CATECHOLAMINERGIC AND PEPTIDERGIC NERVOUS SYSTEMS OF A MERMITHID NEMATODE, ROMANOMERMIS CULICIVORAX, ROSS AND SMITH, 1976

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CATECHOLAMINERGIC AND PEPTIDERGIC NERVOUS SYSTEMS OF A MERMITHID NEMATODE, *Romanomermis culicivorax*, Ross and Smith, 1976

By

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ABSTRACT

The nervous system of the mermithid nematode, Romanomermis culicivorax, was investigated at different developmental stages for the distribution of catecholamine(s) and the neuropeptide FMRF-amide. A glyoxylic acid-induced fluorescence method was used to locate catecholamines, while FMRF-amide was detected by immunohistochemistry.

The distribution of sense organs was elucidated by scanning electron microscope studies. Six cephalic sensory papillae (two lateral, two dorso-lateral and two ventro-lateral) and two amphids were found in the head tips of adult nematodes. In males, each lateral papilla contained two pores and each of the dorso-lateral and ventro-lateral cephalic papillae contained three pores. Three pores were present in each of the six cephalic papillae of females. Several caudal sensory papillae were present in the tails of males, each of which contained one pore. Caudal sensory papillae were absent in the tail of females.

The catecholaminergic and peptidergic nervous systems increased in complexity during development. In the adult nematode, catecholamine(s) occurred in the nerve ring, ganglia of the nerve ring, cephalic nerves, ventral and dorsal nerve cords and mid-body region ganglia. The distribution of catecholaminergic ganglia in the tail region was different for

each sex. The tail region of males contained 16-20 catecholaminergic ganglia, whereas the tail of females contained only two ganglia. The catecholamine widely distributed throughout the nervous system of R. culicivorax is not dopamine, since immunoreactivity to dopamine was observed only in the amphids.

The role of the catecholamine(s) in reproduction was studied by measuring the fluorescence intensity of catecholamine(s) in the nervous systems of males and females under different rearing conditions. A significantly greater concentration of catecholamine was recorded in the nervous system of adult males and females than in post-parasitic juveniles. A significantly higher concentration of catecholamines was observed in adults that were reared in physical contact with the opposite sex than in those reared in isolation. Adult males that were reared with females in the same water rearing medium, but physically separated by a barrier, displayed a significantly greater concentration of catecholamines in their nervous systems than did males reared in isolation, but the catecholamine staining intensity of such males was less than in males that were allowed physical contact with females. In adult males, the fluorescence intensity of catecholamines declined progressively during and after copulation. However, in adult females, the intensity of catecholamines remained constant before, during and after

copulation. It is suggested that the catecholamine(s) may play a role in regulating copulatory behaviour, egg production and/or oviposition.

In adults, FMRF-amide-like peptide was present in the nerve ring, cephalic papillary ganglia, cephalic nerves, amphids, ganglia posterior to the nerve ring, longitudinal nerve cords and several mid-body region ganglia. The distribution of peptidergic ganglia in the tail region was different for each sex. Four clusters of ganglia were present in the tail of females, whereas such ganglia were absent in the tail of males.

The results suggest that catecholamines and FMRF amide like peptides are widely distributed within the nervous system of R. culicivorax. These substances might function as neurohormones and neurotransmitters in controlling physiological and developmental processes.

This thesis is dedicated to the
memory of my father who was
the continuous source of
inspiration for me
in every walk
of life.

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I. INTRODUCTION

The mermithids (Nematoda:Mermithidae) are a family of nematodes that parasitize arthropods, especially insects (Poinar, 1979). Fifteen species of mermithids have been reported from 79 different species of mosquitoes (Petersen, 1984). One of these, Romanomermis culicivorax Ross and Smith, 1976, has been widely regarded as a potential biocontrol agent for mosquitoes.

The nematode is only able to complete parasitic development in mosquitoes (Petersen, 1984; 1985). It is not infective to nontarget organisms (Ignoffo et al., 1973; 1974), is resistant to a variety of chemical insecticides (Finney et al., 1977; Levy and Miller, 1977) and can be mass produced in vivo by propagating it through a mosquito laboratory host (Petersen and Willis, 1972; Platzer and Stirling, 1978). Numerous trials have confirmed that the nematode is able to control field populations of mosquitoes (Petersen, 1985).

Adult females lay eggs in the pond substratum. The development of the eggs is temperature dependent and requires 7-10 days at 26°C (Petersen, 1981; Thornton and Brust, 1979). The first juvenile molt is completed within the egg (Poinar and Otieno 1974). The second stage infective juveniles (pre-parasites) hatch from the eggs and enter the hemocoel of larval mosquitoes by penetration of the host's cuticle

(Shamseldean and Platzer, 1989). Based on the growth pattern of the parasite, it has been suggested that a second molt occurs at approximately four days after infection (Gordon et al., 1974; Poinar and Otieno, 1974). During the parasitic phase, mermithids derive their nutrients directly from the host's hemolymph by transcuticular uptake (Gordon and Burford, 1984; Rutherford and Webster, 1974) and store nourishment in a storage organ (trophosome) for subsequent utilization by the post-parasitic (free-living) stages. Upon completion of parasitic development (usually 6-8 days at 26°C), the nematode emerges as a third stage juvenile from the host by rupturing the host's exoskeleton. This rupturing of the cuticle is lethal to the host. After emergence, the post-parasitic juvenile nematodes burrow into the pond substratum where a final molt to the adult stage occurs, approximately five days after exiting the host at 26-27°C (Vyas-Patel, 1989). As in the case of Filipjevimermis leipsandra, a mermithid parasite of chrysomelid beetle larvae (Poinar, 1968), only three molts have been observed or inferred in the life cycle of this nematode. The life cycle is completed within three to four weeks at 26°C (Petersen, 1984).

Despite its biocontrol potential, research on R. culicivorax has declined during the last decade, because of the difficulty in obtaining sufficient numbers of infective juveniles of the nematode at a commercially-feasible cost for

field applications. The only commercially viable method for mass-rearing R. culicivorax would be an in vitro technique (Finney, 1981; Petersen, 1985). However, it has thus far, not proved possible to culture R. culicivorax in artificial media.

There are a variety of reasons why an obligate parasite such as R. culicivorax may fail to complete development in artificial media. Among the factors that the host makes available to the nematode, which therefore may be absent in vitro, are provision of nutrients, optimal physicochemical conditions for growth and possibly, growth stimuli to trigger specific developmental processes. In addition, the role of the nematode's own endogenous system for controlling development is expected to play a vital role in regulating growth and development both in vivo and in vitro. In all invertebrate phyla thus far studied, neurosecretory cells located within the nervous system constitute the sole or dominant system for controlling long term developmental as well as short term metabolic processes (Laufer and Downer, 1988).

Peptidergic and aminergic types of neurosecretory cells have been reported from several species of nematodes (Davey, 1988). Histochemical techniques were used to identify peptidergic neurosecretory cells in the nervous system of Ascaris lumbricoides (Davey, 1964; 1966), Phocanema decipiens (Davey, 1966; Davey and Kan, 1968; Goh and Davey, 1985) and Dirofilaria immitis (Delves et al., 1989). These cells were

located in various sites: the cephalic papillary ganglia, amphidial glands, ganglia of the nerve ring, cell bodies of the longitudinal nerve cords and rectal and anal ganglia. Ultrastructural studies confirmed the presence of peptidergic neurosecretory cells in various components of the nervous system of Haemonchus contortus (Rogers, 1968) and Capillaria hepatica (Wright, 1974). Recently, immunocytochemical techniques have been employed to identify several neuropeptides in the nervous system of nematodes. Among the neuropeptides so far studied, FMRF-amide (Phe-Met-Arg-Phe-NH2), a peptide originally isolated from the clam Macrocallista nimbosa (Price and Greenberg, 1977) was found to be well distributed in the nervous systems of Caenorhabditis elegans (Li and Chalfie, 1986; Li, 1990), Goodeyus ulmi (Leach et al., 1987), A. suum (Davenport et al., 1988), A. lumbricoides (Sithi,)rngul et al., 1990), Panagrellus redivivus (Atkinson et al., 1988; Davenport et al., 1991), D. immitis and Brugia pahangi (Warbrick et al., 1992). FMRF-amide-like peptide was present in the cells associated with the cephalic papillary ganglia, cephalic nerves, nerve ring, dorsal, ventral and lateral ganglia of the nerve ring, longitudinal nerve cords, rectal and anal ganglia in the tail and several nerve processes in the nematode body.

Using fluorescent, ultrastructural and immunocytochemical methods, aminergic neurosecretory cells

have been demonstrated in the nervous system of C. elegans (Sulston et al., 1975; McIntire and Horvitz, 1985). P. decipiens (Goh and Davey, 1976), Xiphinema americanum (Högger et al., 1978), Prionchulus punctatus, P. redivivus (Wright and Awan, 1978), Nematodirus battus (Sharpe et. al. 1980), Trichinella spiralis (Lee and Ko, 1991), P. redivivus, Nematospiroides dubius (Sharpe and Atkinson, 1980) and G. ulmi (Leach et al., 1987). The staining was generally observed in the amphids (chemosensory organ), cephalic nerves, cephalic papillary ganglia, dorsal, ventral and lateral ganglia of the nerve ring, longitudinal nerve cords, several small nerve fibers and deirids (a pair of lateral, sub-cuticular anterior mechanosensory organs) in some nematodes.

Most of the above listed studies on neurosecretions in nematodes were descriptive in nature and did not examine the functional role of neurosecretions in controlling physiological and developmental processes. In P. decipiens, however, molting from the fourth stage juvenile to the adult stage was shown to be under the control of a secretion from neurosecretory cells of the dorsal and ventral ganglia of the nerve ring (Davey and Kan, 1968). The aminergic neurosecretion noradrenaline, secreted by noradrenergic cells of the cephalic papillary ganglia, is believed to cause the release of a peptide from peptidergic neurosecretory cells of lateral ganglia of the nerve ring. This peptidergic neurosecretion

activates the excretory gland cells to release an enzyme that stimulates ecdysis (Goh and Davey, 1985). The release of a peptidergic neurosecretion from the amphidial nerve cells also controls molting in D. immitis (Delves et al., 1989).

Information on the neurosecretory system of R. culicivorax is lacking. However, based on the earlier limited studies on vertebrate- parasites and free-living nematodes, it seems probable that R. culicivorax should possess neurosecretory sites. Therefore, the primary objective of the present study was to further our understanding of nematode neurosecretory systems in general and to obtain information on the endogenous control system of R. culicivorax that may prove useful for establishing in vitro culture. The presence and distribution of catecholamines and an FMRF-amide-like peptide in the nervous system of different developmental (parasitic and free-living) stages of R. culicivorax was investigated. Secondly, a preliminary investigation in adult R. culicivorax was also undertaken to correlate the secretory activity of catecholaminergic neurosecretory neurons with reproduction.

II. MATERIALS AND METHODS

2.1. Source of Nematodes

The laboratory colony of R. culicivorax was maintained in vivo following the general technique of Petersen and Willis (1972), with the modifications of Platzer and Stirling (1978). Two further modifications were made: (a) larvae of the mosquito Aedes aegypti, rather than Culex pipiens, were used as the laboratory host and (b), instead of sand, distilled water was used to store the post-parasitic stages. To propagate the colony and supply nematodes for experimental use, newly hatched mosquito larvae were exposed to infective pre-parasitic nematodes (10-20 infective juveniles/ mosquito larva) immediately after hatching from the eggs for a period of 12 hours (Bailey and Gordon, 1973). This resulted in an infection rate of approximately 75 to 80%, a mean intensity of infection of approximately 2 parasites per host; and a sex ratio of approximately 1 male: 1 female. Infected mosquito larvae were transferred to casserole dishes (30 X 20 X 5 cm), maintained in an incubator at 27-30°C and fed with guinea pig chow ad libitum. Under these conditions, post-parasitic stages emerged from the host 6-8 days after infection. Post-parasitic nematodes were transferred from the casserole dishes to petri dishes containing distilled water immediately after their emergence from the host and kept in an incubator at 27°C until

maturity.

2.2. Scanning Electron Microscopy of External Sensory Organs of R. culicivorax.

Adult males and females of R. culicivorax were transferred from distilled water into small vials containing Karnovsky's fixative (4% (w/v) paraformaldehyde and 5% (v/v) glutaraldehyde (Karnovsky, 1965) in 0.2M cacodylate buffer (Na [CH3]2 AsO2.3H20/ HCL; pH 7.4; Bozzola and Russell, 1992) and fixed for one hour at room temperature. After several rinses in 0.2M cacodylate buffer, specimens were post-fixed in 1% (v/v) osmium tetroxide (J.B.EM. Services, Inc. Dorval, Quebec, Canada) in 0.2M cacodylate buffer for two hours at room temperature. After several rinses in 0.2M cacodylate buffer, specimens were dehydrated through a graded series of ethanol (35, 50, 70, 80, 90%), spending five minutes in each, then one hour in 100% ethanol. Dehydrated specimens were then critical-point dried using liquid carbon dioxide in a Polaron E 3000 critical point drying apparatus (J.B.EM. Services, Inc. Dorval, Quebec, Canada). Male and female nematodes were then attached to two separate aluminium stubs (J.B.EM. Services, Inc. Dorval, Quebec, Canada), then gold-coated in an Edwards Model 150A sputter coater (Edwards High Vacuum, South Service Road, West Oakville, Ontario, Canada). Gold-coated specimens were examined with a Hitachi S570 Scanning Electron Microscope (SEM) operated at an accelerating voltage of 20 kV.

Photographs were taken of the sensory organs in the head and tail regions of male and female nematodes.

2.3. Preliminary Exploratory Experiments

Preliminary exploratory experiments were conducted in an attempt to locate the neurosecretory cells in R. culicivorax by use of histochemical (performic acid-resorcin fuchsin and aldehyde fuchsin) and ultrastructural (transmission electron microscope) methods.

2.3.1 Histochemical Methods

2.3.1.1. Performic acid-resorcin Fuchsin Technique.

The whole mount method described by Ittycheriah and Marks (1971) for the detection of neurosecretory cells in insect brains, was used, with slight modifications in duration of treatments. Adult nematodes were collected from distilled water, fixed in modified Bouin's fluid (Halmi, 1952) for 24 hours at room temperature, washed with distilled water containing a few crystals of lithium carbonate, then oxidized in performic acid solution for 15 minutes. The oxidized nematodes were washed thoroughly with distilled water 3 times for a total of 30 minutes, then dehydrated by transferring them through 30, 40, 50, 60 and 70% alcohol for 5, 10, 15, 20 and 60 minutes, respectively. Dehydrated specimens were then stained with resorcin-fuchsin for 24 hours, then differentiated repeatedly in 70% alcohol until no more superfluous stain was evident. The stained specimens were then

dehydrated by transferring them successively through 35, 50, 70, 80% alcohol for 5 minutes in each case, then through 95 and 100% alcohol for 20 and 60 minutes, respectively. The specimens were then cleared in cedar oil for 4 hours. The cedar oil was removed with xylene and nematodes were mounted in Permount(R) (Fisher Scientific, Denison Street, Toronto, Canada) and observed under the light microscope for the presence of neurosecretory cells in the nervous system. Photographs of presumptive neurosecretory cells were taken using a photomicroscope.

2.3.1.2. Aldehyde Fuchsin Technique.

Post-parasitic nematodes were collected from distilled water, fixed in modified Bouin's fluid for 12 hours, then the head region of the nematodes was cut (ca. 1-2 mm length) and fixed in the same fixative for another 12 hours. After fixation, specimens were dehydrated and embedded in paraffin wax (Clark,1986). Serial sections were cut from the tip of the head through the nerve ring (ca. 3-5 μm thickness) using a glass knife, fitted on a Sorvall(R) "Porter Blum" Ultramicrotome M 1 (Ivan Sorvall Inc. Newtown Connecticut, U.S.A.). The sections were placed serially on microscope slides that had been smeared with an albumin fixative (Fisher Scientific, Canada). The sections on the slide were then dried on a slide dryer overnight at about 40°C (Clark, 1986). The slides were then subjected to Ewen's paraldehyde fuchsin

technique (Ewen, 1962) with slight modifications as described below. The sections were deparaffinized by transferring them to xylene for 10 minutes and rapidly hydrated by transferring them sequentially through 100, 90, 70% alcohol to distilled water. The sections were oxidized in Gomori's fluid for 5 minutes, rinsed with distilled water and decolorized with 1.5% potassium metabisulphite for 1 minute. Decolorized sections were thoroughly rinsed with distilled water, followed by 70% alcohol, for 1 minute. These sections were stained with paraldehyde fuchsin for 45 seconds, destained in 90% alcohol for 4 minutes, differentiated in acid alcohol for 20 seconds, rinsed with 70% alcohol followed by distilled water. The slides were transferred into mordant (0.4 g% phosphotungstic acid/1.0 g% phosphomolybdic acid in distilled water) for 5 minutes and rinsed with distilled water. The sections were counterstained for 10 minutes, differentiated in 0.2% (v/v) acetic acid for 15 seconds. These slides were rinsed with absolute alcohol and finally transferred to xylene for 5 minutes. The sections were mounted in Permount(R) and viewed at 400X with a light microscope. Cells resembling putative neurosecretory were photographed using a photomicroscope at 400X.

2.3.2 Ultrastructural Localization of Neurosecretory Cells.

Adults of R. culicivorax were transferred from distilled water to a small vial containing Karnovsky's fixative (4%

(w/v) paraformaldehyde and 5% (v/v) glutaraldehyde in 0.05M potassium phosphate buffer, pH 6.9; Karnovsky, 1965) and fixed for 12 hours at 4°C. The tip of the head of adult males and females and the tail portion of adult males were cut (ca. 1.00 mm length) and fixed in the same fixative for another 24 hours at 4°C. These specimens were then washed thoroughly with three changes of 0.05M potassium phosphate buffer (pH 6.9) for a period of 30 minutes and post-fixed in 1% (v/v) Osmium tetroxide (J.B.EM. Services, Inc. Dorval,Quebec, Canada) for 24 hours at room temperature. The post-fixed specimens were washed with six changes of 0.05M potassium phosphate buffer (pH 6.9) for a period of 1 hour. Following this they were dehydrated through a graded series of ethanol (25, 35, 50, 70, 80%), spending 20 minutes in each, then 60 minutes each in 95 and 100% ethanol. Dehydrated specimens were transferred at 30 minute intervals for infiltration through a sequence of 3:1, 1:1 and 1:3 ethanol : Spurr's embedding resin (Spurr, 1969) and finally into Spurr's embedding resin for 24 hours under a vacuum (20 PSI) in a desiccator at room temperature.

Infiltrated specimens were transferred and aligned in small flat "moulds" containing Spurr's embedding resin. These "moulds" were then kept in an oven for 24 hours at 70°C for polymerization. The polymerized blocks were removed from the flat "moulds" and trimmed to expose the head tips of the nematode for sectioning. The sections were cut with a glass

knife mounted on a Sorvall(R) "Porter Blum" Ultramicrotome MT-1. Initially, thick sections (ca. 1-1.5 µm thick) were cut transversely and stained with 1% (w/v) toluidine blue (B.D.H. Chemicals Laboratory Division, England) to ascertain the proper plane of the head of the nematode being sectioned and to determine the location of the nerve ring. From these blocks ultrathin, mostly silver coloured, sections (ca. 60-70 nm thick) were cut transversely through the nerve ring and transferred onto 75 X 300 mesh copper grids (J.B. EM. Services Inc., Canada). Sections were stained with 2% (w/v) aqueous uranyl acetate for 2 minutes, followed by 1% (w/v) aqueous lead citrate for another 2 minutes. The stained sections were observed under a Zeiss EM 9 A Transmission Electron Microscope for the presence of neurosecretory cells/granules. In general, the peptidergic neurosecretory cells are distinguished by the membrane bound electron-dense granules with diameter of 100-300 nm located in both the cell bodies and axons (Davey, 1988). Photographs were taken of the cells of interest.

