4

STUDIES ON THE BIOLOGY AND ULTRASTRUCTURE OF NEMATODES PARASITIC IN INSECTS

by

Irene L. Riding, B.Sc., A.R.C.S.

A thesis submitted for the degree of Doctor of Philosophy in the faculty of Science, London University.

Department of Zoology & Applied Entomology,
Imperial College of Science & Technology,
London, S.W.7.

March, 1971.

ABSTRACT

A new species of Howardula (Tylenchoidea: Sphaerularidae: Allantonematinae) is described, the life cycle is elucidated and it is compared with related nematodes. Experiments on bionomics of Howardula sp. and its effect on Megaselia halterata Wood (Diptera: Phoridae) are reported and discussed regarding its potentiality for biological control.

Ultrastructure of the Nematoda body wall is reviewed and electronmicroscopic studies on some of the stages of Howardula sp. Deladenus siricidicola Bedding (Tylenchoidea: Neotylenchidae), D. wilsoni Bedding and Contortylenchus sp. (Tylenchoidea: Sphaerularidae: Allantonematinae) show that the free living females resemble other nematodes, but the parasitic forms lack a cuticle and all except Contortylenchus possess microvilli. The cuticle transformation occurs by different methods in Howardula and Deladenus and microvilli formation in other animals is discussed.

A nutritional function for the body wall is postulated, with reference to previous assumptions about these mouthless, flaccid-bodied nematodes. Discussion includes current ideas on uptake of nutrients by parenteral-feeding parasites as well as by absorptive gut cells, and some of the techniques for investigating its occurrence in tylenchids are outlined. Morphological and biological peculiarities of sphaerularids are reviewed and the evolutionary implications, together with the taxonomic position of Deladenus, are discussed.

Details of the contents are summarised at the end of each section.

A C K N O W L E D G E M E N T S

I wish to thank Professor T.R.E. Southwood in whose department this work was carried out, for providing laboratory facilities and for arranging a staff appointment which enabled me to gain university teaching experience while doing research.

I am indebted to my supervisor Dr. N.G.M. Hague for directing this study; for his wisdom in encouraging a more flexible approach to research, and for his understanding during the difficult period of my mother's illness.

I thank Dr. N.A. Croll also of this department for the exhilerating discussions we have had and for advice on presentation of papers and publication techniques.

I am most grateful to Dr. D. L. Lee, Head of Parasitology, Houghton Poultry Research Station, for his expert advice on electronmicroscopy and for the stimulation provided by his keen interest in the development of this work.

I am indebted to Dr. N.W. Hussey, Head of Entomology, Glasshouse Crops Research Institute, for initiating research on nematodes in phorids and for providing me with the original Howardula material and sundry items of equipment. Also to Mr. J. J. Hesling, G.C.R.I., for giving me his notes on Bradynema.

I am equally indebted to Mr. F. Wilson, Head of the C.S.I.R.O., Sirex Unit, Silwood Park and his staff including Dr. R.A. Bedding, Dr. P. Spradbury and Mr. A. Kirk for providing all the Deladenus material and for their willingness to discuss mutual research interests.

I also wish to thank Dr. W.R. Nickle, Research Nematologist, Crops Research Division, U.S.D.A., Beltsville, Maryland, for lending me type specimens of Howardula and Bradynema; for his

advice on these genera and for confirming the morphological observations on Howardula sp.

I am grateful to Messrs. Darlington & Sons Ltd., Worthing for donating regular supplies of mushroom spawn and to Mr. D. Tolhurst, Clockhouse Mushroom Co., and Mr. J. Tunney, Bushell's Ready Mix Ltd., for donating compost.

I thank Dr. Suad Al-Rabiai for allowing me to use her Contortylenchus material; Mr. A. Presland for electronmicroscope facilities and Dr. C.T. Lewis for ultramicrotomy equipment.

Collaboration with undergraduate students Keith Probert, Ingrid Travis, Anne Goodings and Ruth Lawson is acknowledged and I am grateful to them for preparing some of the material used in this study.

I appreciate the advice of Dr. G. Murdie on statistical analysis of results and I am indebted to Mr. H.A. Devitt, Mrs. C.A. Steel, and Mrs. G. Murdie for their invaluable help during the preparation of this manuscript.

CONTENTS

		Page
ABSTRACT	......	i
ACKNOWLEDGEMENTS	......	ii
CONTENTS	......	iv
GENERAL INTRODUCTION	......	1

SECTION I. ASPECTS OF THE BIOLOGY OF *HOWARDULA* sp.

		Page
I. 1. INTRODUCTION	......	16
Summary of objectives	......	19
I. 2. MATERIALS & METHODS		
2. 1. Source of material	......	20
2. 2. Breeding the flies	......	21
2. 3. Rearing the parasite	......	23
2. 4. Sectioning parasitised flies	......	24
I. 3. OBSERVATIONS		
3. 1. The life cycle of *Howardula* sp.	......	26
3. 2. THE MALE NEMATODE		
3. 2. 1. Description and development .		31
3. 2. 2. Effect of temperature on development	......	33
3. 3. THE INFECTIVE FEMALE		
3. 3. 1. Description and development		37
3. 3. 2. Effect of temperature on development	......	41
3. 3. 3. Moulting inside the host	.....	41
3. 4. THE MATURE FEMALE		
3. 4. 1. Description and development		56
3. 4. 2. Reproduction in relation to host age		64
3. 4. 3. Intraspecific competition	....	68
3. 4. 4. Incidence of parasitism	......	71

3.5. HOST-PARASITE RELATIONSHIPS

3.5.1. Effect of parasite on host internal anatomy 74
3.5.2. Effect of parasite on host copulation 77
3.5.3. Effect of parasite on host egg output 78
3.5.4. Elimination of host population 79

I.4. DISCUSSION 82

I.5. SUMMARY 98

SECTION II. BODY WALL STRUCTURE, ITS FORMATION AND PROBABLE NUTRITIONAL FUNCTION IN THE PARASITIC FEMALE HOWARDULA Sp., WITH COMPARATIVE OBSERVATIONS ON DELADENUS SIRICIDICOLA Bedding 1968, D. WILSONI Bedding 1968 AND CONTORTYLENCHUS Sp., INCLUDING SOME OF THEIR FREE-LIVING STAGES.

II.I. INTRODUCTION 100

Summary of objectives 104

II.2. MATERIALS & METHODS 107

II.3. OBSERVATIONS

3.1. The free-living infective Howardula sp. 114
3.2. Moulting of the young parasitic Howardula sp. 125
3.3. The adult parasitic Howardula sp. 139
3.4. The mycetophagous adult female Deladenus siricidicola 157
3.5. The young parasitic Deladenus siricidicola 163
3.6. The mature parasitic Deladenus siricidicola 176
3.7. The mature parasitic Deladenus wilsoni 189
3.8. The mature parasitic Contortylenchus sp. 206

II.4. DISCUSSION 216

II.5. SUMMARY 242

GENERAL DISCUSSION 244

LITERATURE CITED 253

APPENDIX 266

GENERAL INTRODUCTION

Entomophilic nematodes.

More than 3000 nematodes from thirteen different families are known to be associated with at least sixteen orders of insects (Welch, 1965). The nematodes are frequently referred to as 'entohelminths' or 'entomophilic' and outstanding contributors to the subject include pioneers, nearly a hundred years ago, such as Leuckart, zur Strassen, von Siebold and Leidy whose work was followed by Cobb, Christie, Steiner, Fuchs, Wuelker and Filipjev in the first half of this century. The major publications of Wachek & Ruhm in the 1950's and more recently by Nickle and Welch have done much to restore interest in these often neglected but potentially economically important nematodes.

Entomophilic nematodes belong to the superfamilies Rhabditoidea, Oxyuroidea, Tylenchoidea, Aphelenchoidea and Mermithoidea and the relationship with insects is usually either phoretic or parasitic. Among the Tylenchida and Rhabditids are many examples of a phoretic association where the insect is utilised as a dispersal agent because translocation from one breeding site to another - from tree to tree or to a fresh dung pat after depletion of nutrients - would be sporadic or impossible for the unaided nematode. Inherent hazards of a relationship which necessitates exposure to an adverse environment when the insect is in flight, are largely overcome by adaptations of the nematodes to attach firmly to the vector and resist desiccation. Perhaps the simplest solution is to be transported inside the 'host' because endo- as well as ectophoresis occurs as was recently indicated in Poinar's (1969a) review. Both relationships are

observed in the bark boring beetle Ips sexdentatus where Cryptaphelenchus is found under the elytra and Parasitorhabditis is in the gut lumen (Al-Rabiai, 1970).

The ectophoretic nematodes are invariably transported as third stage 'dauer' larvae enclosed by the moulted second cuticle which together with a reduced surface area, plugged apertures, anabiosis and a sheltered niche on the insect, facilitates resistance to desiccation. Attachment to the vector is passively secured by the hydrophobic nature of the external surfaces of the two animals although some nematodes have evolved modifications like Panagrolaimus which exudes viscous material from the mouth.

The Steinernematidae are unusual among the rhabditids because they have a temporary endoparasitic relationship which ultimately kills the insect. Poinar and Thomas (1966) described the interdependence of Neoaplectana carpocapsae and the bacterium Achromobacter nematophilus, an association probably occurring in other members of the genus which have been frequently employed in biological control trials. Many of the neoaplectanids attack and kill a wide variety of insects which are economically important pests and relevant literature was reviewed by Welch (1962) and more recently by Campion (1969). It appears that these nematodes can be manipulated either as control agents or insecticides but their success critically depends on environmental conditions and host abundance.

The oxyurid families Thelastomatidae and Rhigonematidae occur exclusively in arthropods but the life cycle is the same as those in vertebrates; they are endoparastic and usually only the egg is exposed to the external environment. Oxyurids are commonly

found in the Blattidae (Orthoptera) where all stages occur in the anterior region of the hind gut, feeding on bacteria and not affecting the host adversely except in rarely occurring heavy infestations (Dale, 1964).

Mermithids have the widest host range, occurring in fifteen orders of terrestrial and aquatic insects although each member is usually restricted to species of a single genus. Parasitism may reach a high incidence locally but there is usually marked discontinuous distribution within a single population (Poinar and Gyrisco, 1962). Mermithids are obligate parasites in the host haemocoel but are free living as adults except for the small group of tetradonematids in which all stages occur in the insect and transmission is by egg or first stage larvae. The relatively large size of these nematodes inevitably causes death of the host when they emerge; as many mermithids parasitise pests and vectors of human disease such as simulids and mosquitos it is hoped that they may eventually be used for biological control (Welch, 1963, 1965).

The remaining group of nematode associates of insects, the Tylenchida, include a repetition and elaboration of the range of relationships already described and is the group to which the nematodes in the present study belong. Nickle revised the taxonomy of insect parasitic Tylenchoidea (1967a) and Aphelenchoidea (1970) after recording personal observations on the majority of genera for several years, producing two outstanding contributions to the systematics of Tylenchida. Aphelenchs have a strong affinity for insect association and about two-thirds of the genera have either a phoretic or an obligate parasitic relationship; the rest are free living fungal feeders, predators or plant parasites (Nickle, 1970).

Many of the entomophilic aphelenchs are parasitic in the larval stages but a particularly interesting life cycle, *Praecocilenchus rhaphidophorus*, was recently reported by Poinar (1969b) who described larvae maturing and copulating inside the uterus of parasitic females from the haemocoel of weevils. Intrauterine adult nematodes were frequently surrounded by retained cuticles and also a membrane which may represent a stretched egg shell. Several life cycles may be completed within one beetle and the free living females are released via the host vulva or anus but the rest of the life cycle was not reported. Perhaps the nematodes have feeding and reproductive cycles in the external environment as in *Deladenus* (Bedding, 1968) which is described in Section II. *Praecocilenchus* (Aphelenchoidea) resembles *Scatonema wulkeri* (Tylenchoidea) because Bovien (1932) observed copulating nematodes inside the uterus of adult parasites from the haemocoel of *Scatopse* flies.

The Sphaerularidae (Tylenchoidea) includes two monogeneric sub-families and two which are more widely known; the Sphaerulariinae and the Allantonematinae. The group is characterised by its aberrant heteromorphic females which in the sphaerularids have increasing degrees of uterine prolapse which may greatly exceed the size of the original nematode, as described by Khan (1957). A prolapsed uterus never occurs in the allantonematids but the parasitic females become large, sausage-shaped reproductive sacs; the oesophagus and intestine usually degenerate and the gonad proliferates, often filling 80% of the body (Nickle, 1967a). In the sphaerularids and many of the allantonematids the life cycle is essentially the same: only the female is parasitic and reproduction occurs in the insect haemocoel; larvae leave the host with varying

degrees of maturation and further development leading to copulation of the nematodes occurs in the external environment. Usually the hosts are characterised by having overlapping insect generations and by occupying a small ecological niche in moist surroundings (Welch, 1965) such as provided by the tunnels of bark boring beetles and the galls of midges.

The hosts of allantonematids are mainly Coleoptera, Lepidoptera, Diptera and Hymenoptera and it is interesting that the outlined life cycle of the nematodes is variously modified. Two sexual generations occur in Parasitylenchus as outlined by Ruhm (1956) and Welch (1959); the fertilised female enters the host haemocoel, reproduces and the resulting larvae quickly mature, copulate, and lay eggs which hatch into nematodes predestined to be free living. Outside the host these larvae develop into adult males and infective females which mate before the parasitic cycle can recommence. Allantonematids normally produce a few hundred progeny but by adding a second sexual generation, Parasitylenchus is able to produce over 4000.

A similar adaptation to parasitism by increasing fecundity is the alternation of sexual and parthenogenetic cycles which occurs in Heterotylenchus where the two methods of reproduction occur in the insect haemocoel (Nickle, 1967b) and in Fergusobia which reproduces sexually inside the host and parthenogenetically in the external environment. Fertilised infective female Heterotylenchus enter a host, mature and produce larvae which develop into parthenogenetic females and subsequently release many offspring destined to develop outside the host. Males copulate with infective females which then resume the parasitic cycle. Fergusobia infective

females enter maggots of the Eucalyptus "gall midge", mature and reproduce. Larvae are released during host oviposition and they mature inside the gall which subsequently forms around the young insect. They reproduce parthenogenetically and it is interesting that only male nematodes are formed for 2-3 months; eventually females are produced to coincide with the final maggot instar which is parasitised after the free living nematodes have mated, thus completing the cycle.

There is only one record of juvenile parasitism in the allantonematids, in contrast to the aphelenchs. Chondronema adults are free living nematodes in beetle frass but their larvae occur in abundance inside the host haemocoel; adults have never been found inside the insect and Nickle (1967a) suggests that this group merits further investigation.

Howardula, Deladenus and Contortylenchus are the tylenchid nematodes to be examined in this study. The life cycle of Howardula will be elucidated and compared with other allantonematids and because of the peculiar non filariform shape and flaccid nature of the mature parasite, an ultrastructural study of the body wall of the three tylenchs is undertaken. The free living states are similarly examined and the cuticle will be compared with that of other nematodes so far described.

NEMATODE BODY WALL ULSTRASTRUCTURE.

Investigation of the structure of nematode cuticle began over a hundred years ago and most of the early papers are reviewed by Chitwood and Chitwood (1950) who concluded that in all nematodes it is a multilayered structure differing in thickness but essentially similar in all groups. Since that time, the application of the

electron microscope to culticular studies has vastly increased our knowledge and rapid progress is now being made which, while not refuting the earlier ideas, emphasizes the great structural diversity and evolutionary plasticity of the nematode cuticle. Fundamental differences occur not only between members of different families and genera but within a single species at different stages of development, particularly when a change of habitat is encountered as Lee (1965b, 1966a) showed in Nippostrongylus brasiliensis and Bird and Rogers (1965) and Bird (1968a) showed in Meloidogyne javanica. Cuticular discrepancies may also occur between the sexes as in Syphacia obvelata (Dick, 1970) and even in different regions of the body of the same animal (Watson, 1962).

Comparative studies on nematode cuticle at the light microscope level were made by Inglis (1964a, b) but more recently excellent comprehensive reviews on structure and composition have been given by Bird & Bird (1969) and Lee (1966b). Consequently a repetition of their work would be superfluous here, so only a brief outline will be presented and only one worker on a given topic will usually be mentioned.

Essentially the cuticle is made up of three layers; the outer cortex, the middle matrix and the inner basal layer. Often it is difficult to distinguish the boundaries of these three regions and in many nematodes each layer is subdivided. On the outside of the cortex is usually (always, according to Roggen, 1967) a thin osmiophilic layer which is currently controversial because it resembles a triple layered membrane (Lee, 1965b) and as such would indicate that the cuticle is intracellular (intrahypodermal) as was recently stated by Bonner et al. (1970). Other workers

disagree and point out that it is wider than the normal plasma membrane and its outer layer is trilaminate in some cases (Jenkins, 1969). Bird (1957) and others have shown that it contains lipid and it is thought to have a role in determining cuticle permeability.

The cortex is made up of two layers in many nematodes and the outer region is often very electron dense and contains sulphur which is present in disulphide and sulphydryl groups (Carbonell and Apitz, 1960). This probably confers resistant properties and keratin has been suggested but has not been verified. Also there is some evidence for polyphenol-quinone tanning (Monne, 1960) which presumably helps to stabilise the outer regions.

The matrix is often described as a homogeneous and rather plastic layer because of its great diversity, but in adult females of the family Heteroderidae (Bird and Rogers, 1965) and in encapsulated larval *Trichinella spiralis* (Beckett and Boothroyd, 1961) it is absent. It has fewer fibres than the other regions of the cuticle and may be fluid-filled as in *Nippostrongylus brasiliensis* (Lee, 1965b, and others) or be traversed by a series of rods, rings and canals, particularly in the Aphasmid orders Chromodorida and Enoplida, as has been demonstrated by several workers including Wright and Hope (1968) and Inglis (1964b). Commonly the matrix contains albumen-like and collagen-type proteins with small amounts of carbohydrate, lipid and several enzymes which indicates metabolic activity; ribonucleic acid was found (in the inner cortex) by Anya (1966a, b) who suggested protein synthesis was occurring, so the cuticle can no longer be considered inert. Indeed Watson (1965) showed that the cuticle of adult *Ascaris* grows considerably and the thickness increases in direct proportion to the length of the worm as it develops from about 3 cm to 25 cm or more.

The basal layer, particularly in the large nematodes, is usually composed of fibrils organised into 2 or three layers of fibres which form a lattice of parallelograms. These were considered by Harris and Crofton (1957) together with the elasticity of the cuticle and the high turgor pressure of the body, to be of fundamental importance for locomotion in *Ascaris*. The fibrils have the characteristics of collagen but only a few nematode electronmicrographs show the typical banding which was seen by Hinz (1963) in *Parascaris*. Inglis (1964b) pointed out that a different system for locomotion must be operative in nematodes which lack these fibres and suggested that toughened rings in the outer cortex of some of the smaller worms might serve the same function of opposing diametrical extension. Wisse and Daems (1968) suggest that the cuticle acts as a coiled spring to antagonise muscular contraction and their hypothesis is supported by excellent electron-micrographs.

The basal layer may or may not be separated by a lamella from the hypodermis which underlies the cuticle, and a plasma membrane is sometimes present.

Bird & Bird (1969) drew attention to the recent investigations of larval nematode cuticles and intimated that a striated layer, in addition to the cortex, honogeneous and basal layers, is common to all larvae. However, it is not obvious from their comments that the striated layer has also been seen in several adult nematodes, including plant parasitic species (Johnson et al., 1970), animal parasitic species (Wright, 1968) and free living ones (Watson, 1962). It has been suggested by Inatomi et al. (1963) that this striated layer may be concerned with moulting, and the possibility of this role occurring in *Howardula* sp. will be discussed later. However,

Lee (1966a) proposed that it confers resistance to changes in the external environment because the close regular spacings indicate protein molecules with very close linkages, and Wisse and Daems (1968) support this suggestion from their observations on second stage Heterodera larvae. They reported that Heterodera has a heavily armoured resistant layer, impermeable to lethal fluids such as fixatives and in a long erudite discussion on the nature of the striated layer, they concluded that it is composed of rods arranged in a geometric pattern with interconnected membranes and went on to theorise about the role of the cuticle in locomotion, as previously mentioned.

The striated layer may be viewed as a modified fibre layer and this relationship is seen in the lateral cords of, for example, Hirschmanniella gracilis (Johnson et al. 1970) where 4 fibre layers are contiguous with the striated zone. These authors commented that the striated layer of free living larval nematodes persists through to the adult stage when a change in environment is not encountered; otherwise it is frequently replaced by fibre layers as seen in many adult parasitic forms, and they suggested that if ontogeny recapitulates phylogeny then the striated layer is more primitive. Lee (1966b) points out that the differences may reflect adaptations to a free living and a parasitic mode of life respectively. It is interesting that Inglis (1964b) attributes the great success of parasitic nematodes to this ability to change the structure of the cuticle during the different stages of the life cycle, which occurs, of course, at the moult.

Moulting.

