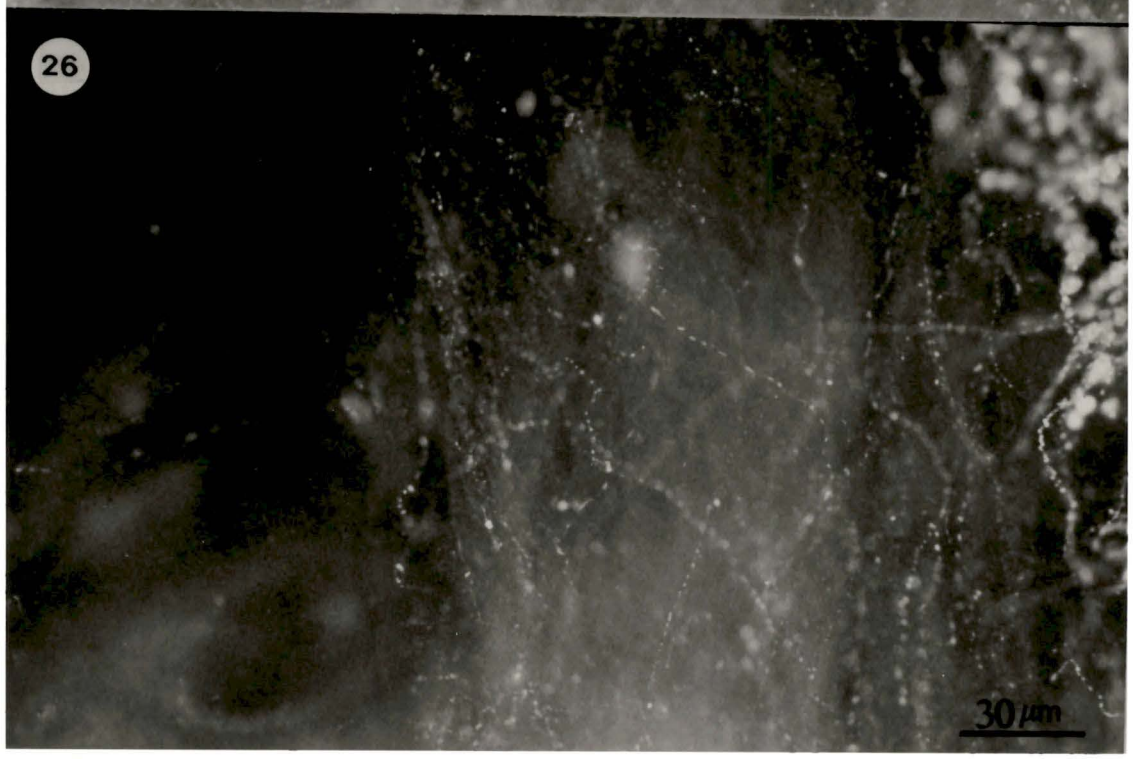

24
R
S
O
20 μm

Figure 25. Circular arrangement of the GnRH-immunoreactive fibres over the anus in *Chelyosoma. (U705-23, FITC)*

Figure 26. Ramifications of the fibres of the dorsal strand plexus over the gonoduct in *Chelyosoma*. (*U705-23, FITC)*


25
30μm

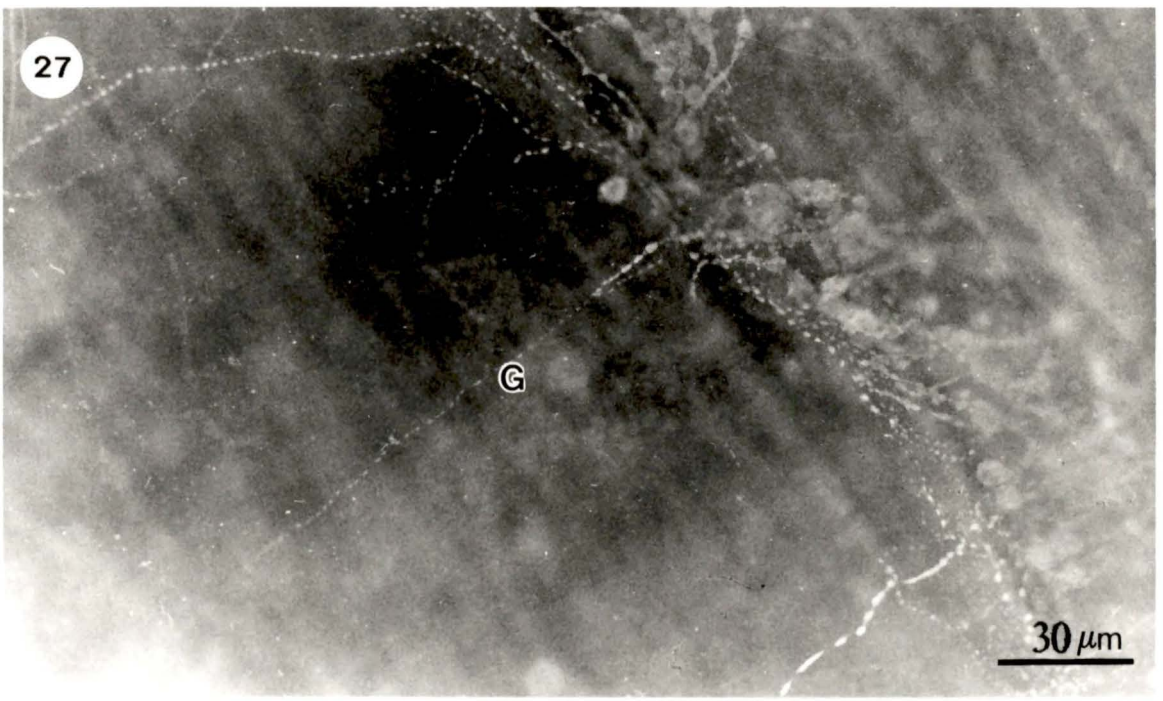

26
30μm

From the main body of the plexus GnRH-positive axons ramify over the entire surface of the gonad in a seemingly random pattern of distribution in both the animals (Fig. 27). The density of axons varies from region to region, but this may be a result of improper penetration of the reagents through the tissue. Despite this variation, it is quite evident that the ramifications of the GnRH immunoreactive neurites extend over the entire surface of the gonads. Although it is difficult to achieve a good preparation of the gonad tissue, several individual fibres have been observed running along the collecting ducts within the gonad itself.

In cryosections of the neural complex of *Chelyosoma*, a group of GnRH-positive cells and axons occupy a medial position between the neural ganglion and the neural gland (Fig. 14). These cells are difficult to observe in whole mount preparations because the neural ganglion partly overlaps the neural gland, thus obscuring the space between the two structures. In preparations where part of the neural gland has been removed, or where the two structures have been somewhat separated, these cell bodies can be seen extending up to the junction of the ciliated funnel duct and the neural gland. Axons extend anteriorly from this aggregation of neurons and ramify over the surface of the duct (Fig. 29). At the base of the ciliated funnel, the axons diverge from the duct and spread over the body of the funnel. Only a few axons are present around its anterior rim. Axons can also be traced anteriorly over the branchial basket to the level of the peripharyngeal band, where they spread circumferentially around the band to the endostyle. None have been observed around or within the branchial tentacles.

In both animals another population of GnRH-positive neurons is present in the peripheral region of the neural ganglion (Fig. 28). GnRH-immunoreactive axons are seen in the peripheral region of the ganglion and in the neuropil of the neural ganglion. Occasional GnRH-immunoreactive cell bodies appear to be

Figure 27. In adult *Chelyosoma* the dorsal strand plexus extends over the gonads. GnRH-positive fibres can be seen extending over the surface of the gonad (*G*), close to the stomach.


27
G
30 μm

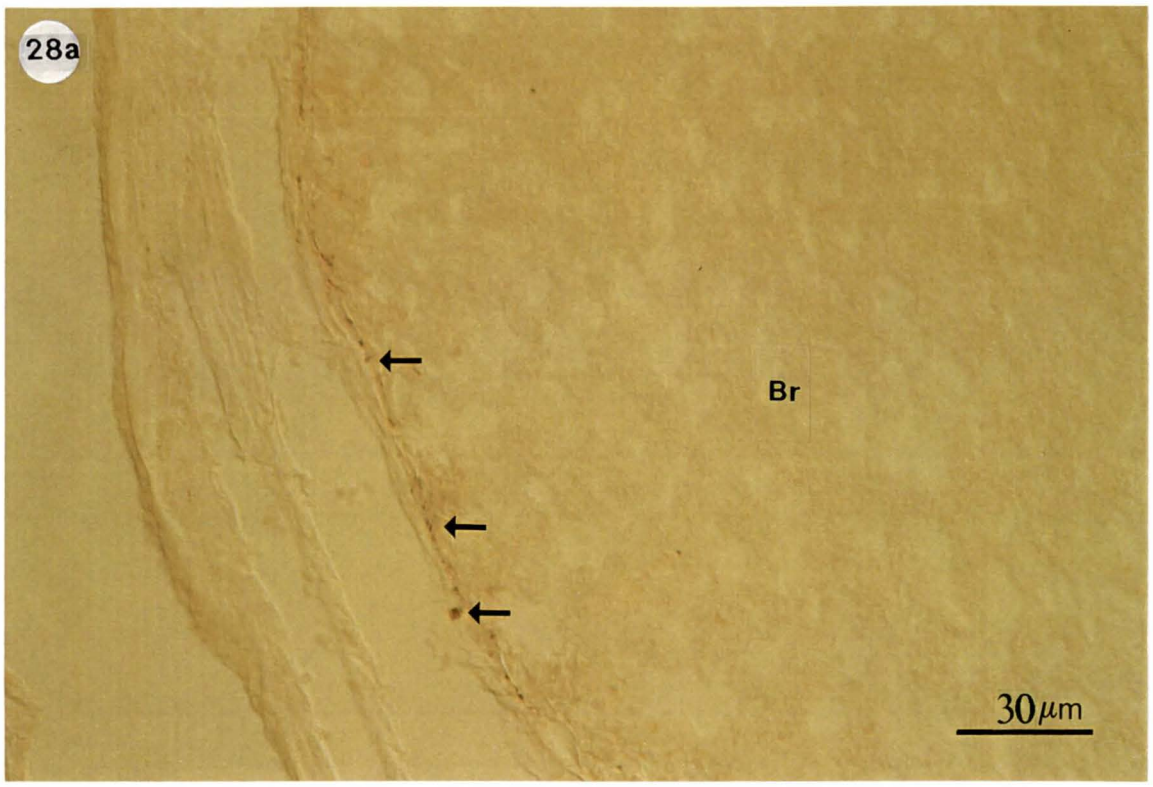
present in the periphery of the ganglion. Although still not determined, it is possible that the axons in the neuropil are projections of these peripheral cells. A noticeable aggregation of GnRH-immunoreactive axons and occasional cell bodies is present in the AM3 root, in the region where it exits the ganglion. GnRH-positive axons have also been observed in the anterior roots and other posterior roots, though much less concentrated in comparison with the AM3 roots.