2.4. Catecholaminergic Nervous System

The catecholaminergic nervous system was studied in pre parasitic, parasitic and post-parasitic stages. Pre-parasitic juveniles were obtained by transferring mature eggs of R. culicivorax with the aid of polystyrene transfer pipet into a beaker containing newly hatched mosquito larvae. Pre-parasites hatched within 2-4 hours. The parasitic juveniles were

dissected out of their hosts at 4, 5, 6 and 7 days post-infection in half strength Phosphate Buffer Saline (Na_2HPO_4/NaCl; PBS)[1]. Post-parasitic nematodes were collected from the casserole dishes immediately after emergence from the host and from distilled water after molting into adults. Mature eggs were also examined for the presence of catecholamines. The glyoxylic acid (GA)-induced fluorescence method, described by Sharpe and Atkinson (1980) for staining of catecholamines in nematodes, with slight modifications, was used in this study. A solution of 2% (w/v) GA in 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4; pH 7.0) was used for staining the catecholaminergic nervous system. Best results were obtained when nematodes were killed quickly and transferred to GA. It was observed that if live nematodes were put into cold GA they usually shrank and became distorted. Killing of the nematodes was performed by placing them in a small beaker containing water, which was then plunged into another beaker filled with boiling water (100°C) for 2-3 minutes, or until the specimens assumed the almost straight form characteristic of heat death (Hooper, 1986). The killed nematodes were incubated at room temperature (24-27°C) in a beaker containing 2% GA for 5 minutes and transferred in small drops of GA onto

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1. Immunohistochemistry Instructional manual, INCSTAR(R) Corporation, Stillwater, Minnesota, USA

slides. The excess GA was removed immediately with filter paper and the slides dried rapidly by a stream of hot air from a hair-dryer for 5 minutes to reduce background fluorescence. The slides were heated at 100°C in an oven for 10 minutes. Nematodes were mounted in Histoclad(R) (Clay Adams, Parsippany, U.S.A.) under a coverslip and examined immediately. To verify that the GA was responsible for staining the catecholamines, control specimens were treated as above, except that distilled water was substituted for glyoxylic acid at all stages of the procedure.

The fully matured eggs (i.e. before hatching) were incubated at room temperature in 2% GA on the slides for 5 minutes. The excess GA was removed immediately with filter paper, then the eggs were dried, heated and mounted as described for the postembryonic stages and examined under a fluorescence microscope for the presence of catecholamines. Photographs were taken of the catecholaminergic nervous system at various stages of development.

2.4.1 Functions of Catecholamines in R. culicivorax

To investigate the possibility that catecholamines may play a role in the nematode's reproductive activity, the relative amounts of catecholamines in the nervous system were compared under the following sets of conditions: (a) males only; (b) females only; (c) males and females together, and (d) males and females separated physically, but connected via

the sterilized water (Figure 1). These sets of conditions were maintained in an incubator at 27°C.

Immediately after emergence from the host, post-parasitic nematodes were collected in a petri dish containing sterilized water, sexed (Imbriani et al., 1982), then distributed among four sets of petri dishes according to conditions (a) to (d). As shown in Figure 1, each experimental set-up consisted of a covered glass petri dish (diam. 9.0 cm) containing sterilized water. The base of a smaller diameter (5.5 cm) plastic petri dish was placed upright inside the larger dish; the sides of this smaller diameter petri dish were perforated with small holes. Thus, nematodes placed inside the smaller petri dish were physically separated from those placed in the larger dish, but chemical communication between the two groups of nematodes could occur via the sterilized water that connected them. In conditions (a) and (b), one hundred nematodes (males or females) were transferred into each perforated dish; in (c), one hundred males and one hundred females were transferred into each perforated dish; in (d), one hundred males were transferred into each glass petri dish and one hundred females into each perforated petri dish. Each petri dish set-up was considered as a separate replication and there were four replications for each condition.

The fluorescence intensity of catecholamines in the

Figure 1. Photographs of experimental design for four different experimental conditions (a-d). Nematodes were placed in sterilized distilled water, contained by the base of a perforated plastic petri dish. The double petri dish system was covered with the lid of glass petri dish. Condition (d) allowed no physical contact between opposite sexes of R. culicivorax, but it permitted chemical mediation between the sexes through the sterilized water in which the separate sexes developed.


a. MALES ONLY
b. FEMALES ONLY
c. MALES AND FEMALES TOGETHER
d. MALES AND FEMALES SEPARATED PHYSICALLY BUT CONNECTED VIA STERILIZED WATER

nervous system of post-parasitic nematodes was measured for all experimental conditions using an epi-fluorescence photomicroscope equipped with photometer head and digital voltmeter. The fluorescence intensity of catecholamine(s) in the nerve ring of male and female nematodes was measured, for all conditions (a-d), two days before and immediately after the last molt. Since mating occurred for nematodes reared under (c), the catecholamine staining intensity in the nerve ring of these nematodes was also measured during (on first or second day of mating) and immediately after mating. The fluorescence intensity of catecholamines in ganglia present in the tail region was only measured for male nematodes. It was found that the tail of females contained only two ganglia that stained inconsistently with GA, whereas the tail region of males contained an abundance of heavily and consistently stained ganglia. The staining intensity in the tail region ganglia of males was measured for nematodes from conditions (a), (c) and (d) after the last molt; staining of the ganglia did not occur before the molt. For male nematodes, reared under condition (c), the fluorescence intensity of tail region ganglia was also measured during and after mating. Fifteen nematodes were selected for the measurement of catecholamine intensity from each replication at all developmental stages examined.

The measurement of percent fluorescence intensity of

catecholamines was based on the method of Björklund et al. (1973), in which a synthetic catecholamine (dopamine) was used as a standard for the measurement of catecholamine fluorescence intensity. To prepare the synthetic standard, dopamine (3-Hydroxytyramine; Sigma Chemical Company, St. Louis, Mo., U.S.A.) was dissolved to a concentration of 2.0 X 10^{-3} M in a 5% (w/v) solution of human serum albumin (Sigma), buffered to a pH 7.0 with 0.1 M phosphate buffer. A small drop of solution was placed on a glass microscope slide and dried at room temperature. The dried droplet containing dopamine was treated with 2% (w/v) GA for 5 min, dried by a stream of hot air from a hair dryer for 5 minutes. The slide was heated at 100°C in an oven for 10 minutes. The dried dopamine droplet was mounted in Histoclad(R).

Before measurement of percent catecholamine fluorescence intensity in the actual specimens, the digital voltmeter was calibrated to 100 by measuring the fluorescence intensity of dopamine in the processed droplet of synthetic dopamine standard. Value obtained for the nematodes were expressed as a percentage of the standard.

2.5. Peptidergic Nervous System

The peptidergic nervous system was studied in parasitic (4, 5, 6, 7 days post-infection) and post-parasitic stages. The peptidergic nervous system was examined by determining the distribution of a peptide with immunocytochemical properties

analogous to FMRF-amide (Phe-Met-Arg-Phe-NH2), a molluscan cardio-excitatory tetrapeptide (Price and Greenberg, 1977).

Immunocytochemical staining was performed on small pieces of nematodes using an indirect immunofluorescence technique (Coons et al., 1955). Nematodes were fixed immediately after removal from the host (parasitic stages), or from water (post-parasites) in 4% (w/v) paraformaldehyde buffered with 0.1 M phosphate buffer (pH 7.0) for 24 hours at 4°C. The nematodes were cut laterally, using a scalpel, into two halves and fixed in the same fixative for another 24 hours at 4°C. In order to soften the cuticle for easy penetration of antibody, fixed specimens were transferred to PBS containing 0.3% (w/v) Triton X-100 for 5-6 hours at 4°C, then to PBS containing 0.4% Triton X-100 plus 1% (w/v) Bovine Serum Albumin (BSA) for 24 hours at 4°C. The nematodes were rinsed 4 times with PBS and transferred to goat serum (Cedarlane Laboratories, Hornby, Ontario, Canada), diluted 1:25 in PBS, for 24 hours at 4°C to block the non-specific binding of conjugate. The specimens were washed with PBS and cut into segments of about 0.1 mm in length. The segments of worm were then transferred to the primary antibody (Rabbit anti-FMRF-amide; INCSTAR® Corporation, U.S.A.) which was diluted 1:100 in PBS. The specimens were kept under a vacuum (20 PSI) for 1 hour to facilitate infiltration of the antibody, and again incubated in the same antibody for 24 hours at room temperature. The

incubated specimens were kept under a vacuum (20 PSI) for 1 hour at room temperature, in an incubator at 37°C for a further 1 hour period (Leach et al., 1987), then thoroughly washed with PBS for a total of 5-6 hours at room temperature. The secondary antibody, goat anti-rabbit IgG conjugated with fluorescein isothiocynate (FITC; Cedarlane Laboratories, Canada), was diluted to 1/50 in PBS. The specimens were transferred to secondary antibody and kept under a vacuum (20 PSI) for 1 hour, then incubated at room temperature for 24 hours. After incubation, specimens were transferred to a vacuum of 20 PSI for 1 hour at room temperature, then to an incubator at 37°C for a further 1 hour period. After incubation, specimens were washed in PBS 4 times for a total of 5-6 hours, then mounted in PBS : glycerol solution (1:1). In the case of control specimens, instead of primary antibody (Rabbit anti-FMRF-amide), normal rabbit serum diluted 1:50 in PBS was used to block the specific antigenic sites; the rest of the procedure was the same as described above. Specimens were observed under an epi-fluorescence microscope and photographs taken of the peptidergic nervous system at various stages of development.

2.5.1. Dopaminergic Nervous System

An indirect immunofluorescence technique was used to localize the distribution of the catecholamine, dopamine, in the nervous system of adult R. culicivorax. The indirect

immunofluorescence technique used for localization of the peptidergic nervous system was followed (Coons et al., 1955) except a double incubation with primary antibody, rabbit anti-dopamine (diluted 1:50 in PBS), was substituted for the single exposure to rabbit anti- FMRF-amide (Gu et al., 1983).

2.6. Fluorescence Microscopy

Specimens were viewed and photographed with a Zeiss Photomicroscope II, equipped with a 50W mercury UV epifluorescence lamp and Plan-Neofluar 25/0,8 Imm. objective. The wide-band interference filters 09 (excitation: band pass 450-490 nm; barrier filter: long pass 515-565 nm) and 18 (excitation: band pass 390-440 nm; barrier filter: long pass 470 nm) were used for indirect immunofluorescence and GA-induced fluorescence methods, respectively.

Fluorescence intensity of catecholamines in the nervous system of the nematode was measured with a MPM3 photometer head and SF14 digital voltmeter controlled by shutter switching system, that was connected to the Photomicroscope II. The measuring field was restricted by a series of interchangeable pin-hole diaphragms (apertures) in the photometer head. For the measurement of percent fluorescence intensity of catecholamines in the four ganglia of the nerve ring of adult nematodes, a 0.32 mm diameter pin-hole aperture was used, whereas the pin-hole aperture used for the tail of males was 0.16 mm in diameter, because ganglia present in the

tail were small in size compared to those present in the nerve ring.

2.7. Statistical Analysis

ANOVA was used to determine the significance of mean staining intensities within and between the rearing conditions.The condit.ons were compared using Duncan's multiple range test.

III. RESULTS

3.1. Scanning Electron Microscopy of External Sensory Organs of R. culicivorax.

The head tip of male (Figure 2A) and female (Figure 2B) nematodes contained six hexagonally arranged cephalic sensory papillae (two dorso-laterally, two ventro-laterally and two laterally). There were two laterally placed amphids (chemosensory organs) in both the sexes. Each cephalic sensory papilla of the female nematode had three pores (Figure 2C). Like females, each dorso-lateral, and ventro-lateral cephalic sensory papilla of males had three pores (Figure 2D), but each of the lateral cephalic sensory papillae of males had only two pores (Figure 2E). No caudal sensory papillae were observed in the female tail (Figure 2F), whereas several caudal sensory papillae were located on the ventral surface of the male tail (Figure 2G). Each of the caudal sensory papillae of the male had only one pore (Figure 2H).

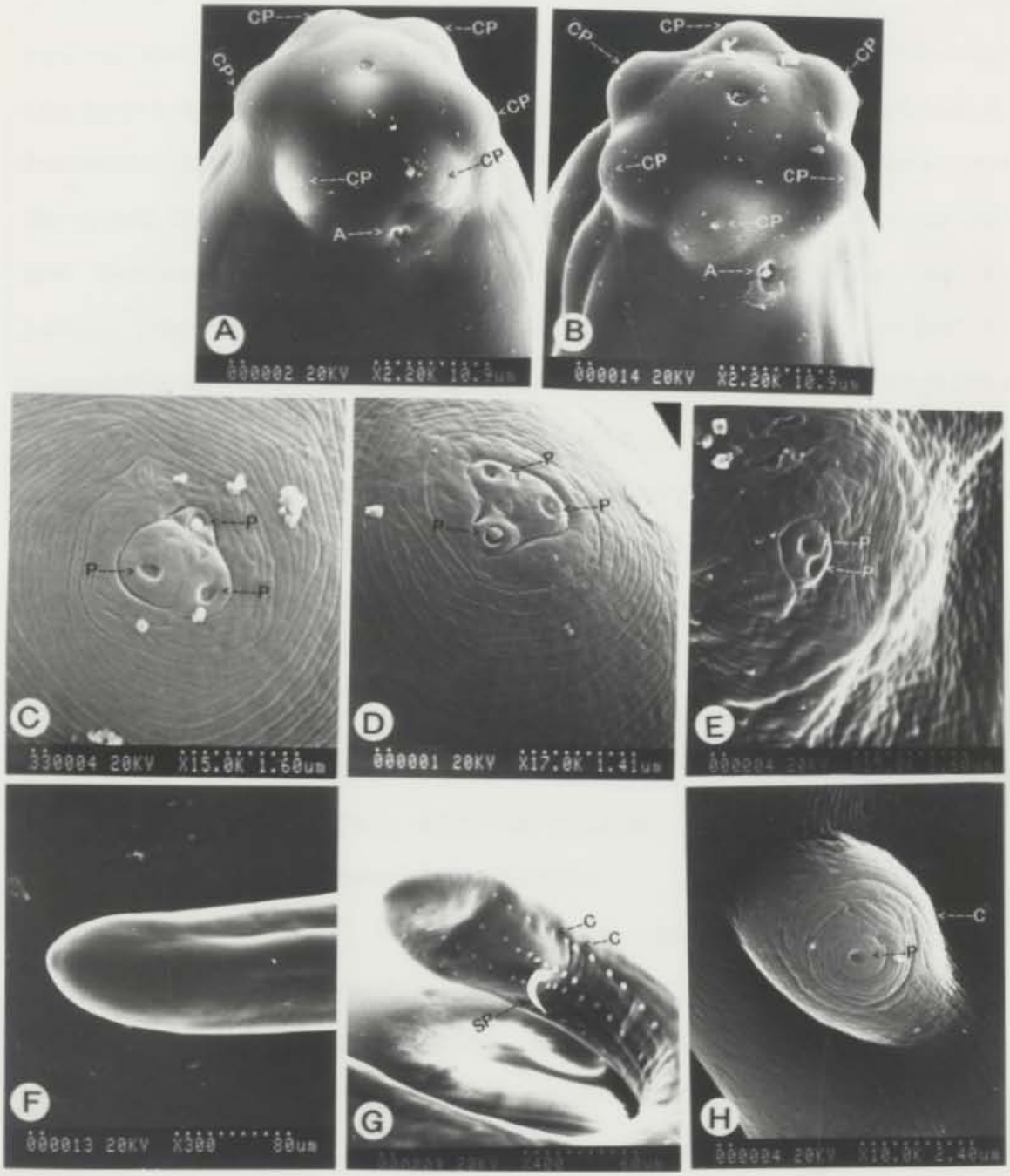
3.2. Preliminary Exploratory Experiments

Neurosecretory cells were not identified in adult R. culicivorax by standard histochemical and ultrastructural methods.

3.2.1. Histochemical Methods

The performic acid-fuchsin technique was useful for staining the outline of the nerve ring, which was located

Figure 2. A and B, SEM photomicrographs of head regions of adult male and female R. culicivorax, respectively, showing six cephalic sensory papillae and one amphidial pore. Another amphidial pore, not visible on the photomicrograph, is located on the other lateral side of the nematode's body. One cephalic papilla is situated in close proximity to each of the amphids; the other four papillae are located in dorso-lateral and ventro-lateral positions. C, SEM photomicrograph of cephalic sensory papilla of female. Note three pores in the papilla. D, SEM photomicrograph of ventro-lateral cephalic sensory papilla of male. Note three pores in the papilla. E, SEM photomicrograph of lateral papilla of male. Note two pores in the papilla. F, SEM photomicrograph of tail of female. Note the absence of sensory papillae. G, SEM photomicrograph of tail of male, showing several caudal sensory papillae. H, SEM photomicrograph of a caudal sensory papilla of male. Note one pore in the caudal papilla. A= Amphid; C= Caudal sensory papillae; CP= Cephalic sensory papilla; P= Pore(s) in sensory papilla; SP= Spicule.


CP
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approximately 200 μm posterior to the head tip of adult nematodes. No staining of other components of the nervous system was observed (Figure 3A). The cross sections through the nerve ring did not show positive staining with paraldehyde fuchsin. However, paraldehyde-fuchsin positive granules were observed in the cross sections through the ovaries (Figure 3B) and testes (Figure 3C). Thick cross sections were cut to locate the nerve ring under the light microscope, prior to ultrathin sectioning for electron microscopy. These sections revealed four hypodermal cords and the circular arrangement of nerves comprising the nerve ring (Figure 4A). The ultrathin cross sections failed to reveal peptidergic and aminergic granules in the nerve ring of both sexes (Figure 4B) and in the tail of males (Figure 4C). However, unidentified electron dense granules were observed in the ultrathin cross sections through the ovary (Figure 4D).

3.3. Catecholaminergic Nervous System

3.3.1. Egg and pre-parasitic Stages

Glyoxylic acid- treated mature eggs of R. culicivorax showed an intensely fluorescent concentration of catecholamine(s). Subsequent studies indicated that the catecholamine(s) is located in the head region of the pre-parasitic second stage juveniles developing within the eggs (Figure 5A). A similar concentration of catecholamine(s) was also present in the head region of pre-parasitic juveniles.

Figure 3. A, Head region of adult R. culicivorax, showing the nerve ring stained with performic acid-fuchsin stain. B, Light photomicrograph of cross section through the ovary of adult female, showing granules stained with paraldehyde-fuchsin stain. C, Light microphotograph of cross section through the testes of adult male, showing granules stained with paraldehyde-fuchsin stain. G= Granules; NR= Nerve ring; O=Ovary; SM= Somatic muscles; T= Testi; TR= Trophosome (scale bars= 25 μm).