Moulting was first described at the ultrastructural level by Watson (1962, Turbatrix aceti) but as her work is still confined

to a Ph.D. thesis her discoveries are not readily available. Since that time only a few other moulting processes have been examined electronmicroscopically; they include Meloidogyne javanica (Bird & Rogers, 1965) Trichinella spiralis (Lee, 1966b), Panagrellus silusiae (Samoiloff & Pasternak, 1968), Hemicycliophora arenaria, Aphelenchus avenae & Hirschmanniella gracilis (Johnson et al., 1970), Nematospiroides dubius (Bonner et al., 1970) and Nippostrongylus brasiliensis (Lee, 1970a). Moulting will be discussed after the relevant observations on the nematodes in the present study have been reported, but it is pertinent to state here that each worm appears to follow a different procedure and, even more remarkable, the process may vary at each moult in the life cycle of a single species (Samoiloff & Pasternak, 1968). The initial stage of thickening and granulation of the hypodermis is common to all, but the subsequent deposition of the osmiophilic line, the separation of the larval cuticle and its resorption or otherwise, the formation of the adult layers and the degree of involvement of the muscle cells, are all diverse processes. This versatility, together with probable differences produced by the fixation techniques of the investigators, presumably led to the current controversy regarding the morphogenesis of nematode cuticle; Roggen et al. (1967) found it to be an extra cellular structure in Xiphinema index but Bonner et al. (1970) are adamant that in Nematospiroides dubius the cuticle is intracellular.

The initiation of moulting was postulated by Rogers & Sommerville (1963) as being due to secretions from glands activated by the products of neurosecretory cells, and later Rogers (1965) showed that leucine aminopeptidase was an important constituent of the "exsheathing fluid" in Haemonchus & Trichostrongylus. Roggen et al. (1967) agreed that this enzyme was associated with moulting in Xiphinema index

and Davy & Kan (1967; Kan & Davy, 1968) found the same in Phoconema decipiens: they also demonstrated that leucine aminopeptidase was produced by the excretory gland under the control of neurosecretory cells in the dorsal and ventral ganglia.

Hypodermis.

The hypodermis which underlies the cuticle surrounds the body and is a narrow layer except where it is expanded to form cords, usually in the dorsal, central and lateral regions which extend into the pseudocoelom. It may be cellular or syncytial and the nuclei are generally confined to the cords but are sometimes present along the intercordal hypodermis as Wright (1968) reported in Trichuris myocastoris. Nerves are associated with the cords as are the lateral excretory canals when present, but Lee (1966a) showed that in the third stage larva of Nippostrongylus brasiliensis the nerves are not actually embedded in the hypodermal tissue. Indication that the hypodermis is metabolically very active, particularly in the lateral cords, is seen by the presence of mitochondria, ribosomes, rough endoplasmic reticulum, golgi-zones, glycogen, lipid and vesicles containing enzymes such as the leucine aminopeptidase which was mentioned earlier. Undoubtedly one of the chief functions of the hypodermis is to produce and maintain the cuticle and it may also be involved in osmoregulation as has been suggested by Wright (1963a) and Roggen (1966).

Somatic musculature.

Beneath the hypodermis in its intercordal regions are the muscle cells of the body wall. The somatic musculature of nematodes is unique in having only a single longitudinal layer of cells, and in its method of innervation; this consists of elongated projections from the

body of each muscle cell synapsing with the main longitudinal nerves in the hypodermal cords. Desmosomes connect the muscle cells to the hypodermis and fibres pass through to the cuticle for firm attachment, so that forces can be transmitted to facilitate locomotion. Nematodes have two basic types of muscle cell but both are spindle-shaped, elongated about the long axis and have clearly demarked contractile and non-contractile regions. Platymyarian muscle cells are present in the majority of small nematodes and they are characterised by having myofilaments arranged only along the hypodermal side of the cell; coelomyarian muscle cells frequently occur in the large worms and have myofilaments arranged in a 'U' shape when viewed transversely. Usually there are fewer than 5 platymyarian cells in one muscle quadrant and this arrangement is described as meromyarian, but coelmyarian cells are abundant and known as polymyarian. Wright (1966) reported the presence of cytoplasmic bridges between muscle cells in a variety of polymyarian nematodes, particularly at the anterior end of the worms, and said that this would significantly increase the co-ordination of movement and account for the greater mobility in this region.

Whether the somatic musculature of nematodes is striated or smooth has been a controversial topic for many years but Rosenbluth (1965) made a very significant contribution with his Ascaris investigations. He showed that the coelomyarian muscles have both smooth and striated characteristics because the myofilaments are staggered, but by ingeniously constructing three-dimensional diagrams he demonstrated that they were obliquely striated.

Two types of myofilaments are present in most nematodes; thick ones which probably contain myosin and thin ones which probably

contain actin, and these may occur together in close association or they may be isolated. The non-contractile part of the muscle cell contains the nucleus, endoplasmic reticulum, mitochondria, ribosomes, glycogen and lipid but of course lacks myofilaments. The muscle cells are separated from the pseudocoelum by a basement membrane in most nematodes.

In concluding this General Introduction it is pointed out that Section I is concerned with the morphology and biology of *Howardula* while Section II includes ultrastructural studies on this and three other parasitic Tylenchid nematodes. The objectives are listed at the end of the introduction to each section and the contents are summarised after the two relevant discussion chapters. The General Discussion integrates observations from both sections.

SECTION 1

ASPECTS OF THE BIOLOGY OF *HOWARDULA* sp.

(Nematoda : Tylenchida)

SECTION I ASPECTS OF THE BIOLOGY OF HOWARDULA sp (Nematoda: Tylenchida)

I 1. INTRODUCTION

The first published report of a Tylenchid nematode parasitising the mushroom phorid Megaselia halterata Wood 1910 (Diptera) was made by Hussey (1959) who found that some dissected flies were full of 'eelworms'. A few of the specimens were subsequently examined by H. E. Welch (University of Manitoba, Winnipeg) who suggested that the nematode might be a new species of the genus Bradynema. (Ref. private correspondence between Hussey and Welch, 1963). After collaboration with J. J. Hesling (G. C. R. I., Rustington) who was also familiar with the material, Welch decided not to publish a description of the new species until a more detailed investigation could be made on the morphology and life history. Hussey (1959) referred to the nematode as Bradynema and continued to use this name in subsequent papers which will be mentioned later. No further attempt was made to describe the nematode and in order to avoid confusion it is necessary to anticipate the data presented in this thesis to point out that Hussey's "Bradynema" is here referred to as Howardula sp. The nematode cannot be given a specific name because a Ph. D. thesis does not constitute a publication within the meaning of the International Code of Zoological Nomenclature 1964, (Article 8) but a paper describing the species is currently being prepared.

Megaselia halterata is a small black fly frequently found on mushroom farms and may reach epidemic proportions such as

occurred in West Sussex in 1961 and in 1953 (Moreton and John, 1955). Eggs are laid in the mushroom compost and the maggots hatch in 2-7 days (Hussey 1961) and feed on the mycelium. If the beds are attacked soon after spawning the mycelial growth can be entirely inhibited (Hussey, 1960) with resulting crop failure. The three larval instars last 4-33 days according to the temperature and this is followed by pupation for 1-9 weeks before the flies emerge (Hussey, 1961). Mating occurs within 3 days and a flight period is thought necessary to induce copulation because frequently the flies leave the breeding site (Hussey, 1964a). Unlike the maggots, the flies do not feed and the females return to the mushroom mycelium to lay eggs for about 10 days (Hussey and Gurney, 1964).

Hussey (1959) found up to 83% of Megaselia halterata were parasitised by Bradynema (Howardula sp.) and this high incidence occurred in flies associated with mushroom beds 17-19 weeks old, at the end of the cropping season. Individual flies contained 1 to 18 mature female nematodes and as many as a thousand parasitic larvae. Moreton and John (1955) reported that M. halterata produce about 40 progeny and Hussey (1967) found that fecundity of parasitised flies was greatly reduced. Where one mature female nematode was present the flies laid fewer than six eggs and when several parasites occurred, the phorids were completely infertile. As M. halterata can reduce mushroom crop yield and transmit virus, bacterial and fungal pathogens as well as irritate the pickers, the potentiality of "Bradynema" as a biological control agent was immediately apparent. Chemical control of phorids is effective but costly and the inherent danger to the mushroom consumer from toxic residues in addition to the threat of fly resistance to pesticides are reasons for seeking alternative methods. In many of the above papers,

Hussey drew attention to the need for a biological control agent and explained his interest in "Bradynema"; unfortunately not being a nematologist he has been unable to give a detailed account of the parasite's life history and biology so one of the aims of the present study is to elucidate these aspects.

As far as Hussey (1959, 1964b, 1967) has been able to ascertain, the life cycle of "Bradynema" is as follows. Second stage female nematodes and "mature males" are liberated from the female fly during oviposition but there is no means of exit from the male host. The free living nematodes are non feeding and they mate in the mushroom compost. The third stage female nematode subsequently enters a first or second instar maggot "through the host cuticle by a characteristic spiral entrance hole". Inside the maggot the young parasite at first contracts, develops a central swelling and loses its worm-like appearance, becoming lemon-shaped then elongating to a "sausage-like form with a characteristic 'foot' shape at one end". By the time the fly emerges from its puparium the female nematode releases eggs which later hatch inside the host haemocoel.

The idiosyncrasies of this life cycle provided a stimulus for the present study. Assuming that Welch's preliminary investigations led to the correct placing of this new nematode among the allantonematids, a group characterised by bizarre life histories, it nevertheless seems very odd that a 3rd stage larval female should be fertilised and even more peculiar that it should mature without two more ecdyses. Similarly the accelerated development of parasitic larvae to "mature males" before they leave the insect is unusual, as is the metamorphosis described for the young parasitic female inside

the maggot.

Other aspects of "Bradynema" seemed equally worthwhile investigating in view of the reported biological control potential. The effect of the parasite on the host particularly with regard to factors influencing fly fecundity and biological performance such as egg production, copulatory behaviour, dispersal and longevity; the optimum conditions required for development of the free living nematodes, their ability to survive adverse conditions and colonise host populations are also relevant. Other incidental observations on this unusual nematode are included and the broad aims of this section are outlined below.

SUMMARY OF OBJECTIVES FOR SECTION I

1. To make a detailed morphological study of the undescribed "Bradynema" to clarify its taxonomic position and to provide sufficient data for subsequent publication, should it prove to be a new species.
2. To elucidate the salient features of life cycle of "Bradynema" to amend the observations previous reported.
3. To investigate the effect of "Bradynema" on *Megaselia halterata* particularly with regard to the insect's fecundity and the parasite's potential as a biological control agent; to observe other aspects of the host-parasite relationship.

I 2 MATERIALS AND METHODS

I 2.1. Source of Material

Dr. N.W. Hussey of the Glasshouse Crops Research Institute, Rustington, very kindly supplied the original group of parasitised flies in January 1968 and consequently it was assured that valid "Bradynema" material was obtained.

Other Megaselia halterata were subsequently collected from a variety of mushroom farms including:-

Berry's Mushroom Co., Turville Heath, Bucks.
Bushell's Ready Mix Ltd., Darby Green, Hants.
Clockhouse Mushroom Co., Egham, Surrey.
Denham Mushroom Company, Denham, Bucks.
Field & Son, Arborfield Cross, Berks.
Hardy's Mushroom Company, Didcot, Berks.
Lawrences Mushroom Company, Goodworth Clatford, Hants.
Newsteads Nurseries, Climping, West Sussex.

Samples were most frequently collected from Lawrences and Field's but parasitised flies were usually present at each of the above localities except Berry's and Clockhouse, where phorids rarely occurred.

Initially it was difficult to distinguish Megaselia halterata with the naked eye from M. nigra Meigen and a variety of Sciarids, but the smaller size of M. nigra and the slow movement of the Sciarids became adequate criteria for a quick identification of these concurrent species. The flies were usually sucked into a pooter and later transferred to 3 x 1 inch tubes for transporting back to the laboratory.

A hand operated bellows was fitted to the pooter to avoid discomfort caused by a throat irritant, presumably associated with M. halterata (personal communication with Miss B. Gurney, G.C.R.I. and subsequently verified!). When thousands of flies were required for experimental purposes a large five litre pooter was used with a modified attachment to a domestic vacuum cleaner. It was necessary to incorporate a 'T' tube between the pooter and the vacuum motor to allow a regulated volume of air to by-pass the fly collection chamber so that excessive suction pressure did not damage the flies. In addition, a pad of cotton wool had to be placed inside the pooter to prevent concussion of insects otherwise colliding against the glass base. In general this method was not satisfactory when the flies were required for breeding purposes because of the inherent pootering trauma, but it was invaluable for sampling the population and acquiring large numbers of adult parasites and larvae, which were unharmed and later released when the flies were dissected.

I 2.2. BREEDING THE FLIES

Hussey and Gurney (1964) described rearing techniques for Megaselia halterata which were based on the newly emergent adult requirements of flight space, rapidly growing mushroom mycelium and a moist environment. A 34-mesh Tygan cage had removable Kilner jars containing spawned mushroom compost, screwed into the floor. The cage was kept at 20-24°C above a heated tray of water and partially covered by a polythene box which facilitated a high relative humidity with some fresh air circulation. Eggs, rather than adults, were used to establish the phorid populations, ensuring

the elimination of "Bradynema". Successive generations of flies were encouraged to oviposit by adding fresh jars of "running" spawn to the base of the cage; spent compost was removed simultaneously.

One of these cages was borrowed from Dr. Hussey in the expectation that it would prove to be equally successful for rearing parasitised phorids. Unfortunately infected M. halterata colonies failed to establish under these conditions and even 'clean' flies were unable to produce significantly increased numbers. Several modified cages were designed, generally following the principles outlined by Hussey and Gurney, but primarily concentrating on water-proofed materials which would not warp in the high humid conditions, consequently reducing fly escape. Mechanisms were incorporated for looking into the flight chamber which was unnecessary for Hussey and Gurney, who maintained overlapping generations at weekly intervals, but essential for controlling irregular fluctuating populations of parasitised flies which required either the addition of excess compost or 'clean' flies almost every generation, according to the host density and parasite incidence.

Eventually the most efficient breeding cage was found to be a sealed transparent perspex chamber identical to a sterile innoculation cabinet but the two glove entrances replaced by removable framed gauze discs. The jars of compost were added or removed after unscrewing the discs and flies were attracted to the opposite end of the chamber by a bright light so that they did not escape during this operation. The jars of compost were simply placed on the floor of the chamber on top of slightly wetted absorbent paper which maintained a suitable humidity level. The cage was kept in a 25°C C.T. room and the gauze discs allowed a limited amount of air

circulation but this was insufficient to cause drying inside the cage.

Much smaller breeding chambers were designed for observations on fly fecundity and host-parasite interactions. Usually the mushroom mycelium was grown on sterile 2% Malt Agar in petri dishes and a perspex container was inverted over the exposed mycelijm and the flies were introduced through a small aperture at the top. This hole was then plugged with a water moistened dental roll and limited ventilation was provided by an adjacent gauze-covered aperture. When individual flies were being observed only a small perspex container was required, usually about five inches high with a diameter sufficiently narrow to fit inside the rim of the Petri dish and sink into the agar, to prevent fly escape. After oviposition the fly and chamber were removed so that the developing maggots and nematode larvae could be observed in situ with a binocular microscope.

I 2.3. REARING THE PARASITE

Adult female Megaselia halterata were dissected in 0.75% Clark's entomological saline and larvae of Howardula sp. were gently teased free from the host tissue. Debris and nematodes were transferred to a 100μm sieve which was supported at the surface of 0.75% saline in a Baermann funnel (Flegg & Hooper, 1970). After about 10 minutes the active larvae had penetrated the mesh and collected at the bottom of the funnel from where they were removed on a drop of saline. They were placed in a Petri dish on coarse black filter paper which had previously been wetted with water to dilute the saline. A lid was placed over the dish which was further covered by a black polythene bag.

Using this method it was possible to observe the development of free living larvae and to examine the influencing factors such as the effect of different temperatures, salinity, substrate, light intensity and moisture content. In addition, a constant supply of adult male nematodes and infective females was available for further experimental investigations, but it must be admitted that the percentage yield was very low.

No attempts were made to culture the parasitic stages *in vitro* because preliminary observations indicated that the adult female *Howardula* was extremely sensitive.

I 2.4. SECTIONING THE PARASITISED FLIES

One of the reasons for sectioning parasitised flies was to investigate the exit route of nematode larvae which could not be established by direct observation because of the close proximity of the anus and vulva in these tiny insects. Also the distribution and effect of the parasite inside *Megaselia halterata* could only accurately be determined with a compound microscope, for which sections are required.

The techniques used for fixing, dehydrating, embedding, sectioning and staining the material were usually those outlined by Pantin (1946) unless otherwise stated. Attempts were made to ensure that some of the flies to be processed were parasitised but live insects gave little indication of infection other than a bloated abdomen which was frequently found to be equally caused by the presence of eggs in females and excessive fluid in males. Occasionally active nematode larvae were visible inside the abdomen of anaesthetised flies when brightly illuminated from below and viewed through a binocular microscope, but usually the insects were selected at random from populations known to

have a high parasite-incidence to ensure that infected flies were present.

Fixation was affected by using alcoholic Bouin's, Carnoy's or Mukerji's solutions after the legs and wings had been removed from etherised flies and the tough cuticle pierced by a finely pointed tungsten wire. The material was left 2-4 hours in Bouin's and Carnoy's fixatives and for 1-3 days in Mukerji's which contains nitric acid to soften the sclerotised exoskeleton. The flies were then washed in 96% alcohol with four changes over 12 hours and the Mukerji-fixed material was further treated with five changes of 70% alcohol over 24 hours. Several changes of supercedrol were used to dehydrate and clear the flies for 1-3 days or for longer periods (Shute and Maryon, 1966). Half an hour before embedding the specimens were placed in an oven at 60°C to heat the supercedrol temperature of the paraffin wax which was composed of two parts 50°C melting point to one part 63°C melting point. The material was transferred to a 1:1 mixture of supercedrol and wax for 15 minutes before being exposed to pure wax for impregnation under vacuum. Three changes of wax at 30 minutes intervals was allowed for embedding before groups at 1-4 flies were aligned in separate containers and the wax allowed to cool.

Serial sections 5-7μ thick were cut on a hot plate where they were subsequently dried.

The sections were coated with 1% celloidin in an alcohol-ether mixture before the wax was removed by xylene prior to staining. Mayer's haemalum/Eosin, Mann's Methyl Blue/Eosin or Mallory's Triple stain were used to diffentiate the sections before they were finally dehydrated in absolute alcohol, cleared in Xylene and mounted with Canada Balsam.

I 3. OBSERVATIONS

I 3.1. The Life Cycle of Howardula Sp.

The mature male Howardula sp. is free living but the female is a parasite in the haemocoel of Megaselia halterata. Nematode eggs are laid during development of host pupae and larvae hatch about the time of eclosion. The first exsheathment occurs a few hours after hatching which infers that a first stage nematode was released but although a moulted cuticle was never observed inside the egg, the difficulty of seeing this phenomenon prevents a catagorical denial of its occurrence. The second stage larvae rapidly accumulate fat globules which suggested that they feed inside the host; they can be further distinguished from first stage nematodes by the less blunt anterior end and increased size although overlapping lengths were occasionally recorded. (FIGURE 1 and 2). There is some development of the genital primordia in second stage larvae but it was impossible to differentiate the sexes; conflicting evidence was provided by sections of larvae inside the host where densely stained nuclei resembling spermatocytes were frequently observed but these were not visible in living specimens or permanent preparations of whole mounts.

No further development of larvae occurs inside the host and it is interesting that in male flies the nematodes are unable to grow beyond the first or immediately post-exsheathed second stage; when these nematodes are removed by dissection they are incapable of further development in the external environment.

Female flies release second stage larvae via the genital tracts during abortive attempts at oviposition, after the few eggs present have been laid. Some larvae escape through the gut and anus but are unable to get out by other routes; nematodes still inside the fly perish

about 24 hours after the host dies, as happens to all the larvae in male flies from which there is no mode of exit.

After being liberated into the mushroom compost the second stage larvae develop into fourth stage females or mature males in 1-2 days at optimum temperatures. The female nematodes moult twice during this period and the males moult three times but both sexes only escape from the moulted cuticles at a single exsheathment. Copulation was never observed and fertilised females were not recovered from the rearing experiments although sperm was seen in females which had become parasites.

The method by which the fourth stage infective female enters a new host was not established despite numerous attempts to investigate this process using a variety of experimental techniques. First instar maggots of Megaselia halterata were never found to be parasitised but fourth stage female nematodes were frequently present in second and third instar maggots and even in young pupae.

The fourth stage female nematode undergoes a final moult within 1-2 days of entering the host haemocoel. Very rapid growth occurs before the parasite becomes sexually mature, usually mid way through the host's pupation period, and eggs are laid until shortly after the fly emerges.

FIGURE 1. MEASUREMENTS OF *HOWARDULA* sp.

		MEAN	STANDARD ERROR	RANGE	COEFFICIENT VARIATION (%)
INFECTIVE FEMALE (n = 10)	Body length	398	12.88	363-470	10.2
	Body width	15.65	0.60	12-18	12.0
	a	25.6	0.72	21-30	8.9
	Tail length	40.05	1.64	29-48	12.9
	c	10.00	0.19	9.4-11.2	5.9
	V (%)	82	0.20	81-83	0.7
	Stylet length	14.55	0.65	12-18	14.2
	Distance of dorsal gland from anterior	29.9	0.83	26-33	3.6
	Distance of hemizonid from anterior	76.44	2.63	64-86	9.7
MALE (n = 4)	Body length	355	11.80	337-387	6.6
	Body width	15.75	0.24	15-16	3.0
	a	22.58	0.62	21-24	5.5
	Tail length	30.75	1.81	29-33	11.8
	c	11.60	0.54	10.2-12.9	9.3
	Extent of testis	197	6.78	181-204	6.8
	T (%)	55.54	1.66	52-59	6.0
	Spicule length	11.32	0.33	10.6-12.2	5.8
PARASITIC FEMALE (n = 100)	Body length	625	-	364-13330	-
	Body width	91.4	-	60-192	-
	a	7.1	-	3.5-11.7	-
	V (%)	85	-	76-96	-
FIRST STAGE	Body length (n = 50)	271	3.26	223-317	8.5
SECOND STAGE	Body length (n = 100)	379	6.23	314-460	10.6

All measurements given in μm.

