A STRUCTURAL STUDY OF THE CEREBRAL GANGLIONIC COMPLEX AND RETROCEREBRAL SYSTEM IN THE ADULT OF CHIRONOMUS RIPARIUS

by

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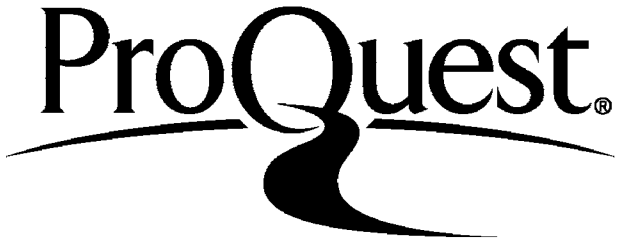

ABSTRACT

A Structural Study of the Cerebral Ganglionic Complex and Retrocerebral System in the adult of Chironomus riparius.

Descriptions are given of the cerebral ganglionic complex and retrocerebral system of neoimaginal Chironomus riparius reared at 20°C. Changes resulting from differences in age, rearing temperature and reproductive state are also presented.

The anatomy of the cerebral ganglia, including the organisation of neuropile masses, of male neoimagines, and the fine structure of the neural sheath, cortical layer and neuropile is described for the first time in an adult chironomid. Two types of perineurial cells and three types of glial cells are distinguished on ultrastructural criteria. A classification of five non-neurosecretory neuron types, based upon size, nuclear to cytoplasmic proportions and appearance, is given. Four paired groups of neurosecretory neurons were found in the brain and two paired groups in the suboesophageal ganglion. Five neurosecretory cell types are classified on the basis of their ultrastructural characteristics. The axon pathways of the cerebral neurosecretory cells and the elements of the retrocerebral system, including the paired corpora cardiaca, corpora allata, peritracheal tissues and "glandes post-cérébrales

antérieures", are described. The nervous and endocrine structures of the neoimaginal male are compared to those of the female.

Age related changes occurring within the brain and endocrine system are described in relation to fat body depletion, behavioural activity and appearance of the dorsal longitudinal flight muscles.

The effect that differences in rearing temperature have upon the longevity, size and weight of the adult is presented. The influence of rearing temperature upon the ultrastructure of the brain is described for the first time in an insect.

Evidence suggesting an association between the activity of the cerebral neurosecretory cells and female reproduction is given.

In the light of the results functional roles for several elements within the endocrine system are postulated. The possible significance that the observed structural changes in the nervous system have upon its functioning is discussed.

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ABBREVIATIONS

The following abbreviations are used:

GPCA - "Glandes post-cérébrales antérieures"

LNC - Lateral neurosecretory cells

MNC - Medial neurosecretory cells

NCA - Nervi corporis allati

NCC - Nervi corporis cardiaci

ONC - Outer neurosecretory cells

VNC - Ventral neurosecretory cells

SNC_1 - Suboesophageal ganglion neurosecretory cells (group I)

SNC_2 - Suboesophageal ganglion neurosecretory cells (group 2)

mm - millimetre (IO^{-3} metre)

µm - micrometre (IO^{-6} metre)

nm - nanometre (IO^{-9} metre)

CHAPTER I

INTRODUCTION

Much of the research upon dipterans is restricted to species of Cyclorrhapha which can be bred under laboratory conditions with minimal attention. Nematocerans, of which far more are of medical importance than cyclorrhaphans, tend to be much more difficult to culture. Chironomids are among the easiest of nematoceran species to maintain under laboratory conditions. Although they are not vectors of disease, their propinquity to mosquitoes makes them important in investigations into the biology of this suborder. Chironomids are important, not only as useful laboratory animals, but also as biological detectors of water quality (JANKOVIC, 1975), and as pests in areas where the adults occur in vast numbers (MULLA et al, 1971).

The ecology (review OLIVER, 1971) and salivary gland polytene chromosomes (review ASHBURNER, 1970) of chironomids, have been the subjects of numerous studies. There has also been some research on the physiology and behaviour of these midges but there is a paucity of structural observations, especially upon the nervous system. The larval

chironomid brain has been referred to by several authors (HOLMGREN, 1904; CREDLAND & PHILLIPS, 1974 and others) but that of the adult has received only brief mentions (MIALL & HAMOND, 1900). None of these papers describes the anatomy of the brain in depth. Detailed anatomical studies upon the dipteran brain, (with the exception of Aedes (CHRISTOPHERS, 1960),) have mainly been restricted to the cyclorrhaphans Drosophila (POWERS, 1943, 1946) and Musca (GROTH, 1971; STRAUSFELD, 1976). An anatomical analysis of the brain of Chironomus riparius would not only advance the knowledge of the nematoceran brain but could also form a useful foundation for ultrastructural observations.

There are few overall descriptions of the fine structure of the various elements within an insect brain, as most ultrastructural studies are restricted to specialized regions (review LANE, 1974). SOHAL et al (1972) have described the fine structure of the neural sheath, glia and neurons within the brain of Musca domestica but there have been no comparable studies on any other dipteran.

The anatomy of the endocrine system of several adult nematocerans have been described (BURGESS & REMPEL, 1966; MEOLA & LEA, 1972; TARGA, 1974 and others) but there have been no detailed observations upon an imaginal chironomid. The retrocerebral glands of larval Chironomus riparius (CREDLAND & PHILLIPS, 1974) were found to be unfused and it was thought that this would make physiological investigation

easier than upon the partially or completely fused glands of culicids or cyclorrhaphous dipterans (CAZAL, 1948; THOMSEN, 1951). If the adult C. riparius has a similar retrocerebral system to that of the larva, it would be ideally suited for structural investigations into the functional roles of the various glands. The adult's retrocerebral structures could be identified by analogy to those of the larva (CREDLAND & PHILLIPS, 1974) and of other insects (review SMITH, 1968). The identification of the cerebral neurosecretory neurons, integral elements of the endocrine system, would have to be based upon the criteria used in the recognition of such cells in insects.

The earliest definitions of neurosecretory cells were based largely upon their appearance after staining with Gomori stains (SCHARRER, 1954; BERN, 1962, 1966; GABE, 1966). The straightforward staining of cells with stains such as chrome haematoxylin-phloxine and paraldehyde fuchsin cannot be regarded as proof of their neurosecretory function (NORMANN, 1965; BERN, 1966; KNOWLES & BERN, 1966; DOANE, 1973). The converse is also true, that some neurosecretory cells do not show a selective response to these stains (BERN, 1962; THOMSEN, 1965). In recent years neurosecretory cells have been characterised on the basis of their ultrastructural features (BERN, 1966; MADDRELL, 1970). NORMANN (1965) contended that the quantity of granules within a neurosecretory cell must be sufficient

to indicate that their production is the major function of the cell. This is not a useful criterion for their recognition, as the interpretation of SCHARRER & BROWN (1961) implies that very active neurosecretory cells will contain only a small number of granules. MADDRELL (1974) considered that in its broadest meaning all neurons are "neurosecretory" as they all synthesize specific substances. He chose to restrict the term to those neurons which release the substance at some distance from the target cells. This, however, presupposes that the functional significance of these neurons, which in the majority of cases has not yet been elucidated, is already known. For conclusive statements to be made about the neurosecretory nature of any cell it has to be demonstrated that the substance they synthesize has a measurable physiological activity (BERN, 1962; MORRIS & STEEL, 1975). In a structural study, using the electron microscope as the primary tool for investigation, a useful and simple way to distinguish a neurosecretory neuron is by finding similar granules in the cells' perikarya and in their axons (LANE, 1974). Additional evidence for the neurosecretory nature of such cells can be gained by tracing the axons to neurohaemal areas which are known to contain swollen nerve endings, lack glial investment and show evidence of membrane recapture (MADDRELL, 1974).

The cerebral neurosecretory system of the larval Chironomus riparius (CREDLAND & PHILLIPS, 1974) was

thought to comprise of a group of paired medial cells. The comparative simplicity of this system, if it occurred in the adult, would facilitate functional analysis. Correlated ultrastructural and cytological observations upon the cerebral neurosecretory cells of imaginal chironomids have not previously been reported, as such studies on adult dipterans have been confined almost exclusively to Calliphora (THOMSEN, 1965; BLOCH et al, 1966), Musca (RAMADE, 1966, 1969) and culicids (BURGESS & REMPEL, 1966; MEOLA & LEA, 1972).

A previous study on Chironomus riparius (CREDLAND, 1971) showed that the temperature of the preimaginal aquatic environment had a marked effect upon the adult's body size and weight. Within limits, a lower temperature was found to produce a larger adult. Although such changes are well known in insects (CLARKE, 1967), the related structural differences in the nervous and endocrine systems have not been investigated.

Age related changes are known to occur in the neurons of several cyclorrhaphous dipterans (review ROCKSTEIN & MIQUEL, 1973) and also within the cerebral neurosecretory system of Musca (ROCKSTEIN et al, 1971). There have been no reports on age related changes in the nervous and endocrine systems of nematocerans based upon ultrastructural observations. Several Cyclorrhapha, such as Calliphora, Drosophila and Musca, are obtainable in large numbers as

genetically pure strains whose emergence can be synchronised. This makes these flies very popular for use in ageing studies. The inbreeding of a laboratory culture of Chironomus riparius, originating from a single egg rope, for over five years, makes it reasonable to assume that individuals would exhibit a considerable genetic homogeneity. Although midges are not available in such large numbers, nor can their emergence be synchronised, they have the advantage over Musca, Calliphora and Drosophila in having a briefer life span when kept under similar conditions, so that experiments can be replicated in shorter periods. Chironomids also have the advantage of not feeding as adults, meaning that nutritional influences, which are known to be important factors in modifying longevity (review ROCKSTEIN & MIQUEL, 1973), can be ignored. Most insect tissues are composed of specialised systems of post-mitotic cells, so that structural changes can be age related. The insect brain comprises such cells, and is particularly suitable for ageing studies as it lacks blood vessels, so that changes resulting from vascular pathology which occur in mammals can be excluded.

There is little evidence to suggest that the spermatogenesis in insects is under the control of the cerebral neurosecretory system. Some neurosecretory cells of the pars intercerebralis of the female are, however, thought to influence the fecundity of Calliphora (THOMSEN, 1948, 1952), culicids (LEA, 1967) and several other dipterans (review ENGELMANN, 1970). The

neurosecretory cells in other regions of the brain have also been suggested to influence egg maturation in Calliphora (THOMSEN, 1952) and in locusts (STRONG, 1965). These reproductive influences are associated with activity differences in the neurosecretory cells, which are reflected in their perikaryal contents and especially in the amount of neurosecretory granules they contain (ENGELMANN, 1970). The cerebral neurosecretory system of the imaginal female midge therefore seems to lend itself to a comparative ultrastructural study related to the reproductive state of the specimen.

Structural studies on the nervous and endocrine systems can give anatomical bases for considering their functional roles. Additional information can be obtained by investigating the changes in microscopical appearance occurring in these systems resulting from differences in the animal's physiological state. It is the structure of these controlling systems and their changes in microscopical appearance resulting from differences in age, rearing temperature and reproductive state which have been investigated and are reported herein.

CHAPTER 2

MATERIALS AND METHODS

A. The Experimental Animals

1. The permanent cultures

The source of all the specimens in this study were the permanent cultures of Chironomus riparius maintained in the animal room at Bedford College. The animals are kept at 20 $\pm$ 2°C with a lighting regime providing 12 hours light each day in a regular cycle. They are maintained using the technique of CREDLAND (1973). These cultures were started from a single egg-rope taken from the ornamental pond in the grounds of "The Holme", Regent's Park in the summer of 1970. Over this period there has been no significant change in the external characters exhibited by this laboratory strain as compared with the wild type, when both are raised at a similar ambient temperature (CREDLAND, personal communication).

2. Incubation of Specimens

For the experiments, specimens were reared in "Griffin cooled" and "Gallenkamp illuminated cooled"

incubators which provided some control of environmental variables.

(a) Incubation at 20°C

Two egg ropes which had been laid in the previous 18 hours were removed from the permanent cultures and placed in a plastic tank, 30 x 10 x 15 centimetres, filled with tap water at 20°C. Filter paper and washed filamentous algae were introduced to provide tube building material for the larvae. Ground "Tetra Min" was used as a food source, the specimens being reared as described by CREDLAND (1973). Several tanks prepared in this way were placed in an incubator at 20 ± 1°C with a controlled lighting regime providing 14 hours light each day in a regular cycle. The water level was maintained to within 2 centimetres of the plastic tank's cover, restricting the vertical space, which was found by DOWNE (1973) to prevent swarming and mating. Aeration was provided by a central air brick and two outer narrow gauge air tubes.

Adults emerging in the first hour of the photophase were collected using an aspirator and transferred without etherization to individual specimen tubes which were then covered with nylon gauze. The sex of the specimen was determined by the appearance of the antennae, which are sexually dimorphic, being longer in the male (CHINERY, 1973). These tubes were then returned to the incubator, and a

drop of water placed on the gauze. The midges were kept in the incubator for 7 hours to allow recovery from emergence. The internal space of the incubator remained at 70% relative humidity, but it is likely that because of evaporation of the water drop, the humidity within the specimen tubes was much higher.

(b) Incubation at 10 and 15°C

Egg-ropes were obtained and put into prepared tubes as described above. The tanks were then cooled at a rate of just over 1°C per hour by gradually changing the thermostat settings of the incubator, until the required temperature was reached. Transferring the egg-ropes directly from 20°C to the lower incubation temperature resulted in few, or no larvae hatching. The emerging imagines were isolated for 7 hours, as described above, at the temperature of incubation.

3. Ageing of Specimens

Emerging imagines were collected and isolated in specimen tubes as described above. These specimens were returned to the incubators in which they were reared. Frequent periodic checks were made to replace the drop of water upon the gauze, and so ensure a constant high level of humidity, which affects the longevity of Chironomus (HILSENHOFF, 1966). At the same time, observations upon the resting position of each specimen were made and

it was also ascertained whether the midge was flight immobile, i.e. tactile stimulation applied by tapping the tube would not elicit flight, or dead, i.e. when no intrinsic movement occurred even when stimulated. From these results life tables were constructed for both sexes. Some living specimens were removed for fixation between the sixth and eighth hour of the photophase after 1, 3, 5 and 7 days of age. It was recorded if these specimens were flight immobile.

Imagines reared and kept at 10, 15 and 20°C were treated in this way. Some emerging males reared at 20°C were isolated and transferred to an incubator at 10°C. The converse experiment was also performed, males reared at 10°C were kept as imagines at 20°C. The longevity of these two groups of males was recorded.

4. The Reproductive state of the Specimens

(a) Mated, oviposited and virgin females

In this group of experiments, midges reared in the "no-swarm" tanks at 20°C, similar to those of CASPARY & DOWNE (1971), were used. Imagines were collected in the first 14 hours after emergence, the last two hours of which were in the photophase. One female and four males were transferred into transparent plastic containers, 30 x 10 x 15 centimetres, containing tap water to a depth of 2 centimetres and filter paper to act as swarm markers (DOWNE & CASPARY, 1973) and oviposition sites

(CREDLAND, 1973). It has been shown by CASPARY & DOWNE (1971) that swarming is essential for mating in laboratory cultures of C. riparius. The tanks were covered with blue tissue paper, except for a small observation hole, and disturbance was kept to a minimum so that swarming was not prevented. The prolonged intermittent observations during daylight hours, to ascertain when mating had ocurred, necessitated that the tanks be kept upon a laboratory bench. Variations in temperature in the laboratory were in the range 18 to 25°C, with an approximately 12 hour photophase per 24 in a regular cycle. The males were removed from the mating tanks at night and four males were returned in the morning if oviposition had not occurred overnight. The period between copulation and oviposition would mean that mated females would be retained in the observation tanks until the egg-ropes had been laid, but as they are monogamous (DOWNE, 1973) mating would not recur. Females were removed from the tanks as soon as mating was observed and kept in an aspirator for 3 hours. The brain and abdomen were then prepared for electron microscope observations and a histological investigation respectively. Fixation was carried out at about the middle of the photophase.

In females where oviposition occurred, without the observation of mating, the brain and retrocerebral complex

were prepared for electron microscope observation and the abdomen for a histological investigation of the ovaries. Fixation was carried out three to seventeen hours after oviposition in the middle of the photophase. The egg-ropes were kept for observation.

Virgin females of similar age to the mated and oviposited specimens were used for comparisons. These individuals were prepared as described above in section A.3.

(b) Ligated and sham operated females

Female imagines in the first 14 hours after emergence were lightly etherized in an aspirator and transferred to the stage of a dissecting microscope. Using a fine hair, a ligature was tied between the tergites of the first and second abdominal segments. Pekinese dog hair was found to be particularly suitable, as it is supple enough to tie a small knot without breaking. Other females were treated in a similar way, except that the ligature was tied using a slip knot which was removed after approximately 15 minutes. The sham operated and ligated females were kept in individual tubes for either 3 or 4 days in an incubator at 20°C as described above in section A.2. After this period, the brain was prepared for electron microscope observations. The abdomen was removed for a histological investigation. The anterior one third was subsequently sectioned in the longitudinal plane to observe the effectiveness of the ligation, whereas the posterior region was cut in transverse section to study the ovaries. Fixation was again carried

out in the middle of the photophase.

B. Light Microscope Procedures

1. Fixation, Dehydration and Embedding

Fixation of specimens was carried out in the middle of the photophase, so that if a circadian cycle occurred in the neurosecretory cells, as reported in other insects (BRADY, 1974) all the animals used would be in a similar phase of the cycle. Before fixation the midges were lightly etherized and the legs and wings removed. The body was then dipped into dilute detergent and quickly rinsed in distilled water, this removed the hydrophobic properties of the cuticle and ensured submergence in the fixative. Two techniques of fixation were employed, that of MEOLA (1970) and of ROCKSTEIN et al (1971). These differ only in the type of Bouin's fixative used and subsequent storage in alcohol. In all cases they were found to give identical results with the stains employed. The specimens were dehydrated in a graduated series of ethanols. If the yellow colour of the fixative was not removed by the end of the 70% alcohol stage, the specimen was bleached in 2.5% aqueous sodium thiosulphate and redehydrated, as described by PANTIN (1964). The specimens were cleared in butanol, gradually infiltrated with Ester 19 47 wax and finally embedded in this wax, using a modified form of ROCKSTEIN

et al's (1971) procedure. Butanol was thought to soften the specimens more than xylene. This, coupled with the use of ester wax, which is much harder than paraffin wax of similar melting point, minimized sectioning difficulties caused by the cuticle. Great care was taken in block making, to minimise air infiltration using the technique described by STEEDMAN (1947). Details of the timings for each stage of the procedure are given in the appendix.

2. Whole Mounts of the Head Capsule

The heads of specimens within the first day as imagines were removed, fixed and dehydrated as described above. The tissue was cleared for several days in xylene at 50°C, which was found to give better definition of the brain than butanol. The capsule was viewed in the clearing agent under a binocular microscope. The brain was found to be visible when a combination of transmitted and incident light was used.

3. Section Cutting and Mounting

The blocks, which are brittle, were trimmed using the procedure described by STEEDMAN (1947). Sections were cut at a 6 um thickness on a motor driven "Jung rotary microtome" with the knife angle set at 5°. Both the block and the knife were heated by a 40 watt electric light bulb positioned between 5 and 10 centimetres from

the knife edge. Heating was commenced at least 15 minutes before sectioning and was found to facilitate ribbon formation and minimise fracturing.

Sections were mounted upon albuminized slides as described by PANTIN (1964). All the slides were precleaned by storage in 70% alcohol containing 1-2% glacial acetic acid. To ensure adequate adhesion of the ribbons to the slide, the slides were dried for at least 18 hours in an oven at 38°C before dewaxing.

4. Staining Methods

The preliminary procedures of dewaxing and hydration were carried out as described by GURR (1962) before staining.

(a) Mallory's trichrome

Mallory's trichrome stain was used for a routine histological investigation. This stain, which is quick and easy to use, was found to give consistent results with good definition. The method outlined by PANTIN (1964) was used, optimum results being obtained by the staining times of CREDLAND (1971). Fading of the colour of this stain reported by PANTIN (1964) was not apparent after two and a half years. The neutral colourless mountant DPX which was used in all cases, is reported (GURR, 1956) not to cause fading of stains.

(b) Ewen's aldehyde fuchsin

Aldehyde fuchsin has commonly been utilized to detect the

presence of neurosecretion in insect tissues (GABE, 1966), which it typically stains with an intense purple colour. In 1962, EWEN, developed a technique using this stain which provides a better colour differentiation of insectan neurosecretory products.

The sections were always stained within 48 hours of being cut, to prevent auto-oxidation by air reported by EWEN (1962). The stain was prepared and used according to his description, with slight variations in the times of some of the staining baths, details of which are given in the appendix, to give optimal definition of neurosecretory material. Some specimens were not oxidised in acid permanganate, which is assumed to act as a control of the specificity of the stain (GABE, 1966). The temperature of the staining baths was maintained at $22 \pm 2^{\circ}C$, and the timings rigidly adhered to, so as to minimise variations between batches of slides stained at different times. DPX was again used as a mounting agent and not Canada balsam as in EWEN'S (1962) procedure. This stain was found to give good definition of neurosecretory material and was therefore used in comparative studies.

(c) Meola's paraldehyde fuchsin

A sensitive aldehyde fuchsin technique was developed by MEOLA (1970) for the neurosecretory system of mosquitoes. It was thought that, as in mosquitoes, this technique would give more reliable results than obtained by EWEN'S (1962).

The stain was prepared in the dry form according to the method of ROSA (1953). Difficulty was encountered in

obtaining enough precipitate for use in histological staining. The amount of precipitate obtained was less than 30% of the expected dry weight for the quantities used. The staining was carried out according to the procedure of MEOLA (1970), except that DPX and not permount was used as a mounting media. The stain failed to give any definition of neurosecretion in the material examined and was therefore not employed in comparable studies.

(d) Chrome haematoxylin-phloxin

Another Gomori stain which is commonly used to detect neurosecretion in insect cells is chrome haematoxylin-phloxin (GABE, 1966).

The sections were refixed and stained using the procedure of GABE (1966). The results were found to be unreliable and not comparable to EWEN'S (1962) aldehyde fuchsin as usually reported (GABE, 1966). For this reason the stain was not used in comparative studies.

(e) Alcian blue

A non-Gomori stain which has been used to detect neurosecretory materials in insects (GABE, 1966) is alcian blue. This stain was primarily employed to detect neurosecretory cell groups found by electron microscopy but which could not be distinguished by the Gomori stains. This stain was prepared as described by PEARSE (1953) and used in conjunction with periodic acid Schiff's test, details of which are given in Appendix 1.

(f) Heidenhain's Azan stain

Heidenhain's Azan stain was similarly used to detect

neurosecretory cells not distinguished by the Gomori stains. It was thought that the colour distinction of cytoplasmic granules of red, yellow or blue, given by the stain would make it particularly suitable for distinguishing neurosecretory cell types. The stain was prepared and used according to the procedures described by GURR (1958). It was found not to distinguish neurosecretion but it gave by far the clearest definition of neuropilar masses as compared with the other stains employed.

5. Optical Photomicroscopy

A Zeiss photomicroscope was used for taking the optical photomicrographs. The best definition of stained neurosecretory material for monochrome studies was obtained using preobject yellow filters. Pan F film was employed and developed in "Microphen".

For colour studies Kodak High Speed Extachrome, tungsten, reversal film was used, employing a preobject neutral density filter. The prints presented in this thesis were produced from slides by Max Spielmann Ltd., Birmingham and show a distinct blue bias.

C. Electron Microscope Procedures

1. Fixation and Dehydration

As for light microscopy, the specimens were fixed in the middle of the photophase. The brains, anterior

ventral nerve cords and retrocerebral complexes were removed whilst being viewed by a binocular microscope using tungsten needles sharpened electrolytically. The structures were dissected whilst immersed in cold 3% glutaraldehyde in Sörensen's phosphate buffer, made up according to DAWES' (1971) to give a 0.1 molar solution with a pH of 7.2. This molarity was found to give the best preservation of organelle structure. Fixation was continued in a fresh quantity of the same solution for 2 hours, washed overnight in the buffer, then post-fixed for 1 hour in 1% osmic acid in the same buffer. Dehydration was accomplished using a modified ethanol-propylene oxide schedule of DAWES' (1971). The tissue was stained in 1% uranyl acetate in absolute ethanol during dehydration, this solution was gradually allowed to reach room temperature. All steps prior to this stage were conducted at 4°C to prevent excess lipid extraction. Isolated systems were subsequently embedded in LUFT (1961) epon.

The development by SPURR (1969) of a low viscosity resin has enabled tissue containing hard components, such as cuticle, to be prepared for electron microscopy using conventional techniques. This resin was used to study the cephalic nervous system and retrocerebral complex *in situ*. Midges were lightly etherized, dipped in a dilute solution of detergent and then quickly

rinsed in buffer. The antennae, legs, wings and meta-thorax were removed whilst the specimen was immersed in the cold glutaraldehyde solution. Several small holes were made in the cuticle to facilitate solution penetration. The head and anterior thorax were fixed and dehydrated as described above, except that staining with uranyl acetate during dehydration was omitted, as this was found to cause fine particulate dirt in the tissue.

Details of fixation and dehydration are given in the appendix.

2. Infiltration and Embedding

(a) LUFT'S (1961) Epon

The proportions of the plastics components were adjusted to produce blocks of medium hardness. Details of this, together with infiltration and embedding are given by DAWES (1971).

(b) SPURR'S (1969) Epon

A minimal amount of the plasticizer (flexibilizer), DER 736, was used in the resin mixture to produce very hard blocks. Oven dry capsules were used for embedding to prevent any uptake of water which is reported by SPURR to cause the castings to become brittle. Infiltration and embedding followed the procedure described by DAWES (1971).

It was found that less cytoplasmic detail was retained when SPURR'S resin was used as the embedding media. For this reason descriptions of cytoplasmic components

are all based upon tissue embedded in LUFT'S resin, except for the peritracheal tissue which was only observed in blocks of the whole prothorax.

3. Ultramicrotomy

The blocks were trimmed to the smallest trapezoid size possible without producing compression in the tissue. The large size of the block face and the hardness of the resin when cutting whole heads often caused "chatter" on the sections. This could sometimes be overcome by using slow cutting speeds and frequently changing the cutting edge of the knife. If this was unsuccessful the block was retrimmed removing as much cuticle as possible. A Cambridge "Huxley" automatic ultramicrotome was used with glass knives usually set at an angle of 7°. Sections of Luft's epon were expanded using either trichloroethylene or chloroform, for Spurr's epon chloroform was found to be the most effective agent. Sections producing a silver interference colour, after expansion, were collected on 200 mesh uncoated grids, or 100 mesh grids coated with formvar or formvar and carbon. Sections giving a white (grey) interference colour were found to be too thin to give sufficient contrast for analysis. Intermittently, sections of approximately 0.5 or 1.0 um thickness were taken for comparative optical microscopic examination.

4. Staining epon Sections

(a) Thin sections

The low intrinsic electron scattering power of biological material is enhanced by the use of heavy metals. The osmium used in the fixation procedure does not produce sufficient contrast in the specimen image for photographing. For Luft's epon to increase contrast 1% uranyl acetate was used in dehydration, described above. The sections were subsequently stained with REYNOLD'S (1963) lead hydroxide chelated with citrate, for four minutes at room temperature, using the general staining procedure of DAWES (1971). Using lead citrate after uranyl acetate gives an added effect, the uranyl acetate acting as a mordant for the lead stain. Obtaining sufficient contrast in thin sections of Spurr's resin proved to be difficult. The problem was overcome by staining the sections for two hours in 2% aqueous uranyl acetate, thoroughly washing the grids in boiled distilled water and then staining with lead citrate for twenty minutes, all steps being carried out at room temperature. Prolonging the staining times further produced a minimal increase in contrast but a great increase in crystalline "dirt" on the sections. The contrast obtained was still not as great as for Luft's epon, and possibly results from the differing hydrophilic properties of the resins. Omission of uranyl acetate staining, with both plastics greatly reduced the electron opacity of neurosecretory granules.

(b) Thick Sections

For correlated optical microscope studies, thick sections of approximately 0.5 to 1.0 µm were transferred from the boat to a clean slide using an aluminium foil ring. To ensure adequate adhesion, the sections upon the slides were air dried for at least half an hour. The sections were then stained with 0.1% toluidene blue in 1% borax solution as described by PEASE (1964).

PANOV and MELNIKOVA (1974) used p-phenylene-diamine for distinguishing neurosecretory neurons in epon sections. Details of the staining procedure were, unfortunately, not given. The most successful results were obtained using 2.5% of the diamine in borax solution, and heating the slide as for toluidene blue. This stained the tissue light brown and some neurosecretory material brown.

A direct comparison was made between the electron microscope appearance of cells in thin sections with the same cells in subsequent thick sections stained with EWEN'S (1962) aldehyde fuchsin, as described above. Thick sections were transferred to slides as described above. After thorough drying the material was "deresinized" using the technique developed by LANE (1965). The saturated alcoholic sodium hydroxide solution was found to take two weeks to mature, not three days as expected. Details of the procedure are given in the appendix. It was found that staining sections of 2 µm gave a more intense colouration than

in thinner sections. Halmi's counterstain was always completely removed from the tissue by differentiation in acetic alcohol, even when only briefly dipped in this solution. It is therefore assumed that the counterstain may be omitted without any change to the end result.

5. Electron Microscopes

The majority of grids were examined and photographed in an A.E.I. Corinth 275 electron microscope. The remainder were viewed in an A.E.I. 6B microscope, with the acceleration voltage at 60 KV.

D. Direct Measurements

1. Dimensions

Neoimagines were caught in an aspirator, etherized and then transferred to the stage of a dissecting microscope. The total length, excluding the antennal apparatus, and the widest parameter of the head were measured by the use of a calibrated eye-piece graticule. The width of the head capsule represents the width of the brain plus the ommatidia of the compound eye. As the ommatidia are but a small percentage of this width, any difference in head capsule size would be reflected by a similar difference in the brain, even if there were quite large discrepancies in ommatidia sizes. This was thought to give a quick and a more accurate measure than could be gained from wax sections because of the shrinkage caused in preparation

and the difficulty in obtaining truly transverse sections.

2. Weight

The same neoimagines whose dimensions had been obtained were transferred onto preweighed filter papers whilst etherized. The specimens were weighed to the nearest 10^{-5} gram on a "Sartorius analytical" balance with an accuracy of 10^{-5}gram.

These specimens were not used for further experimentation as the effect of prolonged etherization and disturbance may have affected the endocrine system.

E. Analysis of Microscope Data

1. Light Microscope

(a) Size Calculations

Actual sizes of tissue components on microscope slides were obtained by using a calibrated graticule in the eyepiece field stop of the microscope. Sizes of structures on micrographs were measured by comparison with a 2 millimetre, twohundred part, calibration slide photographed at the same magnification and enlarged to the same extent as the specimen.

(b) Diagrammatic Representations of the Cerebral Ganglionic Complex and Retrocerebral Structures

Representations of the cortex and neuropile masses of the cerebral complex, and of the retrocerebral structures were made by tracing projected images of serial sections on a

"Gilbert and Sibert Conference Microscope". Facilitated by comparisons with micrographs, representations of the spatial arrangements were constructed in diagrammatic form.

(c) Amounts of Neurosecretory Material

The number of cells containing selective stained material with EWEN'S (1962) aldehyde fuchsin, in the ventral and medial neurosecretory cell groups in both sides of the brain were counted. A subjective assessment of the relative amount of neurosecretion based upon the amount of selective staining of the cells was then given. A rating of between 0 and 4 was given, by comparison with two reference slides which contained a detectable amount, rated 1, or was packed with neurosecretory material, rated 4. The rating was based upon the stain uptake of the majority of the cells. The product of the number of neurosecretory cells and the rating gave an index reflecting the total amount of neurosecretion in these groups within the brain.

A subjective relative assessment rated as 0, + or ++ was given for the axons of the medial neurosecretory cells, the corpora cardiaca and the peripheral neurosecretory cells of the prothorax.

(d) Population Density of Neurons

Population density counts of neurons were based upon the sum of the number of nuclei often counts included in a 20 μm^2 circle. This circle, which was in fact the spot

photometer on a Zeiss microscope, was further divided by an eye-piece graticule to facilitate counting. Ten counts of the cortical region of a number of sections of each brain stained with aldehyde fuchsin were made. Only neurons of the cerebral lobes were counted, not those of the optic lobes which are much smaller and have a greater density. Nuclei bisected by the circle, but with the majority of their mass lying within it were included in the count. Nuclei of some glial cells would also be counted, as at the light microscope they are difficult to distinguish from neurons. This would be true of every brain, the inclusion of glial cells in the count should be cancelled out in comparisons as the glial to neuron ratio appeared to be constant. It must be noted that brain cells are always uninuclear. The field density number, which is the sum of the ten counts, represents the size of the internuclear space, and is not a true measure of the total number of neurons in the brain as intimated by many authors (SOHAL & SHARMA, 1972; ROCKSTEIN & MIQUEL, 1973 and others), although it may reflect it. A decrease in field density can result from an increase in the volume of cytoplasm and/or the volume of extracellular spaces, or a reduction in the number of nuclei caused by cell death. It is concluded that for such measurements to be meaningful, they must be coupled with ultrastructural observations.

(e) Ovary Assessment

The size of five of the largest oocytes and their nurse cells, per specimen, in wax sections were measured by a calibrated graticule. These oocytes are the most developed in a specimen, being in a terminal position in the ovary. Their mean size can be compared in different specimens to give some indication of the reproductive state of the animal. An assessment was also made of the relative amount of yolk droplets in these terminal oocytes as compared with the amount in neoimaginal females.

2. Electron Microscope

(a) Size Calculations

Measurement of the sizes of various components was calculated from the product of the magnification of the negative and the subsequent enlargement of that negative in the resultant print. The true magnification of the negative was found by comparison with micrographs of graticule grids, which give a more accurate result than that given by the manufacturer's stated magnifications.

(b) Neurosecretory Granule Populations

Prints of neurosecretory cells of known magnification were divided into 1 centimetre squares using a felt-tip pen to facilitate measurement. The size of each granule was then measured using a x 10 magnifier with an incorporated graticule. From this result the actual size of each granule was calculated. Several photographs of each

cell, taken at various sectional depths at a total magnification in the order of 25,000, were used. Between 150 and 600 granules per cell were measured, except in exceptional circumstances when very low numbers of granules were present. From these results, the average of the true mean diameters of the granules within the various cell types could be calculated as described below.

(c) Relative amounts of Neurosecretory Material

A subjective method was used to assess the relative quantity of neurosecretory granules in each cell. A typical representative of each type of neurosecretory cell from a group was chosen. Micrographs were arranged by a subject in the order of the total amount of secretion present. The subject was asked to take into account both the size of the granules and the numbers, but to disregard any density differences. Having so ordered the photographs, a rating of between 1 and 10 was given to each cell. In this way a comparison of the amount of neurosecretion present within different cells within a single brain, and of the same cells in different brains was made. The merit of this method is discussed in Chapter 7.

(d) Nuclear to cytoplasmic ratios

Nuclear and cytoplasmic ratios of various cells were found from enlarged prints by employing an "Allbrit" disc planimeter. The outline of the plasma membrane and nuclear membrane was traced using the instrument. Mean

values were calculated for each cell type based upon the measurement of several cells. Ten repetitions were carried out upon one micrograph, from which it was calculated that the instrument was accurate to within $\pm$ 3%.

F. Mathematical Methods

1. Calculation of True Mean Diameters

The diameter of a granule in a micrograph, because of sectioning, may only represent a fraction of the true diameter. Calculations of means are particularly affected when the diameter of the granule is the same order of size as the section thickness, which is true of neurosecretory granules. To compensate for this, the formula of FROESCH (1973) was employed, which calculates the true diameter from the apparent mean diameter and the section thickness. Silver sections were cut, which according to PEASE (1964) are 60 to 90 nm in thickness. No account was taken for the shrinkage of section width which has been reported to occur on exposure to the electron beam (BENNETT, 1974).

2. Life Tables

Life tables were constructed as outlined in PEARL (1940).

3. Statistical Methods

Standard statistical methods were employed to test

the significance of the results. The analysis of data was carried out using the formulae and tables in BAILEY (1959) and MORONEY (1951). As no confidence limits could be set and because of the nature of the subjective assessment of amounts of neurosecretion, statistical analysis could not be applied to these results.

CHAPTER 3

OBSERVATIONS UPON NEOIMAGINES REARED AT 20°C

INTRODUCTION

Section I of this chapter deals with male specimens reared at 20°C which have been fixed in the first day after emergence. A description of the gross morphology of the brain based upon light microscopy is followed by the fine structural details of its various elements. The anterior region of the ventral nerve cord is similarly described in terms of light and electron microscope data. This is followed by observations on the endocrine and stomatogastric systems. Finally in this section, a description of a group of heterogeneous neurosecretory structures is given.

Section II is devoted to observations on neoimaginal females reared under the same conditions as the males in Section I. A comparison with those observations on the male is made, to avoid repetition of the details in the previous section.

Whenever possible, the results have been condensed into figures or tables, when this does not omit important details. The results are detailed in the appendix, and a résumé only is presented whenever necessary in the text. The implications of these results are discussed in Section I of Chapter 7.

SECTION I

THE MALE

A. The Brain

1. Light Microscope Observations

(a) Gross Morphology

Figures 1 and 2 show the position of the brain within the head capsule based upon cleared preparations. The brain lies midway between the front and back of the capsule and is equidistant from the top and the bottom. The ommatidia converge on each lateral and laterodorsal extremity of the brain and are joined to the ganglionic plates of the optic lobes. Anteriorly, a large nerve connects each side of the brain to the ipsilateral antennal apparatus.

The brain of the neoimagine, (Plates 1 and 2), like that of Aedes ægypti (CHRISTOPHERS, 1960) consists of supra-oesophageal ganglion, and two cerebral crurae which serve as connections to the composite suboesophageal ganglion. External to the supra-oesophageal ganglion per se, and not conspicuously separated from it, are the large optic lobes. A narrow tunnel in the midline of the brain accommodates the foregut, aorta and recurrent nerve.

The supra-oesophageal ganglion of an insect is often divided into the protocerebrum, the deutocerebrum and the tritocerebrum. The delimitations of these regions are not

LIGHT MICROGRAPHS

The cerebral ganglionic complex of the neoimaginal male (20°C)

Plate 1 : Medial sagittal section of the head.

Plate 2 : Transverse section of the head.

Plate 3 : Transverse section of the supraoesophageal ganglion.

The micrographs show the situation of the ganglia within the head capsule and the relative positions of several of the neuropile masses.

KEY:

a - aorta

c - central body of the central complex

e - ellipsoid body of the central complex

f - fat body

g - gut

h - haemolymph

n - NNC

o - ommatidia

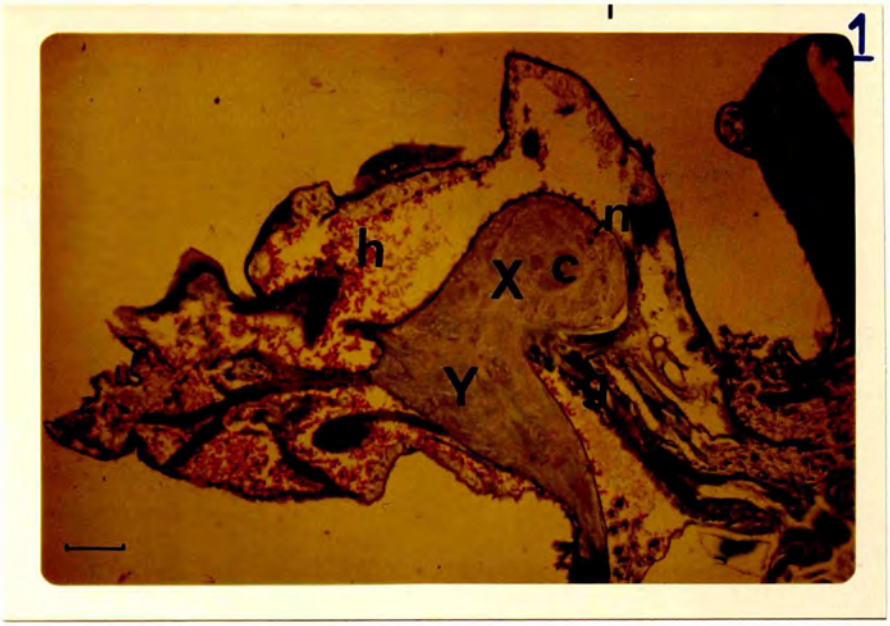
X - supraoesophageal ganglion

Y - suboesophageal ganlion

Arabic numerals refer to those used in the text for the neuropile masses.

SCALE BARS : 50 µm

(Stain: Ewen's aldehyde fuchsin)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n
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2
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10
a


3
a

Figure 1. Frontal view.

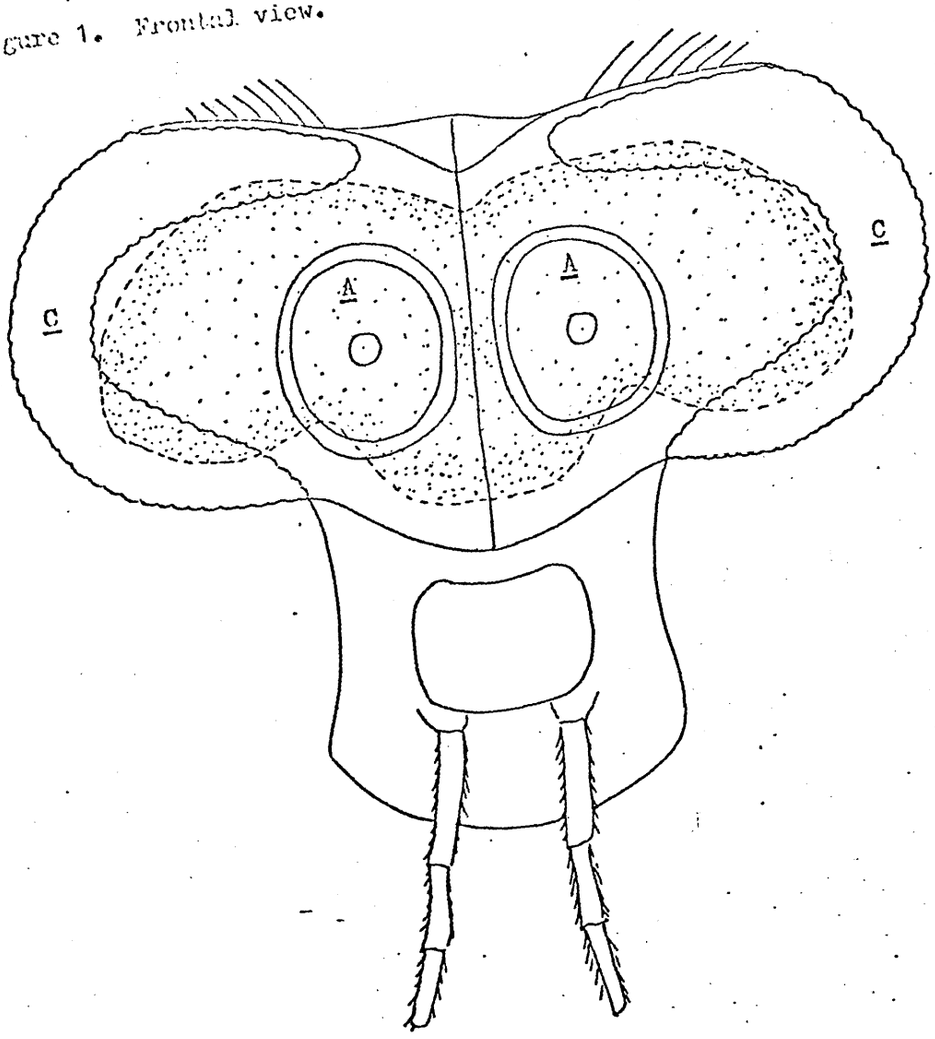
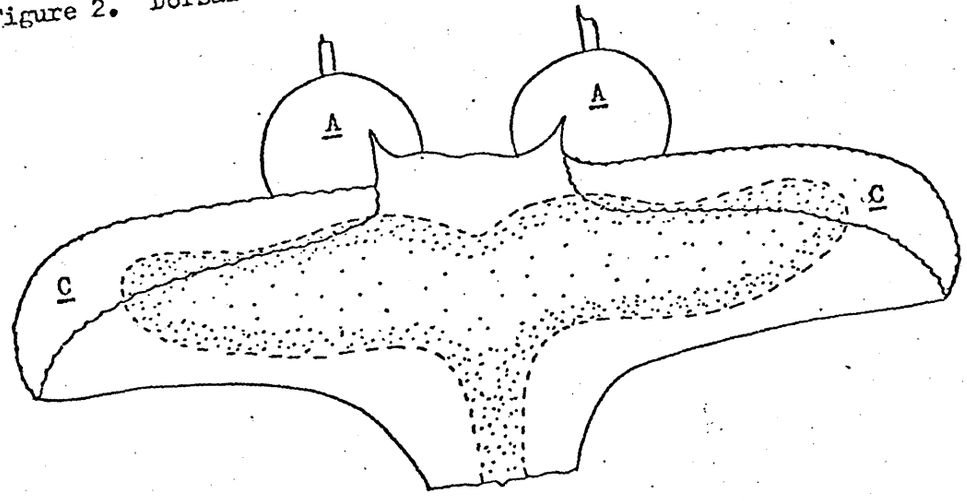


Figure 2. Dorsal view.



Diagrams showing the position of the cerebral ganglionic complex (stippled) relative to the compound eyes (C) and antennal apparatuses (A) in cleared preparations of the head capsule.

clearly differentiated in the brain of the adult. These three regions, adopting the terminology of CHRISTOPHERS (1960) are conveniently grouped and referred to as the cerebral lobes.

Enclosing the cerebral ganglionic complex and the large nerves which issue from it, is a thin neural sheath, the structure of which cannot be discerned in detail with the light microscope. Under the neural sheath in the brain is the "cortical layer", which consists of the cell bodies of neurons. Internal to this is the "neuropile" formed by the ramifications of the axons which originate from the cell bodies of the cortical layer.

(b) Cortical layer

The cortical layer is not continuous, and varies considerably in thickness, in some regions being over ten cells in depth. The dorsal cortical layer is reduced in thickness in the dorsal median fissure and on the latero-dorsal extremity of the cerebral lobes, (Figure 3), but there is no break in continuity as in Aedes (CHRISTOPHERS, 1960). In the ventral median fissure of the brain, the neuropile is exposed to the neural sheath, except at the extreme anterior where a covering of cell bodies exists.

The cell bodies of the cortical layer vary in size, similarly sized cells often occurring grouped together. The lateral extremities of the cerebral lobes and the entire optic

Figure 3. Frontal view.

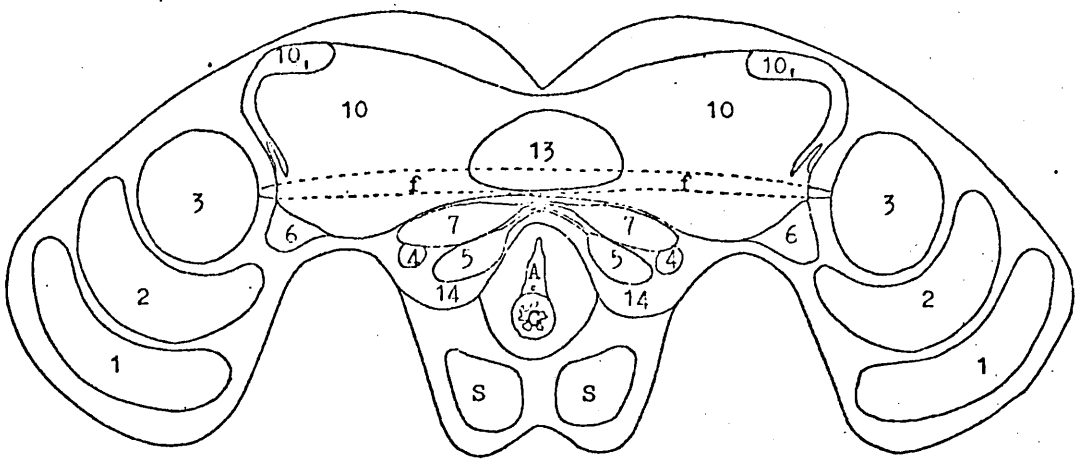


Figure 4. Dorsal view.

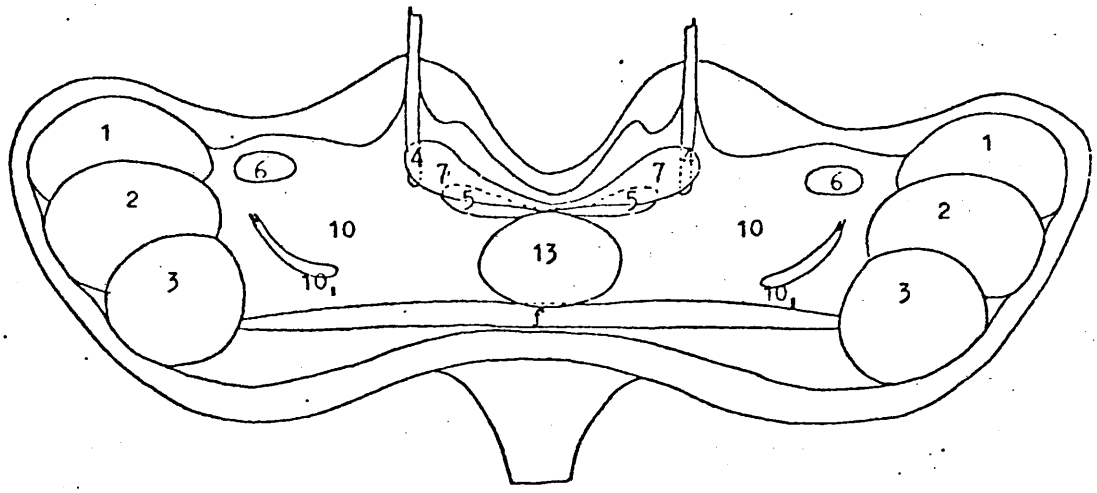


Figure 5. Detail of neuropile mass 13, the central complex, showing the central body (CB) and the ellipsoid bodies (E).

Diagrammatic representations of the neuropile masses within the cerebral ganglionic complex. Numbers refer to those used in the text. The alpha fibre tract (f), the suboesophageal neuropile masses (S) and the central canal containing the gut (G) and the aorta (A) are shown.

lobes have a thick cortical layer composed of "small" neurons. Centrally, the cortical layer of the cerebral lobes is composed largely of "medium" sized neurons. Neurosecretory neurons are usually slightly larger than medium sized cells, which they are typically adjacent to. Scattered throughout the cortical layer of the cerebral lobes are very large cells, which shall be referred to as "giant" neurons. It is difficult to differentiate glial cells with the light microscope. The perikarya of brain cells show a similar staining reaction which, for example taking up "light green" in Halmis' counterstain, shows them to be basophilic. When this stain is employed in EWEN'S (1962) procedure, the nuclei stain a lighter green than the cytoplasm with aggregations of chromatin appearing orange.

(c) The Neuropile

The axons of the neuropile show some organization into aggregates, (Plate 3), which adopting the terminology of CHRISTOPHERS (1960) will be referred to as "masses". The brain was found to have a morphological similarity in its neuropile masses to those of Aedes aegypti. For this reason the numbers in Figures 3 and 4, which show the neuropile masses, and in the text, follow as closely as possible those designated by CHRISTOPHERS (1960) for the mosquito.

The Optic Lobes

The optic lobes consist, as in many insects, of three neuropile masses, (Plate 2). From the lateral extremity towards the midline these are termed ganglionic plate, external medullary mass and internal medullary mass.

Mass 1: Ganglionic plate

The ganglionic plate is a convexo-concave disc, the convex surface forming the extreme lateral cortical layer of the optic lobes. The ommatidia of the compound eye are separated from the optic lobes by a space, typically 25µm across, which often contains fat-body cells. As in Aedes, numerous thick bundles of nerve fibres pass from the surface of this plate and traverse the space to form a plexus under the basement membrane of the ommatidia. Chironomids have no ocelli (COE, 1950) and no remnants of an ocellar nerve arising from this plate, as occur in Aedes, were found. Into the ganglionic plate's concave inner surface fits the convex region of the external medullary mass. These two neuropile masses are connected by a wide outer chiasma.

Mass 2: External medullary mass

The external medullary mass is also concavo-convex, but it is not as flattened as the ganglionic plate. Internally the concave surface fits part of the spherical shape of the internal medullary mass to which it is joined by a narrow chiasma.

Mass 3: Internal medullary mass

The internal medullary mass is a spherical body with a diameter of 60-70 µm, positioned exteriorly to the cerebral lobes. On its internal surface it is connected by a thick fibre tract, which traverses the cerebral lobes, to a similar mass on the other side of the brain.

The Cerebral Lobes

Mass 4

A distinct neuropile mass, (Plate 3), which tends to stain more darkly than other masses, excepting the central body, is seen. In transverse section it is round, with a diameter typically of 12 µm. It is formed by the entering antennal nerve and is connected by fibres on its lateral internal surface to "Mass 5".

Mass 5: The antennal neuropile centre

The antennal centres are situated at the level of the aorta, just dorsal to the crura. They are typically 25 µm across. They are not the most distinct of neuropile masses, as in the similar mass in Aedes. The two antennal centres of the brain are joined by fibres which loop over the ventral medial fissure.

Mass 6

As in Aedes, this is a considerable mass forming a lobe internal to the internal medullary mass from which it receives fibres, and external to the antennal neuropile centre, (Plate 3).

Mass 7

A large neuropile mass situated internally to Mass 6 is observed. It is situated at the level of the posterior limits of the antennal centres, (Plate 3), but is unlike the similar mass in Aedes in not being posterior to them. It lies just dorsal to the antennal centre, and is connected to a similar mass in the other half of the brain by fibres which loop over the ventral median fissure dorsal to those from the Mass 5s. In structure and associations, it is similar to the accessory lobe described by BULLOCK & HORRIDGE (1965) for a generalized insect.

Mass 10

This large mass, (Plate 2), forms the greater part of the cerebral neuropile. It receives fibres from the optic lobes, and many commissural fibres connect the Mass 10s of both sides of the brain, conspicuously in the anterior of the cerebral lobes near the decussation of the fibres from the medial neurosecretory cells.

Mass 10_1

This mass lies in the dorso-lateral extremity of Mass 10 in the medial anterio-posterior region of the brain. It resembles a bent cylinder, the fibres from which emerge ventrally into the ventro-lateral region of Mass 10. It was not described in Aedes.

Mass 13: The Central Complex

This is a single very distinct neuropile mass, situated

centrally in the supraoesophageal ganglion, (Plate 3). It is not isolated by "clefts" from the surrounding neuropile, as is the comparable mass in Aedes. It is divisible into three elements and is less complex than Mass 13 of Aedes. Figure 5 shows the central complex, which adopting the terminology of POWER (1943) for Drosophila melanogaster may be referred to as a single central body per se and a pair of smaller elements, which fit into the ventro-anterior concavity of this body, as do the ellipsoid bodies of Drosophila.

Mass 14

This mass lies in the ventral region of the cerebral lobes and is composed of several neuropile masses, which as in Aedes possibly form part of the crura. The small size of its component masses and the difficulty in delimiting them has prevented their structure from being elucidated.

Masses 8, 9, 11 & 12

These four masses described by CHRISTOPHERS (1960) in Aedes are not found in the brain of Chironomus.