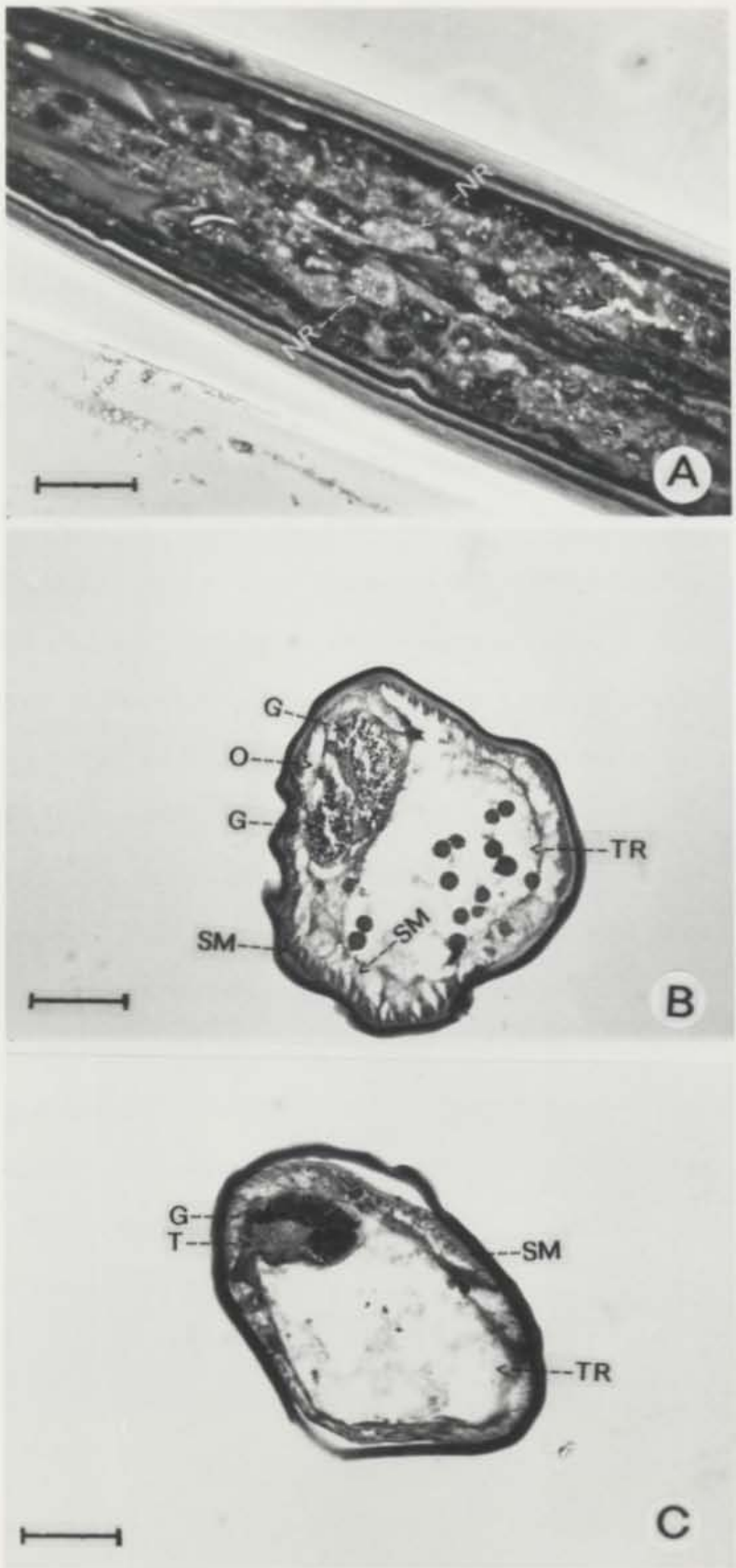
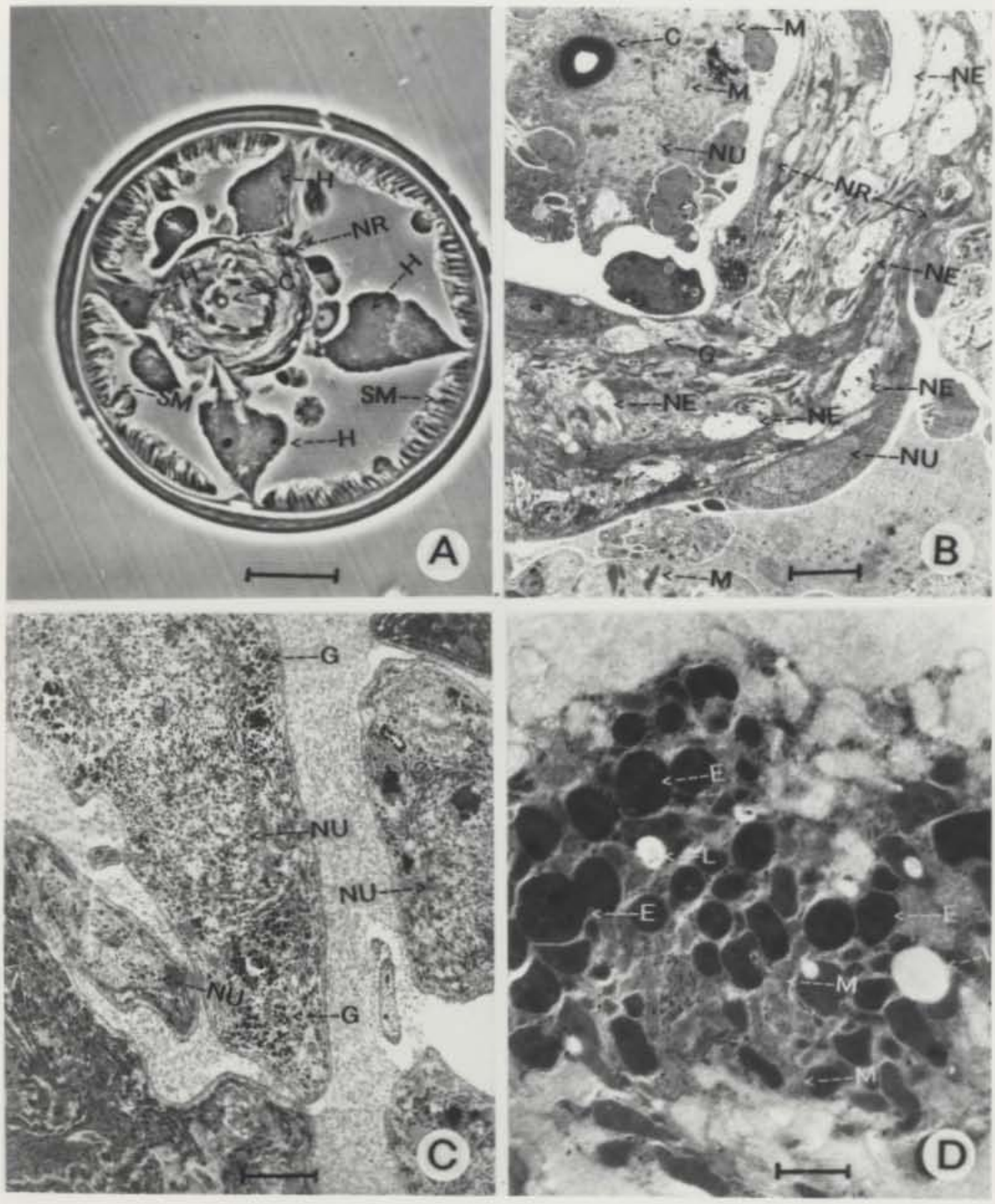

NR
NR
A
G
O
G
TR
SM
SM
B
G
T
SM
TR
C

Figure 4. A, Light photomicrograph of a cross section through the nerve ring of R. culicivorax, Note the four hypodermal cords and the circular arrangement of nerves in the nerve ring (scale bar= 25 μm). B, An electron micrograph of a cross section through the nerve ring of R. culicivorax. Note the absence of peptidergic and aminergic electron dense granules. C, An electron micrograph of a cross section through the tail of adult male. Note the absence of peptidergic and aminergic electron dense granules. D, An electron micrograph of a cross section through the ovary of female, Note the electron dense granules. C= Cuticular tube; E= electron-dense granules; G= Glycogen granules; H= Hypodermal cords; L= lipid body; M= Mitochondrion; NE= Nerve endings; NR= Nerve ring; NU= Nucleus; SM= Somatic muscles (scale bars= 1.0 μm).


H
NR
H
H
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SM
SM
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NE
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M
B
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NU
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G
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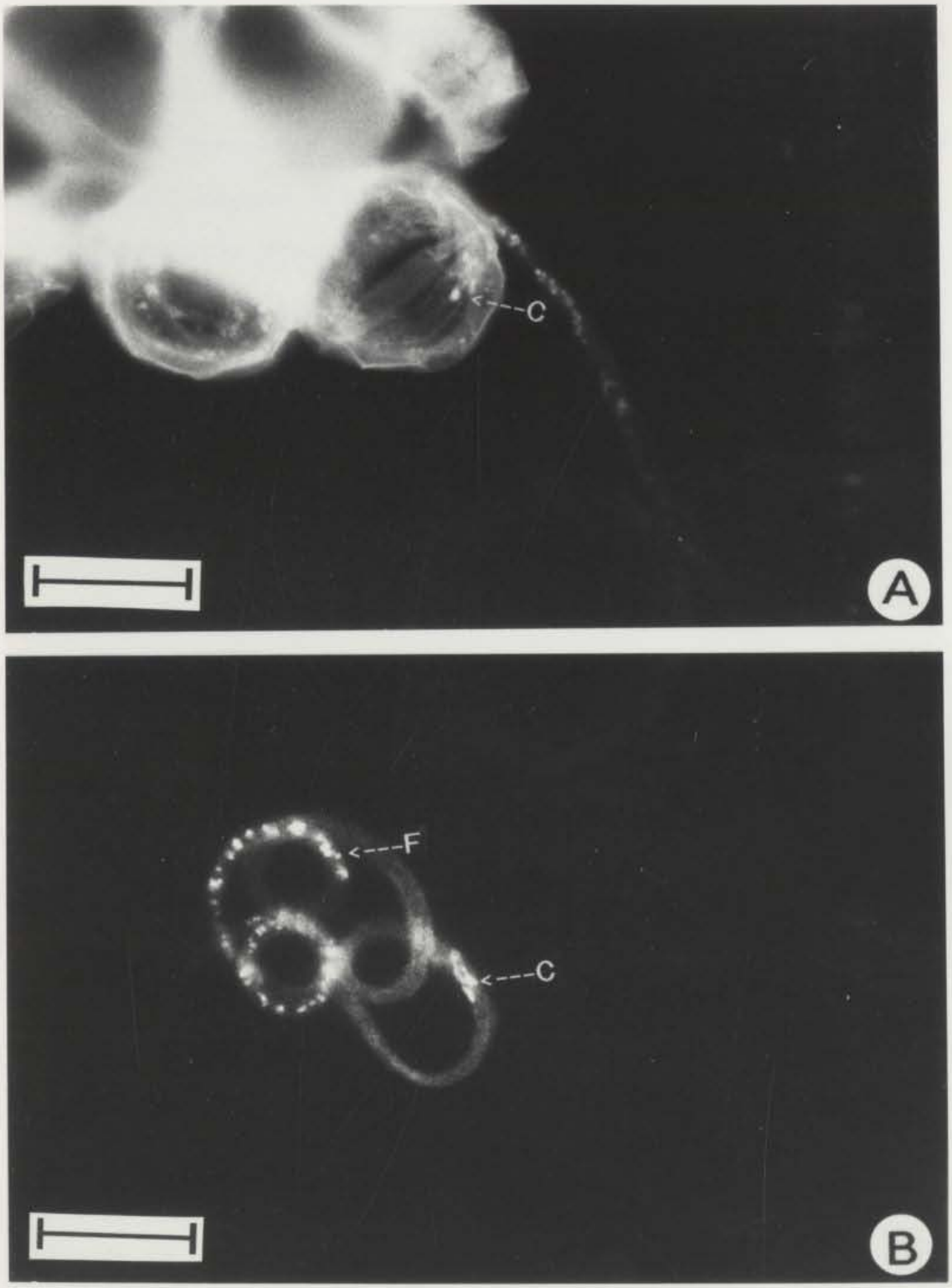
D

Figure 5. A, Mature eggs of R. culicivorax stained with glyoxylic acid. One egg shows a concentration of catecholamines; others show autofluorescence, unrelated to the presence of catecholamines. A pre-parasitic juvenile can be seen close to the egg that contains the catecholamines. B, Pre-parasitic second stage juvenile of R. culicivorax, showing concentration of catecholamines in the head region and fluorescent material, towards the posterior end of the nematode, in the posterior 'intestinal' region. C= Concentration of catecholamine(s); F= Fluorescent Material; J= Pre-parasitic juvenile (scale bars= 100 μm).

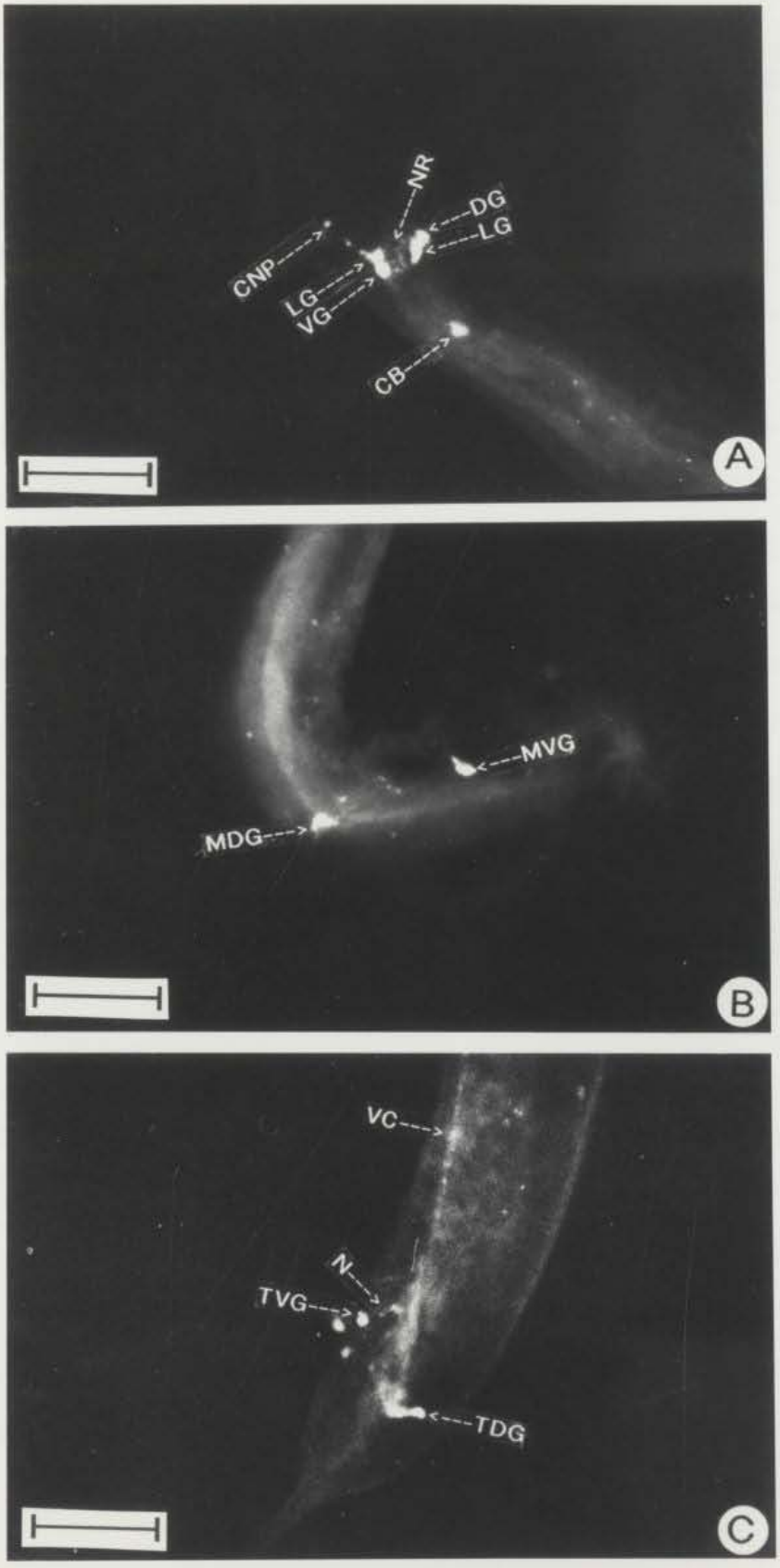

C
A
F
C
B

Fluorescent material, probably not associated with the nervous system, was also present in the posterior region of the pre-parasites, described by Poinar and Hess (1974) as the 'intestinal' region (Figure 5B).

3.3.2. Parasitic Stages

Four catecholaminergic ganglia were present in the head region, two in the mid-body region and two in the tail region of parasitic juveniles, four days post-infection (Figure 6A, B, C). The ganglia in the head region were connected by nerve fibers to form the nerve ring around the cuticular tube, approximately 125 to 200 μm posterior to the head tip. One of these four ganglia was located dorsally, one ventrally and the other two laterally. The cephalic nerves did not fluoresce with the GA, except for a short nerve projection (Figure 6A) that originated from the anterior surface of each of the four ganglia of the nerve ring. Dorsal and ventral nerve cords were stained with the GA. Although not visible on the photomicrographs, these nerve cords originated from the bases of the dorsal and ventral ganglion in the nerve ring. They ran posteriorly into the tail (Figure 6C). The two catecholaminergic ganglia present in the mid-body region were located dorsally and ventrally. The two concentrations of catecholaminergic ganglia present in the tail tip, each composed of several small cell bodies, were located at the dorsal and ventral sides of the tail. These ganglia were

Figure 6. Parasitic juvenile of R. culicivorax, four days post-infection stained with glyoxylic acid. A, Head region, showing nerve ring with four catecholaminergic ganglia. B, Mid-body region, showing two catecholaminergic ganglia. C, Tail region. Note the ventral nerve cord connecting catecholaminergic cell bodies. CB= cell body in ventral nerve cord; CNP= Cephalic nerve projection from ventral ganglion; DG= Dorsal ganglion; LG= Lateral ganglia; MDG= Mid-body dorsal ganglion; MVG= Mid-body ventral ganglion; N= Nerve from the ventral nerve cord; NR= nerve ring; TDG= Tail dorsal ganglion; TVG= Tail ventral ganglion; VC= Ventral nerve cord; VG= Ventral ganglion (scale bars= 100 μm).