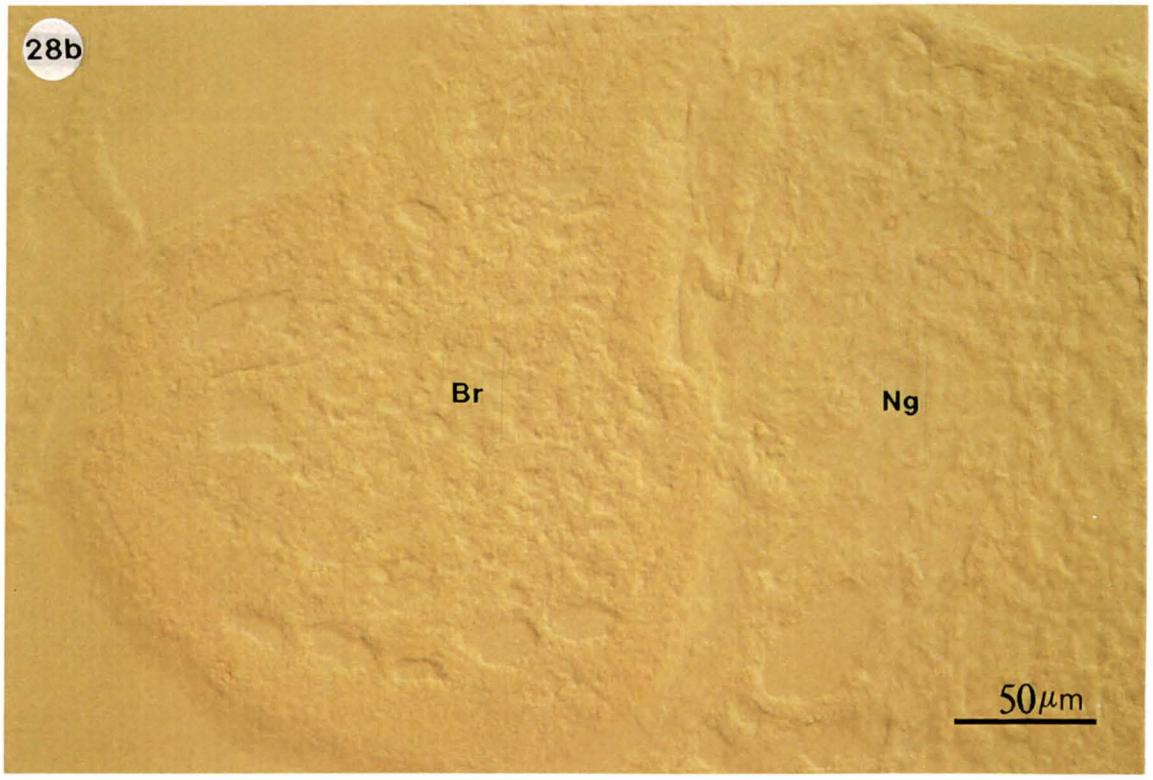
There appear to be some GnRH-positive axons in the peripheral region of the neural gland at the ganglion-gland interface in cryosections. These axons appear at the same levels at which GnRH-positive cell bodies and axons are present in the space between the neural gland and the neural ganglion. This probably results from a displacement of tissue during processing and sectioning of the neural complex. No GnRH-immunoreactivity is observed in the neural gland itself.

(B.2b) Immunocytochemical Results (anti-tubulin)

Incubation with antiserum against tubulin proves an effective way of labelling the cholinergic fibres, as discussed earlier (see Fig. 9). However, in preparations that were double labelled with anti-tubulin and anti-GnRH antisera, the labelled microtubular tracts did not coincide with the labelled neurites of the dorsal strand plexus, though label for tubulin appeared in the cholinergic fibres (Figs. 22, 23, 30). All cell bodies and axons of the dorsal strand plexus were devoid of any label for tubulin. On the other hand, the label for tubulin was present in the neural ganglion and its major roots, including the visceral root. This indicates recognition of tubulin by the antibody. Therefore, absence of label in the components of the dorsal strand plexus could be interpreted as (i) complete

Figure 28. **(a)** Cryosections, incubated with U705-23 and visualised using avidin/biotin-DAB reveal a peripheral population of GnRH-immunoreactive axons and cell bodies (*arrows*) in the neural ganglion (*Br*) of *Chelyosoma*. **(b)** Controls, incubated following same procedures as used in (a), but without the GnRH antiserum, show a lack of label around the peripheral region of the neural ganglion (*Ng*).


28a
Br
30μm


28b
Br
Ng
50μm

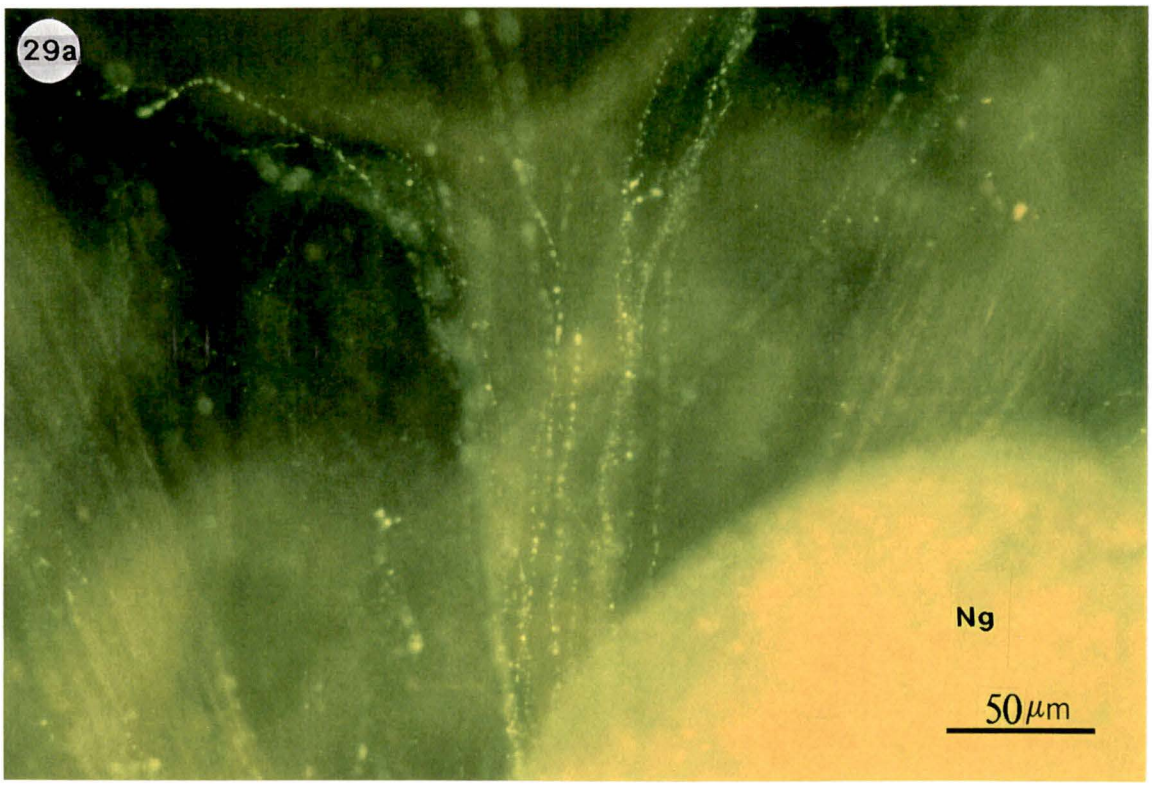
absence of tubulin or its presence in undetectable quantity; or, (ii) presence of other forms of tubulin.

(B.2c) Summary of New Results

The cells and axons that form the dorsal strand plexus are immunoreactive with anti-GnRH antiserum. This facilitates tracing the distribution of the GnRH-immunoreactive dorsal strand plexus. Furthermore, the dorsal strand plexus' lack of immuno-reactivity with anti-tubulin allows a comparison of its overlap of innervation with the innervation of the cholinesterase-positive fibres.

The dorsal strand plexus is distributed throughout the animal, extending from a peripheral population of GnRH-positive cells around the neural ganglion to the gonoducts and the gonads. GnRH-immunoreactive fibres also extend over the ciliated funnel, the branchial siphon and the branchial basket. In addition to the plexus around the dorsal strand, a group of GnRH immunoreactive cells and fibres exists in the region between the ganglion and the neural gland. These are an extension of the dorsal strand plexus. The posterior concentration of GnRH-positive cell bodies, around the AM3 root continues, posteriorly, into the dorsal blood sinus in close proximity to the dorsal fold and the visceral nerve roots. The cell bodies and the axons form a scaffolding-like arrangement around the dorsal strand. Some axons can be seen entering the transverse blood vessels of the branchial basket. Axons have also been observed near the atrial surface of the branchial basket, but none were seen to make any synaptic contact with the stigmatal ciliated cell clusters. Ramifications of the GnRH-positive axons are found over the gonoducts. The GnRH neurons have been observed in the blood spaces between the outer (atrial) epithelium and the internal organs like gut, gonoducts etc. A more intimate contact has not been revealed in the sections. At

Figure 29. A population of GnRH-positive cell bodies and axons (**a**) extends between the neural ganglion and the neural gland (*Ng*). Shown here are these axons over the duct of the ciliated funnel (*Cf*) in *Chelyosoma*. Figure 29(**b**) show the same region visualized using 5-A6 (anti tubulin). (*BM1, BM2: Branchio-medial roots.*)