Neuropile tracts

Stained material and brains which have been dissected out in buffer show a complex array of branching interconnections. The thousands of fibres involved makes assessment of the connections between various parts of the brain difficult. Only the most conspicuous fibre tracts will be described. These are designated with greek letters to avoid confusion with the tracts of other dipterans which they show little resemblance to.

Tract α

This is the thickest fibre tract of the brain, it lies dorsal to the ventral medial fissure and just posterior to the central body. It connects the internal medullary masses of the two optic lobes, traversing the cerebral lobes.

Tract β

This tract lies anterior to the central complex and dorsal to the ventral medial fissure. It contains fibres which connect the accessory lobes and antennal centres to those of the other side of the brain.

Tract γ

Anterior to the decussation of the medial neurosecretory fibres, in the extreme frontal region of the brain is a thick [illegible] commissure. On either side of the medial region of the brain these fibres fan out, and appear to connect large areas of Mass 10 to the corresponding mass in the other side of the brain.

Tract δ

A fibre tract laterally connects the posterior dorsal region of Mass 10 to the three neuropile masses of the optic lobes.

Tract ϵ

This tract arises from the internal ventral region of Mass 10, and passes through the crura of that side to the ventral nerve cord.

(d) Tracheal System of the Brain

At the front of the brain, on the ventro-lateral margin of

each cerebral lobe, three branches of the ventral cephalic trachea enter. From these, a complex branching system of trachea and tracheoles ramifies through the cerebral and optic lobes as well as the crura and sub-oesophageal ganglion. Tracheoles are most common in the deeper cortical layers and are much less frequent in the neuropile.

2. Electron Microscope Observations

(a) The Neural Sheath

The neural sheath consists of an outer acellular layer and a subjacent cellular layer, termed by SCHARRER (1939) the neural lamella and perineurium respectively.

Neural lamella

The neural lamella is continuous with the sheath extending over the ventral nerve cord and the peripheral nerves. It consists of a single component consisting of a matrix of finely granular material, (Plate 6), in which fine fibrils with no specific orientation occur. These fibrils have a diameter of 4 - 8 nm, with no periodic banding, which although typical does not preclude them from being collagenous in nature (LANE, 1974). The lamella, which in the brain has a thickness of 300 - 400 nm, is similar in structure to a basement membrane. It is not associated with fat body cells.

Perineurium

Underlying the neural lamella is a single layer of specialized glial cells (WIGGLESWORTH, 1965), forming the

perineurium. It is approximately 2 µm in depth and completely surrounds the ganglia. Exteriorly the perineurial cells' membranes face onto the neural lamella. The cells are connected by tight junctions and, to a lesser extent, by septate desmosomes. There is considerable lateral overlapping between the cells giving the appearance of multiple layers in many sections, (Plate 4). Internally, the perineurial cells are usually separated from the neurons by a layer of glial (type 1) cells to which they are joined by tight junctions. Inpushings of perineurial cell cytoplasm into the cortical layer occur.

Two types of perineurial cells can be distinguished which are termed type I and type II after MADDRELL & TREHERNE (1967). Type I perineurial cells are characterised by their relatively large oval mitochondria, typically 2.0 x 0.4 µm, and the numerous free and aggregated deposits of glycogen, (Plate 6). Ribosomes are numerous, both free in the cytoplasm and encrusting cisternae of endoplasmic reticulum. Microtubules occur infrequently and Golgi units are rarely encountered. "Empty" areas of cytoplasm, possibly representing degenerative foci, which together with clear vacuoles, are frequently observed in these cells. The nucleus is large, cylindrical and often irregular in outline, (Plate 5). It typically measures 9 µm in length with a diameter of approximately 1.5 µm. The nucleus contains diffuse and aggregated chromatin, making the

ELECTRON MICROGRAPHS

The margin of the brain of the neoimaginal male (20°C).

Plate 4 : (SCALE BAR = 2µm), section of a type 1 glial cell (1) which separates the multiple overlapping perineurial layers from the cortex. Note that the type I perineurial cells contain many glycogen rosettes, whereas the type II perineurial cells contain a dense population of microtubules.

Plate 5 : (SCALE BAR = 200nm), section of a type I perineurial cell nucleus (i), which like that of the type 1 glial cell (1), has a grey nucleoplasmic background.

Plate 6 : (SCALE BAR = 200nm), section of the single component neural lamella which consists of a granular matrix in which fine fibrils occur.

The cortex of the brain of the neoimaginal male (20°C).

Plate 7 : (SCALE BAR = 1µm), section of a type 2 glial cell body, the cytoplasmic processes of which ramify between the neurons perikarya.

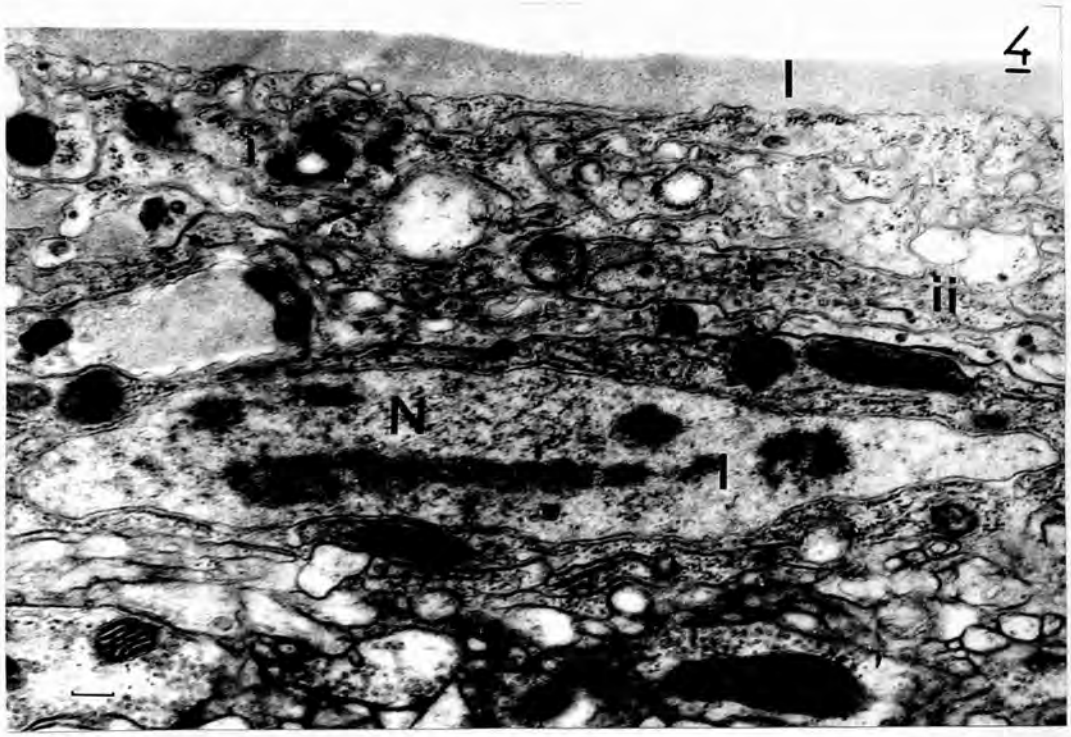
The neuropile of the brain of the neoimaginal male (20°C).

Plate 8 : (SCALE BAR = 1µm), section of a type 3 glial cell body, the cytoplasmic processes of which invest axons.

KEY:

a - axons in the neuropile
f - fibrils
g - glycogen
l - neural lamella
m - mitochondrion
N - nucleus
p - neuronal perikaryon
t - microtubules
i - type I perineurial cell
ii - type II perineurial cell
1 - type 1 glial cell
2 - type 2 glial cell
3 - type 3 glial cell

(Preparation procedure: Luft's epon)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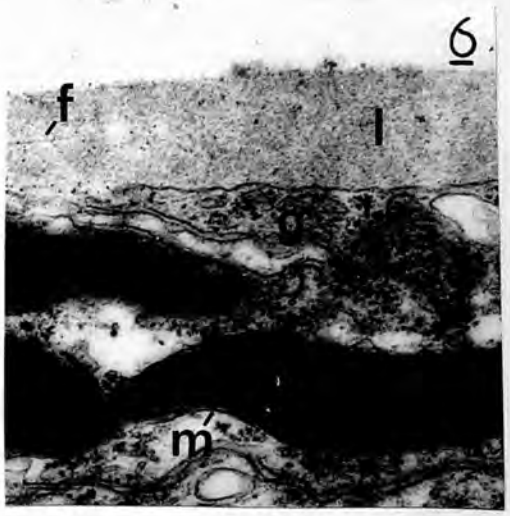
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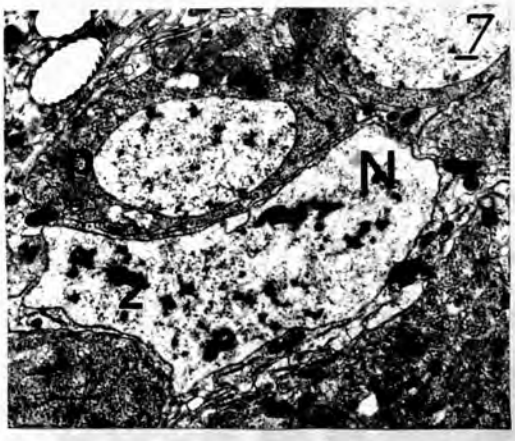
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8
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nucleoplasm electron-dense. There is little electron contrast between the nucleoplasm and the cytoplasm. The rarity with which the nuclei are encountered suggests that both types of perineurial cells are large and plate-like.

Type II perineurial cells have a similar nucleus to those of type I. The cytoplasm contains few mitochondria, which are usually smaller than those encountered in the other type of perineurial cells. They contain large numbers of microtubules with a diameter of 20 nm and multivesicular bodies are commonly encountered, (Plate 4). Glycogen rosettes occasionally occur but are not usually aggregated. The mass of the cell body tends to adopt a deeper position than those of type I perineurial cells.

(b) The Glial cells

Glial cells, in insects as in vertebrates, form a protective sheath around neurons. The glial cells may be recognised by their multiple processes with which they invest cell bodies and axons of neurons (WIGGLESWORTH, 1965). Not all axons of insects are surrounded by glioplasm (review LANE, 1974). The cytoplasmic processes are often very fine, the identification of glial cells can therefore only be carried out with certainty with the electron microscope. The nucleus of these cells was found to contain large, numerous aggregates as well as diffuse chromatin. This, together with the lack of electron contrast between the nucleoplasm and the cytoplasm, was found to be a useful

TABLE 1. Size, proportion and relative frequency of brain and suboesophageal ganglion (SOG) cells of male neoimagines, and of corpora allata medullary cells (CA) in specimens, reared at 20°C.

Cell type	No*	Size in um**		N/C ratio	SD ±	Relative frequency of neurons		Neuron polarity
		Soma	Nucleus			Brain	SOG	
Glial 1	30	M 0·3	4x1	0·43	0·16			
Glial 2	30	M 0·2	8x2·5	2·11	0·81			
Glial 3	30	M 3·0	4x3·5	0·57	0·13			
Neuron 1	50	3·5x3·5	2·5x2·5	1·83	0·22	C	-	Mono
Neuron 2	20	3x3	2·5x2·5	1·90	0·04	R	-	Mono
Neuron 3	30	8x5	6x3·5	0·63	0·19	C	C	Mono
Neuron 4	10	4x4	3·5x3·5	0·63	0·24	V	-	Mono
Neuron 5	15	15x10	4x4	0·20	0·05	R	I	Multi
NSC α	30	12x9	5x4	0·32	0·07	V	V	Mono
NSC β	30	9x6	3x3	0·29	0·19	V		Mono
NSC γ	5	12x7	5x4	0·26	0·05	-	V	Mono
CA N' ♂	10	7x7	3x3	0·19	0·09			
CA N' ♀	10	7x7	3x3	0·21	0·03			
CA A' ♀	10	7x7	3x3	0·20	0·11			
CA O' ♀	10	7x7	3x3	0·23	0·04			

* Number of cells measured

** Size to nearest 0·5µm excepting the average cytoplasmic width (M) of the glial cells which was measured to the nearest 0·1µm.

The ratio of nuclear to cytoplasmic (N/C) planimetry readings is expressed as a fraction followed by the standard deviation (SD) of the results.

Neuron, including neurosecretory cell (NSC), frequency was estimated subjectively: Common(C)>Infrequent(I)>Rare(R)>Very rare(V).

Dimensions of CA of neoimagines(N'), 7 day old (A') and oviposited(O') individuals are shown.

aid in their identification.

In the brain of the neoimagine, glial cells may be divided into three types both upon their appearance and position. These types are given the arabic numerals 1, 2 and 3, so as not to be confused with the roman numeration of SOHAL et al (1972) for the glial cells of Musca, to which they show little resemblance. Agranular endoplasmic reticulum is rare or absent from the glial cells of the neoimagine. Table 1 includes a summary of the average dimensions and nuclear to cytoplasmic ratios of these cells.

Type 1 glial cells

Type 1 glial cells occur on the outer margin of the cortical layer forming a non-continuous border separating the perineurium from the perikarya of the neurons, (Plate 4). The nucleus is usually cylindrical, typically 4 µm long with a diameter of 1 µm. The nucleoplasm usually has a grey background. The cytoplasm contains a dense population of ribosomes, both free and incorporated into the abundant granular endoplasmic reticulum. A few oval mitochondria occur and microtubules are occasionally encountered. The processes of these cells ramify between the neurons of the outer cortical layer. In regions where the cortical layer is thin, the processes penetrate into the neuropile.

Type 2 glial cells

These cells usually occur deep within the cortical layer

of the brain, (Plate 7). The nucleus is large and irregularly shaped with a profile measuring 6 x 2 µm to 10 x 3 µm. The nucleoplasm typically has a white background. These cells have a high proportion of cytoplasm. The cytoplasm resembles that of type 1 cells, except for ribosomes which are less abundant. The cytoplasmic branches of these glial cells separate the perikarya of the neurons and also extend into the neuropile where they invest axons.

Type 3 glial cells

These are the most numerous type of glial cells. They form a non-continuous border on the inner margin of the cortical layer and also occur scattered within the neuropile. The indented nucleus is oval or round with a profile measuring 3.5 x 3.5µm to 7 x 4.5µm. The nucleoplasmic background is typically white, (Plate 8). The cytoplasm is similar to type 1 glial cells, except that the cisternae of the granular endoplasmic reticulum often occur in parallel rows. In areas of cytoplasm with a low density of ribosomes, numerous microtubules can be seen. These have a diameter of 20nm and are similar to those occurring in the perineurium and also to the axonal neurotubules. The microtubules are orientated parallel to the neurotubules in the adjacent axons. The processes of these cells penetrate between the axons of the neuropile.

(c) Extracellular spaces

Few extracellular spaces occur within the brain, and those which do are not usually associated with glioplasm. In the cortical layer, extracellular spaces are associated with the rare type 2 and type 4 neurons described below. Areas of cytoplasmic degeneration, or fixation damage in axons give the appearance of being extracellular spaces. They are, however, surrounded by an integral plasmalemma which is usually unbroken and often contain remnants of neurotubules and mitochondria, which indicates that these spaces are intracellular.

(d) The Tracheal system

Trachea and tracheoles can easily be identified in nervous tissue in the electron microscope by their dense cuticular linings (HESS, 1958). In the trachea (Plate 17), the cuticular lining is spirally thickened forming the taenidia. These trachea branch into tracheoles which can be distinguished, as in other insects (IMMS, 1947) by their lack of spiral thickenings and their small size, being less than 1 um in diameter. The cuticular tube of the tracheal system is surrounded by cytoplasm of tracheal cells. Glioplasm further separates the plasmalemma of these cells from the neurons. In tracheoles, the surrounding sheath of glioplasm is often extremely thin, sometimes less than 20nm.

(e) The Neurons

In Musca domestica a classification of non-neurosecretory neurons, based on size, nuclear to cytoplasmic ratio and relative development and distribution of cell organelles was described by SOHAL et al (1972). Using the same criteria, five types of non-neurosecretory neurons can be distinguished in the brain of the neoimaginal midge. Arabic numerals will be used to denote the various types to avoid confusion with the Roman numeration used in Musca. The specialised neurosecretory neurons of the endocrine system will be described below. Table 1 includes the average sizes and proportions of the neuron types.

Type 1 neurons

These relatively small neurons are the most numerous in the brain. Their distribution was described using the light microscope, where they were referred to as "small" neurons. They are polygonal in shape, (Plate 9), probably resulting from the close approximation of adjacent cells. The soma is almost completely occupied by a rounded nucleus, the chromatin of which occurs in both aggregated and diffuse forms. In the optic lobes, a greater proportion of the chromatin occurs in the aggregated form than in type 1 neurons of the cerebral lobes. The nucleoplasmic background is typically white. The nuclear envelope is crenulated with a variable thickness. The cytoplasm is densely populated with free ribosomes, but cisternae of

ELECTRON MICROGRAPHS

Neuron types of the brain of the neoimaginal male (20°C).

Plate 9 : Type 1 neuron, note the high nuclear to cytoplasmic ratio.

Plate 10 : Type 2 neuron, note the irregular shape of the nucleus and the lack of cellular continuity with other neurons.

Plate 11 : Type 3 neuron which is larger and has a lower nuclear to cytoplasmic ratio than type 1 neurons.

Plate 12 : Type 4 neuron, note the irregular shape of the nucleus and electron density of the cytoplasm.

KEY:

a - aggregated chromatin

d - diffuse chromatin

m - mitochondrion

n - nucleus

nl - nucleolus

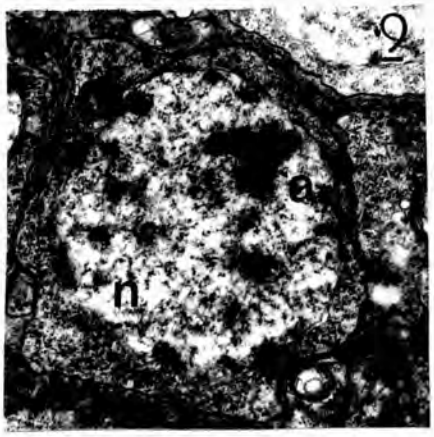
r - granular endoplasmic reticulum

3 - type 3 neuron

4 - type 4 neuron

SCALE BARS : 1µm

(Preparation procedure: Luft's epon)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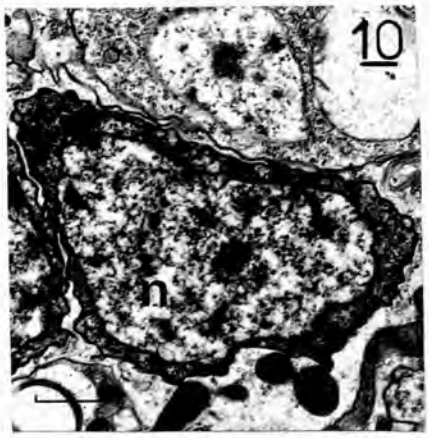
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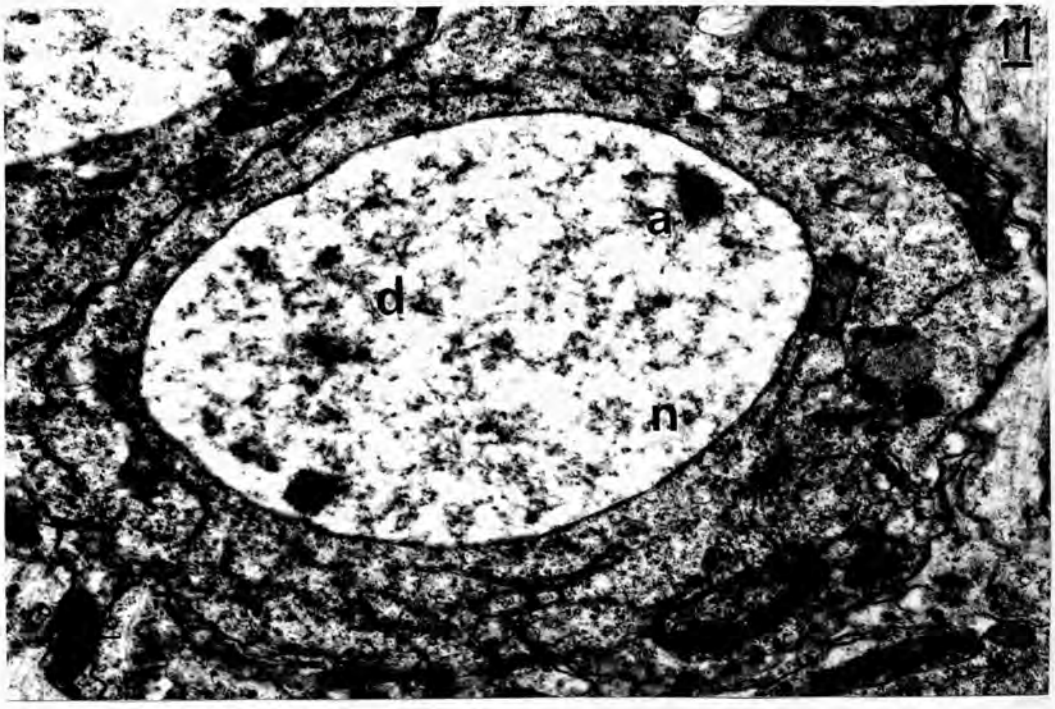
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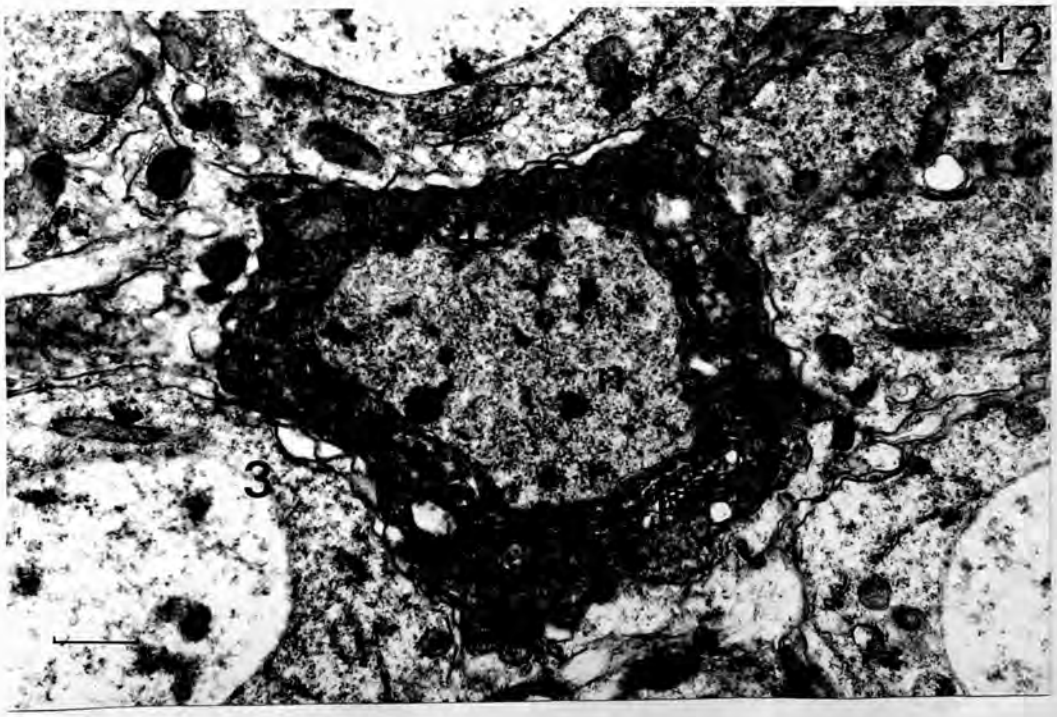
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granular endoplasmic reticulum and Golgi units are rarely encountered. A few small rounded mitochondria, approximately 250nm in diameter, occur. Transparent membrane-bound vesicles, 70 - 100nm in diameter are occasionally encountered, but they do not occur in the axons.

Type 2 neurons

These cells occur adjacent to type 1 neurons, but are much less frequent than them. The nucleus is irregular in shape and contains both diffuse and aggregated chromatin in a nucleoplasm with a grey background (Plate 10). The nuclear envelope is crenulated with a variable thickness. The cytoplasm is electron-dense, resulting from the high population of free ribosomes and the scattered glycogen rosettes that these cells contain. Granular endoplasmic reticulum and Golgi units are encountered more frequently than in type 1 neurons. Mitochondria are of similar size and occurrence to those of type 1 neurons. The plasmalemma, with a surrounding sheath of glioplasm, is often partially separated from adjacent cells by extracellular spaces.

Type 3 neurons

The distribution of these cells was described above using the light microscope, where they were referred to as "medium sized neurons". Typically, the perikaryon is oval surrounding an oval nucleus, (Plate 11), which contains one or two nucleoli. The remainder of the chromatin is usually in diffuse form. The nucleoplasmic background is white and the nuclear envelope is more regular

in width than that of other neurons. The cytoplasm contains many free ribosomes and much granular endoplasmic reticulum, the cisternae of which are often in parallel arrays. Golgi units are often encountered and are frequently found in association with electron-lucent membrane-bound inclusions, 30 - 100 nm in diameter. These inclusions are restricted to the perikaryon and resemble those in aggregation in multivesicular bodies which are encountered in these cells. These bodies appear to be homologous to the multivesicular lysosomes of LANE (1974). Mitochondria are frequent and are usually round, 250 - 500 nm in diameter or oval 250 x 500 - 500 x 1000 nm in profile.

Type 4 neurons

No more than 7 of these neurons have been found in the brain of a neoimagine and usually only 4 are encountered. They occur singularly adjacent to type 3 neurons in the dorsal pars intercerebralis. The cell and the nucleus are irregular in shape (Plate 12). The nuclear envelope has a variable thickness and is often crenulated. The chromatin occurs in diffuse and aggregated forms, and the nucleoplasmic background is grey. The cytoplasm has a high electron-opacity resulting from the dense population of ribosomes. Granular endoplasmic reticulum is common, the cisternae often ocurring in parallel arrays and are frequently distended. Golgi units are rarely encountered. Mitochondria are similar to those occurring in type 3 neurons, but some show a reduction in matrical density

and a loss of cristae. Several "empty areas" or degenerative foci occur in the cytoplasm. These cells are often partially separated from adjacent neurons by extracellular spaces, as are type 2 neurons.

Type 5 neurons

These large cells were termed "giant neurons" in light microscope observations. They occur throughout the cortex, usually singularly and are most frequently encountered in the anterior ventral region of the cerebral lobes adjacent to type 3 neurons. The rounded or oval nucleus has a membrane with a regular width and a white nucleoplasmic background. The chromatin is usually diffuse and typically no nucleoli occur. Numerous mitochondria, which are similar in size and appearance to those of type 2 neurons, occur in the cytoplasm (Plate 13). Ribosomes are common, both free in the cytoplasm and incrusting cisternae of granular endoplasmic reticulum which often occur in loose parallel arrays. Several Golgi units are usually encountered which are frequently found in association with membrane-bound inclusions similar to those of type 3 neurons. Unlike other neurons, mitochondria, cisternae and ribosomes occur in the axon hillock region of these cells. These cells have several axons and are possibly the only multipolar neurons in the brain.

(f) The Axons

The swelling of the cell body in the axon hillock region

ELECTRON MICROGRAPHS

Neuron types of the brain of the neoimaginal male (20°C).

Plate 13 : Type 5 neuron, note the large size of the perikaryon compared to those of adjacent neurons and the dense population of mitochondria.

KEY:

AX - neuropile
C - cortex
m - mitochondrion
no - nucleus
r - ribosomes
SCALE BAR : 1μm

Axon types of the brain of the neoimaginal male (20°C).

Plate 14 : Section of the posterior median fissure region of the brain, showing the four axon types. SCALE BAR : 1um.

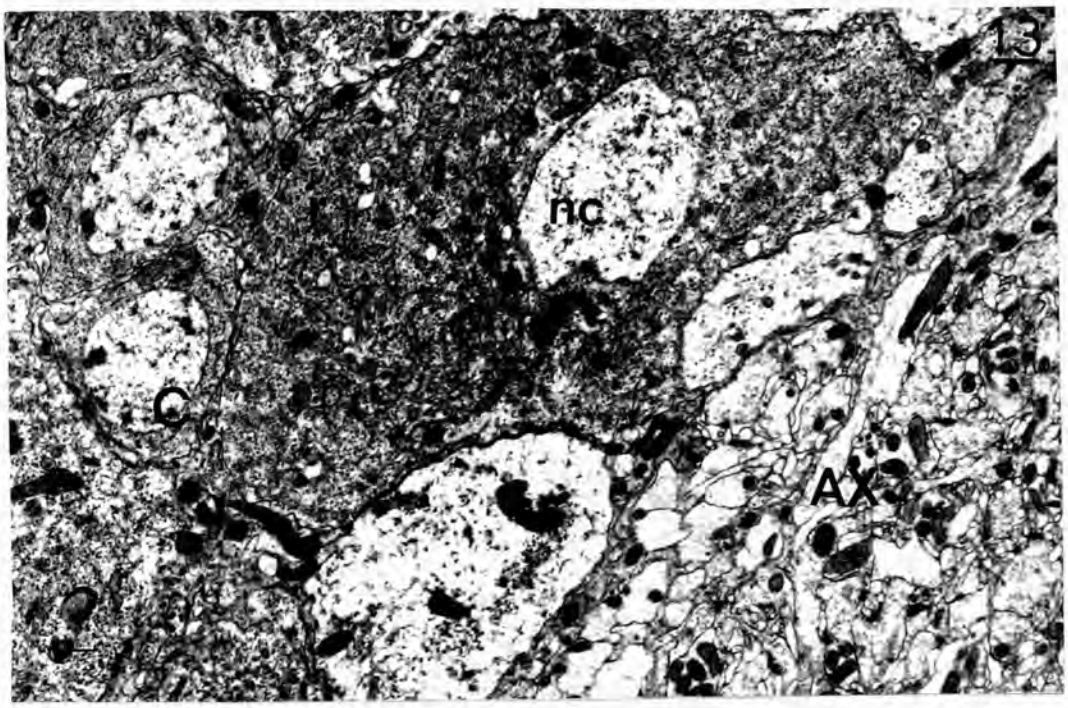
Plate 15 : Transverse section of a type D axon, showing the spacing of neurotubules and their radiating filamentous project -ions. SCALE BAR : 0·5 μm.

Plate 16 : Oblique section of several type C, neurosecretory, axons. SCALE BAR : 1μm.

KEY:

A - type A axon
B - type B axon
C - type C axon
D - type D axon
g - granule of neurosecretion
m - mitochondrion
n - neurotubule
s - saccules of smooth endoplasmic reticulum

(Preparation procedure: Luft's epon)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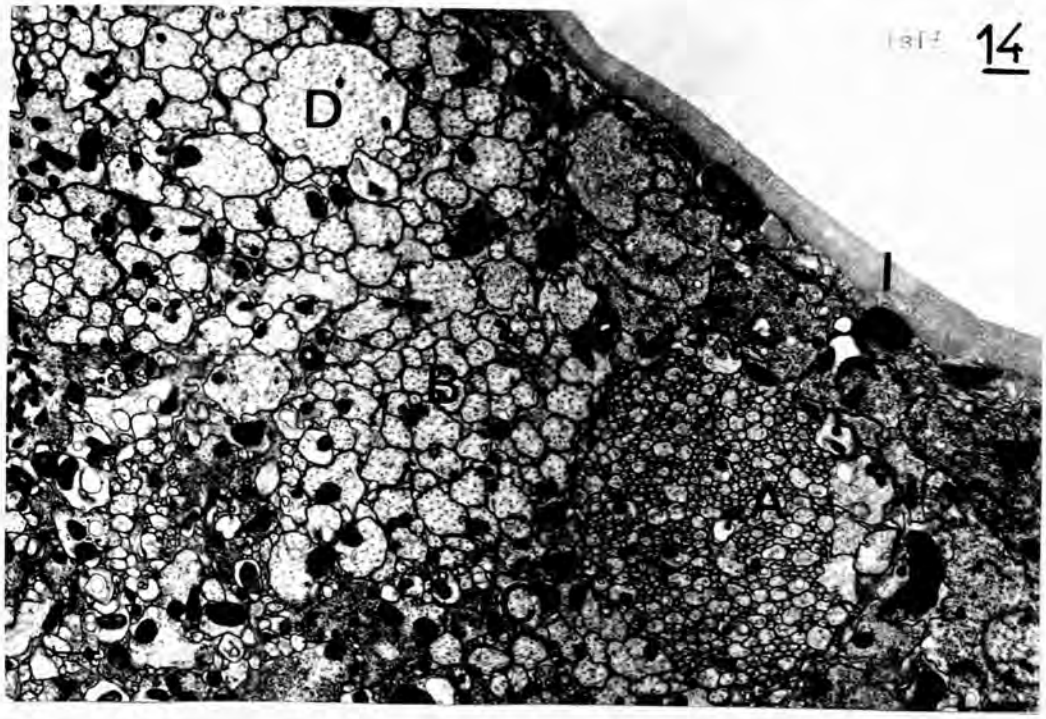
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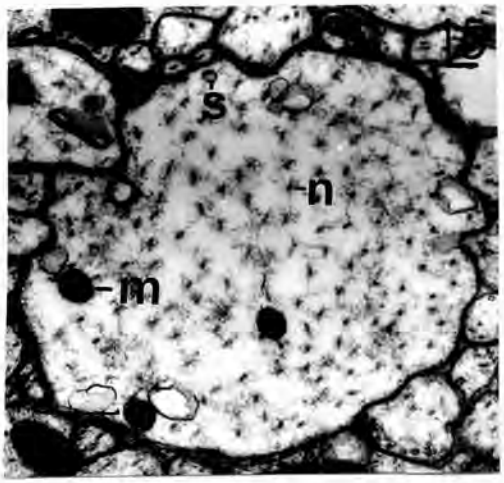
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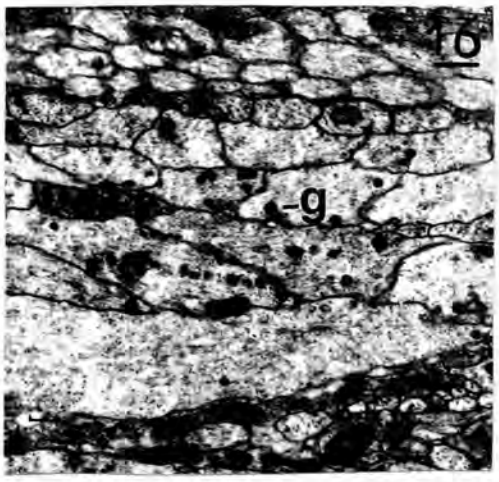
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g

where the cytoplasm extends into an axon, exhibits a cytological difference from the perikaryon, except in type 5 neurons described above. The hillock and the axonal process contain neurotubules, mitochondria and saccules of smooth endoplasmic reticulum and lack other organelles.

Neurotubules, 20 ± 2 nm in diameter, are equidistantly spaced and aligned parallel to the plasmamembrane of the axon. Delicate filamentous projections (Plate 15) 6 µm or less in thickness, radiate from individual neurotubules to join with similar projections from adjacent ones. These projections appear to form the ground substance of the axon. With combined uranyl acetate and lead citrate staining, the neurotubules appear as an electron-dense ring, 20nm in diameter, surrounding an electron-lucent core approximately 10nm across.

The mitochondria appear to have a greater proportion of their volume composed of cristal membranes than occur in the same organelles of the perikarya. The cristae are orientated parallel to the longitudinal axis of the axon.

Infrequently, membrane profiles are observed which may be saccules of smooth endoplasmic reticulum.

At the axon's termination, a swelling occurs in which electron-lucent "synaptic" vesicles, 30 - 50 nm in

TABLE 2. Summary of characteristics of axon types and their usual origins in the cerebral ganglia of neoimaginal males reared at 20°C.

Axon type	Diameter in nm	Average No. of neurotubules*	Usual No. of mitochondria*	Frequent origin
A	100-250	2	0	N1 & N2
B	400-1000	20	1	N3 & N4
C	700-1600	60	0	NSC
D	2000-4000	150	4	N5

* Per transverse section of an axon, based upon 50 observations of each axon type. Neuron (N) types are given the same numerals as used in the text. The neurosecretory axons (type C) originate from neurosecretory cells (NSC).

diameter are found.

A classification of axons into four types (Plates 14, 16), based upon size and axonal organelles is given in Table 2. There were no observations indicating a multicellular origin for any of the axon types. At the proximal end, the size of an axon was found to be directly proportional to the size of the neuron from which it originates. The aggregation of axons appears to mirror the organization of the cortex, this is clearly demonstrated by a type "D" axon, which is usually surrounded by type "B" axons reflecting the cortical arrangement of a type 5 neuron being adjacent to type 3 neurons. Observations upon the non-neurosecretory axons in the nervi corporis cardiaci II indicate that there is little change in the diameter over the majority of the axon's length. The formation of collateral branches, which are smaller in diameter than the main axon, means that the axon size cannot always be related to the size of the perikaryon.

The axons are found in the neuropile either in "synaptic regions", where axons of differing size occur randomly orientated, or in "pathways" where axons of similar size are aggregated with others showing the same orientation into discrete bundles.

B. The Anterior Region of the Ventral Nerve Cord

The suboesophageal and prothoracic ganglia and the connectives between them have been observed in this study.

I. Suboesophageal Ganglion

Two thick circum-oesophageal connectives or crura, attach the suboesophageal ganglion to the brain, (Plate 1). Anteriorly on each side of the suboesophageal ganglion issue the mandibulo-maxillary and labial nerves, reflecting its compound nature (CHAPMAN, 1971). It is joined posteriorly by a pair of connectives to the prothoracic ganglion. The ganglion is shaped such that its interior surface is dorsal, and its exterior comprises a single ventral and two lateral surfaces, as shown in Figure 3.

(a) Light Microscope observations

There is no clear division between the tritocerebrum and the suboesophageal ganglion. The ganglion has no dorsal cortical layer, the neuropile abuts onto the neural sheath, which forms the floor of the canal containing the oesophagus and aorta. The cortical layer of the ventral and lateral surfaces may be up to five cells in depth. Ventrally, a median shallow cleft occurs resulting from a depression in the neuropile and not from a reduction in the thickness of the cortical layer, as are the fissures in the brain. The cortical cells are "medium" sized with several "giant" neurons occurring amongst them.

The neuropile is composed of a single mass on either side of the mid-line. Anteriorly, there is a considerable amount of axonal bridging between these two masses. Posteriorly, they become divided, which is reflected in the formation of the ventral cleft described above. The axons of these masses extend into the two connectives of the nerve cord, which arise from the posterior extremity of the ganglion.

(b) Electron Microscope observations

i. Neural Sheath

The neural sheath is similar to that of the brain in being composed of a single component lamella and perineurium (cells recognisable as the types described above). The neural lamella is continuous with the brain's, having a constant thickness of approximately 1 µm. The fine fibrils occurring in this layer are more frequently encountered than in the lamella of the brain. The single layer of perineurial cells overlap laterally. The perineurium of the dorsal region of the ganglion, which lacks a cortical layer, is composed entirely of type II perineurial cells, (Plate 19) whose characters were described above. The lateral and ventral perineurial layer resembles that of the brain in being composed of both types of perineurial cells.

ELECTRON MICROGRAPHS

The brain's tracheal system of the neoimaginal male (20°C).

Plate 17 : Oblique section of a trachea, showing the spiral thickening of the intima. SCALE BAR : 0·5μm.

KEY:

a - axons
c - tracheal cell cytoplasm
o - lumen of trachea
t - taenidium

The ventral nerve cord of the neoimaginal male (20°C).

Plate 18 : Section of a dense body in the neuropile of the suboesophageal ganglion. SCALE BAR : 1μm.

KEY:

e - electron-dense vesicles
g - glycogen
m - mitochondrion
v - multivesicular body

Plate 19 : The neural sheath of the suboesophageal ganglion showing the underlying type II perineurial cell cytoplasm. SCALE BAR : 0·5μm.

KEY:

f - fibrils
l - neural lamella
M - mitochondrion
t - microtubules in type II perineurial cells

Plate 20 : Part of the prothoracic ganglion showing the neuron cell bodies and the extracellular spaces. SCALE BAR : 1μm.

KEY:

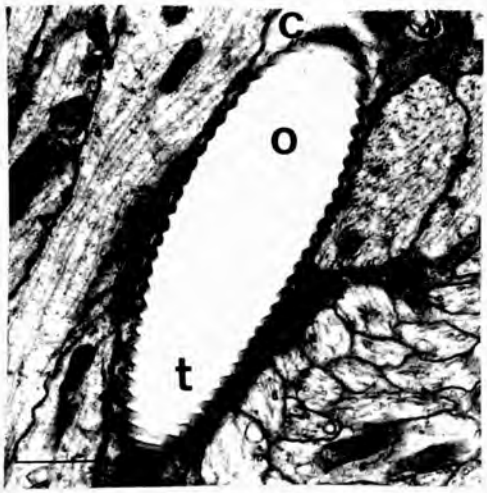
n - nucleus of a neuron
u - extracellular spaces

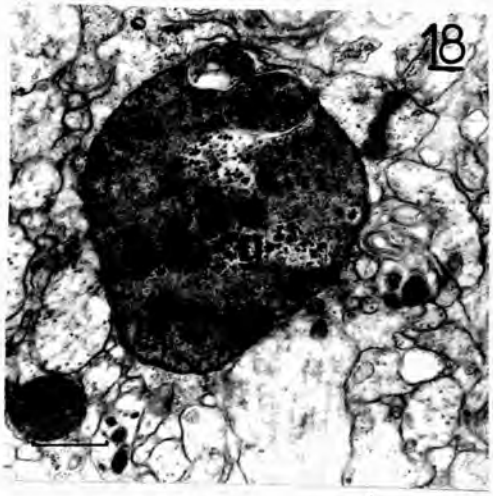
Plate 21 : The periphery of the prothoracic ganglion, showing the close association between the neural lamella and fat body cells. SCALE BAR : 1μm.

KEY:

d - lipid droplets
F - fat body
g - glycogen
l - neural lamella
PG - prothoracic ganglion

(Preparation procedure: 17-19, Luft's epon, 20-21, Spurr's epon)





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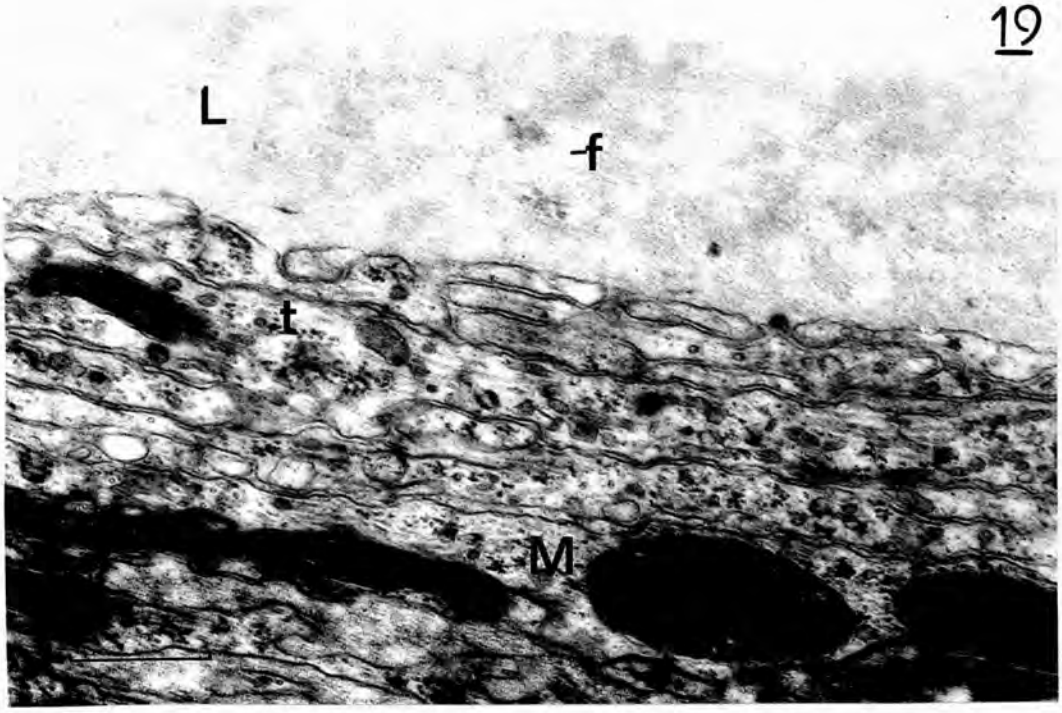
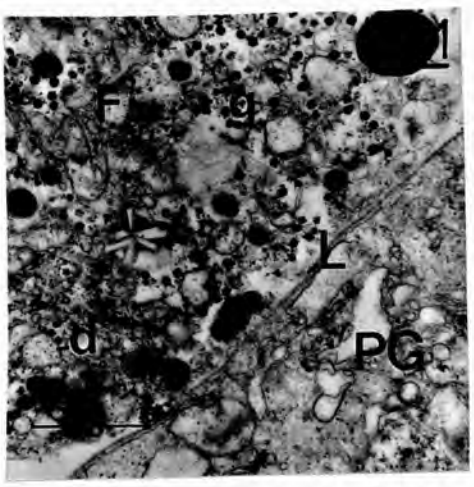




ii. Glial cells

The glial cells fit into the three categories described in the brain. Type 1 glial cells do not occur between the perineurium and the cortical layer, as in the brain. On the dorsal, internal surface of the ganglion, type 1 glial cells separate the perineurium from the neuropile. Type 2 glial cells, as in the brain, occur between the perikarya of the cortical layer. Type 3 glial cells, as in the brain, occur in the neuropile. They are infrequently encountered on the internal margin of the cortical layer, where they are commonly found in the brain.

iii. Extracellular spaces

Extracellular spaces are of infrequent occurrence in this ganglion.

iv. The Tracheal system

As described above, the tracheal system shares a common origin with that occurring in the brain. The structure and distribution of tracheoles is similar to that described in the brain, except that they are more frequently encountered in the neuropile of the suboesophageal ganglion than they are in the brain.

v. Neurons

Using the same classification as applied to the

brain, it is found that most of the neurons in the suboesophageal ganglion are of type 3. Type 5 neurons are found throughout the ganglion and are encountered more frequently than in the brain. These type 5 neurons are unlike those of the brain in containing up to two nucleoli. Two types of neurosecretory cells occur in the ganglion and will be described below.

vi. Axons

The axons of the neuropile are mainly of the type B, as described in Table 2, intermingled with which are many type D axons. The aggregation of axons into discrete bundles is not as marked as in the neuropile of the brain.

vii. Dense bodies

In the neuropile of the suboesophageal ganglion, dense bodies 2 - 3 µm across are occasionally encountered, (Plate 18). Serial sections show that these bodies are spheroid. They are invested with glioplasm. On the internal margin of the bounding membrane, dense and translucent vesicles occur, which are approximately 50 nm in diameter and surrounded by a membrane. These bodies are densely populated with ribosomes and contain many mitochondria. Aggregates of glycogen deposits and multivesicular bodies are often found. Other organelles, including the nucleus, appear to be absent.

2. The Connectives

The two adjacent connectives, each approximately 75 µm in diameter, are situated ventrally along the median longitudinal axis of the insect's body. They connect the suboesophageal ganglion, via the neck region, to the prothoracic ganglion, (over a distance of approximately 350 µm.) Just anterior to the prothoracic ganglion, the two connectives fuse.

The neural sheath is composed of a single component lamella with a perineurium with the two cell types having a similar appearance and association with those described in the brain. The lamella is 100 nm in thickness, consisting, as in the brain, of a granular matrix in which fine fibrils occur. In some areas, there is a close association between the neural lamella and surrounding fat body cells.

Type 3 glial cells are the only cell bodies which occur in the connectives subjacent to the perineurium. They are found amongst the axons which they invest.

Extensive extracellular spaces occur between the axons of the neuropile, which otherwise resembles that of the suboesophageal ganglion, except that no dense bodies are found.

Tracheoles, having a structure similar to those in the brain, occur infrequently.

3. The Prothoracic Ganglion

The prothoracic ganglion is situated mid-ventrally in the first thoracic segment. In transverse section it is oval, approximately 100 µm across by 75 µm high, with a depth of over 150 µm, as seen in longitudinal sections. The neuropile of the ganglion is completely surrounded by a cortical layer. The ventral and lateral cortical layers are up to 8 cells in depth, whereas the dorsal layer is usually no more than 4. The neurons of the cortical layer are similar in size and distribution to those of the suboesophageal ganglion. The neuropile is divided into a single mass on either side of the midline. Each mass is composed of many smaller masses, whose boundaries are not clearly delimited. Considerable axonal bridging occurs between the masses of each side of the ganglion.

The neural sheath has the same appearance as described for the connectives, and is also often associated with fat body cells (Plate 21). It is continuous with the connectives.

The glial cells may be divided into the three types already described in the brain, which they resemble in both position and appearance.

Extensive extracellular spaces occur, both between the perikarya of the cortical layer and between the axons of the neuropile, (Plate 20).

The distribution and structure of tracheoles is similar to that described in the suboesophageal ganglion.

The neurons are of type 3 and type 5 and occur in a similar frequency to those of the suboesophageal ganglion. The large neurosecretory cells that this ganglion contains will be described below. The mitochondria of the neurons often have a low matrical density with irregularly orientated cristae and the cisternae of the endoplasmic reticulum are often swollen. In other respects, the organelles are similar to those described for the same cells in the brain.

The axons of the neuropile resemble those of the suboesophageal ganglion, but no dense bodies are present.

C. The Stomatogastric and Endocrine Systems

The stomatogastric system of the neoimagine comprises two small ganglia and their associated nerves. The median "frontal ganglion" is joined by bilateral connectives, the labrofrontal nerves, to the tritocerebral lobe of the brain. The hypocerebral ganglion is connected to the frontal by a median recurrent nerve which for much of its length lies in the lumen of the aorta. The more posterior hypocerebral ganglion is connected by nerves to the paired corpora cardiaca.

The endocrine organs consist of neurosecretory cells within the central nervous system and specialised glands, the corpora cardiaca, corpora allata and peritracheal tissues. The paired "glandes post-cérébrales antérieures" which are associated with these structures, may also have an endocrine role. Some of the neurosecretory axons from cells in the brain appear to innervate the hypocerebral ganglion and several pass through it before entering the corpora cardiaca.

As there is a close spatial association between the stomatogastric and endocrine systems, they will be described in the same section to avoid repetition.

1. Neurosecretory cells of the Brain

(a) Light Microscope observations

Table 3 includes a summary of the staining responses of the various neurosecretory cell groups, using different staining techniques. In the neoimaginal male, the only group to be detected by these histological procedures were the medial neurosecretory cell groups. With chrome haematoxylin-phloxin, the perikarya of a few of the cells of the MNC infrequently exhibited a reddy colour which is referred to as phloxinophila (GABE, 1966). The MNC usually appear greeny-turquoise after treatment with alcian blue, (Plate 35) combined with periodic acid Schiff's test. This colour does not occur in the controls for alcian blue, and there is no indication of

TABLE 3. Summary of neurosecretory cell groups, their constituent types and staining reaction, of male neoimagines reared at 20°C.

Cell group	Position	No. of cells of each type/ group					Staining reaction				
		α_1	α_2	α_3	β	γ	M'PAF	E'PAF	CHP	AB	HA
MNC	Either side of the midline in anterior dorsum of pars intercerebralis	8	-	-	-	-	-	+	*	+/-	-
VNC	Either side of the midline in posterior ventral region of the pars intercerebralis	3	3	-	-	-	-	-	-	-	-
LNC	In the doso-lateral cortex of the pars intercerebralis of each protcerebral lobe	-	-	-	3-4	-	-	-	-	-	-
ONC	At the lateral margin of the protocerebrum adjoining each optic lobe	-	2-3	-	-	-	-	-	-	-	-
SNC_1	At each side of the suboesophageal ganglion in the dorso-lateral cortex	-	-	-	-	1	-	-	-	-	-
SNC_2	Either side of the midline in the ventral cortex of the suboesophageal ganglion	-	-	1-2	-	-	-	-/+	-	-	-

* Occasional phloxinophilia.

Meola's (M') and Ewen's (E') paraldehyde fuchsin (PAF), chrome haematoxylin-phloxin (CHP), alcian blue (AB) and Heidenhain's azan (HA), stains were used.

purple staining with the Schiff's test. Differential staining was regularly detected in cells of this group when EWEN'S (1962) paraldehyde fuchsin procedure was used, (Plate 22). The maximum number of MNC which stains in a brain is 8 and the average is 5, (refer to Appendix 3), the mean staining indices are given in Table 5. The other stains employed failed to give any definition of neurosecretory cells in the material examined.

(b) Electron Microscope observations

With the electron microscope four paired groups of neurosecretory cells were identified in the brain of the adult midge. Their location, sizes, constituent cell types and relative granule quantities are indicated in Figures 6 and 7 and Tables 1, 3 and 6.

i. Common features

Brain neurosecretory cells were found to exhibit several common features. They all appear to be unipolar, as are most other neurons of the brain. The perikarya has a dense population of ribosomes, both free within the cytoplasm and encrusting the cisternae of endoplasmic reticulum. The granular endoplasmic reticulum is commonly arranged as a system of parallel lamellae. Numerous mitochondria occur, which are variable in size and shape. Several Golgi units are invariably present

Figure 6. Transverse section.

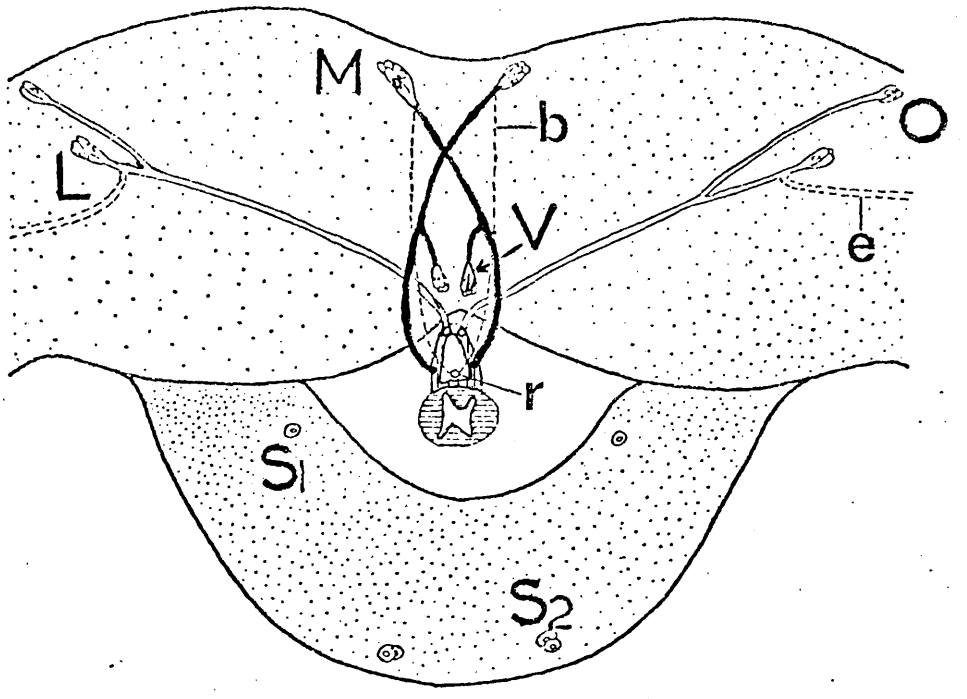
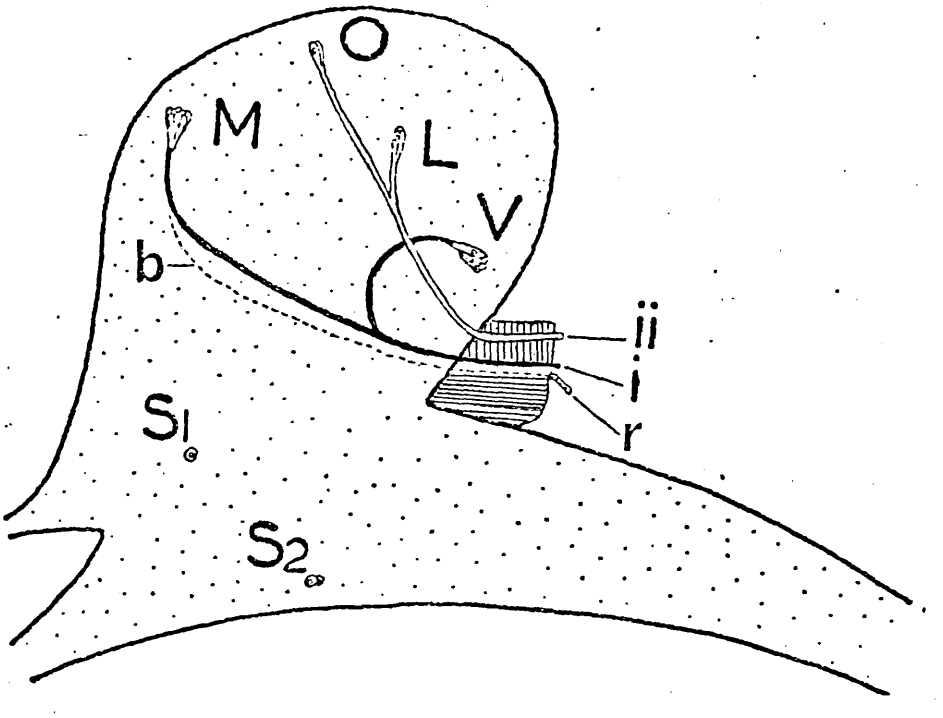


Figure 7. Sagittal section.