NR
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MDG
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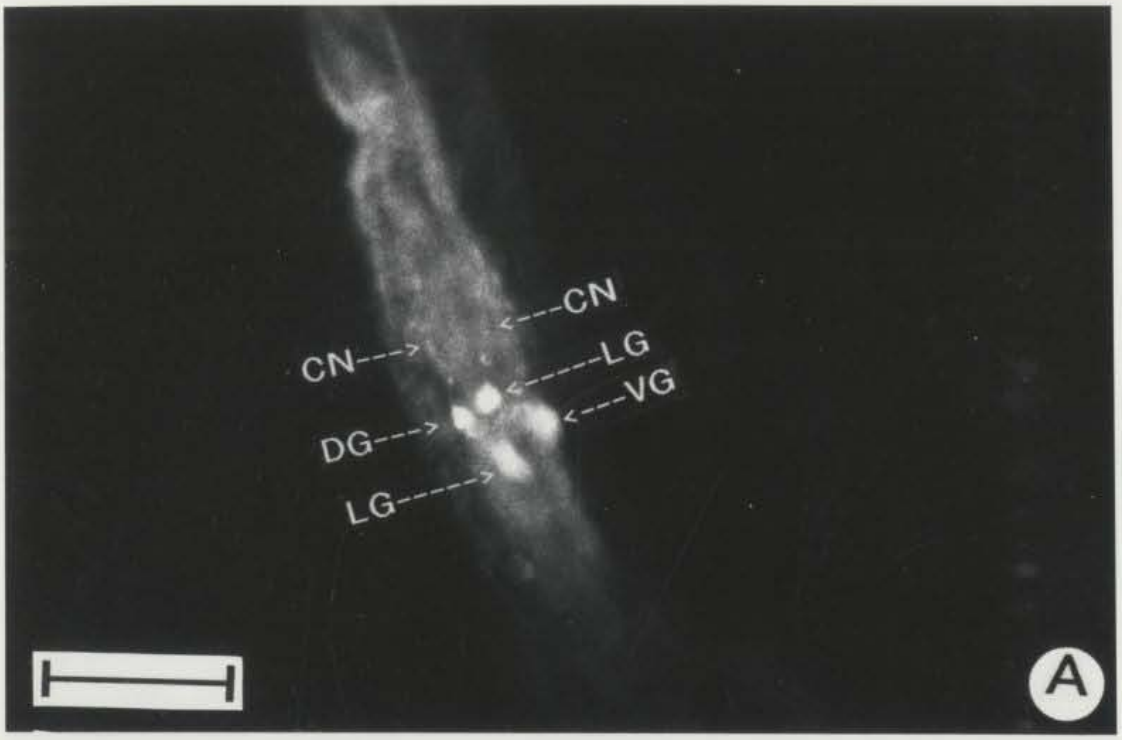
N
TVG
TDG
C

innervated by the ventral nerve cord and linked with each other by small catecholaminergic nerve fibers.

The catecholaminergic nervous system was more extensive by five days post-infection (Figure 7A). A cephalic nerve originated from each of these four ganglia and ran anteriorly toward the head. It was observed that the anterior tips of these nerves were bifurcated at the extreme tip of the head, presumably innervating the anterior sensory organs that had been identified in adult nematodes by SEM (Figure 2A, B). The dorsal and ventral nerve cords previously observed were not visualised by the fluorescent catecholamine staining procedure at this stage. Moreover, ganglia observed in the mid-body region of the four day old parasitic juvenile, were not discerned again with GA staining until development of the adult stage. The various catecholaminergic cell bodies that had been located in the tail at four days post-infection had coalesced into two distinct ganglia, located dorsally and ventrally.

The distribution of catecholaminergic neurons, ganglia and nerve processes in the nervous system of six day old parasitic juveniles was identical to that of five day old juveniles (Figure 8), except that the ventral nerve cord was now visible again. The cephalic nerves were more strongly stained making them easier to follow than at five days after infection (Figure 8A).

Figure 7. Parasitic juvenile of R. culicivorax, five days post-infection stained with glyoxylic acid. A, Head region, showing the nerve ring with the associated ganglia and cephalic nerves. B, Tail region, showing two catecholaminergic ganglia. It was observed under a different focal plane than that shown in the photomicrograph that these ganglia were innervated by the dorsal and ventral nerve cords. CN= Cephalic nerve; DG= Dorsal ganglion; LG= Lateral ganglia; TDG= Tail dorsal ganglion; TVG= Tail ventral ganglion; VG= Ventral ganglion (scale bars= 100 μm).


CN
CN
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DG
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A

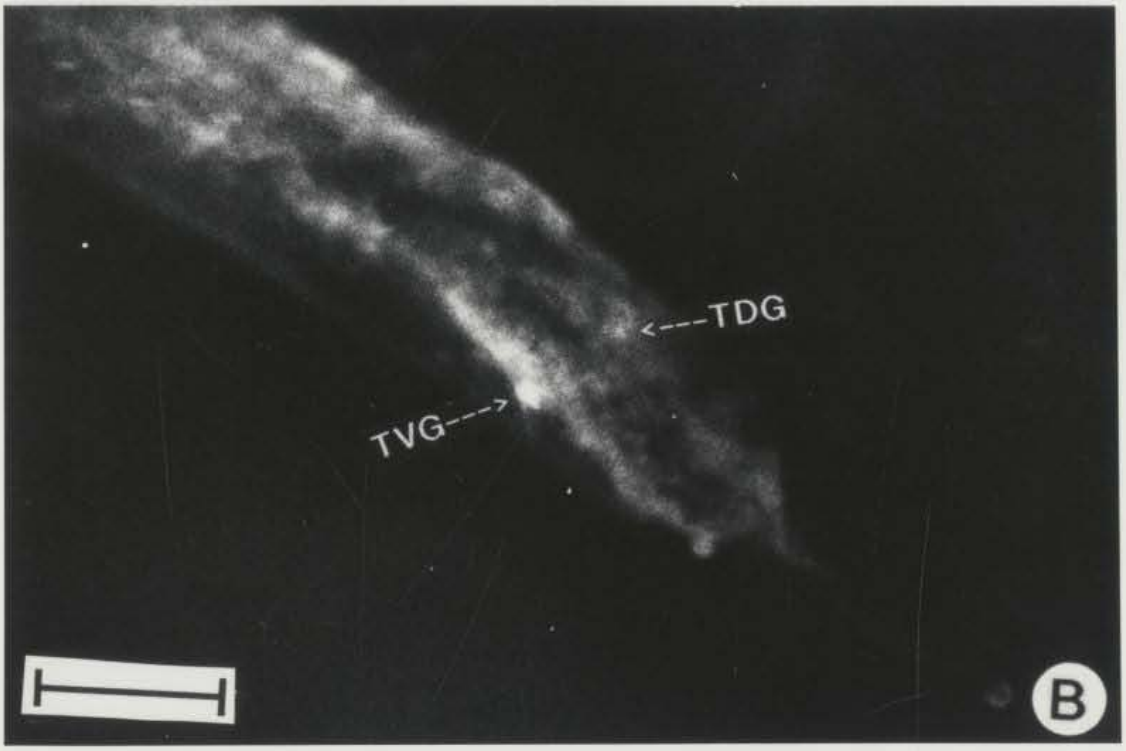
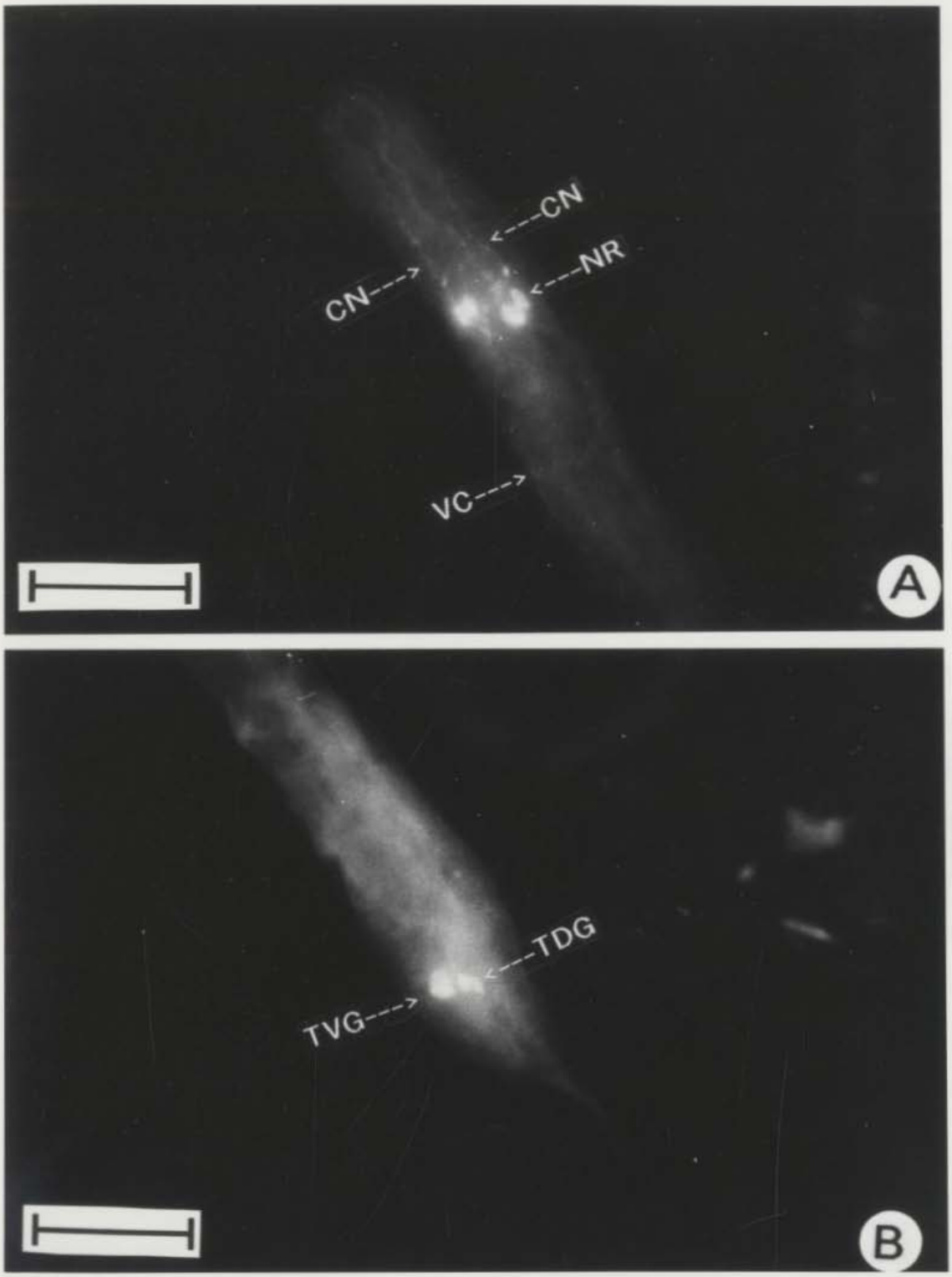

TDG
TVG
B

Figure 8. Parasitic juvenile of R. culicivorax, six days post-infection stained with glyoxylic acid. A, Head region, showing nerve ring with four catecholaminergic ganglia, associated cephalic nerves and ventral nerve cord. B, Tail region, showing the two catecholaminergic ganglia. It was observed under a different focal plane than that shown in the microphotograph that these ganglia were innervated by the dorsal and ventral nerve cords. CN= Cephalic nerve; NR= Nerve ring; TDG= Tail lateral ganglion; TVG= Tail ventral ganglion; VC= Ventral nerve cord (scale bars= 100 μm).


CN
CN
NR
VC
A
TDG
TVG
B

The catecholaminergic nervous system of seven day old parasitic juveniles was identical to that of six day old parasitic juveniles. However, all the components of the nervous system, i.e. the cephalic nerves (Figure 9A), the dorsal (Figure 9A and B) and ventral (Figure 9A and C) nerve cords and ganglia in the nerve ring (Figure 9A) and tail region (Figure 9D) were clearly visible. In addition to this, several catecholaminergic small cell bodies were present in the cephalic nerves and the dorsal and ventral nerve cords (Figure 9A, B, C and D).

3.3.3. Post-parasitic Stages

The catecholaminergic nervous system in the head region of post-parasitic male and female juveniles, adult males and females, and in the tail region of adult females (Figure 10C) was identical to that of seven day old parasitic juveniles. However, the tail region of post-parasitic juveniles (males and females) and adult males was different from that of seven day old parasitic juveniles. The ganglia in the tail regions of both the post-parasitic juvenile males (Figure 10A) and females (Figure 10B) were not visualized by the catecholamine staining procedure. However, the tail of the adult males contained approximately 16 to 20 heavily stained and ventrally located catecholaminergic ganglia (Figure 10D). Each of these ganglia was innervated by small nerve processes emanating from the ventral nerve cord. Small nerves emanated from each

Figure 9. Parasitic juvenile of R. culicivorax, seven days post-infection stained with glyoxylic acid. A, Head region, showing nerve ring with the associated four ganglia, cephalic nerves, dorsal and ventral nerve cords. B and C, Mid-body region of the same specimen, showing dorsal and ventral nerve cords with small cell bodies. D, Tail region, showing two catecholaminergic ganglia and ventral nerve cord. CB= cell bodies in the cephalic nerve, the ventral and dorsal nerve cords; CN= Cephalic nerve; DC= Dorsal nerve cord; NR= Nerve ring; TDG= Tail dorsal ganglion; TVG= Tail ventral ganglion; VC= Ventral nerve cord (scale bars= 100 μm).

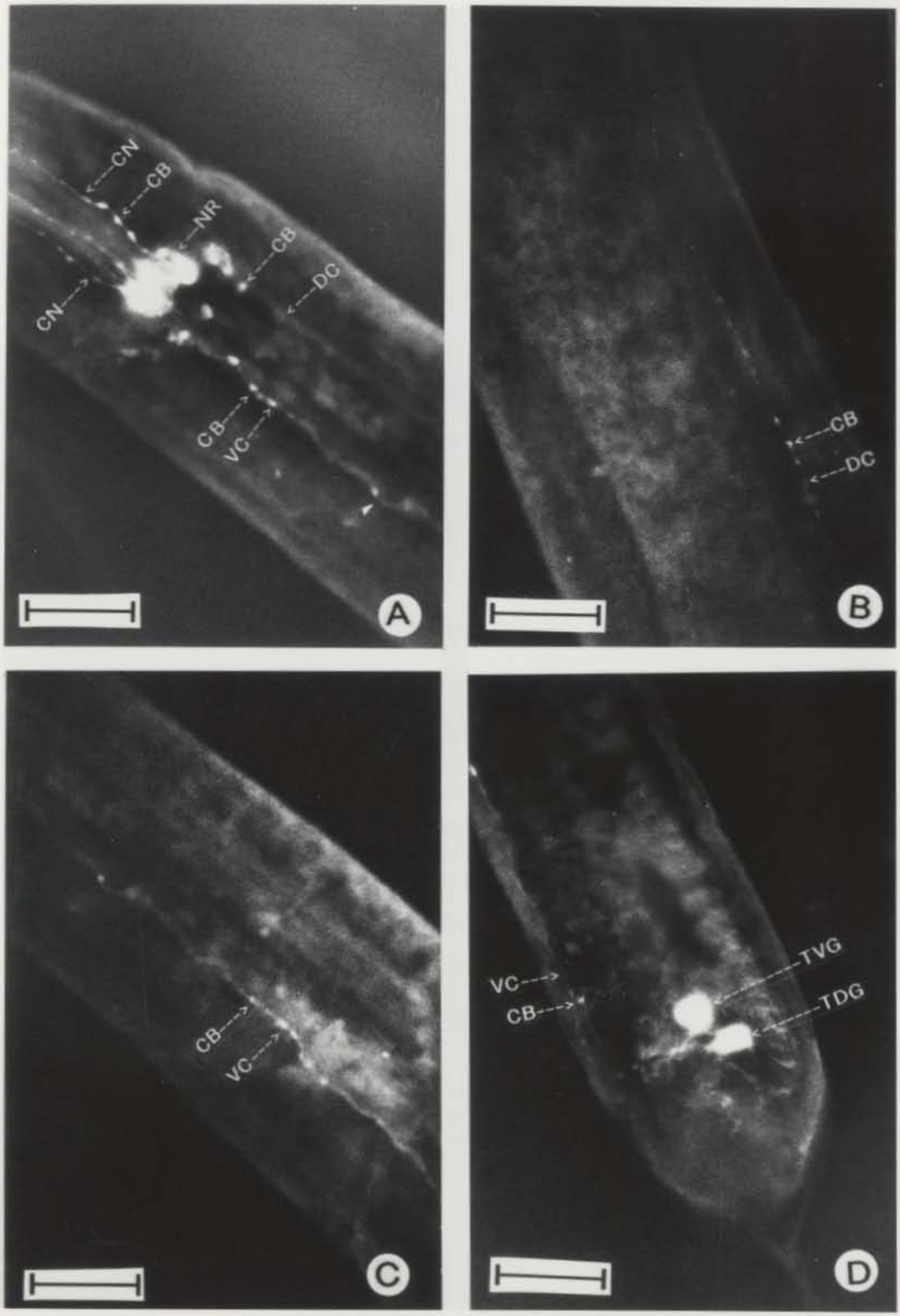

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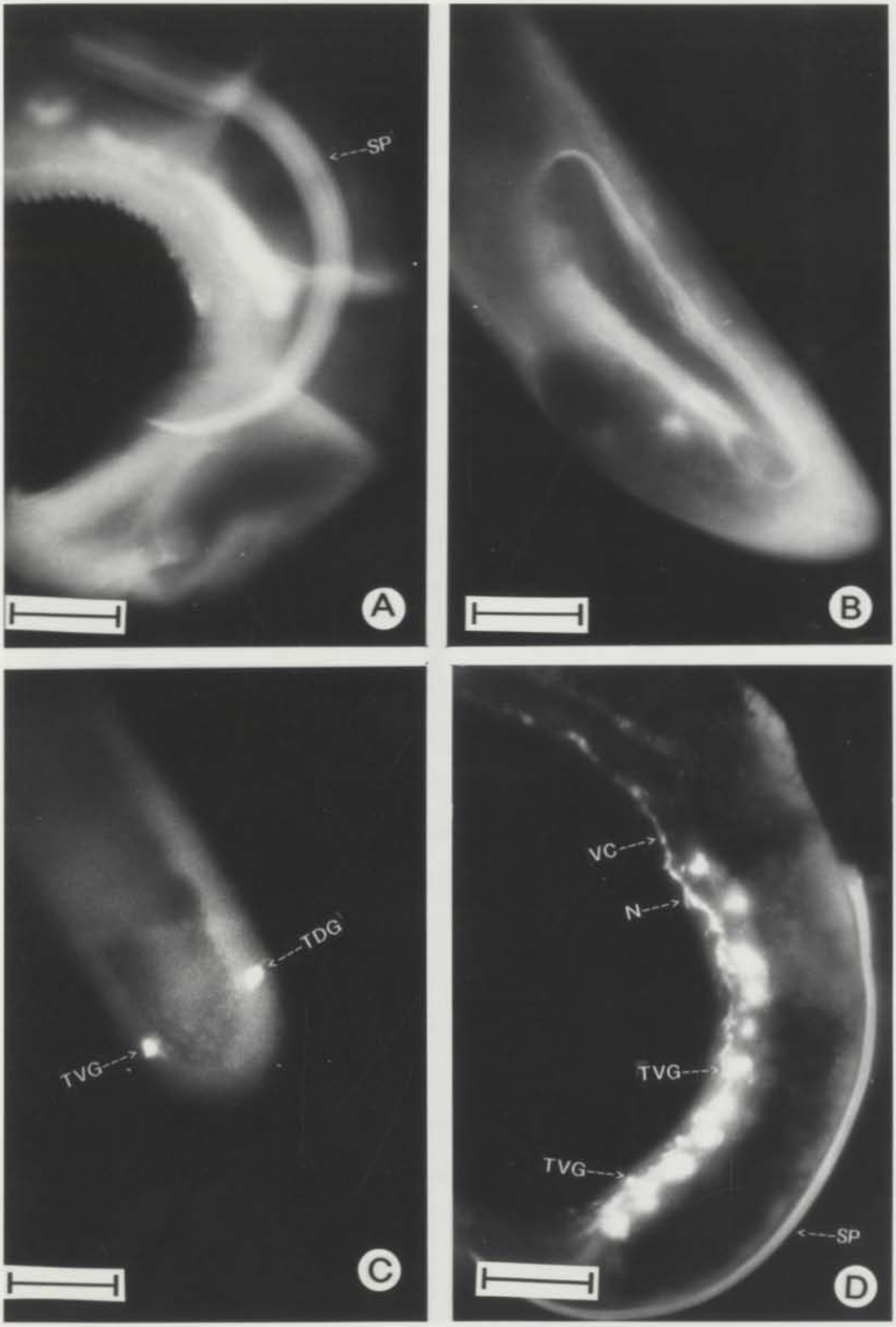
Figure 10. Tails of post-parasitic R. culicivorax stained with glyoxylic acid. A, Tail of post-parasitic male juvenile. Note the absence of catecholaminergic ganglia. B, Tail of post-parasitic female juvenile. Note the absence of catecholaminergic ganglia. C, Tail of adult female, showing two catecholaminergic ganglia. D, Tail of adult male, showing several ganglia innervated by nerve fibers emanating from the ventral nerve cord and also innervating tail papillae. N= Nerve innervating tail papillae; SP= Spicule; TDG= Tail dorsal ganglion; TVG= Tail ventral ganglion; VC= Ventral nerve cord (scale bars= 100 μm).