29a
Ng
50 μm

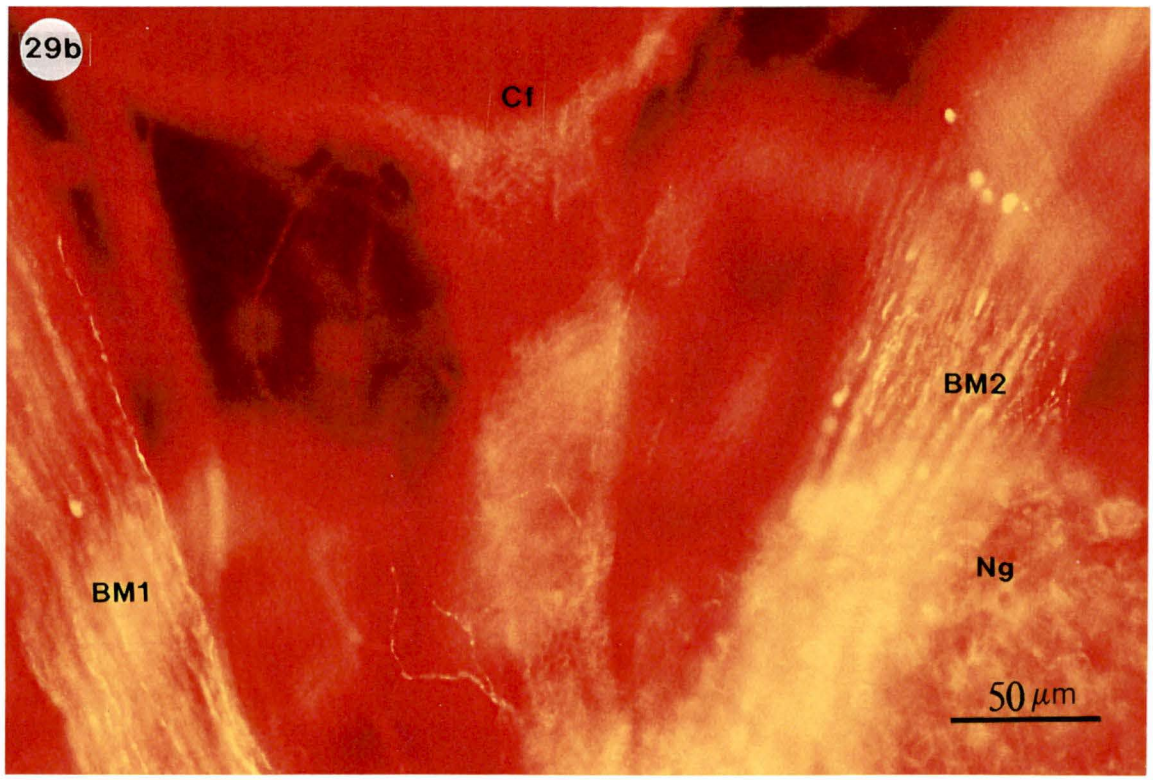
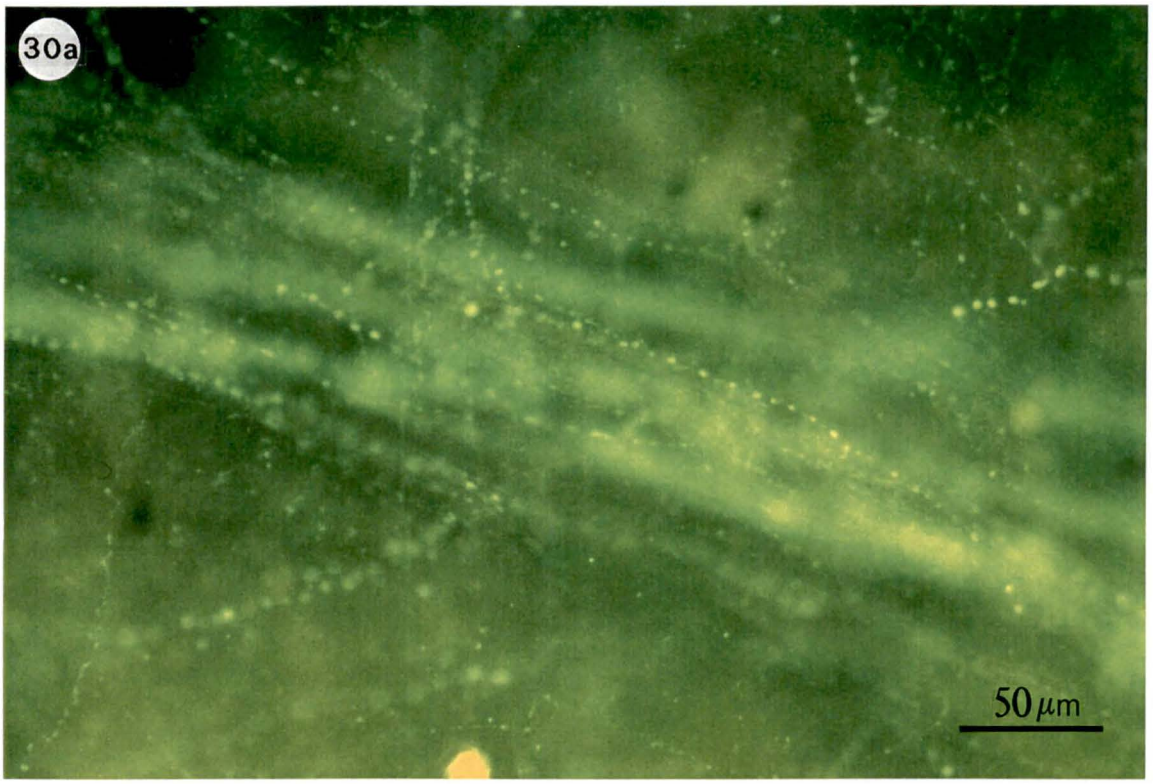

29b
Cf
BM2
Ng
BM1
50 μm

Figure 30. Double label with anti-GnRH antisera (a) and anti-tubulin (b) reveal the relative overlap of the cholinergic system and the dorsal strand plexus in *Chelyosoma*.


30a
50 μm


30b
50 μm

the base of the branchial basket the dorsal strand plexus loops below the oesophagus and extends over the gonads. This is most prominent in juvenile *Chelyosoma*.

Anti-tubulin does not reveal any microtubular tract in the dorsal strand plexus. This absence (or undetectable quantity) of tubulin in the cell bodies and axons of the dorsal strand plexus sharply contrasts with the rich tracts observed in the cholinergic system of the animals; it may serve as an extremely convenient method of visualizing and differentiating between the two systems.

(II) EM Evidence regarding Location of Peptide; Ultrastructure

(1) Previous Findings

Various researchers have associated the dorsal strand and the plexus of cells that accompany it with gonadogenesis. Irons (1986) describes the ultrastructure of the dorsal strand and its role during gonadogenesis in immature *Corella*. During this stage of development the dorsal strand consists of single cells, about 2 μm in diameter, which possess a large elliptical nucleus. Their cytoplasm contains mitochondria, Golgi complex, lamellar rough endoplasmic reticulum and abundant free ribosomes. Some regions of the cytoplasm show an aggregation of filaments with a 4 to 7 nm diameter. These are thought to be actin filaments. Dense core vesicles, 120 to 200 nm in diameter, are also present in the cytoplasm of some dorsal strand cells. Dense core vesicles are a characteristic of neurosecretory cells and are usually associated with neurosecretions, the dense core being electron dense and often a neuropeptide (Shepherd, 1983). These vesicles are most abundant in cells of the strand that lie in close contact with the ovotestis during gonadogenesis. Despite the presence of these vesicles in the cells of the dorsal strand, Irons does not suggest the strand to be a nervous structure. She proposes the dorsal strand to be a conduit for trophic factors responsible for initiation of gonadogenesis, and therefore suggests that contact between the dorsal strand and the gonad is necessary for maturation of the gonads.

Irons (1986) also observed another cord of cells accompanying the dorsal strand. This cord, presumably the dorsal strand plexus, is composed of multiple processes, 0.3 μm to 2.5 μm in diameter. Though separate from the dorsal strand, in some regions the cell membranes of the cord cells lie in direct contact with the

plasmalemma of the dorsal strand. In these regions, both the structures are enveloped by the atrial epithelium.

In *Corella* the plexus first appears as an irregular layer of cell processes, just prior to cavitation of the ovotestis during gonadogenesis (Irons, 1986). Processes of these cells vary from 0.3μm to 2.5 μm in diameter. In addition, some filamentous structures, 25 to 30 nm in diameter, were occasionally seen in these sheath cells and are considered microtubules by the author. The cytoplasm of these cells also contain mitochondria, secondary lysosomes, and lamellar endoplasmic reticulum. Irons did not find the perikarya of these cells in the gonadal region of *Corella*; however, she presumed them to be located in the neural ganglion.

Moderately dense, membrane bounded vesicles, 100-150 nm in diameter, and small electron opaque vesicles, 60-80 nm in diameter are also present in these cells. Some ovoid vesicles possess a long axis up to 250 nm in diameter. It should be noted that some somatic cells of the developing gonads in contact with the nerve cell processes also posses similar vesicles. Irons attributed a neurosecretory function to these vesicles. As they are not present in adult animals, she proposed that the dorsal fold plexus was probably involved in the later stages of the continuation of gonadogenesis in juvenile animals. Yet, it could not be responsible for the initiation of gonadogenesis, as it is absent in the early stages. Fedele (1938) considers the dorsal strand as a site of neuroblastogenesis, giving rise to the neurons of the dorsal strand plexus. Mackie (1995) also refers to some dorsal strand plexus neurons as neuroblasts. Goodbody (1974) further suggests that the dorsal strand may also perform a nutritive function, serving as 'glial cells' to the neurons and fibres of the dorsal strand plexus. However, the exact nature of the functional relationship of the strand and the plexus is still not known (Bone and Mackie, 1982).

2. New Findings

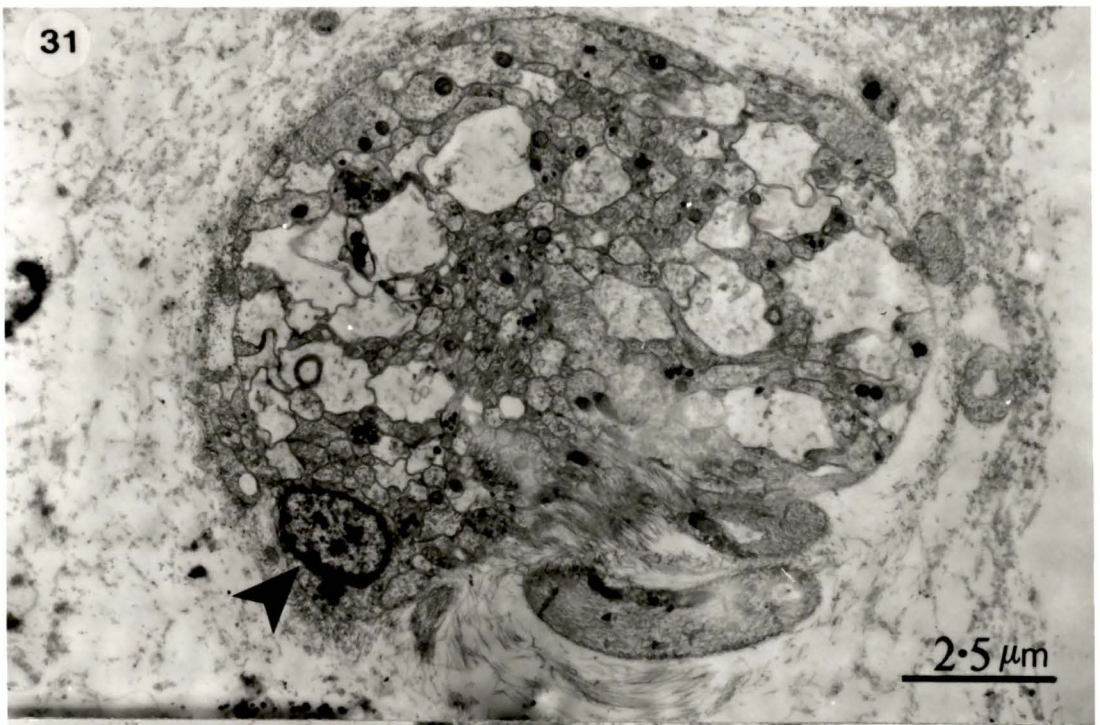
(2a) TEM and Immunocytochemical Results

The GnRH-like peptide is localized in the dense core vesicles found in the fibres and cell bodies of the dorsal strand plexus.