Diagrammatic representations of sections through the cerebral lobes and suboesophageal ganglion to show the relative positions of the MNC (M), LNC (L), ONC (O), VNC (V), SNC_1 (S_1) and SNC_2 (S_2). Axons from the VNC and MNC (blocked lines) enter the aorta wall (vertically stripped), forming the NCC I (i), ventral to those of the LNC and ONC (open lines) which constitute the NCC II (ii). Collateral branches of the MNC (b) run into the reccurent nerve (r), whereas those of the LNC (e) go to the optic lobes. The gut is horizontally stripped.

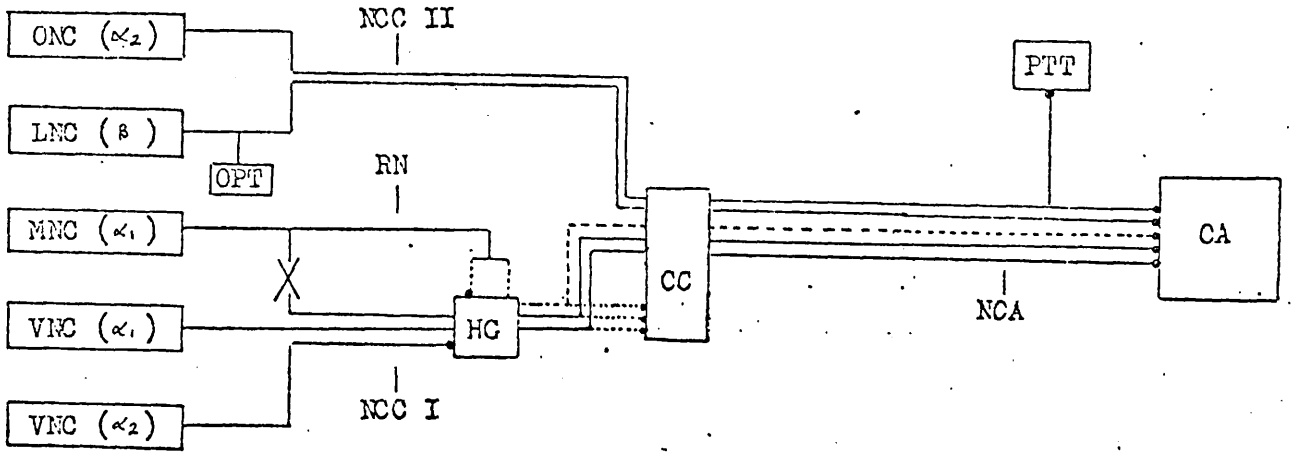


Figure 8. Schematic diagram of the axonal pathways of the neurosecretory cells in one side of the brain in relation to the hypocerebral ganglion (HG), and the epsilateral corpus cardiacum (CC), peritracheal tissue (PTT) and corpus allatum (CA). Greek lettering and Arabic numerals refer to the types of neurosecretory cells described in the text. Unbroken lines show traced pathways, whereas broken lines indicate possible connections, to termination points (·). The collateral branches of the LNC are shown passing to the optic lobes (OPT), whereas those of the MNC run to the reccurent nerve (RN). As the fibres of the MNC decussate (X) those from the contralateral group will constitute the MNC axons in the NCC I on this side.

which are frequently associated with membrane bound granules, possibly representing the secretory product. The size of the neurosecretory granules varies in the different classes of cells, but the populations of sizes of granules have a unimodal distribution (Figure 11 and Appendix 4). The nucleus is typically round or oval in shape, with a nuclear envelope which, although frequently crenulated, is usually of constant width. The nucleoplasmic background is white.

ii. Classes of neurosecretory cells

Roman lettering (KNOWLES, 1965, RAMADE, 1969, CHALAYE, 1974 and others) and numerals (STEEL & HARMSEN, 1971; KONO & KOBAYASHI, 1972; MORRIS & STEEL, 1975 and others) have been given to the neurosecretory cells in the brains of a number of insects. The neurosecretory neurons in different species, which are dissimilar in many respects have often been designated with the same character. To avoid confusion with the classifications of other authors, the neurosecretory classes in the brain of the adult midge have been designated with Greek letters.

α cells

The pear-shaped perikarya of these cells are larger than those of the adjacent non-neurosecretory cells, which are usually type 3 neurons. The central

ELECTRON MICROGRAPHS

Neurosecretory cell types in the brain of the neoimaginal male (20°C).

Plate 22 : The ventral neurosecretory cell group of the brain which includes both α_1 and α_2 types of neurosecretory cells. Note the prominent nucleoli displayed in two of the cells.
SCALE BAR : 1µm.
KEY:

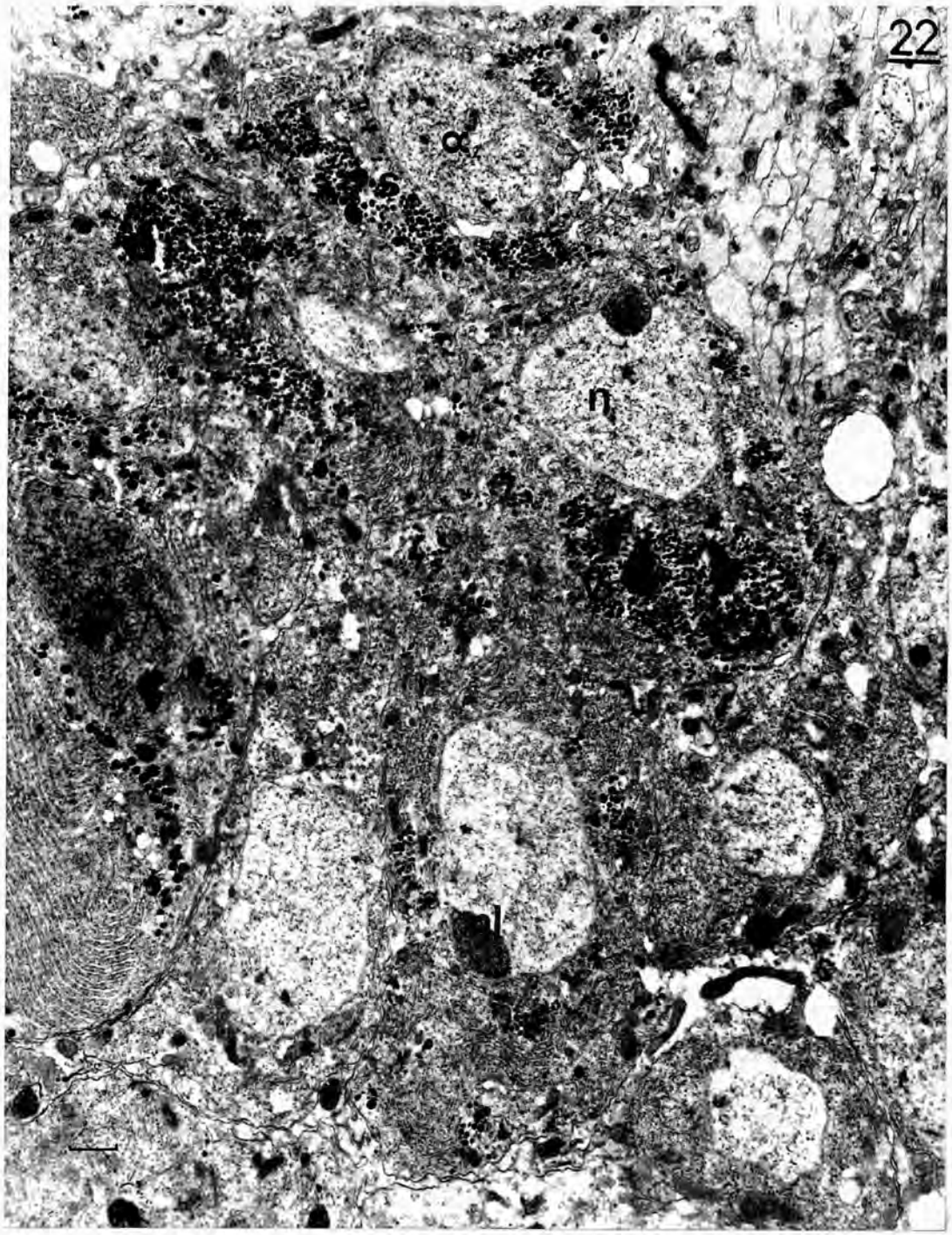
n - nucleus
nl - nucleolus
r - granular endoplasmic reticulum
s - neurosecretion
v - multivesicular body

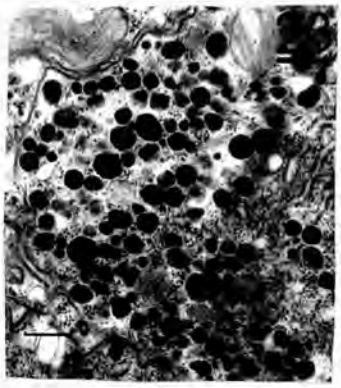
Plate 23 : Granules of α_1-type neurosecretory cell.
SCALE BAR : 200nm.

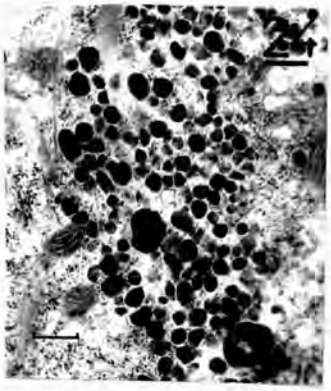
Plate 24 : Granules of α_2-type neurosecretory cell.
SCALE BAR : 200nm.

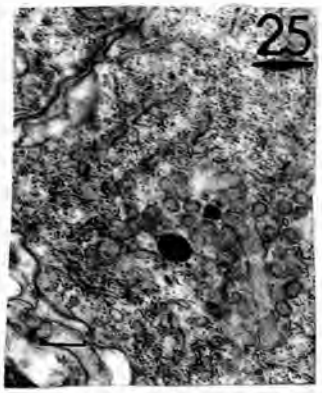
Plate 25 : Granules of α_3-type neurosecretory cell.
SCALE BAR : 200nm.

(Preparation procedure: Luft's epon)


22
α
n
l




24


25

nucleus contains a single, prominent, electron-dense, eccentrically situated nucleolus, 1 μm in diameter, the remaining chromatin being mainly diffuse. The cytoplasm of these cells is often rich in glycogen, which is frequently aggregated (Plate 29). The adjacent type 3 neurons also frequently contain glycogen deposits which occur less frequently in these cells in other parts of the brain. On the basis of the electron density, size and shape of their contained granules, three types of α cells, two of which occur in the brain, have been recognised, (Plates 23, 24, 25), details of which are given in Table 4. In other respects, these cells are similar, although α_2 type cells of the ONC group contain more numerous multilamellate bodies and rather fewer Golgi units than the others.

β cells

The perikarya of the β cells is smaller than those of α cells and usually has an oval or polygonal outline, (Plate 30). The nucleus contains both diffuse and aggregated chromatin, but lacks a prominent nucleolus. The characteristic granules (Table 4) further distinguish these cells from those in the α category.

TABLE 4. Granular characteristics of each neurosecretory cell type within the cerebral ganglia of neoimaginal males reared at 20°C.

Cell type	Granules		
	Profile	Appearance	Size* (nm)
α_1	round	electron-dense	140-200
α_2	irregular	electron-dense	70-100
α_3	round	electron-lucent	70-120
β	round	electron-lucent	80-100
γ	round flattened	electron-dense electron-dense	130-160 150-250x 70-150

* Range of true mean diameters.

TABLE 5. Mean staining indices of the two cell groups, within the brain, which selectively stain with Ewen's paraldehyde fuchsin (summary of data from Appendix 3).

Cell group		Rearing temperature 20°C: Days after emergence 0(1hr)	1	3	5	6-7	10°C: 1
MNC	♂	10	16	19	35	33	2
	♀	-	15	21	25	28	8
VNC	♂	0	0	0	0	0	0
	♀	-	1	1	1	3	0
No*		2	30	4	3	10	15

Staining indices

* Number of specimens of each sex used.

TABLE 6. Summary of relative granule quantities of the brain neurosecretory cells.

Cell group		Neoimagines: Rearing temperature 10°C ♀	10°C ♂	20°C ♀	20°C ♂	6-7 days old 20°C ♀	20°C ♂
MNC	α_1	10 (3)	10 (3)	8 (3)	6 (4)	1 (5)	1 (3)
VNC	α_1	8 (3)	8 (3)	8 (5)	6 (4)	7 (5)	8 (3)
	α_2	4 (3)	4 (3)	10 (5)	10 (3)	10 (4)	10 (6)
ONC	α_2	1 (4)	1 (3)	10 (4)	10 (4)	3 (3)	- (6)
LNC	β	4 (2)	3 (3)	5 (2)	5 (4)	2 (4)	2 (3)

Granule quantities

Amounts are based on a subjective assessment (0-10) of each cell type in different experimental groups. So that comparisons between cell types can be made some figures have had to be adjusted. Figures in parentheses refer to the number of specimens used in the assessment.

LIGHT MICROGRAPHS

Transverse sections showing the medial neurosecretory cell group.

Plate 26 : The MNC, of the neoimaginal male reared at 20°C, containing fine granular material.

Plate 27 : The MNC, of a neoimaginal male reared at 10°C, containing coarse granular material.

Plate 28 : The MNC, of a male reared and kept at 20°C, 6-7 days after emergence, some of which contain a single mass of purple stained material whereas a single cell upon each side has stained orange.

KEY:

A - neuropile

C - cortex

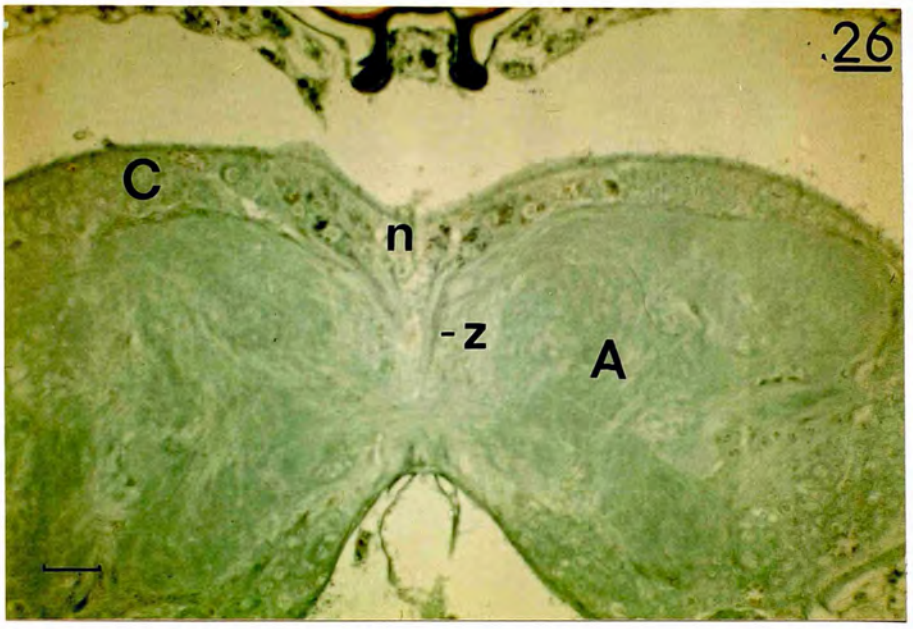
n - neurosecretory cell

o - orange staining cell

z - axons containing neurosecretory material

SCALE BARS : 20µm.

(Stain: Ewen's aldehyde fuchsin)


26
C
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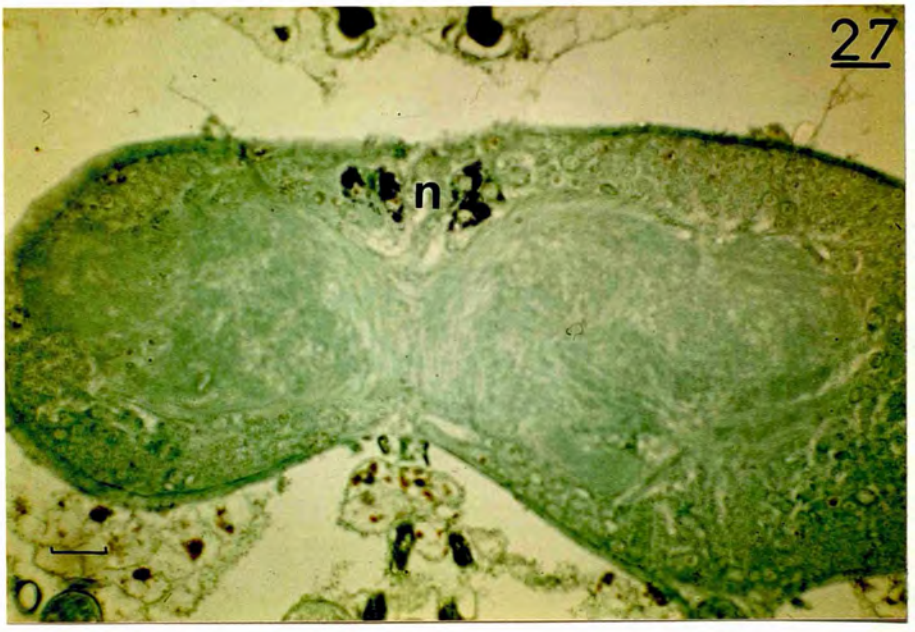
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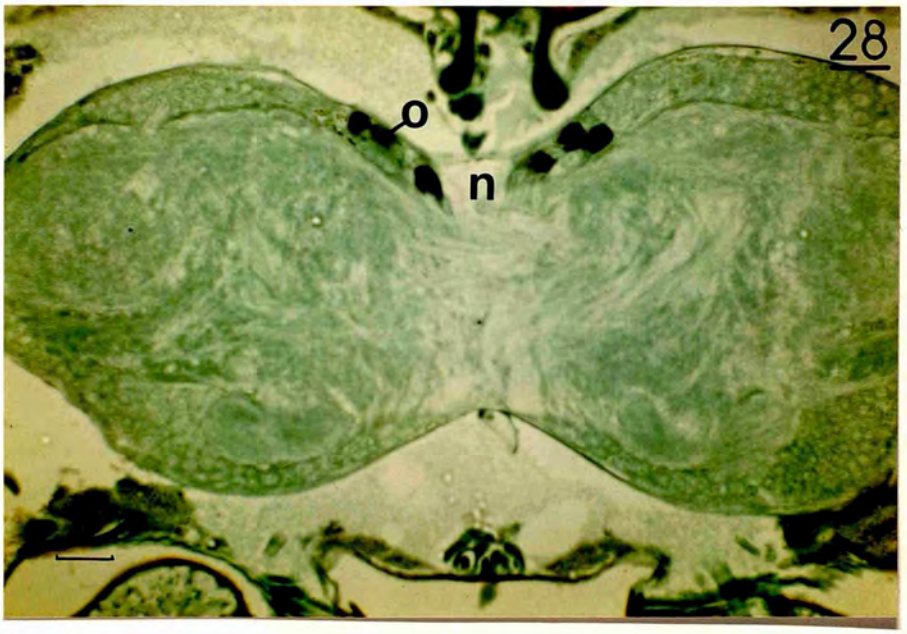
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ELECTRON MICROGRAPHS

The brain's cortex of the neoimaginal male (20°C).

Plate 29 : Part of a MNC containing glycogen deposits and showing the neurosecretory granules apparently budding off from a Golgi unit. SCALE BAR : 200nm.

KEY:

G - glycogen

R - granular endoplasmic reticulum

S - neurosecretion

U - Golgi unit

Plate 30 : A β cell showing electron-lucent granules.

SCALE BAR : 200nm.

KEY:

M - multilamellate body

N - nucleus

Other labels as Plate 29

The aorta of the neoimaginal male (20°C).

Plate 31 : Section of part of the aorta, showing the different neurosecretory granule types within the aorta wall and the recurrent nerve in the aortic lumen. SCALE BAR : 1μm.

KEY:

α_1 - α_1 neurosecretory cell granules

α_2 - α_2 neurosecretory cell granules

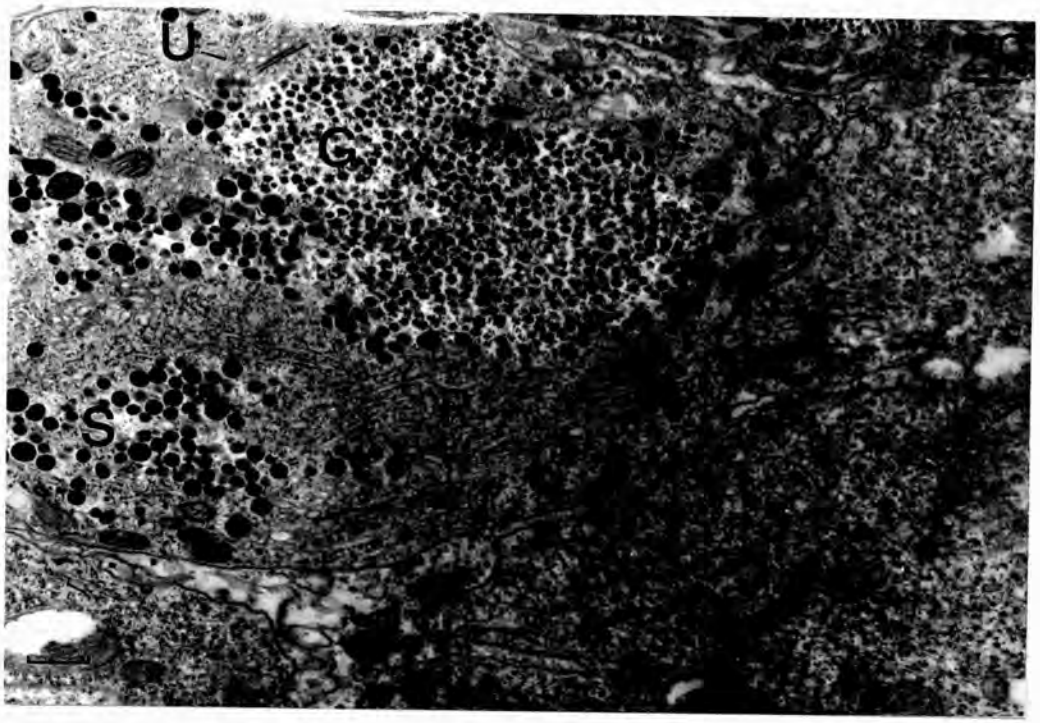
β - β neurosecretory cell granules

a - aorta wall

rn - recurrent nerve

l - aortic lumen

(Preparation procedure: Luft's epon)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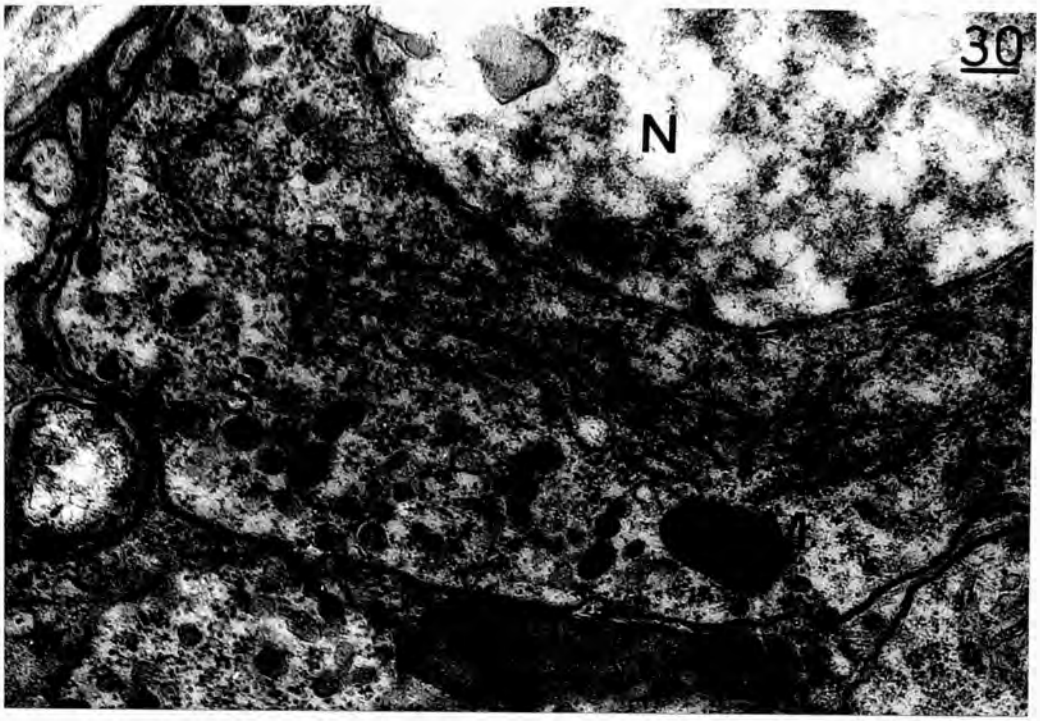
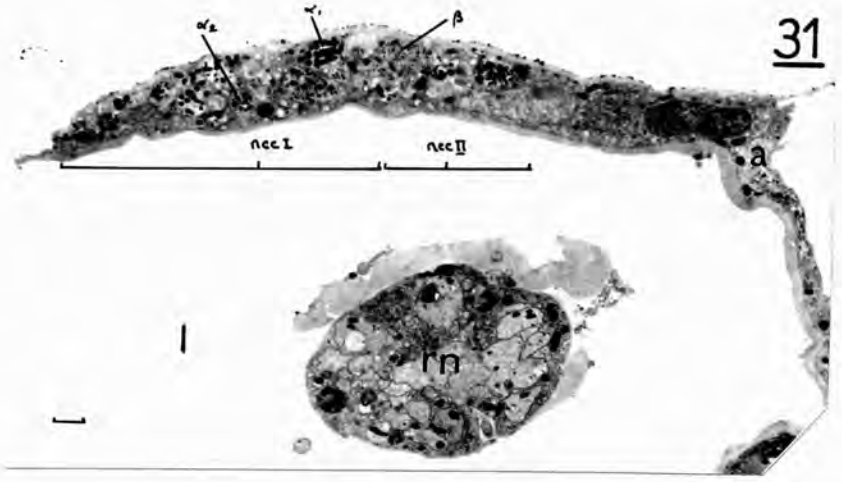

30
N


31
α2
α1
β
nec I
nec II
a
l
rn

2. Axon Pathways of the Brain's Neurosecretory Cells

The neurosecretory pathways within the brain are shown in Figures 6 and 7. The connections of these neurosecretory axons with retrocerebral structures are shown in Figures 9 and 10 and represented schematically in Figure 8. The axons forming the nervi corporis cardiaci posterior to the brain are surrounded by an acellular sheath 100 nm in depth and similar in appearance to the brain's neural lamella, which it is continuous with. There is no subjacent cellular layer to this sheath. The size of the granules at the distal end were found to be of similar size to those at the proximal end of the axon and to those within the perikaryon.

As in other insects (review ENGELMANN, 1970), the nerve issuing from the median neurosecretory groups within the brain, in this case the MNC and VNC, is termed the NCC I. Similarly, the nerves of the lateral groups, comprising in the midge the LNC and ONC, are termed the NCC II. Unless otherwise stated, the descriptions below are based upon electron microscope observations.

(a) Nervi Corporis Cardiaci I

Axons of the MNC groups run anterio-ventrally decussating in a frontal median chiasma before turning posteriorly to emerge from the ventral surface of the brain as the paired NCC I which enter the lateral walls of the aorta.

Figure 9. Diagrams of transverse sections through the gut (g), aorta (a) and retrocerebral structures. Anterior to posterior: the NCCI (;) and NCCII (;;) fuse with the wall of the aorta which becomes progressively muscular (stripping). The aortic lumen contains the recurrent nerve (r) which fuses with the NCCI anterior to the hypocerebral ganglion (h). The two nerves running posteriorly from the ganglion fuse anterior to the corpus cardiacum (cc) which lies adjacent to the "glande post-cérébrale antériere" (gp) and the peritracheal tissue (pt), the latter being attached to a branch of the dorsal cephalic trachea (t).

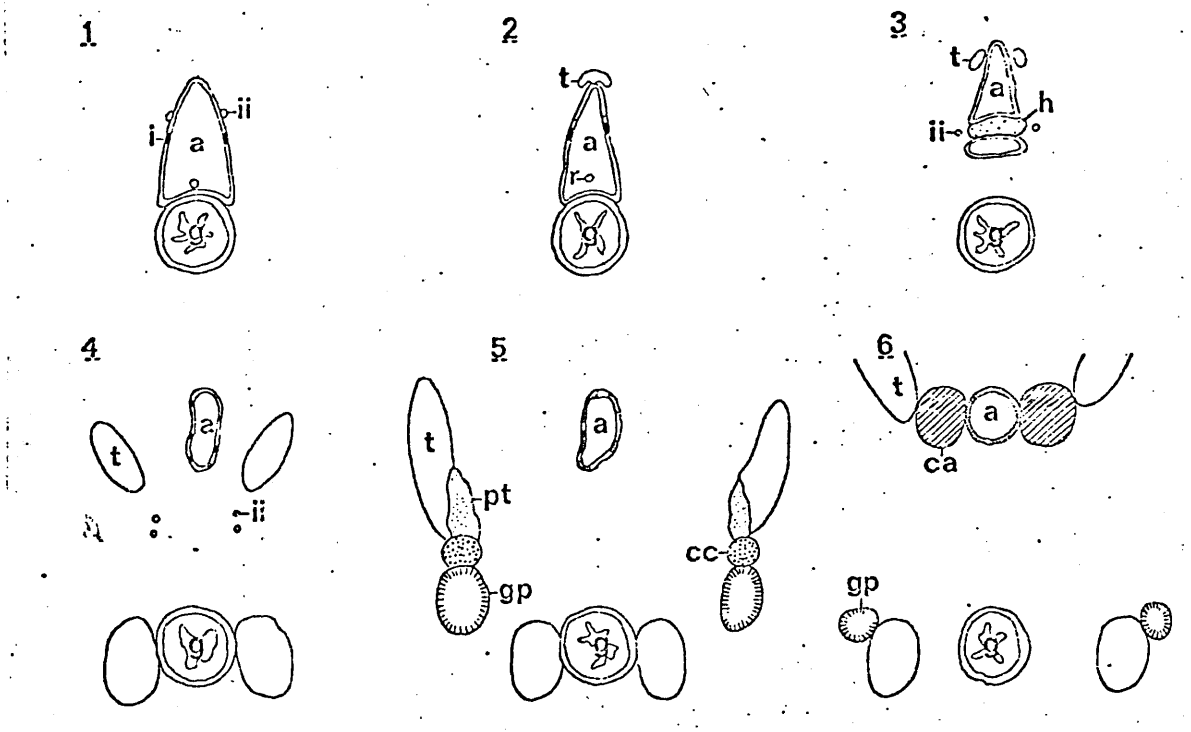
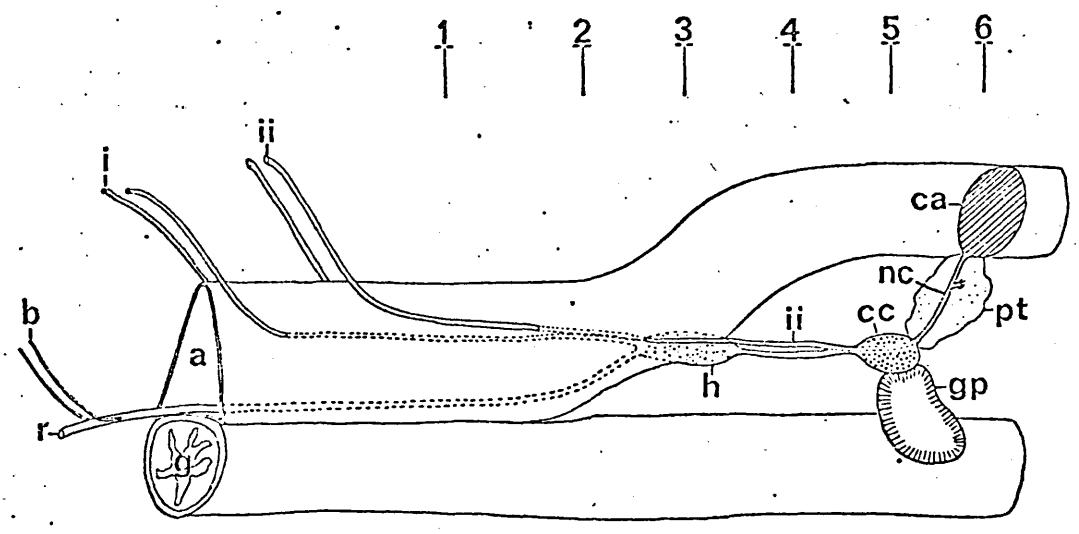


Figure 10. Schematic representation of the retrocerebral structures in relation to the gut and the aorta as seen from the left side. The Arabic numerals correspond to the depths of sections shown in Figure 9. The collateral branches of the MNC (b) fusing with the recurrent nerve, and the NCA (nc) running from the corpus cardiacum to the corpus allatum, are shown. Other labelling is as in Figure 9.



The MNC axons are the only neurosecretory nerves which can be detected in the brain by light microscopy, because of their selective staining with EWEN'S (1962) aldehyde fuchsin.

The axons of each group of VNC run anterio-dorsally to join those of the contra lateral MNC posterior to the chiasma and so comprise part of the NCCI.

The NCC I becomes an integral part of the ventro-lateral aorta wall (Plate 31). Each NCC I typically comprises 13 neurosecretory axons. Of these axons, 10 contain α_1-type granules, 7 or 8 of which issue from the MNC and 2 or 3 from the VNC. The other 3 axons contain the smaller α_2-type granules and issue from the VNC which produce this type of neurosecretory granule. The axons containing the α_2-type granules usually maintain a dorsal position to the other axons of the NCC I. Between 5 and 10 non-neurosecretory, type B axons, also occur in the NCC I. The origin of the axons appears to be the type 3 neurons of the pars intercerebralis.

Before running into the hypocerebral ganglion, the two NCC I fuse with the median recurrent nerve.

(b) Collaterals to the MNC axons

Axons from the MNC bifurcate into large branches over 1 µm across which run to the NCC I and smaller collaterals approximately 700 nm in diameter. The eight

collaterals, containing α_1-type granules, from each MNC group, become aggregated into an axon bundle. The bundle of each side runs dorsally, leaving the brain without decussating through the lower lateral wall of the central canal. It then runs posteriorly on the dorsal surface of the gut, just lateral to the aorta. The axon bundle of each side then passes through the wall of the aorta, entering the lumen, where they both fuse with the single recurrent nerve, which, up to this position, has not contained neurosecretory granules. The axons no longer remain aggregated but become distributed amongst the non-neurosecretory axons of the recurrent nerve. After the fusion of the NCC I with the recurrent nerve, these collaterals are indistinguishable from other axons containing α_1-type granules, making it impracticable to differentiate their terminations.

(c) Nervi Corporis Cardiaci II

Axons from both the LNC and the ONC run towards the midline of the brain, entering the neuropile where they combine to form the NCC II of each side. These nerves leave the brain's ventral surface posterior to the emergence of the NCC I. The NCC II runs posteriorly along the lateral wall of the aorta (Plate 32) before fusing with it behind the brain. It maintains a dorsal position in the aorta wall to the NCC I (Plate 31), even though they

ELECTRON MICROGRAPHS

The aorta of the neoimaginal male (20°C).

Plate 32 : Transverse section of the aorta and gut in the neck region, showing the NCC I as an integral part of the aorta wall and the right NCC II just anterior to fusion.

SCALE BAR : 1µm.

KEY:

A - aortic lumen

L - gut lumen

R - recurrent nerve

W - gut wall

i - NCC I

ii - NCC II

Plate 33 : Transverse section of the hypocerebral ganglion showing aggregates of neurosecretory granules.

SCALE BAR : 1µm.

KEY:

A - aortic lumen

b - glial cell

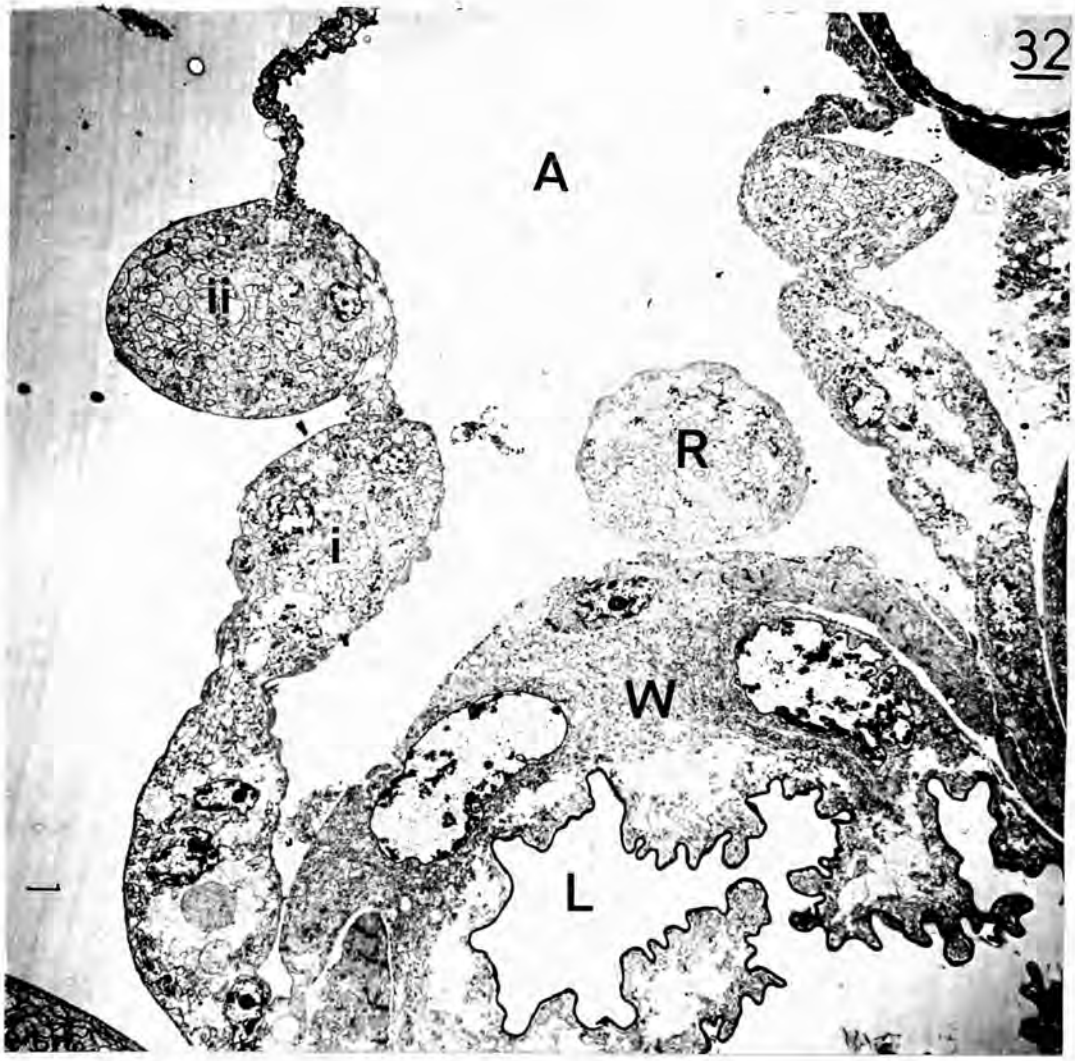
c - neuron

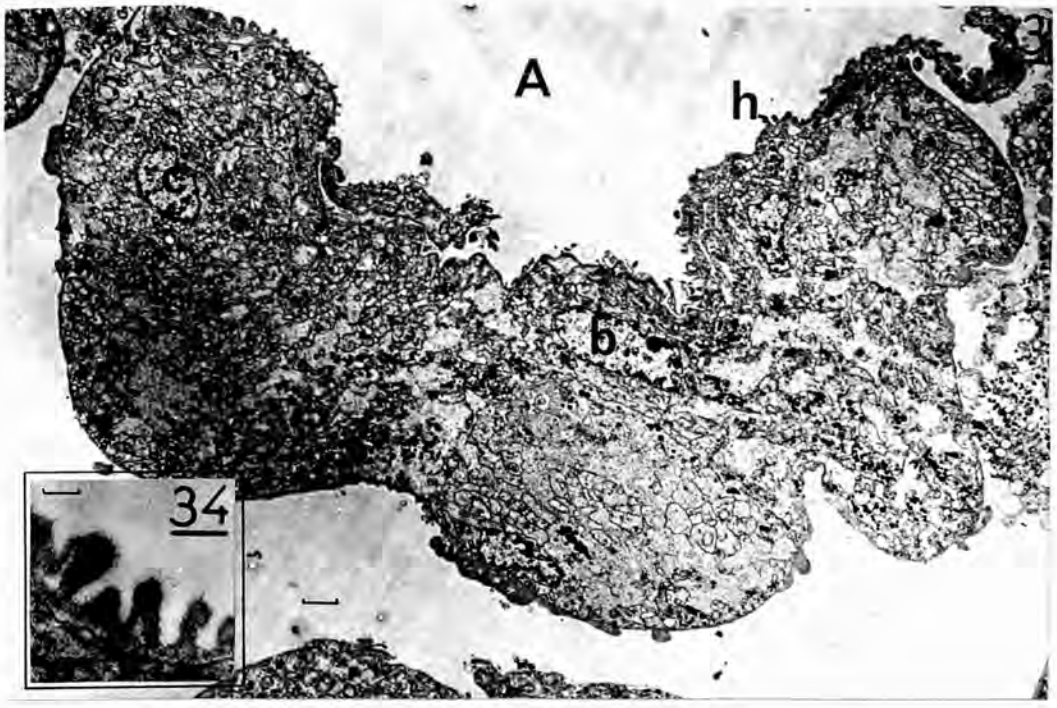
h - bounding sheath

Plate 34 : Detail of the sheath bounding the aortic lumen showing villi containing dense granular aggregates.

SCALE BAR : 200nm.

(Preparation procedure: Spurr's epon)


32
A
ii
i
R
W
L


3
A
h
b
34

may be adjacent. Of the 6 - 10 neurosecretory axons of each NCC II, a maximum of 4 contain β-type granules, the remainder containing α_2-type granules. As there are often twice as many axons containing α_2-type granules as there are α_2-type cells in an ONC group, it is possible that the axons from these neurons bifurcate. Although the axons have been traced to the ONC region, it is impracticable to find the origin of each axon, to discover with certainty that two axons issue from a single cell. The NCC II also contains between 10 and 15 non-neurosecretory type-B axons, whose origins are unknown. Anterior to the fusion of the NCC I with the recurrent nerve, most, if not all, the axons of both NCC II leave the aorta walls and run laterally on each side of the hypocerebral ganglion in the haemocoel. Each NCC II fuses with an epsilateral nerve issuing from the posterior of the hypocerebral ganglion, just anterior to the corpus cardiacum.

(d) Collaterals to the LNC axons

The axon of each LNC bifurcates into two equal branches each approximately 750 nm across. One branch runs to the NCC II, the remaining branch runs with 2 or 3 other similar collaterals containing β-type granules, into the optic lobes, where they were traced for a short distance.

3. The Frontal Ganglion

The frontal ganglion is a single median mass of approximately 100 cells, attached to the mid-dorsal surface of the anterior foregut (Plate 40), measuring about 25 x 40 x 40 µm. Laterally a frontal connective on each side joins the ganglion to the tritocerebrum of the brain. A single recurrent nerve issues from the posterior of the ganglion.

The anterior head capsule, where this ganglion is situated, is heavily cuticularized, which prevented the cutting of ultrathin sections from embedded heads. The brain and much of the retrocerebral complex can be dissected from the head capsule, but it was found that the anterior region of the foregut could not be removed without destruction of the ganglion. Descriptions of this ganglion are therefore based upon light microscope observations. The occurrence and distribution of glial cells which can only be identified with certainty, in the midge, with the electron microscope, is unknown. The cells of the ganglion are similar to the "medium" sized neurons of the brain and are therefore, most probably, type 3 neurons. Gomori stains do not indicate the presence of neurosecretory cells and there is no electron microscope evidence of neurosecretory granules in the anterior of the recurrent nerve. The two frontal connectives which have been observed with the light microscope, show no indication of neurosecretion.

4. The Recurrent Nerve

The single recurrent nerve (Figures 8, 9, 10) joins the frontal to the hypocerebral ganglion. At its anterior, it is composed of approximately 40 non-neurosecretory type B axons and it is surrounded by a 50 nm thick neural lamella with a subjacent layer of glioplasm. After leaving the frontal ganglion, it runs through the haemocoel before becoming attached to the mid-dorsal surface of the gut in the anterior region of the brain. It runs into the gut musculature and becomes intimately associated with the epithelium of the gut wall. Before entering the anterior opening of the aorta, it runs on the mid-dorsal surface of the gut. The recurrent nerve forms an integral part of the ventral wall of the anterior aorta. Whilst passing through the central canal of the brain, collaterals from the MNC groups of each side of the brain fuse with it. Posterior to the brain, the recurrent nerve leaves the ventral wall and migrates dorsally in the lumen of the aorta (Plate 32). The surrounding neural lamella in this region is 500 nm in depth and lacks a subjacent glial layer. In the neck, axons of the NCC1 and possibly a few from the NCC II fuse with the recurrent nerve just anterior to the hypocerebral ganglion.

5. The Hypocerebral Ganglion

The hypocerebral ganglion contains as few as twenty cell bodies, it measures approximately 15 x 40 x 40 µm. The anterior half of the ganglion forms an integral part of the ventral aorta wall (Plate 33). Its posterior region is situated ventral to the aorta wall, as shown in Figures 9 and 10. The ganglion is surrounded by an acellular neural lamella which has an irregular thickness and is reduced on the border of the aortic lumen. In the posterior half of the ganglion, fat body cells often occur adjacent to this lamella. Perineurial cells, resembling those of the brain are not found, but an incomplete investment with cells similar to glial type 2 occurs. The ganglion is not clearly divided into a neuropile and cortical layer. The non-glial cells and their nuclei are irregular in shape. Each nucleus is approximately 5 µm across and contains diffuse and aggregated chromatin, the latter often being marginal. The grey nucleoplasmic background is surrounded by a crenulated membrane with a variable thickness. Ribosomes are numerous, both free in the cytoplasm and encrusting cisternae of endoplasmic reticulum. A few mitochondria and many glycogen rosettes are distributed in the cytoplasm. Golgi units are infrequently encountered. Although somewhat similar in size to type 3 neurons of the brain, they

do not resemble them in distribution or frequency of their organelles.

The ganglion contains numerous aggregates of neurosecretory material, mainly of α_2-type granules. The nerves leaving the ganglion posteriorly contain only α_1-type granules, indicating that the axons from the α_2-type cells of the VNC terminate here, as shown in Figure 8.

In the anterior half of the ganglion the neural lamella, 100 nm or less in thickness, which forms the ventral boundary of the aortic lumen, forms numerous short villi. When adjacent to axons with neurosecretion in them, the villi often contain single, round, electron-dense bodies, 100 - 150 nm in diameter. These bodies are similar in size to α_2-type granules, but larger than glycogen rosettes measuring 50 - 75 nm across which also occur subjacent to the neural lamella. At high magnifications it is revealed that the bodies are comprised of finely granular material and are not delimited by a surrounding membrane, (Plate 34). Under some staining conditions, the neurosecretion in the adjacent axons also appears to be composed of finely granular material, but it is surrounded by a limiting membrane.

A pair of nerves issues from the posterior of the ganglion and joins it to the paired corpora cardiaca.

Each nerve contains approximately 30 axons, many of which contain α_1-type granules. Anterior to the corpora cardiaca, the nerve of each side fuses with the ipsilateral NCC II.

6. The Corpora Cardiaca

The position and innervation of the corpora cardiaca are shown in Figures 8, 9 and 10. Each cardiacum consists of a neuropile bounded on its outer lateral surface by a single layer of loosely associated cells. The complete structure is enclosed within an acellular sheath, usually 100 nm in thickness, but in some areas as much as 500 nm, which is similar to the brain's neural lamella.

Each cardiacum (Plate 36) contains up to 10 similar pear-shaped cells measuring approximately 10 x 10 x 40 µm. In wax sections stained with EWEN'S (1962) aldehyde fuchsin, the 5 µm diameter nucleus appears a brilliant orange surrounded by a pale green cytoplasm, which frequently contains large quantities of purple granules representing neurosecretion (refer to Appendix 3). The electron microscope reveals a marginal nucleolus, 2 µm across, as well as smaller aggregations and much diffuse chromatin. The nucleoplasmic background is typically white and surrounded by a smooth regular membrane. Small, intra-nuclear particles similar to those described by NORMANN (1965) were not observed. The cytoplasm has the same characters as the common

LIGHT MICROGRAPHS

Plate 35 : Horizontal section of the brain of a neoimaginal (20°C) male stained with alcian blue/Schiff's, showing the green colouration of the MNC.

SCALE BAR : 10µm.

KEY:

arrow - MNC

x - central complex

Plate 36 : Transverse section of the retrocerebral glands of a neoimaginal (20°C) male stain with Ewen's aldehyde fuchsin.

SCALE BAR : 10µm.

KEY:

a - corpus allatum

c - corpus cardiacum

g - 'gpca'

l - aortic lumen

p - peritracheal tissue

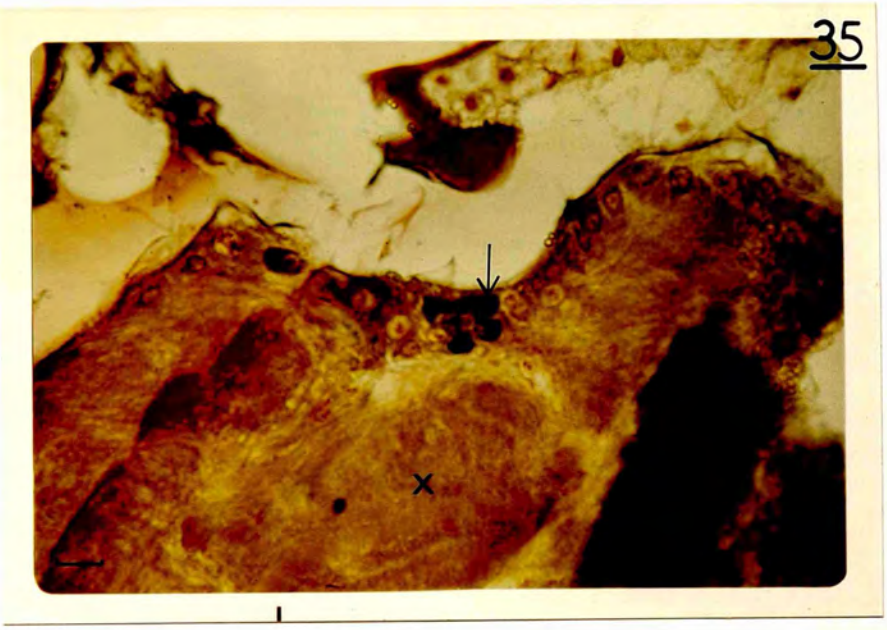
w - gut

Plate 37 : Transverse section of the 'gpca' and corpus cardiacum of a (20°C) male, 6-7 days after emergence, stained with Ewen's aldehyde fuchsin. Note the granular appearance of the 'gpca' as compared to the neoimagine (Plate 36), and the dark staining of the corpus cardiacum cells.

SCALE BAR : 10µm.

KEY:

labels as plate 36.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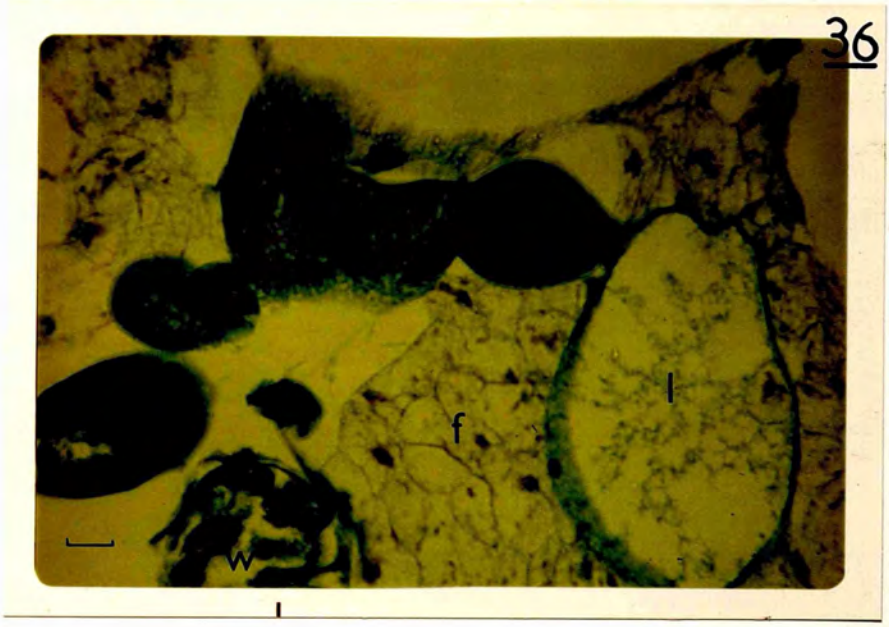
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features of the brain's neurosecretory cells, in addition to which the cisternae of the endoplasmic reticulum are often distended and the mitochondria are frequently long and thin with dense matrices. The electron-dense granules which the cytoplasm contains vary from 70 to 550 nm in diameter (Plate 41) and the population shows a bimodal distribution of sizes (Figure 11). Cell bodies of glial cells and non-neurosecretory neurons do not occur in the cardiaca. The axons of the neuropile region include those from the hypocerebral ganglion, the NCC II and the cardiacum's intrinsic neurosecretory cells. Aggregates of neurosecretory granules (Plate 42), many having a diameter larger than 300 nm (Figure 11), occur in the intrinsic cells' axons. The smaller granules in the axons of the extrinsic neurosecretory cells are not usually aggregated. Evidence for the release of neurosecretory granules was not observed in the cardiacum.