FIGURE 2.

HOWARDULA: LARVAE AND ADULT MALE

FIG. 2A First stage larvae from host haemocoel.

FIG. 2B Second stage larvae from host haemocoel.

FIG. 2C Adult male: entire nematode.

FIG. 2D Adult male: posterior regions showing the caudal ala, spicule without a gubernaculum, testis(on left) and gut (on right).

FIG. 2E Infective female (L_4): entire nematode.

FIG. 2F Infective female (L_4): anterior region showing the thin walled stylet with a wide lumen; oesaphagus; two ampullae into which the ducts of the three oesaphageal glands open; nerve ring; hemizonid (on left) and gut (centre, posteriorly).

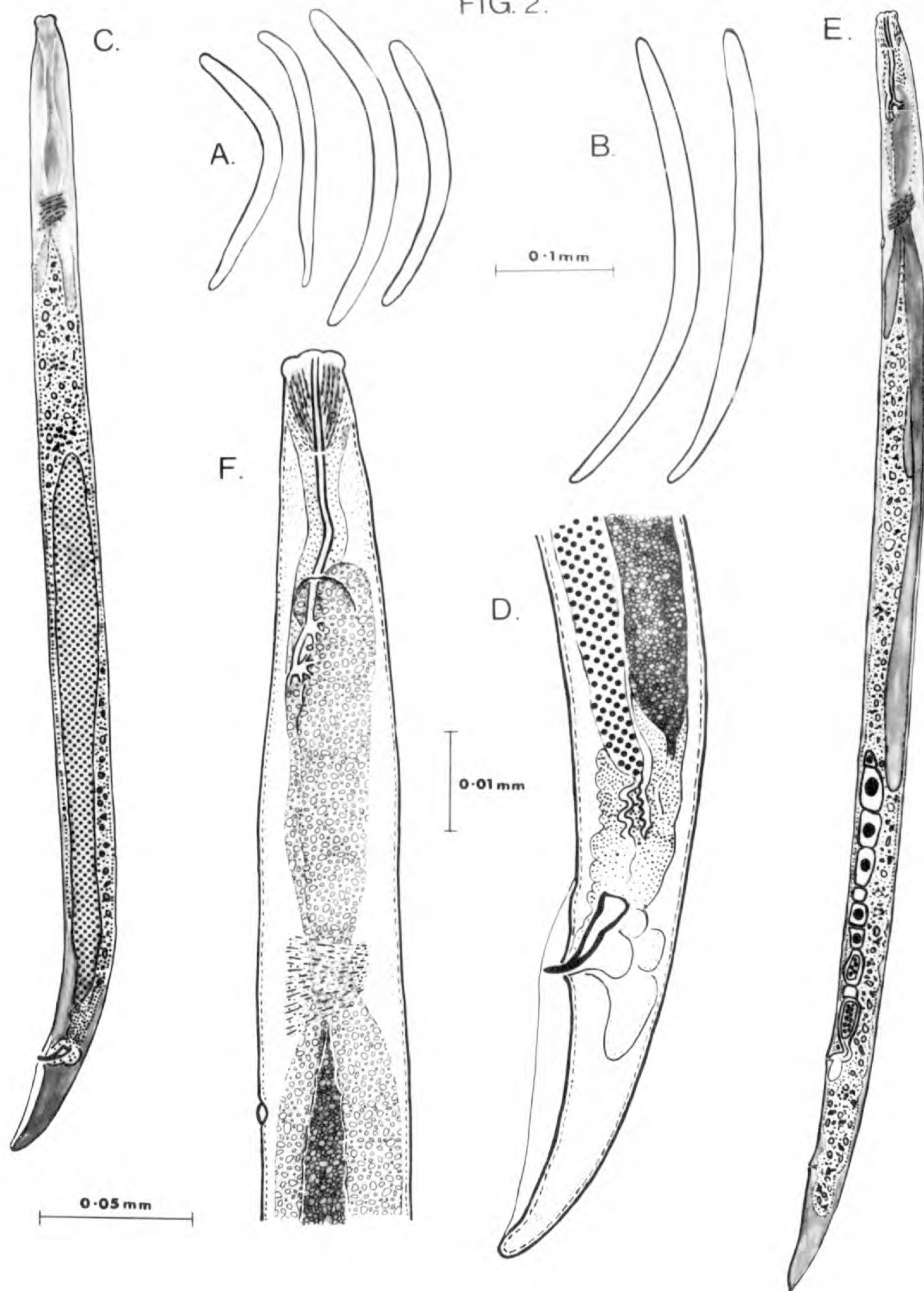


FIG. 2.
A.
B.
C.
D.
E.
F.
0·1mm
0·01mm
0·05mm

I 3.2. THE MALE NEMATODE

3.2.1. Description and Development

The following measurements were taken from drawings at 2-3000 X magnification of specimens which had been fixed in TAF, dehydrated in Seinhorst (1959) solutions 1 and 11 and mounted in glycerine. A Wild Research microscope which incorporated a drawing tube and an additional 2·5 x magnifier was used to trace the nematodes.

The free living male Howardula sp. is 337-387 µm long (mean = 356 $\pm$ 11.8) and 15-16 µm wide (mean = 15.7 $\pm$ 0.2). The paired spicules are 10.6-12.2 µm in length (mean = 11.3 $\pm$ 0.3) and there is no gubernaculum. The only conspicuous internal structure is the single testis which is packed with highly refractile nuclei of amoeboid sperm and occupies 52-59% of the total body length (mean = 55.5 $\pm$ 1.7), as illustrated in FIGURE 2C. The gut is represented by a collection of lipid droplets and the anterior region shows little differentiation except for a faint indication of two oesophageal glands which obscure the oesophagus-intestine junction. It is doubtful if the latter connection exists because the free living male does not feed and the weakly developed oesophagus has no stoma or mouth opening. A stylet is not present and the excretory pore was not seen. The tail is approximately one tenth of the total body length and has paired narrow peloderan-type caudal alae which are usually not visible in living specimens even when viewed under phase contrast. The alae extend posteriorly to the end of the tail from a short distance anterior to the cloaca. The data obtained from measuring male Howardula sp. is summarised in FIGURE 1 and

includes the de Mann ratios a (body length / body width) and c (body length / tail length).

Male nematodes were only occasionally recovered when compost from cultivated mushroom beds known to contain Howardula sp. was extracted by Baerman techniques. Further evidence that males are rare or short lived was obtained after rearing second stage larvae by the method described in the previous section. Counts of nematodes a few days after removal from Megaselia halterata showed that the mean number of males present was 5%, with a maximum individual record of 14%, and they died in 1-3 days according to the temperature.

Remarkably rapid development of gonad and spicules occurs as soon as the parasitic larvae enter the external environment. The rudimentary testis is visible by the time the second stage cuticle is moulted; a few spermatocytes and the spicule primordia are visible at the third moult which respectively differentiate into spermatozoa and spicules when the fourth cuticle is separated. The three retained moulted cuticles are best seen either at the fore or hind end of the nematode, depending on whether the male is lying anteriorly or posteriorly inside the sheaths, and there is always a much bigger space between the second and third larval cuticles than between the third and fourth, which are often very difficult to distinguish. The male escapes from the three cuticles at a single exsheathment and resumes the constant activity which was abruptly arrested when the parasitic larvae were removed from the host and put into water.

I 3.2.2. EFFECT OF TEMPERATURE ON DEVELOPMENT OF THE MALE NEMATODE.

Assuming that males are required to fertilise fourth stage females before the parasitic cycle can commence, and the relevance of parasitism to host control, an investigation of factors influencing development of free living stages of *Howardula* sp. could indicate optimum conditions for artificial propagation and dispersal of nematodes. The temperature of mushroom compost varies but usually it is 22-25°C when the spawn is growing and then cools to 15-18°C during the cropping period. Information about the effect of temperature on the nematode larvae may indicate the period in the mushroom cycle when *Howardula* sp. could most effectively be introduced to phorid populations.

METHOD

Approximately 1000 adult *Megaselia halterata* with an estimated parasite incidence of 27%, were killedwith ether and roughly dissected in 0.75% saline to release *Howardula* larvae. The nematodes were extracted from host debris in a seive above a Baermann funnel and it was necessary to use 0.75% saline for this process because water inactivates the second stage larvae. The worms were concentrated by centrifugation and the fat-body polluted supernatant liquor was replaced by tap water. After a second centrifugation the volume of water was made up to 60 ml in a 100 ml measuring cylinder and the nematodes were evenly dispersed through the medium using Peter's (1952) method. Five 0.15 ml samples were placed in McMaster counting chambers and the number of nematodes recorded by using a binocular microscope; from this it was estimated that 81-86,000 larvae were present and that 0.4 ml would isolate about

550 nematodes, which was considered to be a suitable number for each replicate.

Subsequently a 1 ml hypodermic syringe calibrated in 0.1 ml units was sealed to a length of glass tubing and the plunger modified so that 0.4 ml portions could be rapidly withdrawn at each upward stroke. Eighty-four two inch diameter perspex dishes were prepared for the experiment by placing moistened filter paper in the lids to prevent drying, and 1 x $\frac{1}{2}$ inch triangles of coarse black filter paper were placed in the bottom; this size was found to hold 0.4 ml of water without flooding and it was a convenient shape for rinsing off the nematodes to be examined at the end of each experiment.

The larvae were transferred to each dish from 60 ml suspension which was agitated for even dispersal before each 0.4 ml sample was removed. Fourteen of these replicates were each placed in a constant temperature at 5, 10, 15, 20, 25 or 30°C and one sample from each temperature was sacrificed at 24 hour intervals.

Two counts were made from one dish at each temperature every day for two weeks. The filter paper containing the nematodes was rinsed into a tube which was centrifuged to concentrate the worms. The supernatent water was removed and the volume made up to 1.5 ml. Two separate 0.15 ml samples were examined in a McMaster counting chamber under a binocular microscope and the number of 1st, 2nd, moulting and infective female larvae and adult males was recorded. The total number of nematodes counted, multiplied by ten, gave an estimation of the worms present so that the number of each stage could be recorded as a percentage.

RESULT

The percentage male *Howardula* sp. developed from second stage larvae at the various temperatures is shown in Appendix TABLE 2. No development occurred at 5°C, 10°C and 30°C and the larvae died within 1-2 days at 30°C. At 10°C the larvae survived for 1-4 weeks but they remained unchanged.

FIGURE 4 shows the mean percentage male nematodes present in each daily sample at 15-25°C. The majority had developed by the second day at 20°C and 25°C; the decline in numbers after this time is indicative of the short life of male nematodes at high temperatures. At 15°C most of the larvae took about 5 days for maturation but it is interesting that as many males were produced as at 20°C; FIGURE 5, where cumulative data from the mean daily samples at each temperature is presented, illustrates this phenomenon. The maximum yield of male nematodes was 14% and this was strikingly reduced by half when the larvae were reared at 25°C.

FIG. 4.

DEVELOPMENT OF MALE NEMATODES FROM LARVAE AT DIFFERENT TEMPERATURES

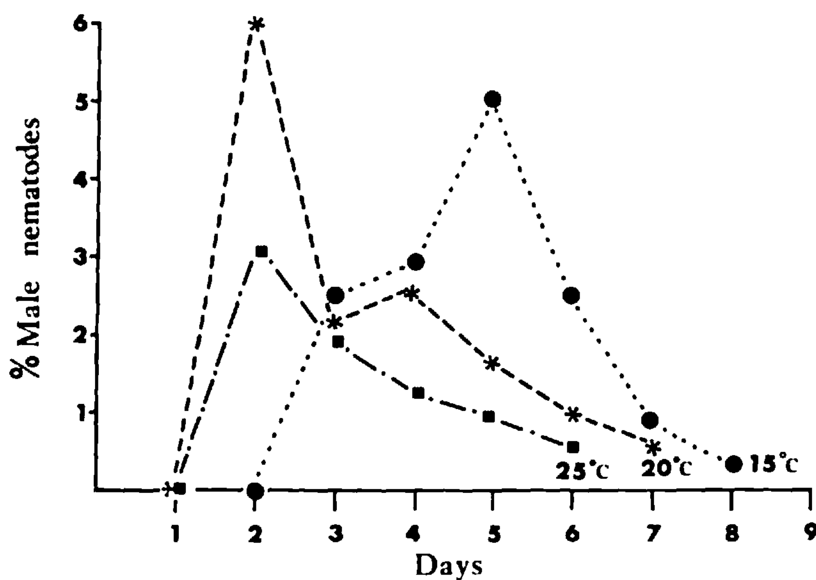
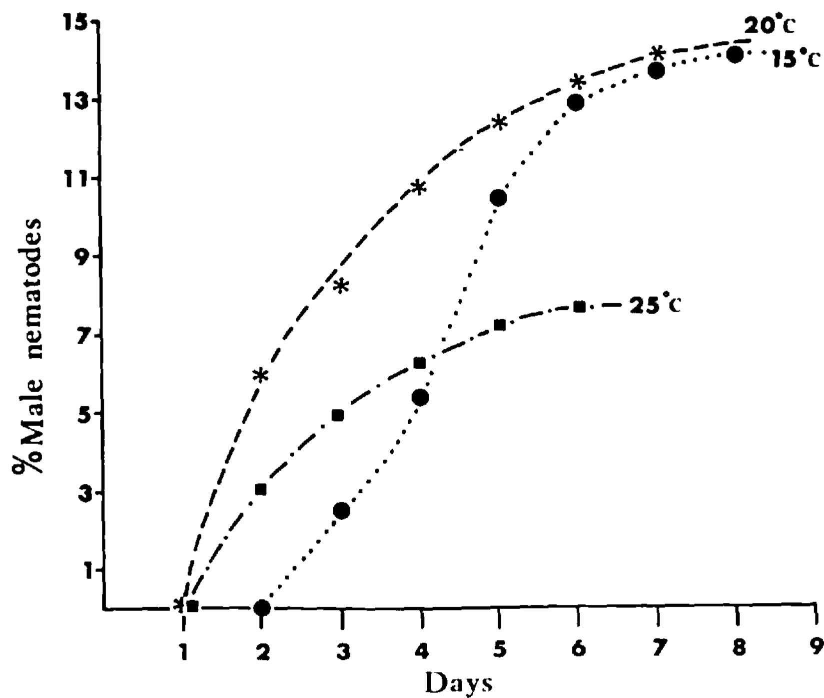


FIG. 5.

CUMULATIVE DEVELOPMENT OF MALE NEMATODES FROM LARVAE AT DIFFERENT TEMPERATURES



I 3.3. THE INFECTIVE FEMALE NEMATODE

3.3.1. Description and development

The fourth stage female Howardula sp. is 363-470 μm long (mean = 398 $\pm$ 12.9) and 12-18 μm wide (mean 15.6 $\pm$ 0.6) with a de Mann ratio a = 21-30 (mean 25.6 $\pm$ 0.7). The anus and vulva in these sexually immature, non-feeding larvae, are not visible in many specimens but occasionally their positions are marked by a slight swelling near the posterior end of the ventral surface as shown in FIGURE 2E. The tail length is 29-48 μm (mean = 40 $\pm$ 1.6), approximately equal to the distance the vulva lies in front of the anus; the distance of the vulva from the anterior end of the nematode, expressed as a percentage of the body length, is 81-83% (mean = 82 $\pm$ 0.2). The hemizonid is frequently visible 64-86 (mean 76 $\pm$ 2.6) from the anterior end but the excretory pore has never been observed although the moulted excretory duct lining is invisible in exsheathed cuticles; from its position the excretory pore is deduced to be coincident with the hemizonid. The single prodelphic gonad has developed to the 5-10 celled stage with a posterior narrow tube usually encompassing two lumina, which probably represent morphogenesis of oviduct and uterus. Each lumen frequently contains sperm when the larvae are examined after recently entering a host, but the free living females were never found after copulation.

The oesophageal glands are the most conspicuous features of the internal anatomy and at least one of the glands extends over half way down the body, usually overlapping the rudimentary gonad. It is impossible to apply the normal nematode terminology of "dorsal" and "ventral" to these glands because although three are present,

two of the glands lie dorsally and the other is ventral but fusion between the three may occur anteriorly. Another unusual feature is the collection of ducts where the glands empty their contents into the oesophagus; these are arranged in prominent clusters which radiate from two large ampullae on the dorsal side of the oesophagus, in the antero-ventral region of the nematode (FIGURE 2F). Two of the oesophageal glands are connected to the first ampulla which lies 26-33 (mean = 30 $\pm$ 0.9) from the anterior end, and each gland has a lobe which extends beyond its oesophageal connection. The third gland mainly lies ventrally and appears to contribute ducts to the second ampulla. It is doubtful if the oesophagus extends posteriorly to the ampullae and there is no indication that it is connected to the intestine whose outline is indicated by highly refractile lipid droplets.

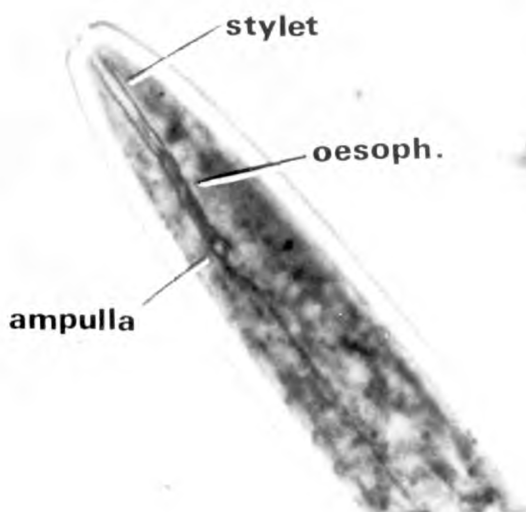
A weakly developed stylet is present which is rarely visible in fixed material and often difficult to detect in living specimens until they are relaxed with propylene phenoxetal, when it can be seen protruding from the anterior end. (PLATE 1). Knobs or other thickenings do not occur at the base of the stylet which has a wide lumen throughout its length and is not tapered anteriorly. Frequently it is slightly curved and appears flexible in living specimens; the stylet walls are not appreciably thicker than those of the contiguous oesophageal lumen which is very prominent and consequently the limit of the stylet is difficult to detect, which probably accounts for the apparent variation in the recorded stylet length of 12-18μm (mean = 14.5 $\pm$ 0.6). The posterior end of the stomal musculature was used to give an approximation of the stylet base but unfortunately these muscles are also weakly developed. There is no head skeleton and the lips are fused. A summary of

the measurements describing the fourth stage larval female *Howardula* sp. is given in FIGURE 1 and the various features are illustrated in FIGURE 2E and PLATE 1A-C.

The second stage larvae destined to become females develop a rudimentary ovary of 2-3 cells during the moult which occurs a few hours after they have been released from the host. The sheath is retained and as a third larval cuticle is being separated, the oesophageal glands differentiate and the prospective oviduct can be distinguished from the ovary which now comprises 4-6 cells. The stylet develops at this stage, but the shrivelled column of cuticle at the anterior end of the moulted sheath suggests that the younger larvae shed a thick stomatal lining which may represent a primordial stylet.

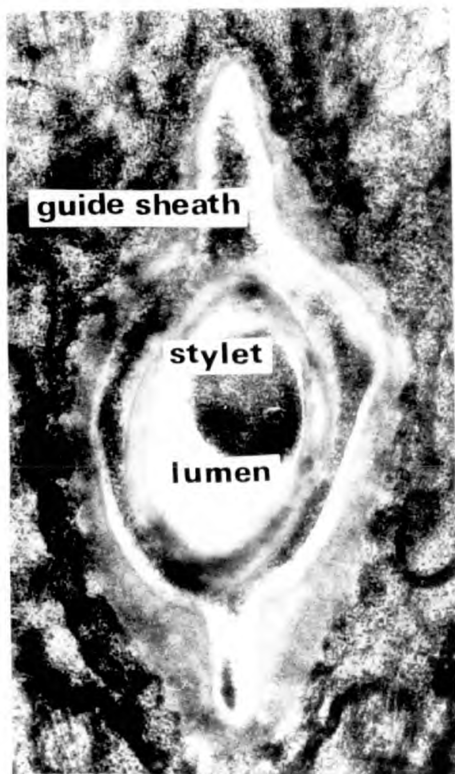
The infective female nematode escapes from its two cuticles at a single exsheathment after 2-4 days in the external environment. A split occurs in the sheaths near the anterior end of the worm and it incompletely circumscribes the body so that a cap is eventually pushed off to one side of the head. As the nematode slowly squeezes out of the cuticles the cap remains attached and is a convenient marker for observing the forward progression of the worm during exsheathment, which otherwise could not be seen because the sheaths are closely applied to the body of the worm. The female now becomes continually active although only slow movements are performed and this behaviour is not modified under experimental conditions even when male nematodes and host maggots are encountered. Presumably, in nature, mating and penetration of *M. halterata* occurs at this stage because no further development ensues if the fourth stage females remain free living, and they die in 1-20 days.