SP
A
B
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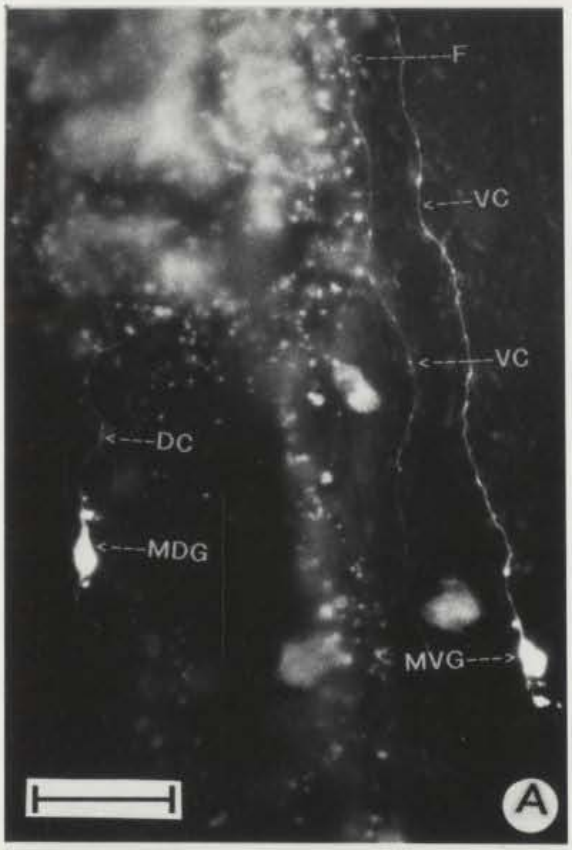
TVG
C
VC
N
TVG
TVG
SP
D

ganglion, presumably innervating the sensory papillae that had been localized by SEM on the ventral surface of the male tail (Figure 2G). In addition to these ganglia, the male tail tip also contained three or four intensely stained and dorsally located catecholaminergic ganglia. These ganglia were also innervated by small commissures to the ventral nerve cord. The catecholaminergic ganglia in the mid- body region, which were clearly visible in the four day old parasitic juveniles but not in the five, six, seven day old parasitic and post-parasitic juveniles, were again observed in adult males and females (Figure 11A). It was also noticed that the ventral nerve cord of adult nematodes contained two catecholaminergic nerves rather than one as in the earlier stages of development (Figure 11B). Fluorescent material was also present in the trophosome of adult nematodes (Figure 11A and B).

3.3.4. Functions of catecholamine(s) in R. culicivorax.

There were no significant differences in the mean intensity of catecholamine(s) between the nerve ring of male and female post-parasitic juveniles (Table 1). The catecholamine staining intensity was unaffected by the presence of the opposite sex, regardless of whether physical contact between the sexes was possible or the sexes were physically separated, but potentially chemically connected via the water medium. The concentrations of catecholamine(s) in

Figure 11. A and B, Mid-body region of adult nematode stained with glyoxylic acid, showing two catecholaminergic ganglia, two nerves in ventral nerve cord and fluorescent material in the trophosome. DC= Dorsal nerve cord; F= Fluorescent material; MDG= Mid-body dorsal ganglion; MVG= Mid-body ventral ganglion; VC= Ventral nerve cord (scale bars= 100 μm).


F
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VC
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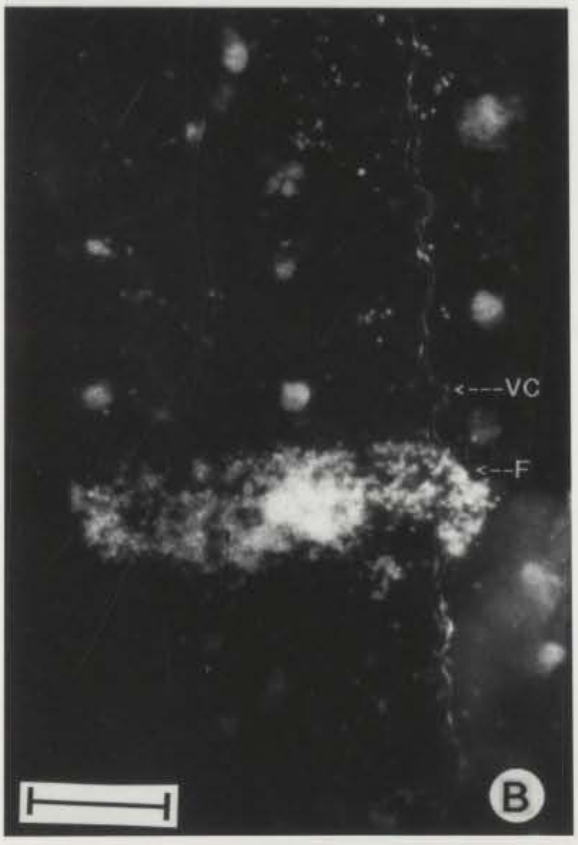

VC
F
B

the nerve rings of adult males and females (Table 2) was greater than in the nerve rings of post-parasitic juveniles (Table 1), when comparable sexes and rearing groups were compared.

The catecholamine staining intensity in adult males and females was increased by maintaining the nematodes together rather than in complete isolation of the opposite sex (Table 2). The concentration of catecholamines in the nerve ring of adult males, maintained in physical contact with females, was almost double that determined for males that were maintained alone. Similarly, the concentration of catecholamines in the tail ganglia of adult males, maintained in physical contact with females, was 50% higher than values obtained for males maintained in isolation of the opposite sex. Even when the males and females were separated by a barrier but in the same water, the, catecholamine staining intensity in the nerve ring and tail ganglia was significantly greater than for males that were maintained alone. However, these values were significantly less than obtained for nematodes in which physical contact between the sexes was possible.

The catecholamine staining intensity in the nerve ring of adult females, maintained in physical contact with the males, was approximately 50 per cent higher than for females maintained alone, or separated from males by a barrier in the same water medium. Unlike males, female nematodes did not

Table 1. Effect of presence of the opposite sex on the concentration of catecholamine(s) in the nerve ring of post-parasitic juvenile R. culicivorax.

Experimental conditions	Concentration of catecholamines	
	Male	Female
a. Males only	14.50 ± 0.48	-
b. Females only	-	13.71 ± 0.54
c. Males and females together	14.18 ± 0.52	13.72 ± 0.46
d. Males and females separated	13.90 ± 0.56	13.55 ± 0.36

Values are expressed as percent intensity of the dopamine standard and are the means of four replicates (n = 60) ± SE. Means in each vertical column and horizontal row were not significantly different (P> 0.05) as determined by Duncan's multiple range test.

Table 2. Effect of presence of the opposite sex on the concentration of catecholamine(s) in the nerve ring and tail ganglia of adult males and in the nerve ring of adult females of R. culicivorax.

Experimental conditions	Concentration of catecholamines		
	Male		Female
	tail	nerve ring	nerve ring
a. Males only	3.62 ± 0.11 D	20.86 ± 0.65 z	-
b. Females only	-	-	22.98 ± 0.34 zu
c. Males and females together	5.87 ± 0.12 A	34.73 ± 0.94 w	31.13 ± 1.16 x
d. Males and females separated	4.05 ± 0.08 B	27.48 ± 0.65 y	24.01 ± 0.79 u

Values are expressed as percent intensity of the dopamine standard and are the means of four replicates (n = 60) ± SE. Means with the same lower case letter (down and across the columns) and means with same upper case letter (down the single column) were not significantly different (P> 0.05), as determined by Duncan's multiple-range test.

significantly increase the concentration of catecholamines in their nerve rings above "females only" when chemical mediation between the sexes via the water medium was allowed.

In view of the finding that the catecholamine staining intensity was significantly enhanced in each sex by the presence of the opposite sex, additional determinations of catecholamines were made on males and females during and after mating, to investigate the possibility that the production and/or secretion of such neurosecretions may be linked to reproductive development. The concentration of catecholamines in both the nerve ring and tail ganglia of adult male nematodes declined progressively during and after copulation; the catecholamine concentration of males after copulation was the same as in post-parasitic juveniles (Table 3). By contrast, the concentration of catecholamines in the nerve ring of adult females remained approximately double that of post-parasitic juveniles, both during and after copulation. However, it is apparent from the data that the catecholamine staining intensity decreased marginally, albeit significantly, during copulation, then increased to the same concentration as in the nerve ring of newly molted adult females (Table 3).

3.4. Peptidergic Nervous System:

3.4.1. Parasitic Stages

At four days post-infection, FMRF-amide like peptide was detected in the nerve ring. Several small peptidergic cell

Table 3. Effect of presence of the opposite sex on the concentration of catecholamine(s) in R. culicivorax at different developmental stages.

Stage of development	Concentration of catecholamine(s)		
	Male		Female
	Tail	Nerve ring	Nerve ring
1. Post-parasitic juveniles	-	14.18 ± 0.52 d	13.72 ± 0.46 d
2. Adults	5.87 ± 0.12 A	34.73 ± 0.94 a	31.13 ± 1.16 b
3. During copulation	3.30 ± 0.08 B	22.12 ± 0.55 c	27.67 ± 0.73 b
4. After copulation	3.22 ± 0.10 B	14.93 ± 0.83 d	31.76 ± 1.06 b

Values are expressed as percent intensity of the dopamine standard and are the means of four replicates (n = 60) ± SE. Means with the same upper case letter (down the single column) and means with the same lower case letter (down and across the columns) were not significantly different (P> 0.05), as determined by Duncan's multiple-range test.

bodies were present at intervals in a single nerve within the nerve ring (Figure 12A). However, the ganglia attached to the nerve ring that stained positively for catecholamine(s) did not stain for FMRF-amide. The ventral nerve cord (Figure 12B) emanated from the ventral side of the nerve ring as a single nerve, then after a short distance bifurcated into two nerves that ran posteriorly toward the tail tip. In the tail tip, these two nerves divided into branches and innervated several peptidergic ganglia (Figure 12C). The ventral nerve cord contained several immunoreactive cell bodies at intervals along its length. Another bifurcated nerve designated as the ventro-lateral nerve originated from the ventro-lateral side of the nerve ring, ran posteriorly for a short distance, then innervated two large ventro-lateral peptidergic ganglia. The ventro-lateral nerve also contained several peptidergic cell bodies along its length. The cephalic nerves did not show positive immunoreactivity at this stage.

The peptidergic nervous system of five (Figure 13A) and six (Figure 13B) day old parasitic juveniles was identical to that of four day old parasitic juveniles.

The peptidergic nervous system of seven day old parasitic juveniles was identical to that of earlier stages except that eight cephalic papillary ganglia, interposed between the nerve ring and the anterior head tip, showed positive immunoreactivity. Each of these ganglia was innervated by a

Figure 12. Parasitic juvenile of R. culicivorax four days post-infection incubated in primary antibody, Rabbit anti-FMRF-amide. A and B, Head region, showing nerve ring with associated ventral nerve cord and ventro-lateral nerve innervating two ventro-lateral ganglia. C, Tail region, Showing ventral nerve cord innervating tail peptidergic ganglia. N= Branch of ventral nerve cord; NR= Nerve ring; TG= Tail peptidergic ganglia; VC= Ventral nerve cord; VL= Ventro-lateral nerve; VLG= Ventro-lateral ganglia (scale bars= 100 μm).

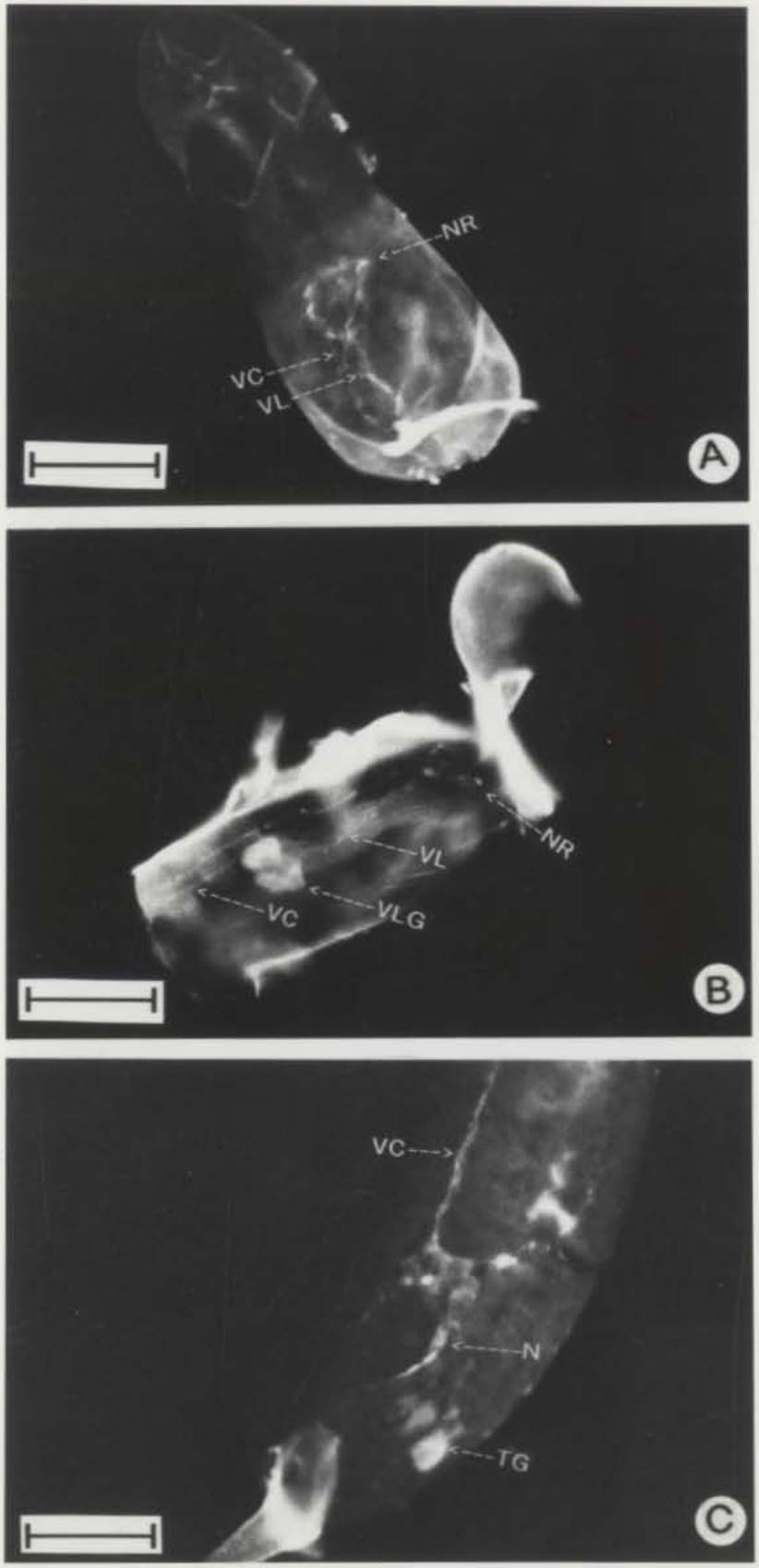
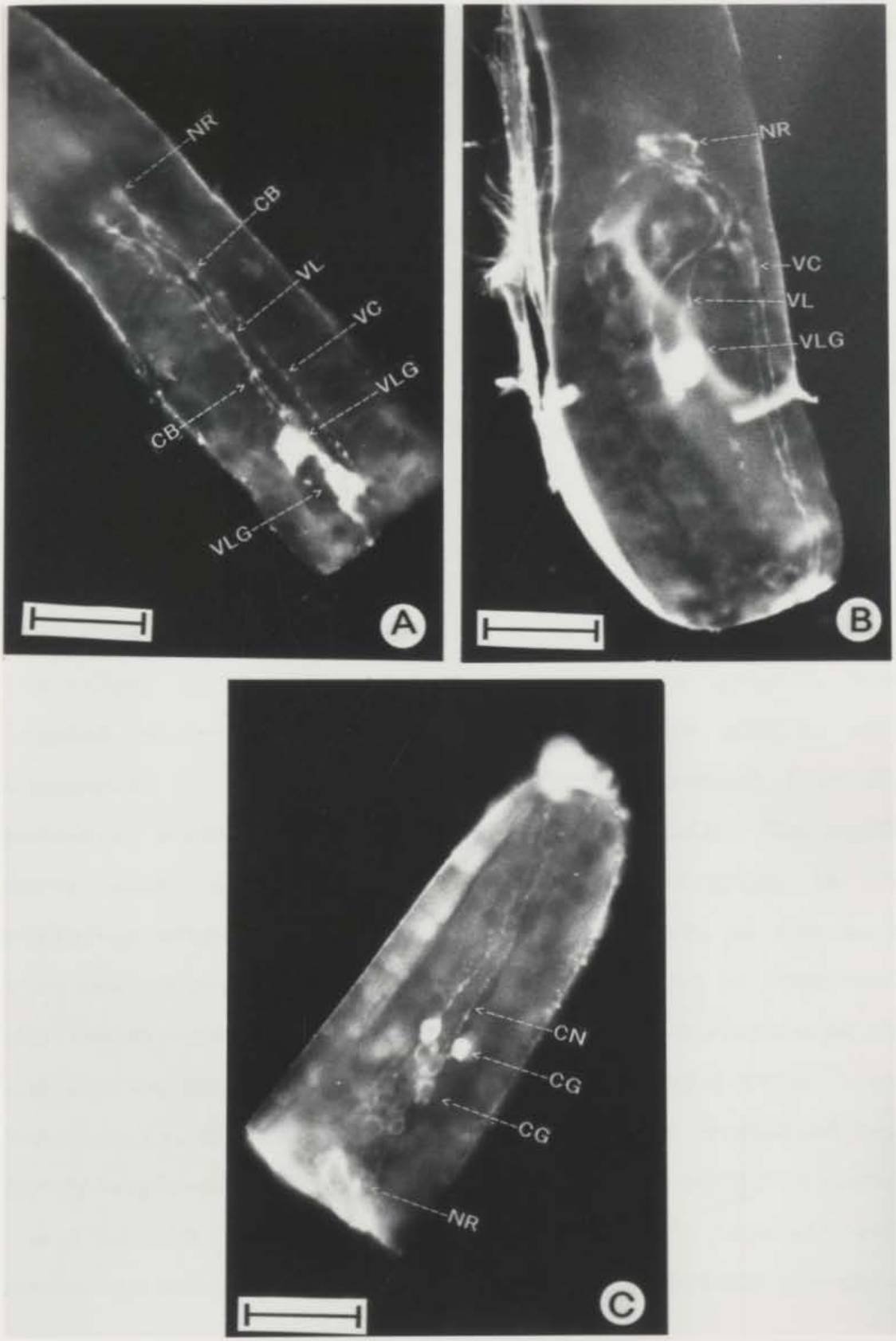

NR
VC
VL
A
VL
NR
VC
VLG
B
VC
N
TG
C

Figure 13. Parasitic juveniles of R. culicivorax five, six and seven days post-infection incubated in primary antibody, Rabbit anti- FMRF-amide. A, Head region of five day old parasitic juvenile showing the bifurcated ventral nerve cord, ventro-lateral nerve and two ventro-lateral ganglia. B, Head regions of six day old parasitic juvenile showing ventral nerve cord, ventro-lateral nerve and two ventro-lateral ganglia. C, Head region of seven day old parasitic juvenile, showing nerve ring with cephalic papillary ganglia and associated cephalic nerves. CB= Cell bodies in the ventral verve cord and ventro-lateral nerve; CG= Cephalic papillary ganglia; CN= Cephalic nerves; NR= Nerve ring; VC= Ventral nerve cord; VL= Ventro-lateral nerve; VLG= Ventro-lateral ganglia (scale bars= 100 μm).