7. Nervi corporis allati

From the dorsal posterior of each of the corpora cardiaca, a nerve, (Plate 38), the nervi corporis allati, runs dorsally, adjacent to the peritracheal tissue to the epsilateral corporus allatum as shown in Figures 9 and 10. Each nerve is surrounded by an acellular sheath, 100 nm thick, resembling the brain's neural lamella. It contains approximately 40 axons, of which up to 4 contain α_2-type granules,

LIGHT MICROGRAPHS

Plate 38 : Section of a corpus cardiacum, stained with Heidenhain's azan, showing the the NCA, of a neoimaginal (20°C) male.

SCALE BAR : 10µm.

KEY:

c - corpus cardiacum cell

h - haemolymph

n - NCA

Plate 39 : Transverse section of the neoimaginal (20°C) male stained with Mallory's trichrome showing the paired corpora allata.

SCALE BAR : 10µm.

KEY:

a - corpus allatum

b - fat body

l - aorta

Plate 40 : Transverse section of the extreme anterior of the foregut, of a neoimaginal (20°C) male, stained with Ewen's aldehyde fuchsin, showing the frontal ganglion.

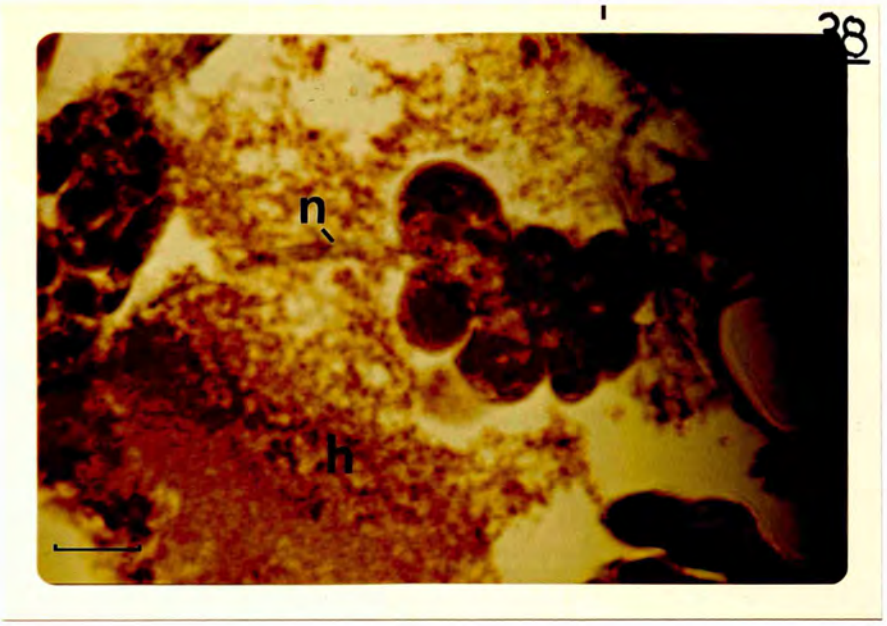
SCALE BAR : 10µm.

KEY:

e - external cuticle of the head

f - frontal ganglion

o - fore gut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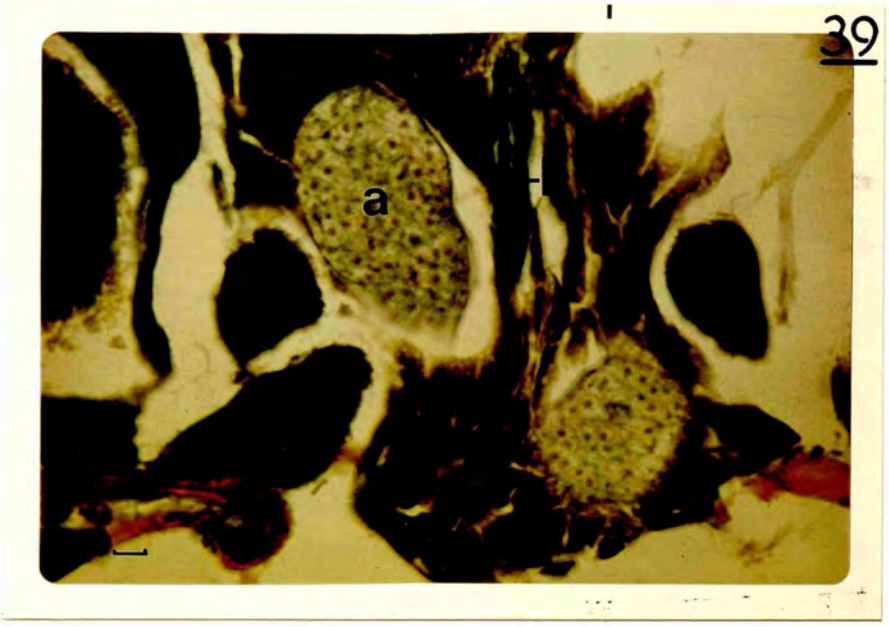
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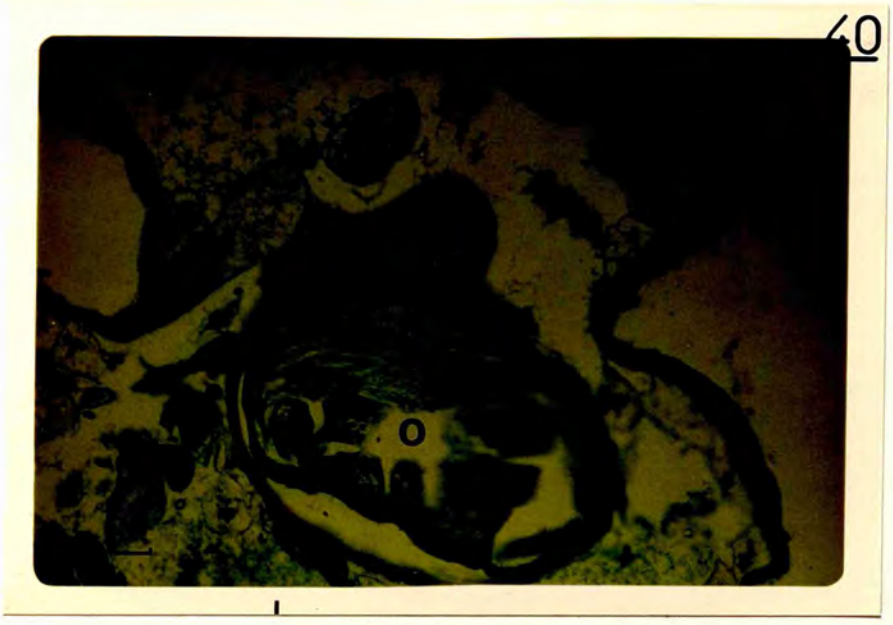
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ELECTRON MICROGRAPHS

The corpora cardiaca of the neoimaginal (20°C) male.

Plate 41 : Section of a corpus cardiacum showing an intrinsic secretory cell and the neuropile region.

SCALE BAR : 1µm.

KEY:

A - neuropile

C - corpora cardiaca cell

n - nucleus

r - granular endoplasmic reticulum

s - intrinsic secretion

Plate 42 : The neuropile region of a corpus cardiacum showing the granule aggregations.

SCALE BAR : 1µm.

KEY:

α_1 - α_1 type neurosecretory granules

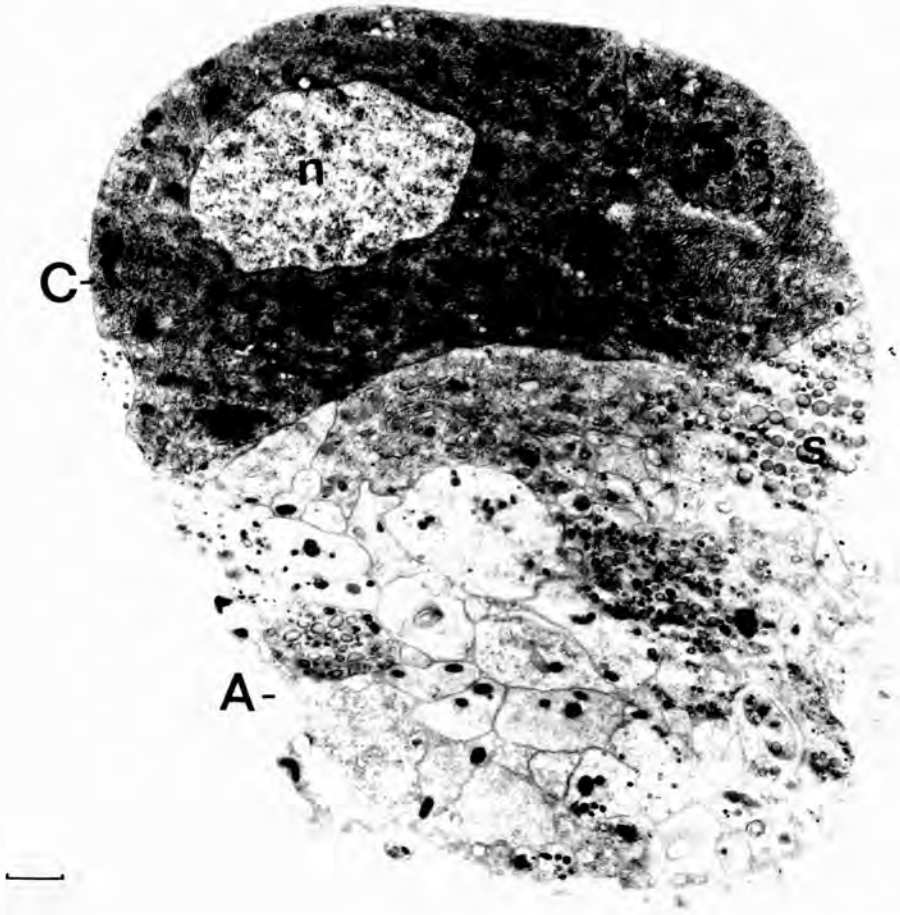
α_2 - α_2 type neurosecretory granules

β - β type neurosecretory granules

s - intrinsic secretion

(Preparation procedure: Luft's epon)

41



42

3 β -type granules and 9 α_1-type granules. The probable origins of these axons are indicated in Figure 8. No axons containing large granules similar to those of the intrinsic cardiacum cells occur.

A small branch from this nerve of up to 10 axons innervates the epsilateral peritracheal tissue. Two or three of the axons have been found to contain α_2-type granules.

8. The Corpora Allata

The spherical corpora allata, approximately 40 µm in diameter, are situated on either side of the aorta at the dorsal extremities of strands of peritracheal tissue, as depicted in Figures 9 and 10.

With the light microscope each allatum appears as a solid ball of approximately 100 small cells, (Plate 39) and resembles the pseudolymphoid type of NOVAK (1975). In wax sections stained with EWEN'S (1962) aldehyde fuchsin the nuclei of these cells appear orange and the cytoplasm green without any purple granules.

The electron microscope reveals that each allatum is surrounded by an acellular sheath, approximately 400 nm thick, which is composed of fine granular material lacking any fibrillar elements. This sheath does not penetrate between the subjacent cells, (Plate 43). Two

ELECTRON MICROGRAPHS

The corpora allata of the neoimaginal (20°C) male.

Plate 43 : Transverse section of the posterior region of a corpus allatum showing the cortical and medullary layers.

SCALE BAR : 1µm.

KEY:

c - cortex

m - medulla

s - sheath

Plate 44 : Section of the marginal zone of a corpus allatum showing the cellular interdigitations.

SCALE BAR : 1µm.

KEY:

g - glycogen deposits

n - nucleus

r - ribosomes

s - sheath

Plate 45 : Detail of the medullary region showing several neurosecretory axons.

SCALE BAR : 1um.

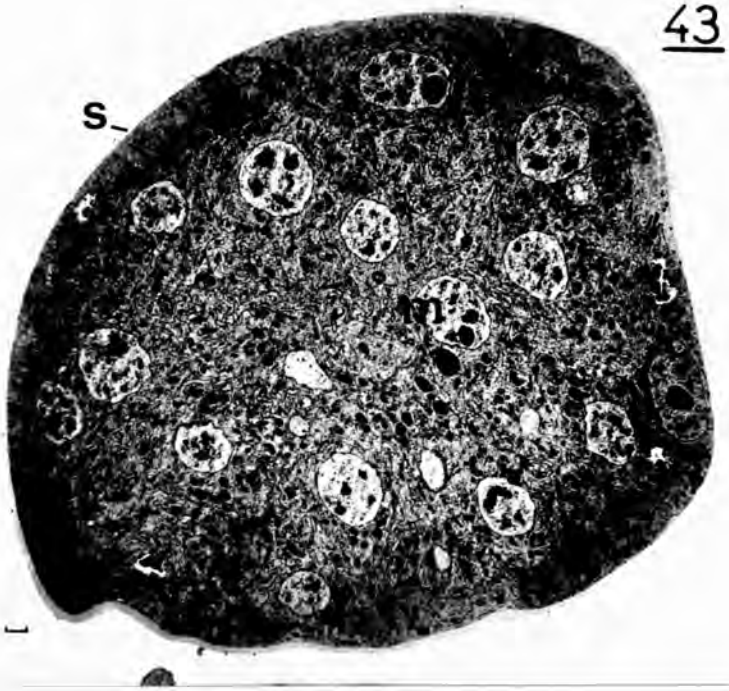
KEY:

α_1 - α_1 type neurosecretory granules

β - β type neurosecretory granules

(Preparation procedure: Luft's epon)

43



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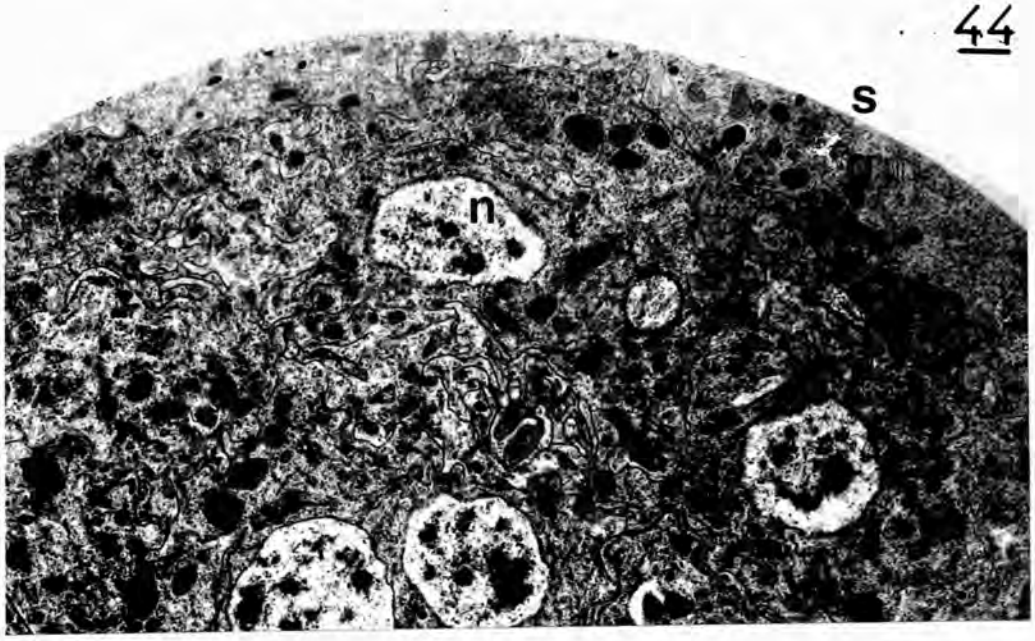

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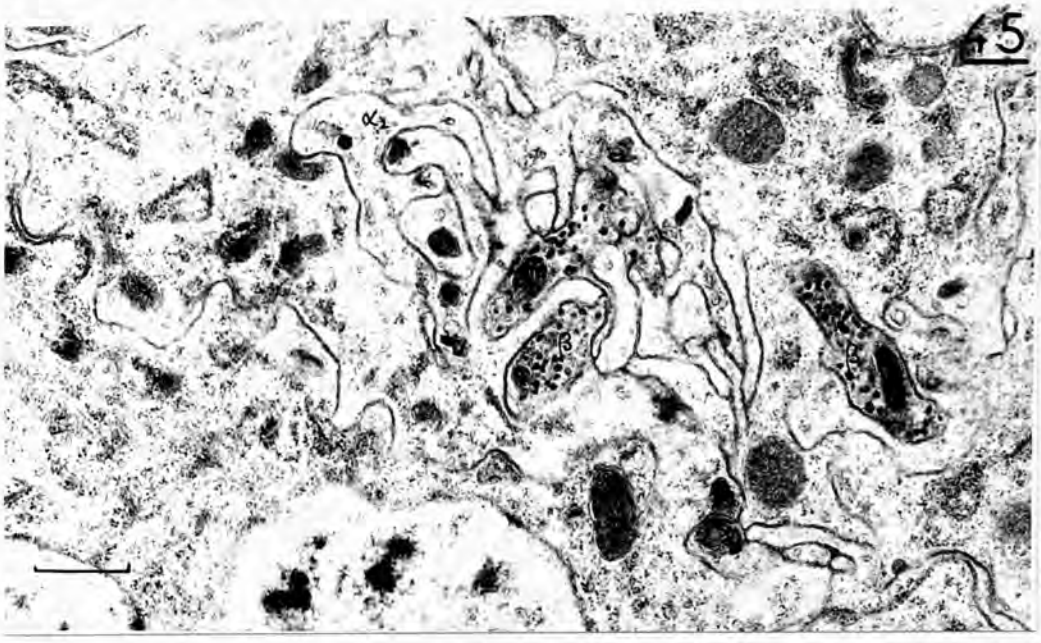


background surrounded by a crenulated membrane of regular width.

Subjacent to the cortical layer are about 100 spheroid medullary cells with a diameter typically of 7 μm. The cytoplasm of the medullary cells has a lower density of ribosomes and contains fewer mitochondria than the cortical cells, but lysosome-like bodies occur more frequently. The other organelles and inclusions resemble those of cortical cells both in appearance and frequency of occurrence. The nucleus resembles that of a cortical cell, except that there is less diffuse chromatin and the nucleoplasmic background is lighter. A considerable proportion of the plasma membrane is linked to that of adjacent cells by tight junctions. No extracellular spaces occur in the allata. Processes of the medullary cells form complex interdigitation between which axons from the nervi corporis allati ramify. The plasma membranes of the axons are linked to the cortical cells by tight junctions. The axons have no glial investment. Axons containing β-type granules are frequently encountered in the medulla (Plate 45) suggesting that the three axons containing this type of granule undergo considerable branching upon entering the allata. Axons containing α_2-type granules (Plate 45) are less frequently encountered. The large α_1-type granules have not

been found in the allata, even though they are present in axons of the nervi corporis allati.

The corpora allata are attached to the aorta wall by a short stalk containing cytoplasm of cortical cells and surrounded by the acellular sheath. The aorta wall is muscular and up to 2 µm thick in this region.

9. The Peritracheal Tissue

The position and innervation of the peritracheal tissue in relation to other endocrine structures is depicted in Figures 8, 9 and 10. It consists of paired strands of tissue, the anterior ends of each are attached to a branch of the dorsal cephalic trachea and the posterior extremity lies adjacent to the epsilateral corpus allatum. Each strand is cylindrical, approximately 30 µm in diameter and 40 - 60 µm long, typically in the shape of an upright "U". Anteriorly, the middle of the "U" lies adjacent to the epsilateral corpus cardiacum and posteriorly to the "glandes post-cérébrales antérieures" of that side.

In wax sections stained with EWEN'S (1962) aldehyde fuchsin, each strand can be seen to consist of approximately 50 cells forming a diffuse tissue, (Plate 36). The cytoplasm of these cells stains pale green with a lighter green nuclei. Each nucleus contains several orange staining deposits, most probably

representing aggregated chromatin. Investing the tissue is a sheath which stains green. The peritracheal tissue is easily distinguished from the adjacent fat body cells, which have colourless transparent cytoplasm surrounded by a distinct cell membrane, when stained with aldehyde fuchsin.

Removal of the retrocerebral complex by dissection invariably destroyed the peritracheal tissue. Electron microscope studies are therefore based upon midges embedded with SPURR'S (1969) epon, in which the tissue may be observed in situ. The cells of the peritracheal tissue are found to vary in electron-density (Plate 46). Those with the greatest electron-lucency appear to have undergone considerable cytoplasmic degeneration, whereas the electron-opaque cells have condensed cytoplasm. The nuclei are of variable irregular shapes, the maximum width typically being 6 µm. The crenulated nuclear membrane has a variable width and encloses a nucleoplasmic background, varying from grey in electron-opaque cells to white in electron-lucent cells. Each nucleus contains much aggregated chromatin, which has a greater electron density than any other so far encountered in the neoimagine. Usually several of the aggregates in each nucleus encompass round areas of electron-lucency (typically 200 - 600 nm in diameter), containing centrally placed granular material. The undegenerated cytoplasm contains many free ribosomes and a few, small, round mitochondria. Cisternae of

smooth endoplasmic reticulum are frequently encountered whereas ribosome-studded cisternae are rare. A conspicuous characteristic of these cells is the large numbers of clear vesicles, 100 - 300 nm in diameter, which are similar to those contained in adjacent fat body cells and, presumably represent lipid droplets. Several lysosome-like multivesicular and multilamellate bodies usually occur in each cell. The cells have long cytoplasmic processes which interweave in the considerable extracellular space of the tissue. An acellular sheath, composed of fine granular material termed external lamina (NOVAK, 1975), completely surrounds the tissue. It varies from 50 to over 400 nm in thickness and in some areas is thrown into finger-like projections, presumably to increase the surface area.

10. The "Glandes Post-Cérébrales Antérieures"

The "glandes post-cérébrales antérieures" are paired structures lying on either side of the gut adjacent to the corpora cardiaca and peritracheal tissue, as shown in Figures 9 and 10. The glands are ellipsoid in shape, approximately 30 x 30 x 50 µm. With EWEN'S (1962) aldehyde fuchsin (Plate 36), the gland stains pale green with a central lighter coloured zone, creating the impression of a lumen. No nuclei are apparent in stained wax sections.

ELECTRON MICROGRAPHS

Plate 46 : Section of the marginal zone of peritracheal tissue, of a neoimaginal (20°C) male, showing the dense chromatin and variable electron-opacity of the cytoplasm of different cells.
SCALE BAR : 1µm.
KEY:

a - neurosecretory axons

b - multilamellate body

c - dense chromatin

d - lipid droplets

n - nucleus

arrows - electron-lucent nuclear areas surrounded by dense chromatin.

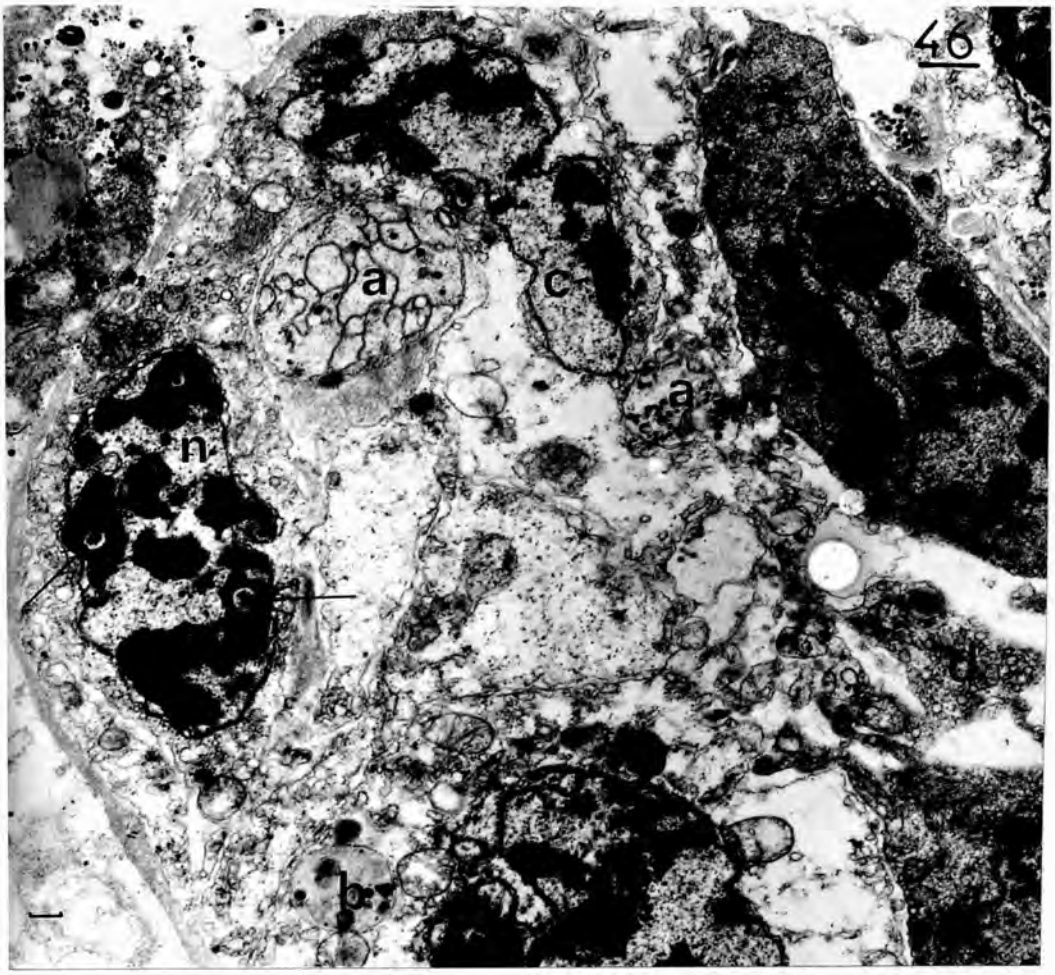
Plate 47 : Section of part of a 'GPCA', of a neoimaginal (20°C) male, showing the infoldings of the external membrane into the body of the gland. Note the encrusting of these infoldings with fine granular material (arrows) in the gland's marginal zone.
SCALE BAR : 1µm.
KEY:

M - matrix

s - saccules of smooth endoplasmic reticulum

v - membrane bound vesicles

(Preparation procedure: Spurr's epon)


46
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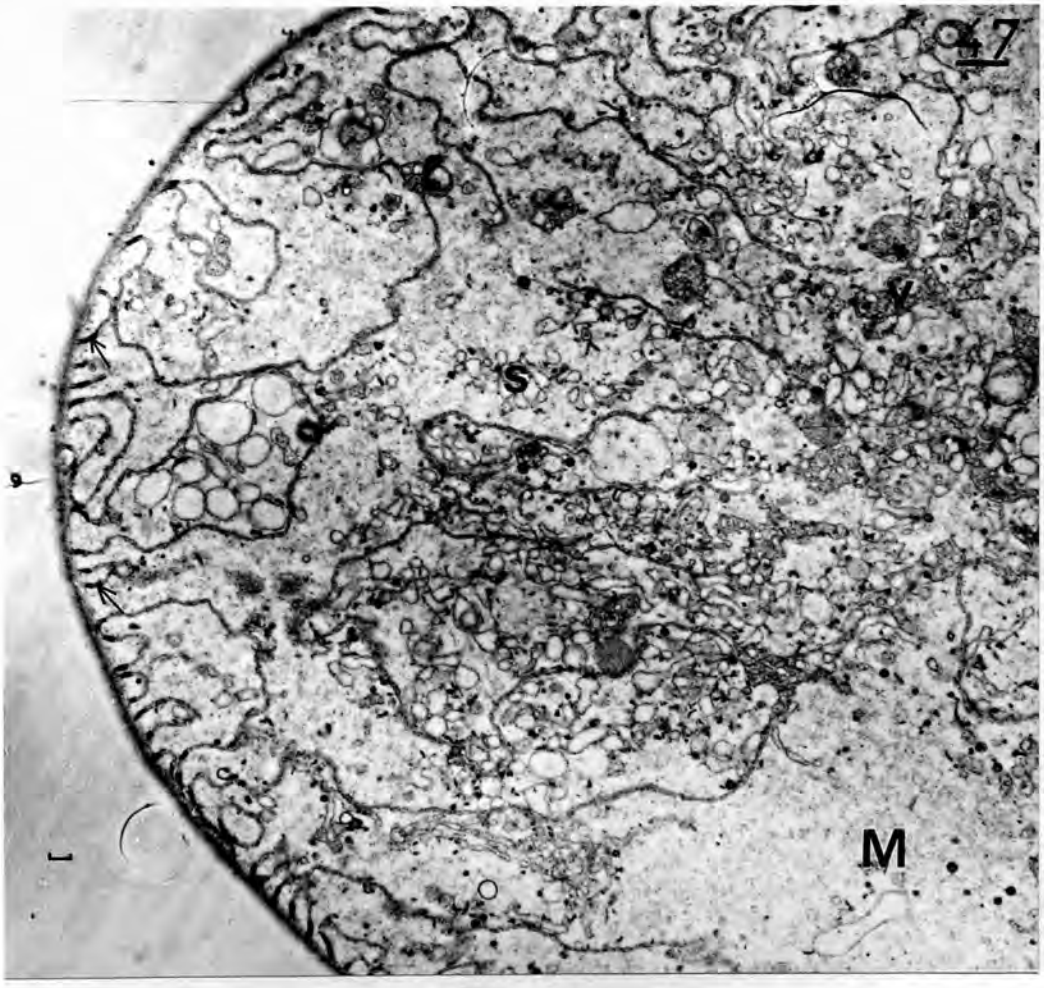
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As with peritracheal tissue, difficulty was encountered in removing these glands, so that the electron microscope observations are based upon tissue embedded in SPURR'S (1969) epon. Fine structural results show that these glands contain no nuclei and are not divided into cells by plasma-membranes. Surrounding each gland is an external sheath, approximately 100 nm in thickness, composed of fine granular material. Subjacent to the sheath, the external membrane of the gland is folded into processes which project deep into the gland (Plate 47), forming a tortuous system of interconnecting channels. In the marginal region of the gland, both cytoplasmic sides of these infoldings are frequently incrusted with fine granular material. The lumen of the processes contains small quantities of granular material. Vesicles varying in size are dispersed in the granular matrix of the gland. Vesicles of 100-250 nm diameter are often found in association with the prolific saccules of smooth endoplasmic reticulum. Frequently, aggregates of these vesicles are enclosed within a double unit membrane which can often be seen to be confluent to the membrane of the processes from the gland's periphery. Larger electron-lucent vesicles with a diameter of as much as 1 µm occur throughout the gland. Particles, the size of ribosomes, occur throughout the granular matrix of the gland but rough endoplasmic reticulum, mitochondria and discrete Golgi units are absent.

These glands are not innervated. Some of the cells of the corpora cardiaca have cytoplasmic projections which abut onto the surface of the gland but do not fuse with it.

D. Other Neurosecretory Structures

Several structures, apparently neurosecretory, have been found in the neoimagine. Their association with the classical insect endocrine system is unknown. For convenience, these heterogeneous structures will be described in the same subdivision.

1. Neurosecretory cells of the suboesophageal ganglion

Two groups of neurosecretory cells have been found in the suboesophageal ganglion. Their size, location, constituent cell types and staining responses are indicated in Figures 6 and 7 and Tables 1, 2, 3 and 4. The cells of both groups exhibit the common features of the neurosecretory neurons of the brain, described above. The groups were arbitarily designated SNC_1 and SNC_2. The SNC_1 cells occur singularly on each side in the anterior region of the ganglion, in the internal dorso-lateral cortical layer of the crura. As the crura is not delimited, these cells may possibly be situated in the ventral tritocerebrum of the brain. In wax sections they were not differentiated by the staining techniques employed. Electron microscope observations show

that these cells differ in several respects from the two classes of brain neurosecretory cells described above. The SNC_1 cells are termed γ class cells. The perikaryon of these cells is irregular in outline and contains a marginal nucleus with a crenulated nuclear membrane of variable width. Most of the chromatin is in diffuse form, a few small aggregates also occur but a discrete nucleolus is absent. The cisternae of the granular endoplasmic reticulum differs from those of brain neurosecretory cells in appearing distended (Plate 48). The γ cells are unusual, as they are thought to contain two types of granules based upon the following reasoning. Cutting a cylindral or flattened granule would result in two types of profiles, being approximately round or oblong in shape. If it is assumed that there is an equal chance of cutting a flattened granule in one of the three spatial planes, there would be twice the chance of obtaining an oblong as opposed to a round shape. As the sections are about 80nm in thickness and the flattened granules appear to measure approximately 200 nm long and 90 nm in diameter, there are four chances of obtaining a round profile and two chances of obtaining an oblong profile in each of the other two spatial planes. Therefore, if only flattened granules were present, there would be an equal chance of obtaining round and oblong profiles. As round profiles are four times as common as oblong, it is concluded that spherical

ELECTRON MICROGRAPHS

Plate 48 : Part of a ɣ-type cell of the SNC$_1$ group, of a neoimaginal (20°C) male, showing the spherical and flattened granules and the distended cisternae of endoplasmic reticulum.

SCALE BAR : 200nm.

KEY:

M - mitochondrion

R - granular endoplasmic reticulum

S - neurosecretory granules

Plate 49 : Section showing several neurosecretory cells which occur in the prothoracic ganglion of neoimaginal (20°C) males. Note the whorled configuration of the endoplasmic reticulum displayed in one of the cells.

SCALE BAR : 0·5µm.

KEY:

N - nucleus

S - secretory granules

U - Golgi unit

W - whorled granular endoplasmic reticulum

Plate 50 : Release site of the prothoracic ganglion's neurosecretory cells axons of a neoimaginal (20°C) male. Note the swollen ending of one of the axons and the reduced thickness of the neural sheath (arrow).

SCALE BAR : 1µm.

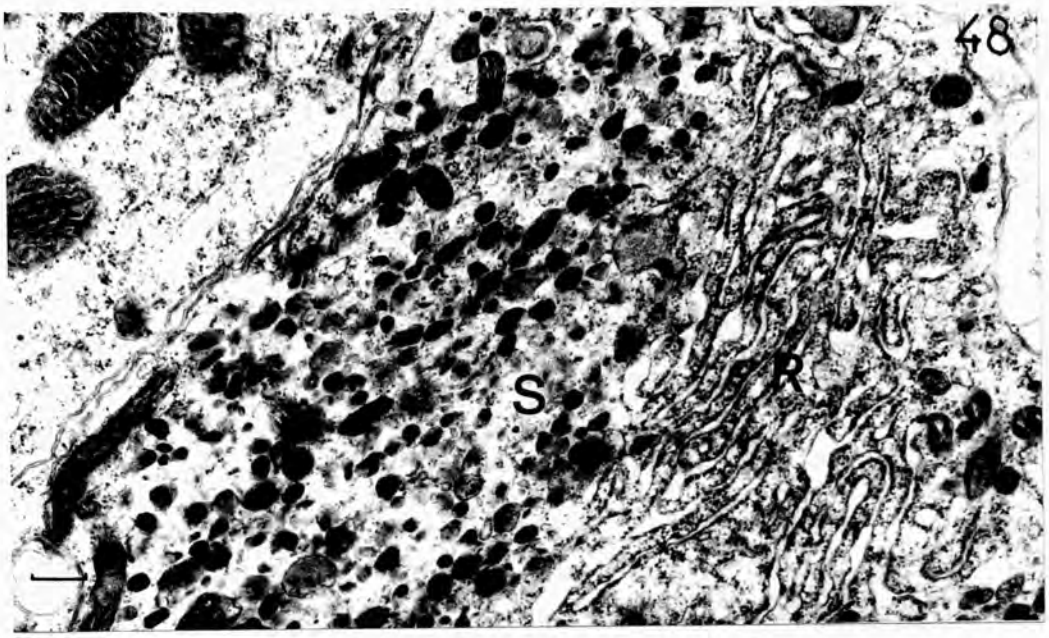
KEY:

H - neural sheath

S - secretory granules

T - trachea

(Preparation procedure: 48, Luft's epon; 49 & 50, Spurr's epon)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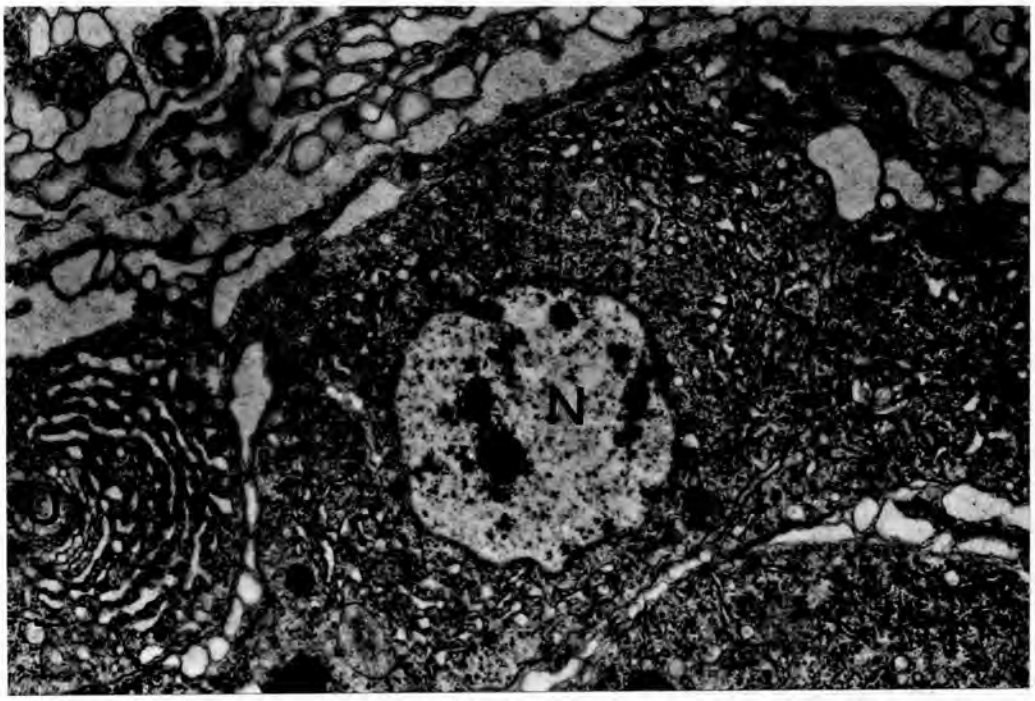
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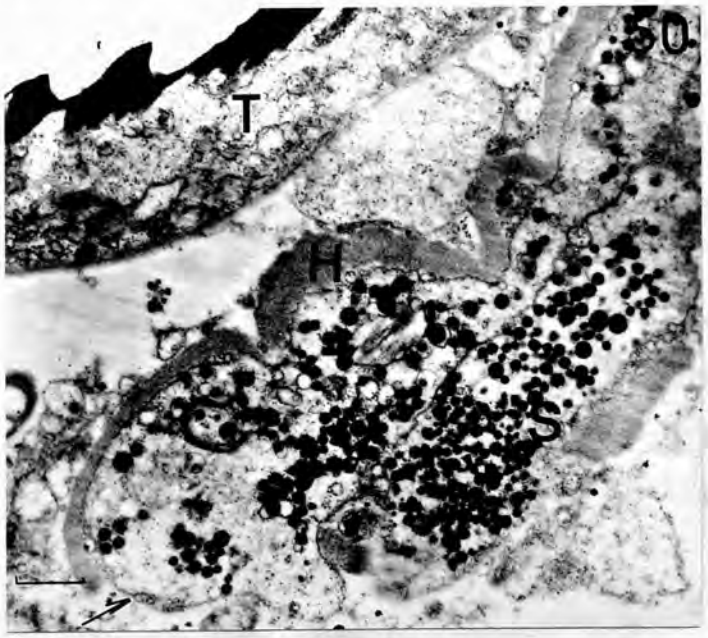
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as well as cylindrical granules occur in the ratio 3 : 1. Axons from these cells run posteriorly and may connect to epsilateral nerves, which contain similar granules in the dorso-lateral walls of the stomadaeum.

The SNC_2 cells occur singularly or in pairs on either side of the midline in the ventral cortical layer of the suboesophageal ganglion. In wax sections, these cells have been found to infrequently contain purple granules when stained with EWEN'S (1962) aldehyde fuchsin. These cells were designated as α_3-type on electron-microscope observations, for although resembling the α cells of the brain, they differ in granule characters (Table 4), especially in electron-density (Plate 28). The axons run posteriorly, but it was impracticable to trace them to their release sites.

2. Neurosecretory cells of the Prothoracic Ganglion

In each side of the anterior prothoracic ganglion in the dorsal, cortical layer, just interior to a large nerve running to the proleg, are a group of 4 or 5 cells resembling the α_1-type neurosecretory neurons of the brain. The cisternae of the granular endoplasmic reticulum is more distended than in the α_1-type cells of the brain and often occurs in whorls at the centre of which are Golgi units (Plate 49). These cells contain very few α_1-type granules which may account for their lack of selective staining with EWEN'S (1962) aldehyde

fuchsin. The characteristic changes in shape of the nerve cord and the size of the perikarya of these cells, allows them to be identified in wax sections treated with aldehyde fuchsin, which stains the cytoplasm a darker green than that of the adjacent type 3 neurons. The greater basophila that this staining reflects, probably results from the denser population of ribosomes in these cells as compared with the adjacent neurons. The axons of these two groups of cells run posteriorly and leave the dorsal surface at the rear of the prothoracic ganglion as a pair of nerves. Each nerve comprises 20 axons, all of which contain considerable quantities of α_1-type granules, surrounded by a sheath, 400 nm in thickness, resembling the neural lamella of the brain. Each nerve runs posterio-dorsally through the haemocoel to the lateral median side of the large "lateral longitudinal" trachea of the same side. Each nerve runs parallel to the trachea, terminating just ventral to it and adjacent to fat body cells in the mesothorax. The axon endings are swollen, typical of neurohaemal areas (MADDRELL, 1974) and the surrounding lamella is thin or absent, which may facilitate release of neurosecretory material (Plate 50). Similar cells were not found in homologous positions in the meso or metathorax.

3. Peripheral neurosecretory cells of the Prothorax

Pear-shaped cells, typically 16 x 8 µm in profile, occur singularly in the lateral fat body on either side of

the posterior prothorax. These cells exhibit the common features of the neurosecretory neurons of the brain, described above. The cisternae of the granular endoplasmic reticulum appear more distended than occurs in the brain's neurosecretory cells. The nucleus, approximately 4 µm in diameter contains a peripheral nucleolus, typically 1 µm across, as well as several smaller aggregates and a little diffuse chromatin. The root apparatus of a scolopidium runs into the rounded pole of these cells, (Plate 52). The single scolopidium of each side, 1 mm or more in length, runs dorsally through the haemocoel and epidermal cell layer to join the cuticle at the posterior-lateral extremity of the pronotum. The electron-dense granules in the cytoplasm (Plate 51), are similar in both range and bimodal distribution of sizes to the corpora cardiaca cells (Figure 11). Each of these peripheral cells gives rise to a group of 3 - 5 axons which contain granules with diameters in the same range as the smaller mode of the cells' bimodal distribution. Each cell and its axon bundle, are surrounded by a sheath, which varies in thickness between 250 and 400 nm, and is similar in structure to the brain's neural lamella. The axons run anteriorly to the ventral surface of a large transverse muscle block in the anterio-ventral region of the prothorax. The axons of each cell branch into several smaller fibres which terminate, subjacent to the muscle block on the ipsilateral side of the midline, in

ELECTRON MICROGRAPHS

The peripheral neurosecretory cells of the prothorax of neoimaginal (20°C) males.

Plate 51 : Cell body of a peripheral neurosecretory cell containing a bimodal distribution of granule sizes.

SCALE BAR : 1μm.

KEY:

A - axons

H - surrounding sheath

N - nucleus

S - secretory granules

Plate 52 : Part of a peripheral neurosecretory cell showing the root apparatus of a scolopidium in close association with the cell.

SCALE BAR : 1μm .

KEY:

C - peripheral cell cytoplasm

R - root apparatus of the scolopidium

Plate 53 : Termination site of the axons from a peripheral neurosecretory cell. Note the swollen ending of the axon covered with a thin neural sheath (arrow), and the small electron-lucent vesicles which are thought to be part of a membrane retrieval system.

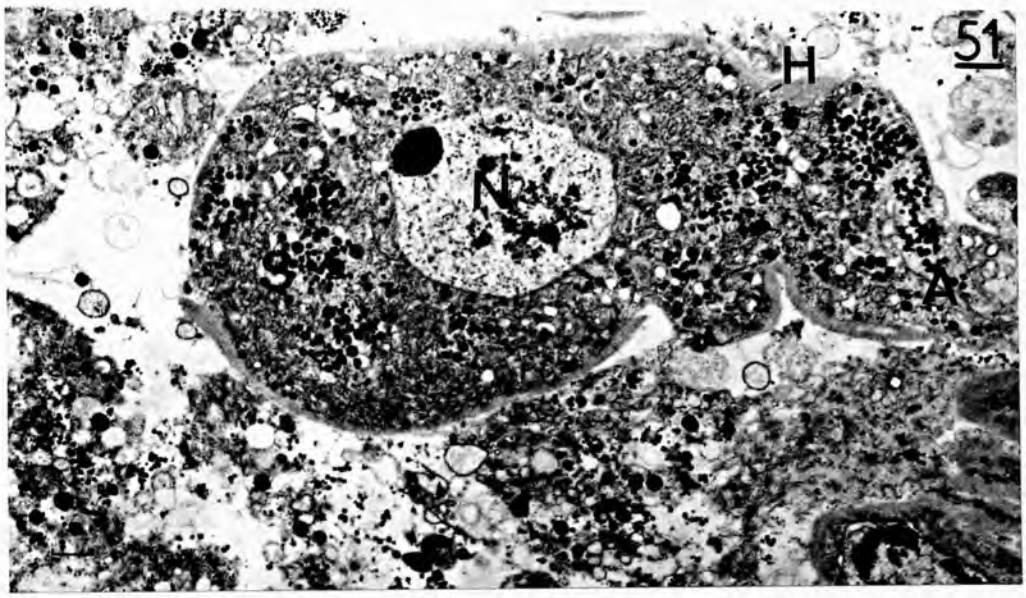
SCALE BAR : 1μm.

KEY:

V - electron-lucent vesicles

Other labelling as Plate 51

(Preparation procedure: Spurr's epon)


51
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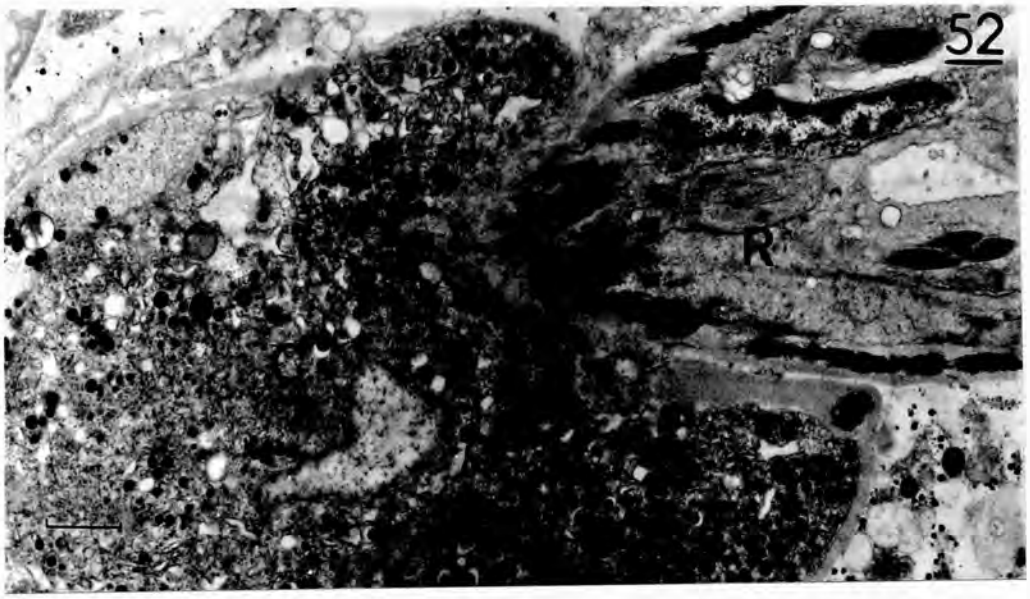
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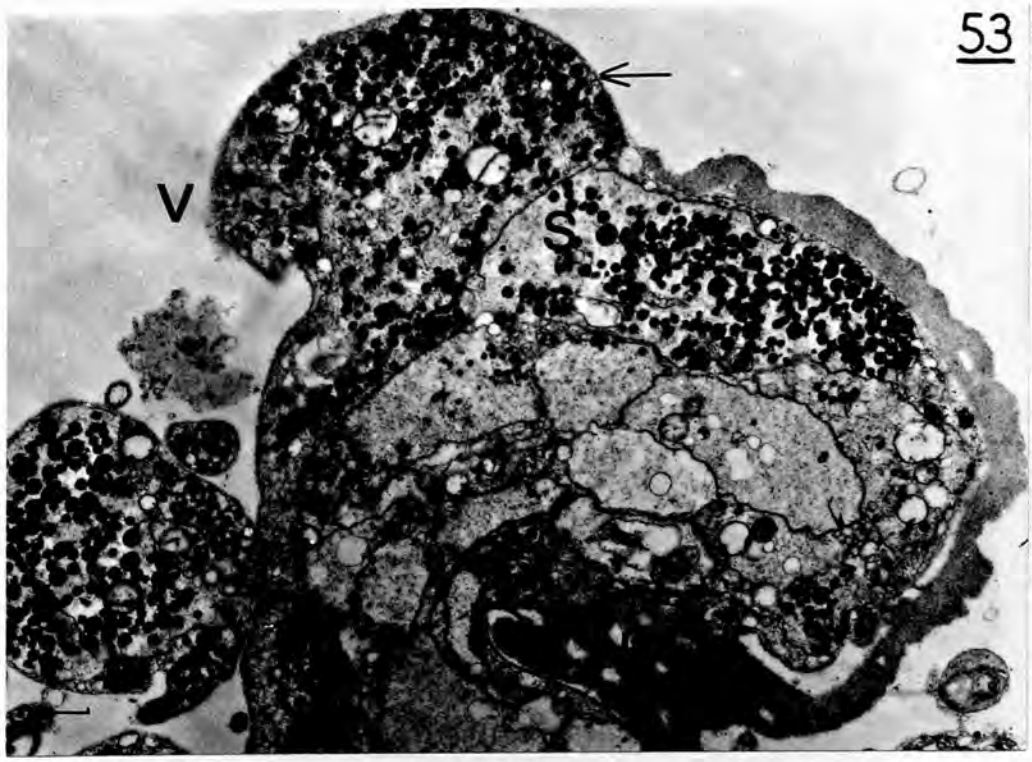
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V
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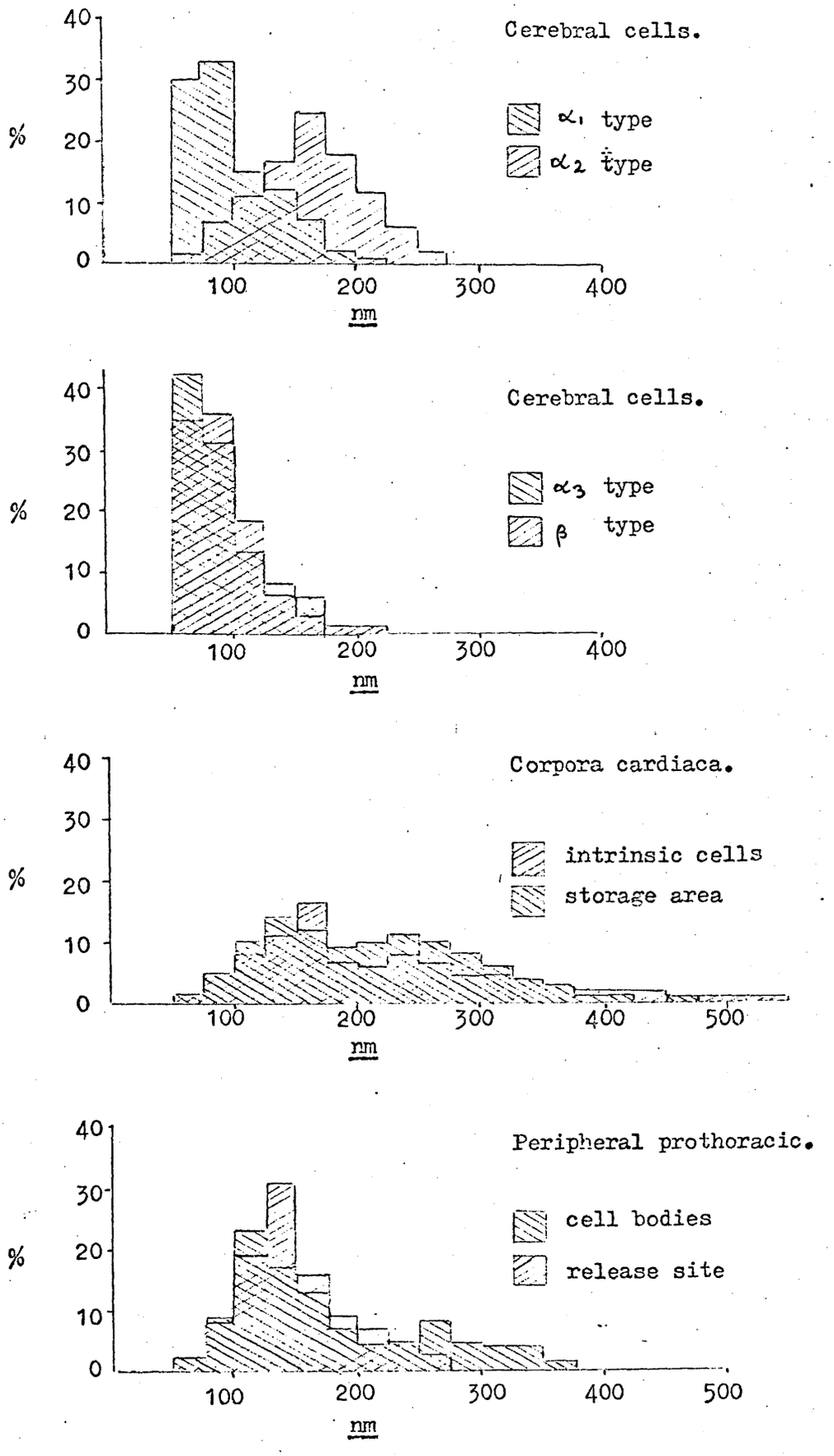


Figure 11. Histograms of percentages (ordinates) of granules within a size range (abscissae in nm) of several neurosecretory structures of the neoimaginal male (summated data, refer to Appendix 4).

swollen endings, the surrounding lamella of which is reduced or absent (Plate 53). The branching of axons and their swollen endings increase the surface area, and are typical characteristics of neurohaemal organs (review MADDRELL, 1974). The swollen endings contain granules in the same size range as present in the axons (Figure 11), and numerous smaller electron-lucent vesicles, 40 - 50 nm in diameter, which are thought to be part of a membrane retrieval system (review MADDRELL, 1974).