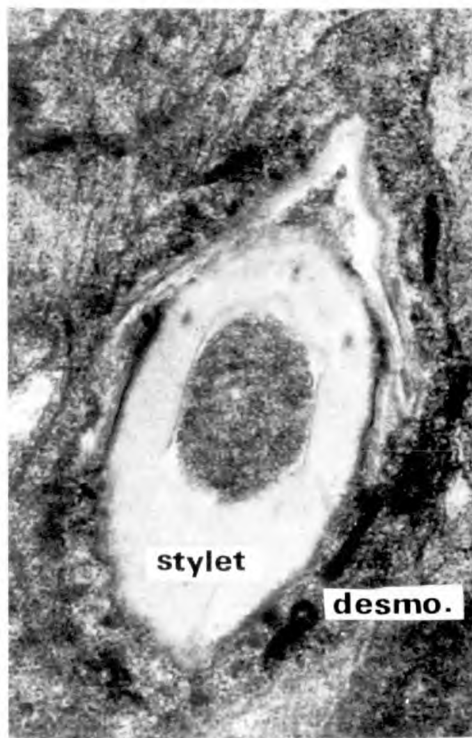
PLATE 1



STYLET of

HOWARDULA





I 3.3.2. Effect of Temperature on Development of the female nematode.

The experimental methods were the same as those described in section I 3.2.2.

The results of this experiment are summarised in Appendix TABLE 1 and illustrated in FIGURE 3. It can be seen that no second stage larvae developed into infective females at 5°C, 10°C and 30°C and the nematodes died within 2 days at 30°C but survived as long as 1-4 weeks at 5°C and 10°C although they were inactive and unchanged. Most larvae matured in 36 hours to 4 days at 20°C and 25°C and there was no significant difference between the results obtained at these temperatures, although there was a tendency for a more speedy development at 25°C. A longer maturation period of 3-6 days was required at 15°C and it can be seen that there was a lower mean percentage yield. Incidental data collected during this experiment showed that some of the nematodes which developed slowly at 15°C were able to survive up to about 20 days, while the rapidly matured worms at 20°C and 25°C rarely lived longer than a week and appeared to weaken after 1-2 days.

I 3.3.3. Moulting inside the Host.

The infective female *Howardula* sp. enters a second or third instar maggot of *M. halterata*, or occasionally a newly formed pupa and undergoes the final exsheathment. Penetration was never observed but the presence of a stylet and the marked decrease in volume of oesophageal glands, which was noticeable in fourth stage larvae recovered from hosts, suggests that this is the mode of entry. The time sequence for moulting and development of the young parasitic females was deduced from daily examination of

infected maggots and pupae in which the duration of parasitism had been approximately estimated. This material was obtained by allowing unparasitised flies to oviposit in jars of mushroom compost for 8 hours and after 2 days, when the eggs had hatched, several thousand second stage Howardula sp. were added to the mycelium. The compost was kept at 26°C and it was assumed that the nematodes would be ready to enter new hosts when the maggots had developed to the second instar and by sampling the population to find the incidence of parasitism, the six day old maggots were designated "1 day old hosts". It was found that although some of the nematodes were at different stages, a rough approximation of the maturation sequence was made from the mean development of 40-50 parasites each day.

Many of the fourth stage Howardula sp. had moulted after 24 hours inside the host. The nematodes are motionless inside the haemocoel and gradually decrease a little in length and increase in width while the cuticle is separating. The gonad is more conspicuous and contains many cells but the oesophagus and its glands become diffuse and are no longer visible by the time exsheathment occurs. It was observed that nematodes which had reached this stage of development burst immediately they were placed in water, while the newly parasitic larvae were unaffected. Subsequent examination showed that there was a marked decrease in tolerance to salinity changes as the duration of parasitism increased; all of the larvae were unaffected by 0.75% saline but only the youngest could survive exposure to water; a few hours later they burst in 0.3% saline but not in 0.5-0.75% and eventually they burst in 0.5% and 0.7% saline.

It is interesting that exsheathment could be induced after the

larvae had been in the host about 18 hours by transferring the parasites to 0.75% saline; prior to this the larvae simply burst and disintegrated anywhere along the length of the body. The induced exsheathment procedure was identical in every worm; a transverse dorso-lateral split appeared a little below the mid line, in the region of the ovary, and almost encompassing the body; a swelling of the nematode immediately projected out of the split, causing a slight latero-ventral flexure (FIGURE 6A) which continued to distend as more of its length emerged from the sheath. Further bending was apparent (FIGURE 6B) and the head and tail were forced towards each other and the body became 'U' shaped as the nematode jack-knifed out of the cuticle. The liberated region was much longer than the rest of the body and this increase spread towards the extremities (FIGURE 6C) which were ultimately entirely freed from the sheath about a minute after the first appearance of the initial split. Because of the uniformity of this unique exsheathment procedure it was assumed to be identical to the natural process which normally occurs after the larvae have been inside the host about 24 hours, and not an artifact caused by artificial conditions. The 'natural' exsheathment was not observed, which is not surprising considering the speed at which it occurred under experimental conditions.

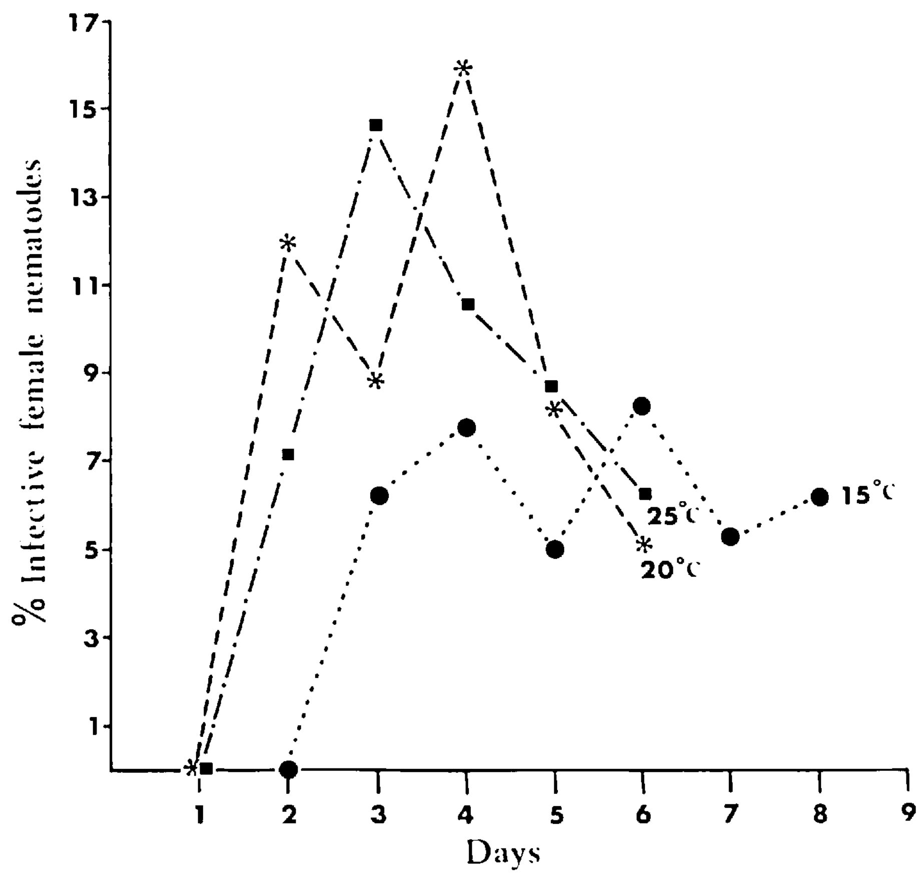
The process of exsheathment could be arrested by increasing the salinity of the medium but this frequently occurred spontaneously, probably due to incomplete development of artificially stimulated nematodes.

The subsequent development of these nematodes is recorded in the relevant section (I 3.4.1.) later.

The ultrastructure of the cuticle as revealed by the electronmicroscope and the nature of the changes which occur during the peculiar moulting process, will be described in a later section.

FIG. 3.

DEVELOPMENT OF INFECTIVE ♀ NEMATODES FROM LARVAE AT DIFFERENT TEMPERATURES



Erratum. A pagination error occurs here; numbers 47-52 have inadvertantly been omitted.

FIGURE 6.

FINAL EXSHEATHMENT OF THE FEMALE *HOWARDULA* sp., WHICH OCCURS INSIDE THE HOST.

FIG. 6A Initiation of exsheathment; a split occurs midway down the body and the nematode immediately swells in this region.

FIG. 6B (Next page) A later stage in exsheathment; the anterior and posterior ends of the nematode are still inside the fourth stage cuticle.

FIG. 6C (Next page) Final stage of exsheathment prior to the nematode escaping and straightening.

c cuticle of fourth stage female
m microvillous surface of adult
cg. cells representing gut
o rudimentary ovary
s sperm
p posterior end of nematode

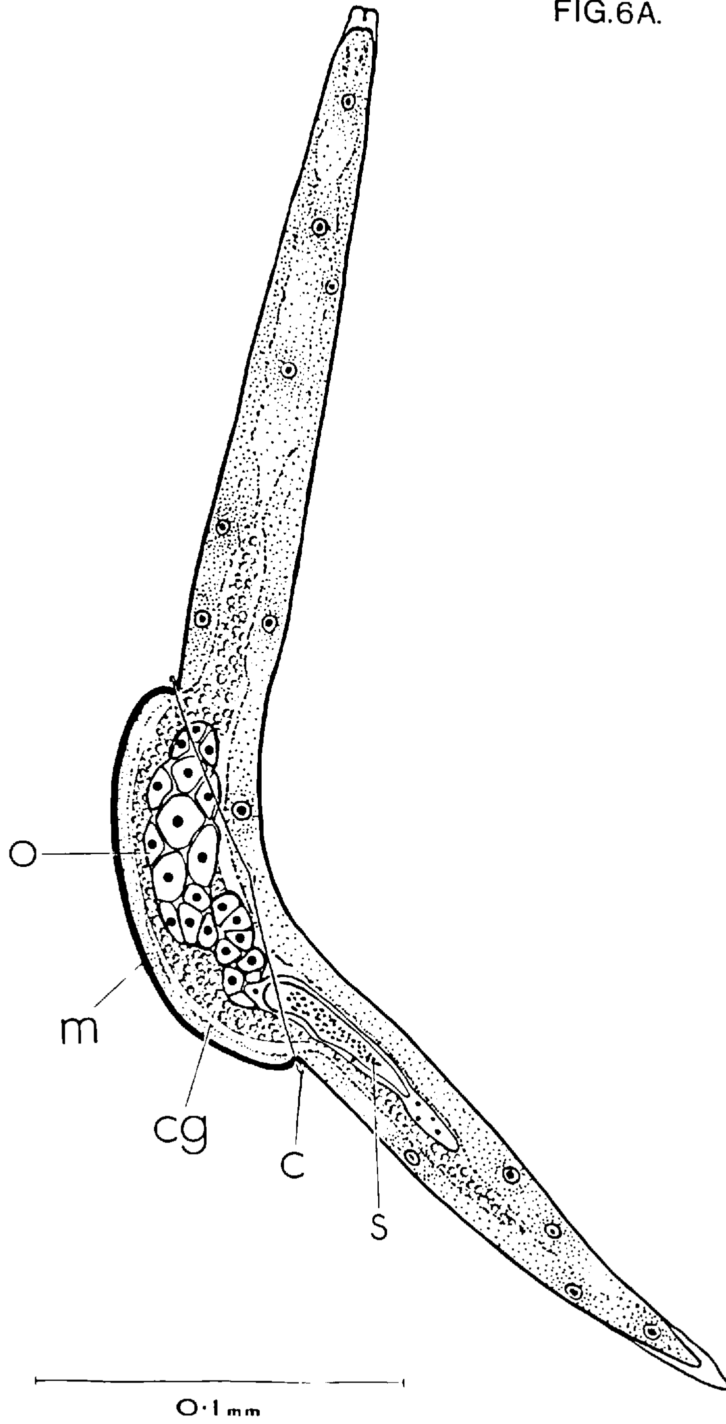


FIG.6A.
o
m
cg
c
s
0·1 mm

FIG.6B.

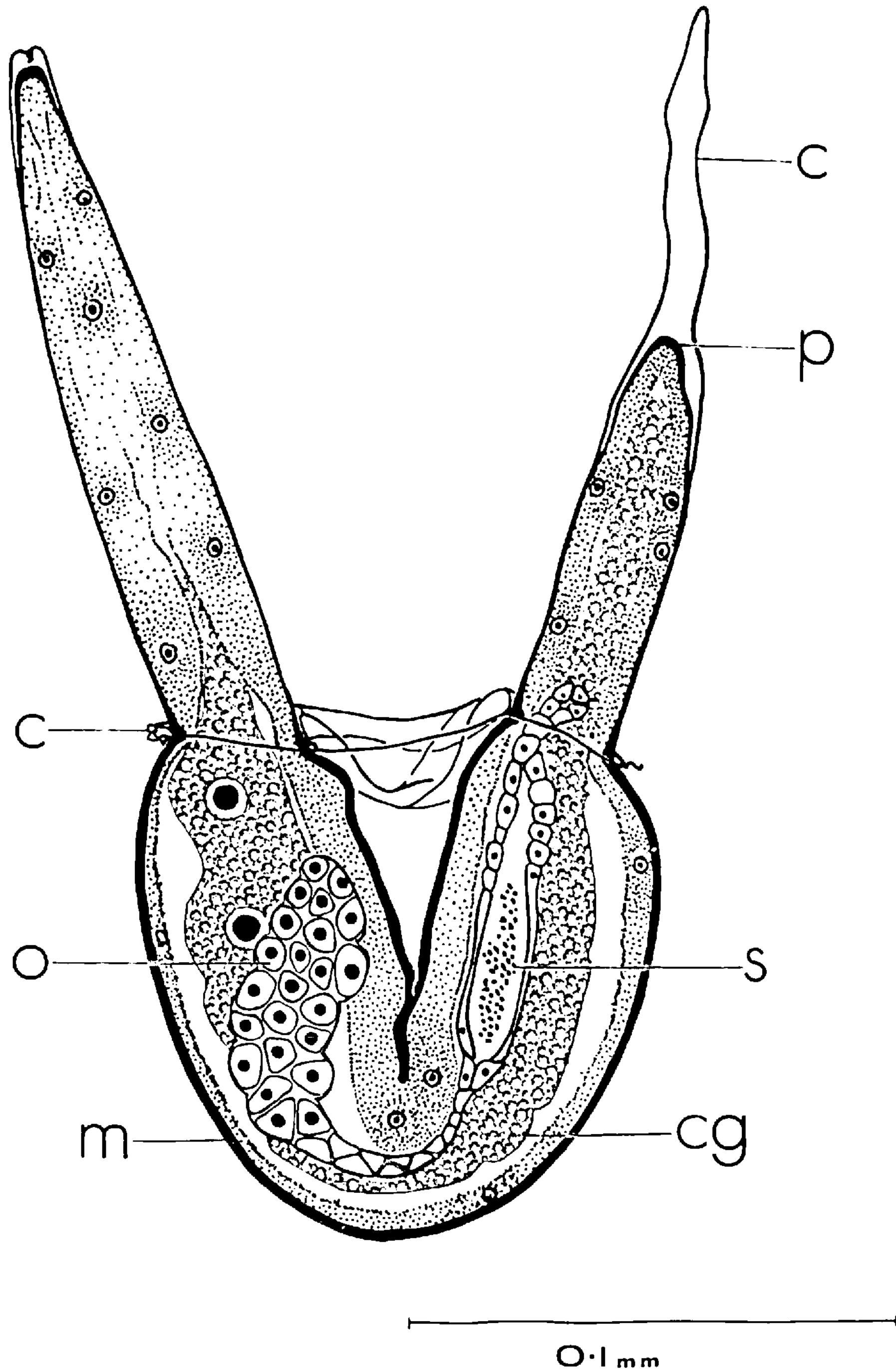
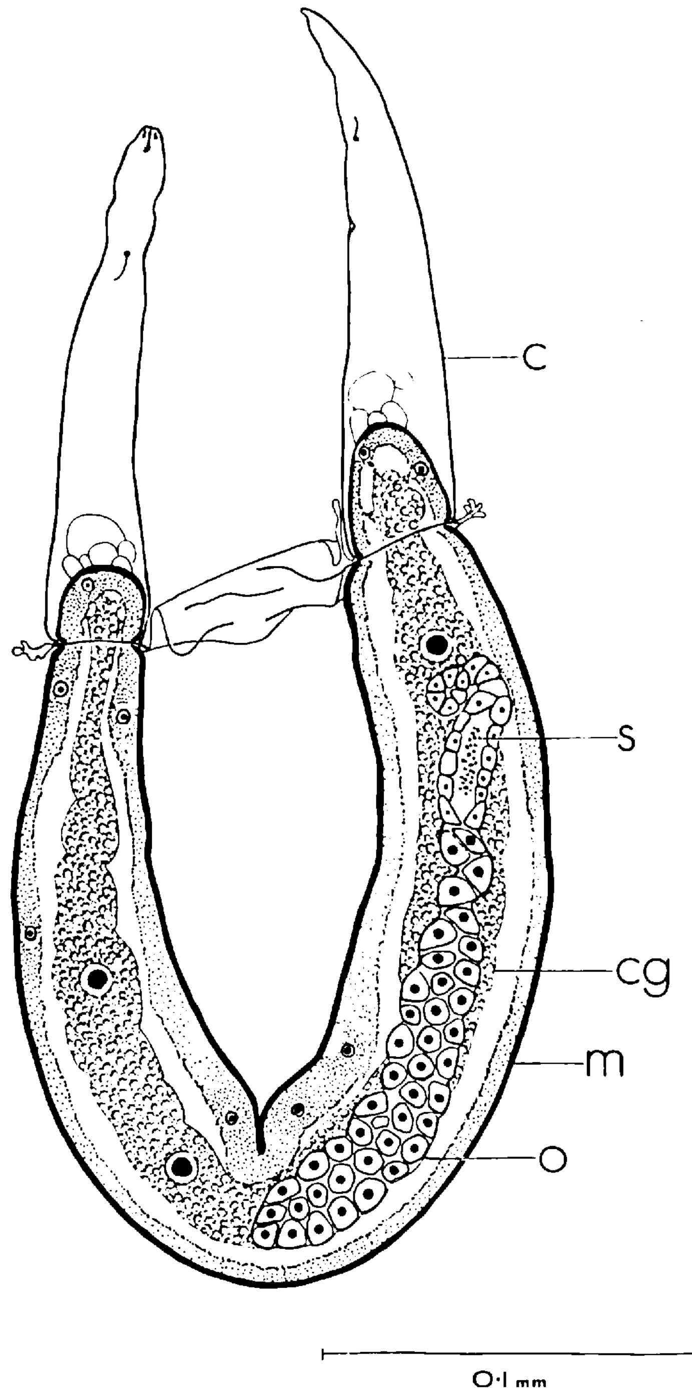


FIG. 6C.



I 3.4. THE MATURE FEMALE NEMATODE

3.4.1. Description and development

The sexually mature parasitic *Howardula* sp. is characterised by marked polymorphism; outlines of a variety of shapes are drawn to the same scale in FIGURE 7 and two of the most commonly occurring forms are illustrated in more detail in FIGURE 8A and FIGURE 8B. There is no mouth, stylet, anus and the gut is merely represented by a meandering chain of large cells with prominent nuclei which are often attached to the body wall in the anterior and posterior regions. The cytoplasm of these cells may be a faint khaki colour and they never encompass a lumen.

The ovary oviduct and uterus fill most of the pseudocoelomic cavity and in the simplest form reflexes occur at the junction of each region so that the ovary and uterus extend from the anterior end to the posterior, with the interconnecting oviduct lying in the opposite direction. Increased proliferation of the gonad superimposes several additional flexures (FIGURE 8A) but this is a functional adaptation of no taxonomic significance. The well-developed musculature associated with the vagina often produces external swellings which form the tips of the vulva. Usually the posterior vulval lip is the most prominent (FIGURE 8B) and this may distort the hind end of the nematode into a "heel" shape, in contrast to the other specimens in which the position of the vulva may be difficult and frequently impossible to locate. On first inspection the vulva appears to be dorsal because the body is frequently arched, and the convex surface tends to be dorsal in other nematodes. In *Howardula* sp. the convex side must be ventral because the observations on

development which were described earlier gave no indication of a change in vulval position. The nematode is oviparous but occasional ovoviviparous specimens have been found.

The mature parasites are flaccid and immobile but occasional movements may be observed in the posterior region, when stimulated artificially. They are almost impossible to transfer with bristles or hairs because they stick to the surface and all containers must be thoroughly siliconised if the nematodes are to be removed. The significance of the adhesive nature of the body wall was only appreciated after the ultrastructural observations, which are reported later, were made. Death ensues a few minutes after mature females are removed from flies regardless of the salinity into which they are placed, but by drawing worms mounted in 0.75% saline immediately after the host was dissected it was hoped that an approximation to living dimensions would be obtained. After measuring a hundred parasitic females, the range in body length was found to be 364-1330 (mean =625) and the width range was 60-192 (mean =91). The de Mann ratio a = 3.7-11.7 (mean = 7.1) and V% = 76-96 (mean = 85%).

FIGURE 9A illustrates the young parasitic Howardula sp. a few hours after the final exsheathment which was depicted in FIGURE 6C. As soon as it is freed from the old cuticle, the nematode shrinks, becoming more rounded, and it takes about two days to achieve the immediate post-exsheathed length, but there is a considerable increase in volume during this period. The body wall grows thicker in the anterior and posterior regions and consequently less distension occurs at the extremities so that nematodes frequently appear lemon-shaped. Differential growth of the gonad causes the elongating oviduct and uterus to displace the ovary

posteriad but the original orientation is regained 1-2 days later, as illustrated in FIGURE 9B. The cells representing the larval gut are the most conspicuous feature of developing parasites because the gonad is comparatively small; the cells appear to increase in size rather than in numbers and are ultimately concealed by the proliferating reproductive system. Maximum length is achieved during the next 2-3 days and in a further 3-4 days the gonad has matured and the first eggs are laid.