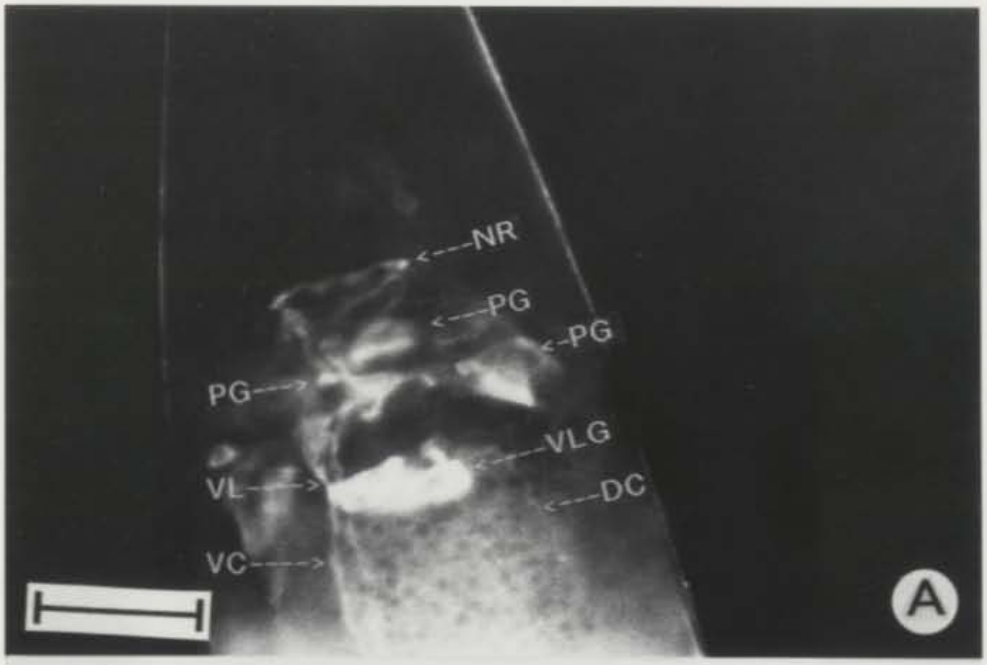

NR
CB
VL
VC
VLG
CB
VLG
A
NR
VC
VL
VLG
B
CN
CG
CG
NR
C

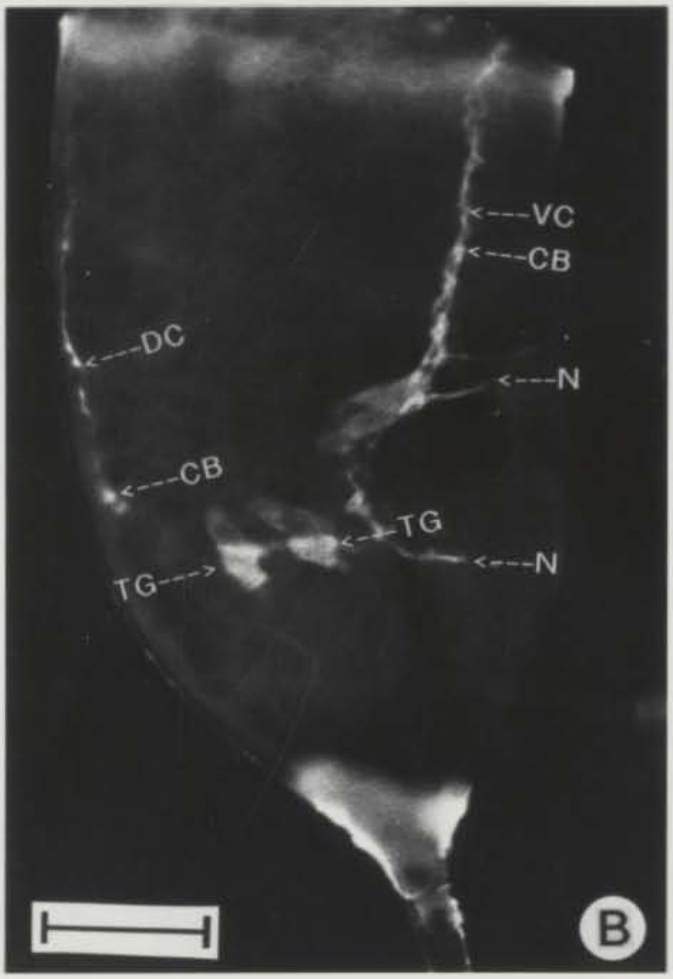
nerve emanating from the anterior surface of the nerve ring (Figure 13C). Cephalic nerves originated from the anterior surface of each of these cephalic papillary ganglia and ran anteriorly toward the head tip, presumably innervating six cephalic sensory papillae and two amphids (chemo-sensory organs) that had been identified at the head tip of adult nematodes by SEM (Figure 2A, B). The cephalic nerves also contained several small peptidergic cell bodies along their lengths.

3.4.2. Post-parasitic Stages

The peptidergic nervous system of post-parasitic juveniles was identical to that of seven day old parasitic juveniles, except that several peptidergic ganglia were located posterior to the nerve ring. These ganglia were innervated by peptidergic nerve fibers emanating from the posterior surface of the nerve ring (Figure 14A). The dorsal nerve cord, which did not stain for FMRF-amide in the parasitic stages, emerged from the dorsal side of the nerve ring and ran posteriorly into the tail tip where it innervated peptidergic ganglia (Figure 14B). Although not visible on the photomicrographs, four lateral (one ventrolateral, one dorsolateral and two lateral) nerve cords also originated from the lateral sides of the nerve ring and ran posteriorly toward the tail tip. These nerve cords (dorsal and lateral) each contained only one nerve and several small peptidergic cell

Figure 14. Post-parasitic juvenile of R. culicivorax incubated in primary antibody, Rabbit anti- FMRF-amide. A, Head region, showing several peptidergic ganglia posteriorly attached to the nerve ring, ventral nerve cord, ventro-lateral nerve and two ventro-lateral ganglia. B, Tail region, showing ventral and dorsal nerve cords innervating peptidergic ganglia. CB= Cell bodies in ventral and dorsal nerve cords; DC= Dorsal nerve cord; N= Nerves from the cell bodies of the ventral nerve cord innervating outer body surface; NR= Nerve ring; PG= Posterior peptidergic ganglia; TG= Tail peptidergic ganglia; VC= Ventral nerve cord; VL= Ventro-lateral nerve; VLG= Ventro-lateral ganglia (scale bars= 100 μm).


NR
PG
PG
PG
VLG
VL
DC
VC
A


VC
CB
DC
N
CB
TG
N
TG
B

bodies along their lengths.

The peptidergic nervous system of both adult males and females was identical to that of post-parasitic juveniles, except that the number of peptidergic nerves in the nerve ring of adult nematodes had increased from one (at previous stages of development) to several nerves (Figure 15A). Moreover, the number of peptidergic ganglia attached posteriorly to the nerve ring of adult nematodes was greater than in the post-parasitic juveniles. However, there was no clear separation of these ganglia; all of the ganglia were joined together like a bunch of grapes (Figure 15A). In adult nematodes, the ventro-lateral nerve and the ventro-lateral ganglia did not show immunoreactivity. Strong immunoreactivity was observed in the amphids (Figure 15B). Several immunoreactive ganglia were scattered throughout the body posterior to the nerve ring (Figure 15C). These ganglia were more densely distributed near the female reproductive system and formed a network of cell bodies and commissures (Figure 15D). The ventral nerve cord of adult nematodes contained three peptidergic nerves. Small cell bodies in the longitudinal nerve cords were connected to each other by peptidergic nerves (Figure 16A). Peptidergic nerves from cell bodies of the dorsal and ventral nerve cords also innervated the dorsal and ventral surface (outer) of the nematode body (Figure 16B).

The peptidergic nervous system in the tail region of

Figure 15. Adult, R. culicivorax incubated in primary antibody, Rabbit anti-FMRF-amide. A, Head region, showing nerve ring with anteriorly attached cephalic papillary ganglia, associated cephalic nerves and posteriorly attached peptidergic ganglia. B, Head region, showing strong immunoreactivity in amphids (anterior chemo-sensory organs). C, Mid-body region, showing a network of peptidergic ganglia. D, Mid-body region near female reproductive system, showing densely distributed peptidergic ganglia. A= Amphids; CB= Cell bodies in the longitudinal nerve cords; CN= Cephalic nerve; CG= Cephalic papillary ganglia; DC= Dorsal nerve cord; LC= Lateral nerve cord; MG= Mid-body peptidergic ganglia; NR= Nerve ring; PG= Posterior peptidergic ganglia; VC= Ventral nerve cord (scale bars= 100 μm).

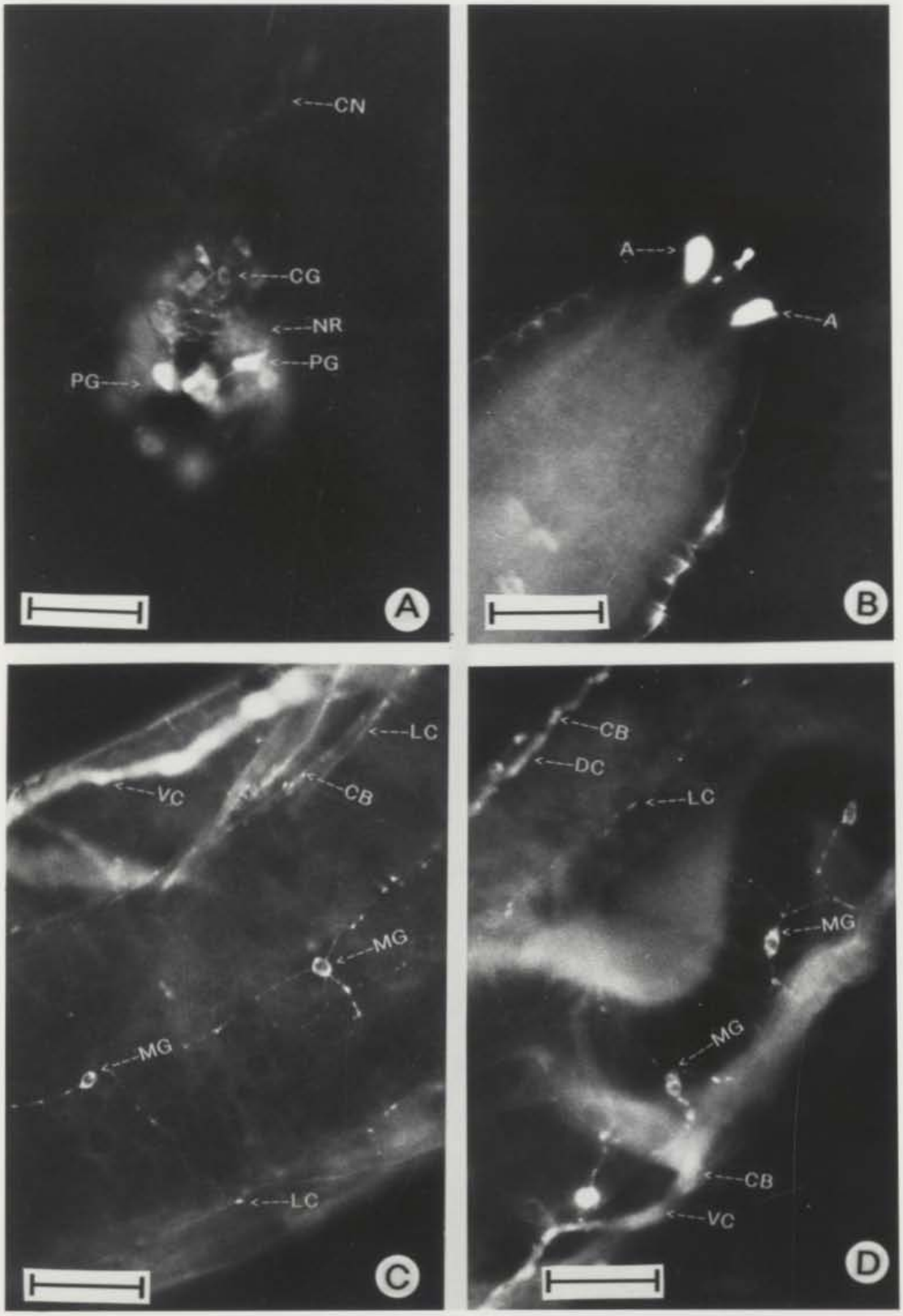
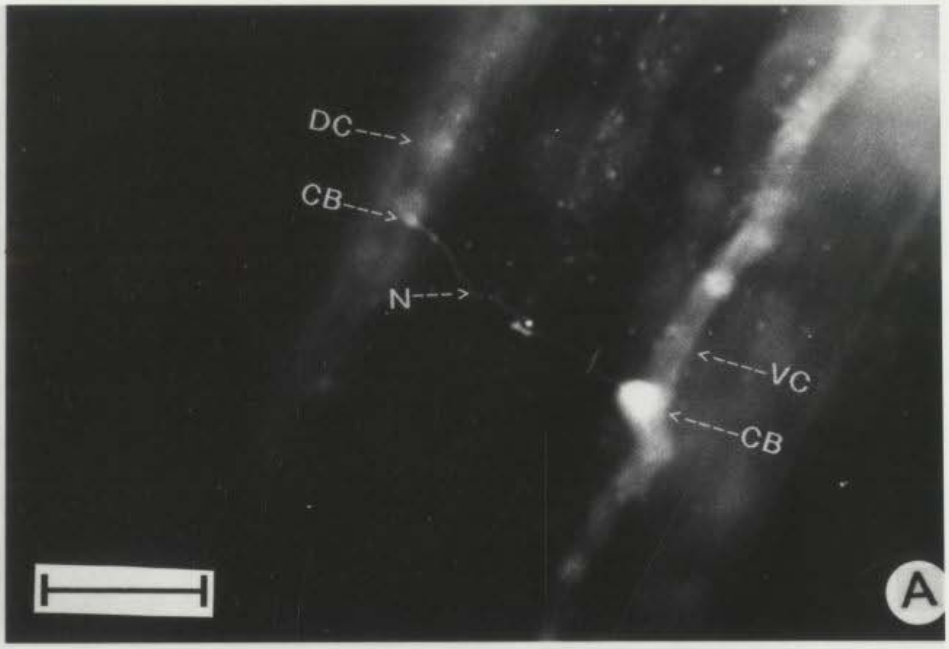
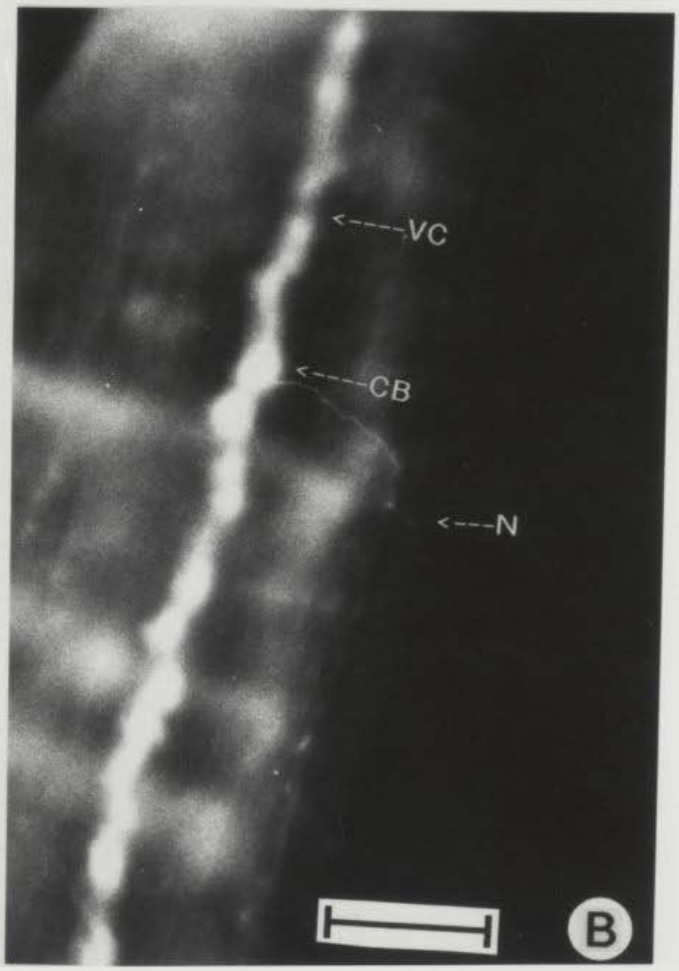

CN
CG
NR
PG
PG
A
A
A
B
LC
VC
CB
MG
MG
LC
C
CB
DC
LC
MG
MG
CB
VC
D

Figure 16. Mid-body region of adult female R. culicivorax incubated in primary antibody, Rabbit anti- FMRF-amide. A, showing a nerve emanating from a cell body in the ventral nerve cord and innervating a cell body in the dorsal nerve cord. B, showing a nerve emanating from the cell body of the ventral nerve cord and innervating the outer surface of the nematode body. CB= Cell bodies in the ventral and dorsal nerve cords; DC= Dorsal nerve cord; N= Nerve connecting outer body surface of nematode; VC= Ventral nerve cord (scale bars= 100 μm).