In wax sections stained with EWEN'S (1962) aldehyde fuchsin, both cells and neurohaemal sites contained considerable quantities of deep purple material, (refer to Appendix 3), reflecting the large quantities of neurosecretion present. Stained wax sections did not reveal similar peripheral structures in the meso and metathorax.

SECTION II

THE FEMALE

The female lacks the feathery antennae of the male, and although of similar length, is over 150% of its weight (Table 9), probably resulting from the fatter abdomen.

A. The Brain

There are no significant differences in the sizes of the head capsule, the central complex, nor of neuropile masses 4 and 5 between the sexes (Table 10). In morphology, the brain is similar to that of the male, Figures 1 to 4 being equally applicable to the female. Table 7 shows that no difference was detected in field density counts of brain cells between the sexes. There is a great similarity to the types and frequency of the constituent brain cells of the male, as there is in all the fine structural aspects of the non-neurosecretory elements.

B. The Anterior Region of the Ventral Nerve Cord

The regions of the ventral nerve cord studied show a great similarity in morphology and fine structure to those of the male, except for the neurosecretory cells described below.

C. The Stomatogastric and Endocrine Systems

In anatomy, the stomatogastric and endocrine systems are similar to those of the male, Figures 6 - 10 being equally applicable to the female.

The female's brain neurosecretory cells differ in their staining reaction to EWEN'S (1962) aldehyde fuchsin (Table 5 and Appendix 3), and the relative quantities of neurosecretory granules they contain (Table 6). The axons from the MNC groups invariably contain purple granules when treated with aldehyde fuchsin, unlike those of the males where only half the specimens exhibit selective staining (Appendix 5).

The histology and fine structure of the remaining components of the two systems are similar to those of the male. Table 1 shows that the medullary cells of the corpora allata have a nuclear to cytoplasmic ratio similar to that of the male.

D. Other Neurosecretory Structures

1. Neurosecretory cells of the Suboesophageal Ganglion

No selective staining, indicating the presence of neurosecretion, was observed in the neurons of the suboesophageal ganglion. No cell bodies containing neurosecretory granules were observed with the electron microscope in this ganglion, but axons containing γ-type

granules were found in the neuropile indicating the occurrence of SNC_1 cells. The inability to detect neurosecretory cells may result from them containing only small numbers of granules.

2. Neurosecretory Cells of the Prothoracic Ganglion

In wax sections treated with EWEN'S (1962) aldehyde fuchsin, a group of cells in the comparable position, showed a similar staining reaction to that of the male.

3. Peripheral Neurosecretory Cells of the Prothorax

In wax sections stained with aldehyde fuchsin, isolated cells in the fat body having close associations with scolopidia contained no purple material, nor did the release sites of these cells (refer to Appendix 3).

CHAPTER 4

THE EFFECT OF AGEING UPON ADULTS AT 20°C

INTRODUCTION

This chapter is mainly concerned with the effects that ageing has upon the brain and endocrine system in specimens reared and kept at 20°C. To assist in the functional interpretations of the results, observations have also been made upon some aspects of behaviour, fat body depletion and flight muscles. Structural observations upon "old" adults are compared with those of the neoimagines already described in Chapter 3. The implications of these results are discussed in Section II of Chapter 7.

A. Life Span Records

The mean life span of the male was found to be 115 hours (Figure 13), significantly shorter than the 143 hours of the female. The survivorship curves for both sexes (Figure 12), based upon life span data (refer to Appendix 6) are similar in shape to the intermediate type of ROCKSTEIN & MIQUEL (1973).

B. Behavioural Changes

When isolated in specimen tubes, young midges usually cling, upside down, to the gauze covering, although some

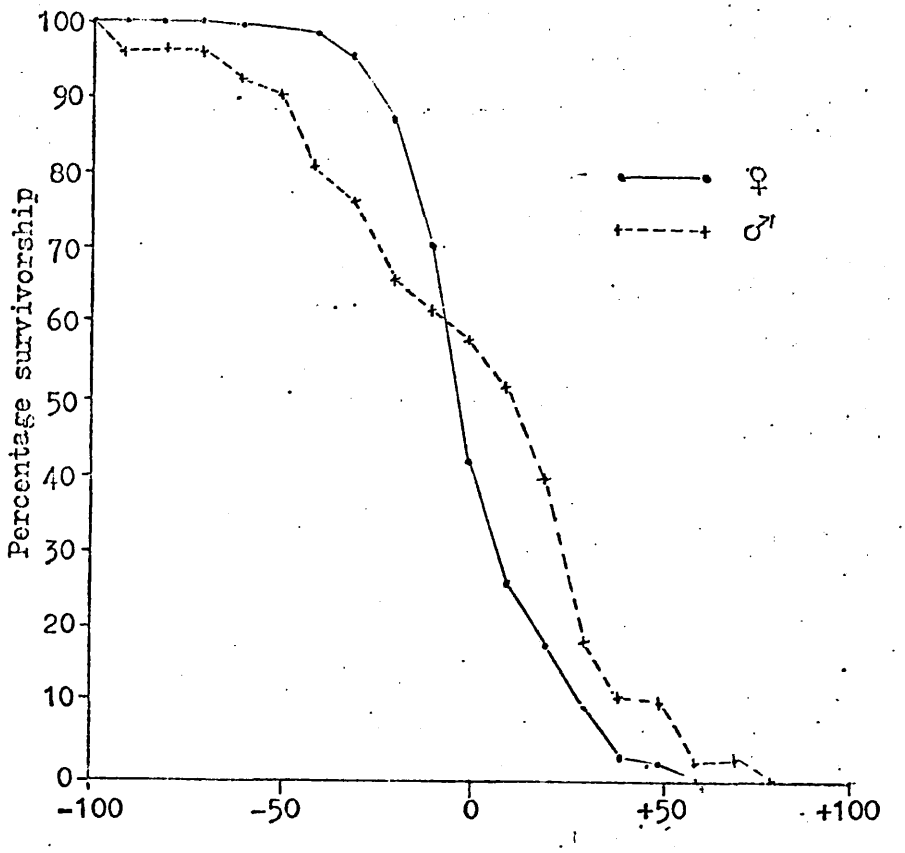


Figure 12. Survivorship curves of males and females reared and kept at 20°C. These life graphs are of the intermediate type, the common characteristic of which is that the curve is concave to the time axis in the first moiety of the life cycle and convex to the same axis in the last moiety.

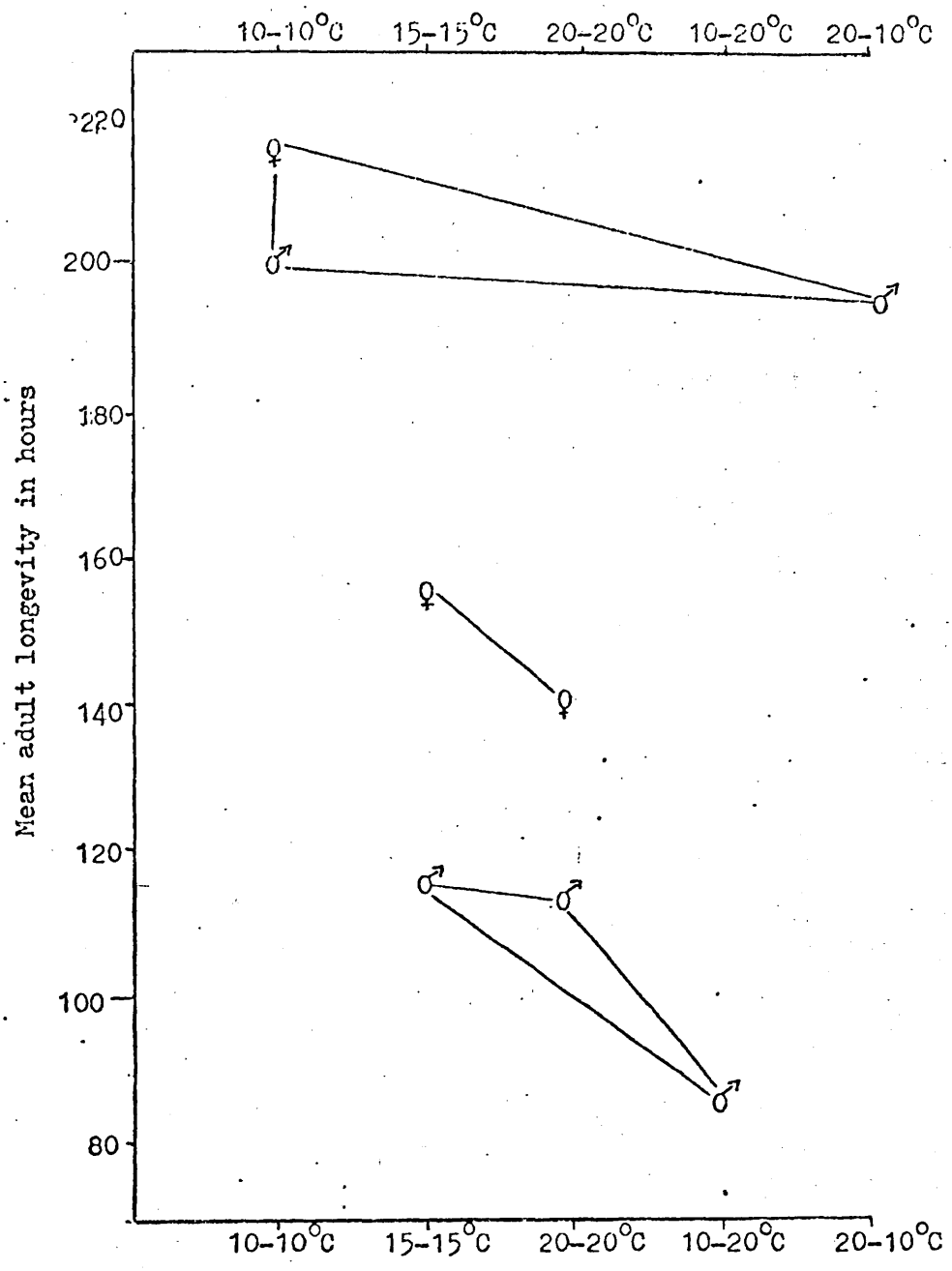


Figure 13. Diagram showing mean longevity of specimens reared (first number) and kept as adults (second number) at several temperatures. The lines connecting the points indicate no significant difference in results at the 5% level.

walking and flying activity occurs. Tapping the side of the tube frequently results in the midge flying for a few seconds before settling on the gauze again. Older animals tend to rest, right side up, on the bottom of the tube. Tapping the side of the tube usually results in a short period of flight, followed by the specimens resuming their former positions. A stage is sometimes observed when no amount of tapping will encourage an "old" midge to fly, the animal tries to maintain itself in an upright position and has great difficulty in righting itself when thrown onto its back. This flight immobility phase lasts for approximately one day, being terminated by death (refer to Appendix 6).

C. External Structural Changes

The abdomen of both sexes becomes narrower and wrinkled with increasing age. Abrasion of the setae and wings is not apparent during the life span.

D. Fat Body Depletion

Stained wax sections of neoimagines show an extensive region of fat body cells in the posterior of the head capsule and the anterior prothorax. With increasing age, these regions become less extensive, which appeared to initially result from a decrease in the cytoplasmic volume, followed by the complete dissolution of the fat body cells. Flight immobile midges showed an almost total lack of this tissue.

TABLE 7. Summary of mean field density counts of cells in the brain.

	Neoimagines				6-7 day old	
	Rearing temperature 10°C		20°C		20°C	
	♀	♂	♀	♂	♀	♂
FD	54	50	55	52	51	52
SD±	10·6	8·7	9·4	8·7	10·0	7·0
No*	10	15	24	30	10	10

FD= mean field density
SD= standard deviation
* Number of specimens used

TABLE 8. Characteristics of the brains' neural lamellae of female specimens reared and kept at 20°C.

Lamella	Days after emergence 1 (6)	3 (2)	7 (6)
Width* nm	300–400	400–500	800–1000
No. of layers	1	1	2
Fibril orientation	Random	Random	oriented

* Measured to the nearest 50nm.
Figures in parentheses refer to the number of specimens used.

E. Structural Changes in the Brain

As no sexual differences have been detected to occur with ageing in the structure of the non-neurosecretory components of the brain, the following descriptions refer to both males and females. Most of the observations on aged specimens are based upon 7 day old midges, the term "old" will therefore refer to animals of this age, unless stated otherwise.

1. Light Microscope Observations

Old midges have a cortical layer of similar thickness to that of neoimagines. The "sponginess" of the neuropile reported in Drosophila melanogaster (MIQUEL, 1971) does not occur in midges of any age.

The staining reaction of non-neurosecretory neurons is similar in old and neoimaginal specimens and field density counts (Table 7) show no significant difference, (refer also to Appendix 3).

2. Electron Microscope Observations

(a) Neural Sheath

Neural lamella - Table 8 shows that the neural lamella increases in thickness arithmetically with age. In old specimens, the lamella is divisible into an external lamina, approximately 200 nm in thickness, composed of finely granular material subjacent to which is a layer up to 800 nm

in thickness (Plate 54). The latter comprises an amorphous matrix in which are embedded fine fibrils, 4 - 6 nm across and up to 1 μm in length. These fibrils have no periodic banding and resemble the randomly orientated fibrils of the single layer neural lamella of neoimagines. In the lamella of old specimens, the fibrils adopt a specific pattern of orientation. Subjacent to the external lamina, a matrical zone of approximately 300 nm in depth contains fibrils whose long axis runs parallel to the anterior-posterior contour of the brain. Below this is a central zone, usually less than 200 nm in depth, the fibrils of which run parallel to the transverse contour of the brain. The most internal zone, lying exterior to the perineurial cells is similar in width and fibril orientation to the external zone.

Perineurium - The perineurium of "old" specimens is of similar thickness to that of neoimagines, but its cells contain a much smaller population of free ribosomes. The perineurial cells of old specimens contain fewer mitochondria, which are usually round, 1 - 3 μm in diameter and have a greater matrical density than those of the neoimagines, which are typically oval. In old specimens some perineurial cells show extensive focal cytoplasmic degeneration, which does not occur in neoimagines.

(b) Glial cells

All three types of glial cells contain smaller populations of

ELECTRON MICROGRAPHS

The brain of (20°C) male specimens 6-7 days after emergence.

Plate 54 : Section of the neural sheath, showing the neural lamella which unlike that of neoimagines (Plate 6) is divisible into two layers.

SCALE BAR : 100nm.

KEY:

E - external lamina

f - fibril

M - mitochondrion of a perineurial cell

Plate 55 : Part of a Glial type 4 cell, which is not found in the brains of neoimagines, showing the small nucleus and abundant smooth endoplasmic reticulum.

SCALE BAR : 200nm.

KEY:

A - smooth endoplasmic reticulum

M - mitochondrion

N - nucleus

Plate 56 : Section of a type 4 neuron showing areas of cytoplasmic degeneration and disrupted mitochondria.

SCALE BAR : 1µm.

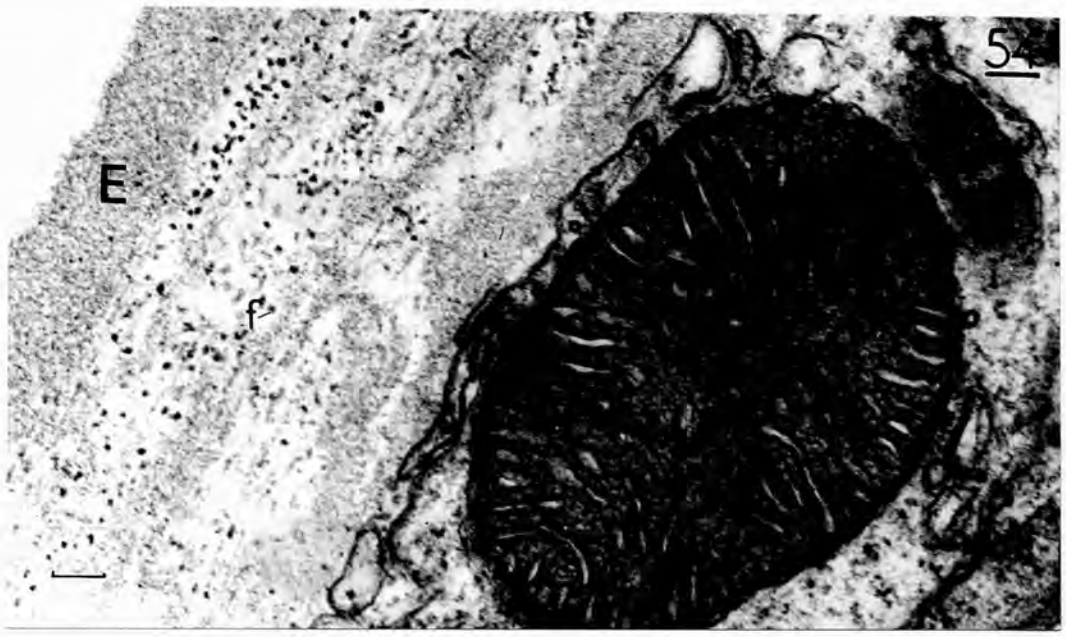
KEY:

D - disrupted cytoplasmic components

M - intact mitochondrion

N - nucleus

(Preparation procedure: Luft's epon)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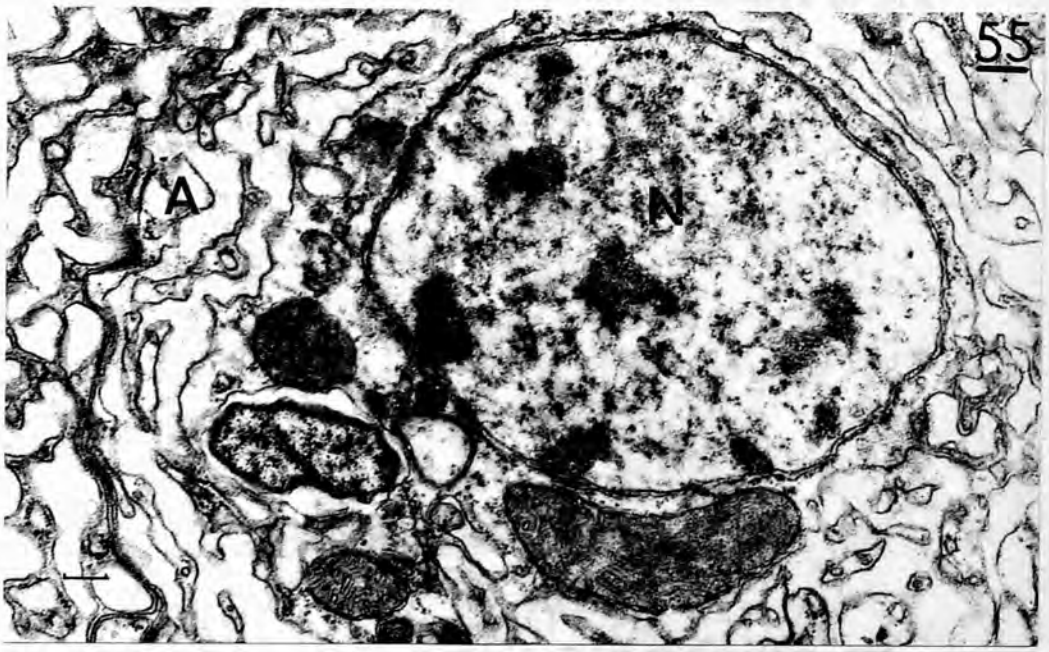
54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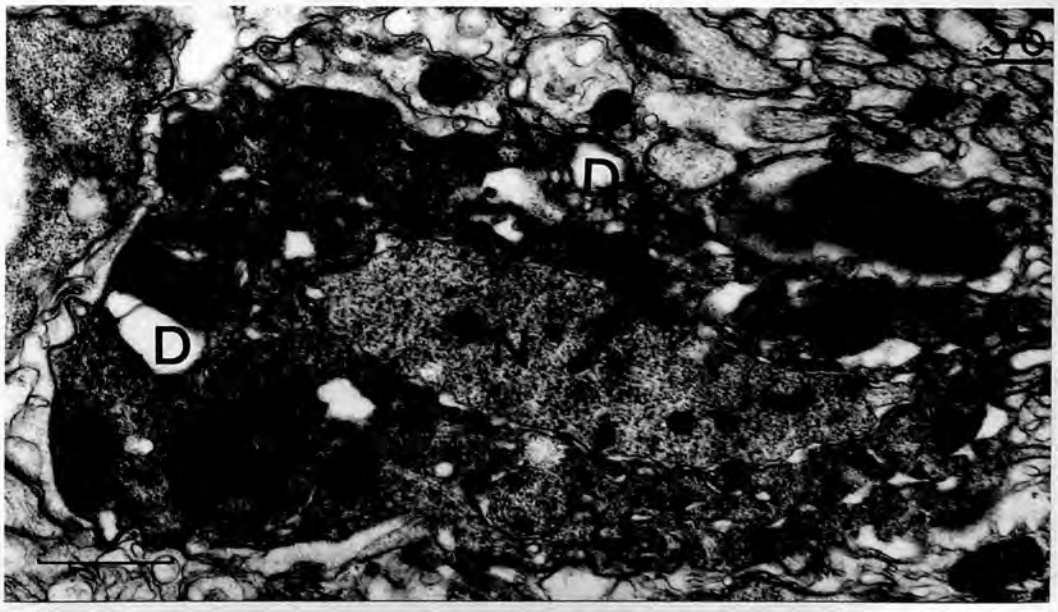

D
D
56

free ribosomes and have mitochondria with denser matrices in the brains of old midges, as compared with neoimagines. Areas of focal cytoplasmic degeneration are much more frequent in the glial cells and the glioplasmic processes between the cortical perikarya are much larger, in the brains of old specimens.

In the dorsal cortex of the pars intercerebralis of old specimens, one or two glial cells occur which are not found in neoimagines. These "type 4" glial cells are unlike those described in Chapter 3, in containing large quantities of smooth endoplasmic reticulum, (Plate 55), the cisternae of which appears distended. The cytoplasm bounding the nucleus, which lacks the smooth endoplasmic reticulum, contains a few small mitochondria, under 200nm across, which have matrices with a low density. This region also contains a few cisternae of granular endoplasmic reticulum. Several Golgi units typically occur. The nucleus is small, 1 - 2 µm in diameter, and contains mainly diffuse chromatin and a few small aggregates. The nucleoplasmic background is white, surrounded by a crenulated membrane with a regular width. The processes of these cells separate perikarya of the cortical layer.

Several cortical cells in the brain of old specimens are surrounded by concentric arrays of double membranes (Plate 58) which may represent multiple glial lamellae.

The width of each unit membrane is typically 200 nm and the separation between the membranes is often the same width. Multimembranous groupings have been found containing a central lumen, or just consisting of an array of concentric membranes. Similar lamellar structures have not been observed in the brains of neoimagines.

(c) Extracellular spaces

Unlike in neoimagines, the brains of old specimens contain extensive extracellular spaces both in the cortical layer and the neuropile. These spaces often contain areas which are electron-lucent surrounded by an amorphous finely granular material. In the neuropile, the largest spaces frequently occur adjacent to type-D, giant axons.

(d) Tracheal system

There is no apparent difference in the brain's tracheal system between neoimagines and old midges.

(e) Neurons

There is no detectable difference in the size of the perikarya of non-neurosecretory neurons between old specimens and neoimagines. Type 2 neurons are more common in the brains of old specimens but there is no marked difference in the frequency of the other types as compared with those of neoimagines.

The cytoplasmic differences between the neurons of neoimagines and old specimens occur in all five types of non-neurosecretory nerve cells. In old midges, focal cytoplasmic degeneration is more frequent (Plate 56), being particularly associated with Golgi units and endoplasmic reticulum, the cisternae of which are often more distended than those of neoimagines. The neuronal mitochondria of old specimens often show a decrease or loss of cristae and a reduction in matrical density, as compared with those of neoimagines. Lysosome-like bodies, containing what appear to be remnants of organelles, are more frequently found in the neurons of old midges.

(f) Neuropile

In old specimens, most of the axonal mitochondria appear similar to those of neoimagines, but a few have denser matrices. Heterogeneous dense structures, resembling those described by HERMAN et al, (1971) in Drosophila melanogaster, have not been found in the neuropile of adult midges.

F. Changes in the Endocrine System

Most of the ultrastructural observations on the endocrine system of old midges are based upon five female and one male specimen (refer to Appendix 7 for numbers of specimens used

in observations). The descriptions of fine structure will therefore mainly refer to the female system, unless otherwise stated.

1. Neurosecretory Cells of the Brain

(a) Light Microscope observations

The time-correlated increase in neurosecretion in the MNC and VNC groups, shown by staining with EWEN'S (1962) aldehyde fuchsin (Plates 22, 24) is indicated in Table 5. Many of the neurosecretory cells which are selectively stained in old midges contain a single mass of purple material, unlike the granular deposits of neoimagines. Frequently, each MNC group of old midges contains a single cell whose cytoplasm appears orange with this stain.

(b) Electron Microscope observations

The neurosecretory cells of old midges (Plates 57, 58 and 59), exhibit the same differences from those of neoimagines as described above for neurons. The relative quantities of granular material in these cells and the relative size of their perikarya is shown in Table 6. Some of the α-type neurosecretory cells of old midges measure only 8 x 4 µm at their widest, as compared with 12 x 5 µm in neoimagines.

The discrepancy between the large amounts of neurosecretion indicated by staining with aldehyde fuchsin and the small

ELECTRON MICROGRAPHS

The brain of (20°C) female specimens 6-7 days after emergence.

Plate 57 : Section showing an α_1 and an α_2 cell of the VNC group. Note that the α_2 cell, on the right, contains slightly more granules than the α_1 cell, on the left, and both contain similar quantities to the comparable cells of neoimagines (Plate 25).

SCALE BAR : 1µm.

KEY:

N - nucleus

S - secretory granules

Plate 58 : Part of a β cell of the LNC group, surrounded by multiple glial lamellae. Note the paucity of granules within the cell.

SCALE BAR : 1µm.

KEY:

L - glial lamellae

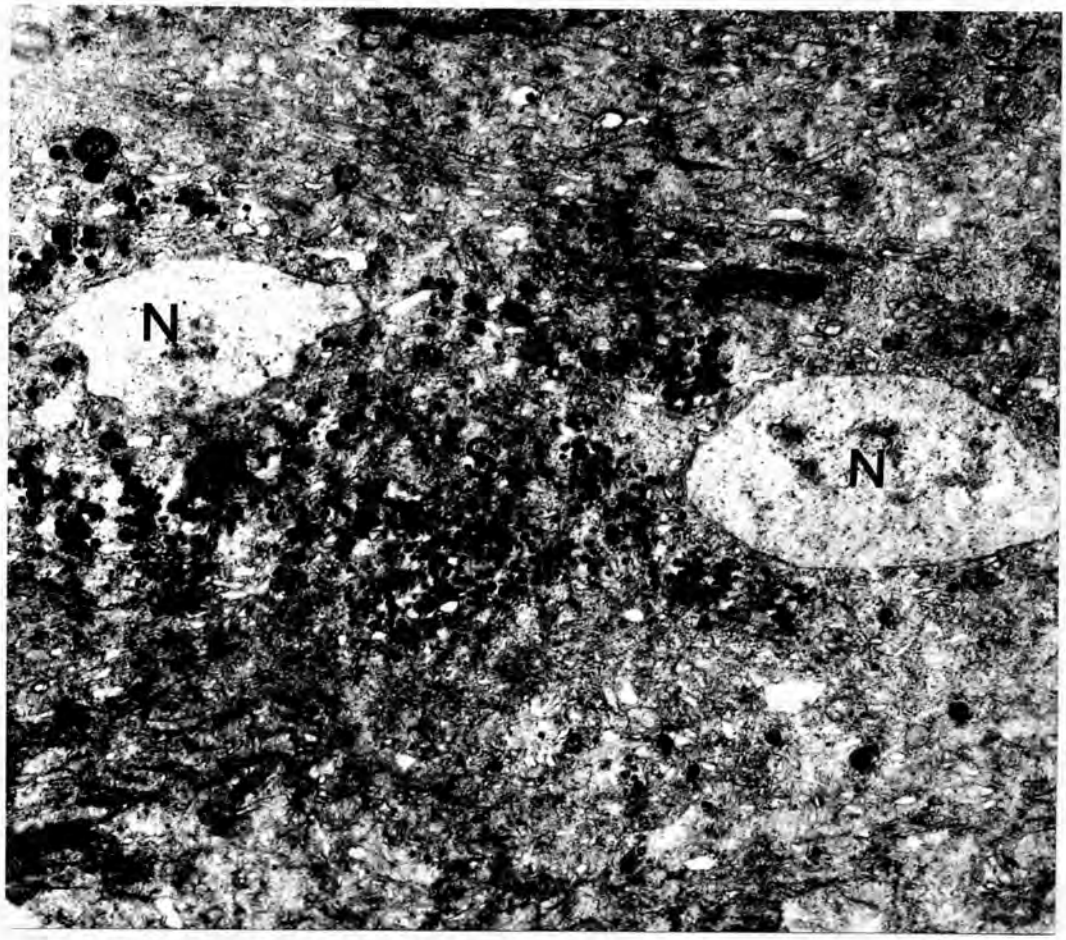
N - nucleus

R - granular endoplasmic reticulum

S - secretory granules

U - Golgi unit

(Preparation procedure: Luft's epon)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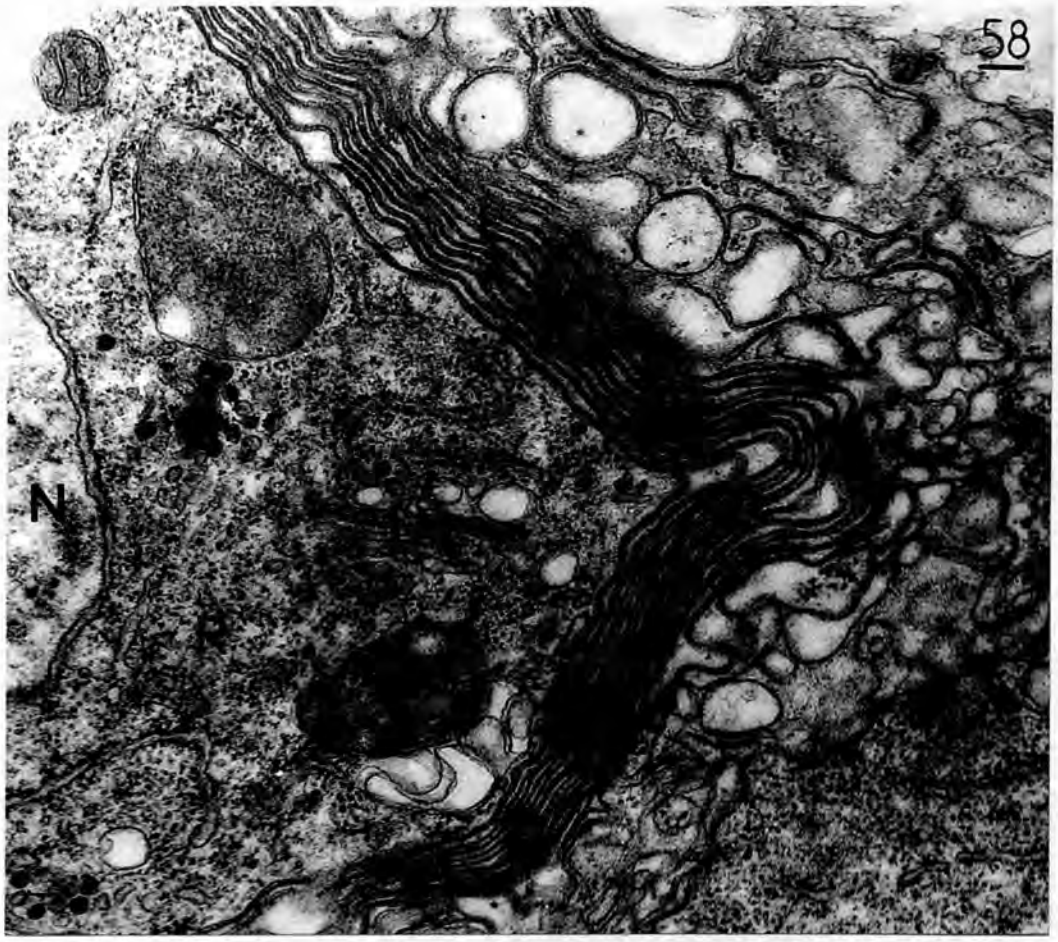
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N

ELECTRON MICROGRAPH

The brain of a (20°C) female specimen 6-7 days after emergence.

Plate 59 : An α_1 neurosecretory cell of the MNC group, showing the paucity of granules contained in these cells in aged specimens.

SCALE BAR : 1µm.

KEY:

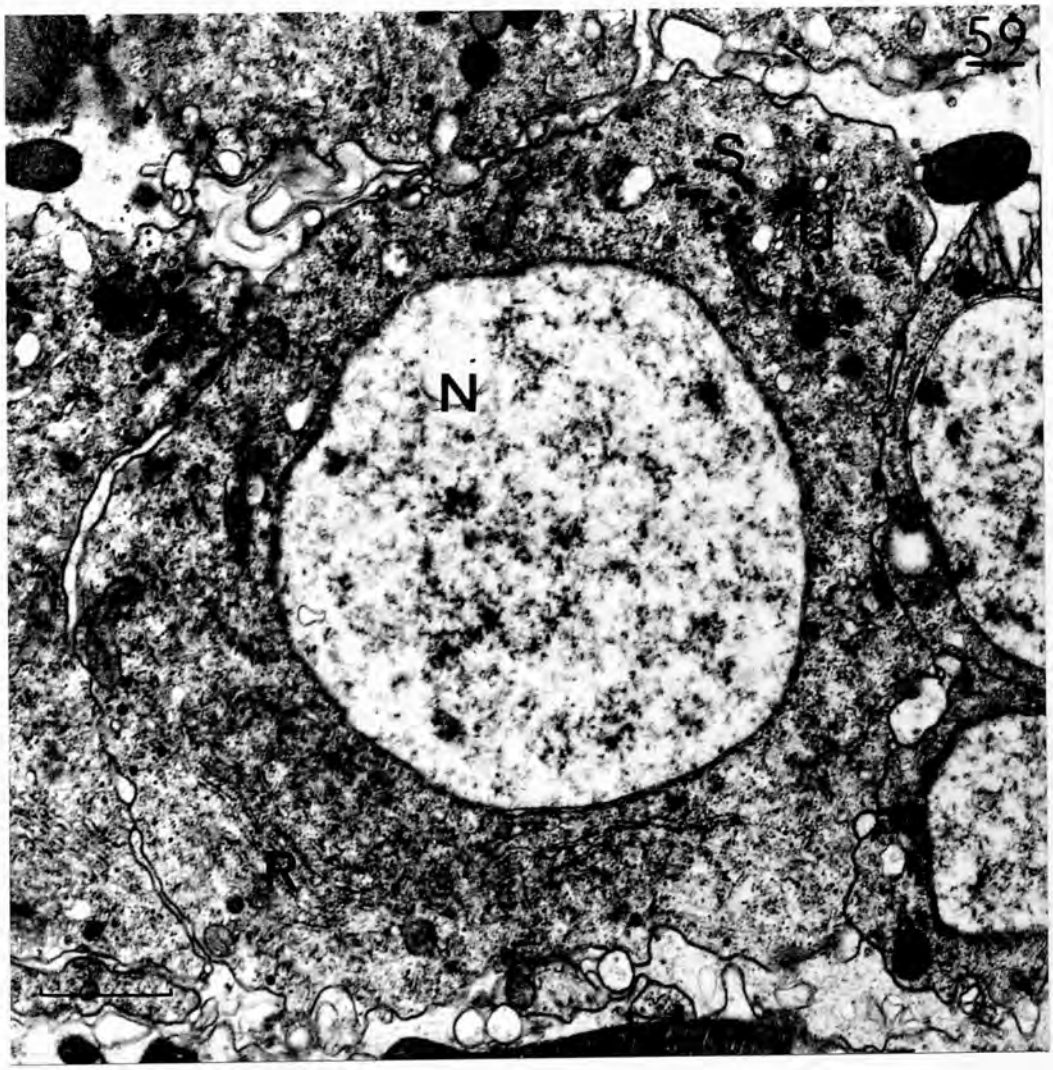
N - nucleus

R - granular endoplasmic reticulum

S - secretory granules

U - Golgi unit

(Preparation procedure: Luft's epon)


59
S
N
R

amounts of electron-dense granules found in the MNC was investigated using the technique of LANE & EUROPA (1965), (refer to Chapter 2). The staining of the MNC of old females with EWEN'S (1962) aldehyde fuchsin was found to be more intense than the cells in the controls which were known to contain electron-dense granules.

2. The Retrocerebral Endocrine Complex

Except for the peritracheal tissue and the "glandes post-cérébrales antérieures", there is no change in the morphology or staining reaction of the retrocerebral endocrine structures observed with the light microscope.

(a) The Corpora Cardiaca

The corpora cardiaca of neoimagines and old specimens of both sexes typically contain much purple granular material when stained with aldehyde fuchsin (refer to Appendix 3).

Electron microscope observations reveal that both the quantity and bimodal population of sizes of granules of the intrinsic cells are similar in old specimens to those of neoimagines. The cisternae of the endoplasmic reticulum of these cells often appear swollen in old specimens (Plate 60), and unlike those of neoimagines, are often associated with focal cytoplasmic degeneration, as are the Golgi units. Mitochondria are fewer in number in

ELECTRON MICROGRAPHS

The retrocerebral glands of (20°C) female specimens 6-7 days after emergence.

Plate 60 : Part of a corpus cardiacum cell showing the distended cisternae of endoplasmic reticulum and cytoplasmic degeneration which occurs in aged specimens. (Compare to the neoimaginal gland - Plate 41).

SCALE BAR : 1µm.

KEY:

D - cytoplasmic degeneration

N - nucleus

nl - nucleolus

R - granular endoplasmic reticulum

S - secretory granules

Plate 61 : The marginal zone of a corpus allatum showing the increased cellular interdigitations (arrows) which occur in aged specimens. (Compare to the neoimaginal gland - Plate 44).

SCALE BAR : 1µm.

KEY:

β - β type granules

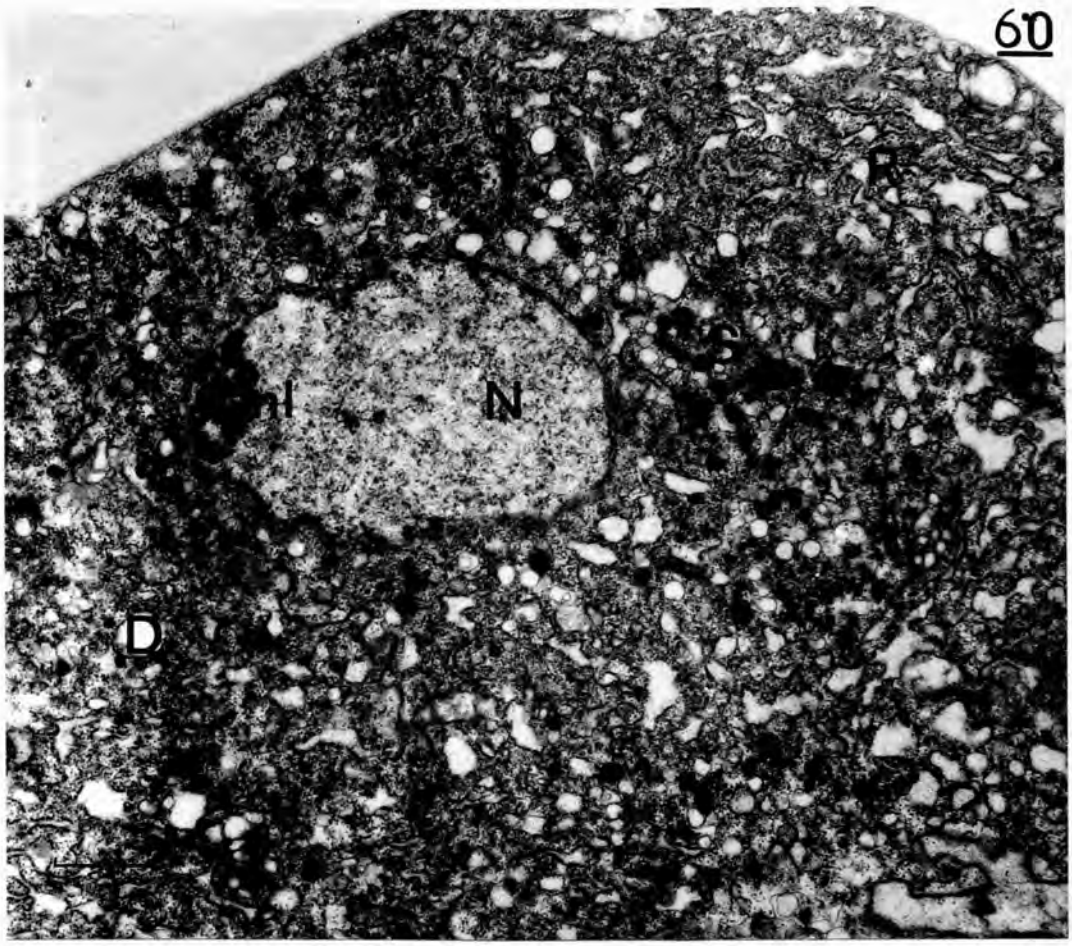
A - medulla

C - cortex

H - sheath

(Preparation procedure: Luft's epon)

60



61

the intrinsic cells of old specimens, the electron-lucent membrane-bound structures which occur in the cytoplasm probably represent mitochondria, which have a reduced matrical density and lack cristae.

(b) The Corpora Allata

The corpora allata of old specimens (Plate 61) show a greater extension of the cortical cells into the medulla and an increase in cellular interdigitation than occurs in neoimagines. Focal cytoplasmic degeneration occurs more frequently in the cortical cells of old specimens and there is less aggregated glycogen than found in neoimagines. The nuclear cytoplasmic ratio of medullary cells is similar in adults of different age (Table 1). In old specimens, the nucleus of the medullary cells contains fewer aggregated and more diffuse chromatin than in neoimagines, and is also different in having a grey nucleoplasmic background. Unlike the cells of neoimagines, glycogen deposits are absent from the medullary cytoplasm of old specimens.

(c) The Peritracheal Tissue

In old specimens, light and electron microscope observations revealed no trace of the peritracheal tissue.

(d) The "Glandes Post-Cérébrales Antérieures"

The "glandes post-cérébrales antérieures" of old midges of both sexes, consistently contain large quantities of finely granular, blue material (Plate 37) which does not occur in those of neoimagines (Plate 36) when stained with EWEN'S (1962) aldehyde fuchsin, (refer also to Appendix 3).

Electron microscope observations show that the glands of old specimens are not nucleated structures and are not divided by cell membranes and are therefore similar to those of neoimagines. The surrounding sheath is 200nm in depth in old specimens, twice as thick as in neoimagines. The infoldings of the external membrane are not incrusted with granular material in old specimens (Plate 62), as are those of neoimagines (Plate 47) and the lumen of the infoldings contains more granular matter. In the glands of old specimens (Plate 63) vesicles, 100 - 250nm in diameter occur, which although similar in size to those of neoimagines, are unlike them in containing granular material. In neoimagines, these vesicles often occur in the lumen of the infoldings, as well as throughout the gland's matrix, as described in Chapter 3. In old specimens most of these vesicles are found in the marginal zone of the gland, usually in close proximity to the infoldings. The membranes of the vesicles are often confluent with those of the infoldings, but are not surrounded by them as in neoimagines. Mitochondria, which do not occur

ELECTRON MICROGRAPHS

The 'GPCA' of (20°C) female specimens 6-7 days after emergence.

Plate 62 : Section of a complete gland, showing the large and small vesicles which occur in the matrix. Note the large vesicle (arrow) apparently undergoing dissolution.

SCALE BAR : 1μm.

KEY:

H - sheath

L - large vesicles

V - small vesicles

Plate 63 : Detail of the marginal zone of a gland showing that some of the small vesicles are confluent with the infoldings of the external membrane.

SCALE BAR : 100nm.

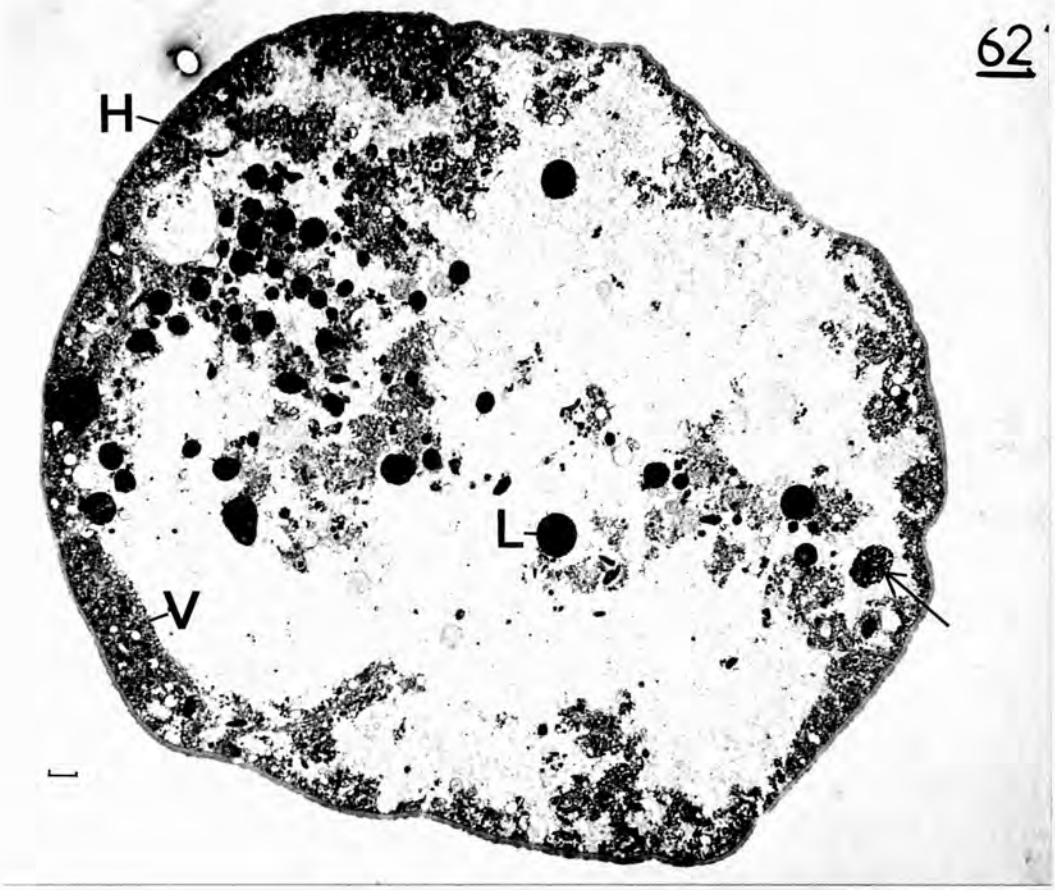
KEY:

H - sheath

I - infoldings of the external membrane

V - small vesicles

(Preparation procedure: Spurr's epon)


62
H
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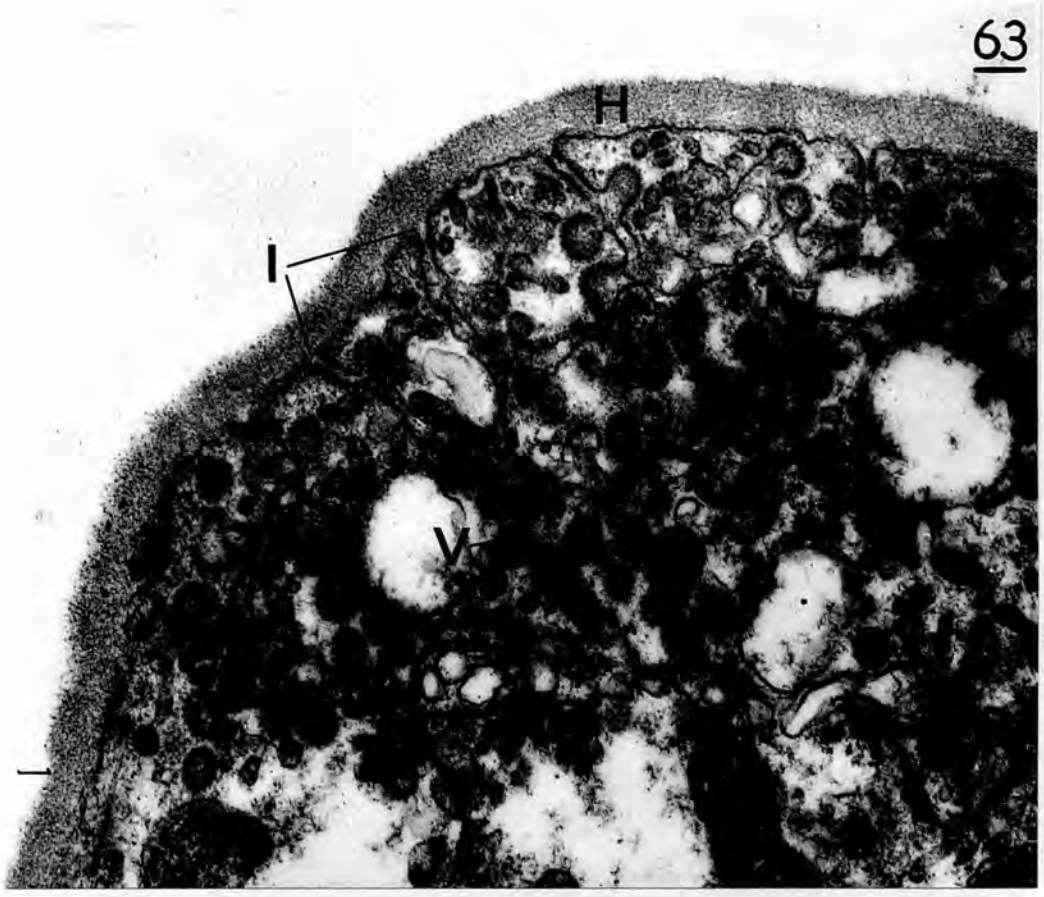
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in the glands of neoimagines, are sometimes found close to the infolded membranes in old specimens. Large vesicles up to 2µm in diameter, filled with granular material similar to that of the small vesicles, occur throughout the matrix of the gland in old specimens. These may be comparable to the large electron-lucent vesicles occurring in the glands of neoimagines. Occasionally, large non-membrane bound granular aggregates are found in the matrices of the glands of old specimens. Cisternae of smooth endoplasmic reticulum is much more frequent in the glands of neoimagines and their matrices contain more granular material than those of old specimens.

G. Peripheral Neurosecretory Cells of the Prothorax

The staining reaction of the peripheral neurosecretory cells of neoimagines and old midges is similar when treated with EWEN'S (1962) aldehyde fuchsin. In females, the cell bodies and release sites rarely stain selectively, whereas in males both usually contain considerable amounts of purple material, indicating the presence of neurosecretion (refer to Appendix 3).

H. The Longitudinal Flight Muscles of the Prothorax

No apparent difference was found in the myofibrilar pattern or mitochondrial arrangement between neoimaginal and old males in the longitudinal flight muscles. Dense bodies, similar to those described by TAKAHASHI et al (1970B), have not been found in these flight muscles.

CHAPTER 5

THE EFFECT OF TEMPERATURE UPON IMAGINES

INTRODUCTION

The first part of this chapter is concerned with the effect that the water temperature, in which the preimaginal phases were "reared" and the subsequent air temperature at which the adults were "kept", has upon longevity, behaviour and size of the imagine. The main part of this chapter deals with the brain and endocrine system of neoimagines reared at 10 and 15°C, as compared with these structures in midges reared at 20°C, already described in Chapter 3. The implications, and possible correlations of these results are discussed in Section III of Chapter 7.

A. Life Span Records

Figure 13 shows that the mean life span of adult females is longer than that of males, when "reared" and "kept" at the same temperature, usually by a significant amount and that the rearing temperature of preimaginal males does not significantly change the mean life span from that of the temperature at which they are kept as adults.

The survivorship curve for males reared at 10°C and kept at 20°C (Figure 14) is of the intermediate type of ROCKSTEIN & MIQUEL (1973) and is similar to that of males reared and kept at 20°C (Figure 12). Midges reared and kept at 10 and 15°C, and males reared at 20°C and kept as adults at 10°C also have a survivorship curve of the intermediate type (Figure 14).

B. Weight and Length Differences

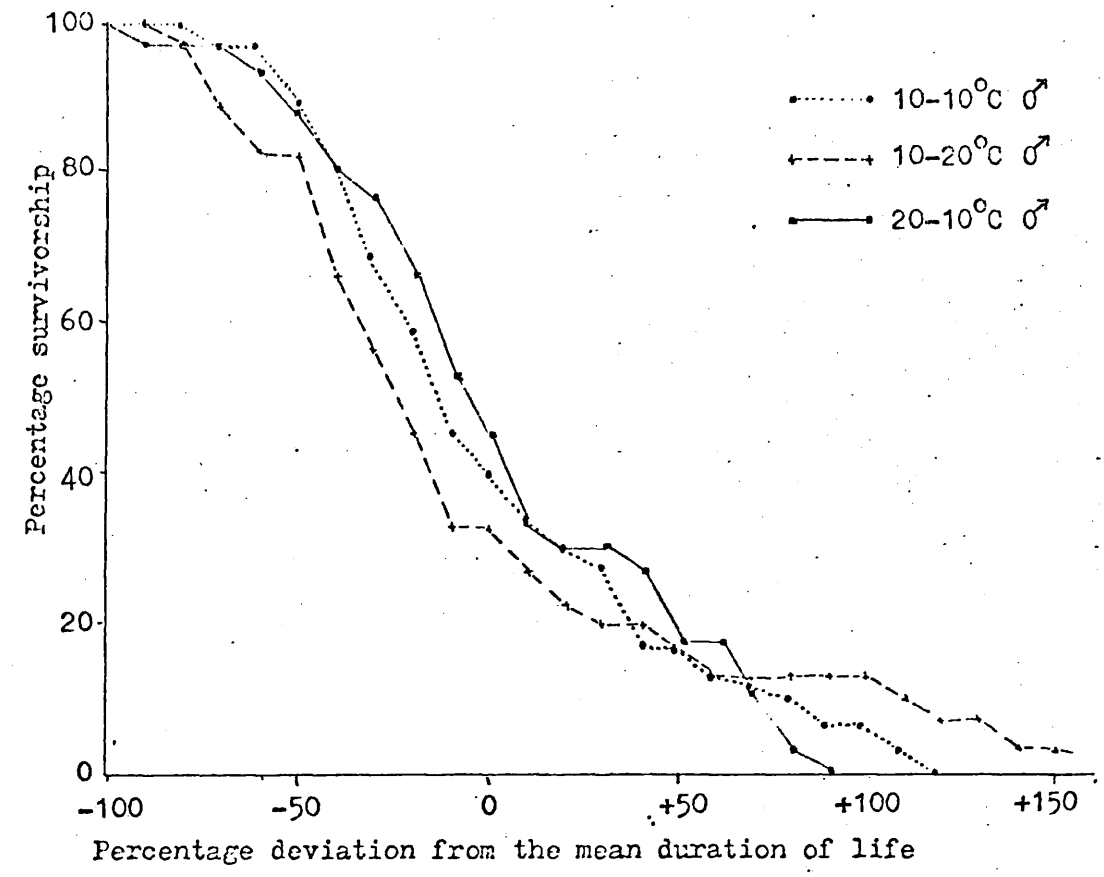
When reared at 10 or 20°C, females are significantly heavier and shorter in length than males reared at the same temperature. Both sexes are significantly heavier and longer when reared at 10°C as compared with those reared at 20°C (Table 9).