FIGURE 7.

HOWARDULA sp. MATURE PARASITES: A VARIETY OF FORMS

FIGURES 8A & B (next 2 pages)

Two mature parasitic *Howardula* sp. showing anatomical details of two commonly occurring forms.

cg	cells representing gut
o	ovary
od	oviduct
m	microvilli
e	eggs
u	uterus
v	vulva

FIGURES 9A & B (next page)

Two stages in the maturation of parasitic *Howardula* sp. which occurs after the exsheathment depicted in Figures 6A-C.

Labelling as in Figure 8 above, plu s:

s sperm

FIG. 7.

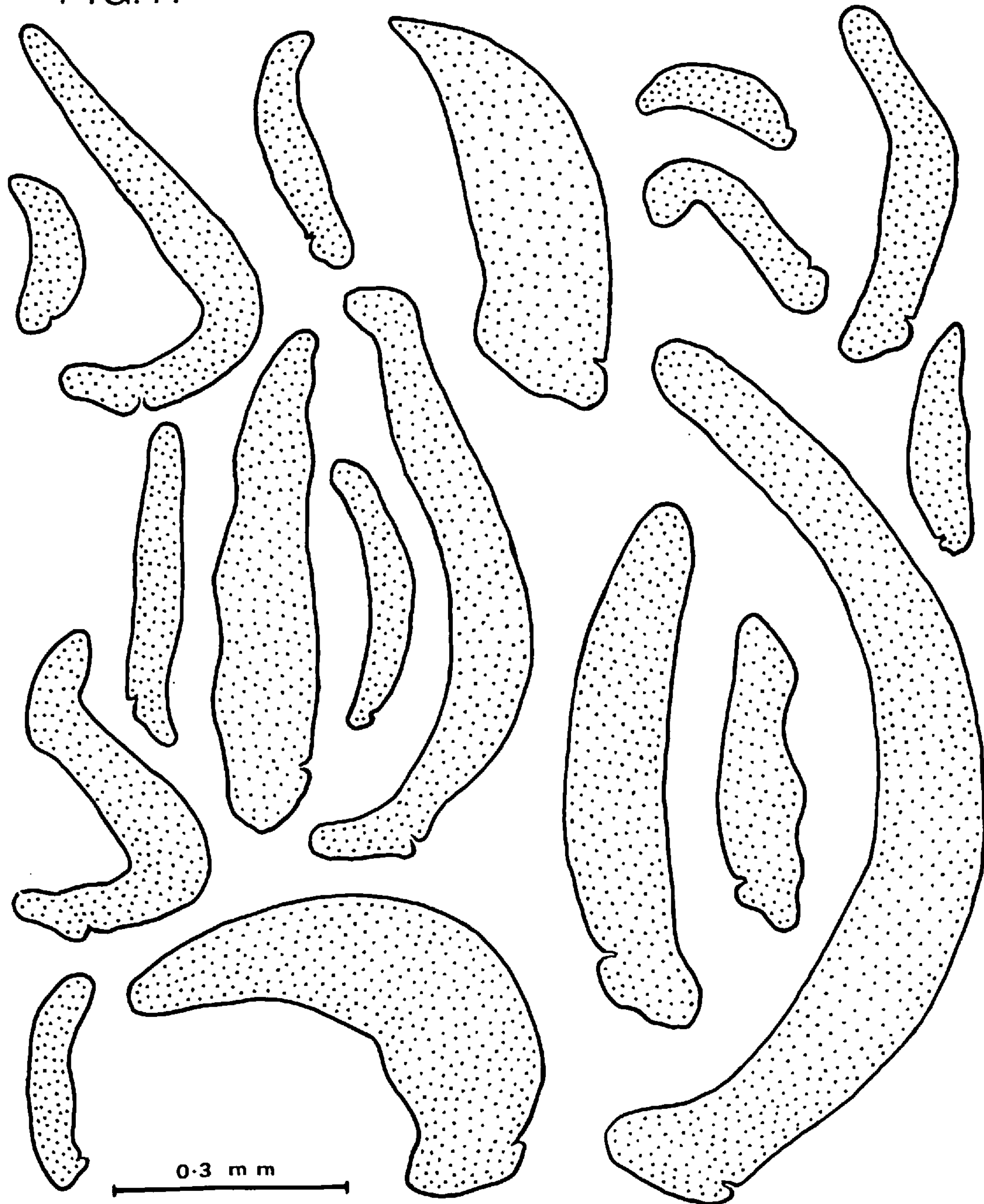
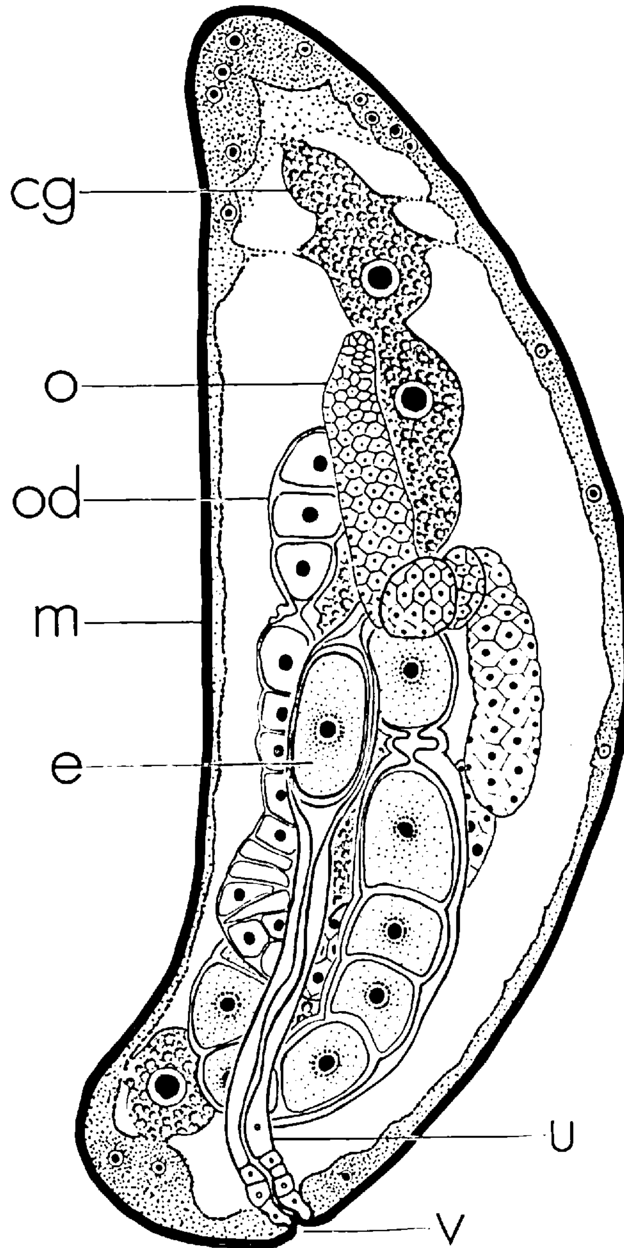


FIG. 8A.



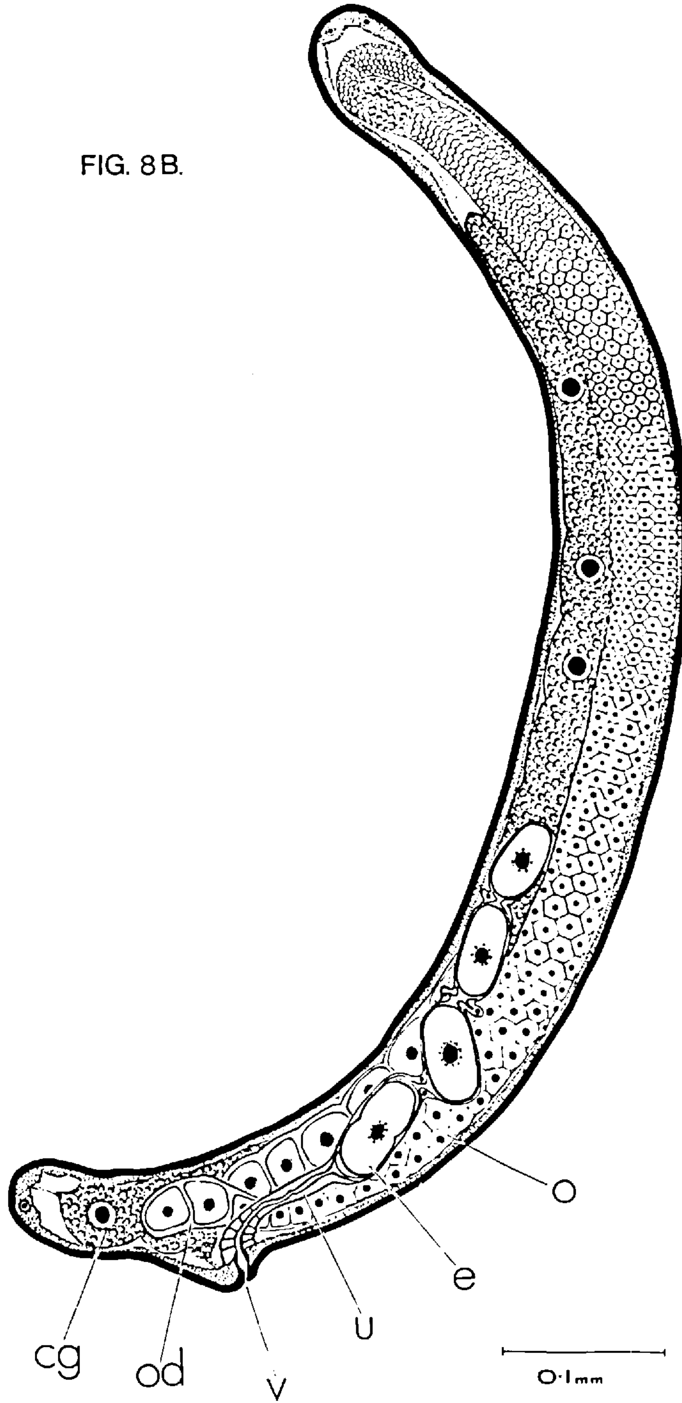


FIG. 8B.
o
e
u
cg
od
v
0·1mm

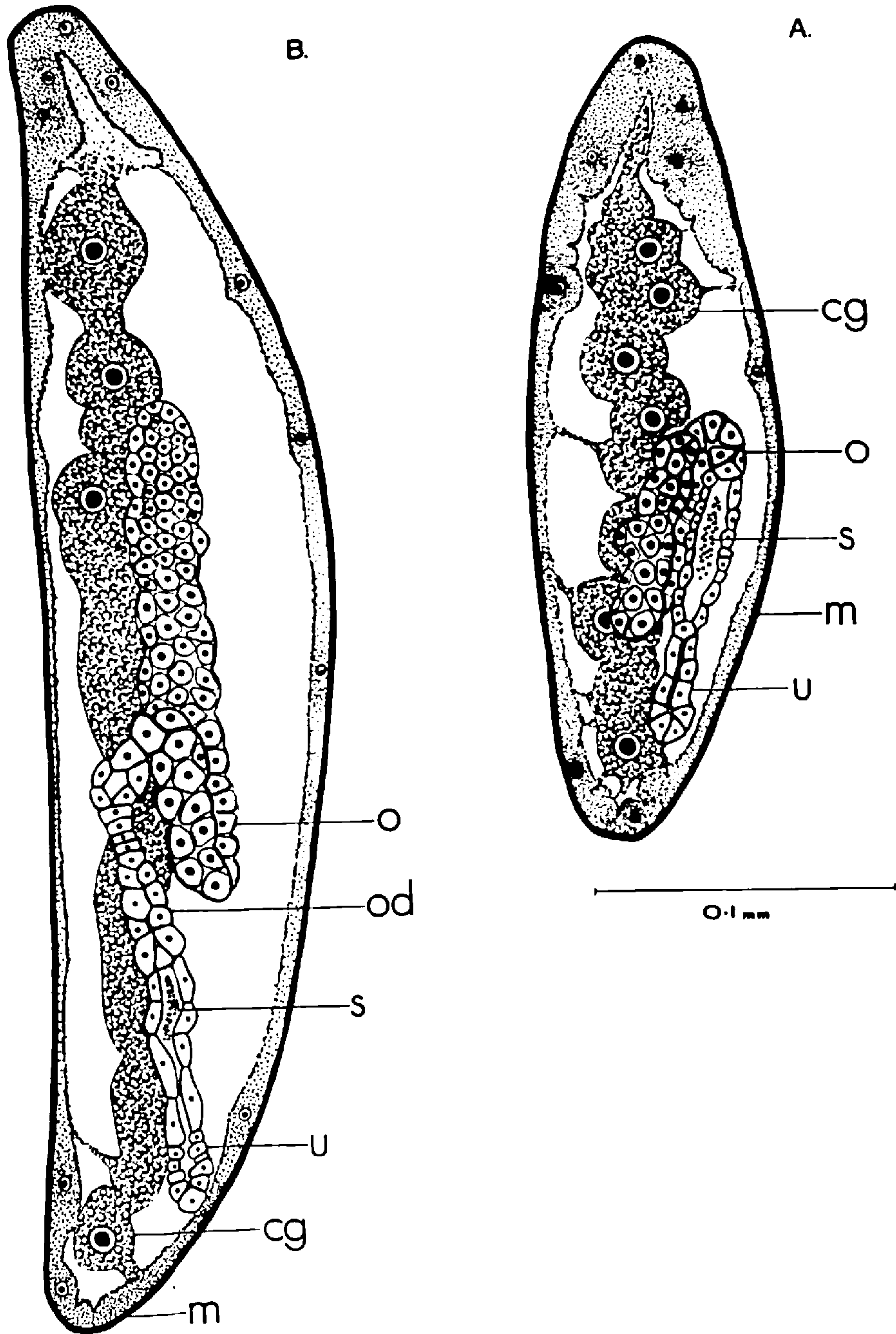


FIG.9.
B.
A.
cg
o
s
m
u
o
od
s
u
cg
m
0·1 mm

I 3.4.2. Reproduction in relation to age of host.

Preliminary observations on the female *Howardula* sp. suggested that there might be a relationship between the time of the parasite's exsheathment, maturation, egg production and subsequent larval development and the major events in the life cycle of *Megaselia halterata* such as ecdyses, pupal eclosion, fly copulation and oviposition. To investigate the feasibility of such an interrelation it was necessary to organize a large source of infected insects of known age. The previous experiment in which aged maggots were dissected to observe the development of parasites showed that *Howardula* maturation was better related to the length of time it had spent inside the host rather than to the age of the insect because frequently a variety of parasitic stages were observed in one host, whether 2nd or 3rd instar maggot, or pupa. Consequently it was decided to concentrate on the development of the progeny of *Howardula,* which must be sufficiently advanced by the time of fly oviposition to be able to develop in the external environment.

METHOD. Approximately a hundred pupae of *M. halterata* were recovered from infested cultures by spreading the compost on heated trays for half an hour and then shaking in a 1/8 inch mesh sieve. The pupae were collected underneath the sieve and this procedure was repeated four times at intervals of 2-3 days to obtain pupae of different ages. Several groups of about 50 pupae were separated according to development; maturation at 27°C had previously been established by examining pupae of known ages and the salient features are listed:-

AGE of PUPAE in DAYS	DEVELOPMENT at 27°C
1	white; no respiratory horns.
3	white; respiratory horns fully developed.
5	pale yellow.
7	light brown; parts of fly visible.
9	legs brown and eyes orange.
10	legs and eyes black.
11	eclosion.

To obtain 2 and 4-8 day old pupae it was necessary to retain 1 and 3 day old specimens on damp filter paper at 27°C for the required period. Pupae were dissected until at least ten infected ones were examined for each age and the number of adult parasites was recorded, together with absolute numbers of eggs and larvae when they were present.

Similar observations were made on groups of flies that emerged between 10 a.m. and 1 p.m. for 1-14 days. A black filter funnel was inverted and sealed to a dish containing about 1000 pupae, and a 3 x 1 inch tube was attached to the stalk of the funnel. As the flies emerged they were attracted to the tube by a bright light above the apparatus. After the daily three hour collection period, which ensured a constant time interval between groups of flies, the insects were kept at 21°C and a ventilation hole, plus a dental roll moistened with sugar solution were fitted into the stopper of the 3 x 1 inch tube. All the flies were killed on day 15 and up to 10 parasitised specimens were dissected from each age group. The number of adult Howardula was recorded and the parasite eggs and larvae from each fly were fixed in TAF and transferred to a centrifuge tube. After concentrating the eggs and larvae they were removed with minimum liquid to a 5 x 1 cm

stoppered tube for storage. The resulting 119 samples were eventually examined in a McMaster counting chamber; water was added to each tube to make a 3 ml volume and after agitating the suspension the number of eggs, first and second stage larvae present in 0.15 ml was recorded so that the initial parasitic burden of each fly could be calculated. Although ten parasitised specimens were obtained from each of the groups up to ten days old, the number of flies surviving for longer periods was greatly reduced and consequently data from the older flies has not been included.

RESULT. A summary of the data from these two experiments is tabulated in Appendix TABLES 3 & 4 and the mean number of parasite progeny is plotted against the age of the host in FIGURES 10 and 11.

The parasitic Howardula began to lay eggs when the pupae were 5-6 days old and oviposition rapidly increased during the next two days, reaching a maximum in 9-11 day old pupae. The flies emerged during the 11th-12th day and the first parasitic larvae hatched immediately prior to this as illustrated in FIGURE 10. The eggs of Howardula continues to hatch in 1-3 day old flies as was verified by the results of the second experiment, using different material, shown in FIGURE 11. The larvae increased in numbers until the flies were 6 days old and by this time only second stage larval nematodes were present, except in male flies.

Comments regarding the reproduction of the parasite in relation to host age will be included in the discussion at the end of Section I.

FIG.10.

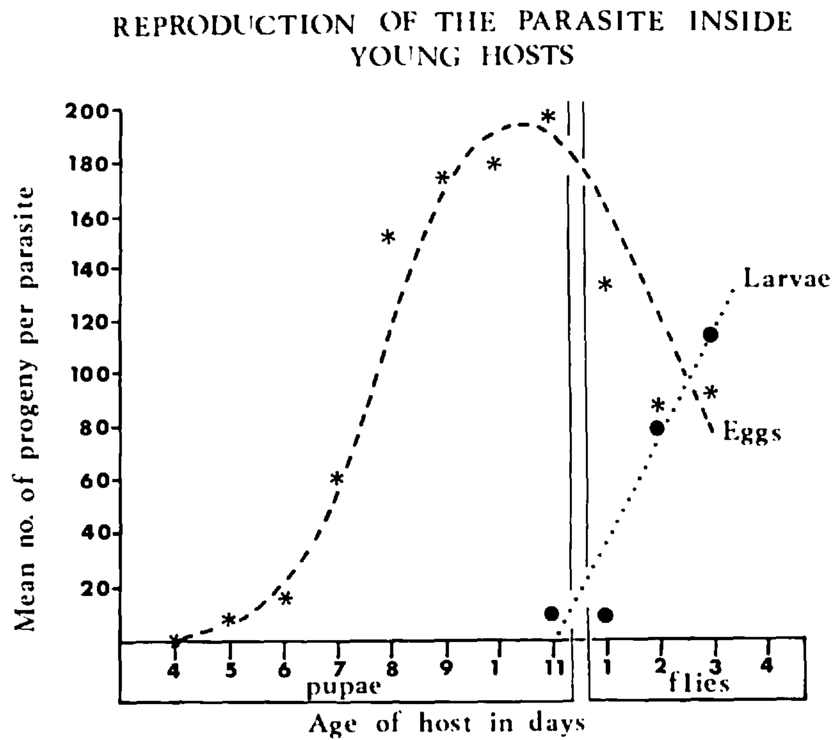
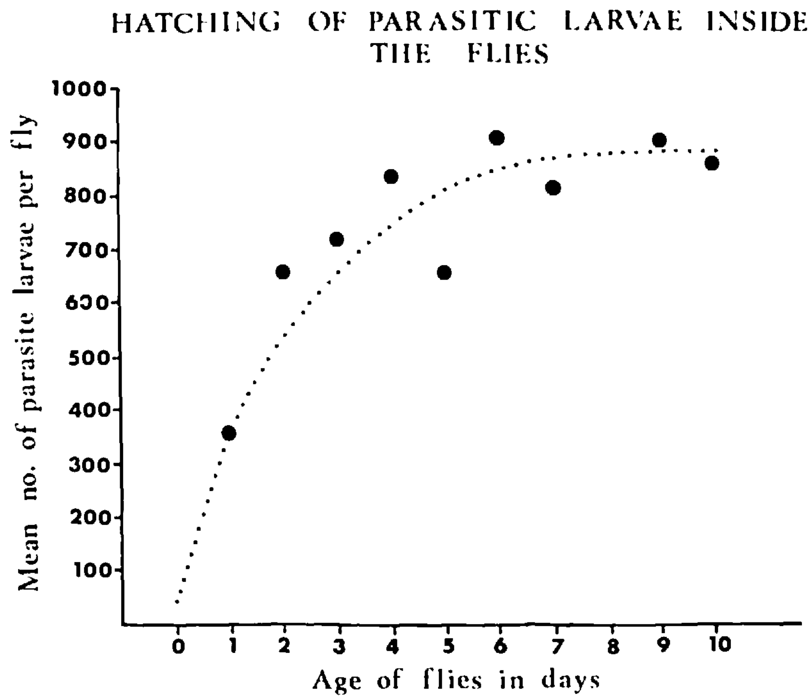


FIG.11.