At the electron microscope level, it is difficult to distinguish the cells of the dorsal strand plexus from other tissue. However, axons with dense core vesicles and dense vesicles have been observed in various regions of the dorsal fold (Fig. 31) and the rectum-gonoduct region (Fig. 33). Figure 32 shows a section through the oviduct, characterized by numerous cilia projecting into its lumen. A single axon lying outside of the oviduct possesses dense core vesicles. Dense core vesicles (dcv) are vesicles with an electron opaque core with a clear space between the core and the organelle membrane: dense vesicles (dv), on the other hand, do not show the clear space between its content and the membrane. In electron micrographs of the neural ganglion, there are a few cell bodies where dense vesicles and the nucleus are visible together. As expected, axons constituting the neuropil possess clear vesicles, dense vesicles and dense core vesicles (Fig. 34). In the anterior and posterior regions of the ganglion, it appears that the concentration of the dense core and dense vesicles is generally in cell bodies and axons lying in the peripheral region of the ganglion (Fig. 35). This peripheral population of axons containing dcvs and dvs is also found in the visceral nerve immediately posterior to the neural ganglion (Fig. 31). In Figures 35 and 36 the core of the dcvs exhibit varying degrees of electron density, from electron opaque to moderately dense. This variation can depend on the angle and the region of section. GnRH-immunoreactive axons and cell bodies were also observed in the space between the neural ganglion and the neural gland. Furthermore, several synaptic junctions between axons in the neuropil of the neural ganglion were observed (Fig. 37).

Figure 31. The visceral nerve bundle immediately posterior to the neural ganglion in *Corella*. Note several axons with dense and moderately dense vesicles. A cell body (*arrow-head*) with a large nucleus is present in close vicinity of the nerve bundle, but is separated by a layer of connective tissue.

Figure 32. An axon with dense vesicles in the vicinity of the oviduct (*O*) in *Corella*. Cilia in cross section can be seen in the lumen of the oviduct.


31
2·5 μm

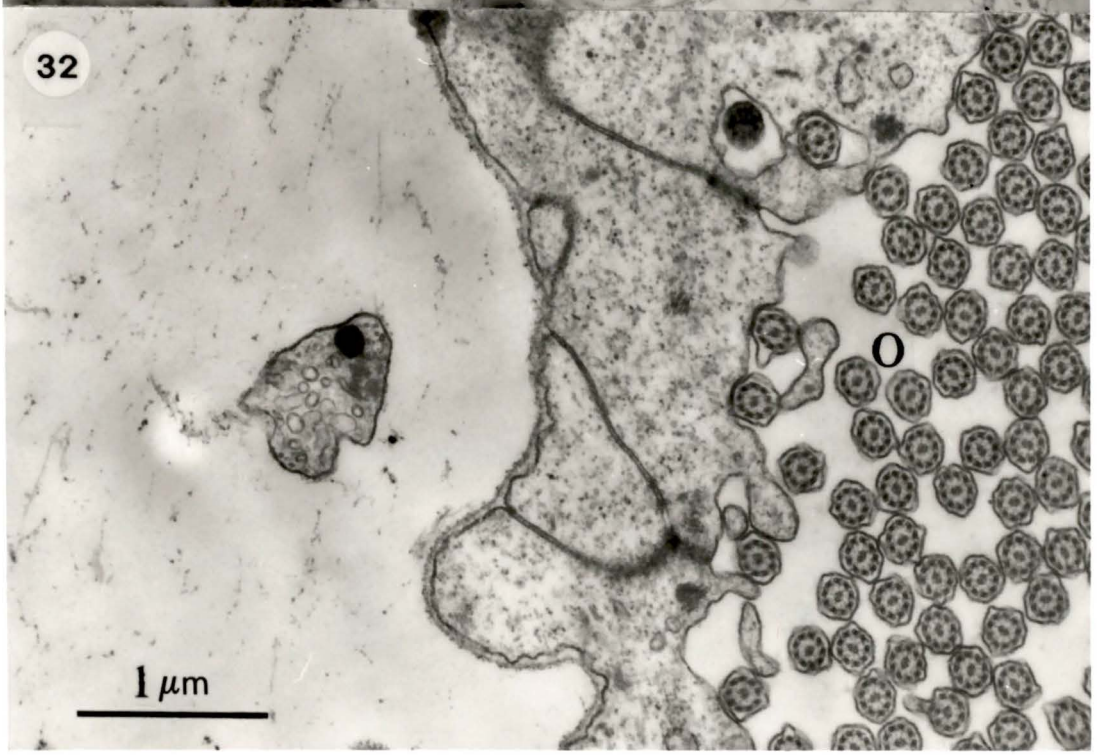
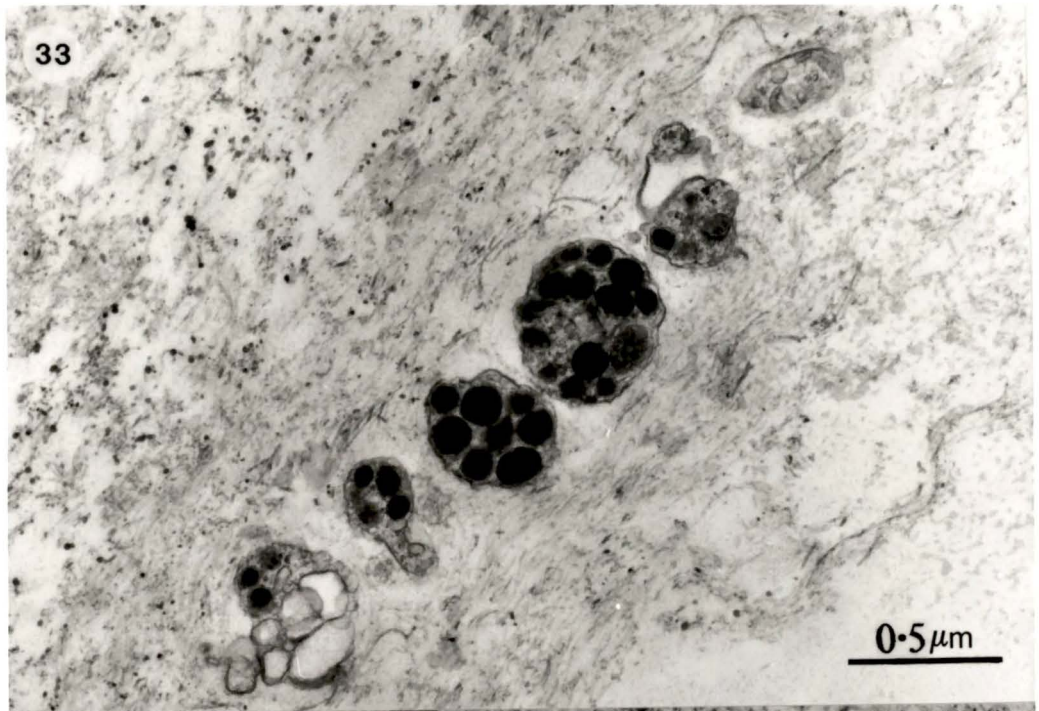

32
O
1 μm

Figure 33. Axons containing dense vesicles in the gonoduct/rectum region of *Chelyosoma*, observed using conventional TEM procedures.

Figure 34. TEM section through centre of the neural ganglion of *Corella*. Dense vesicles (*large arrow-heads*), dense core vesicles (*small arrow-heads*) and clear vesicles (*arrows*) can be seen in the axons. Note the abundance of microtubules in the axons.


33
0·5μm

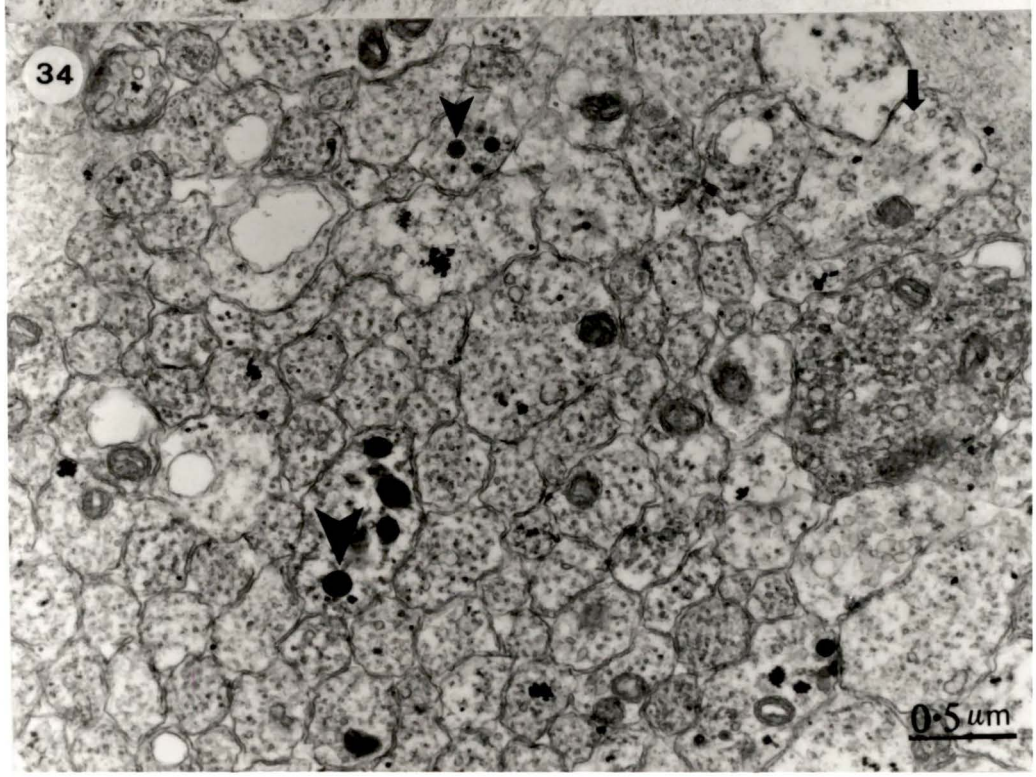

34
0·5μm

Figure 35. TEM section showing axons containing dense core vesicles in the peripheral region of posterior neural ganglion in *Corella (gl:glycogen granules)*.

Figure 36. TEM section of the visceral nerve bundle in *Corella*. A few axons lying on the periphery of the bundle possess numerous dense core vesicles.