C. Behavioural Differences

Midges kept at 10°C as adults, regardless of rearing temperature exhibit much less flying and walking activity than specimens reared and kept at 20°C. Midges reared at 10°C and transferred as adults to 20°C show an increase in activity, but still appear less active than specimens reared and kept at 20°C. Midges reared and kept at 15°C show no apparent difference in activity from those of 20°C.

D. Structural Differences in the Brain

No sexual differences have been found in the brains of adult midges reared at the same temperature, the following



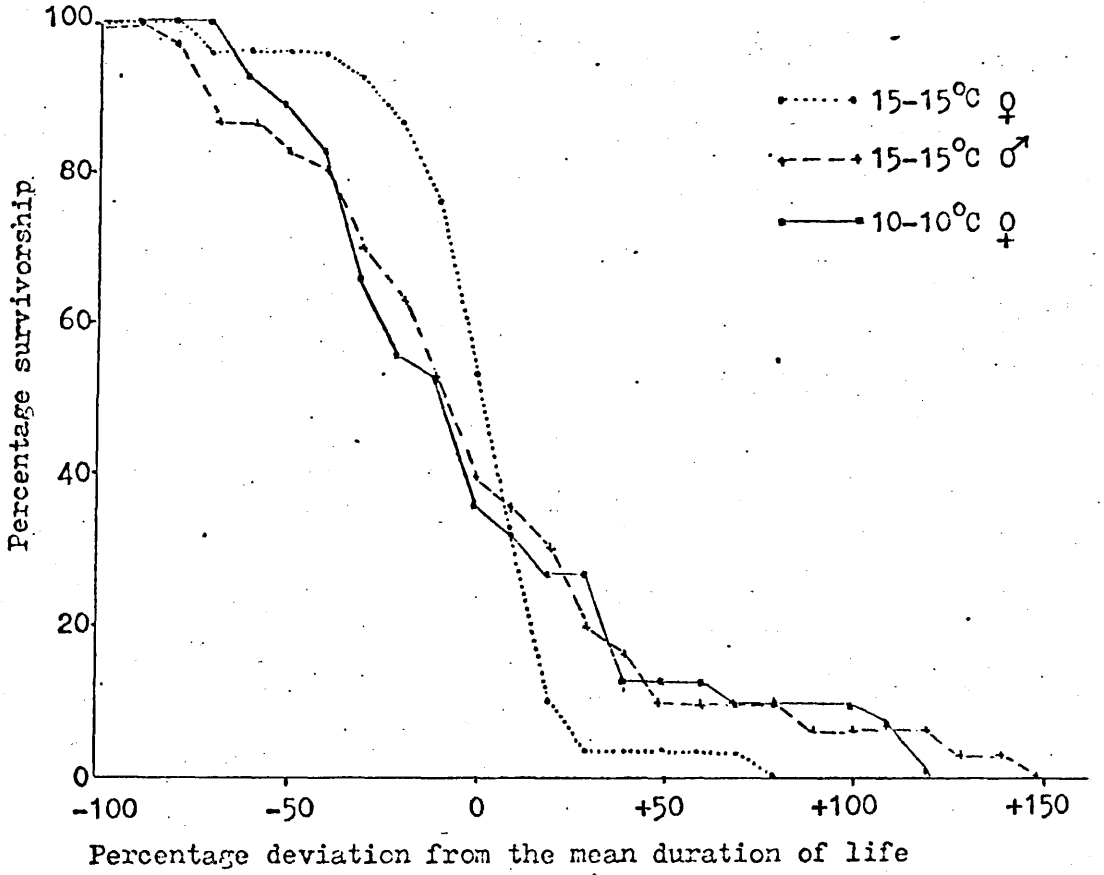


Figure 14. Survivorship curves of males and females reared (first number) and kept as adults (second number) at several temperatures. These life curves are of the intermediate type as shown in Figure 11.

description will therefore refer to both males and females. As a pilot study on midges reared at 15°C showed that there was no apparent difference in either morphology or fine structure of the brains with those of specimens reared at 20°C, only comparative differences between neoimagines reared at 10 and 20°C are therefore described.

1. Light Microscope Observations

The size of the brain, reflected in the width of the head capsule (Table 9) is significantly larger in specimens reared at 10°C as compared with 20°C. There is no significant difference in the size of the central complexes (Table 10) or in the field density counts of neurons (Table 7), at the two rearing temperatures. The larger brains of specimens reared at 10°C as compared with 20°C appear to result mainly from cortical layers of greater depth. Extracellular spaces occur throughout the neuropile and cortical layer of midges raised at 10°C, whereas they are rarely found in specimens reared at 20°C.

2. Electron Microscope Observations

The descriptions below refer to the fine structure of the brains of specimens reared at 10°C.

(a) Neural Sheath

The neural lamella is up to 600 nm thick, 50% wider than in specimens reared at 20°C, but resembling it in structure.

TABLE 9. Summary of average body lengths, head capsule widths and weights.

	Rearing temperature 10°C		20°C	
	♀	♂	♀	♂
Body length (mm)*	6·4(0·22)	7·1(0·53)	5·9(0·32)	6·2(0·29)
Head width (µm)**	770(40)-----	-790(24)	720(36)-----	-740(79)
Weight (mg)†	3·7(0·34)	3·3(0·21)	2·4(0·31)	1·5(0·27)
No.††	30	30	30	30

Numbers in parentheses are the standard deviations(±).

Dotted lines connect results which are not significantly different at the 5% probability level.

* To the nearest 0·1mm, excluding the antennal apparatuses.

** To the nearest 10µm.

† To the nearest 0·1mg.

†† Number of specimens used.

TABLE 10. Summary of average sizes of the antennal centres, neuropile masses 4 & 5, and the central complex, neuropile mass 13, of neoimagines.

Neuropile Mass No.	Rearing temperature 10°C ♀	10°C ♂	20°C ♀	20°C ♂	
4*	6(0·71)	6(0·71)	6(0·40)	6(0·00)	Size in µm
5*	16(2·38)	16(1·51)	16(2·50)	16(2·72)	
13**	125(11·1)	130(10·4)	125(9·8)	125(8·3)	
Sample size	10	10	10	10	

* Mean size to the nearest 2µm.

** Mean size to the nearest 5µm.

Numbers in parentheses are the standard deviations (±).

The subjacent perineurium is 2 µm in depth, similar to that of specimens reared at 20°C, but differing in containing large, round (up to 1 µm in diameter) mitochondria, as compared with small, oval mitochondria, many of which have a lower matrical density and distribution or absence of cristae. The external mitochondrial membranes are sometimes not continuous so that the interstructural space is confluent with the cytoplasmic matrix, which rarely occurs in these organelles in specimens reared at 20°C. Areas of focal cytoplasmic degeneration and heterogeneous electron-dense bodies resembling collapsed mitochondria are more frequently found than in the perineurial cells of specimens reared at 20°C.

(b) Glial Cells

There is little difference in the appearance of the three types of glial cells as compared with those of specimens reared at 20°C, described in Chapter 3, except that the endoplasmic reticulum sometimes adopts a whorled configuration and its cisternae are occasionally more distended. Concentric multimembranous arrays are often found investing cortical cells, as well as surrounding central lumens, and probably represent multiple glial lamellae. They are similar to those, described in Chapter 4, occurring in the brains of old specimens, but they have not been observed in those of neoimagines reared at 20°C.

(c) Extracellular Spaces

The extensive extracellular spaces, sometimes over 1 µm

across, usually contain areas of electron-lucency surrounded by amorphous granular material, resembling the spaces found in "old" specimens described in Chapter 4. These spaces are less pronounced in the central region of the neuropile.

(d) Tracheal system

The structure of the tracheal system is similar to that of specimens reared at 20°C, described in Chapter 4. The lumen of the trachea of some specimens contains an intima (Plate 64), presumably left by incomplete moulting.

(e) Neurons

All types of neurons, including neurosecretory cells, have mitochondria which have lower matrical densities and disruption or loss of cristae (Plate 68), as compared with those of midges reared at 20°C. The mitochondrial intercstructural space is sometimes confluent with the cytoplasmic matrix because of the surrounding membranes' disruption, which rarely occurs in the neurons of midges reared at 20°C. The cisternae of the granular endoplasmic reticulum appear more distended than occurs in specimens reared at 20°C and is unlike it, often being found as whorled arrays at the centre of which are Golgi units. Lysosome-like bodies containing what appear to be remnants of cell organelles occur more frequently than in the neurons of midges reared at 20°C. Type 3 neurons are the only brain

ELECTRON MICROGRAPHS

The brain of female (10°C) neoimagines.

Plate 64 : Section of the brain showing considerable extracellular spaces, multiple glial lamellae and trachea containing an intima.

SCALE BAR : 1μm.

KEY:

2 - neuron type 2

3 - neuron type 3

A - neuropile

C - cortex

e - extracellular spaces

l - glial lamellae

t - trachea

Plate 65 : Section of the medial dorsal region of the brain containing α_1 neurosecretory cells of the MNC group and very electron-dense type 4 neurons. Note the extracellular spaces associated with the type 4 neurons.

SCALE BAR : 1μm.

KEY:

4 - neuron type 4

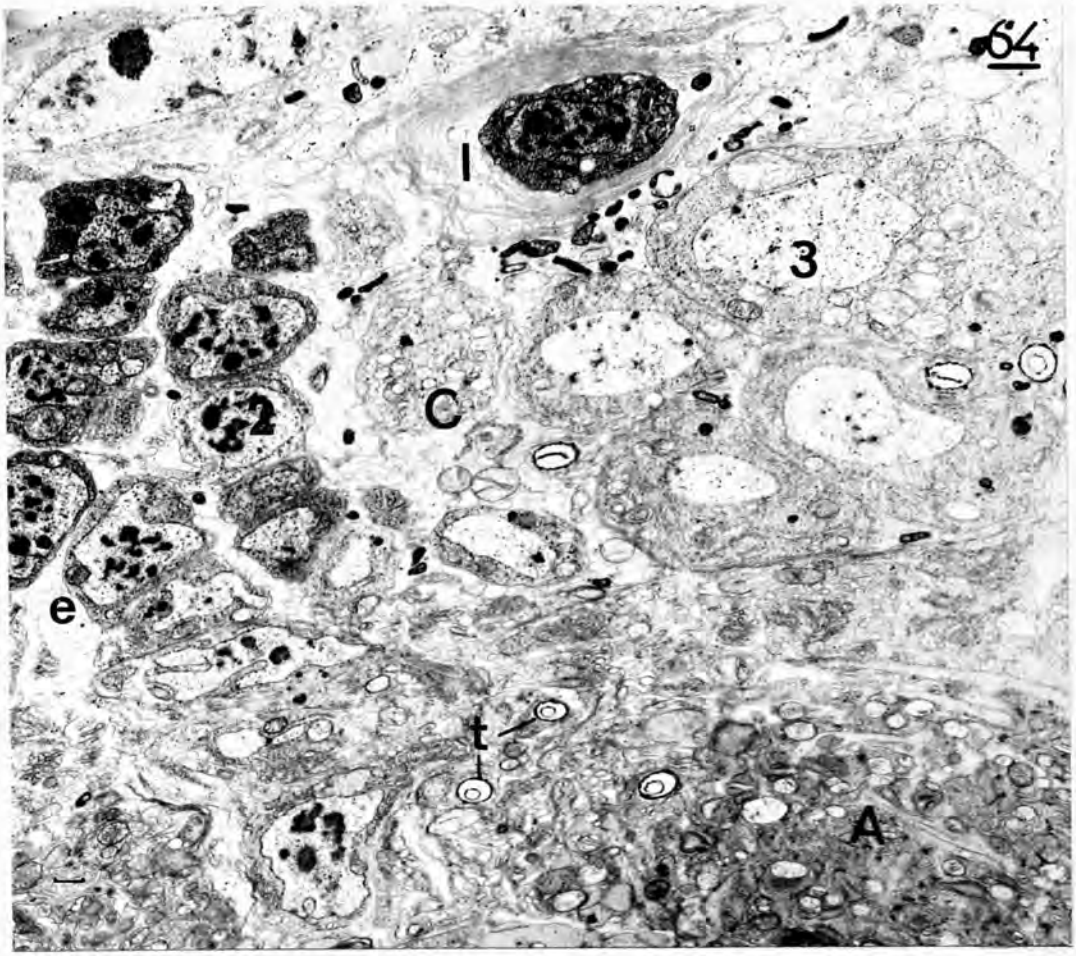
m - disrupted mitochondrion

n - nucleus

S - neurosecretory cell

Other labels as Plate 64

(Preparation procedure: Luft's epon)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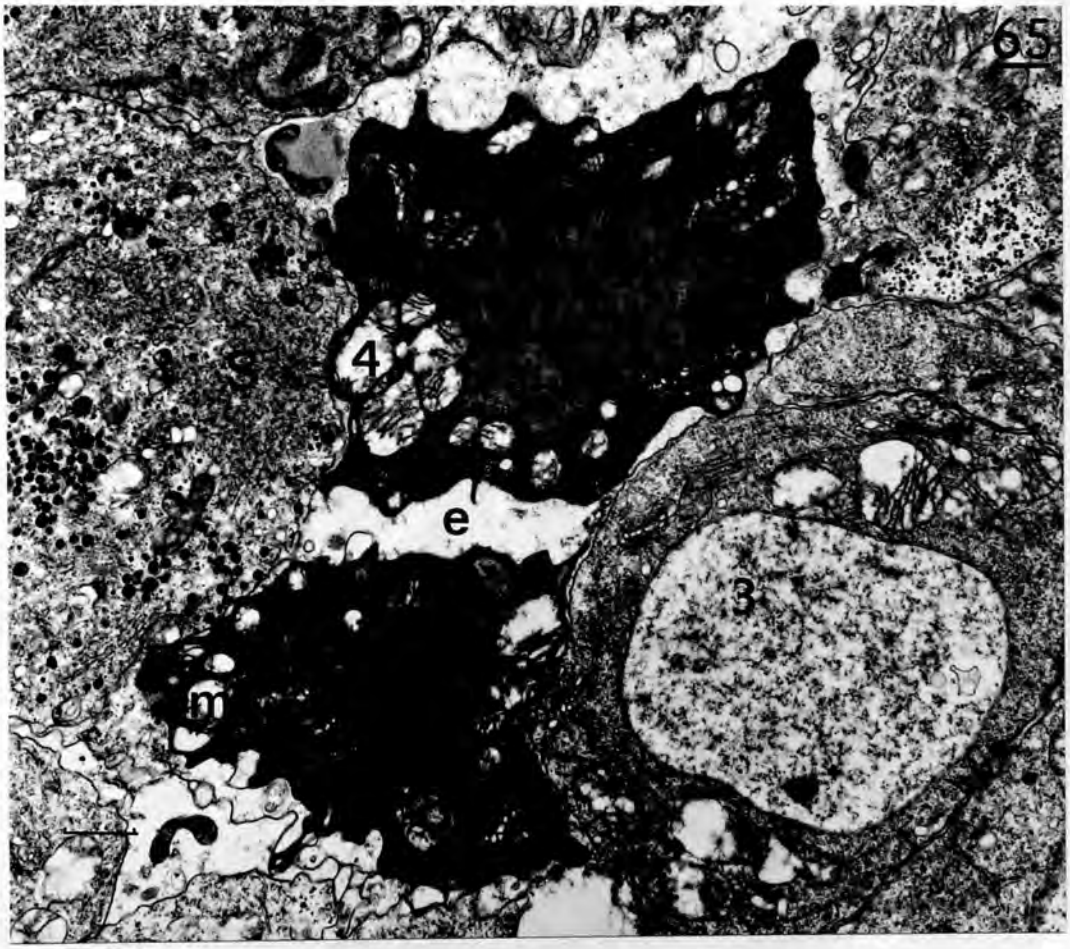
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cells to exhibit electron-lucent nuclear vacuoles which have not been found in the brains of specimens reared at 20°C.

The frequency of type 2 neurons is much higher than in the brains of midges reared at 20°C, and many more intermediates between type 1 and type 2 occur (Plate 64). Type 4 neurons are also more frequent but not as common as type 2. In the pars intercerebralis of most specimens reared at 10°C, cells resembling type 4 neurons occur, but with a much greater electron-density (Plate 65). Similar electron-opaque cells have not been found in the brains of midges reared at 20°C.

(f) Neuropile

Many of the axonal mitochondria show a similar difference to those of specimens reared at 20°C, as exhibited by these organelles in the neural perikarya. Axons in the central region of the neuropile have mitochondria similar in structure and matrical densities, to those of specimens reared at 20°C.

E. Differences in the Endocrine System

The following descriptions refer to specimens reared at 10 and 20°C.

1. Neurosecretory Cells of the Brain

(a) Light Microscope observations

The mean staining indices of the MNC and VNC groups,

ELECTRON MICROGRAPHS

The brain of female (10°C) neoimagines.

Plate 66 : An α_1 neurosecretory cell of the MNC group, which contain slightly more granules than are found in neoimagines reared at 20°C.

SCALE BAR : 1μm.

KEY:

h - neural sheath

n - nucleus

nl - nucleolus

r - granular endoplasmic reticulum

s - secretory granules

u - Golgi unit

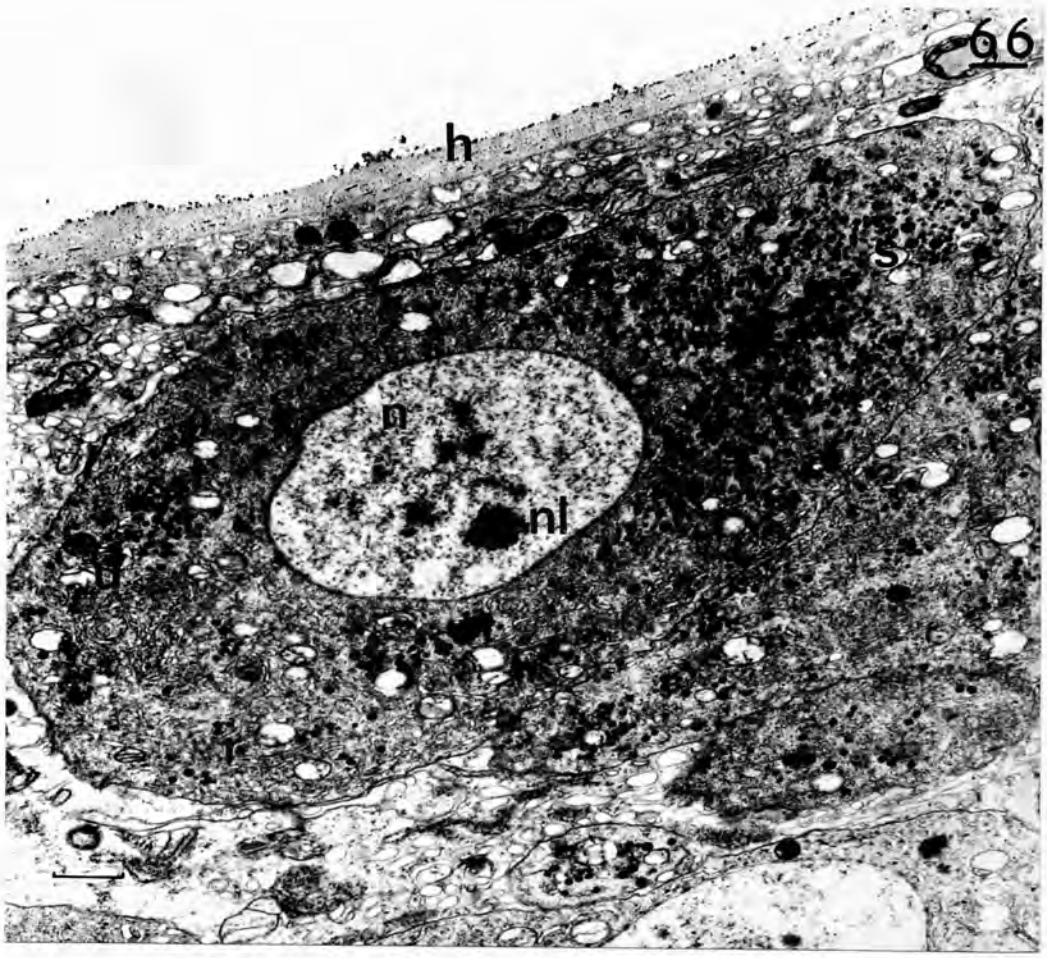
Plate 67 : A β neurosecretory cell of the LNC group, which contain a similar quantity of granules to the comparable cells of neoimagines reared at 20°C (Plate 30).

SCALE BAR : 0•5μm.

KEY:

Labelling as Plate 66

(Preparation procedure: Luft's epon)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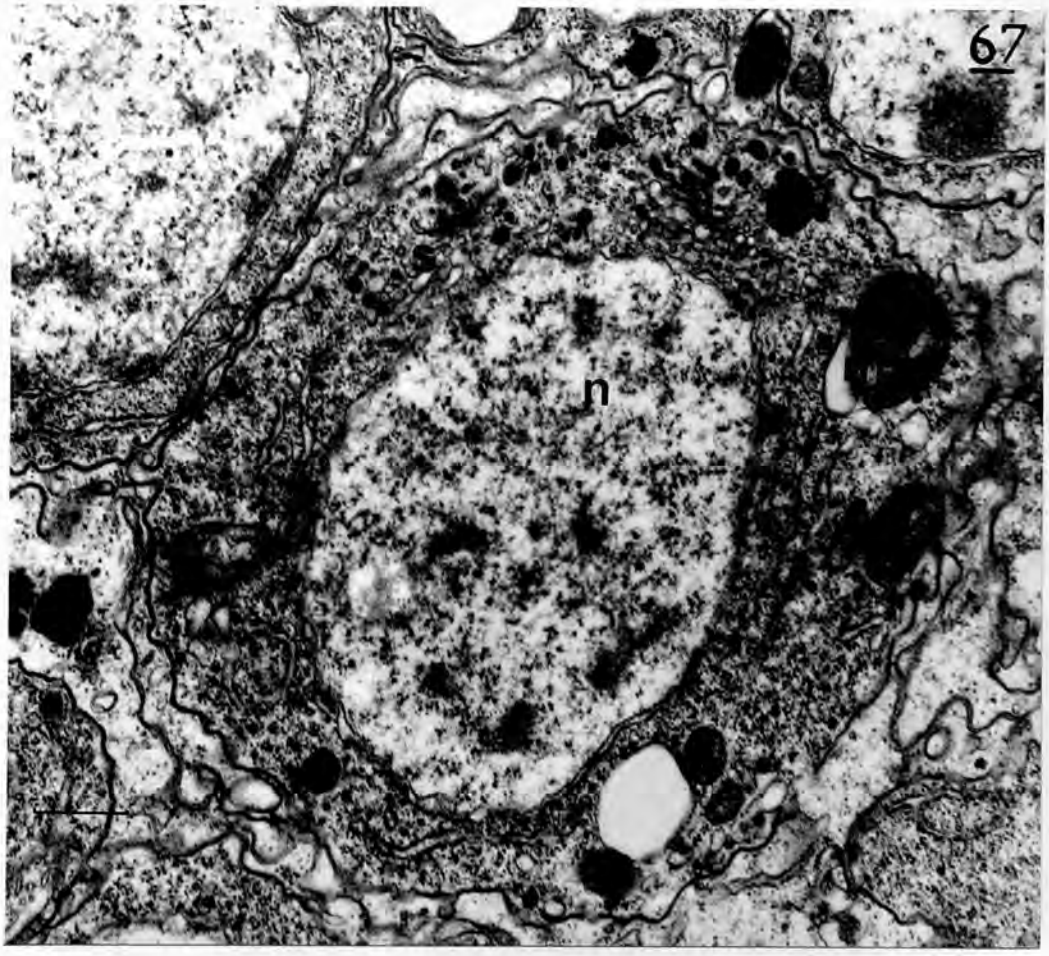
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when treated with EWEN'S (1962) aldehyde fuchsin of neoimagines reared at 10 and 20°C are given in Table 5, which shows that the latter specimens appear to contain more neurosecretion. Selectively stained cells contain coarser purple granules (Plate 23) in specimens reared at 10°C as compared with 20°C (Plate 22). The axons from the MNC rarely show selective staining in midges reared at 10°C, which usually occurs in those specimens reared at 20°C (refer to Appendix 3).

(b) Electron Microscope observations

The relative quantities of granular material in the cerebral neurosecretory cells of specimens reared at 10°C (Plates 66 - 69) and 20°C (Plates 25 - 30) and the relative sizes of their perikarya, is included in Table 6. The β-type cells of the LNC group of midges reared at 10°C (Plate 67) contain both electron-dense and electron-lucent granules, unlike those of specimens reared at 20°C where only the latter type occur (Plate 30). The similarity in the amount of granules in the cells of the MNC at both rearing temperatures does not reflect the larger amount of neurosecretion, indicated by staining with aldehyde fuchsin, in specimens reared at 20°C.

2. Retrocerebral Endocrine Complex

Observations upon the retrocerebral endocrine structures are based upon wax sections stained with EWEN'S (1962) aldehyde fuchsin. Detectable differences were found

ELECTRON MICROGRAPHS

The brain of the female (10°C) neoimagine.

Plate 68 : Part of the VNC group, showing a single α_1 neurosecretory cell containing many granules and an α_1 cell (arrow) with few granules.

SCALE BAR : 1um.

KEY:

n - nucleus

nl - nucleolus

r - granular endoplasmic reticulum

s - secretory granules

Plate 69 : An α_2 neurosecretory cell of the VNC group containing several Golgi units but few secretory granules.

SCALE BAR : 1µm.

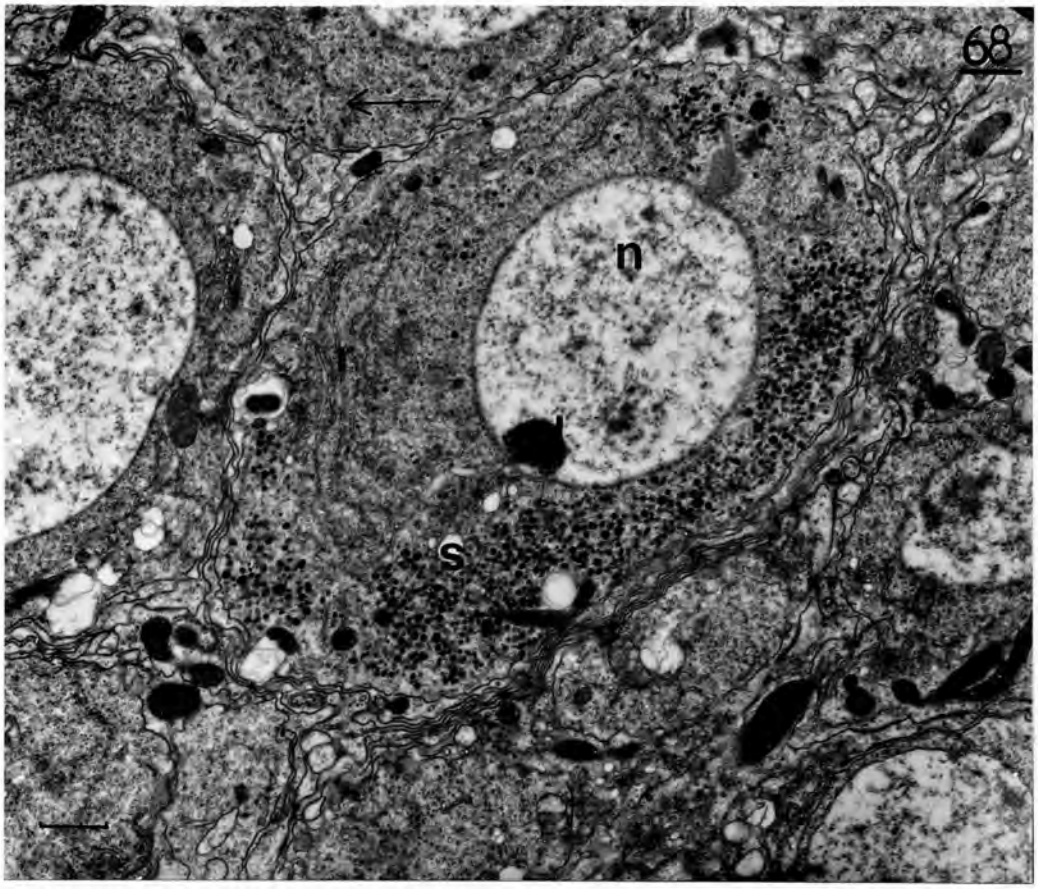
KEY:

u - Golgi unit

v - multilamellate body

other labelling as Plate 68

(Preparation procedure: Luft's epon)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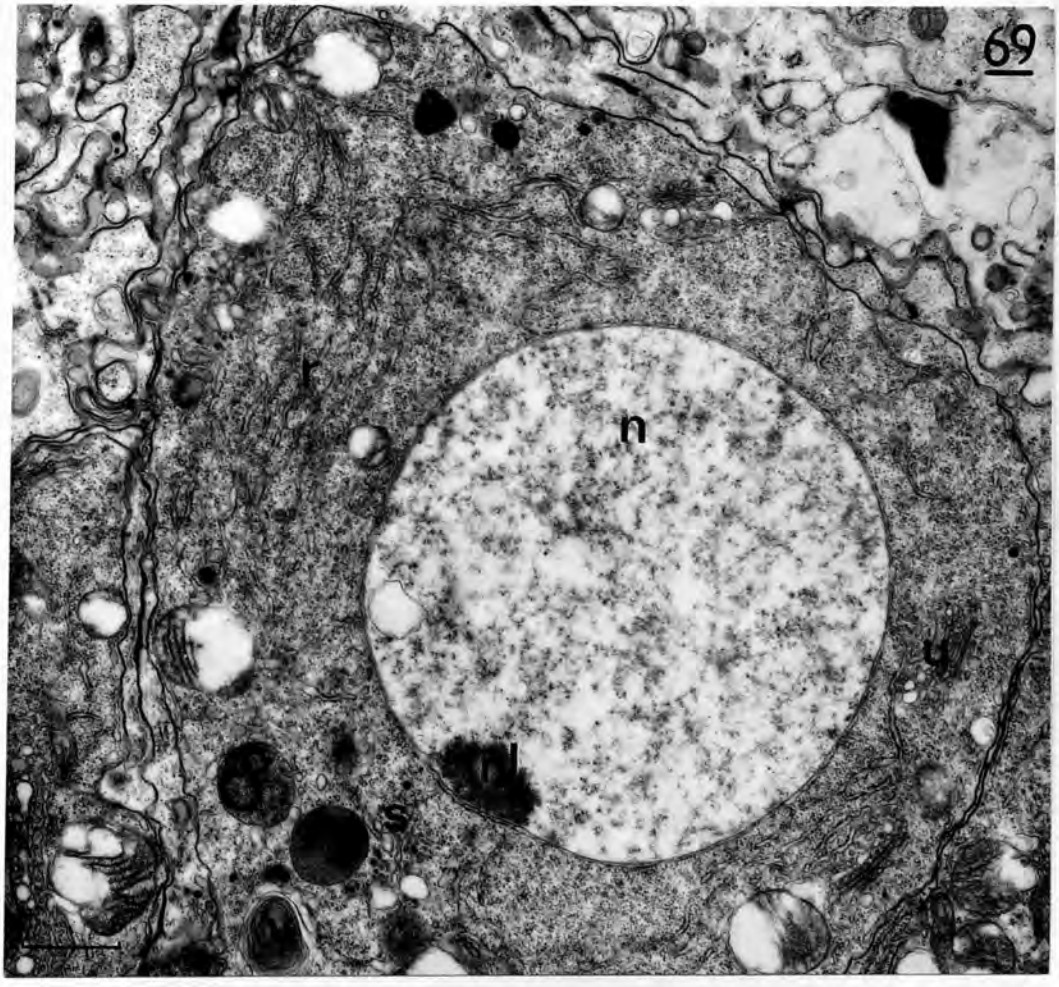
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ELECTRON MICROGRAPHS

The brain of a female (10°C) neoimagine.

Plate 70 : Section of the dorsal cortex, in the region between the optic and central lobes, showing the cytoplasmic degeneration of neurons and the extracellular spaces associated with type 2 and type 4 neurons.

SCALE BAR : 1µm.

KEY:

1 - neuron type 1

2 - neuron type 2

3 - neuron type 3

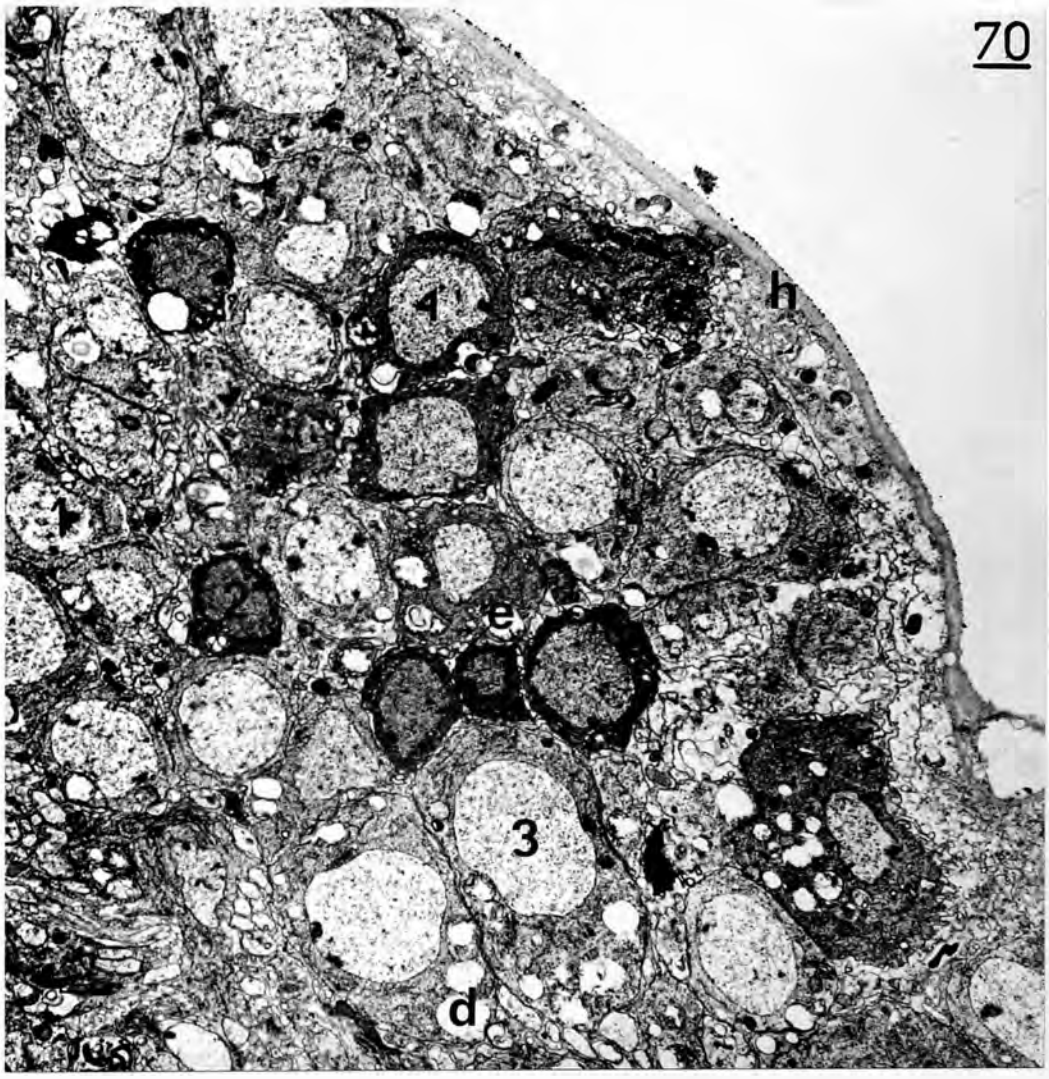
4 - neuron type 4

d - cytoplasmic degeneration

e - extracellular spaces

h - neural sheath

(Preparation procedure: Luft's epon)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in the corpora cardiaca, which show selective staining less frequently and to a smaller extent (refer to Appendix 3) and in the peritracheal tissues, whose cells have a more intimate association and contain less extracellular space, in neoimagines reared at 10°C as compared with those reared at 20°C.

G. Differences in staining of the Peripheral Neurosecretory Cells of the Prothorax

The peripheral neurosecretory cells of neoimagines reared at 10°C have not been found to exhibit selective staining with EWEN'S (1962) aldehyde fuchsin in either sex (refer to Appendix 3). The staining reaction of the female's cells is therefore similar to those of specimens reared at 20°C, but differs from those of 20°C males, which frequently contain purple granules in both the cell bodies and the release sites at this temperature.

CHAPTER 6

CHANGES ASSOCIATED WITH REPRODUCTION IN THE FEMALE

INTRODUCTION

The last chapter of results is concerned with changes in the cerebral neurosecretory cells associated with different states of reproduction in the female. Results on the ultrastructure of the neurosecretory cells are correlated with light microscope observations upon stained wax sections of the ovaries. The ultrastructure of the retrocerebral endocrine complex was investigated in mated, oviposited individuals which showed the greatest difference in the cerebral neurosecretory cells, as compared with those of females in other reproductive states. The implications of these results are discussed in Section IV of the next chapter.

A. Egg Fertility

The egg-ropes of all oviposited individuals used in this study were found to be fertile. It is therefore assumed that these specimens were mated, as virgins are known to lay sterile eggs (CREDLAND, 1971).

B. Light Microscope Observations

Longitudinal sections of the anterior abdomen of ligated specimens, showed that the ligature did not cause complete constriction of the haemocoel.

The morphology of the reproductive organs were identified with comparison of those of Chironomus plumosus (WENSLER & REMPEL, 1962). Table 11 shows that the largest oocytes and their nurse cells undergo a reduction in size and number of yolk droplets with age, which is not prevented by mating, (Plates 73 and 74). The ovaries of oviposited individuals are found to contain few, if any, oocytes (Plate 75), and the nurse cells are surrounded by masses of diffuse tissue which is probably derived from degenerated follicular epithelial cells.

C. Electron Microscope Observations

1. Neurosecretory Cells of the Brain

Table 11 shows that, with the exception of oviposited individuals, there are no marked differences in the relative amount of granules or the relative perikarya sizes of the neurosecretory cells in the brains of old virgin midges with those of the experimental reproduction groups which were kept under less controlled conditions of temperature and lighting. One or two of the β-type cells of the LNC group in oviposited individuals

TABLE 11. Relative quantities of granules in the brains' neurosecretory cells and the relative sizes of their perikarya, in females reared at 20°C, and the sizes of their terminal oocytes, in relation to reproductive state.

	Adult temperature 20±1°C			22±4°C			
Cell group	1	7	3-4	4	3-4	3-4	Days after emergence
	V	V	M	MO	VL	VS	Reproductive state
MNC α_1	10R (5)	1R (4)	3R (2)	8R (5)	2R (4)	1R (2)	Relative quantity of granules* and relative sizes of perikarya
VNC α_1	10N (5)	7R (4)	10R (2)	4R (5)	10R (4)	10R (2)	
VNC α_2	10N (5)	10R (4)	10R (2)	10R (5)	10R (4)	10R (2)	
ONC α_2	10N (2)	3R (3)	4R (2)	3R (3)	4R (3)	- (0)	
LNC β	2N (2)	2N (4)	3N (2)	10I (3)	2N (3)	2N (1)	
	N (4)	R (4)	R (2)	A (4)	R (4)	R (2)	Amount of yolk droplets
	80x 100 (4)	50x 50 (4)	50x 50 (2)	- (4)	50x 50 (4)	50x 50 (2)	Terminal oocyte size**

Relative values are based on subjective assessment.

Figures in parentheses show the number of specimens used.

* Granule quantities are rated 0-10, relative quantities are only comparable horizontally.

** Size, in μm, of the largest oocyte and its nurse cell present, measured to the nearest 10μm.

A=Absent.

N=Similar to those of neoimaginal virgins.

R=Reduced, in size (neurosecretory cells) or, in quantity (yolk droplets), as compared to those of neoimaginal virgins.

I=Increased size of perikarya of neurosecretory cells as compared to those of neoimaginal virgins (refers to 1 or 2 cells in each LNC group only, the remainder being N).

Key to Reproductive States:-

L=Ligated M=Mated O=Oviposited S=Sham ligated V=Virgin

ELECTRON MICROGRAPHS

The neurosecretory cells of mated oviposited females.

Plate 71 : Part of the VNC group showing two α_2-type cells which contain more granules than the α_1-type cell.

SCALE BAR : 1μm.

KEY:

n - nucleus

r - granular endoplasmic reticulum

s - secretory granules

Plate 72 : A β neurosecretory cell of the LNC group containing a considerable quantity of aggregated granules and having a large perikaryon.

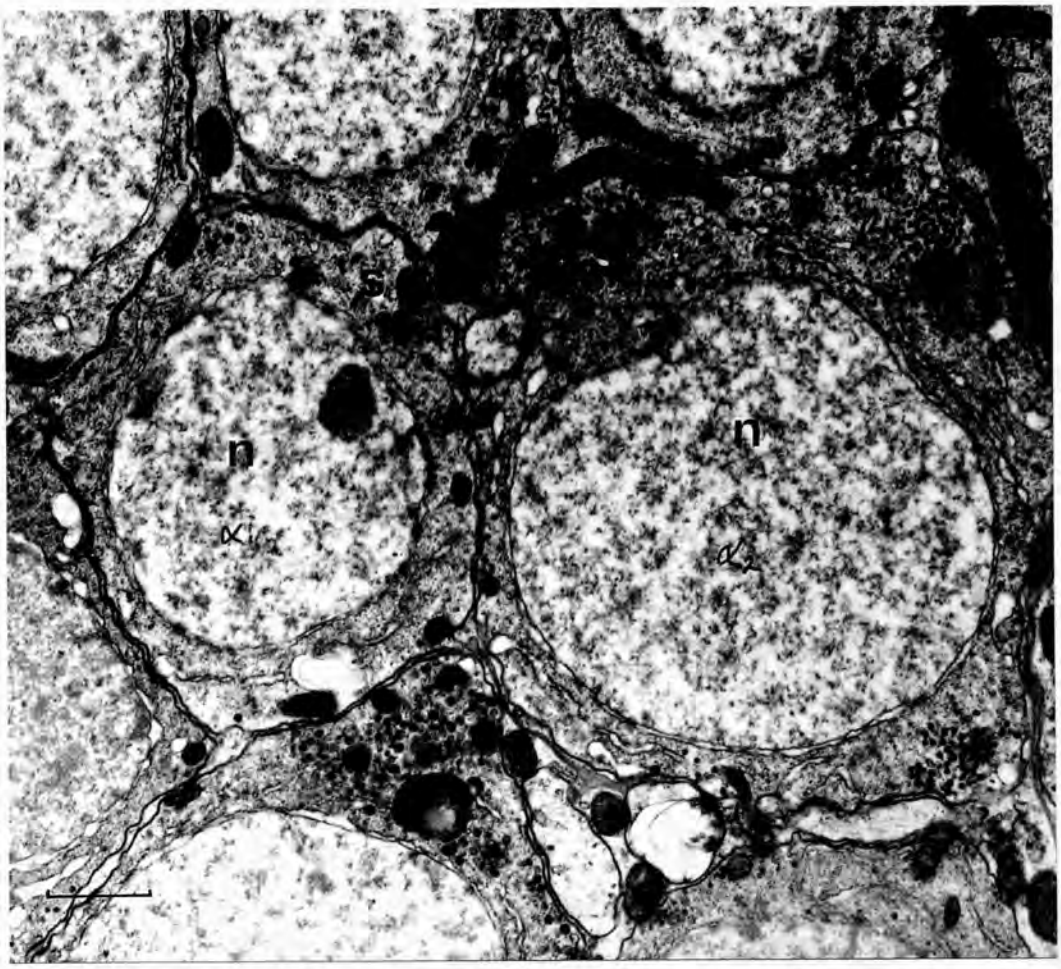
SCALE BAR : 1μm.

KEY:

u - Golgi unit

other labelling as Plate 71

(Preparation procedure: Luft's epon)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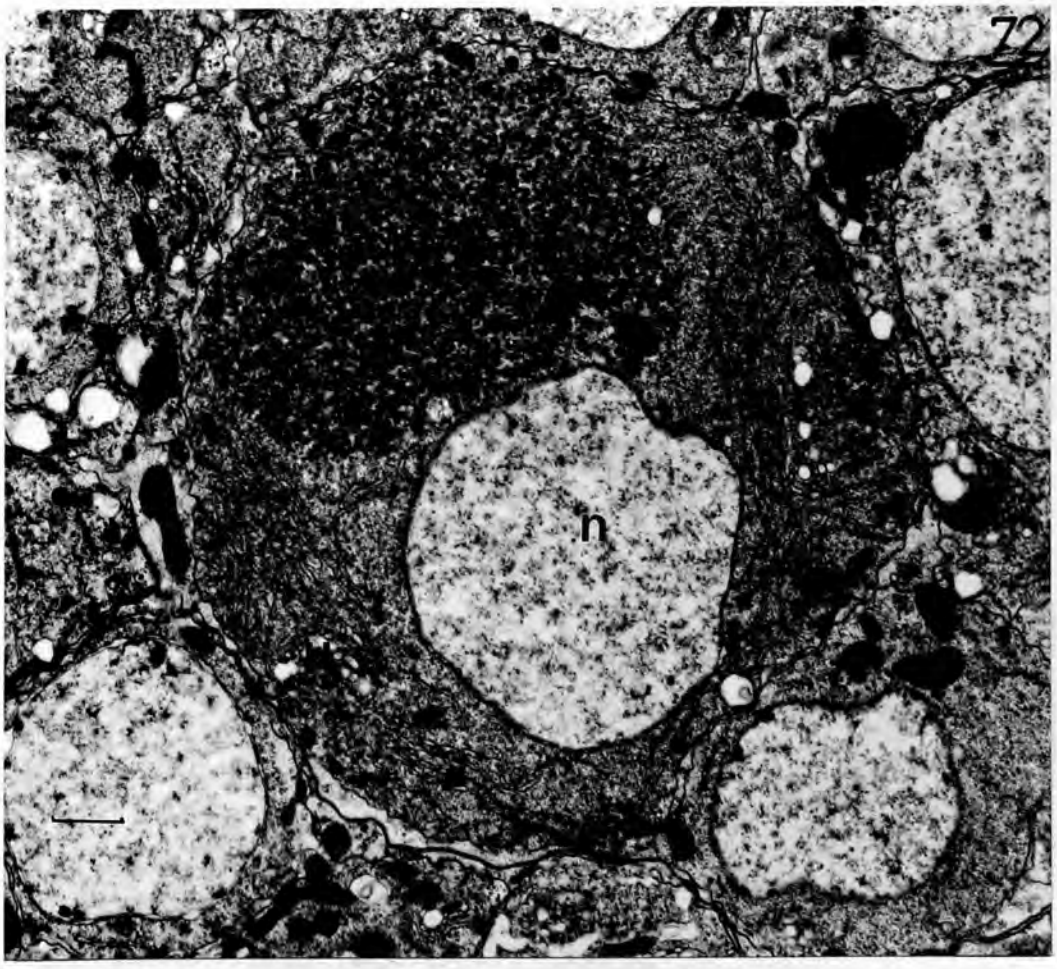
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are exceptional in having a perikarya measuring as much as 12 x 10 µm, larger than that for these cells in any other specimens investigated (Plate 72). The cytoplasm contains a greater quantity of granules than found in the β-type cells of any other specimens. The granules, which vary in electron-density, are often aggregated into a large mass at the proximal pole of the cell. Parallel lamellae of granular endoplasmic reticulum and Golgi units are more frequent in the cytoplasm of β-type cells in oviposited individuals, than in these cells in any other specimens. In oviposited specimens, the α_1 cells of the MNC contain more granules, and those of the VNC less granules (Plate 71), than in similarly aged virgins.

2. Retrocerebral Endocrine Structures of Oviposited Females

The corpora cardiaca, corpora allata and the "glandes post-cérébrales antérieures" of oviposited individuals have a similar ultrastructural appearance to those of aged virgin females described in Chapter 4. The nuclear to cytoplasmic ratio of the medullary corpora allata cells (Table 1) is similar, and both groups lack peritracheal tissues. The corpora cardiaca contain amounts of neurosecretory granules, and the intrinsic cells have a granule size distribution, similar to those of virgin neoimagines.

LIGHT MICROGRAPHS

The ovaries.

Plate 73 : A terminal oocyte of a neoimaginal virgin showing the nurse cell and yolk droplets.

Plate 74 : A terminal oocyte of a virgin 7 days after emergence. Note the smaller size of the oocyte as compared to that of the neoimagine (Plate 73).

Plate 75 : Section of the ovary of a mated oviposited specimen, in which the nurse cells but no yolk droplets can be distinguished.

SCALE BARS : 10µm.

Key:

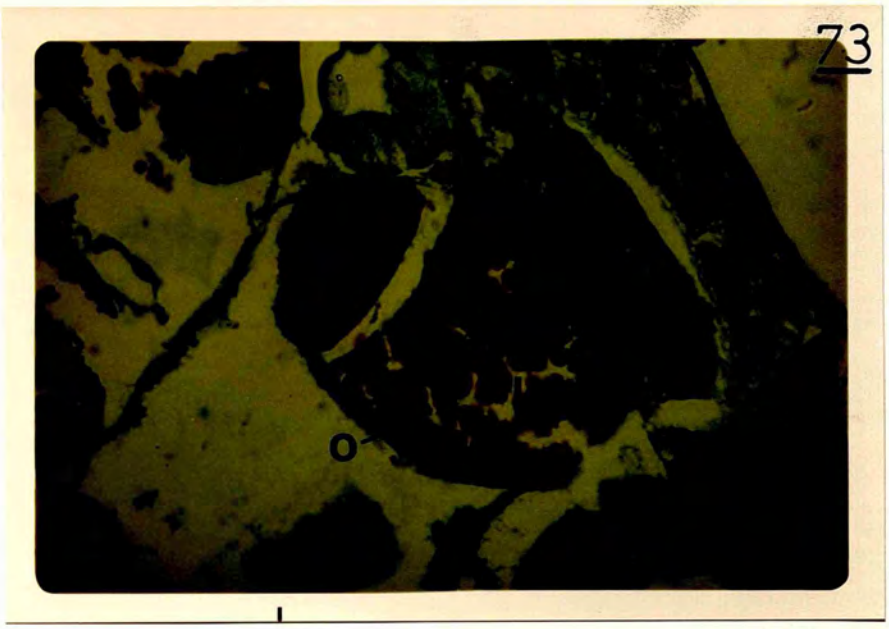
f - degenerating follicular epethelium

l - yolk droplets

n - nurse cell

o - oocyte

(Stain: Ewen's aldehyde fuchsin)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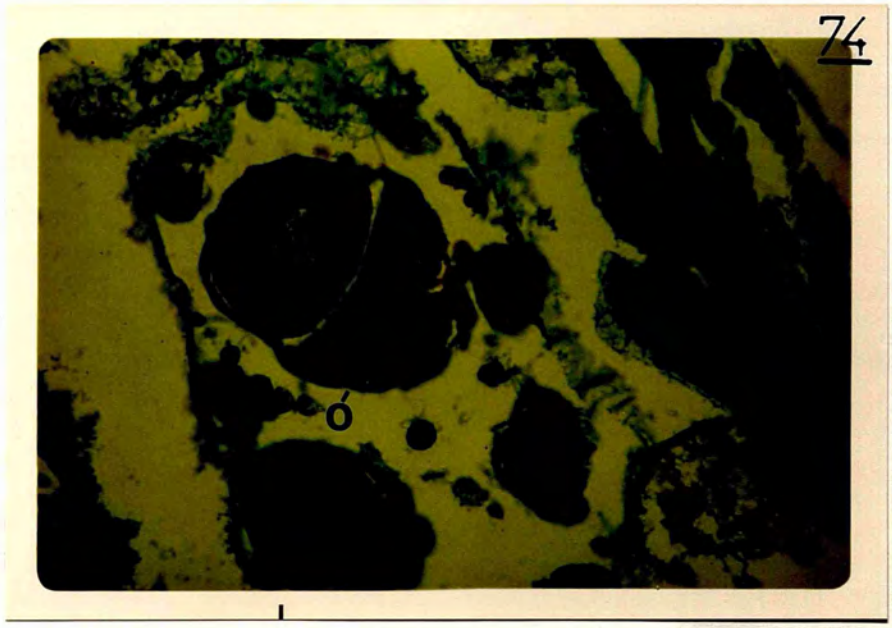
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CHAPTER 7

THE DISCUSSION

Introduction

The information gathered in this study mainly concerns the structure of the nervous and endocrine systems. The co-ordination and integration of cellular activities in insects, as in other multicellular organisms, is under the control of these two systems. A detailed description of these systems based upon observations in neoimaginal male specimens reared at 20°C is given in Chapter 3. Studies of the two systems in the female (Chapter 3) and in specimens differing in age (Chapter 4), rearing temperature (Chapter 5) and reproductive states (Chapter 6) are intrinsically interesting. These observations however, by comparison, enable functional inferences to be made based solely upon structural criteria.

SECTION I

NEOIMAGINES REARED AT 20°C

A. THE MALE

The Brain

The gross morphology of the brain and suboesophageal ganglion in comparison with other adult dipterans, has been partially discussed in Chapter 3. The cell bodies of neurons within the central nervous system of the midge, as in all

insects, form a peripheral cortical layer enclosing a central neuropile. This is the converse to the arrangement found in vertebrates. The positioning of the perikarya in insects is thought to be correlated with the external type of organ nutrition (CAZAL & SANCHEZ cited in POWER, 1943). The vertebrate brain, unlike that of insects, is vascular with penetrating capillaries supplying the centrally placed cell bodies with their nutritional and respiratory requirements. In Chironomus riparius regions where the cortical layer is thickest are usually composed entirely of neurons with small cell bodies. The larger surface to volume ratio, for exchange, of smaller cells may enable the development of a thicker cortical layer than in areas comprising of larger cell bodies. The perikarya of neurons and other brain cells were found to give a basophilic staining reaction typical of insect nervous tissue. HESS (1958) suggests that the large quantities of ribosomes visible with the electron microscope accounts for the acidity of these cells.

The clarity of division of neuropile masses and fibre tracts obtained by the histological procedures employed, made the use of specialized silver stains unnecessary. CHRISTOPHERS (1960) found that silver stains were not requisite in the demonstration of the 14 neuropile masses found in the brain of Aedes aegypti adults. Ten of these masses are comparable to those found in Chironomus riparius. The four masses which are not found in the midge (masses 8, 9, 10 and 11) are peripherally situated within the mosquito's brain. No additional neuropile masses to those occurring in Aedes were found in Chironomus.

The large mass 10 had a divisible element, termed 10_4 not found in Aedes, which resembled in position the posterior stalk of the corpora pedunculata of Drosophila melanogaster (POWER, 1942). The element 10_4 did not comprise of three concentric bands of fibres, as occur in the stalks of those dipterans which possess corpora pedunculata (HOWSE, 1974). These mushroom bodies, which are prominent structures in the brains of many insects, are much reduced in dipterans (GROTH, 1971). As in the brain of Aedes (CHRISTOPHERS, 1960), the midge, excepting the possible stalk region, lacks clearly divisible corpora pedunculata. These structures, in other insects, are operative in the selection of motor patterns and formation of behavioural sequences, their volume being proportional to the behavioural complexity (HOWSE, 1975). Whether this is true of nematocerans is, at present, unknown.