DC
CB
N
VC
CB
A

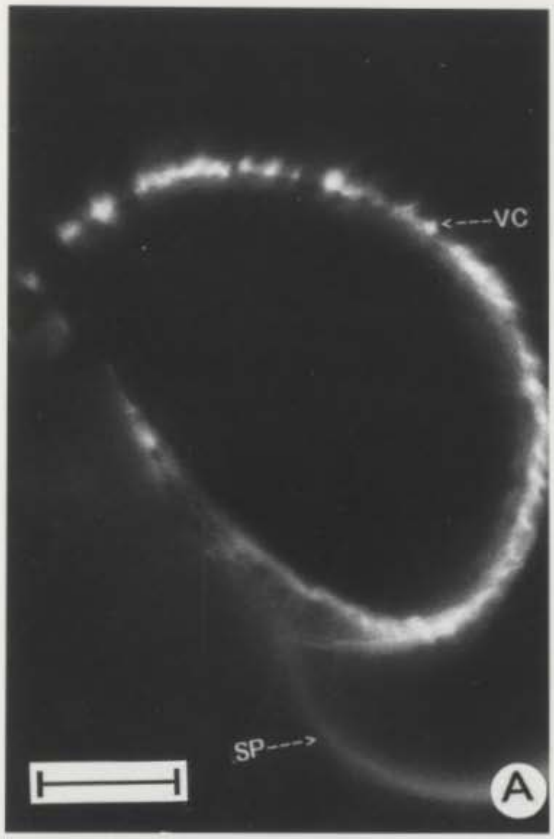

VC
CB
N
B

males was different from that of females. No peptidergic ganglia were present in the tail of adult male nematodes; only the ventral nerve cord showed strong positive immunoreactivity in the tail region (Figure 17A). However, the several ganglia that had been located in the earlier stages had coalesced into four separate clusters, located dorsally (one), ventrally (one) and laterally (two) in the female tail tip. These four clusters of peptidergic ganglia were innervated by the dorsal, the ventral and two lateral nerve cords respectively (Figure 17B).

3.4.3. Dopaminergic Nervous System

Dopamine immunoreactivity was observed only in the amphids of adult, R. culicivorax (Figure 18). The major components of the nervous system, such as nerve ring, cephalic nerves, ganglia within the nerve ring, longitudinal nerve cords and ganglia in the tail region, did not show positive immunoreactivity to dopamine.

Figure 17. Tails of adult male and female R. culicivorax incubated in primary antibody, Rabbit anti-FMRF-amide. A, Male tail region, showing the ventral nerve cord and the absence of peptidergic ganglia. B, Female tail region, showing ventral nerve cord and two clusters of peptidergic ganglia. Two additional clusters of ganglia, other than those shown in the microphotograph, were observed under a different focal plane. CB= Cell body in the ventral nerve cord; SP= Spicule; TG= Clusters of tail peptidergic ganglia; VC= Ventral nerve cord (scale bars= 100 μm).


VC
SP
A

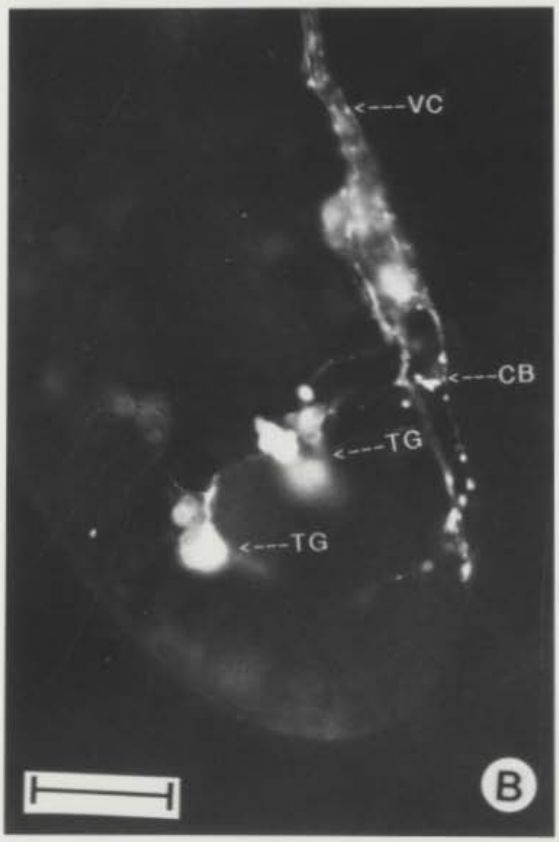
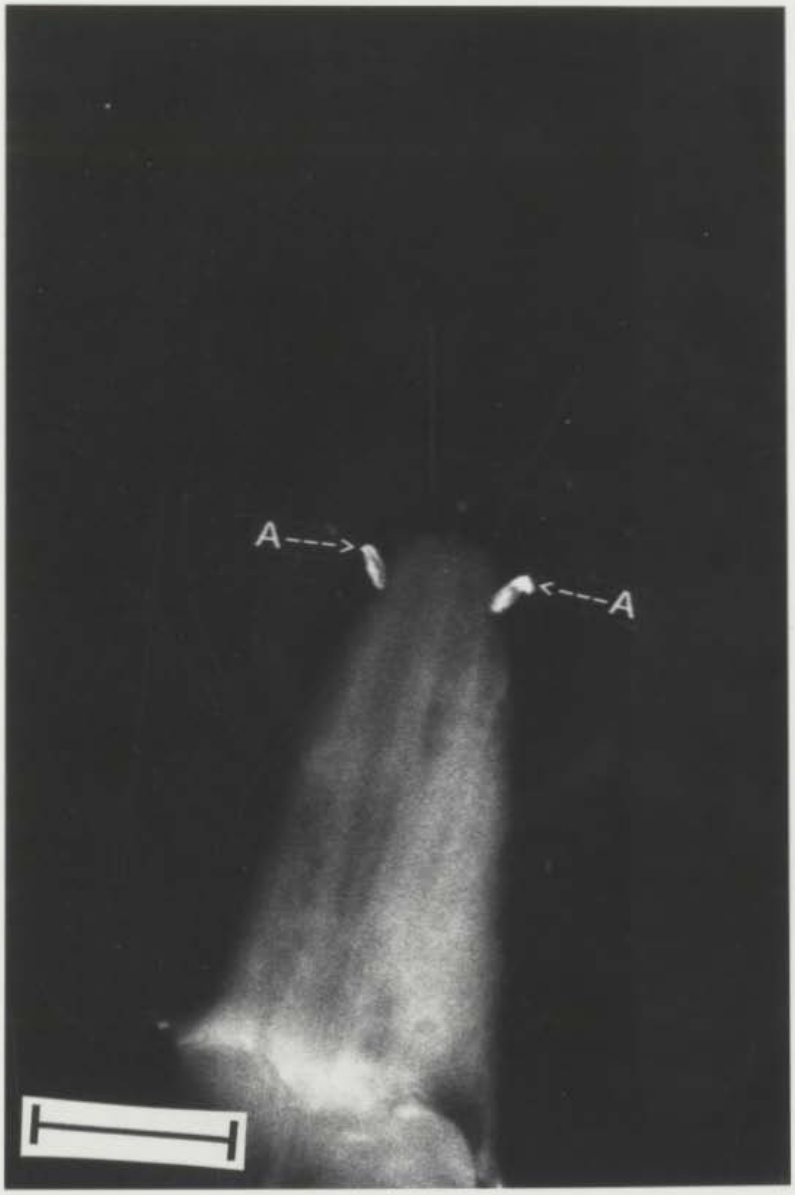

VC
CB
TG
TG
B

Figure 18. Head region of adult, R. culicivorax incubated in primary antibody, Rabbit anti-dopamine; showing strong dopamine immunoreactivity in the amphids. A= Amphid (scale bar= 100 μm).


A
A

IV. DISCUSSION

4.1. General Overview

This study has shown that catecholamine(s) and FMRF-amide-like peptide are widely distributed in the nervous system of R. culicivorax and the external sensory organs are innervated by the catecholaminergic and peptidergic components of the nervous system.

Scanning electron microscopy confirmed the findings of Wright and Richter (1982), that the head tip of both sexes contained six hexagonally arranged cephalic papillae and two amphids (cephalic sensory organs). My observations are at variance, however, with previous SEM studies which identified two pores in each of the two lateral cephalic papillae and three pores in each of the remaining four papillae in both males and females of R. culicivorax (Wright and Richter, 1982). This arrangement of pores was only present in adult males; three pores were observed in each of the six papillae of females. Several caudal sensory papillae were observed on the ventral surface of the male tail and each papilla contained only one pore. Caudal sensory papillae were absent in the female tail. The presence of caudal sensory papillae has been reported in several other parasitic as well as free-living nematodes (McLaren, 1976). It has been suggested that the caudal papillae in males of Dipetalonema viteae function

both as chemoreceptors and mechanoreceptors in regulating copulatory behaviour (McLaren, 1972). In R. culicivorax, the cephalic sensory organs are innervated by both the catecholaminergic and peptidergic cephalic nerves, whereas caudal papillae are innervated only by the catecholaminergic nerves that emanated from the tail ganglia, providing circumstantial evidence that such substance(s) may be involved in regulating or mediating sensory information from the environment as well as copulatory activity.

Conventional histochemical and ultrastructural techniques were not suitable for localizing neurosecretory sites in the nervous system of R. culicivorax. These techniques revealed positively stained and electron dense granules in the ovaries and testes, but not in the nervous system of the nematode. The size of these electron dense granules (400-1000 nm diameter) was greater than is characteristic of neurosecretory granules (100-300 nm diameter; see Davey, 1988). Further studies would be needed to determine the chemical nature and the role of these granules in gametogenesis and related processes.

By contrast, fluorescence microscopy and immunohistochemical techniques proved extremely valuable for locating neurosecretory centres in R. culicivorax. The general organization of the catecholaminergic and peptidergic nervous systems of adult R. culicivorax are shown in Figures 19 and 20, respectively. Differences between the catecholaminergic

Figure 19. Schematic diagram of anterior and posterior regions of adult, R. culicivorax, showing organization of catecholamionergic nervous system. CB= Cell bodies in cephalic nerves, ventral and dorsal nerve cords; CN= Cephalic nerve; CP= Caudal papilla; DC= Dorsal nerve cord; DG= Dorsal ganglion; LG= Lateral ganglia; MDG= Mid-body dorsal ganglion; MVG= Mid-body ventral ganglion; N= Nerve innervating caudal papilla; NR= Nerve ring; SP= Spicule; T= Trophosome; TDG= Tail dorsal ganglion; TVG= Tail ventral ganglion; V= Vulva; VC= Ventral nerve cord; VG= Ventral ganglion.

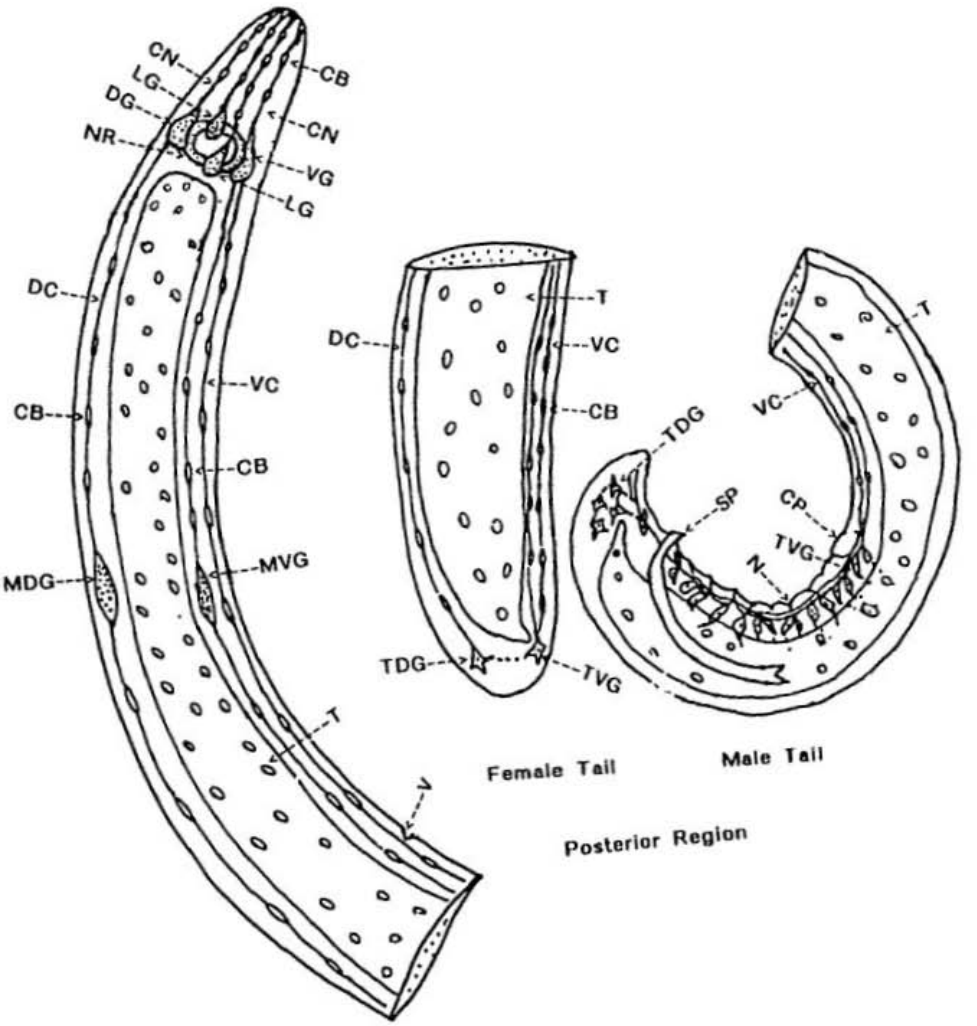
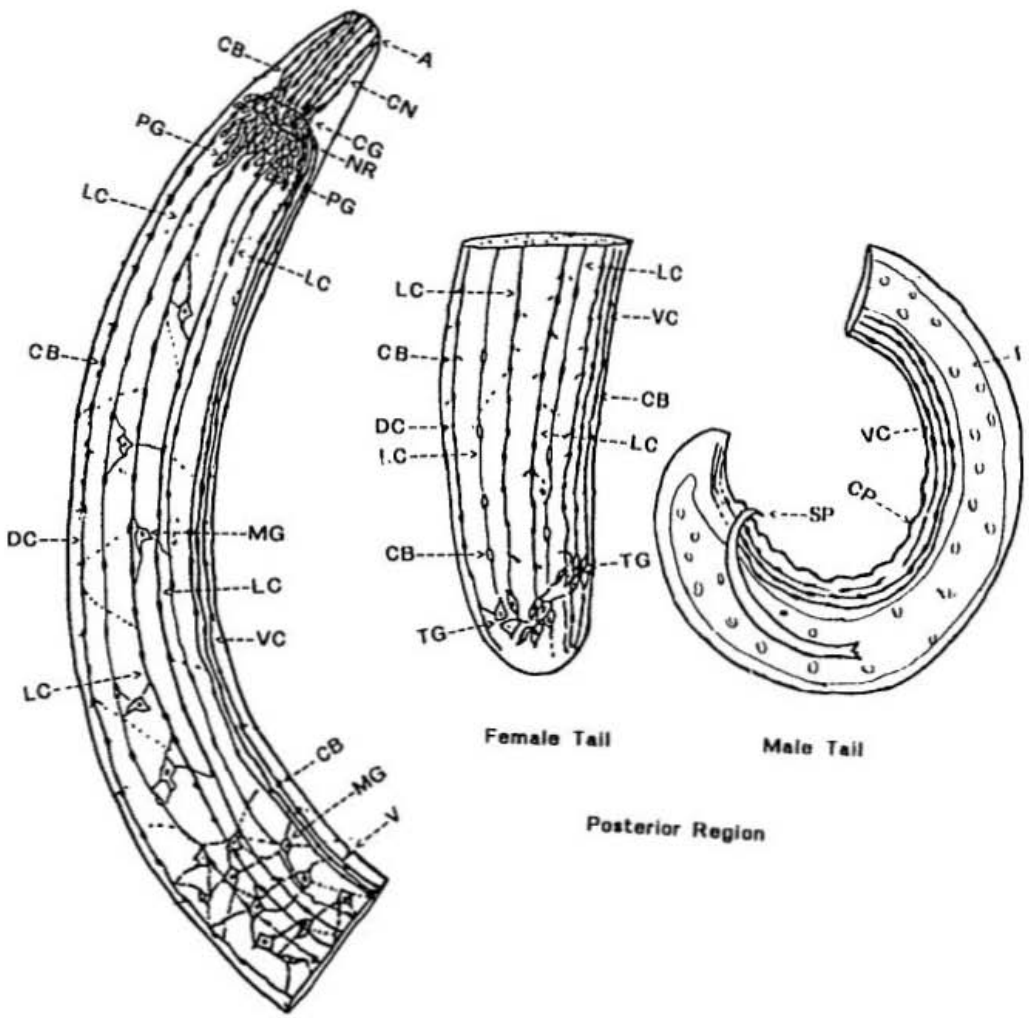

CN
LG
DG
NR
CB
CN
VG
LG
DC
VC
CB
CB
MDG
MVG
T
V
Anterior Region
DC
T
VC
CB
TDG
TVG
Female Tail
TDG
VC
T
SP
CP
TVG
N
Male Tail
Posterior Region

Figure 20. Schematic diagram of anterior and posterior regions of adult, R. culicivorax, showing layout of peptidergic nervous system. A= Amphid; CB= Cell bodies in the cephalic nerves and longitudinal nerve cords; CG= Cephalic papillary ganglia; CN= Cephalic nerve; CP= Caudal papillae; DC= Dorsal nerve cord; LC= Lateral nerve cord; MG= Mid-body peptidergic ganglia; NR= Nerve ring; PG= Posterior peptidergic ganglia; SP= Spicule; T= Trophosome; TG= Clusters of tail peptidergic ganglia; V= Vulva; VC= Ventral nerve cord.


CB
A
CN
PG
CG
NR
LC
PG
LC
CB
DC
MG
LC
VC
LC
CB
MG
V
Anterior Region
LC
LC
VC
CB
DC
CB
LC
LC
CB
TG
TG
Female Tail
VC
CP
SP
Male Tail
Posterior Region

and peptidergic nervous systems of the nematode are summarized in Table 4. The catecholaminergic nervous system consisted of a nerve ring with four strongly stained ganglia, four cephalic nerves, two longitudinal nerve cords (dorsal and ventral) and two ganglia in the mid-body region of the nematode. The ventral nerve cord was comprised of only two catecholaminergic nerves. The adult female tail included only two ganglia, whereas the male tail contained several (16-20) ganglia. The peptidergic nervous system comprised a nerve ring with more than four immunoreactive ganglia, eight cephalic nerves, six longitudinal nerve cords (one dorsal, one ventral and four lateral) and several ganglia in the mid-body region of the nematode. The ventral nerve cord contained three peptidergic nerves. The adult female tail contained four clusters of peptidergic ganglia, whereas such ganglia were absent in the adult male tail.

4.2. Catecholaminergic Nervous System

Biogenic amines have been biochemically identified from tissue extracts of A. suum (Bargiel et al. 1970), Aspiculuris tetraptera (Anya, 1973a, b), Aphelenchus avenae (Wright and Awan, 1978), C. elegans (Sulston et al., 1975; Horvitz et al., 1982), C. briggsae (Kisiel et al. 1976) and H. contortus (Rogers and Head, 1972). Moreover, such amines have been localized within the nervous systems of several species of parasitic (Goh and Davey, 1976; Högger et al., 1978; Sharpe

Table 4. Differences between catecholaminergic and peptidergic nervous systems of adult, R. culicivorax.