I 3.4.3. Intraspecific Competition.

a. Parasite morphometrics and multiple parasitism.

During the description of the adult parasitic *Howardula* sp. in section I 3.4.1. it was stated that measurements were made from drawings of 100 living specimens. A general impression was obtained that the size of the parasites decreased with an increase in numbers present, consequently the data was organised into groups according to the numbers of parasites which occurred in each host. In wild populations of flies normally only one or two parasites are found but this material was collected from breeding cages where as many as 26 females were present in one insect. However, occurrences of nine or more parasites in a single host were infrequent, therefore only the dimensions of 1-8 are included and the number of records for each group varied from 10-17.

A summary of the data is tabulated in Appendix TABLE 5 and the mean length of parasites from each frequency is plotted against the mean width in FIGURE 12. Numbers 1-8 indicate mean dimensions of the parasites in each frequency group. It can be seen that there is a linear relationship between length and width and when only one adult female occurs in a host it is considerably larger than specimens from multiple infections. Two or three parasites in one insect are individually much smaller than an isolated female but they are bigger than specimens occurring in groups of four. However, there does not seem to be much difference between nematodes which are recovered from flies containing 4 to 7 worms but it is interesting that when 8 parasites occur in one host, another marked decrease in size occurs.

b. Parasite fecundity and multiple infections

Attention has not yet been drawn to the number of Howardula eggs, L_1 and L_2 recovered from flies of different ages which was reported in Section I 3.4.2. Although it was easy to distinguish eggs and larvae with a binocular microscope, it was very difficult to identify L_1's from young L_2's at the level of magnification demanded by the use of a McMaster counting chamber. Consequently the possible inaccuracies of this segregation led to the summation of larvae and by including the number of eggs, a figure representing the total parasite progeny in each fly was obtained. The number of adult nematodes per fly had been recorded, so the mean offspring per parasite was calculated to find out if the fecundity of individual females was influenced by the presence of others.

Appendix TABLE 6 summarises the relevant data and the $\log_{10}$ mean number of offspring per parasite is plotted against the number of parasites per fly in FIGURE 13. It can be seen that there is a reduction in the mean offspring produced by each female with increasing numbers of parasites per fly and a best fitting regression line was calculated as shown in Appendix TABLE 12. It was found that for every additional parasite, the progeny of each adult female decreased by a factor of 0.829.

The absolute reduction of progeny can be calculated from the data in Appendix TABLE 12, but there is little point in calculating the actual number of offspring suppressed because a rough approximation can easily be obtained by an examination of the mean difference of progeny/parasites per fly. The absolute

reduction in offspring varies with each increased female so that the difference in number of progeny produced by parasites occurring in groups of 6 and 7 is very slight, compared with the decrease in progeny between females occurring individually and in pairs. Consequently the most useful expression is the factor of 0.829 by which offspring is suppressed as a result of one more parasite being present in the host.

FIG. 12.

PARASITE MORPHOMETRICS IN MULTIPLE INFECTIONS

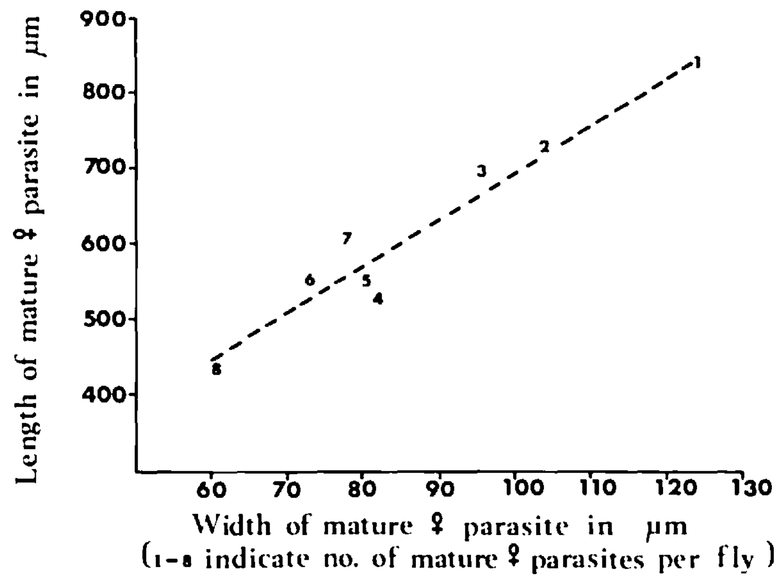
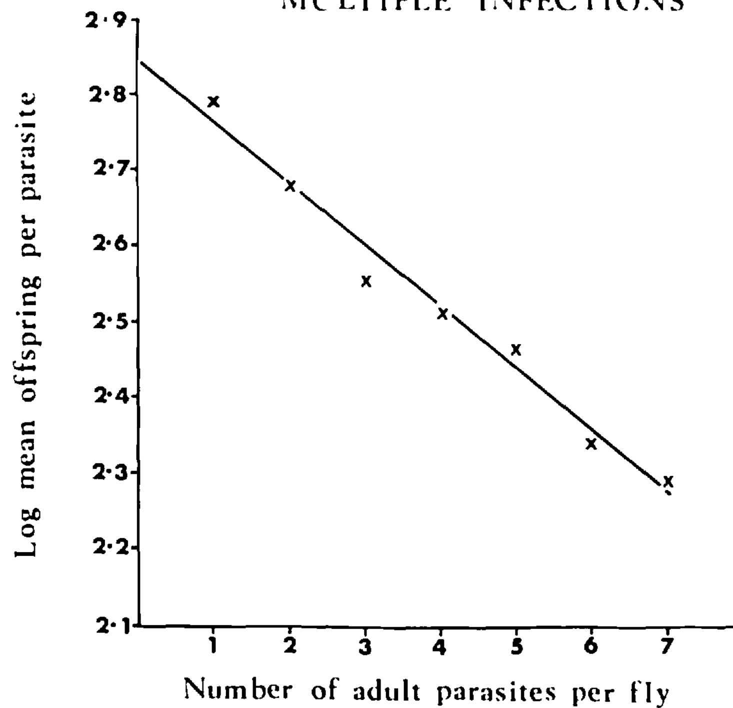


FIG. 13.

DECREASE IN PARASITE FECUNDITY IN MULTIPLE INFECTIONS



I 3.4.4. Incidence of Parasitism.

Megaselia halterata adults were collected at the localities listed in section I 2.1. at intervals from January 1968 to August 1970 and after dissecting about 100 flies from each sample the overall incidence of Howardula sp. was recorded. The data is summarised in appendix TABLE 7 and illustrated in FIGURE 14.

It can be seen that there was an annual fluctuation in the level of parasitism which was at a minimum in Spring and reached a maximum in Autumn. This coincided with changes in the fly population density; although no attempt was made to estimate the numbers of M. halterata it was apparent that the flies were rare in March to May but abundant in September to November.

A comparison of the incidence of parasitism in male and female flies showed no significant difference between the host sexes although there was a tendency for females to be more frequently infected.

A record was kept of the number of adult Howardula sp. which occurred in male and female flies and the data is presented below together with stages of non parametric analysis required to investigate statistical significance using the Wilcoxon (1949) matched pairs signed-rank test. A sign is given to each pair of flies according to whether the females contain more, equal or fewer parasites than male flies. The difference (d) between each pair is ranked disregarding the sign and identical figures are given the same rank. The number of observation (n) is the total matched pairs minus the ones which have the same number of parasites ($n = 12$). T is the smaller sum of like-signed ranks,

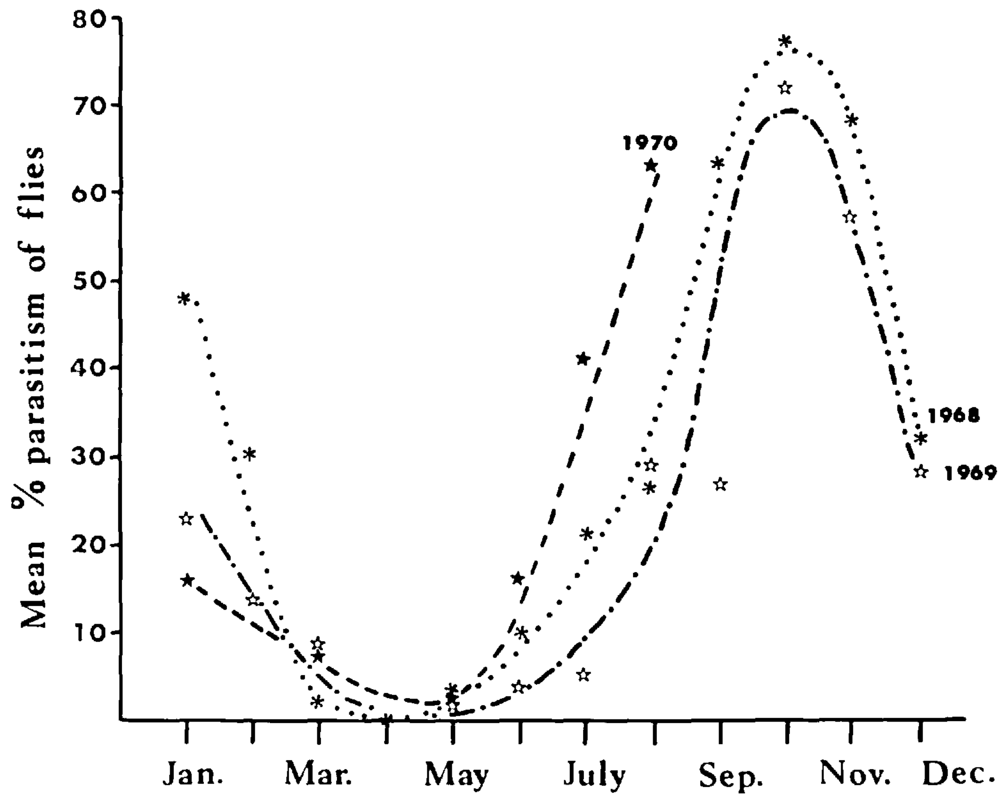
either positive or negative; in this case the sum of the negative ranks is the smaller and T = 12. The Wilcoxon signed rank table gives values for T under a particular significance level for the observed value of n. It was found that the expected value for T when n = 12 was greater than the observed value (T = 12) at the 5% level, and the Null Hypothesis that there was no difference between the flies must be rejected at this level of significance.

Matched Pairs of Flies	Mean Adult Parasites per fly (n = 50)													
FEMALES	3.4	3.3	1.0	2.3	1.6	2.5	2.3	1.4	1.5	1.3	5.5	1.3	2.6	
MALES	2.0	1.9	1.7	2.0	1.5	2.5	1.5	1.0	1.3	2.0	3.9	1.0	1.4	
	+	+	-	+	+	=	+	+	+	-	+	+	+	Sign
	1.4	1.4	0.7	0.3	0.1	0	0.8	0.4	0.2	0.7	1.6	0.3	1.2	d
	10	10	6	3	1	0	8	5	2	6	12	3	9	Rank
	1	2	3	4	5	-	6	7	8	9	10	11	12	n

The results showed that there was a highly significant difference ($0.05 > p > 0.02$) in the occurrence of Howardula sp. and that female M. halterata contain more parasites than male flies.

FIG. 14.

% PARASITISM OF FLIES COLLECTED FROM MUSHROOM FARMS OVER 3 YEARS



I 3.5. HOST-PARASITE RELATIONSHIPS

3.5.1. Effect of parasite on host internal anatomy

An examination of the flies processed as described in Section I 2.4. showed that Mukerji's fixative was unsatisfactory. Many of the internal organs had been damaged and distorted by the corrosive reagent which is probably only required to fix insects with extremely tough exo-skeletons, such as beetles. Carnoy-fixed material was best preserved and Mayer's haemalum/eosin was the most effective stain, being progressive and self differentiating and producing blue nuclei in pink cytoplasm.

Longitudinal sections through unparasitised and parasitised *Megaselia halterata* flies are shown in PLATE 2A and B respectively. The most obvious effect of *Howardula* sp. on the host is the damage done to the fat body which in uninfected flies completely fills the abdominal cavity as a mass of tightly packed polyhedral cells or trophocytes. Individual trophocytes are large, frequently vacuolated with granular inclusions and they ramify between the internal organs as shown in PLATE 2A. In contrast, parasitised flies have a depleted fat body which is often disintegrated and sparse and the abdomen is swollen as shown in PLATE 2B. In many infected flies the fat body is completely destroyed, probably due to mechanical damage caused by the constant locomotory activity of nematode larvae and also by their feeding which is indicated by nests of parasites in eroded trophocytes.

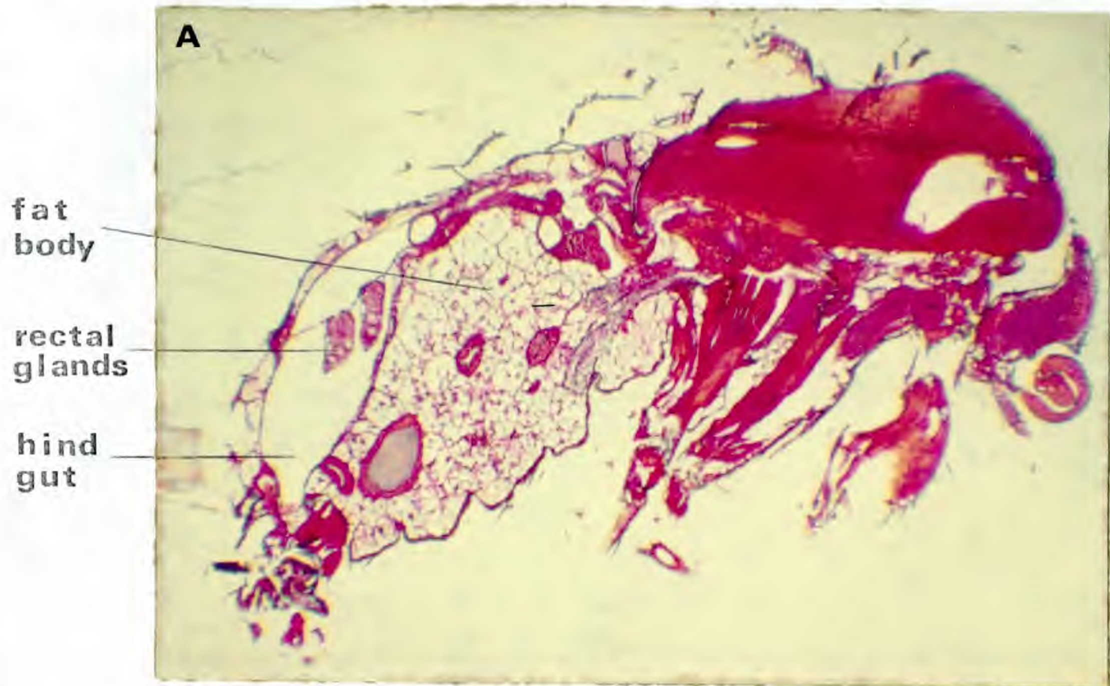
The larval nematodes are usually orientated along the longitudinal axis of the host in the antero-lateral regions but they mainly lie transversely at the posterior of the fly. Frequently a few larvae are present in the thoracic region where they cause

dispersion of the flight muscles; others have been observed in the legs where they also damage the muscles and occasionally nematodes have been found in the haemocoelomic cavities of the insect's head. The adult parasites occur in the abdomen and are often surrounded by trophocytes in such close proximity that it is difficult to determine the boundary of the host-nematode interface.

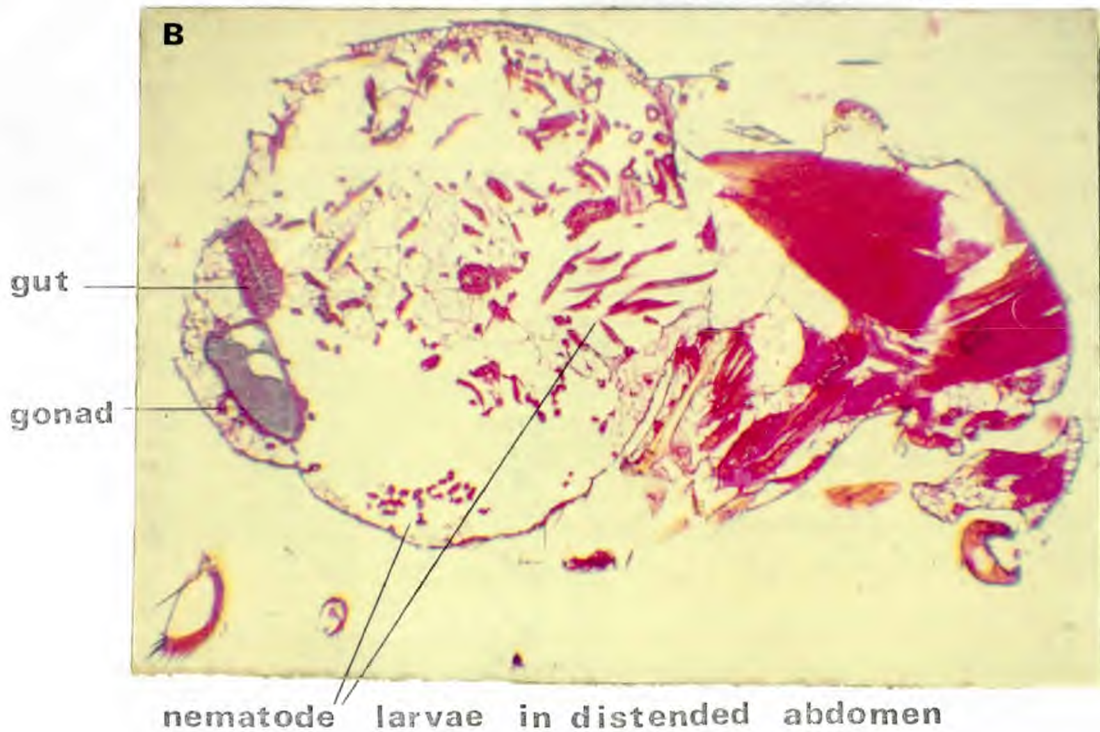
The gonads are reduced and in female flies the membranes of the polytrophic ovarioles which are well developed in unparasitised specimens are thin and frequently perforated so that larval nematodes can pass freely between the oocytes from the abdominal cavity. The ovary never contains as many oocytes as in uninfected flies and there appear to be less sperm in parasitised male flies.

PLATE 2.

LONGITUDINAL SECTION THROUGH UNPARASITISED M. HALTERATA



LONGITUDINAL SECTION THROUGH M. HALTERATA INFECTED WITH HOWARDULA



I 3.5.2. Effect of Parasite on Host Copulation

In order to investigate the possibility of Howardula sp. affecting the copulatory ability of M. halterata, a random sample of phorids was obtained by collecting all the flies which alighted in 30 minutes on a one square yard area on the external surface of a mushroom shed door. The flies were pooted into two bottles according to whether they were in copulation or not and subsequently dissected to find out if they were parasitised.

Copulating flies had an incidence of 41% parasitism in comparison with 74% parasitism in non copulating individuals. The results are given below in the form of a 2 x 2 Contingency Table to test for significance.

	FLIES					
	COPULATING		NOT COPULATING			
FLIES PARASITISED	38	(a)	55	(b)	93	(a+b)
NOT PARASITISED	54	(c)	19	(d)	73	(c+d)
	92	(a+c)	74	(b+d)	166	(N)

By substituting these figures in a Chi Squared equation it was found that $X^2 = 18.3663$ which is significant at the 0.1% level. Therefore the Null Hypothesis that there was no difference between the two groups of flies must be rejected because the probability of these results being obtained at random is less than 0.001. Consequently the difference between the flies is highly significant and it was concluded that Howardula sp. effects M. halterata by reducing its ability to copulate.

I 3.5.3. Effect of Parasite on host egg production

Individual female *M. halterata* were allowed to oviposit in the small breeding containers described in Section I 2.2. and each was dissected after a natural death in order to find out if *Howardula* sp. was present. Often parasitism had previously been detected by the presence of nematodes on the agar plates but it was not always possible to see the larvae among the thick weft of mushroom mycelium. Originally 1-6 male flies were enclosed with the female but copulation rarely occurred under these confined conditions and it was necessary to allow 1-3 days with many newly emerged flies of both sexes in a large flight chamber before the females could be isolated, exposed to mycelium and induced to oviposit. The total number of eggs produced by each fly was recorded after the female had died, by which time several maggots had hatched.

Appendix TABLE 8A shows the number of eggs laid by parasitised flies and Appendix TABLE 8B, unparasitised flies, but the results are summarised in Appendix TABLE 13 together with the 'Student's *t* test used to examine the statistical significance of this data. The highly significant difference ($p = 0.001$) demonstrates that the presence of *Howardula* sp. has a marked effect on *Megaselia halterata* by reducing the number of eggs laid by female flies.

Attempts were made to find out if the reduction in host egg production was related to the number of parasites inside the fly and as this data was not available from the previous experiment, other material was examined. 100-200 seven day old flies which had been allowed sufficient time for copulation and egg maturation but had not been exposed to mushroom compost, were dissected and the number

of eggs and adult parasites (if present) were counted in each specimen.

The results are presented in Appendix TABLE 9 and it can be seen that as many as 13 Howardula females were found inside each fly. 1-5 parasites were commonly present but 1 or 2 adult nematodes occurred most frequently. The mean number of eggs recovered from 'clean' flies was 25 but the mean eggs of parasitised M. halterata was much lower than this, although individual phorids contained as many as 38 eggs when only one parasite was present. A summary of the data is shown in FIGURE 15 where the mean percentage reduction in fly eggs is plotted against the number of adult parasites that occurred in each fly.