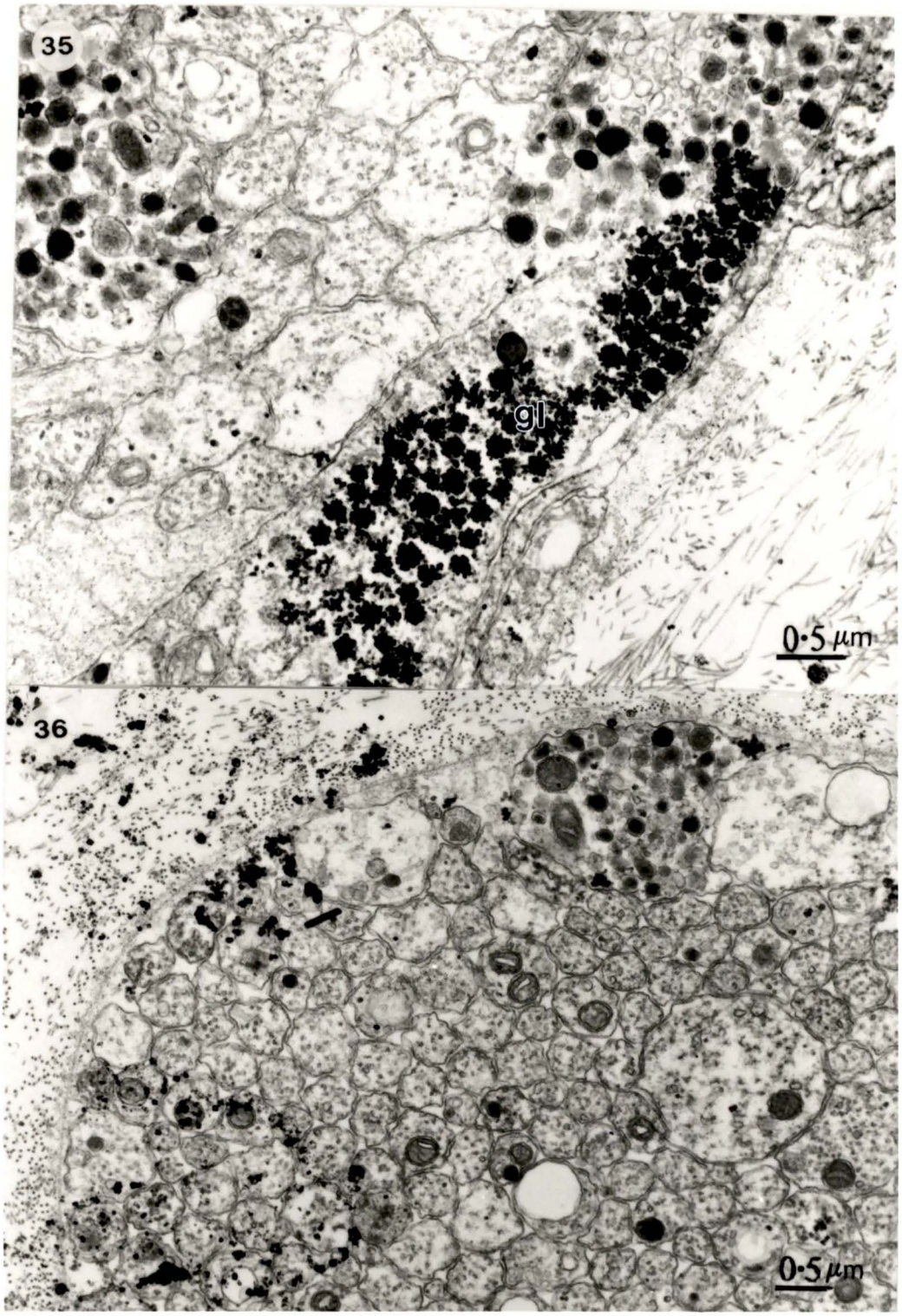
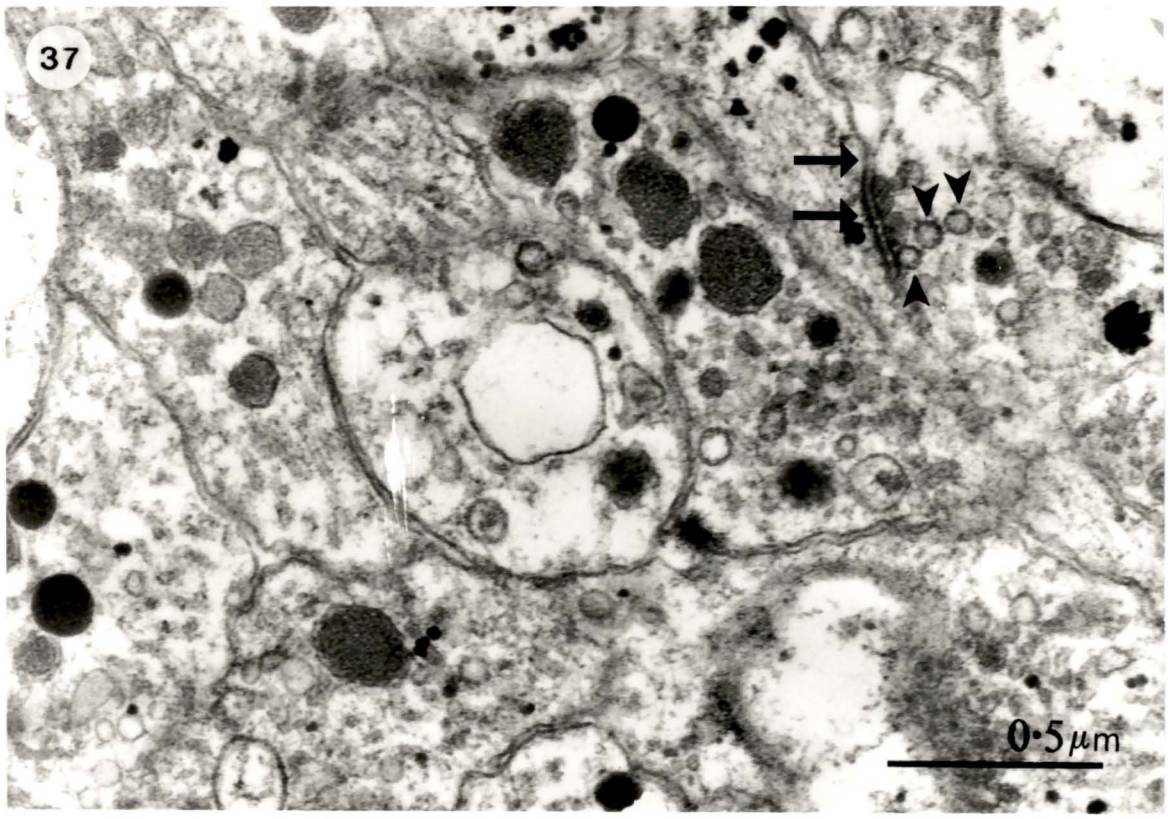

35
gl
0·5 μm
36
0·5 μm

Figure 37. A synapse in the posterior mid-region of the neural ganglion of *Corella*. The synaptic surfaces (*arrows*) and three pre-synaptic vesicles (*arrow-heads*) are well defined.


37
0·5 μm

Although I found several axons containing dcvs and dvs in the vicinity of the gonoducts, I was unable to locate synaptic contact between these axons and the gonoducts. To verify whether these axons and cell bodies are constituents of the dorsal cord plexus, the tissue was pre-incubated with U705-23 (anti-LHRH) antibody and biotin/peroxidase conjugated strepavidin. Thick sections of the treated tissue revealed two to five cells and several axons with label in the vicinity of the gonoducts (Fig. 24). Few axons were discerned close to the rectum. Electron microscopy of these cells and axons show that label resides in the dense core vesicles (Fig. 38). However, in some cells the GnRH immunoreactivity is localized in the cytoplasm, with clear vesicles suspended within the labelled area (Fig.39). Also, in these cells the label seems to be restricted to one end of the cell. These findings confirm the localization of the GnRH-like peptide in the dense core vesicles of the axons found in the periphery of the gonoducts. It is possible that, in cells with label in the cytoplasm, the peptide was leached from the vesicles during the processing of the tissue. In controls, where the primary antibody was omitted, no label was observed (Fig. 38b).

The diameter of axons with GnRH immunoreactive vesicles is between 0.2 μm to 2.9 μm (±1.0). These axons exhibit a density of the labelled vesicles ranging between one to 36 (**Appendix 1**). The mean density of dcvs per unit area of axonal section profile is 15.47/μm² (±27.08). There appears to be no correlation between the size of the axon (measured here as the area of cross-section) and the number of dense core vesicles observed in the section. While some axons show no dense core vesicles, they are comparable to axons with labelled vesicles, as they contain very few microtubules. The mean size of the cells containing GnRH-immunoreactive vesicles is 6.8 μm (±1.5) in length and 4.79 μm (±1.2) in width, with an average nuclear diameter of 2.55 μm (±0.5) (**Appendix 2**, also see: Fig. 40).

(2b) Ultrastructure Differences Between the Two Systems

In electron micrographs of the neural complex and the posterior portions of the dorsal fold, including the gonoducts, two types of axons can be differentiated based on their inclusions. One type contain large quantities of filamentous structures, 25 to 35 nm in diameter, and are predominant in the neural ganglion and in the visceral bundles (Fig. 36). These filamentous structures are microtubules and they are usually a characteristic feature of axons. The second type of axons generally contain dense vesicles and dense core vesicles, but appear to possess very few microtubules (Fig. 33, 38).

In tissue preparations that have been pre-treated with DAB to label for GnRH-immunoreactivity the cell membranes and organelles are damaged (Fig. 38), due to long incubations in solutions containing Triton X-100. In some cases an external layer of connective tissue may permit an estimation of approximate size and shape of the axon or cell body. In these pre-treated and labelled tissues microtubules can be easily discerned in most axons. However, almost all axons with labelled dense vesicles show no structure resembling microtubules. These results are somewhat consistent with the conventional TEM results where a few microtubules are seen in axons containing dense vesicles and dense core vesicles. These results also substantiate the immunocytochemical studies using anti-tubulin at the light microscopy level (see Section **B.2b**). However, in conventional TEM sections, there is no way of determining if the axons containing the dense vesicles and dense core vesicles are part of the dorsal strand plexus, and their vesicular inclusions immunoreactive with the anti-GnRH antiserum.

Figure 38. (a) An axon containing several GnRH-immunoreactive vesicles, in the vicinity of the gonoducts in a juvenile *Chelyosoma*.

(b) In control preparations, where the primary antibody was omitted, the label is absent in the vesicles (*arrowheads*) of this axon near the gonoducts in *Chelyosoma*.

(c) A cell containing several GnRH-immunoreactive vesicles observed near the gonoducts in *Chelyosoma*.