Characteristics	Catecholaminergic nervous system	Peptidergic nervous system
Number of ganglia in the nerve ring	Four	Several
Number of cephalic nerves	Four	Eight
Number of longitudinal nerve cords	Two	Six
Ventral nerve cord	consists of two nerves	consists of three nerves
Number of lateral nerve cords	Absent	Four
Number of ganglia in the mid-body region	Two	Several
Number of ganglia in the adult female tail	Two	Four clusters
Number of ganglia in the adult male tail	Sixteen to twenty	Absent

and Atkinson, 1980; Sharpe et al., 1980; Lee and Ko, 1991) and free-living nematodes (Sulston et al., 1975; McIntire and Horvitz, 1985; Wright and Awan, 1978; Leach et al., 1987). The present studies showed a widespread distribution of catecholamine(s) in the nervous system of R. culicivorax. In general, the catecholaminergic neurosecretory sites of R. culicivorax correspond with those identified in other nematodes, with some differences in points of detail. In R. culicivorax, catecholamine(s) was observed in four ganglia which were located within the nerve ring (one dorsal, one ventral and two lateral). However, catecholamines were observed only in the ventral ganglion of C. elegans (Sulston et al., 1975), N. battus (Sharpe et al., 1980) and P. redivivus (Sharpe and Atkinson, 1980) and in the ventral and dorsal ganglia of X. americanum (Högger et al., 1978). In contrast to R. culicivorax, the location of catecholaminergic ventral ganglia of all these nematodes was posterior to the nerve ring. Also, in X. americanum, catecholamines within the dorsal ganglion was anterior to the nerve ring rather than, as in R. culicivorax, within the nerve ring. In R. culicivorax, catecholaminergic cephalic nerves originated from each of the four ganglia within the nerve ring. This differs from C. elegans (Sulston et al., 1975), P. decipiens (Goh and Davey, 1976), X. americanum (Högger et al., 1978), P. punctatus, P. redivivus (Wright and Awan, 1978), N. battus (Sharpe et al.,

1980), N. dubius (Sharpe and Atkinson, 1980) and T. spiralis (Lee and Ko, 1991) in which catecholaminergic cephalic nerves originate from sub-ventral and sub-dorsal cell bodies that are connected to, but not within, the nerve ring.

In R. culicivorax, the dorsal and ventral nerve cords stained positively for catecholamine(s), whereas in P. decipiens (Goh and Davey, 1976), N. battus (Sharpe et al., 1980), P. redivivus, N. dubius (Sharpe and Atkinson, 1980) and P. punctatus (Wright and Awan, 1978), only the ventral nerve cord contained catecholamine(s). Aminergic cell bodies have been localized near the vulva of adult female P. redivivus (Sharpe and Atkinson, 1980), X. americanum (Högger et al., 1978) and G. ulmi (Leach et al., 1987); no such secretory sites were identified in R. culicivorax, however. Two catecholaminergic ganglia were present in the mid-body region of adult females of R. culicivorax, but these were considerably anterior to the vulva and were also present in adult males. The tail region of adult males of R. culicivorax contained 16-20 catecholaminergic ganglia, whereas the tails of males of A. lumbricoides, C. elegans (Sulston et al., 1975) and P. redivivus (Sharpe and Atkinson, 1980) contained only 2, 6 and 8 dopaminergic cell bodies, respectively. However, like X. americanum (Högger et al., 1978), females of R. culicivorax contained two catecholaminergic ganglia in their tail tips; the tail of females of P. punctatus and P. redivivus contained

only one aminergic cell body/ ganglion (Sharpe and Atkinson, 1980; Wright and Awan, 1978).

The approach taken in the current study of systematically detailing the distribution of neurosecretions/ neurotransmitters throughout development has been undertaken for only a few nematode species. With respect to amines produced by the nervous system, Leach et al. (1987) showed that there were no substantial differences among developmental stages in the distribution of serotonin within the nervous system of G. ulmi; additional serotonergic neurons were observed near the vulva of adult females as well as the spicule of adult males. Sharpe et. al. (1980) only examined the infective and adult stages of N. battus, but concluded that the nervous system of both these stages contained a similar distribution of catecholamine; catecholaminergic neurons, not present in the infective stage, were present near the vulva of adult females and the copulatory bursa and spicule of adult males, however. My observations for R. culicivorax contrast with the above isolated studies, since pronounced changes in the distribution of catecholamines were observed during the development of this species.

In general, the complexity of the catecholaminergic nervous system of R. culicivorax increased throughout development. As was found to be the case for C. elegans (Sulston et al., 1975) catecholamine(s) was present in the

juveniles of R. culicivorax before and after hatching from the eggs. In four day old parasitic juveniles of R. culicivorax, catecholamine staining was observed in the nerve ring, dorsal and ventral nerve cords, mid-body region ganglia and tail region ganglia (two). In five day old parasitic juveniles, catecholamine(s) was also observed in cephalic nerves. Further elaboration of the catecholaminergic nervous system was evident in adult males of R. culicivorax, as tail ganglia containing catecholamine(s) were visualized.

It was also observed that some components of the catecholaminergic nervous system became temporarily inactive, then became active again during more advanced developmental stages. For example, ganglia observed in the mid-body region of four day old parasitic juveniles were not detected again through catecholamine staining until development of the adult stage. The two catecholaminergic ganglia observed in the tail tip of parasitic juveniles were not visualized in the tail region of post-parasitic juvenile males and females, but the catecholaminergic ganglia were present in adult males and females. The reason for the disappearance and subsequent reappearance during development of catecholamine(s) in some ganglia of the nervous system of R. culicivorax is not clear at present.

The identity of the catecholamine(s) is not known. The GA- induced fluorescence is not designed to differentiate

between the various possible catecholamines, e.g. dopamine, noradrenaline and adrenaline. Use of a dopamine-specific immunofluorescence technique showed the presence of dopamine only in the amphids of adult R. culicivorax, indicating that the catecholamine(s) of more widespread distribution in the nematode's nervous system are not dopamine. Further studies are needed to determine whether the catecholamine(s) is noradrenaline, adrenaline and/ or other unidentified catecholamines.

The catecholamine(s) widely distributed within the nervous system of R. culicivorax could function as neurotransmitters and/or neurosecretions. Catecholamines are important neurotransmitters in a wide variety of invertebrates and vertebrates (Withers, 1992; Walker and Kerkut, 1978). The possible role of these compounds as neurosecretory substances in nematodes has been proposed (Davey, 1988). While the present study did not directly address the issue of whether or not the catecholamine(s) in R. culicivorax function as neurotransmitters and/or neurosecretions; it demonstrated that the presence of these substance(s) within the nervous system was correlated with reproductive development.

4.2.1. Functions of catecholamine(s) in R. culicivorax

The experimental studies showed that the physical presence of the opposite sex stimulated the production of catecholamine(s) in the nerve ring ganglia of both the sexes

and in the tail ganglia of the adult males. In males, such increased catecholamine production was partially achieved, in the absence of physical contact with females, by chemical mediation between the sexes through the water. This suggests that the catecholamine(s) are produced to facilitate and/or as a consequence of copulatory behaviour. The possibility that catecholamine(s) may play a role in sex attraction deserves further investigation, especially in view of the finding in R. culicivorax that sex attractants of an undetermined nature are secreted by female nematodes to attract male nematodes (Hominick; personal communication). The production of sex attractants by female nematodes has been documented for a variety of nematode species (Bone, 1982; Green, 1980).

The observations that the concentration of catecholamine(s) declined progressively in the males during and after copulation suggests that the role of catecholamine(s) in males may be limited to sex attraction and the initial stages of copulatory behaviour. The presence of catecholaminergic ganglia in the tail of adult males and their connection with the caudal sensory papillae re-inforce the hypothesis that the catecholamine(s) may be functioning, at least in part, to regulate copulatory behaviour. In the case of females, the fluorescence intensity of catecholamine(s) remained approximately double that of post-parasitic juveniles, both during and after mating. This suggests that

catecholamine(s) could, directly or indirectly, be involved in regulating egg formation and/or oviposition, a suggestion that agrees with the finding that the exogenous application of biogenic amines altered egg laying in C. elegans (Horvitz et al., 1982; Croll, 1975).

The roles of catecholamine(s) in physiological or developmental processes of the nematode, other than reproduction, are not known. It has been suggested that aminergic neurons in the nerve ring of C. elegans (Sulston et al., 1975), P. punctatus (Wright and Awan, 1978) and P. redivivus (Sharpe and Atkinson, 1980) are concerned with mechanoreception, those associated with the sensilla of T. spiralis may be chemosensory (Lee and Ko, 1991) and those in the tail region of G. ulmi (Leach et al., 1987), P. redivivus, N. dubius (Sharpe and Atkinson, 1980) and N. battus (Sharpe et al., 1980) may play a role in the coordination of copulatory behaviour. It is likely that catecholamine(s) may be functioning in a similar fashion in R. culicivorax. The strong dopamine immunoreactivity in the amphids and GA-induced catecholaminergic staining in the cephalic papillary nerves suggests that the cephalic sensory organs (cephalic papillae and amphids) may be functioning through catecholamine(s) as mechanoreceptors or chemoreceptors.

4.3. Peptidergic Nervous System

Peptidergic neurosecretory cells are widely distributed

among different species of nematodes (Davey, 1988). Certain sites within the nervous system of R. culicivorax that produce a peptide with immunohistochemical staining properties of FMRF-amide are the same as those known to produce peptide neurosecretions in other nematode species. Based on histochemical and ultrastructural studies, it has been determined that peptidergic neurosecretory cells are located in the amphidial ganglia or amphidial nerves of A. lumbricoides (Gresch and Scheffel, 1958), D. immitis (Delves et al., 1989), D. viteae (McLaren, 1970) and Capillaria hepatica (Wright, 1974), in cephalic papillary ganglia, dorsal, ventral and lateral ganglia of A. lumbricoides (Davey, 1964; 1966) and P. decipiens (Davey, 1966; Goh, 1975) and in the ventral ganglia of H. contortus (Rogers, 1968). All of these sites were found to produce FMRF-amide-like peptide in R. culicivorax.

The finding that the nervous system of R. culicivorax produces a substance similar or identical to FMRF-amide, a peptide first isolated from the clam Macrocallista nimbosa (Price and Greenberg, 1977), is consistent with recent knowledge of neuropeptides in nematodes. Immunocytochemical studies have revealed the presence of a large number of neuropeptide , analogous to hormones of vertebrate and invertebrate origin, in the nervous system of several nematode species. In A. lumbricoides, such neuropeptides include

cholecystokinin octapeptide, gastrin-17, α-melanocyte stimulating hormone, calcitonin gene related peptide, corticotrophin releasing factor, vasoactive intestinal peptide, luteinizing hormone-releasing hormone, Aplysia peptides L11 and 12_B, and small cardioactive peptide B, (Sithigorngul et al., 1990). Cholecystokinin has been localized in the nervous system of C. elegans (McIntire and Horvitz, 1985), adrenocorticotrophic hormone in G. ulmi (Leach et al., 1987) and adipokinetic hormone in P. redivivus (Davenport et al., 1991).

FMRF-amide-like peptide immunoreactivity has been visualized in the major components of the nervous system of adults of A. suum (Davenport et al., 1988; Sithigorngul et al., 1990; Stretton et al, 1992), P. redivivus, C. elegans (Atkinson et al., 1988; Li and Chalfie, 1986) , G. ulmi (Leach et al., 1987), D. immitis and B. pahangi (Warbrick, et al., 1992). The present study has shown that FMRF-amide like peptide is widespread in the nervous system of parasitic as well as post-parasitic stages of R. culicivorax.

The distribution of this neuropeptide in the nerve ring, cephalic papillary nerves, cephalic papillary ganglia, longitudinal nerve cords and ganglia posteriorly attached to the nerve ring is the same as in A. suum (Davenport et al., 1988), D. immitis and B. pahangi (Warbrick et al., 1992). However, there were a few minor differences between R.

culicivorax and other nematodes in the location and position of the FMRF-amide immunoreactive neurons. In A. suum (Davenport et al., 1988), P. redivivus (Atkinson et al., 1988), D. immitis and B. pahangi (Warbrick, et al., 1992) the FMRF-amide positive ventral and lateral ganglia were separately attached to the posterior surface of the nerve ring. However, in R. culicivorax, ganglia posterior to the nerve ring that stained for FMRF-amide were grouped together in a single mass and jointly attached to the posterior surface of the nerve ring like a bunch of grapes.

In contrast to R. culicivorax, the amphids and amphidial nerves of A. suum (Davenport et al., 1988), P. redivivus and C. elegans (Atkinson et al., 1988) do not contain FMRF-amide-like peptide. Like R. culicivorax, FMRF-amide-like peptide was observed in the cell bodies of the ventral cord near the vulva of adult females of P. redivivus (Atkinson et al., 1988) and C. elegans (Li and Chalfie, 1986). However, in R. culicivorax, a network of ganglia and nerve processes was also observed near the female reproductive system. In R. culicivorax, the organization of the peptidergic nervous system in the tail region of females was different from that of males. Four clusters of peptidergic ganglia were present in the female tail tip, whereas only the ventral nerve cord in the tail region of males showed positive immunoreactivity to FMRF-amide. Like R. culicivorax, FMRF-amide-like peptide was

present in the tail ganglia of females of A. suum (Davenport et al., 1988), P. redivivus and C. elegans (Atkinson et al., 1988; Li and Chalfie, 1986). Unlike R. culicivorax, FMRF-amide positive neurons were visualized in the tail region of males of P. redivivus, C. elegans (Atkinson et al., 1988; Li and Chalfie, 1986) and A. suum (Davenport et al., 1988).

With respect to developmentally associated changes in neuropeptides, my findings for R. culicivorax agree, in general, with those of Li (1990), who reported that in C. elegans, as the nematode progressed through the four juvenile stages into the adult stage, more neurons and nerve processes containing FMRF-amide-like peptide became visible. However, Leach et al. (1987) did not observe any differences among the various developmental stages of G. ulmi in the distribution of FMRF-amide-like peptide within the nervous system. The incremental development of the peptidergic nervous system of R. culicivorax is similar to that of C. elegans (Li 1990). The main components of the peptidergic nervous system (nerve ring, ventral nerve cord, ventro-lateral nerve, ventro-lateral ganglia and several ganglia in the tail region) were visible in the four day old parasitic juvenile stages of R. culicivorax. In seven day old parasitic juveniles, FMRF-amide was also detected in eight cephalic papillary ganglia and associated cephalic nerves. Subsequently, in post-parasitic juveniles, several peptidergic ganglia posterior to the nerve

ring and one dorsal and four lateral nerve cords showed positive immuoreactivity to FMRF-amide. After the last molt to the adult stage, more FMRF-amide-like peptide containing nerves were present in the nerve ring, the number of peptidergic ganglia posterior to the nerve ring had increased and a network of ganglia and nerve processes became visible throughout the nematode body, especially near the reproductive system of females.

Although FMRF-amide-like peptide appears to be widely distributed in the nervous system of nematodes, its function is not known. Such peptides are known to have cardioregulatory roles in molluscs and leeches (Kuhlman et al., 1985; Lehman and Greenberg, 1987), stimulate contraction of muscle preparations of coelenterates (Anctil, 1987) and modify contraction of locust- skeletal muscle (Evans and Myers, 1986). It has been found that the FMRF-amide-like neuropeptides, AF1 (Lys-Asn-Glu-Phe-Ile-Arg-Phe-NH2) and AF2 (Lys-His-Glu-Tur-Leu-Arg-Phe-NH2), isolated from A. suum, inhibit locomotory movements when injected into intact nematodes, suggesting that the peptide(s) may be playing a role in nematode locomotion (Cowden et al. 1989; Cowden and Stretton, 1990). Thus, it is possible that FMRF-amide-like peptides may regulate muscular activity associated with locomotion in R. culicivorax. Further, in R. culicivorax, the presence of peptidergic ganglia near the female reproducti

system suggests that FMRF-amide-like peptide may be acting as a neurohormone and controlling egg production and/or oviposition. The manner by which a tetrapeptide such as FMRF amide may act is a matter of conjecture. Peptides are known to act as neuorosecretory substances in nematodes (Davey, 1988). However, it is also possible that the compound could function as a neurotransmitter, since neruopeptides are known neurotransmitters in many invertebrates (O'Shea and Schaffer, 1985; Withers, 1992).

4.4. Concluding Remarks

In conclusion, scanning electron microscope studies confirmed the presence of external sensory organs (amphids, cephalic and caudal sensory papillae) in the nematode. It is suggested that these organs function both as chemoreceptors and mechanoreceptors. The present study also shows that catecholamine(s) and FMRF-amide-like peptide(s) are widely distributed in the nervous system of R. culicivorax. In general, both the catecholamine(s) and the peptide became more widely distributed within the nervous system of R. culicivorax as the nematode developed. These compounds may be acting as neurotransmitters or neurohormones. The experimental evidence suggests that the catecholamine(s) may be involved in regulating copulatory behaviour, egg formation and/or oviposition.

Additional research is required to determine the precise

chemical nature of both the catecholamine(s) and FMRF-amide-like peptide and to more fully assess their role(s) in physiological and developmental processes of the nematode. A full understanding of the nature of the regulatory system(s) used by the nematode to control such processes constitutes important fundamental information, needed to devise an *in vitro* culture system that best simulates *in vivo* conditions.

V. SUMMARY

1. Scanning electron microscope studies demonstrated the presence of six cephalic sensory papillae (two lateral, two dorso-lateral and two ventro-lateral) and two amphids in the head tip of adult nematodes. In males, each lateral papilla contained two pores and each dorso-lateral and ventro lateral cephalic papilla contained three pores. Three pores were observed in all of the six cephalic papillae of females. The tail of males contained several caudal sensory papillae, each of which contained one pore. Caudal sensory papillae were absent in the tail of females.

2. The distribution of catecholaminergic and peptidergic components of the nervous system of the mermithid nematode, R. culicivorax, was determined by using GA-induced fluorescence and FMRF-amide-specific immunofluorescence techniques, respectively.

3. As R. culicivorax progressed through successive juvenile stages and into the adult stage, more catecholaminergic and peptidergic neurons became visible.

4. Both the FMRF-amide-like peptide and catecholamine(s) were observed in parasitic as well as post-parasitic stages. Catecholamine(s) was also present in the egg and pre-parasitic stages.

5. The catecholamine(s) was evident in the cephalic papillary

nerves, nerve ring, four ganglia in the nerve ring, ventral and dorsal nerve cords, mid-body region ganglia, two ganglia in the tail tip of adult females and 16-20 ganglia in the tail region of adult males.

6. Immunoreactivity to dopamine was observed only in the amphids, indicating that the catecholamine of wider distribution within the nervous system was not dopamine.

7. Experimental studies indicated that the physical presence of the opposite sex or chemical mediation between the sexes enhanced the intensity of catecholamines in the nervous system of adult males and females. The concentration of catecholamine(s) declined progressively in males during and after copulation, whereas in females it remained relatively constant during and after copulation. The findings suggest that the catecholamine(s) may be controlling copulatory behaviour, egg production and/or oviposition.

8. FMRF-amide-like peptide was present in the amphids, cephalic papillary nerves, cephalic papillary ganglia, nerve ring, ganglia posterior to the nerve ring, longitudinal nerve cords and mid-body region ganglia of male and female nematodes. Four clusters of peptidergic ganglia were observed in the tail tip of adult females. Such ganglia were absent in the tail of males.

9. Results are discussed with respect to the distribution of the catecholamines and FMRF-amide-like neuropeptide in the

nervous system of R. culicivorax and their roles as putative neurotransmitters and/or neurohormones in controlling development of the nematode.

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