It was concluded that an increase in parasitism causes a correspondingly greater suppression of M. halterata eggs and that when ten or more female Howardula sp. are present, the fly is completely unable to reproduce.

I 3.5.4. Elimination of host populations

It was mentioned in Section I 2.2. that considerable difficulty was experienced in breeding populations of parasitised phorids because of the effect of Howardula sp. on host fecundity. Approximately 1000 flies were used to establish each breeding cage and usually the life cycle of M. halterata took 21 days at 25-27°C, so the population was sampled every 3-4 weeks to find the incidence of Howardula sp. As the incidence of parasitism approached 50% unparasitised flies were added to the breeding cage together with 18 jars of mushroom compost when otherwise only 12 jars would have been added. Dilution of the parasite enabled continuation of infected breeding stock but when the population was not manipulated in this way the flies were eliminated

in 4-6 generations, as shown in Appendix TABLE 10.

FIGURE 16 illustrates the increasing incidence of parasitism in flies from three different breeding cages which were initially collected at different localities and were 3.7-8.5% parasitised. In each cage a large population of flies was produced and the incidence of parasitism exceeded 90% by the third generation but subsequently in two of the colonies, fewer than a dozen flies were produced in the fourth generation and these were unparasitised. The other cage continued to maintain a high level of parasitism until the sixth generation when a similar crash of parasites and hosts were recorded.

Comments about the results of Section I are included in the following discussion.

FIG. 15.

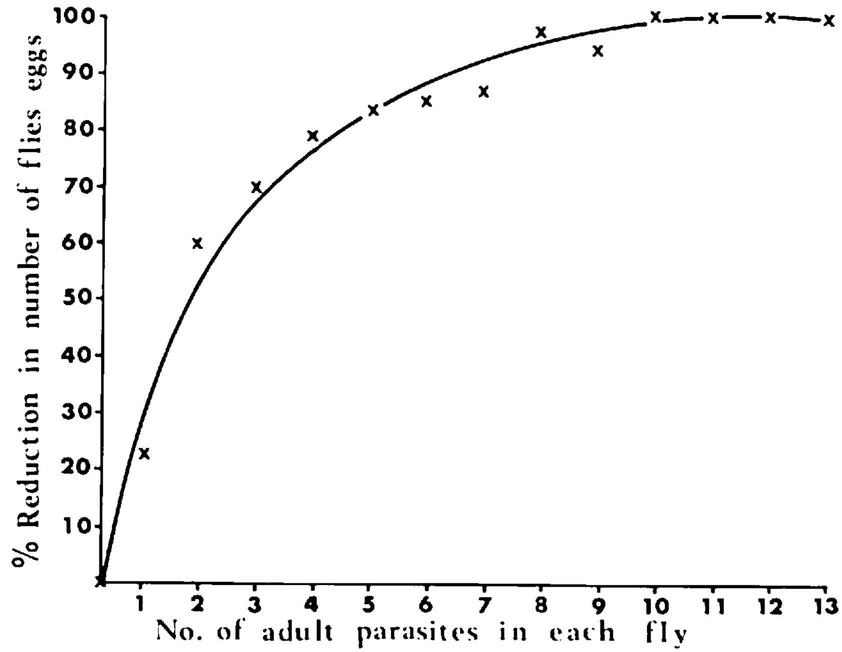
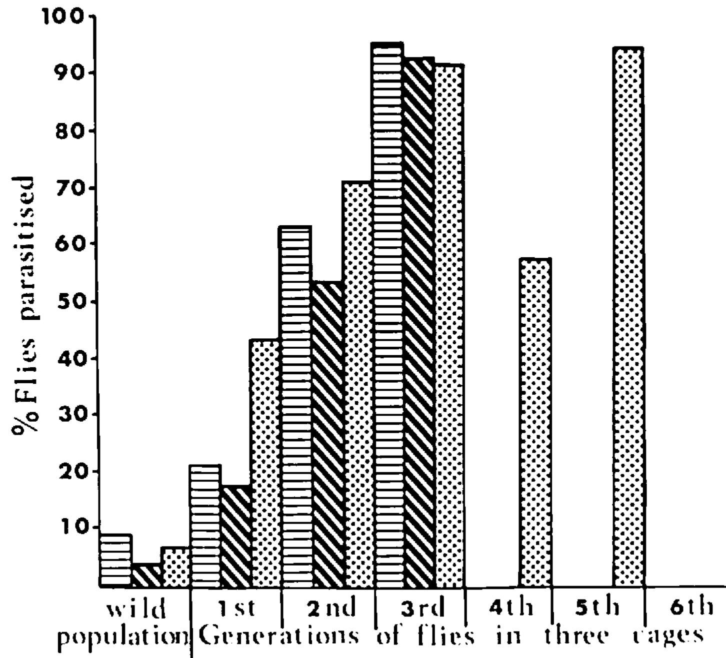


FIG. 16.



I 4. DISCUSSION

TAXONOMIC STATUS OF HOWARDULA sp; comparative morphology and biology of the genus.

The nematode which parasitises Megaselia halterata closely resembles the seven known species of the genus Bradynema and eleven known species of Howardula, as can be seen from Nickle's (1967a) summary of the diagnostic generic characteristics. However, the presence of a stylet in the fourth stage female, reported in Section I 3.3.1, unquestioningly determines that this nematode should be placed in the genus Howardula. No stages in the life cycle of Bradynema possess a stylet but among the six species of Howardula, where the free living female has been described, a stylet is always present and it has also been reported in five of the mature parasitic females. A stylet is never present in the male nematodes.

The salient features of the nematodes comprising the genus Howardula are summarised in Appendix TABLE 11 and the relevant data on the present Howardula sp. is included for comparative purposes. It can be seen that there is inadequate information about four of the species: H. cuneifer, H. claviger, H. terribilis and H. hirstum, all of which were described by Warren in 1941 and given a new combination by Wachek in 1955. Only the adult parasitic females are known and these are incompletely described; Wachek (1955) suggested that as they occurred in Acarina they were probably identical to the mite-parasitising H. acarinorum which he had just described but he did not name them species inquirendae.

Howardula dubium (Christie 1938) Nickle 1965 is similarly lacking in data because only the free living female is known but this is sufficient for the present purposes. Although the openings of the

oesophageal glands closely resemble the arrangement in the infective Howardula sp. and the body length has an overlapping range, they are obviously not the same species; the differences in V%, stylet length and the amount of stored sperm, which in H. dubium occupies over half the length of the body, are readily distinguishing features.

Of the six remaining species, all except H. aoronymphium Welch 1959 are obviously not synonymous with Howardula sp. because a stylet is present in the adult parasitic females. H. aoronymphium also resembles the phorid nematode in arrangement of oesophageal glands but they can be separated by the larger dimensions of the male and females, the asymmetric spicules and by the absent caudal alae of H. aoronymphium.

All the members of the genus Howardula where the life history is known have direct cycles with free living mature males fertilising an infective female, which becomes parasitic and produces larvae that ultimately leave the host. A very brief period is spent in the external environment in contrast to several other tylenchid parasites of insects such as Deladenus and Contortylenchus which are examined in the next section. To facilitate a short duration outside the host the development of parasitic larvae is accelerated in several species. In H. benigna third stage larvae are released (Wachek 1955) and in H. oscinellae larvae become sexually differentiated inside the host (Goodey 1930); in H. aeronyphium the males are more advanced than the females (Welch 1959) and in H. aptini the nematodes are liberated as mature, free living forms (Nickle and Wood, 1964). With the exception of H. benigna where undifferentiated larvae leave the host (Cobb, 1921), Howardula sp. differs from the other species in releasing second stage nematodes with little genital primordia development. Surprisingly, in the external environment

these larvae are able to mature faster than the other species of which the nearest rival, H. acarinorum, takes 4-6 days (Wachek, 1955).

Presumably rapid development of Howardula free living stages, producing a truncated life cycle, is related to the life history of insect hosts. (Host list for Howardula species is given in Appendix TABLE II). Drosophila, mites, frit flies, thrips and phorids are all capable of producing several generations in one year and have a brief larval period. As most Howardula infective females can only enter the host's larval instars, a limited time is available for nematode maturation, copulation and host location; consequently a truncated development was an essential adaptation for survival. Support for this theory is provided by H. phyllotretae which differs from the other species by being unable to mature outside the host in tap water (Oldham, 1933) and by parasitising a "flea beetle" Phyllotreta which only has one generation each year. The larvae of H. phyllotretae over-winter inside the hibernating beetle and are presumably deposited during oviposition in Spring because Oldham observed nematodes in the insect oviducts. Because of staggered egg laying, beetle grubs are available for about five months and consequently the nematodes probably mature at an imperceptibly slow rate. Alternatively, as it is unlikely that Oldham overlooked this development, H. phillotretae may have larvae which feed in the external environment before being able to mature which would account for their unchanged condition in tap water, in contrast to other species of Howardula.

There are discrepancies in the number of moults that occur in the various species. H. oscinellae larvae are reported to moult once inside the host and twice in the external environment (Goodey, 1930)

with the unobserved fourth moult most probably occurring in the uterus of the adult female nematode, prior to release. Wachek (1955) offers the curious explanation that the second moult of the 'cultured' free living nematodes resulted from the larvae having been removed prematurely and that Goodey had unwittingly observed a moult which would normally have occurred inside the host. Wachek states that H. benigna larvae moult twice before leaving the host and apparently he assumes that this usually occurs in the other species but Sharga (1932) found that H. aptini has "several ecdyses" which seems understandable in view of the maturity of the liberated nematodes. Howardula sp. appears to be exceptional because the parasitic larvae only moult once, as reported in Section I 3.1.

Until H. aoronymphium was described there was no mention of exsheathment after the infective female has become parasitic, except for Goodey (1930) who says that growth is accomplished "without further ecdyses" in H. oscinellae "as it has already attained the adult condition before entering the larvae", Welch (1959) found that the final moult of H. aoronymphium occurred in the "haemocoel of the definitive host" and pointed out that this was unusual because in the other known species it takes place in the free living state. Unfortunately he overlooked Sharga's (1932) account of the developing young parasitic H. aptini which "moult several times"; Lysaght (1936) was more specific when she clarified the life cycle and described one "post-nuptial moult" in the body cavity of the host.

In view of the significance of the final moult of Howardula sp. leading to an aberrant cuticle-lacking, microvilli-covered parasite (Riding, 1970) described at the ultrastructural level in the next section, the moult observed by Lysaght is of particular interest.

The cuticle of the infective female H. aptini fails to separate from the body after exsheathment inside the host, and it remains attached at the anterior end of the parasite as a long appendage. "Sinious movements", show that some part of the muscular system is still functioning and after the moult, swelling occurs with a simultaneous decrease in length. Ultimately the parasite becomes much fatter although it never regains its initial length (mean adult length = 0.21 mm; mean infective female length = 0.25 mm). The gut degenerates as it does in Howardula sp. into a "formless mass of cells more or less central in position" but the stylet persists at the base of the exsheather cuticle.

Lysaght does not explain how the old cuticle arrives at the anterior end or where the first rupture occurs but it appears to differ from the exsheathment described for Howardula sp. in Section I 3.3.3. (see FIGURE 6A, B & C). Presumably a split initially forms near the posterior of H. aptini and the old cuticle could be forced forward by "sinuous movements" but it is not clear if the swelling Lysaght reported immediately after the moult actually commences during exsheathment. If so, this would facilitate withdrawal from the cuticle as it does in Howardula sp. where exsheathment is entirely passive because the nematode is unable to move, unlike H. aptini. The reduced musculature of Howardula sp. which excludes a 'normal' sheath exit is apparently compensated by osmotic phenomena where water rapidly enters the exposed regions of the adult body with subsequent enlargement of tissues. Osmoregulation in nematodes has been outlined by Lee (1965a) and experimental data of the type described by him is required before the phenomenon which occurs during the exsheathment

of Howardula sp. can be understood. From the observations described in Section I 3.3.3. it appears that the newly parasitic female can osmoregulate and survive in water and up to 0.9% saline solutions but this ability is gradually lost as it progresses towards exsheathment. Presumably ions and non electrolytes enter the nematode from the host haemolymph so that when moulting specimens are removed, they burst in increasing concentrations of saline with increased duration inside the host, because of a sudden influx of water by osmosis. A continuation of a unidirectional passage of material from the host could ultimately produce a greater water potential (diffusion pressure deficit) inside the parasite than that of the surrounding insect haemolymph. Consequently when the split occurs in the old cuticle a sudden influx of water could effect exsheathment. The subsequent swelling which occurs immediately after the sheath is lost in Howardula sp. and H. aptini could be explained by diffusion processes which would be necessary to attain equilibrium with the host tissues; the further slow increase in size being due to growth.

Unfortunately Welch (1959) gives no information about the nature of the final moult of H. aeronymphium but from the processes recorded in the other two species it seems likely that a similar atypical exsheathment occurs and probably an investigation of the other members of Howardula would reveal a comparable moult inside the host. Failure to observe this stage of development in the young parasite should not be interpreted as being absent because it is a rapid process which could easily have been overlooked. Evidence that the female has a final moult inside the host is provided by all eleven species of Howardula having enlarged flaccid sac-like

adults which bear no relationship in appearance to the infective females and it is inconceivable that a persisting cuticle could be thus modified. Also the fact that none of the authors observed a triple moult in the free living male (Section I 3.2.) which must occur to complete the accepted five staged life cycle of nematodes, suggests that other moults could equally have been overlooked. The discovery of microvilli on the outside of Howardula sp. led to an examination of the mature H. benigna and Bradynema rigidum (kindly loaned by Dr. W.R. Nickle) and not surprisingly it was observed that these related nematodes are similarly modified because microvilli were discernable with the light microscope in these comparatively large nematodes.

The evolutionary implications of the highly specialised modifications of Howardula and Bradynema will be mentioned in the general discussion at the end of this study. It is relevant to conclude this comparative examination of the Howardula species by pointing out that there is sufficient justification for considering the Megaselia parasite to be a new nematode. It does not belong to the genus Bradynema because it possesses a stylet so it is included with Howardula, the only other similar genus. Distinguishing morphological and biological features which can separate the new species from the other known members of the genus have been indicated and a paper describing Howardula sp. will shortly be submitted for publication. At the suggestion of Dr. W.R. Nickle, the specific name will be chosen to honour Professor H.E. Welch for his contribution to the subject of entomophilic nematodes and to acknowledge his previous association with the M. halterata parasite.

EXPERIMENTAL DATA AND BIOLOGICAL CONTROL POTENTIAL

From the observations reported in Section I 3. the life cycle of *Howardula* sp. is apparently well synchronised with the stages of *Megaselia halterata* and the host is adversely affected by the presence of the parasite.

It was disappointing that two of the significant events in the host-parasite relationship, entering and leaving the insect, were not observed neither were copulating nematodes found. Circumstantial evidence that parasitic larvae exit during the fly's abortive attempts at oviposition was provided by the presence of nematodes among the ovarioles of sectioned insects but they were never observed lower down in the oviducts nor associated with freshly laid eggs. Clusters of nemas were present in drops of fluid exuded from the fly as was also seen by Hussey (1959) but these could have left via the anus because although nematodes were not found in the rectum, many larvae aggregated in the mid gut region of the haemocoel suggesting that they may later penetrate the gut. It is possible that both routes of exit (oviposition and defaecation) are available to the nematodes but several other *Howardula* species utilise only one pathway; *H. acarinorum* and *H. oscinellae* for example, leave their respective hosts through the anus. However the fact that *Howardula* sp. is unable to get out of male flies infers that the vulva is the most likely way out for the larvae but the situation is further complicated by the inability of parasites to reach the second larval stage in male hosts and presumably they are not able to react to the stimuli which motivates a directional migration in older worms.

It is perhaps not surprising that experiments designed to observe infective females entering the maggots failed, in view of

the fact that copulation could not be induced under artificial rearing conditions. As all newly parasitic females contained sperm, either protandrous hermaphroditism occurs coincident with host entry, which would account for the sparsity of male nematodes or else fertilisation took place in the external environment. It is more likely that copulation occurs and probably this absent stimulus in the experimental animals could explain their complete lack of "interest" in host maggots and pupae; presumably copulation is a necessary trigger which leads to a change in behaviour, enabling location and penetration of an insect. Further investigation using fertilised females extracted from mushroom compost may elucidate the mechanics of host penetration; entry per os is excluded because of the damage which would be inflicted by the relatively large mandibular sclerites of the maggots and the contra-indication of a stylet-bearing nematode which has diminished oesophageal glands after entering the host.

Multiple parasitism or hyperinfestation (Cross, 1966) is widely known to adversely affect the size and fecundity of parasites as was observed in *Howardula* sp. (Section I 3.4.3.). The "crowding effect" is more marked in parasites of invertebrates because the mass of parasites is closer to the mass of host than it is in vertebrates, and the food reserves are limited (Welch, 1963). Mature *Howardula* sp. occurring in isolation were two or three times bigger than individuals recovered from phorids containing several nematodes and they had a corresponding increase in gonad proliferation which was related to an increase in the number of progeny. This manifestation of intraspecific competition is probably associated with the amount of food available inside the host although

the fact that the total larvae increased with each extra parasite (calculated from Appendix TABLE 6) even though individual parasites were producing fewer eggs, suggests that the flies resources were not exhausted. Confirmation that phorids can tolerate a much greater parasite population is obtained from observations of as many as 26 adult Howardula sp. in one fly. However, it is significant that the phorid is unable to reproduce when more than nine parasites are present.

The effect of tylenchid nematodes on host fecundity is widely documented. Zondag (1969) showed that Deladenus reduces the ovary of Sirex noctilio and both the male and female wood wasps are rendered sterile; Stoffolano and Nickle (1966) found the ovaries of the face fly Musca autumnalis were completely destroyed by Heterotylenchus; Massey (1962) estimated a 70% reduction in the broods of bark boring beetles infected with Aphelenchulus elongatus and there are many other similar records including several among the literature cited earlier on Howardula species. Frequently the authors have indicated the biological control potential of these nematodes and Hussey (1967) reported preliminary experiments on the interaction of Howardula sp. and M. halterata to investigate the efficacy of the parasite. He found that the proportion of phorid larvae parasitised decreases as their density increases and inferred that some mechanism other than random contact enables nematodes to reach the maggots, resulting in hyperparasitism of some hosts while others remain uninfected. Departures from a Poisson Distribution can be seen in Section I 3.4.3. and it is a common feature of nematode infections that the frequency of parasite number per host departs from chance distribution (Welch, 1963) and that exposure or susceptibility to parasitism is

uneven within an insect population. In Howardula sp, this is presumably explained by the slow locomotion and poor dispersal ability of the infective female which is deposited in clumps, and unless the host density is high many of the nematodes will enter the same maggot. It is particularly interesting that female hosts should contain more parasites than males, and that this was similarly recorded by Welch (1959) for Howardula aoronymphim in Drosophila. Possible explanations include predestined "female" maggots having different physical, chemical or behavioural characteristics which would favour selection by infective nematodes, or else differential mortality of the parasites could occur because it was shown that male phorids are poor hosts which never harbour viable second stage larvae. Further investigation is required to elucidate the operative mechanism.

It is unusual for tylenchid parasites to kill their hosts; for this reason they are considered to have less biological control potential than the mermithids and steinernematids and Ruhm (1956) concluded that they are of little importance in controlling scolytid beetles. In general this is probably true, but in particular examples the sphaerulardids may be significant regulatory factors, such as Tripius sciarae in Bradysia paupera (Poinar, 1965) and Contortylenchus elongatus in Ips confusus (Nickle, 1963). Hussey and Gurney (1964) suggested that the phorid epidemic of 1953 may have been due to the absence of Bradynema but it was found in the present study that greater host densities have a corresponding higher incidence of parasitism and many other nematodes appear to be host density dependent. Saunders and Norris (1961) demonstrated this

phenomenon with Parasitaphelenchus oldhami in Scolytus multistriatus, as did Welch (1959) with Parasitylenchus diplogenus in Drosophila subobscura. However in view of the characteristic discontinuous distribution of tylenchid nematodes (Ruhm, 1956) which occur in foci, absence of parasites in one locality could lead to a population explosion such as occurred with phorids in West Sussex in 1953.

Assessing the role of nematodes in the natural regulation of insect populations is fraught with problems (Sweetman, 1963) but from the observations on breeding infected phorids (FIGURE 16) there can be little doubt that Howardula sp. was directly responsible for the phorid population crash which occurred in 3-5 generations because cages of unparasitised flies in similar conditions were maintained for several months at a high density level. The parasitised phorids which survived for five cycles is probably explained by old mushroom compost being given at the emergence of the 3rd generation flies. Hussey (1961) showed that mushroom compost is most attractive to phorids about ten days after spawning at 75°F and normally compost 1-2 weeks old was given to the flies, but in the absence of younger mycelium they will readily oviposit in three week old compost. Although older compost is adequate for the phorids, as the mushroom colonises the medium it becomes drier and consequently the nematodes are at a disadvantage; presumably this was reflected in the decreased parasitism of the 4th generation flies which subsequently increased to eliminate the phorids after the 5th cycle.

This experiment emphasises the need to consider such factors as moisture requirement and optimum temperatures for nematode

development and dispersal before attempting to manipulate the parasite to increase its performance as a biological control agent. It was shown in Section I 3. that temperature influences the rate at which free living Howardula sp. mature in the external environment, and their longevity. Although no development occurs at 5°C, 10°C and 30°C it was hoped that the larvae would be able to survive for long periods at the lower temperatures; survival ability, a prerequisite property for biological control, facilitating storage, accumulation and subsequent application to host populations as was utilised by Dutky et al (1964) with juvenile Neoaplectana which have a shelf life of up to five years at 7°C. Unfortunately the second stage larvae only survived 1-4 weeks at 5°C and 10°C and less than three weeks after they had become adult males and infective females.