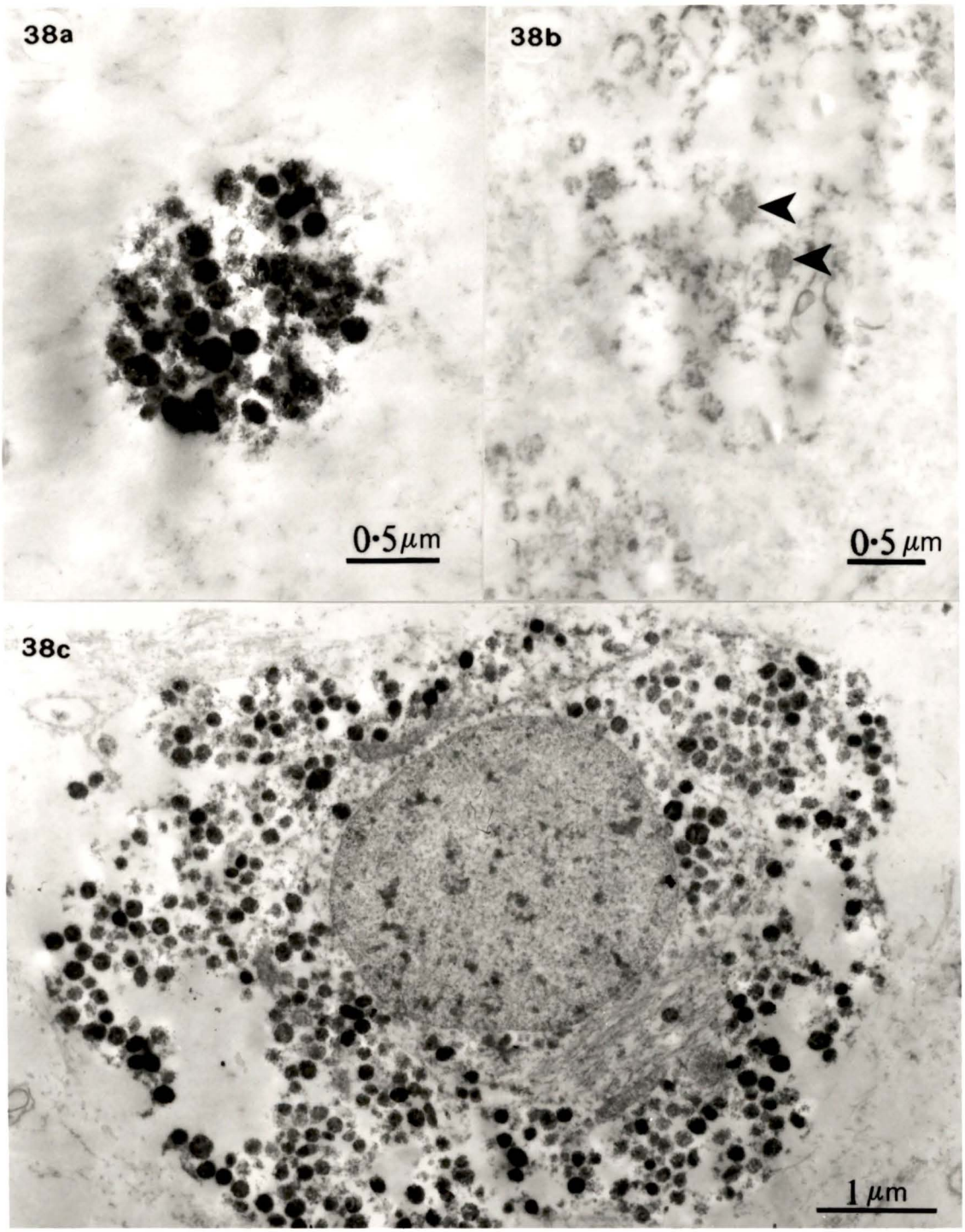

38a
0·5 μm
38b
0·5 μm
38c
1 μm

Figure 39. (a,b) Cell bodies of the dorsal cord plexus containing positive labelling for GnRH-immunoreactivity. The label is dispersed in the cytoplasm of the cell with several clear vesicles present in the region. This may be due to the leaching out of the vesicular contents during the processing of the tissue. Note the localization of the label to one end of the cell.

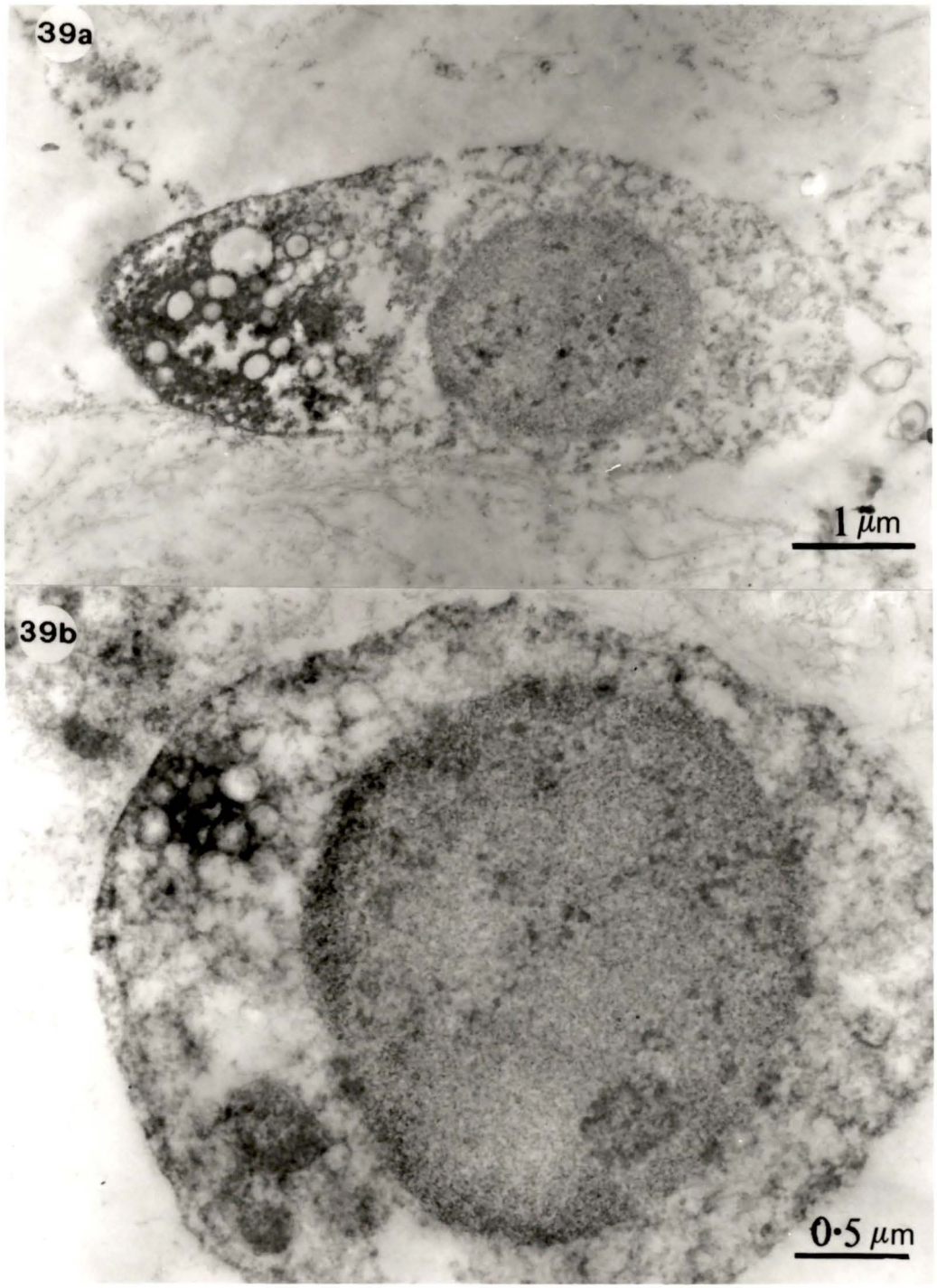
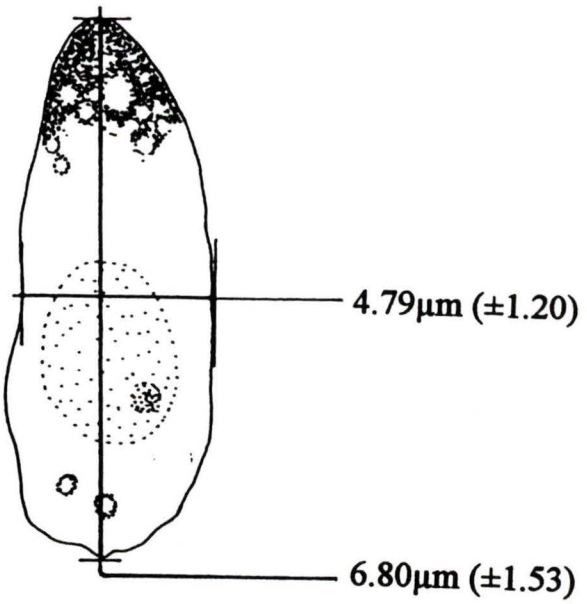
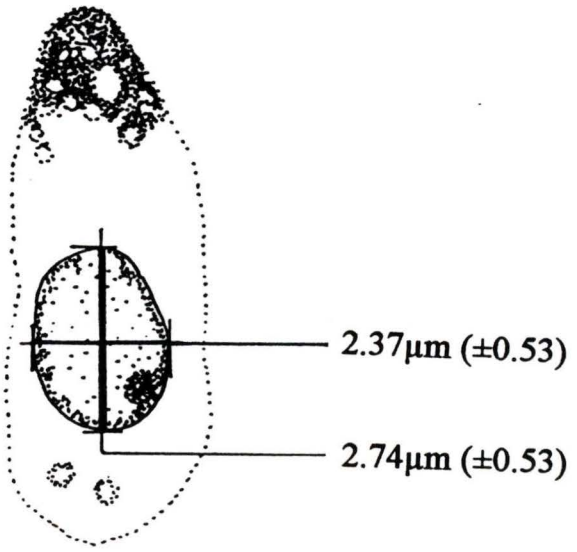

39a
1 µm
39b
0·5 µm

Figure 40. Diagrammatic representation of the mean cell size, nucleus size and axonal diameter of the components of the dorsal cord plexus of *Chelyosoma*.

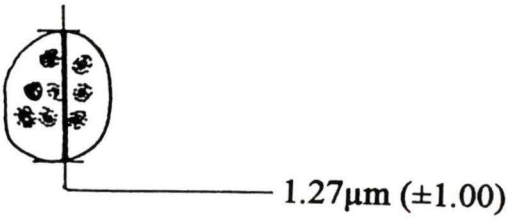
Cell



Nucleus



Axon



(2c) Summary of New Results

TEM studies of tissue labelled for GnRH confirm that the peptide is localized in the dense vesicles of axons and neurons belonging to the dorsal strand plexus. Ultrastructural observations of the dorsal fold region also reveal a structural difference between the cholinergic axons and the GnRH-immunoreactive axons. Most axons possess large quantities of microtubules; however, axons with GnRH-immunoreactive inclusions appear to have comparatively lower quantities of microtubules and, in most cases, no microtubules. These results agree with the immuno-cytochemical observations on whole mounts.

Synapses have not been observed between either set of neurons and the gonoducts. However, several axons containing GnRH-immunoreactive dense vesicles are localized around the sperm duct and the oviduct. Occasionally, these GnRH-immunoreactive axons were observed within the branchial basket. It may be noted that neuropeptide transmitters are typically released from varicosities along the neurites rather than from 'classical' synapses at nerve terminals.

DISCUSSION

The Dorsal Strand Plexus and the Dorsal Strand

The dorsal strand plexus reveals a rich and relatively consistent distribution pattern of neurons and their processes in the dorsal blood sinus, with a fair degree of overlap of innervation with the cholinergic system. The sheath of cells surrounding the dorsal strand as described by Huus (1924), Fedele (1938) and Millar (1953) is GnRH immunoreactive. It is also a part of the system described in *Ciona* (Georges and Dubois,1980) and *Chelyosoma* (Kelsall et al.,1990) and similar to the plexus described by Mackie (1995) in *Ciona* and *Dendrodoa glossularia*.