The central complex is simpler than that of Aedes (CHRISTOPHERS, 1960), comprising of only three elements, a large central body subjacent to which are a pair of structures resembling in position the ellipsoid bodies of Drosophila (POWER, 1943). The ventral tubercles which occur in the central complex of Drosophila have not been found. A comparison of the width of the head capsule with that of the central complex has been made for a number of dipterans by GROTH (1971). Average measurements for the midge are in harmony with this author's data giving added support to the suggestion of HOWSE (1974) that the size of the central complex is proportional to the dimensions of the head and is not related

to behavioural complexity. Lesion experiments have shown that the central complex is involved in the selection of levels of behavioural responsiveness (HOWSE, 1974). The neuropile masses of the optic lobes and antennal centres are presumed to be involved in processing of sensory inputs. There is little information regarding the functional significance of the other neuropile masses.

Except for tract α, which is similar to the "optical component of the central commissure" of Drosophila (POWER, 1943), the conspicuous fibre tracts of Chironomus bear little resemblance to those described in other dipterans (CHRISTOPHERS, 1960; GROTH, 1971; STRAUSFELD, 1976 and others).

The Neural Sheath

Fine structural observations indicate that the components of the brain of Chironomus are similar in many respects to those of Musca domestica described by SOHAL et al (1972). The neural lamella of the neoimaginal midge resembles a basement membrane being similar to the outermost division of the sheath, the external lamina, of Musca and of other insects (SMITH, 1968). The neural lamella of the midge's brain, unlike that of the ventral nerve cord, is not associated with fat body cells. Subjacent to the lamella is a single layer of perineurial cells which WIGGLESWORTH (1956) suggested secretes the lamella, although the actual site of its production is unresolved (review LANE, 1974). The

perineurium is similar in position, and in containing numerous mitochondria, to the "glial type i cell layer" described by WIGGLESWORTH (1959) in Rhodnius, but unlike it in lacking regularly spaced nuclei. In Chironomus the perineurial cells appear to be large and plate-like with considerable lateral overlapping. As in Musca (SOHAL et al, 1972), the perineurium is separated from the neurons by glial cells. Two types of perineurial cells are distinguished in the midge comparable to those described by MADDRELL and TREHERNE (1967) in Carausius morosus and Periplaneta americana. SOHAL et al (1972) description of the brain of Musca indicates that it has only type I perineurial cells. Type I perineurial cells are characterized by numerous large mitochondria and many free and aggregated deposits of glycogen, which are also found in the perineurium of the cockroach (SMITH and TREHERNE, 1963). In Periplaneta, WIGGLESWORTH (1960) noted that the mitochondria are often found in large numbers immediately below the lamella and in clusters deep within the cytoplasm, indicating high energy demands for these regions. In the midge, because of the large size of the mitochondria and the shallow depth of the perineurium, no such spatial relationship is discernable. Numerous free and cisternae bound ribosomes occur but Golgi units are rarely found, characters not noted in the type I perineurial cells described by MADDRELL and TREHERNE (1967). Unlike the cells described by these authors, microtubules are not totally absent. Vacuoles observed in the cytoplasm of these cells are

suggested by SOHAL et al (1972) to be the site of lipid removed by preparation procedures and the degenerative foci are probably areas where glycogen has been washed out.

Type II perineurial cells like those described by MADDRELL and TREHERNE (1967) adopt a deeper position than type I and contain large numbers of microtubules and few mitochondria. They are dissimilar in possessing some glycogen granules.

Supportive, storage and trophic functions have been ascribed to the perineurium of insects (review LANE, 1974). In the midge there is a structural basis for considering different functional roles for the two perineurial cell types. Type II cells contain numerous microtubules and are bound to the subjacent glial cells by many tight junctions, indicating a supportive role. The tight junctions of the subjacent glial cells are suggested by LANE (1974) to be the structural basis for the blood-brain barrier described by TREHERNE and PICHON (1972). Type I perineurial cells appear to be concerned with storage of glycogen and lipids, and the transfer of nutrients facilitated by energy supplied by the numerous mitochondria. Their position external to the suggested blood-brain barrier would be an advantage in allowing diffusion of nutrients from the haemolymph which could subsequently be transferred, via their cytoplasmic processes, to the cortical cells.

Glial cells

The glial cells within the brain of the midge have a similar

spatial relationship with the neurons as do the comparable cells of Musca (SOHAL et al 1972), although their fine structure is somewhat different. Type 1 glial cells correspond in position to the "peripheral subperineurial glial layer" of WIGGLESWORTH (1960) and of "Class 1 neuroglia" described by STRAUSFELD (1976), who used a positional classification in the brain of Musca. They are smaller than the "type I glial cells" described by SOHAL et al (1972) but are similar in having a cytological organisation indicative of synthesis of proteins, as well as supportive and nutritive functions. These cells, as in Musca (SOHAL et al, 1972) appear to form a link in the transfer of nutrients from the perineurium to the neurons, a hypothesis propounded by WIGGLESWORTH (1959).

Type 2 glial cells occur between the neuronal perikarya in a comparable position to the "Class 2 neuroglia" of Musca (STRAUSFELD, 1976). Their cytological organisation indicated that they are involved in protein synthesis but to a lesser extent than type 1 glial cells, as they contain fewer cisternae of granular endoplasmic reticulum. They differ from the lipid synthesizing "type II glial cells" of Musca (SOHAL et al, 1972) in lacking smooth endoplasmic reticulum. It is possible that they have a similar functional role to type 1 glial cells and may act in supplying nutrients to the perikarya deep within the cortical layer.

Type 3 glial cells occur on the inner margin of the cortex, corresponding in position to the "inner subperineurial layer" of

WIGGLESWORTH (1960) and "Class 3 neuroglia" of Musca (STRAUSFELD, 1976). They do not totally define the outline of the neuropile, as generally occurs in insects (SMITH and TREHERNE, 1963), as axons often abut onto the neuronal perikarya. These cells are similar in appearance to the "type III glial cells" of Musca (SOHAL et al, 1972) and are also similar in occurring within the neuropile, corresponding in position to "Class 4 neuroglia" described by STRAUSFELD (1976). The presence of glial cells amongst axons has been intimated in defining the synaptic pathways within the neuropile (LANE, 1974). The cytoplasmic organization is again indicative of protein synthesis and similar to that of the "type III glial cells" of Musca (SOHAL et al, 1972). The presence of large numbers of microtubules in the type 3 glial cells, which have been observed in the glia of other insects (review SMITH and TREHERNE, 1963), suggests that these cells may have a supportive, as well as nutritive role. The microtubules are orientated parallel to the neurotubules in the adjacent axons which may facilitate intercellular transfer of substances, as tubules are thought to be involved in axonal transport (review WUERKER & KIRKPATRICK, 1972).

Smooth endoplasmic reticulum which often occurs in the glia of insects (review SMITH, 1968) has not been found in these cells in the neoimaginal midge.

Extracellular Spaces

The presence of extensive extracellular spaces, termed "glial lacunar system" by WIGGLESWORTH (1960), have been confirmed by numerous electron microscope studies (review SMITH & TREHERNE, 1963). Most of the physiological information of extracellular spaces is obtained from studies of the central nervous system of Periplaneta and usually relates to its nerve cord. There is little information regarding the "glial lacunar systems" of Diptera. In the brain of Musca, SOHAL et al (1972) mention the presence of several distended extracellular spaces but do not describe them as an extensive system. In Calliphora, BLOCH et al (1966) describe the glial lacunae as being much less extensive than those of Periplaneta and the authors express some doubt as to whether the system is truly extracellular. In the neoimaginal midge, very few spaces occur in the brain. It is possible that an osmotic fixation artefact could cause the loss of extracellular spaces in prepared material. This possibility is discounted because the mitochondria and cisternae of endoplasmic reticulum, which are highly susceptable to osmotic effects (BAKER, 1966), appear normal. Small electron-lucent areas, which initially appear to be extracellular, usually on closer examination are found to be intracellular resulting from cytoplasmic degeneration. Axons often give this appearance but careful inspection invariably reveals fragments of neurotubules surrounded by a plasma membrane. LANE (1974) has reported the occurrence of unbanded fibrillar elements in the lacunar system. Although the size of these elements was not stated, they are apparently associated

with electron opaque material which would prevent confusion between degenerated axons containing neurotubules and glial lacunae. Spaces between perikarya in the cortical layer occur infrequently and are usually associated with two particular types of neurons, discussed below. It, therefore, seems unlikely that there is an extensive system of interconnecting extracellular channels in the brain of the neoimaginal midge. The small size of the brain may make such a system unnecessary for the fulfillment of nutritional demands, which can be supplied by simple diffusion.

SMITH & TREHERNE (1963) state, "A knowledge of the extent of the extracellular space within the insect nervous system is essential for any proper understanding of its physiology." The absence of an extracellular system within the brain of the neoimaginal midge and, possibly that of Musca (SOHAL et al, 1972), suggests that the understanding of neural transmission based upon the extensive glial lacunae of cockroaches, may not be comparable to such processes within the brains of other insects.

Tracheal system

Haemolymph is not involved in gaseous exchange in insects, oxygen being carried directly to the site of utilisation by a system of trachea (CHAPMAN, 1971). The tracheal system in the brain of the midge is similar to that of Aedes (CHRISTOPHERS, 1960). The larger tracheoles do not pass through an extra-

cellular space when entering the neuropile, as they do in the abdominal ganglia of Periplaneta (SMITH & TREHERNE, 1963). As described by HESS (1958), the cuticular lining of the tracheole is always surrounded by the cytoplasm of the tracheolar cell, which is further separated from the adjacent neurons by glioplasm. The greater abundance of tracheoles in the deeper regions of the cortex may indicate a higher oxygen demand for the cells in these areas.

Neurons

Numerous studies have revealed that insect ganglia contain neuronal cell bodies which differ in size, staining reaction and cytological organisation (reviews: SMITH & TREHERENE, 1963 and LANE, 1974). In the brain of Musca, SOHAL et al (1972) distinguished five types of non-neurosecretory neurons on the basis of perikaryon size and organelles, with the added criterion of nuclear to cytoplasmic ratio. Using these characters, five types of non-neurosecretory neurons were distinguished in the brain of Chironomus, four of which resemble those of Musca in structure. Neuron types 1 to 4 are monopolar, as are many of the neurons within the brains of insects (LANE, 1974).

Type 1 neurons are smaller than "type I neurons" of Musca, but are similar in cellular organization. The nuclear envelope is crenulated with a variable thickness, characters not noted by SOHAL et al (1972) but which do appear in their

published micrograph. In the midge, these cells are the main constituents of the optic lobes, whereas in Musca, "type 1 neurons" are restricted to the protocerebrum.

Type 2 neurons are less frequent than type 1. They occur adjacent to type 1 neurons, but have a similar cellular organization. They have a cell body which is smaller and has a greater electron-density than type 1 neurons and is dissimilar in being associated with extracellular spaces. It is suggested that degenerative processes cause type 1 neurons to shrink, resulting in the separation of their plasma membranes from adjacent cells and increasing the electron-density. These type 2 neurons are similar in structure, electron-density and in having the highest nuclear to cytoplasmic ratio of the brain neurons, to the "type II cells" of Musca. SOHAL et al (1972) do not comment upon the occurrence of spaces surrounding "type 11 neurons", but the published micrograph of this cell type exhibits such spaces. In Musca "type II" neurons are the main constituents of the optic lobes cortex, whereas in the neoimaginal midge, type 1, with only a few type 2 neurons, occur in this region. If, as suggested above, type 2 neurons are derived from type 1, it may be possible to obtain by transformation, optic lobes comparable to those of Musca in containing mainly type 2 neurons, a hypothesis which is further discussed below.

Type 3 neurons are similar in structure to the larger "type 111 neurons" of Musca, but have a more restricted distri-

bution, being absent from the lateral extremities of the optic lobes.

Type 4 neurons, although larger than type 2, are similar in electron-density and in being associated with extracellular spaces. Their size and distribution indicates that they may be derived from the type 3 neurons by a similar process to the transformation of type 1 to type 2 cells. The restriction of type 4 neurons to the pars intercerebralis may indicate that the suggested processes of degeneration occurring in type 3 cells begin in this region of the brain. Type 4 cells, although similar in size to the bipolar "type IV neurons" of Musca, do not resemble them in either electron-density or polarity.

The giant, type 5, neurons have the largest cell bodies occurring in the brain. Although smaller than the "type V" neurons of Musca, they are similar in cytological organization, except that the numerous mitochondria are no larger than in other neurons and the endoplasmic reticulum is usually more organized, often occurring in parallel arrays. In Chironomus the giant neurons are found throughout the brain, unlike those of Musca which are localized in specific regions. The giant neurons of both species are the only multipolar nerve cells occurring in the brain.

The functional significance of the various neuronal types is unknown and awaits elucidation by physiological techniques.

The frequent large invaginations of glioplasm into the neuronal perikarya, the "trophospongia of Holmgren", reported in many other insects (review LANE, 1974) have not been found

in Chironomus. BLOCH et al (1966) and SOHAL et al (1972) do not comment on the presence of invaginations into the perikarya in the brains of Calliphora and Musca respectively, nor do such structures appear on their published micrographs. The lack of trophospongia may be a dipteran condition but, as reports of it are usually restricted to larger insects, e.g. Periplaneta (HESS, 1958), Locusta (GIRARDIE & GIRARDIE, 1967) and Carausius (LANE, 1974), its presence may simply be a reflection of the increased nutritional constraints that a more massive system with a lower surface to volume ratio exhibits.

Axons

As in the neurons of other insects (review LANE, 1974), the axon hillock exhibits a difference in cytological organization from the perikarya containing only those organelles present in the axons. An exception occurs in the giant neurons which are similar to those of Musca (SOHAL et al, 1972) in having no marked difference between the perikarya and the axon hillock. The axonal contents of the midge are typical of insects (review LANE, 1974) in lacking neurofilaments, ribosomes and saccules of granular endoplasmic reticulum which are found in most other nervous systems (review WUERKER & KIRKPATRICK, 1972). The neurotubules and their filamentous projections are similar in size and organization to those typical of nervous tissue (review WUERKER & KIRKPATRICK, 1972). Double staining with uranyl acetate and lead citrate results in the neuro-

tubules appearing as an electron-dense ring, very similar to those occurring in Periplaneta (LANE & TREHERNE, 1970) when the same stains are used. The axonal mitochondria are similar in organization and orientation to those typical of nervous tissue (review WUERKER & KIRKPATRICK, 1972). The termination of each axon is marked by the occurrence of vesicles, typical of insect synapses (reviews: SMITH & TREHERNE, 1963 and LANE, 1974).

The axons of Chironomus are unmyelinated as are those of other invertebrates (review LANE, 1974). Most of the smaller axons are naked and even the largest axons, although often having a glial investment, never exhibit the meso-axonal condition which occurs in the large axons of many insects (review LANE, 1974). The results of this study indicate that axons from groups of neurons, usually of the same type, often form discrete bundles within the neuropile. This grouping of neuronal cell bodies reflected in the axon bundles possibly represents a division into different functional units within the brain. The axons referred to as "giant" originate from singular type 5 neurons and are probably not comparable to the much larger ones of other insects which are thought to have a multicellular origin. (CHAPMAN, 1971).

The Ventral Nerve Cord

The anatomy of the suboesophageal ganglion of Chironomus is similar to that of Aedes (CHRISTOPHERS, 1960). Knowledge of the cell types and fine structure of the components of this ganglion in comparison with those of the brain has been partially discussed in Chapter 3. The neural sheath is similar to that of the brain, except that the outer acellular layer is twice as thick. As the neural lamella has a mechanical role (SMITH, 1968), this increased thickness may be a reflection of the need of increased support in this region. In the ventral wall of the central canal the neuropile abuts onto the perineurium, the cortical layer being absent. In this region the perineurium comprises solely of type II perineurial cells, which as suggested above, may perform a supportive role. The presence of type I perineurial cells, which possibly have a trophic role, may be unnecessary as there are no neuronal cell bodies in this region. Type I glial cells are absent from the suboesophageal ganglion, except in this dorsal corticalless region. These glial cells are possibly capable of performing their trophic role without the aid of type I perineurial cells, which as suggested above, may form the initial link in the transference of nutrients from the haemolymph to the neurons. The absence of type I glial cells from the remainder of the ganglion is at present not understood.

The suboesophageal ganglion lacks type 2 and type 4 neurons which, it is suggested, are derived by transformation

from other neuron types. This would appear to indicate that the supposed degenerative processes involved are not occurring in the ganglion of the neoimagine.

The axons within the neuropile of the suboesophageal ganglion tend not to be grouped into discrete bundles. This indicates that axons from the ganglion's cortical cells are not usually grouped and that the axons in bundles from the brain become distributed when passing through the ganglion. Within the ganglion's neuropile, numerous dense bodies of unknown origin and function occur which have not been found in the brain.

The anatomy of the prothoracic ganglion and its connectives to the suboesophageal ganglion are similar to those of Aedes (CHRISTOPHERS, 1960). The neural sheath of these structures resembles that of the brain, except that the neural lamella is thinner. In some areas there is a close association between the surrounding fat body cells and this lamella. This "neural fat body sheath" has been described in many other insects (review LANE, 1974) and it has been suggested (SMITH & TREHERNE, 1963) that the spatial relationship indicates that the metabolic processes of the fat body cells and the perineurial cells are closely linked across the neural lamella. As this lamella is not a barrier to free diffusion (review LANE, 1974), its thinness may facilitate the metabolic linkage by reducing the distance over which diffusion has to occur. The neurons of the prothoracic ganglion are of

the same type as occur in the suboesophageal ganglion but are unlike them in exhibiting classical signs of osmotic damage to their organelles. Unlike in the brain and suboesophageal ganglion, there is an extensive system of extracellular spaces resembling the glial lacunae of other insects which is discussed above. It is difficult to ascertain whether these spaces occur in vivo or are a preparation artefact caused by this region of the nerve cord being more susceptible to osmotic damage than the suboesophageal ganglion. The cortical cells of both the brain and this ganglion are often exposed, by dissection damage, directly to the solutions employed in preparation but still have a similar appearance to those of intact tissue. This suggests that the osmotic effect which may result in the appearance of this ganglion's tissue is not caused by the functioning of the blood-brain barrier, discussed above. It is possible that the osmotic damage to the cells of the prothoracic ganglion, which may also result in the formation of extracellular spaces, is caused by them having a lower osmotic pressure than the cells of the cerebral nervous tissue.
As noted above, much of the work on insect glial lacunae is based upon the ventral nerve cord, suggesting that the extracellular spaces in Chironomus may not be total artefacts.

The Neurosecretory system

Before commenting upon the structure of the neurosecretory system, some discussion of the techniques used in its elucidation are necessary. The methods employed in this study were the classical histological procedures as outlined by GABE (1966) coupled with an electron-microscope investigation. Neither of these methods can provide proof that a cell is neurosecretory, as conclusive evidence must be based upon the demonstration that such cells produce substances with a measurable physiological activity (BERN, 1962; MORRIS & STEEL, 1975). The term "neurosecretory cell" in this study has to be based on structural criteria and is, therefore, given to cells which contain granules of 60-300 nm both within the perikarya and also within the axons leaving these cells, which is characteristic of neurosecretory cells (LANE, 1974). In most cases, the axons from these cells were traced to neurohaemal areas which provides further evidence for their neurosecretory nature. Of the stains employed in this study, EWEN'S (1962) paraldehyde fuchsin was found to be the most successful in selectively staining neurons. The results with this stain were restricted to the MNC group in the neoimaginal male. Why the alternative aldehyde fuchsin technique (MEOLA, 1970), developed for use with mosquito systems, gave no selective staining of neurons has not been resolved. A possible fault lies in the preparation method

(ROSA, 1953) of this stain which did not give the expected amount of precipitate. After staining with EWEN'S (1962) paraldehyde fuchsin technique, the cells in the MNC group contained purple inclusions and thus resembled the classical A-type cells of other authors (review GABE, 1966). The second Gomori stain, chrome haematoxylin-phloxin, which has usually been considered to give parallel results to aldehyde fuchsin (BERN, 1962), failed to give a staining reaction typical of A-type cells. Phloxinophila was rarely encountered and only in a few of the MNC cells, indicating the presence of B-type cells. The chrome haematoxylin-phloxin method is in no way specific (ANONYMOUS, 1954) and there are several reports of it giving unsatisfactory results in Diptera (FRASER, 1959; THOMSEN, 1954, 1965). TIWARA & SRIVASTAVA (1975) found that chrome haemotoxylin-phloxin does not give comparable results to aldehyde fuchsin for neurosecretory material. The staining reaction of the supposed neurosecretory material in Chironomus with chrome haematoxylin-phloxin which initially appears peculiar, is, therefore, not unique. The assumption that it gives comparable results to aldehyde fuchsin must, apparently, be viewed with some sceptiscism. Failure of a neuron to stain selectively with Gamori stains does not preclude it from being neurosecretory (BERN, 1962; THOMSEN, 1965). The inability to detect the other cell groups identified as neurosecretory by electron-microscopical techniques, is therefore not surprising.

It was found that omission of uranyl acetate staining greatly reduced the electron-opacity of neurosecretory granules. As uranyl acetate acts as a mordant for lead citrate by combining with proteins (DAWES, 1971), this increase in contrast indicates that the neurosecretory granules in Chironomus, as in other insects (review MADDRELL, 1974), are of a proteinaceous nature. If the double staining technique is not employed, cells containing granules of low electron-opacity are usually missed in low magnification searches of tissue.

Quantification of the amount of neurosecretory material present within a cell was assessed by a simple subjective method. Densitometric scanning of micrographs would not have given comparable results because of the varying electron-densities of the granules. Such methods do not obviate the worker's discretion of choosing representative micrographs of these cells. The definitive methods which have been used to quantify amounts of neurosecretion in other invertebrates (WENDELAAR BONGA, 1971; ROUBOS, 1975; and others) have been applied to much larger and more homogeneous regions of neurosecretory tissue, than occur in Chironomus. These methods are also based upon the preparation of numerous micrographs from which a random selection can be made, necessitating the consumption of large quantities of materials. Such methods were therefore found to be impracticable in the present study. X-ray microanalysis of sulphur to

estimate the amount of neurosecretion, as used by STEEL & MORRIS (1975) in Rhodnius, could not be employed because the relevant equipment was unobtainable.

The axons of the various neurosecretory cells were traced by using the characters of size and density of their granules. This method is based upon the assumption that the granules do not change in their passage down the axon. Evidence for this is provided by the observations that granules at the distal end of an axon are of similar size and electron-density to those of the proximal end, as shown by the serial sectioning of some axons throughout their length. This further supports the view of NORMANN (1965) that progressive synthesis of neurosecretion along the axon, as postulated by GERSCHENFELD et al (1960), does not occur.

The neurosecretory neurons of Chironomus exhibit many features in common with such cells in other insects (reviews: LANE, 1974; MADDRELL, 1974). As is typical, the neurosecretory granules appear to be budded off from the saccules of Golgi units. There have been no observations to support the view that granules may be formed directly from granular endoplasmic reticulum, as reported in frontal ganglia (BORG et al, 1973; YIN & CHIPPENDALE, 1975). The occurrence of glycogen deposits within the neurosecretory cells' perikarya and their frequent association with glycogen rich neurons, may reflect the high nutritional demands most probably required for the synthesis of neurohormones.

The Medial Neurosecretory Cells of the Brain

As is typical of pterygote insects (GABE, 1966), the midge has a group of MNC in the pars intercerebralis. This group appears to comprise of cells of one morphological type which are similar to the MNC of the larvae of Chironomus riparius (CREDLAND & PHILLIPS, 1974), although with the electron microscope they are found to contain fewer granules (CREDLAND, personal communication). The MNC are the only neurosecretory cells that these authors reported in the brain of the larvae.

The α_1 cells of this group resemble the larger "M.N.C. I" of Calliphora erythrocephala described by BLOCH et al (1966) and like them contain spherical electron-dense granules but with a marginally smaller diameter. The occurrence of classical B-type cells (review GABE, 1966) suggested, as discussed above, by the inconsistent rare staining with chrome haemotoxylin-phloxin could not be demonstrated with aldehyde fuchsin techniques nor were there any fine structural evidence for two cell types in this group. Therefore the MNC of Chironomus appear to be mainly, if not completely, comprised of classical A-type cells, (review GABE, 1966), resembling the medial cells of mosquitoes (BURGESS & REMPEL, 1966; MEOLA & LEA, 1972).

The MNC are the only cerebral neurons to exhibit selective staining with either alcian blue or aldehyde fuchsin, suggesting that some of their chemical constituents differ from those of other neurosecretory cells. The similarity in observations is not unexpected as both reactions are thought to function by the selective oxidation of the amino acids, cysteine and cystine

producing strong acids, which are necessary for staining with the constituent basic dyes at low pH. It is possible that the MNC cells differ from the other neurosecretory cells in containing a higher concentration of these amino acids, however the selectivity of the staining reactions is far from certain.

The negative results with periodic acid Schiff's test for the MNC and the other groups was not unexpected, as neurosecretory cells in other dipterans are known to give a similar reaction (RAMADE, 1969).

The Ventral Neurosecretory Cells

The six cells of each VNC group are similar in position to the two cells occurring in mosquitoes (MEOLA & LEA, 1972) which only occasionally stain with aldehyde fuchsin. The axons from these cells in the midge join those of the contralateral MNC ventral to the chiasma, whereas in the mosquito, their terminations are unknown. These cells resemble in position a group of "other possibly neurosecretory cells" described by THOMSEN (1965) in Calliphora erythrocephala, but lack any association with "vacuolated cells", RAMADE (1969) suggested that the vacuolated cells may appear frequently in higher Diptera, but in the midge, as in other nematocerans, they have not yet been demonstrated.

The Lateral Neurosecretory Cells

The LNC of Chironomus are unlike those of mosquitoes (MEOLA & LEA, 1972) but similar to those of Calliphora (THOMSEN, 1965) and Musca domestica (RAMADE, 1969) in not exhibiting purple staining with paraldehyde fuchsin. The LNC of Calliphora (THOMSEN, 1965) are phloxinophilic, unlike those of Chironomus and of Aedes ægypti (BURGESS & REMPEL, 1966). Two of the three LNC of each side of Musca (RAMADE, 1969) are similar to those of Chironomus in showing no selective reaction with chrome haematoxylin-phloxin, although the other one is phloxinophilic. In position and in having axons which do not decussate but form the NCC II of the epsilateral side, the LNC of Chironomus are similar to those other dipterans. The axons of the LNC of Chironomus differ in having collateral branches which pass into the optic lobes.

The Outer Neurosecretory Cells

The ONC groups in Chironomus are located in similar positions to the "laterale A-zellen" in Drosophila (KÖPF, 1957), the "cellules optiques medianes" of Musca (RAMADE, 1969) and the "optic neurosecretory cells" of Calliphora (THOMSEN, 1965). The term "outer" was selected as these cells are situated at the periphery of the protocerebral lobe and because it avoids confusion which could arise from applying the term "lateral" to two differently situated cells. The term "optic" was not employed as this may imply a functional significance.

The cerebral neurosecretory system of adult Chironomus riparius shows many features to be found in other Diptera. There appears to be a close resemblance to the organization found in Calliphora (THOMSEN, 1965) which is less complex than that described in Musca (RAMADE, 1966; 1969). The cerebral neurosecretory system of Chironomus lacks the anterior groups found in mosquitoes (BURGESS & REMPEL, 1966; MEOLA & LEA, 1972), but is similar in respect of the medial, lateral and ventral groups. The occurrence of the ONC in mosquitoes has not yet been demonstrated and their absence may represent another difference. The possible functional significance of these cells in Chironomus will be discussed in the light of the additional data.

Neurosecretory Nerves

Two pairs of neurosecretory nerves arise from the posterior of the neoimaginal midge's brain, the NCC I and NCC II, which, as occurs in many insects (review ENGELMANN, 1970) run to the corpora cardiaca. The adult condition is different from the larval, where only one pair of nerves have been found (CREDLAND & PHILLIPS, 1974) which appear to be comparable to the NCC I. As in other insects, (review ENGELMANN, 1970) the neurosecretory axons are derived from cells situated in the medial region of the brain (MNC and VNC) and those of the NCC II from the lateral cell groups (LNC and ONC). Both nerves, as do the bifurcated NCC of the larva (CREDLAND & PHILLIPS, 1974),

enter the wall of the aorta as occurs in mosquitoes (BURGESS, 1971; MEOLA & LEA, 1972). The axons of the NCC II appear to pass through the corpora cardiaca, the majority terminating in the corpora allata, as has been reported in Locusta migratoria (HIGNHAM & WEST, 1971). The NCC I and NCC II both contain non-neurosecretory axons, which have also been observed in the NCC I of both Periplaneta americana (WILLEY, 1961) and Blabera fusca (BROUSSE-GAVRY, 1963) but have not been reported in Diptera. The neurosecretory nerves are similar to some other peripheral nerves of both the adult and the larva (CREDLAND, personal communication) in lacking the typical glial investment of insect nerves (SMITH, 1968). The nerves are only surrounded by an acellular neural lamella which is thought to be permeable (review LANE, 1974). The presence of a lamella without subjacent glia is indicative of a neurohaemal area (MADDRELL, 1974), as the axons are partly, or totally, exposed to the extraneural environment, which would imply that neurosecretion may be released anywhere along the NCC in Chironomus. No observations were found to support this idea and the reduction or total absence of the acellular sheath around the swollen endings of axons in supposed neurohaemal areas suggests that the lamella of Chironomus, when over a certain thickness, may act as a barrier to the release of neurosecretion, although it is possibly permeable to small molecules, as in other insects.

The collateral branches of the MNC leave the brain anterior to the NCC and join the recurrent nerve. These axons do not decussate, which has also been observed in some neuro-

secretory axons leaving the pars intercerebralis of Periplaneta americana (WILLEY, 1961), but has not been previously reported in Diptera. The collaterals are indistinguishable from other axons containing α_1-type granules, so that it has proved impracticable to ascertain their terminations. For this reason they were not denoted as an NCC, even though their axons may pass to the corpora cardiaca.

The absence of NCC III and NCC IV which occur in some insects (review ENGELMANN, 1970), was not unexpected as they have not yet been reported in any Diptera so far studied.

The Frontal Ganglion

Technical difficulties resulted in the observations upon the frontal ganglion being restricted to the light microscope. This single median ganglion exhibits the same nervous associations as found in the larva (CREDLAND & PHILLIPS, 1974), which is typical of most insects (CHAPMAN, 1971). The Gomori stains did not indicate the presence of any neurosecretion within the ganglion. BELL et al (1974) suggests that the failure of many investigators to differentiate neurosecretory cells in the frontal ganglia of some insects, may have been due to the selection of very active stages when the amount of available neurosecretory material was too low to be visualized by conventional techniques. If this were so in Chironomus, the cells would be actively transporting neurosecretion along their axons. No evidence of neurosecretion was found by histological procedures in the frontal

connectives and the electron microscope revealed no neurosecretory granules in the recurrent nerve anterior to the brain. It is therefore, tentatively, suggested that no neurosecretory cells occur in this ganglion in Chironomus. The observations do not further elucidate the functional significance of the ganglion, as suggested by PENZIN & STÖLZNER (1971) in controlling water balance or the association with diapause implicated by YIN and CHIPPENDALE (1975).

The Recurrent Nerve

The recurrent nerve, as is typical in insects (CHAPMAN, 1971), connects the frontal ganglion to the hypocerebral ganglion. Its intimate association with the gut wall may reflect the control that the frontal ganglion is thought to have upon gut movements (WIGGLESWORTH, 1965). The loss of glial investment, atypical of insect nerves (SMITH, 1968), is associated with a thickening of the acellular neural lamella surrounding the nerve. The increase in thickness of the lamella may compensate for the loss of glia which are suggested, provide both support and protection from the extraneural environment (review LANE, 1974). The integral association of the recurrent nerve with the aorta wall has also been reported in mosquitoes (BURGESS & REMPEL, 1966; MEOLA & LEA, 1972). Unlike in these mosquitoes, the recurrent nerve does contain neurosecretory axons posterior to the brain.

The Hypocerebral Ganglion

The hypocerebral ganglion differs in organization from that of other insects (review LANE, 1974) in lacking a perineurium *per se*, although an incomplete investment with glia occurs. There is also no clear division into a central neuropile with a surrounding cortex. The close proximity to fat body cells is, however, typical and may, as suggested in the ventral nerve cord, be associated with a metabolic linkage which, in this case, apparently occurs between the glial and fat body cells.

The granular aggregates which occur in the ganglion are similar to the neurosecretory storage sites of other insects (review MADDRELL, 1974). Electron microscope observations indicate that the ganglion is a neurohaemal organ releasing granules into the aorta via the thin acellular sheath, lacking subjacent glia, which separates the axons from the lumen. The presence of non-membrane bound granules in the sheath and the occurrence of small electron-lucent vesicles in the axons suggests that the neurosecretion is released by exopinocytosis and the membrane is subsequently recaptured, as proposed in other insects (MADDRELL, 1974). The granules appear to undergo partial disintegration in the sheath before being released into the aortic lumen. The finger-like processes of the sheath may facilitate the dispersal of the released factor by providing a large surface area in contact with the haemolymph.

The nervous association of the ganglion with the recurrent nerve, NCC I and NCA resembles that of the "neurohaemal organ" of Culiseta inornata (BURGESS, 1971) and the "dorsal mass" of Aedes ægypti (BURGESS & REMPEL, 1966). The suggestion of BURGESS (1971) that in these mosquitoes, these regions correspond to the neurohaemal component of the corpora cardiaca of other insects, may be applicable to Chironomus as such a component appears to be absent. The comparable region in Aedes taeniorhynchus (LEA, 1970) has been implicated in the regulation of egg maturation, in anautogenous specimens. The possible functional significance of this release site in male and autogenous female nematocerans awaits further elucidation.

The hypocerebral ganglion resembles that of mosquitoes (MEOLA & LEA, 1972) in containing no intrinsic neurosecretory cells. The axons from the α_2-type VNC of the brain appear to terminate in this ganglion and the majority of the released and stored neurosecretory material associated with it is probably derived from these cells. As in the larvae (CREDLAND & PHILLIPS, 1974), a single pair of nerves leaves the posterior of the ganglion. In the adult each nerve combines with another which arises from the epsilateral side of the fused recurrent nerve with the NCCs anterior to the hypocerebral ganglion, unlike the larval condition where each nerve combines with one from the ipsilateral anterior region of the hypocerebral

ganglion. In the adult, the axons which are derived from the posterior of the hypocerebral ganglion appear to pass through the corpora cardiaca and terminate in the corpora allata, whereas the comparable nerves in the larva run to the ingluvial ganglion (CREDLAND & PHILLIPS, 1974). The apparent absence of an ingluvial ganglion in the adult is unusual and may be associated with the probable lack of a functional digestive system, as this structure is thought to control gut movements (WIGGLESWORTH, 1965).

The Corpora Cardiaca

The adults possess a pair of unfused corpora cardiaca, as do the larvae (CREDLAND & PHILLIPS, 1974), in agreement with observations on the preimaginal phases of other members of the genus Chironomus (KUMMEL, 1969; POSSOMPÈS, 1948; ZEE & PAI, 1944 and others). This genus differs from other nematocerans studied, which possess partially or completely fused corpora cardiaca (CAZAL, 1948; MEOLA & LEA, 1972; TARGA, 1974 and others). These glands, as is apparently typical of the genus, are separated from the aorta, differing from those of many other nematocerans (MEOLA & LEA, 1972; TARGA, 1974 and others) and of cyclorrhaphan dipterans (NASKAR & NANDA, 1975; THOMSEN, 1951 and others).

The corpora cardiaca are enclosed in an acellular sheath, as occurs in many insects (SMITH, 1968) but are unusual in

lacking subjacent glial cells. Loosely associated intrinsic neurosecretory cells occur resembling those of the larvae (CREDLAND & PHILLIPS, 1974) and of many other insects (review SMITH, 1963). The glands differ from those of mosquitoes (MEOLA & LEA, 1972) in containing no intrinsic non-neurosecretory cells and in possessing neurosecretory cells. The intrinsic cells of Chironomus are similar but larger than the cerebral neurosecretory cells and differ in having granules with a wider range of size and a bimodal distribution of diameters. The possible significance of this distribution is discussed below. These cells bear a marked resemblance to those of Calliphora erythrocephala (NORMANN, 1965; THOMSEN & THOMSEN, 1970), except that no comparable "intra-nuclear particles" have been found.

The neuropile region of the corpora cardiaca contain axons of both intrinsic and extrinsic origins. Within the axons, aggregations of neurosecretory granules occur and thus resemble the storage depots of other insects (review MADDRELL, 1974). Most of these aggregates comprise of large diameter granules, apparently derived from the intrinsic cells. The lack of association with the aorta, the presence of a thick investing sheath and the absence of observations indicating granule release suggest that the corpora cardiaca are not a neurohaemal area in this species. This suggestion, coupled with the observation that no large diameter granules are

found in the axons leaving the aorta, would appear to indicate that either the intrinsic secretion is being stored, or is broken down into smaller granules before leaving the corpora cardiaca. These larger granules may represent the production of a different hormone than that contained in the smaller granules, as suggested in the mammalian pituitary (NAKANE, 1970), or simply be a more efficient utilization of membrane in the storage of secretion, (e.g. a single 250 nm diameter granule requires half the surface area of membrane as six granules 140 nm in diameter containing the same amount of secretion).

The Corpora Allata

The paired corpora allata are associated with the aorta and are not fused to the corpora cardiaca, and therefore resemble those of many nematocerans (BODENSTEIN, 1945; KÜMMEL, 1969; MEOLA & LEA, 1972 and others). TARGA (1975) reported that the midge Rhyncoschiara hollænderi possesses a single fused corpora cardiaca-allata complex. This nematoceran is somewhat unusual as it is a rare exception to the hypothesis of POSSOMPÈS (1953) which suggests that such single structures are characteristic of the higher Diptera. The allata are of the "pseudolymphoid type", which is a phylogenetically lower type than the "large cell type" typical of many Diptera (NOVAK, 1975). The pseudolymphoid type has been reported in the larva of Chironomus riparius (CREDLAND & PHILLIPS, 1974) and in the larva and pupa of

C. melanotus (KÜMMEL, 1969). Ultrastructural observations of the corpora allata of adult dipterans are restricted mainly to species of Calliphora (JOHNSON, 1966; THOMSEN & THOMSEN, 1970 and others) and to Drosophila melanogaster (AGGARWAL & KING, 1969; KING et al, 1966). There have been no published ultrastructural observations upon the adult allata of Nematocera, and the reports of CREDLAND & PHILLIPS (1974) and KÜMMEL (1969) on preimaginal phases are the only fine structural descriptions of the gland of this suborder. Penetration of the investing acellular sheath, which in the allata of many insects (reviews of NOVAK, 1975 and SMITH, 1968) forms a stroma, does not occur. No mention of an allatal stroma is found in the descriptions of the preimagines of C. riparius (CREDLAND & PHILLIPS, 1974) or of C. melanotus (KÜMMEL, 1969).

Ultrastructurally, two cell types have been distinguished in the neoimaginal midge, based upon their position, shape and appearance. The matrix of these cells showed little difference in electron-opacity which results in the "dark" and "light" allata cells which have been observed in a variety of insects (BUSSELET, 1969; DORN, 1973; THOMSEN & THOMSEN, 1970). The single allatal cell type in the preimagines of C. melanotus (KÜMMEL, 1969), most resembles those cells constituting the cortical layer. The similarity between the components of the cortical and medullary cells in C. riparius if present in C. melanotus may have resulted in a failure to distinguish the

two cell types. Detailed fine structural observations upon the corpora allata of C. riparius larvae are not yet available, but the introductory work of CREDLAND & PHILLIPS (1974) shows a marked similarity to those of the adult. SMITH (1968) states that "The surprisingly variable structure of the allatum cells, from one species to the next, provides conflicting indications of the nature of their secretion." In C. riparius the predominance of the smooth endoplasmic reticulum may be indicative of steroid production, as indicated in other dipterans (KING et al, 1966; THOMSEN & THOMSEN, 1970 and others). A few lipid droplets, suggested by THOMSEN & THOMSEN (1970) to contain juvenile hormone, occur in the cytoplasm of the allata cells. Areas of cytoplasmic degeneration may also reflect the presence of lipid lost in preparation. The scarcity of lipid droplets and the absence of distinct aggregations of smooth endoplasmic reticulum may, as in Calliphora (THOMSEN & THOMSEN, 1970), be indicative that the allata are in an inactive phase. Light microscope observations indicate a similarity in size of both allata cells and their nuclei to those present in the larvae (CREDLAND & PHILLIPS, 1974). The relationship of nuclear and cytoplasmic size is thought to be indicative of the activity of the allata cells (ENGELMANN, 1970), as found in Calliphora (THOMSEN & THOMSEN, 1970). The larval observations are based upon the fourth instar, prior to pupal apolysis, when it is probable that the corpora allata are in a phase of inactivity. The similarity in the allata cells of this larval phase with those of the neoimaginal male, suggests

that these glands in the latter may also be inactive.

The neurosecretory axons from the NCA branch profusely upon entering the allata, which is characteristic of a neurohaemal organ (MADDRELL, 1974). The intimate association of these axons with the allata cells, was also noted by KÜMMEL (1969), and may facilitate the transfer of material. There are several suggestions of the antagonistic effects that nervous, as opposed to neurosecretory influences, have upon the activity of the corpora allata (review NOVAK, 1975). In the midge the occurrence of axons from the ONC and LNC containing α_2 and β-type granules respectively suggests that possibly one group may be associated with restraining and the other activation.

In Chironomus melanotus, KÜMMEL (1969) suggests that the allatum hormone is released into the channels which occur between its cells. The allata of C. riparius have no extracellular spaces. The occurrence of tight junctions between the interdigitations of the allata cells indicates that the absence of such spaces is not a preparation artefact. In C. riparius the allatum hormone possibly is released through the acellular sheath via the cortical cells. It is suggested above that a similar sheath prevents release of neurosecretion, which is most probably proteinaceous, (review MADDRELL, 1974) and has a high molecular weight. As the hormone released from the allata of insects is apparently of a much lower molecular weight than a protein (ENGELMANN, 1970), the acellular sheath may not be a barrier to its release. The association of this sheath

with the aorta is most probably just for attachment. The possibility that the allatum hormone is released into the aorta seems very unlikely as the aortal wall is muscular and up to 2 um thick in this region.

As the influence of these glands in male insects is at best only partially understood in only a few species, speculation of their functional significance is inappropriate.

The Peritracheal Tissue

The peritracheal tissues of Diptera are homologous to the prothoracic glands of other insects, and are like them in degenerating at metamorphosis (SLAMA et al, 1974). Observations on the fine structure of these glands have been confined almost exclusively to the ultimate larval instars (BLAZSEK et al, 1975). The occurence in the neoimaginal C. riparius of tissue resembling in both position and ultrastructure, the peritracheal tissue of the larvae (CREDLAND & PHILLIPS, 1974; PHILLIPS, personal communication) is most unusual. A surrounding external lamina, typical of these glands occurs (NOVAK, 1974). The cells do not resemble those of the parenchyma of the corpora allata, as is usual for peritracheal tissue (SLAMA et al, 1974). As in the prothoracic glands of cockroaches (review NOVAK, 1974) the cells' long interwoven processes are separated by extracellular channels. The nuclei, which are irregular in shape and contain many nucleoli, are characteristic of peritracheal tissue (SLAMA et al, 1974). The chromatin of these cells is exceptionally electron-dense but

contains small round areas that are electron-lucent, bearing a similarity to the nucleoli of inactive prothoracic glands of Galleria mellonella (BLAZSEK et al, 1975). The irregular outline of the cellular surfaces, the condensed chromatin and the large number of both lysosomes and lipid droplets that the peritracheal tissue of the neoimaginal male exhibits are in Galleria (BLAZSEK et al, 1975), characters announcing the disintegration of these glands. The peritracheal tissue of the neoimaginal male therefore appear to be inactive and undergoing degenerative changes which will lead to its dissolution.

The "Glandes Post-cérébrales Antérieures"

As observed in the larva (CREDLAND & PHILLIPS, 1974) a pair of "glandes post-cérébrales antérieures" as described by POSSOMPÈS (1948) occur. Although having the same close approximation to the corpora cardiaca which occurs in the larva, they are different in lacking any innervation from the NCC. The glands are syncytial and appear to have no nuclei but in other respects they resemble those of the larvae described by CREDLAND & PHILLIPS (1974) which is the only published ultrastructural report upon these glands. The presence of deep infoldings of the external cell membrane, forming tortuous channels associated with many vesicles may be related to the phagocytic function suggested by PHILLIPS (personal communication).

Other Neurosecretory Structures

As there are no descriptions of neurosecretory cells in the suboesophageal or prothoracic ganglia of any other adult dipterans, the observation of the two discrete paired groups in the suboesophageal ganglion and the single pair in the prothoracic ganglion currently stand in isolation. GABE (1966) and MADDRELL (1974) have reviewed the occurrence of non-cerebral neurosecretory cells in insects, and both explain that the functions of these cells have rarely been elucidated. The possible association of the SNC_1 cells of the suboesophageal ganglion with the foregut may be indicative of their function. The axons of the neurosecretory cells of the prothoracic ganglion terminate in a neurohaemal area resembling the type "transverse évolué" of GRILLOT et al (1971) and RAABE et al (1971). As RAABE and her colleagues have not yet investigated the position and structure of neurohaemal organs in Diptera, it is as yet unknown if this type is of wide occurrence in the order. The close proximity of this neurohaemal area with fat body cells, in the midge, indicates that the possible function of these cells may be associated with fat metabolism.

MADDRELL (1974) postulates that peripheral neurosecretory cells may be of wide occurrence in insects. The pair of isolated cells found in the prothorax of the midge, although multipolar, resemble the corpora cardiaca cells in their appearance and bimodal population of sizes of granules. They are unlike the

peripheral neurosecretory cells of Carausius morosus and Phormia terrae-novae (FINLAYSON & OSBORNE, 1968) and of larval Lepidoptera (HINKS, 1975) in apparently having no connection with the central nervous system. The processes from these cells terminate in an area which shows many of the characters typical of a neurohaemal organ, which is apparently of the type "médian primitif proximale" of GRILLOT et al, (1971). The root apparatus of a scolopidium, which is similar in structure to those of Johnston's organ in, for example, Aedes ægypti (BOO & RICHARDS, 1975), runs into the perikaryon of these cells. The lack of connection with the nervous system usually precludes a cell from being nervous, however the association with a sensory structure is indicative of a sensory nerve cell. It is suggested that these cells have a nervous origin but have become dissociated from other components of the central nervous system. The scolopidia, which are mechanoreceptors, possibly mediate the synthesis and/or release of a factor from the peripheral cells that they are inserted in. Deformation of the cuticle in the region where the scolopidium is attached may initiate changes in the peripheral cell. The functional significance of this isolated, unique system, which lacks mediation from higher nervous centres, is at present unknown.

B. THE FEMALE

The female, although slightly shorter than the male, is almost twice its weight, which is probably associated with its role in egg production. The size and anatomy of the brain is very similar to that of the male. The difference in complexity of the antennae between the sexes was not reflected in the size of the brain's antennal centres. The ultrastructure of the non-neurosecretory components of the brain, showed a marked similarity to those of the male. Both light and electron microscopy, using the interpretation of SCHARRER & BROWN (1961), indicate that the MNC group are more active in the neoimaginal female. The converse is apparent in the VNC group, where the neoimaginal male has the most active cells. The VNC group resembles that of female mosquitoes (MEOLA & LEA, 1972) in exhibiting some purple staining with paraldehyde fuchsin. The electron microscope shows that the sexual difference in the relative amounts of granules is restricted to the α_1 cells of the VNC group. The difference in staining reaction with paraldehyde fuchsin, therefore infers that its selective staining is restricted to α_1 type neurosecretory cells. The remainder of the endocrine components are similar in neoimaginal midges of both sexes. The activity of the corpora allata cells, as reflected by their nuclear to cytoplasmic ratios is similar in both sexes. It has been suggested above that these cells are inactive in the male. In many adult female insects the allata

are important in the control of egg maturation (reviews by ENGELMANN, 1970 and NOVAK, 1974). Females of C. riparius are autogenous and capable of mating soon after emergence (DOWNE & CASPARY, 1973). The inactivity of the corpora allata in the neoimaginal female may therefore simply be a reflection that the processes of egg maturation, under the control of this gland, are already complete.

The inability to obtain selective staining with paraldehyde fuchsin of the peripheral neurosecretory cells in the female may be indicative that these cells are more active than in the male.

SECTION II

The effect of ageing upon adults at 20°C

Life span records show that the survivorship curves of both sexes are of the intermediate type, which ROCKSTEIN & MIQUEL (1973) explain is the most typical for insects and other animals. Females have a longer mean life span than males, which occurs in many insect species, although this is not always the case in Diptera (review ROCKSTEIN & MIQUEL, 1973). This finding is the converse to the results of CREDLAND (1971) for the same species, and probably reflects a different genetic constitution between the two populations used, caused by inbreeding. It is known, for example, that in Drosophila melanogaster, the genetic constitution can effect the sexual differences in longevity (review ROCKSTEIN & MIQUEL, 1973).

Behavioural observations show that mobility is increasingly impeded with age. The inability to fly, appears to be correlated with exhaustion of the fat body. Depletion of the fat body possibly results in the wrinkling and collapse of the abdomen which is apparently the only external sign of senescence. In Drosophila a similar abdominal change has been shown to be correlated with decrease in fat body (review ROCKSTEIN & MIQUEL, 1973). No major ultrastructural changes were found in the flight muscles of the immobile specimens. A number of

authors (TAKAHASHI et al, 1970B; SOHAL, McCARTHY & ALISON, 1972 and others) have described flight muscle degeneration in senescent flies. The findings of WEBB & TRIBE (1974) indicate that no major degenerative changes in the flight muscles of dipterans occur, and these authors intimate that many of the previous observations are due to preparation artefacts. The inability of aged midges to fly appears not to result from degeneration of the flight muscles, which is probably true for other dipterans which have been studied (WEBB & TRIBE, 1974). The sudden and complete lack of attempting to fly that midges exhibit is difficult to explain in terms of depletion of energy supplies alone. This difference in behaviour possibly lies in the physiology of the nervous system and may be reflected in a change of its structure.

The Brain

The brain shows no reduction in the thickness of the cortical layer or "sponginess" of the neuropile, even in advanced old age, both of which are characters of senescent brain of Drosophila melanogaster (MIQUEL, 1971). There is also no loss of basophilia of the giant neurons reported by HERMAN et al, (1971) in Drosophila. The population density of neurons shows no change with age, which together with lack of change of cortical thickness indicates that there are no age related losses of neurons. This is in agreement

with the findings of SOHAL & SHARMA (1972) for Musca domestica, but conflicts with the observations of many workers in both insects and mammals (review ROCKSTEIN & MIQUEL, 1973). As insect neuronal population studies have been largely restricted to Apis and Drosophila generalizations cannot be made.

The neurons of Chironomus show similar changes in subcellular organization as found in Musca (SOHAL & SHARMA, 1972), except that the population of free ribosomes remains unchanged in Chironomus. Many of these changes are indicative of cellular deterioration. It is known that such changes, in mammals, can result in functional deterioration of the nervous system without any change in neuronal numbers (KOHN, 1971). The increased frequency of type 2 and type 4 neurons further supports the suggestion that these types are derived by degenerative processes from type 1 and type 3 neurons respectively.

In the axons of the neuropile no "heterogenous dense bodies", similar to those described by HERMAN et al, (1971) in Drosophila occur. These structures are thought to contain "age pigment" and represent degenerated synaptic boutons. In the midge brain axonal mitochondria appear to undergo an increase in matrix density with age. These density changes may, as in flight muscle of Drosophila (TAKAHASHI et al, 1970A), represent the accummulation of age pigment. The functional significance of

this pigment is unknown, but it possibly represents the accummulation of a non-excretable cellular by-product.

The neural lamella shows an increase in thickness with age. It becomes differentiated into two components on or after the fourth day of adult life, giving it an appearance similar to that of three day old Musca (SOHAL et al, 1972). The specific orientation that the fibrils of the neural sheath adopt in older specimens may give added strength to this layer. These fibrils have no periodic banding but this does not prevent them from being collagenous (LANE, 1974). The thickening and structural organization of this layer in older animals is of unknown significance.

The appearance of the perineurial and glial cells of the aged brain indicate that they are undergoing similar degeneration processes to those of the neurons. The glial mitochondria, unlike those of the neuronal perikarya, shows an increase in matrical density with age. This may represent an accumulation of age pigment as suggested in the axonal mitochondria. The rarity of the type of glial cell found only in the brains of aged specimens, may have resulted in the lack of observations of these cells in neoimagines. ROCKSTEIN & MIQUEL (1973) state that brain cells are post-mitotic, therefore if these glial cells occur only in the brains of aged specimens they must be derived by transformation from other glial cells. The occurrence of the multiple glial lamellae surrounding neurones and vacuoles have also been reported in Drosophila

(HERMAN et al, 1971) where they are thought to be indicative of neuronal generation. As there have been no detailed fine structural reports of changes in glial cells with age, these observations at present stand in isolation.

An extensive system of extracellular spaces develops with increasing age in both the cortex and neuropile of the brain. As no such system occurs in the brain of young specimens it may not be equivalent to the glial lacunal system of WIGGLESWORTH (1960). However, the appearance of these spaces, and the occurrence within them of amorphous material is similar to the glial lacunal system of cockroaches (review SMITH & TREHERNE, 1963). These extracellular spaces are similar, though less extensive, than the vacuoles which occur in the brain of senescent Drosophila (HERMAN et al, 1971). This vacuolation is thought to result from an extracellular oedema. The cytoplasmic degeneration noted in both neurons and glial cells in the midge may be coupled with shrinkage of the cell body. Slight volume changes, which would not be detectable by mensuration, could be compensated for by an influx of fluid to form an oedema. The spaces between neurons could hamper inter-cellular communication and may be functionally more deleterious than the neuronal cytoplasmic degeneration. The structural changes in the brain are not as marked as those occurring in longer lived dipterans, for example Drosophila (HERMAN et al, 1971; MIQUEL, 1971 and others). The termination of life, in Chironomus, therefore appears not to be dependent upon degeneration of the nervous system.

If the age related changes in the brain visible with the electron microscope are a true reflection of those that are occurring in vivo, it is likely that they will be of functional significance to the nervous system. A functional difference may be reflected in changes in behaviour patterns, and could result in the flight immobility that the aged midges exhibit.

Endocrine system

The increase in the amount of neurosecretory material that the medial cells exhibit, using the interpretation of SCHARRER & BROWN (1961) indicates that they are less active in older animals. A similar accummulation of material was reported by ROCKSTEIN et al (1971) in Musca domestica. The findings of these authors indicate that the accummulation is correlated with the absence of axonal neurosecretory material. The axons of the MNC of aged midges still show selective staining, indicating that axonal transport of neurosecretion is still occurring. The suggestion that in Musca (ROCKSTEIN et al, 1971) ageing is correlated with a failure of release of the hormone from the neurosecretory cells, so that it is unavailable to initiate physiological reactions in the old fly, is apparently not true in Chironomus. The accummulation of neurosecretory material does suggest that its synthesis is greater than its transport. The occurrence of cells in the MNC groups of aged specimens, whose perikarya stain orange with paraldehyde fuchsin is indicative that some classical

B-type cells (review GABE, 1966) occur. This change in staining may be associated with the relatively larger amounts of neurosecretion in the MNC of aged individuals as compared with those of neoimagines. There is, however, no ultrastructural evidence for two cell types in the MNC of aged individuals, and the majority of the cells give the typical staining reaction of A-type cells with paraldehyde fuchsin as described by GABE (1966). The VNC of some aged females contain more neurosecretory material, when stained with paraldehyde fuchsin than younger specimens, indicating that accummulation may also occur in these cells. Electron microscope observations reveal that neurosecretory cells of all but the LNC group undergo a reduction in size of the perikarya. The restriction of marked size changes to neurosecretory cells may be a reflection that these cells, unlike other neurons, have a glandular activity. The reduction of cytoplasmic volume, using the interpretations applied to corpora allata cells (see ENGELMAN, 1970) is a further indication of a decrease in activity with age.