Undoubtedly, the dorsal strand is quite distinct from the dorsal strand plexus. The dorsal strand is derived from the embryonic neural tube (Willey (1893). It is a cord of slightly elongated cells, usually six in cross-section (Huus, 1924; Millar, 1953). In *Corella*, a lumen of about 1.0 μm diameter is enclosed within the strand (Mackie et al., 1974). The strand, itself, originates as the posterior extension of the ciliated funnel duct from the neural gland. Serial thick sections of the neural gland in this study reveal that the lumen of the gland is continuous with the lumen of the dorsal strand in *Chelyosoma*. Irons (1986) suggests the dorsal strand may act as a conduit for the passage of hormones from the neural gland to the gonads. However, in young *Corella* the strand exists as a cord of single cells; the lumen forms in adult animals. This suggests that the dorsal strand itself is responsible for hormonal secretion, mediated by the neural gland, which may also explain Irons (1986) findings of dense core vesicles in the cells of the dorsal strand in contact with the ovotestis. It also suggests the dorsal strand is in some way involved with the formation or maintenance of the dorsal strand

plexus, which makes its presence mandatory during gonadogenesis. It should be noted here that the dorsal strand appears in juvenile *Corella* before the plexus. Furthermore, Irons (1986) as well as Huus (1924), Millar (1953) and Mackie et al. (1974) suggest the involvement of the dorsal strand and the dorsal strand plexus in gonadogenesis. However, their speculation is based purely on morphological relationships, GnRH-immunoreactivity of the plexus was not known at the time of their studies.

The neural components of the dorsal strand plexus are first seen (in the anterior part of the dorsal blood sinus) in association with the dorsal strand immediately posterior to the neural ganglion. Here the GnRH immunoreactive neurons and neurites are aggregated around the AM3 root of the ganglion, extending posteriorly to ensheath the dorsal strand. This sheath-like arrangement has been observed in many ascidians (Huus, 1924; In *Ciona* by Fedele, 1938; Millar, 1953). Although the reason for this association is not clear, it is possible that the GnRH neurons actually derive from the dorsal strand (Fedele, 1938). This may explain the presence of some cells around the dorsal strand in stages reminiscent of early neuroblast differentiation, which Fedele (1938) and Mackie (1995) describe in *Ciona*. Based on the fact that the GnRH-immunoreactive neuroblasts in vertebrate embryos delaminate from the olfactory placodes and migrate to various destinations in the brain (Webb and Noden, 1993), Mackie (1995) suggests that a similar process may occur in the adult tunicates. It may also be possible that neurogenesis is limited to embryonic stages. If this is correct, the neuroblasts seen in the adult tunicates are in an arrested state and act as reserves to be utilised during growth, regeneration or reproductive cycles.

Extent of the Dorsal Strand Plexus

This study confirms the presence of a sheath-like arrangement of the dorsal strand plexus around the dorsal strand in both *Corella* and *Chelyosoma*, as has also been described in other ascidians (Huus, 1924; Fedele, 1938; Millar, 1953; Mackie,1995). At the base of the neural complex, where the dorsal strand originates, the dorsal strand plexus is spread around the AM3 posterior root of the ganglion. In both juvenile and adult *Chelyosoma* another group of GnRH-immunoreactive cells are present along the ciliated funnel duct similar to those reported in juvenile *Ciona* by Georges and Dubois (1985). This group of neurons and neurites ramify over the outer surface of the ciliated funnel and its duct to the level of the neural gland. Posterior to this point they merge into the main body of the plexus over the AM3 root. Anterior to the ciliated funnel a few fibres extend over the branchial musculature surrounding the peripharyngeal band, but none extend to the pigmented spots on the branchial siphon of *Corella* (as compared to cholinergic extensions.) Carlisle (1951) suggests an extrinsic stimulation for hormonal release by the neural gland, which mediates gamete release. Although no GnRH immunoreactivity has been observed in the neural gland, if Carlisle is correct, then these branchial projections may be involved in the process of stimulation mediated gamete release.

It is unclear whether the plexus actually originates from the neural ganglion itself, as suggested by Huus (1924), or if its presence around (Georges and Dubois, 1985) and within the visceral nerve (Kelsall et al., 1990) merely indicates a small population of neurons, whose fibres migrate to the ganglion (Mackie, 1995). In addition to this aggregation of GnRH-immunoreactive elements in the ganglionic roots, a peripheral population of GnRH-immunoreactive fibres exists within the

ganglion itself. While cryosections reveal GnRH-immunoreactive fibres spread within the neuropil of the ganglion, it is difficult to determine if these fibres are projections of cell bodies that may be situated within the ganglion itself, or whether they once again represent an immigrant population of neurites that enter the ganglion through the anterior and the posterior roots. In *Ciona* the elements of the dorsal strand plexus delineate the inner margins of the dorsal blood sinus as their outer limit of extension within the sinus (Mackie, 1995; personal communication). This pattern of neurite distribution is exhibited along the entire length of the blood vessel and similar patterns of neuronal distribution can be followed in *Corella* and *Chelyosoma*. The blood sinus is continuous to the level of the ciliated funnel. If the neurites radiate in a similar pattern anterior to the AM3 root, it explains the presence of the peripheral population of GnRH-immunoreactive cell bodies and fibres seen in the space between the neural gland and the neural gland and on the outside of the ganglion. This population of GnRH-immunoreactive elements should, however, be distinguished from those seen within the neural ganglion itself, which is probably an immigrant population (as described earlier).

A few neurites have been observed entering the branchial basket from the dorsal blood sinus via the transverse blood vessels in young *Chelyosoma*. Some are also present within the gill bars of both adults and juveniles. The presence of GnRH-immunoreactive neurites has also been reported in the gill bars of *Dendrodoa* (Mackie, 1995), but no cell bodies have been observed in the branchial sac of any of the animals. Since only a few examples of GnRH-immunoreactive neurites in the branchial basket have been observed, it is not feasible to suggest a correlation between the GnRH-immunoreactive fibres and the branching pattern of the visceral nerves. The GnRH-immunoreactive neurites do not exhibit synaptic

boutons at the base of the ciliated cell clusters of the branchial basket, which is notably different from the innervations of the basket by the cholinergic fibres (also see Arkett et al., 1989).

The dorsal strand plexus accompanies the dorsal strand posteriorly within the dorsal blood sinus to the level of the oesophagus in most ascidians (Huus 1924; in *Ciona*: Millar, 1953; Mackie, 1995) and *Corella* (Mackie et al., 1974). However, in *Chelyosoma* the main axis of the plexus shifts at the level of the anus-gonopores, although some cell bodies are still present in the dorsal fold region. The dorsal strand plexus, itself, loops across the sperm duct and comes to lie in close proximity to the gonoducts. From the rest of its length, running along the gonoducts, numerous neurites transverse the region between the gonoducts and the dorsal fold. As in *Ciona* (Bone, 1959; Mackie, 1995), extensive ramifications of GnRH-immunoreactive neurites are seen over the sperm duct. In the region of the anus and the gonopores a distinct scaffolding-like arrangement is exhibited by the neurites. This rich innervation of the gonoducts by the GnRH-immunoreactive plexus may suggest the involvement of the GnRH-like peptide in gamete discharge, independently or in conjunction with photic factors (Mackie, 1995).

In contrast to Markman's (1958) observations in *Ciona*, extension of the plexus to the posterior proximity of the pericardium is not seen in either *Chelyosoma* or *Corella*. Markman's 'visceral nerve plexus' was probably the branches of the (cholinergic) visceral nerve and not the dorsal strand plexus. It is interesting to note that Bone and Whitear (1958) describe a plexus of nerve fibres in the wall of the pericardium of young *Ciona*, with nuclei at various intervals. They suggest these nuclei may belong to the sheath around the nerve. Mackie et al. (1974) observed protistan parasites in the cholinergic nerve trunks that could have been mistaken for cell bodies by other workers.

In both *Corella* and *Chelyosoma* varicosities are present all along the length of the neurites of the cholinergic as well as the dorsal strand plexus. Although I was unable to trace the extension of the dorsal strand plexus in the regions of the pericardium described by Bone and Whitear (1958), it is possible that the 'nuclei' described by them may be the varicosities seen in the cholinergic neurites. This assumption is further supported by the fact that no ganglion cells were found in the pericardium wall of young *Ciona* (Bone and Whitear, 1958; also see Mackie, 1995).

Continuation of the dorsal strand outside the dorsal blood vessel has not been observed in adult *Chelyosoma* and *Corella*. Consequently, it remains unclear if the plexus is still in association with the strand outside the dorsal blood vessel in adults, or if it represents the tract occupied by the strand in the immature animals. In juvenile *Chelyosoma* the plexus exits the dorsal blood vessel at the level of the oesophagus. It is possible that outside the dorsal blood vessel and the branchial sac, the plexus lies within still another large visceral blood vessel. In the body cavity it extends over the surface of the stomach and comes to occupy a medial position above the gonoducts. It is not known if the plexus is still associated with the dorsal strand over the gonads in juvenile *Chelyosoma*, although Irons (1986) confirms the association in immature *Corella*. In both animals several neurites extend from the main axis of the plexus over the surface of the gonads contained within the loop of the gut. While, contrary to Millar's description of *Ciona* (1953), no posterior aggregation of GnRH-immunoreactive cells over the gonads has been observed in *Chelyosoma* and *Corella*, their presence cannot be ruled out entirely. In adult animals the relative thickness of the tissues in these regions make penetration of reagents more difficult and visibility is poor.

The Cholinergic System

Previous studies by various workers describe two types of nervous elements in tunicates. The results presented in this study agree with the observations made by those researchers and confirm the presence of two distinct components of the peripheral nervous system of *Chelyosoma* and *Corella*: the cholinergic system, comprising the neural ganglion and its peripheral projections, and the dorsal strand plexus.

The cholinergic system previously described by Arkett et al. (1989) shows cell bodies in the neural ganglion and projections into the viscera of the animals. Cholinergic innervation extends into the branchial siphon and its musculature. Markman (1958) also observes nerve endings from the anterior nerves on the pigmented spots that flank the branchial siphon of *Ciona*. This is not the case in *Corella*, where cholinergic neurites are abundant in the region of the branchial siphon, but there is no connection of the neurites and the pigmented spots. The neurites appear to run around these structures and appear to be in association with them; this agrees with Markman's observation, but also draws attention to Bone and Mackie's (1982) reference to light insensitivity of the pigmented spots in *Ciona*.

My investigations reveal numerous neurites coursing though the mantle, innervating the musculature in that area. These results augment the previous descriptions of the cholinergic innervations of the viscera in *Chelyosoma* and *Corella* (Arkett et al., 1989). A rich plexus of cholinergic fibres surround the rectum and most of the cholinergic neurites are observed in the fold of tissue overlying the gonoducts and the rectum. Isolating the rectum from the gonoducts in tissue preparation reveals a few neurites close to the oviduct and the sperm duct. As the visceral bundles consist mostly of motor nerves (Arkett et al., 1989), their

projections over the gonoducts could be, in some way, involved with the contraction and subsequent expulsion of the gametes. However, further study is necessary to confirm this theory.