The relative amount of granules within the VNC and particularly within the MNC, did not reflect the differences in the amount of neurosecretory material indicated by paraldehyde fuchsin staining. The technique of LANE & EUROPA (1965) which enabled concurrent sections of the same cell to be prepared for light and electron microscope work, also showed that the MNC, although containing fewer granules, gave a greater response to paraldehyde fuchsin than the controls which contained more

granules. This conflicts with the concept that there is a granule to granule comparison between light and electron microscope results propounded by BLOCH et al (1966). It is possible that the neurosecretory material (or its carrier) in these cells, although maintaining some of its chemical integrity and therefore staining with paraldehyde fuchsin, is no longer in the form of discrete electron-dense granules. The breakdown of the granules may be associated with the ageing process of these cells.

Light and electron microscope observations indicate that there is apparently no age related change in the amount of neurosecretion in the corpora cardiaca. Fine structural observations show that the cytoplasmic components of the intrinsic cells have undergone similar changes to those of neurons within the brain.

The corpora allata show a slight difference in the association between cortical and medullary cells with age. The nucleus to cytoplasmic ratio of young and old specimens is not indicative of a change of activity in the allata cells. The change in appearance of chromatin that occurs with age may be of functional significance. Glycogen deposits are either absent or much reduced in older specimens, possibly because the synthetic activity of the allatum requires their utilization. The appearance of the allata cells, unlike those of the corpora cardiaca, gives little indication of cytoplasmic degeneration in aged specimens.

The "glandes post-cérébrales antérieures" shows both histochemical and ultrastructural differences in old and young specimens. The interpretation of PHILLIPS (personal communication) based upon his observations of this structure in the larva, is that it has a phagocytic function. If this suggestion is adopted the granular vesicles occurring in these glands may represent the remnants of phagocytosed particles. The appearance of these glands could be interpreted as having a greater activity in older specimens which may be necessitated by the increasing amounts of tissue debris in the haemolymph that older animals are likely to have.

The total absence of peritracheal tissue in older specimens was not unexpected. The degeneration apparent in neoimaginal specimens has most probably resulted in the ultimate dissolution of this tissue in older animals.

The peripheral neurosecretory cells of the prothorax show the same sexual difference in activity based upon their paraldehyde fuchsin staining in young and old specimens.

SECTION III

The effect of temperature upon imagines

The life span records obtained in this study indicate that the aquatic rearing temperature is of less importance to adult longevity than the air temperature to which the imagine is subjected. Differences of 10°C in the rearing temperatures studied did not significantly alter the adult's longevity if the imagines were kept under similar conditions. In Drosophila where all the stages of the life cycle are terrestrial, the preimaginal temperature is known to affect the adult longevity (ALPATOV & PEARL, 1929; LINTS & LINTS, 1971 and others). It is not known whether Chironomus is unusual among dipterans, with aquatic stages of the life cycle, in the influence of preimaginal temperature upon adult longevity as there is a paucity of information on this subject.

In Chironomus adults a 5°C decrease in air temperature from 20°C was found to have no significant effect upon the mean adult life span, and yet the same change between 15°C and 10°C significantly increased the mean life span of both sexes. These results could be explained by the hypothesis that a homeostatic mechanism which maintains a similar rate of ageing between 20°C and 15°C breaks down at lower temperatures. Such an interpretation has been used to account for temperature effects upon ageing in Drosophila (review ROCKSTEIN & MIQUEL, 1973).

At 20°C both sexes exhibit an intermediate type survivorship curve as described by ROCKSTEIN & MIQUEL (1973), which they explain is typical of many animals. Decreasing the temperature to which the imagines are subjected does not change the shape of the survivorship curve.

Increased life spans correlated with a decrease in temperature are typical of insects, the direct effect upon metabolic activity is usually cited as the dependant factor resulting in the change of rate in the ageing process (review ROCKSTEIN & MIQUEL, 1973).

The increase in size and weight of animals reared at 10°C as compared to those of 20°C has been previously noted in this species (CREDLAND, 1971). As in Drosophila melanogaster (ALPATOV & PEARL, 1929), these results were accounted for by the decrease in temperature prolonging the larval period and allowing a greater food intake which is probably particularly important in Chironomus which has a non-feeding adult. As fewer larvae develop from egg ropes at 10°C (CREDLAND, 1971), decrease in larval crowding may also be a significant factor affecting body size as in D. melanogaster (MILLER & THOMAS, 1958). The increase in the oxygen content of water from 6 ml per litre at 20°C to 8 ml per litre at 10°C (extrapolated from data in GORDON, 1968), may also be of relevance.

The decrease in behavioural activity which occurs with decreasing temperature has been observed in many insects,

for example, Musca domestica (RAGLAND & SOHAL, 1973) and it is typical of poikilotherms.

Light and electron microscope observations indicate that the similar but larger brains of midges reared at 10°C as compared with those raised at 20°C, result from both an increase in the number of neurons and the development of extensive extracellular spaces. There were no results indicating an increase in cell sizes. The composition of neuron types in the brain is found to differ with rearing temperature. A greater percentage of type 2 and type 4 neurons is found in young midges reared at 10°C as compared with either young or old specimens reared at 20°C. The increase in density and shrinkage of type 3 neurons in midges reared at 10°C, forming type 4 neurons, has apparently reached a more advanced state in the pars intercerebralis, where cells of high electron-opacity are encountered. This cellular collapsing is suggested to be a degenerative process. Cytoplasmic degeneration, as noted in the majority of cells in the brains of aged specimens, is not apparent in midges reared at 10°C. The swollen or exploded appearance of the mitochondria and endoplasmic reticulum cisternae of these specimens' neurons is typical of an osmotic fixation artefact (BAKER, 1966) and could mask degenerative changes. The ultrastructural appearance occurs in brains fixed at the same time in the same stock solutions as brains from other experimental midges, which did not exhibit any osmotic damage. The cellular appearance could result

from the temperature constraints changing the osmotic pressure of the cell cytoplasm so that the buffer is no longer isotonic to it. The lowering of temperature may also affect the blood-brain barrier (TREHERNE & PICHON, 1972) as it is reported to have an enzymatic component localized in the glial cells (HOUK & BECK, 1976). However, as discussed above, regarding the ventral nerve cord of 20°C specimens, it is unlikely that this barrier could account for all the osmotic damage which still occurs when the neurons are exposed to the bathing solutions.

The occurrence of extracellular spaces within the brain of specimens reared at 10°C has been observed as an ageing effect in midges reared and kept at 20°C. The suggestion that these spaces may inhibit neuronal function may again be applicable. The activity of these 10°C specimens even when transferred to 20°C is apparently less than animals reared and kept at this temperature, based upon non-quantified observations. This difference in activity may result from a change in the nervous system, and could be connected with the occurrence of extracellular spaces and the dense neurons within the brains of midges reared at 10°C. As there have been no comparable observations upon the structure of brains of insects reared under differing conditions of temperature, these results at present, stand in isolation.

CLARKE wrote in 1967 that "There are relatively few observations on the effect of temperature on the endocrine

system of insects." This statement is still of relevance today as there are as yet no published structural observations upon the endocrine systems of adult insects reared at different temperatures. In this study it was found that the MNC of the brain of midges raised at 10°C show considerable variability in staining response with paraldehyde fuchsin. The electron microscope shows that these cells contain neurosecretory granules within the cytoplasm and also isolated in multivesicular type lysosomes. Initial grouping of neurosecretory material into these lysosomes may be reflected in the coarse granular appearance that these cells often exhibit after staining with paraldehyde fuchsin. Subsequent denaturing of the neurosecretory material which may occur within these lysosomes could account for the lack of selective staining with paraldehyde fuchsin that is apparent in some specimens. This may also explain the discrepancy between light and electron microscope results, which indicate that there is more neurosecretory material on average in these cells using the latter. Electron microscope assessment indicates that these cells contain more neurosecretory material than in midges reared at 20°C and are therefore less active, using the interpretation of SCHARRER & BROWN (1961). At 10°C these cells have a similar activity in both sexes, whereas at 20°C the MNC appear more active in the female. Rearing temperature therefore appears to have a more marked effect upon the α_1 cells in the MNC groups of the female's brain. The α_1 cells of the VNC of both sexes have a similar activity at the

rearing temperatures studied. The α_2 cells of this group and of the CNC group have markedly less neurosecretory material, and therefore greater activity in brains of midges reared at 10°C. The β cells of the LNC show little difference in the relative quantities of granules in both sexes at either temperature.

Paraldehyde fuchsin staining indicates that the corpora cardiaca of both sexes reared at 10°C have a greater activity than those of 20°C. The corpora allata and "glandes post-cérébrales antérieures" have a similar appearance in both sexes at the two temperatures. The peritracheal tissue in neoimagines reared at 10°C shows less structural signs of dissolution than occurs in those reared at 20°C. The appearance may result from the slowing of the rate of disintegration processes, caused by the direct effect which temperature has upon enzyme activity (BRANDTS, 1967).

The peripheral neurosecretory cells in the prothorax of specimens reared at 10°C do not exhibit selective staining with paraldehyde fuchsin, which may be indicative of a greater activity than in those midges reared at 20°C.

The functional significance of the changes in the appearance of endocrine structures, occurring with temperature, which infer activity differences is at present unknown. In this study adult midges were kept for 7 hours at the temperature at which they were reared, to allow for recovery from the trauma of emergence, before fixation. The

importance of this period to the appearance of the endocrine system has not been studied, but as a complete cellular depletion of neurosecretion has been reported to occur within 25 minutes (STEEL & HARMSEN, 1971), its relevance cannot be discounted.

SECTION IV

Reproductive effects upon Brain Neurosecretory Cells

Observations indicated that electron microscopical assessment of amounts of neurosecretion within neurons gave similar, repeatable results, whereas light microscope assessment often gave very variable results. Many of the neurosecretory groups in the brain are only detectable by electron microscope techniques. An electron microscope investigation was therefore used to study the effects of reproduction upon the neurosecretory cells in the brain. The amount of time needed to obtain electronmicrographs from living specimens and the difficulty in procurring some of the experimental groups, necessitated that this study be based upon only a small number of animals. The conclusions that are drawn are therefore of a tentative nature.

The observations of ovaries based upon wax sections stained with paraldehyde fuchsin, show that the terminal oocytes undergo a reduction in size with age. This, coupled with the observation of a decreased number of yolk droplets, indicates that oosorption is occurring, as found by DOWNE (1973). The females, which were fixed 3 hours after mating, did not show any change in their oocytes. If copulation has an effect upon the ovaries, it may not occur in such a short time. These females were intermittently kept with males for three days prior to mating. The presence of males, therefore, appeared not to prevent oosorption, and thus, as in mosquitoes, with the exception of Aedes taeniorhynchus (O'MEARA & EVANS, 1976), the presence of

males does not influence ovarian development. The ovaries of the oviposited specimens appeared to be undergoing dissolution. Observations upon the anterior abdomen in ligated individuals showed that complete constriction of the haemocoel had not been achieved. The depletion of fat body cells, and subsequent shrinkage of the abdomen which occurs with age, may have caused the ligatures to be unsuccessful. Therefore, a negative feedback mechanism, if it exists between the ovaries and the endocrine complex, as described in Aedes ægypti (HAGEDORN, 1974), would not be interrupted. As expected from this observation, ligated individuals showed no difference in ovaries or relative amounts of brain neurosecretion to sham-operated specimens, which are similar in these respects to similarly aged virgins. Mated females did not show any marked difference in the relative amounts of neurosecretory material within the various brain cells as compared with virgins of similar age. Mating may not have an effect upon the activity of these cells, or the 3 hour period between mating and fixation may not have been long enough to reflect such a change. In mated oviposited individuals there is an increase in the amount of neurosecretory material in the α_1 cells of the MNC, and a slight decrease in the α_1 cells of the VNC, as compared with similarly aged virgins. The most marked difference occurs in the β cells of the LNC which have larger perikarya and contain more neurosecretion than in the same cells of any other group of Chironomus females studied. The corpora allata of oviposited females showed no marked difference in the nucleus to

cytoplasmic ratio as shown in virgin females, which using the interpretation of ENGELMANN (1970) reflects a similar activity in both groups. Thus the corpora allata of Chironomus riparius may not be directly involved in the production of gonadotrophic hormone, as ENGELMANN (1970) speculates is the case in Calliphora, or its production may only occur in the preimaginal phases.

The period between mating and oviposition is unknown in Chironomus riparius but in C. plumosus it is two days (HILSENHOFF, 1966). If the period is similar in C. riparius the oviposited specimens may have been fixed about two days after copulation. Mating, although not within 3 hours, may subsequently trigger the change in activity of the neurosecretory cells found in the oviposited specimens. The neurohormone produced by these cells may be a gonadotrophin involved in the final stages of egg maturation, or alternatively, the hormone may influence oviposition. ENGELMANN (1970) wrote that "Normally the median neurosecretory cells of the pars intercerebralis liberate a substance which is involved in egg maturation." This has been intimated in a number of dipterans, for example, Calliphora (THOMSEN, 1952), Sarcophaga (WILKENS, 1960) and Aedes (LEA, 1967), and the results suggest that this may also be true in Chironomus.

The increase in size of the perikarya of the LNC using the interpretation of ENGELMANN (1970) for allata cells is

suggestive of a higher activity than is apparent in non-ovipositedspecimens. The presence of larger amounts of neurosecretory material within these cells indicates a lower activity. It would seem most likely that the glandular activity of these cells increases, as reflected by the size of the perikarya, but after oviposition the synthesis and transport of neurosecretory material ceases causing the accumulation of granules. The cells were probably fixed before any reduction in cytoplasmic volume could occur. In Calliphora, THOMSEN (1952) concluded that the LNC may influence egg development. Strong evidence for the production of gonadotrophin from the LNC was found in Schistocerca paranensis (STRONG, 1965), where the medial neurosecretory cells were not implicated in egg maturation. It is possible that in C. riparius these cells produce a gonadotrophic hormone.

The possibility that changes in neurosecretory cells in the brain are associated with the control of oviposition cannot be discounted. Although nervous control is of prime importance in governing oviposition, some observations suggest that brain neurosecretion also plays an integral part (NAYAR cited in ENGELMANN, 1970).

SECTION V

Concluding remarks

The anatomy of the brain and anterior ventral nerve cord were found to closely resemble those of another adult nematoceran (CHRISTOPHERS, 1960) and to be similar in several respects to cyclorraphan dipterans (GROTH, 1971; POWER, 1943, 1946). The fine structure of the neural sheath, glia and non-neurosecretory neurons are comparable to those of Musca domestica (SOHAL et al, 1972), which is the only similar published study in any other dipteran.

The cerebral ganglia differ from the typical insect pattern in lacking an extracellular system, although there is a paucity of observations on these spaces in Diptera. The ventral nerve cord posterior to the head does exhibit large extracellular spaces which may be comparable to those which have been used in the study of neural transmission in Dictyoptera (reviews SMITH & TREHERNE, 1963; TREHERNE, 1974). The cerebral ganglia contain extracellular spaces in old 20°C specimens and in neoimagines reared at 10°C. In both cases such spaces appear to be formed by the collapsing of neurons to form smaller, denser cells. Aged individuals show a decrease in activity which may result from an exhaustion of energy reserves but is also possibly affected by changes occurring in the brain.

Neoimagines reared at 10°C and kept at 20°C appear to be less active than midges reared and kept at 20°C, which is unlikely to result from lack of energy reserves. It is interesting to note that in the brains of both aged 20°C specimens and neoimagines reared at 10°C there is a higher percentage of dense neurons than occurs in the more active neoimagines reared at 20°C. Conclusive statements regarding the functional significance of the neuronal differences occurring in the brain with age and changed rearing temperature must await neurophysiological investigations.

As occurs in many insect species, the neurosecretory cells are situated in medial and lateral positions, the axons from which form the NCC I and NCC II respectively. More neurosecretory cell groups were found than have been described in other nematocerans (BURGESS & REMPELL, 1966; MEOLA & LEA, 1972 and others) but this may simply result from using the electron microscope as the primary instrument, rather than finding selectively stained cells with the light microscope whose ultrastructure was subsequently studied. Of the techniques employed EWEN'S (1962) paraldehyde fuchsin was the most successful stain for the detection of neurosecretion for light microscopy, which although giving somewhat variable results, gave the best selective staining of the MNC and VNC groups. Staining with chrome haematoxylin-phloxine was found to be unsuccessful and atypical but not unique (TIWARARA & SRIVASTAVA, 1975).

When kept under the same conditions the comparable neurosecretory brain cells of both sexes contain similar quantities of granules and therefore have a similar activity. There appears to be a correlation between the physiological state of the midge and the activity of these cells. Rearing temperature has a marked effect upon the α_2-type cells of both the VNC and the ONC which contain fewer granules in specimens reared at $10^{\circ}C$ as compared with those reared at $20^{\circ}C$.

The amount of granules in the α_1 cells of the MNC, α_2 cells of the ONC and β cells of the LNC decreases with advancing age. EWEN'S (1962) paraldehyde fuchsin technique indicated an increased amount of sulphur rich amino acids in the MNC group which, as in Musca (ROCKSTEIN et al, 1971), is thought to indicate the presence of larger quantities of neurosecretion with advancing age. These results do not correlate with the electron microscope appearance which was usually found in these cells. This apparently unique difference may be caused by dissolution of granules linked with ageing processes, although its interpretation is far from clear.

Reproduction in females has a marked effect upon the amount of granules in the α_1 type cells of the MNC and VNC, and on the β-cells of the LNC which also show an increase in the size of the perikarya. These changes appear to be associated with mating and/or oviposition.

The retrocerebral structures of the endocrine system show an anatomical similarity to the preimaginal stages of chironomids which have been described (KÜMMEL, 1969; POSSOMPÈS, 1948 and others) but are unlike mosquitoes (MEOLA & LEA, 1972) in possessing unfused corpora cardiaca. The presence of peritracheal tissue in an adult insect is unusual but in C. riparius apparently results from only partial disintegration of the gland at metamorphosis, complete dissolution occurring in the imago. The "glandes post-cérébrales antérieures" of POSSOMPÈS (1948) were found to be similar to those described in the larvae (CREDLAND & PHILLIPS, 1974) and such structures may be typical of chironomids.

The corpora allata, using the interpretation of ENGELMANN (1970), were found to have similar activities in male and female neoimagines and mated oviposited females. The innervation of the corpora allata with axons from four of the five paired neurosecretory cell groups in the brain must be of functional importance although the significance of this gland in the adult midge is still unresolved.

No comparable neurosecretory cells have been described in other adult dipterans with those found in the suboesophageal and prothoracic ganglia. The peripheral neurosecretory cells of the prothorax, which are associated with a mechanoreceptor and lack direct connection to the central nervous system, have not been described in any other insect. Comparable data on these cells in neoimagines and old midges reared at 20°C

and of neoimagines reared at 10°C is only available in specimens stained with paraldehyde fuchsin. Selective staining was found to be restricted to males reared at 20°C, regardless of age, in which large amounts of neurosecretion were indicated in both the cell body and the release site. Why these structures should show such a clear sexual staining difference in all but the specimens reared at 10°C is at present unknown.

A structural investigation of the nervous and endocrine systems is a necessary step for the analysis of the functional roles of their various elements. It is hoped that the results included in this study will prove to be stimulating to further research upon the nervous and endocrine control of the harlequin fly, Chironomus riparius.

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APPENDIX 1

HISTOLOGICAL PROCEDURES

Fixation techniques .

A. Modified Rockstein et al's procedure (1971).

1. Etherise specimens.
2. Rinse in dilute detergent then in distilled water.
3. Immerse in aqueous Bouin's solution with trichloroacetic acid, under vacuum, for 4 hours at 60°C.
4. Remove legs and wings then continue fixation in a fresh amount of the same solution for 20 hours at room temperature.
5. Immerse in 50% alcohol for 1 hour.
6. Store in 70% alcohol.
7. Continue dehydration in two changes of 90% and 100% alcohol each for 45 minutes.
8. Immerse in butanol/alcohol (1:1) for 30 minutes.
9. Immerse in pure butanol for 30 minutes.
10. Immerse in butanol/ester wax (1:1) under vacuum at 60°C for 30 minutes.
11. Immerse in two changes of fresh wax each for 20 minutes.
12. Embed in more fresh wax.

B. Modified Meola's procedure (1970).

1-2. As above.

3. Immerse specimen in aqueous Bouin's solution at 50°C, allow to cool to room temperature, then hold under vacuum for 15 minutes.

4-12 As above.

Staining procedures

A. Ewen's (1962) aldehyde fuchsin stain.

1. Section and fix to slides not more than 2 days before staining, dewax as usual.

2.	Acidified potassium permangenate*	1 minute
3.	Distilled water	15 seconds
4.	Sodium bisulphite*	1 minute
5.	Distilled water	1 minute
6.	30% alcohol	1 minute
7.	70% alcohol	3 minutes
8.	Aldehyde fuchsin	3 minutes
9.	95% alcohol	30 seconds
10.	Acid alcohol	3 minutes
11.	70% alcohol	1minute

* Freshly made

(continued)

12.	30% alcohol	1 minute
13.	Distilled water	3 minutes
14.	Aqueous phosphomylbdic/phosphotungstic acids*	15 minutes
15.	Distilled water	30 seconds
16.	Halmi's counterstain	2 hours
17.	Acetic alcohol*	dip
18.	100% alcohol, two baths	dip
19.	Xylol	3 minutes
20.	Xylol	15 minutes
21.	Mount with D.P.X.	

Control for selectivity of the procedure:- omit oxidation (stage 2).

B. Chrome haematoxylin-phloxin (after GABE, 1966).

1.	Dewax and bring sections to water	
2.	Refix in Bouin's fluid containing 4% chrome alum, at 37°C.	24 hours
3.	Yellow in running tap water	5 minutes
4.	Oxidize in acidified potassium permangenate*	1 minutes
5.	Tap water	dip
6.	3% aqueous sodium bisulphite*	dip
7.	Running tap water	5 minutes
8.	Chrome haematoxlin*	5 minutes
9.	Tap water	dip
10.	1% aqueous phloxin	2 minutes
11.	Tap water	dip
12.	5% aqueous phosphotungstic acid	5 minutes
13.	Running tap water	5 minutes
14.	Dehydrate rapidly in a graded series of alcohols	
15.	Xylol, two baths each	1 minute
16.	Mount with D.P.X.	

Control for selectivity of procedure:- omit oxidation (stage 4).

C. Combined alcian blue/Schiff's test.

1.	Dewax and bring sections to water	
2.	Oxidize in a solution of 2·5% $KMnO_4$* 10ml and 5% H_2SO_4 10ml, diluted to 60ml with distilled water	1-2 minutes
3.	Distilled water	dip
4.	Bleach in 2·5% potassium metabisulphite	2 seconds
5.	Distilled water	dip
6.	3% alcian blue solution in 2N H_2SO_4	15-30 minutes
7.	Running tap water	5 minutes
8.	Distilled water	5 minutes

(continued)

* Freshly made

9.	Treat with 1% periodic acid	5 minutes
10.	Distilled water	3 minutes
11.	Place in Schiff's reagent	20 minutes
12.	Running tap water	10-15 minutes
13.	Stain nuclei in Delafield's haematoxylin	10 minutes
14.	Differentiate in acid alcohol	
15.	Counterstain in orange G (1%) in phospho -tungstic acid (2%)	15-20 seconds
16.	Rinse and dehydrate rapidly. Clear and mount.	

Control for selectivity of alcian blue stain: omit oxidation (stages 2-5).
Control for Schiff's reagent: omit oxidation with periodic acid (stage 9).
Results:- neurosecretory material may be PAS positive and/or blue in alcian blue. Neurosecretory cells may therefore range from turquoise to deep blue.

D. Mallory's triple stain (after PANTIN, 1964).

1.	Dewax and bring sections to water	
2.	Mordant in a solution of saturated mercuric chloride containing 5% glacial acetic acid*	10 minutes
3.	Rinse in distilled water	10 seconds
4.	1% aqueous acid fuchsin	15 seconds
5.	Tap water	20 seconds
6.	1% aqueous phosphomolybdic acid*	60 seconds
7.	Distilled water	10 seconds
8.	Mallory's stain	5 minutes
9.	Distilled water	dip
10.	90% alcohol (differentiates aniline blue)	3-5 seconds
11.	100% alcohol, two baths each	10 seconds
12.	Clear and mount	

* Freshly made

APPENDIX 2

ELECTRON MICROSCOPICAL PROCEDURES

Tissue preparation techniques

A. Technique for embedding in Luft's (1961) epon.

1. Etherise specimens
2. *Dissect out relevant tissues under 2·5% gluteraldehyde in 0·1 Molar Sörensen's phosphate buffer. Continue fixation in this solution for 2-3 hours.
3. *Wash in the buffer — Overnight
4. *Rinse in fresh buffer — 15 minutes
5. *Postfix in 1% osmium tetroxide made up in the phosphate buffer — 1 hour
6. *Wash in fresh buffer, two changes each — 15 minutes
7. *Dehydrate in an ethanol series (30%, 50%, 70% & 90%) each for — 15 minutes
8. Stain in cold 1% uranyl acetate in 100% ethanol, allow this stage to slowly reach room temperature.
9. Wash in fresh 100% ethanol — 3 minutes
10. Rinse in propylene oxide, two baths each for — 15 minutes
11. Pour propylene oxide/epon (1:1) over the specimens and leave uncovered in a fume cabinet overnight.
12. Prepare moulds with fresh epon and embed specimens. Polymerize in an oven at 60°C for 2 days.
13. Remove from oven, leave moulds to cool before removing the blocks.

B. Technique for embedding in Spurr's (1969) epon.

1. Etherize then quickly remove legs, wings, antennae and abdomen. Puncture thorax with a sharp needle. *Rinse quickly in dilute detergent followed by 0·1 Molar Sörensen's phosphate buffer.
2. *Fix in 2·5% gluteraldehyde made up in the buffer for 2-3 hours.

3-7. As above.

9-10. As above.

11. Propylene oxide/epon (1:1) — 2 hours
12. Propylene oxide/epon (1:2) — 2 hours
13. Epon, two changes each for — 15 minutes
14. Prepare dry moulds with fresh epon, embed specimens. Polymerize in an oven at 70°C — 8 hours
15. Remove from oven and allow to cool.

* At 4°C.

Technique for removal of resin from semi-thin sections (after LANE & EUROPA, 1965).

By adding an excess of sodium hydroxide, prepare a saturated solution in dry absolute ethanol. Leave the solution to mature to a dark brown colour (1-2 weeks). Mount sections, of 1-2 um thickness, to a clean glass slide. Treat the slide as follows:-

1. Flame the slide to ensure adequate adhesion of the sections.
2. Stand the slide in the caustic alcohol — 1 hour
3. Remove the slide and rapidly drain.
4. Rinse in four changes of dried 100% ethanol each for — 10 minutes
5. Rinse in 0·1 Molar Sörensen's phosphate buffer — 5 minutes
6. Rinse in four changes of distilled water each for — 2-3 minutes
7. Treat as dewaxed sections brought to water in histological procedures.

Results:- stain uptake is not as good as in wax sections, possibly because of the difference in section thickness. Sections treated in this way and stained with EWEN'S (1962) aldehyde fuchsin, unlike wax sections, fade after 1-2 months.

APPENDIX 3

Analysis of wax sections stained with EWEN'S (1962) aldehyde fuchsin

The tables on the following pages show the staining responses of several structures to EWEN'S (1962) procedure, and the field density counts of brain cells based upon these sections.

The large number of slides necessitated their staining in several batches. The last character (a-m) of each specimen number indicates the staining batch in which the slides were included.

The staining indices (INDEX) for the MNC and VNC are shown, rated as the product of the number of cells stained (MNC No. and VNC No.) and the amount of purple/mauve staining (0,+,++,+++) they contain, assessed as described in Chapter 2. The axons of the MNC (AXONS), the corpora cardiaca cells (CC), and the peripheral neurosecretory cells' (PNSC) cell bodies (CB) and release sites (RS), are all rated on their purple/mauve staining (0,+,++) for content of neurosecretion. The similar rating of different structures does not represent a similar content of neurosecretion as each of these structures is rated seperate -ly. The green (G) or blue (B) granular appearance of the "glandes post-cérébrales antérieures" (GPCA), after staining, is indicated. Lack of entries indicates that the structures were not found in the specimen, often resulting from loss or damage to sections.

The summated values of 10 counts per specimen of the number of brain cells contained in an area of 20 µm are shown (FD). The statistical analsis of these results follows the tables.

Neoimaginal males reared at 20°C

Specimen number	MNC No.	INDEX MNC	VNC No.	INDEX VNC	AXONS MNC	CC	GPCA	PNSC CB	PNSC RS	FD
MA1 a	4	8	0	0	0	+	B	++	++	50
MA2 a	5	7	0	0	0					51
MA3 a	6	12	1	1	+	++	G	++	++	78
MA4 a	8	16	0	0	+	++	G	++	++	48
MA5 b	7	19	0	0	+	+	G	++	++	51
MA6 b	7	14	0	0	+	++	G	++	++	49
MA7 b	6	12	0	0	0	++	G	++	++	48
MA8 b	4	8	0	0	+					60
MA9 c	1	1	0	0	0	0	G	0	+	63
MA10 c	4	8	0	0	+	+	G	++	++	56
MA11 c	4	8	0	0	0	++	B	++	++	41
MA12 c	5	10	0	0	0	+	G	++	++	44
MA13 d	4	8	0	0	0	+	G	++	++	50
MA14 d	6	12	0	0	0					46
MA15 d	6	12	0	0	+					48
MA16 d	5	10	0	0	+	++	G	++	++	52
MA17 e	3	6	0	0	0	++	G	0	0	55
MA18 e	4	9	0	0	0	++	G	++	++	41
MA19 e	5	10	0	0	+	++	G	++	++	76
MA20 e	4	8	0	0	+	++	G	++	++	59
MA21 e	6	12	0	0	0					42
MA22 e	6	12	0	0	0	++	G	++	++	51
MA23 e	4	8	0	0	+	++	G	++	++	58
MA24 e	8	12	0	0	0	++	G	++	++	50
MA25 e	5	10	0	0	+	+	G	++	++	53
MA26 e	4	8	0	0	+	++	G	++	++	44
MA27 e	6	12	0	0	+	++	G	++	++	57
MA28 e	4	8	0	0	0					45
MA29 f	5	10	0	0	+	++	G	++	++	53
MA30 f	8	16	0	0	+	++	G	++	++	51
Totals	145	306	1	1						1570
Means	5	10	0	0						52•3
									σ	8•70
									σ^2	75•8

σ =population standard deviation of distribution

σ^2=population variance of distribution

Female neoimagines reared at 20°C

Specimen number	MNC No.	INDEX MNC	VNC No.	INDEX VNC	AXONS MNC	CC	CPCA	PNSC CB	PNSC RS	FD
FA1a	9	9	1	1	++		G	0	0	66
FA2a	10	10	2	2	+	+	G	0	0	59
FA3a	8	16	2	2						64
FA4b	6	12	1	1	+	+	G	0	0	54
FA5b	8	16	1	1	+					58
FA6b	9	9	2	2	+					60
FA7b	6	18	3	3	++					42
FA8c	8	24	2	2	+		G	0	0	63
FA9c	10	30	1	1	+	++	G	0	0	73
FA10c	8	24	1	1						45
FA11d	6	18	0	0	+	++	G	0	0	57
FA12d	8	16	1	1	+	+	G	0	0	64
FA13f	10	20	2	2	+					41
FA14f	7	21	1	1	+					67
FA15g	10	20	1	1	+	++	G	0	0	44
FA16g	8	16	1	1	+					49
FA17g	2	2	2	2	+	++	G	0	0	57
FA18g	6	12	1	1	+	++	G	0	0	50
FA19g	10	20	2	2	+	+	G	0	+	43
FA20g	8	16	1	1	+	++	G	0	0	43
FA21g	7	21	1	1	+					54
FA22g	0	0	0	0	+	++	G	0	0	69
FA23g	4	8	1	1	+	+	G	0	0	48
FA24g	6	18	1	1	+	+	G	0	0	46
Totals	174	376	31	31						1321
Means	7	15	1	1						55·0
									σ	9·42
									σ^2	88·7

σ =population standard deviation of distribution

σ^2=population variance of distribution

Midges 6-7 days after emergence reared and kept at 20°C

Specimen number	MNC No.	INDEX MNC	VNC No.	INDEX VNC	AXONS MDC	CC	GPCA	PNSC CB	PNSC RS	FD
MB1k	13	36	0	0	+	++	B	++	++	51
MB2k	12	24	0	0	+	++	B	++	++	43
MB3k	12	24	0	0	+	++	B	++	++	58
MB4k	11	33	0	0	+	++	B	++	++	55
MB5m	12	36	0	0	+	++				47
MB6m	12	36	0	0	+	++	B	+	++	48
MB7m	12	36	0	0	+	++	B			64
MB8m	10	30	0	0	+	++	B	++	++	44
MB9c	12	36	0	0	+	++	B	++	++	51
MB10c	12	36	0	0	+	++	B	++	++	60
Totals	118	327	0	0						521
Means	12	33	0	0						52•1
									σ	6•99
									σ^2	49•0
FB1k	6	18	2	4	+	++	B	0	0	46
FB2k	10	30	0	0	+	++	B	0	0	57
FB3k	10	30	0	0	+	++	B	0	0	42
FB4k	12	36	4	8	+	++	B	0	0	44
FB5m	10	30	0	0	+	++	B	0	0	73
FB6m	9	27	4	8	+	++	B	0	0	59
FB7m	8	16	0	0	+	++	B	0	0	50
FB8m	12	36	0	0	+	++	B	0	0	46
FB9m	10	30	0	0	+	++	B	0	0	41
FB10m	10	30	4	8	+	++	B	0	0	57
Totals	97	283	14	28						515
Means	10	28	1	3						51•5
									σ	10•0
									σ^2	99•8

M=male

F=female

σ =population standard deviation of distribution

σ^2=population variance of distribution

Neoimagines reared at 10°C

Specimen number	MNC No.	INDEX MNC	VNC No.	INDEX VNC	AXONS MTC	CC	GPCA	PNSC CB	PNSC RS	FD
SM1a	2	2	0	0	0	0	G	0	0	52
SM2 a	0	0	0	0	0	+	G	0	0	61
SM3 b	0	0	0	0	0	0	G	0	0	43
SM4 b	6	6	0	0	0	+	G	0	0	63
SM5 b	4	8	0	0	+	+	G	0	0	63
SM6 b	0	0	0	0	0					40
SM7 c	0	0	0	0	0	+	G	0	0	54
SM8 c	2	2	0	0	0	0	G	0	0	47
SM9 f	2	4	0	0	0	0	G	0	0	39
SM10f	0	0	0	0	+	+	G	0	0	42
SM11f	4	4	0	0	+	0	G	0	0	57
SM12f	0	0	0	0	0	0	G	0	0	46
SM13h	2	2	0	0	+	0	G	0	0	41
SM14h	0	0	0	0	0	+	G	0	0	59
SM15h	0	0	0	0	0	+	G	0	0	48
Totals	22	28	0	0						755
Means	1•5	2	0	0						50•3
									σ	8•67
									σ^2	75•0
SF1c	4	12	0	0	0	+	G	0	0	73
SF2c	0	0	0	0	0	0	G	0	0	48
SF3f	4	8	0	0	0					60
SF4f	4	12	0	0	+	+	G	0	0	58
SF5f	0	0	0	0	0	0	G	0	0	54
SF6k	3	9	0	0	+	0	G	0	0	41
SF7k	4	12	1	1	0	0	G	0	0	65
SF8k	4	12	0	0	0	+	G	0	0	43
SF9k	0	0	0	0	0	0	G	0	0	42
SF10k	4	12	0	0	0	+	G	0	0	56
Totals	27	77	1	1						540
Means	3	8	0	0						54•0
									σ	10•6
									σ^2	112

SM=male

SF=female

σ =population standard deviation of distribution

σ^2=population variance of distribution

Comparisons of the means of brain cell field densities

As the number of samples in any group did not exceed 30 a Student's t-test for small samples, as given in BAILEY (1959), was employed.

The equality of the variances was tested statistically by the variance ratio (F) test.

Sample calculation.

Comparison of the mean field density of 30 (n_1) neoimaginal males reared at 20°C (MA) with that of 15 (n_2) neoimaginal males reared at 10°C (SM).

Equality of the variances of MA (σ_1^2) and SM (σ_2^2).

$$\hat{F} = \frac{\sigma_1^2}{\sigma_2^2} = \frac{75{\cdot}8}{75{\cdot}0} = 1{\cdot}01$$

Degrees of freedom in the numerator = $n_1 - 1 = 29$

Degrees of freedom in the denominator = $n_2 - 1 = 14$

The tabulated value for this result in BAILEY (1959) exceeds that of the data indicating that the variances are statistically similar at the 5% probability level.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where S may be calculated from:

$$S^2 = \frac{1}{n_1 + n_2 - 2}\left\{\Sigma_1 x^2 - \frac{(\Sigma_1 x)^2}{n_1} + \Sigma_2 x^2 - \frac{(\Sigma_2 x)^2}{n_2}\right\}$$

$$S^2 = 75{\cdot}58$$

$$\therefore S = 8{\cdot}69$$

Thus $$t = \frac{52{\cdot}3 - 50{\cdot}3}{8{\cdot}69\sqrt{\frac{1}{40} + \frac{1}{15}}} = \frac{2}{2{\cdot}7} = 0{\cdot}73$$

The propability of observing a value of t of 0·73, with 43 degrees of freedom, is greater than 0·10 or 10%. The means are therefore not significantly different.

Similarly the means of all the observed field densities were found not to significantly differ from one another.

APPENDIX 4

Analysis of granule sizes and size distributions

The table on the following page shows the distribution of granule sizes in several types of neurosecretory structures. The range of true mean diameters (TMD) of granules, of structures containing a unimodal distribution of granule sizes, is also shown. The TMD are bigger than the mean of the measured diameters as many of the granules, because of sectioning, will only represent a fraction of their true diameters.

Sample calculation of TMD (after FROESCH, 1973).

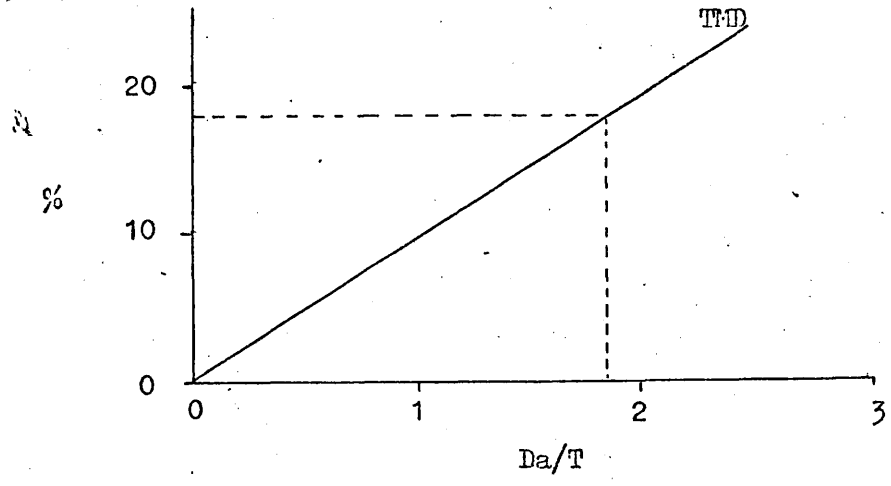
Knowing the apparent mean diameter (Da) and the approximate section thickness (T), the quotient Da/T is found.

e.g. for a particular cell Da= 158·7 nm
in silver sections T ≐ 70 nm

Thus $\frac{Da}{T} = \frac{158{\cdot}7}{70} = 1{\cdot}78$

From the graph below the corresponding percentage proportion can be added to Da, i.e. 158·7 nm, in order to get the true diameter (TMD), of the granule population. In this case the dotted line on the graph indicates that the percentage is 20.

Thus TMD = Da X % + Da = 158·7 X $\frac{120}{100}$ + 158·7 ≐ 190nm.



The difference between the 'apparent' (Da) and the 'full' (TMD) mean diameter in percentages of Da (taken from FROESCH, 1973).

Summary table of distribution of granule sizes

	Cell type	No.* Cells	No.** Granules	Percentage of granule falling within the size range (in nm)																					TMD nm †
				50-75	76-100	101-125	126-150	151-175	176-200	201-225	226-250	251-275	276-300	301-325	326-350	351-375	376-400	401-425	426-450	451-475	476-500	501-525	526-550		
NEUROACTIVES	♂ α_1	6	200	2	7	11	17	25	18	12	6	2													140-200
	♀ α_1	5	200	4	5	10	14	22	17	12	7	4	3												140-210
	♂ α_2	6	200	30	33	15	12	7	2	1															70-100
	♀ α_2	5	200	25	36	17	16	3	3																80-110
	♂ α_3	4	200	42	31	13	8	6																	70-120
	♀ α_3	5	200	38	27	19	10	5	1																60-120
	♂ β	2	100	35	36	18	6	3	1	1															60-100
	♀ β	2	100	26	33	21	12	6	1	1															90-110
	♂ γ Flattened Round — diameter	2	100	7	14	23	30	17	6	2	1														130-160
	♂ γ Flattened Round — length	2	100		3	6	8	16	21	15	15	7	5	3	1										150-250
	♂ γ Flattened Round — width	2	100	12	21	18	17	15	10	5	2														70-150
	♂ PNS c	4	300	2	7	23	17	13	7	3	5	8	5	4	4	2									
	♂ PNS r		300	2	8	19	31	16	9	7	5	3													
	♂ CC c	3	200	1	5	8	11	16	6	5	7	10	8	6	4	3	2	2	2	1	1	1	1		
	♂ CC r		200	1	5	10	14	12	9	10	11	7	5	5	4	3	1	1		1	1				
OVD††	♂ CC c	2	200	2	3	10	12	23	6	4	11	8	7	5	3	2	1		1	1			1		
	♂ CC r		200	1	1	12	13	16	10	4	7	11	8	7	4	2	2		1		1				

Percentages are given to the nearest 1%.

* Number of cells used in the sample.

** Approximate number of granules per cell measured.

† Range of true mean granule diameters given to the nearest 10 nm.

†† 6-7 days after emergence.

The granules of the cell bodies (c) and release sites (r) of the corpora cardiaca and peripheral neurosecretory cells of the prothorax (PNS) were measured. As these structures have bimodal distributions of granule sizes, their TMD are not given.

APPENDIX 5

DIMENSIONS and WEIGHTS

Width of neuropile masses 13, 4 & 5 in neoimagines.

10°C ♀			10°C ♂			20°C ♀			20°C ♂			Neuropile Mass No.
13	4	5	13	4	5	13	4	5	13	4	5	
135	6	18	130	6	20	140	6	14	125	6	16	
120	6	16	120	8	16	115	6	18	130	6	14	
145	8	14	140	8	16	135	6	12	140	6	18	
130	6	12	120	6	16	130	6	16	115	6	18	Width in µm
115	6	18	115	6	14	125	6	18	120	6	12	
125	6	16	145	6	18	115	6	12	125	6	18	
120	6	12	120	6	16	120	6	14	135	6	12	
115	8	18	115	6	14	110	4	16	120	6	16	
135	6	14	130	6	16	115	6	18	115	6	18	
115	6	16	140	6	14	120	6	14	130	6	16	
1255	64	154	1275	64	160	1225	61	152	1255	60	174	TOTALS
125	6	16	130	6	16	125	6	16	125	6	18	MEANS

Neuropile Mass 13 is measured to the nearest 5 µm.
Neuropile Masses 4 & 5 are measured to the nearest 2 µm.

The significance of the above results was found by Student's t-test using the formulae given in Appendix 3. The results for each of the neuropile masses were found to give propability values greater than 5% and therefore the size of each mass did not significantly differ with sex or rearing temperature.

The significance of weight, body length and head capsule width, included in the table on the following page, as the number of samples in each case was 30, was tested using the following formula:

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Where $\bar{x}_1$ and $\bar{x}_2$ are the means of n_1 and n_2 samples with estimated variances of s_1^2 and s_2^2. Based on the normal variable (d) the percentage points for the significance tests were obtained from the table in BAILEY (1959), the results of which are summarised in Table 9 in the text.

Summary table of body lengths (L), head capsule widths (H) & weights (W), of neoimagines.

20°C ♀			20°C ♂			10°C ♀			10°C ♂			
L mm	H µm	W mg	L mm	H µm	W mg	L mm	H µm	W mg	L mm	H µm	W mg	
6·00	750	2·20	6·25	700	1·04	6·50	750	4·46	7·00	800	3·36	
6·00	700	2·84	6·50	750	1·47	6·50	800	3·16	7·50	800	3·23	
5·50	600	2·66	5·50	750	1·95	6·00	800	4·02	7·00	800	3·34	
6·00	700	1·84	6·00	700	1·35	6·50	800	3·81	7·00	800	3·60	
5·00	700	1·75	6·50	700	1·01	6·50	800	3·64	7·00	800	3·27	
6·00	700	2·62	6·50	700	1·47	6·50	750	3·73	7·00	800	3·07	
6·00	750	2·71	6·25	750	1·24	6·50	750	3·57	7·00	800	3·25	
6·00	700	2·74	6·50	700	1·40	6·50	800	3·56	7·50	800	3·52	
5·50	700	2·45	6·25	700	1·71	6·50	800	3·93	7·00	800	3·31	
6·00	700	2·70	6·50	750	1·86	6·00	700	3·24	7·00	800	3·46	
6·00	750	2·46	6·00	700	1·55	6·50	750	3·67	7·00	800	3·34	
6·00	700	2·67	6·00	700	1·21	6·00	750	3·41	7·50	800	3·59	
6·50	750	2·03	6·50	700	1·29	6·50	800	4·18	6·50	800	3·18	
6·00	700	2·32	6·00	750	1·68	6·50	750	3·51	7·00	800	3·35	
6·00	700	2·73	6·00	700	1·57	6·00	800	4·39	7·00	800	3·71	
6·00	700	2·29	6·50	750	1·83	6·50	800	3·80	7·00	700	2·99	
5·50	650	2·62	6·00	650	1·11	6·50	700	3·35	7·00	800	3·46	
6·00	700	2·51	6·00	700	1·49	6·50	800	3·93	7·00	750	3·14	
6·00	700	2·41	6·50	750	1·63	6·50	800	4·19	7·50	800	3·31	
6·00	750	2·19	6·00	700	1·37	6·50	800	3·77	7·00	800	3·63	
6·00	700	2·35	6·00	700	1·70	6·50	700	3·16	6·50	750	3·15	
6·00	700	1·86	6·00	700	1·65	6·00	700	3·11	7·50	800	3·80	
6·50	750	2·23	6·00	700	1·15	6·50	800	3·89	7·00	800	3·57	
6·00	700	2·60	6·50	750	1·89	6·50	700	3·70	7·00	800	3·08	
6·00	700	2·32	6·00	700	1·56	6·00	750	3·54	7·00	750	3·16	
5·00	600	1·93	6·50	750	1·78	6·00	700	3·22	7·00	800	3·54	
6·00	700	2·46	5·50	650	1·09	6·00	750	3·46	7·50	750	3·12	
6·00	700	2·03	6·00	750	1·67	6·50	800	3·98	7·00	800	3·36	
6·00	700	2·59	6·00	700	1·61	6·50	800	3·90	7·00	800	3·22	
6·00	700	2·76	6·50	750	1·74	6·50	800	4·02	7·00	800	3·27	
117·5	21050	71·87	184·8	22250	45·07	191·0	23000	111·75	212	25700	100·34	T
6·0	725	2·40	6·25	750	1·50	6·25	775	3·73	7·0	800	3·35	M

T = Total M = Mean

L (excluding the antennae) measured to 0·25 mm.

H measured to 25 µm.

W weighed to 0·01 mg.

APPENDIX 6

LONGEVITY RECORDS

Duration of flight immobility phase.

	Life span in hours	Hours of flight immobity		
		Period	Confidence(±)	% of Life span
♂	45	24	1	53
	61	7	2	11
	71	8	4	11
	74	11	4	15
	85	24	7	28
	118	18	8	15
	123	22	1	18
	134	17	8	12
	140	42	7	30
	141	35	8	24
	146	21	7	14
	201	25	1	12
♀	133	7	2	53
	142	7	2	49
	170	48	1	28
	171	45	8	26
	185	53	2	29
	190	24	7	13
	217	49	5	23

Average duration of flight immobility is 20 (±12) % for males, and 31 (±14) % for females, of the total life span (numbers in parentheses are the standard deviations).

The significance of the mean life span differences, obtained from the data included in the table on the following page, was tested using the d-test as given in Appendix 5. The results of these tests are summarised in Figure 13 in the text.

LIFE TABLE (continued on the next page).

	♀ 10-10°C			♀ 15-15°C			♀ 20-20°C			♂ 10-10°C			♂ 15-15°C		
%d	T	lx	%s	T	lx	%s	T	lx	%s	T	lx	%s	T	lx	%s
-100	0	30	100	0	30	100	0	50	100	0	30	100	0	30	100
- 90	21•7	30	100	15•7	30	100	14•3	50	100	20•1	30	100	11•7	30	100
- 80	43•4	30	100	31•3	30	100	28•6	50	100	40•2	30	100	23•5	29	97
- 70	65•1	30	100	47•0	29	97	42•8	50	100	60•3	29	97	35•3	26	87
- 60	86•8	28	93	62•7	29	97	57•1	50	100	80•4	29	97	47•0	26	87
- 50	108•5	27	89	78•4	29	97	71•4	50	100	100•5	27	89	58•8	25	83
- 40	130•2	25	83	94•0	29	97	85•7	49	98	120•6	24	80	70•6	24	80
- 30	151•9	20	66	109•7	28	93	99•9	48	96	140•7	21	70	82•3	21	70
- 20	173•6	17	56	125•4	26	87	114•2	44	88	160•8	18	60	94•1	19	63
- 10	195•3	16	53	141•0	23	74	128•5	35	70	180•9	15	50	105•8	16	53
0	217•0	11	36	156•7	16	53	142•8	21	42	200•7	12	40	117•6	12	40
+ 10	238•7	10	33	172•4	10	33	157•1	13	23	221•1	10	33	129•4	11	36
+ 20	260•4	8	27	188•0	3	10	171•4	9	18	241•2	9	30	141•1	9	30
+ 30	282•1	8	27	203•7	1	3	185•6	5	10	261•3	8	27	152•9	6	20
+ 40	303•8	4	13	219•4	1	3	199•9	1	2	281•4	5	17	164•6	5	17
+ 50	325•5	4	13	235•0	1	3	214•2	1	2	301•5	5	17	176•4	3	10
+ 60	347•2	4	13	250•7	1	3	228•5	0	0	321•6	4	13	188•2	3	10
+ 70	365•9	3	10	266•4	1	3				341•7	4	13	200•0	3	10
+ 80	390•6	3	10	282•1	0	0				361•8	3	10	211•7	3	10
+ 90	412•3	3	10	297•7						381•9	2	7	223•4	2	7
+100	434•0	3	10	313•4						402•0	2	7	235•2	2	7
+110	455•7	2	7	329•1						422•1	1	3	247•0	2	7
+120	477•0	0	0	344•7						442•2	0	0	258•7	2	7
+130													270•5	1	3
+140													282•2	1	3
+150													294•0	0	0
+260															
+270															

%d = Percentage deviation from the mean duration of life

T = Time in hours from emergence

lx = The number of specimens surviving

%s = Percentage survival

The first figure of specimen indentification refers to rearing temperature, the second to the temperature at which the adult was kept.

LIFE TABLE (continued).

%d	♂ 20-20°C			♂ 10-20°C			♂ 20-10°C		
	T	lx	%s	T	lx	%s	T	lx	%s
-100	0	50	100	0	30	100	0	30	100
- 90	11•5	48	96	8•9	30	100	19•5	29	97
- 80	23•0	48	96	17•7	29	97	39•0	29	97
- 70	34•6	48	96	26•6	27	89	58•5	29	97
- 60	46•1	46	92	35•5	25	83	78•0	28	93
- 50	57•7	45	90	44•4	25	83	97•5	26	87
- 40	69•2	40	80	53•3	20	66	117•0	24	80
- 30	80•8	38	76	62•2	17	56	136•5	23	76
- 20	92•3	33	66	71•0	15	50	156•0	20	66
- 10	103•9	31	62	80•0	10	33	175•5	16	53
0	115•4	28	56	88•9	10	33	195•2	15	50
+ 10	126•9	26	52	97•8	8	27	214•5	10	33
+ 20	138•5	19	38	106•7	7	23	234•0	9	30
+ 30	150•0	9	18	115•6	6	20	253•5	9	30
+ 40	161•1	5	10	124•5	6	20	273•0	8	27
+ 50	173•1	5	10	133•6	5	17	292•5	5	17
+ 60	184•6	1	2	142•2	4	13	312•0	5	17
+ 70	196•2	1	2	151•1	4	13	331•5	3	10
+ 80	207•7	0	0	160•0	4	13	351•0	1	3
+ 90	219•3			168•7	4	13	370•5	0	0
+100	230•8			177•6	4	13			
+110				186•5	3	10			
+120				195•4	2	7			
+130				204•2	2	7			
+140				213•0	1	3			
+150				319•7	1	3			
+260				328•6	0	0			
+270									

%d = Percentage deviation from the mean duration of life

T = Time in hours from emergence

lx = The number of specimens surviving

%s = Percentage survival

The first figure of specimen identification refers to rearing temperature, the second to the temperature at which the adult was kept.

APPENDIX 7

ELECTRON MICROSCOPE SAMPLE SIZES

Summary table of the number of individuals observed with the electron microscope on which the results are based.

Observed tissue	Neoimagines (20 ± 1°C)		6-7 day olds (20 ± 1°C)		3-4 day old ♀ (22 ± 4°C)				Neoimagines (10 ± 1°C)		Neoimagines (15 ± 1°C)	Number of individuals observed
	♂	♀	♂	♀	M	MO	VL	VS	♂	♀	♂	
Brain SOG	10	5	3	5					3	3	2	
Cerebral NSC	10	5	1	5	2	5	4	2	3	3	1	
Axons of NSC	6	1		1								
Hypocerebral ganglia	3	1										
Corpora cardiaca	6	2	2	4		3						
Corpora allata	6	2	1	4		3						
Peritracheal tissue	3	1	1*	4*		3*						
GPCA	3	2	1	3		3						
SOG NSC	4	2*										
Prothoracic NSC	3											
Peripheral NSC	3											
Longitudinal Flight Muscle	3		3									

* Number of specimens searched in which structure was not found.

GPCA = "Glandes post-cérébrales antérieures"

M = Mated

L = Ligated (abdomen/thorax)

S = Sham ligated

V = Virgin

O = Oviposited

NSC = Neurosecretory cells

SOG = Suboesophageal ganglia