Differentiating between the Two Systems

The dorsal strand plexus differs from the cholinergic system by showing a lack of histochemical reaction for cholinesterase, and a strong reactivity with GnRH antisera. The scanty observations of the GnRH-immunoreactive neurites within the branchial basket contrast sharply with the rich innervation of the basket by the cholinergic nerves. Furthermore, the GnRH-immunoreactive neurites do not form synaptic boutons at the base of the ciliated cell clusters lining the stigma of the basket. Electron micrographs enable further distinction between the cholinergic and GnRH-positive axons in the vicinity of the gonoducts, based on vesicular inclusions and the presence of microtubules.

Another striking characteristic of the GnRH-immunoreactive fibres is the lack of labelling for tubulin. Tubulin is the component of microtubules, an essential element of neurites as microtubules are involved in the transport of cellular secretions within the cell and to the point of discharge. In neither animal did label for tubulin appear in the components of the dorsal strand plexus. On the other hand, the cholinergic fibres and cell bodies reveal richly labelled microtubular tracts. Also, label for tubulin was observed in the cilia of the ciliated cells of the branchial basket and the wall of the gonoducts, etc., which confirms positive recognition by the antibody against tubulin, and implicates the absence of tubulin in the GnRH-immunoreactive nerve elements. The absence of tubulin could suggest immaturity of the cells. The lack of label may also indicate that the quantities of microtubules are negligible in comparison to those observed in the

cholinergic fibres, or can be attributed to the presence of other forms of tubulin. This raises questions about possible mechanisms for the transportation of the secreted peptide within these neurons and to the distal regions of the neurites. Could it be possible that the peptide is locally secreted, in a paracrine fashion? Even if the peptide was being directly discharged into the blood stream, it would be in close vicinity to the target organs, such as the gonads and the gonoducts. Also, the quantity of the GnRH-like peptide produced in the animals is considerably high (Sherwood, personal communication) and hence should be optimal even after the dilution in the blood. This may explain the presence of the multitude of cell bodies and fibres, rich in the GnRH-like peptide, throughout the body of the animals, especially over the reproductive tracts.

Location of the GnRH-like Peptide

The GnRH-like peptide has been localized in the dense and the dense core vesicles present in the axons and cell bodies of the dorsal strand plexus in both adult and juvenile *Chelyosoma*. These labelled vesicles range from 100 nm to 180 nm, figures which agree with the size of the dense core vesicles observed by (Irons, 1986) in immature *Corella*. The diameter of the processes containing these vesicles in *Chelyosoma* is 0.2 μm to 2.9 μm, as compared to 2.5 μm to 3.0 μm in *Corella* (Irons, 1986). Irons did not observe any perikarya of the processes in the gonadal region and presumed it is located in the neural ganglion. However, the present study reveals cell bodies of the GnRH-immunoreactive tract distributed along the gonoducts and over the gonads in both juvenile and adult animals. Average size of these cells range between 4.9 μm to 6.8 μm, with an average nuclear diameter of 2.55 μm. Axons and cell bodies containing GnRH-immunoreactive vesicles are located in the close vicinity of the gonoducts. In

immature *Corella* moderately dense, membrane bounded vesicles, 100-150 nm in diameter, and small electron opaque vesicles, 60-80 nm in diameter, are also present in some somatic cells of the developing gonads (Irons, 1986).

Photic Induction of Gamete Release

Woollacott and Porter (1977) describe the presence of pigmented cells on the distal tip of the sperm duct of *Ciona*. According to them, these may be involved in a light initiated and microfilament mediated gamete release from the sperm duct. No localized aggregation of pigmented cells has been seen on the sperm duct openings of *Corella* and *Chelyosoma*, even though pigmentation is sometimes visible on the main body of the sperm duct. Furthermore, the tunic of adult *Chelyosoma* is dense and almost opaque. Only in very young animals does it have a translucent quality. *Chelyosoma* is found, in its natural habitat, in clumps of several animals, so grouped that only the anterior siphonal disc is exposed. This rules out the possibility of gamete release from the sperm duct being dependent on subtle differences in light intensity. It may be that the distinction between night and day is adequate or that light induced gamete release is limited to more transparent species like *Ciona*. I note here that there seems to be a certain degree of pressure sensitive release of sperm from the sperm duct of both *Corella* and *Chelyosoma*, though electrical stimulation of the gonoduct has not been effective in causing the release of gametes in *Corella* (Mackie, personal communication).

Conclusion

In conclusion, then, it is established that two distinct components of the peripheral nervous system exists in these representatives of ascidians. This leads to further queries regarding the origin of the dorsal strand plexus, its function in reproduction and its fate in evolution. The cells of the dorsal strand continuously undergo mitotic divisions; DNA labelling of these cells would make it possible to follow migratory pathways of these cells and determine their fate. It is evident that there is an alternate control of reproduction, other than the brain. The association of the dorsal strand plexus with the reproductive tract of the animals, and the presence of GnRH-like peptide, reiterate the involvement of the strand in the control of sexual activity in the animals. Further research is necessary to enable a better understanding of the functions of the dorsal strand plexus.

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Appendix I.

Axonal Diameter and number of Dense Core Vesicles (DCVs) in the Dorsal Strand Plexus.

		Axonal diameter (μm)	Number of dcvs	Area of section (μm²)	density per unit area
1.		0.8	-	0.5	0
2.		0.8	-	0.5	0
3.		4.7	-	17.3	0
4.		2.1	-	3.5	0
5.		0.5	-	0.2	0
6.		2.2	-	3.8	0
7.		0.9	5	0.64	7.9
8.		1.3	8	1.33	6.0
9.		0.5	4	0.2	20.4
10.		0.2	3	0.03	96.8
11.		0.5	3	0.2	15.3
12.		1.4	17	1.5	11.0
13.		1.5	18	1.8	10.1
14.		3.8	25	11.3	2.2
15.		0.7	4	0.4	10.4
16.		1.7	7	2.5	3.0
17.		4.8	44	18.1	2.4
18.		1.7	5	2.3	2.2
19.		0.8	5	0.5	10.0
20.		0.2	4	0.03	129.0
21.		1.3	14	1.3	10.5
22.		1.0	12	0.8	15.3
23.		1.8	33	2.5	13.0
24.		1.2	22	1.1	19.5
25.		5.1	64	20.4	3.1
26.		1.0	5	0.8	6.4

Appendix I.

Axonal Diameter and number of Dense Core Vesicles (DCVs) in the Dorsal Strand Plexus.

		Axonal diameter (μm)	Number of dcvs	Area of section (μm²)	density per unit area
27.		0.9	2	0.6	3.1
28.		2.2	22	3.8	5.8
29.		1.0	9	0.8	11.5
30.		2.9	26	6.6	3.3
31.		1.1	16	1.0	16
32.		1.6	25	2.0	12.5
33.		0.5	5	0.2	25
34.		1.7	36	2.3	15.6
35.		0.5	3	0.2	15.3
36.		0.5	1	0.2	5.1
37.		1.2	8	1.1	7.3
38.		0.3	4	0.1	57.1
39.		0.6	1	0.3	3.5
40.		1.2	12	1.1	10.6
41.		0.5	13	0.2	66.3
42.		0.8	2	0.5	4.0
43.		0.4	4	0.1	32.0
44.		1.9	1	2.8	0.3
45.		1.7	29	2.3	12.8
46.		0.8	10	0.5	20.0
47.		0.6	1	0.2	3.5
48.		1.8	12	2.5	4.7
49.		1.9	12	2.8	4.2
50.		0.5	3	0.2	15.3
51.		1.0	4	0.8	5.1

Appendix I.

Axonal Diameter and number of Dense Core Vesicles (DCVs) in the Dorsal Strand Plexus.

		Axonal diameter (µm)	Number of dcvs	Area of section (µm²)	density per unit area
52.		1.8	29	2.5	11.4
53.		1.0	2	0.8	2.5
54.		1.7	3	2.3	1.3
55.		0.4	1	0.1	8.0
56.		0.3	7	0.1	100.0
57.		0.3	0	0.8	0
58.		0.9*L*	83	0.6	130.5
59.		0.3 *L2.3*	10	1.3	7.5
60.		0.4 *L3.6*	10	3.1	3.2
61.		1.7	39	2.3	17.2
62.		1.5	19	1.8	10.7
63.		2.3	34	4.1	8.2
64.		1.6	19	2.0	9.4
65.		1.3	4	1.3	3.0
66.		1.5	5	1.8	2.8
67.		1.6	7	2.0	3.5
68.		1.4	15	1.5	10.0
69.		0.4	1	0.1	8
70.		0.7	0	0.4	0
71.		0.5	1	0.2	3.1
72.		0.6	0	0.3	0
73.		0.4	0	0.1	0
74.		0.5	14	0.2	70

Mean axonal diameter:1.27µm (±1.00)
Mean area of axonal section profile: 2.11µm² (±3.79)
Mean density of dcvs per unit area: 15.74/µm² (±27.08)
(*L: length of the axonal cross-section in a grazing section*)

Appendix II

Dorsal Strand Plexus: cell and nucleus dimensions

	Cell Size(µm)		Nucleus Size(µm)	
	length	width	length	width
1.	7.0	5.1	2.9	2.5
2.	4.1	7.8	3.0	2.5
3.	7.2	6.0	2.9	2.4
4.	7.8	6.5	3.6	2.3
5.	7.3	5.7	3.5	2.7
6.	6.9	5.2	2.0	1.5
7.	6.5	5.1	2.6	2.5
8.	6.6	5.4	2.6	2.6
9.	4.0	3.7	2.5	2.1
10.	6.0	2.5	2.1	2.1
11.	6.7	2.6	1.2	1.2
12.	-	-	2.8	2.4
13.	7.5	4.9	3.0	2.8
14.	6.9	4.6	2.0	1.3
15.	5.1	5.1	2.7	2.7
16.	5.7	5.7	2.3	2.3
17.	6.6	5.5	3.1	3.1
18.	6.8	3.8	2.1	2.0
19.	7.6	4.3	3.4	3.1
20.	-	-	3.1	3.1
21.	-	-	2.4	2.4
22.	8.6	7.4	3.0	1.8
23.	8.0	6.4	3.2	1.9
24.	7.2	4.0	2.7	2.0
25.	4.8	-	3.0	3.0
26.	12.0	4.9	2.8	2.4
27.	5.1	4.7	3.3	2.8

Appendix II

Dorsal Strand Plexus: cell and nucleus dimensions

	Cell Size(µm)		Nucleus Size(µm)	
	length	width	length	width
28.	5.8	3.6	-	-
29.	5.3	2.8	-	-

Mean cell length: 6.80µm (±1.53)

Mean cell width:4.79µm (±1.20)

Mean nucleus length:2.74µm (±0.53)

Mean nucleus width:2.37µm (±0.53)

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Characterization of Two Components of the Peripheral Nervous System of *Chelyosoma productum* and *Corella inflata*

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