Maturation of Howardula sp. larvae was most rapid at 25°C which coincides with the temperature of the spawn-running beds when the mushroom mycelium is most susceptible to phorid invasion (Hussey, 1961), but the reduced yield of male nematodes and the precipitated death in 1-2 days of both sexes at this temperature suggests that it is not optimum. The lower mean yield of infective females at 15°C was compensated by their increased survival of up to 20 days but as phorids do not have overlapping generations, prolongation of life would not compensate for the disadvantage of the longer maturation period at 15°C and is probably of no consequence for control measures because the mushroom sheds are rarely kept at this low temperature. From the results recorded in Sections I 3.2.2. and I 3.3.2. it would appear that the optimum temperature for maturation of free-living

Howardula sp. is between 15°-20°C which would probably produce the most favourable combination of speedy development, liberal yield and adequate survival. If the nematodes were to be utilised at around 18°C in Control experiments as 'biological insecticides' (Welch, 1962) it would necessarily have to be in the cropping sheds when the compost has cooled, and prior to casing. As it was found that infective females only enter the older maggots (in contrast to Hussey's observations) their maturation should coincide with the appropriate stage in the host cycle, assuming that phorid eggs were laid as usual in the spawn running shed. A temporary increase in the moisture content of the compost would probably have to be arranged to facilitate nematode location of the host.

Inherent problems of manipulating Howardula sp. populations at the free living stage are readily apparent. Phorids lay eggs where the mycelial growth is most rapid and as parasitised flies release nematodes at the same site, the chance of infective females encountering maggots is enhanced; the limited dispersal ability of the nematodes would be accentuated if they were applied separately to the compost. In order to overcome this disadvantage it would be necessary to release large numbers of larvae, particularly as the yield of infectives from the second stage is never more than about 25% under artificial conditions, and although it only took a few minutes to obtain thousands of nematodes by shredding flies for the experiments in this study, it would probably be impractical to do it on the scale required for control in mushroom sheds. Release of parasitised flies would be less tedious and more efficient because the larvae would be deposited near phorid eggs and presumably yield high numbers of infective females in the absence of handling trauma and tap water.

Obviously more research is required before conclusions can be drawn about the feasibility of using *Howardula* sp. to control *Megaselia halterata* but there is sufficient evidence from this brief study to suggest that further investigations are warranted. The reduction in egg output (Appendix TABLES 8A and 8B) was statistically highly significant and although it was found that up to 38 eggs could be laid by parasitised flies, in contrast to the 5-6 reported by Hussey (1967), the phorids became completely sterile when several parasites were harboured. Similarly it was found that *Howardula* sp. significantly reduced copulation of *M. halterata* but Hussey (1959) made the opposite observation after finding 83% parasitism in both sexes of mating pairs; as he did not examine non copulating flies from the same population it might be assumed from the results in Section I 3.5.2. that they must have been nearly 100% parasitised. Further investigation is required to clarify this issue but it is probably of more academic than economic interest because the significant effect of the parasite is on host egg production; it was shown that some infected phorids are able to lay fertile eggs.

The seasonal fluctuation of *Howardula* sp. incidence (FIGURE 14) is a common occurrence among parasites; *Heterotylenchus pavlovskii* and *Parasitylenchus diplogenus* (Welch 1965) for example, are similar to *Howardula* sp. in having a host density dependent Autumn peak. Hussey (1959) also found that *Howardula* sp. was most abundant at this time and reached an average parasitism of 80% but he showed that the incidence of the nematode in mushroom sheds tended to rise at the end of the cropping period when phorid numbers were decreasing. This apparent divergence from the usual host density dependent relationship presumably indicates that the parasite was effectively

reducing the fly population at this stage. He suggested that if the autumn and late cropping situation could be artificially shifted to boost the proportion of parasitised flies early in the year, then it may well be possible to prevent the annual outbreaks which are so troublesome in September and October. The present findings support this suggestion but 'early in the season' is judged to be June-July when sufficient phorids are naturally present to avoid the usual biological control procedure of having to introduce the pest before the parasite. If *Howardula* sp. could be released in such quantities that a high incidence of hyper-parasitised flies was produced in one generation, the phorids could be eliminated and subsequently wild populations would be reduced.

The intracacies of planning a complex experimental investigation into how Utopia can be attained are beyond the scope of this study, as is a review of the techniques currently being employed in biological control. However, it is interesting that *Howardula* sp. which has proved to be a fascinating research subject with its truncated life cycle and peculiar metamorphosis into a microvilli-bearing adult, may become economically important in controlling phorid pests. The next Section will examine the ultrastructure of the larval and adult *Howardula* sp. together with three other related nematodes from this interesting group of tylenchs.

I 5. SUMMARY - Section I

1. A tylenchid nematode parasite of *Megaselia halterata* Wood is described and compared with closely related allantonematids, from which it is deduced to be a new species of the genus *Howardula.*

2. The life cycle of *Howardula* sp. is elucidated; second stage larvae leave adult *M. halterata* and exsheath in the external environment to become either mature males or fourth stage females. After copulation, the infective female enters a second or third instar maggot, undergoes a final moult then matures and lays eggs which hatch around the time of pupal eclosion.

3. The life cycle of *Howardula sp.* is truncated to facilitate transmission during the critically brief period when the appropriate phorid larvae are available.

4. The final exsheathment of the female nematode is atypical and is assumed to be the stage at which the cuticle is replaced by microvilli; it is suggested that a similar phenomenon occurs in other species of *Howardula* and probably also in *Bradynema*.

5. Multiple parasitism reduces the size and fecundity of *Howardula* sp.

6. The parasite destroys fat body and follicular membranes of *M. halterata* and reduces copulation and egg output. With increasing hyperinfestation, the host is rendered sterile.

7. *Howardula* sp. is host density dependent and reaches maximum incidence in September and October. Elimination of fly populations in breeding cages is accomplished in 3-5 generations.

8. An attempt is made to discuss the bionomics of the parasite in relation to its possible utilisation as a biological control agent for *M. halterata.*

SECTION II

BODY WALL STRUCTURE, its FORMATION and probable NUTRITIONAL FUNCTION in the PARASITIC FEMALE *HOWARDULA* sp. with comparative observations on *DELADENUS SIRICIDICOLA* Bedding 1968, *D. WILSONI* Bedding 1968 and *CONTORTYLENCHUS* sp. including some of their free living stages.

SECTION II

BODY WALL STRUCTURE, ITS FORMATION AND PROBABLE NUTRITIONAL FUNCTION IN THE PARASITIC FEMALE HOWARDULA SP., WITH COMPARATIVE OBSERVATIONS ON DELADENUS SIRICIDICOLA Bedding 1968, DELADENUS WILSONI Bedding 1968 AND CONTORTYLENCHUS SP., INCLUDING SOME OF THEIR FREE-LIVING STAGES.

II. 1. INTRODUCTION

During the course of the investigations described in Section I, it became obvious that the parasitic Howardula sp. could not feed like other nematodes because it lacked a mouth, oesophagus, intestine and anus. Feeding occurs because the female greatly increases in size after becoming a parasite, and also produces many eggs (Riding, 1970); consequently it was assumed that nutrients must enter through the body wall. An examination of the fine structure of the cuticle was considered a relevant starting point to investigate the feasibility of this phenomenon occurring in Howardula.

Feeding in a variety of nematodes has been described by several workers and the reader is referred to the reviews of Dropkin (1969), Lee (1965a), Fairburn (1960) and Rogers (1962) to appreciate the magnitude of this subject and for further references. Although the nature of the food obtained and the mechanism by which it is acquired, digested, absorbed and assimilated varies, each nematode has the same basic process of ingestion through the stoma and absorption into an intestine. The exceptions of course are the

"non-feeding" stages which simply rely on stored material for their survival, as has been pointed out for example by Jaskoski (1960) and Rogers and Sommerville (1963).

It is the presence of a thick complex cuticle forming the outer covering of nematodes which is thought to prevent uptake of food through the body wall, and this idea has recently been reinforced by Warwick and Chia's (1969) experiments in which labelled glucose was absorbed by two marine nematodes, but it was never found in the cuticle. Also Colam (1969) reported that there was no evidence of cuticular absorption of nutrients in his histo-chemical, biochemical and ultrastructural studies on representatives of some of the main groups of nematodes selected from various habitats.

Deladenus (Tylenchida : Neotylenchidae) was chosen as the second nematode for investigation because, like *Howardula*, the parasitic female is simply "a reproductive tube filled with developing eggs and larvae, has a thick hypodermis and the oesophagus and associated glands are degenerate" according to Bedding (1968). Also it provided an opportunity to observe adaptations to contrasting modes of life in adults of the same sex within a single species, because the life cycle of *Deladenus siricidicola* and *D. wilsoni* is unique in producing three entirely different female forms; each has characteristic morphological features which reflect their contrasting habits and habitats. Zondag (1962) first found these parasites in the wood wasp *Sirex noctilio* in New Zealand. Subsequently Wilson & Spradbury extended the host range to include other siricid species in Europe and their ichneumonid and cynipid parasitoids, as reported by Bedding (1967) who described the life cycle briefly.

The body wall of the young parasitic *Deladenus siricidicola*

was examined, rather than that of the mature parasite, because it was suspected that the older worms may have deteriorated after some 9 months or more inside the host, being subjected to the action of the larvae they contain. Many worms were processed, ranging from one to three months duration inside the host, but it was obvious from the inconsistencies observed that the body wall was still differentiating and that older parasites would have to be examined.

Ultrastructural studies were also made on the mature parasitic Deladenus wilsoni and Contortylenchus sp. Al-Rabiai (1970) described the life cycle of Contortylenchus sp. which is basically the same as Howardula; the mature female is parasitic and reproduces inside the host's haemocoel and the male is free-living. The infective female Contortylenchus penetrates the pupa of the bark-boring beetle Ips sexdentatus and often becomes encapsulated during maturation. Eventually it releases larvae into the capsule and these ultimately escape into the host's haemocoal, penetrate the gut and escape from the beetle as ensheathed fourth stage larvae. Moulting and copulation occur in the frass before the infective female is ready to penetrate another host.

During her investigation of the body wall at the light microscope level, Al-Rabiai (1970) examined transverse sections of the adult parasitic female and reported the presence of three conspicuous folds; two in the dorsal and one in the ventral region. She comments that Contortylenchus has no functional mouth and gut but a cuticle is present, and she suggests that if feeding takes place through the body wall, it must be in the region of the folds where the hypodermis is very thin. These discrepancies in a nematode closely related to Howardula stimulated the current ultrastructural

study, but it is regretted that only a preliminary investigation has yet been made.

Considerable difficulty was experienced with most of the worms examined in this study during the various processes required to prepare the material for electronmicroscopic examination. However, in order to demonstrate that the techniques used in this study would have produced structures in cuticles, myofilament patterns in muscles and recognisable nerves had they been present, PLATE 3 is included; it shows the 3 layers of the body wall of Chromadorina viridis, but it must be emphasised that this nematode is in no way related to the Tylenchs of the present study.

In concluding this introduction to Section II, the objectives are briefly summarised:

1. To investigate the body wall structure of the mature female Howardula sp. (Tylenchida : Sphaerularidae), Deladenus siricidicola, D. wilsoni (Tylenchida : Neotylenchidae) and Contortylenchus sp. (Tylenchida : Sphaerularidae), all of which live in the heaemocoel of insects and do not possess a functional mouth or gut.

2. To relate these morphological and anatomical observations with the probability of the body wall of each worm having a nutritional function.

3. To examine the ultrastructure of their free-living stages for comparative purposes, to investigate possible adaptations to different environments similarly encountered by these related genera.

4. To observe the nature of the metamorphosis which occurs when the free-living larvae become parasites.

5. To consider if the information obtained has any phylogenetic significance; particularly with regard to Deladenus sp. which according to Bedding (1968) is a Neotylench but which Nickle (personal communication) maintains is a Sphaerularid, as are Howardula and Contortylenchus.

PLATE 3.

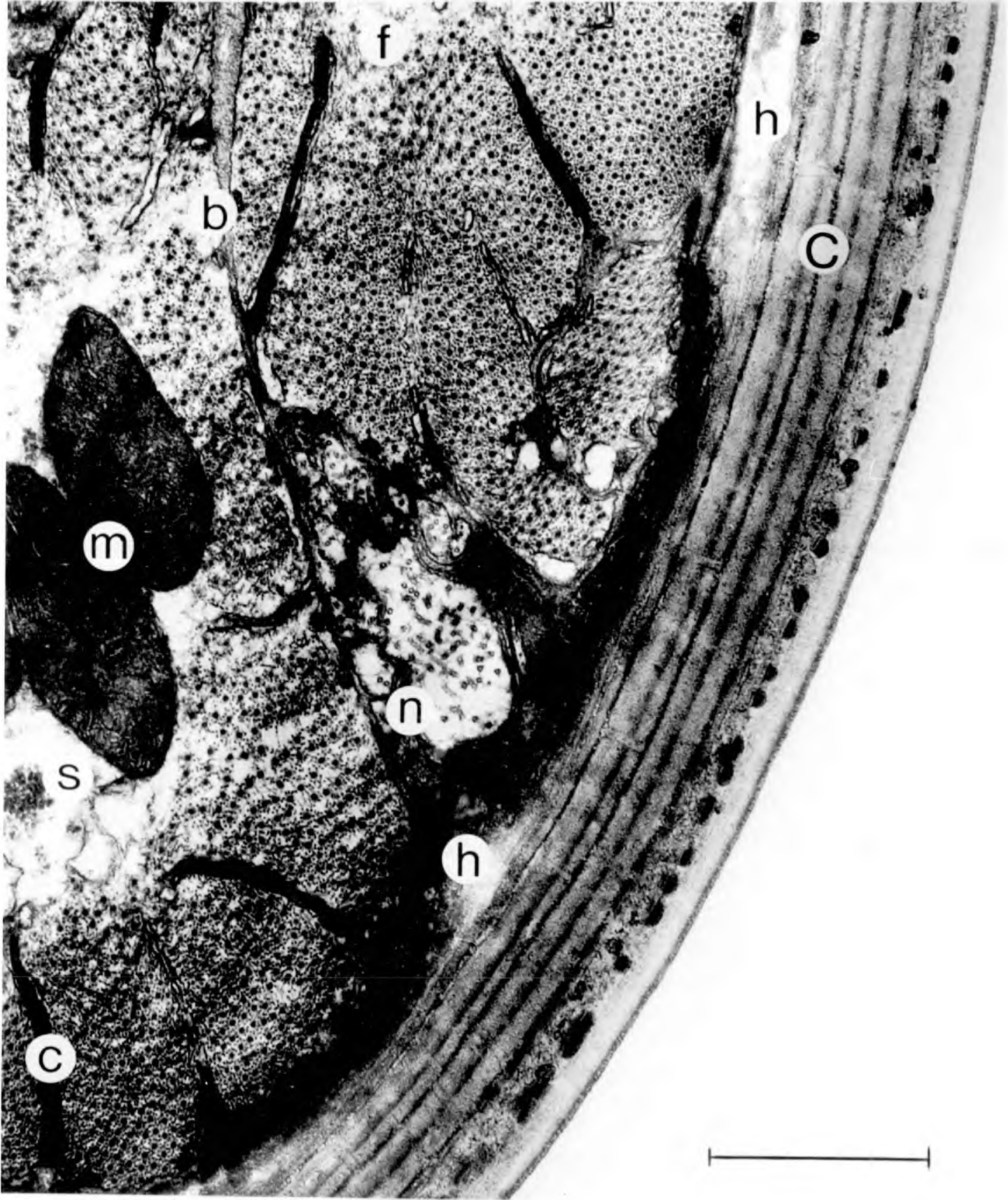
TRANSVERSE SECTION THROUGH THE BODY WALL OF *CHROMODORINA VIRIDIS*.

This electronmicrograph is included to show some of the features normally observed in the nematode body wall.

C	multilayered cuticle
h	hypodermis
f	myofilaments of somatic muscle cell
c	cytoskeletal structure of muscle cell
s	sarcoplasmic region of muscle cell
m	mitochondria
b	basal lamina
n	nerve axon

(The scale on this, and all other electronmicrographs represents 1 um.)

PLATE 3



II. 2. MATERIALS AND METHODS

Parasitic and free-living stages of Howardula sp. were obtained from the sources described in Section I.

Mr. F. Wilson of the C.S.I.R.O. Sirex Biological Control Unit, Silwood Park, Ascot, supplied the Deladenus parasitic stages in insects collected at Ave et Auffe, Winenne, Belgium. These were the wood wasp Sirex noctilio F. infected with D. siricidicola Bedding and the Ichneumonid parasitoids Pseudorhyssa sternata Merrill and Rhyssa persuasoria (L) infected with D. wilsoni Bedding. Dr. Robin Bedding of the C.S.I.R.O. Tasmanian Regional Laboratory confirmed identification of the parasites and also provided the mycetophagous and infective female forms of D. siricidicola which had been cultured from insects collected in Greece and Spain.

Dr. Suad Al-Rabiai allowed me to use Contortylenchus sp. from the bark-boring beetle Ips sexdentatus Boerner which she bred (Al-Rabiai 1970) from insects originally supplied by the Alice Holt Forestry Commission Station near Farnham, Surrey.

A variety of fixatives was used for each group of nematodes; many trials were made varying the concentration of solutions and using different buffer combinations as well as altering the time spent in each. Osmium tetroxide, glutaraldehyde and potassium permanganate were the main fixatives used with sodium veronal-sodium acetate, phosphate or cacodylate as the buffers, with or without sucrose or glucose.

Ultimately the most consistently satisfactory results were obtained by fixing for 3-4 hours at 4°C with 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) with added calcium chloride, followed by several washes in cacodylate buffer containing 7.5%

sucrose. This method was based on the investigations of Gordon et al. (1963) and gave the best fixation for the parasitic stages. Details of how the solutions were made up may be considered superfluous, but it is probably worth recording that errors can unwittingly be made during this procedure, particularly with the glutaraldehyde. TAAB Laboratories at Emmer Green, Reading supply a processed "ready to use" 25% glutaraldehyde which is recommended as a time-saver but it was found that the bottle must be shaken for several minutes immediately prior to use; otherwise inconsistent results were obtained with this fixative. The 0.1 molar cacodylate buffer was prepared from 2.14 gm sodium cacodylate in 100 ml distilled water and a few drops of concentrated hydrochloric acid were added to adjust the pH to 7.2-7.4; this was stored in the refrigerator until required and then diluted with equal parts distilled water to make the 0.05M buffer. 5 ml. of the 25% glutaraldehyde were added to 45 ml. of the cacodylate buffer, followed by 0.25 ml of 1% aqueous calcium chloride solution to complete the 2.5% glutaraldehyde fixative. The presence of calcium chloride appeared to give better fixation of membranes.

Many of the specimens were "postfixed" in Millonig's (1961) phosphate buffered osmium tetroxide for 1-3 hours at room temperature but no significant difference in fixation was noticed although there was some indication of improved contrast. Some of the parasites were treated with this fixative alone, in which case a longer exposure was usually required and up to 18 hours appeared to give improved results. On the contrary it is interesting to note that prolonged treatment with glutaraldehyde did not enhance fixation and it was suspected that leaching had occurred.

The free-living stages of the nematodes were similarly fixed but marginally better results were produced by using sodium acetate-sodium veronal buffered solutions. Palade's (1952) and Zetterqvist's (after Kay 1967) veronal buffered osmium tetroxide gave reasonable fixation but Rosenbluth's (1965) was possibly slightly superior. Essentially the same basic buffer was used with potassium permanganate were distinct, the cytoplasm was markedly granular.

Osmium tetroxide were also buffered with disodium phosphate and glucose was added (Caulfield, 1957) but the results were not substantially improved. As experience was gained, it became increasingly obvious that the fixative/buffer combination was not critical. Many nematodes are poorly fixed regardless of the process used and discrepancies occur between different regions of the same worm and even in adjacent areas of the same section! Consequently specimens are always selected from the best material in each group and the electron micrographs from each treatment are often indistinguishable. This observation is supported by Malhotra's experiments (1962a, b; 1963) in which he investigated the effects of various buffers on osmium fixation and concluded that the quality of preservation was in no way superior to that produced by simple distilled water solutions of osmium tetroxide alone.

The greatest single factor contributing to improved fixation was undoubtedly cutting the nematodes into small pieces (Bird, 1964), ideally 0.1-0.3 mm long. Most fixatives had little effect on the free-living stages and many worms could survive several hours, particularly in glutaraldehyde, so penetration of the solution was facilitated by piercing the cuticle. The worms were chopped up after being in the fixative for several minutes; it was necessary to use a dissecting microscope and each nema was cut with the sharp edge of a

hypodermic needle point. The pieces were then left in the fixative for a further 3 to 4 hours either at $4^{o}C$ or at room temperature; cooling did not appear to affect fixation but the pungent solutions were less unpleasant to use in the cold.

The parasitised insects were frequently dissected in the fixative because entomological saline often killed the adult nematodes. Rapid penetration of the solution occurred but better fixation was achieved when the worms were cut into several portions.

After being fixed, the nematodes were washed in the appropriate buffer with added sucrose and then embedded in agar, following the method described by Wright and Jones (1965). This was essential not only to enable orientation of the specimens for sectioning, but because it was rarely possible to collect sufficient material to concentrate the pieces by the less troublesome centrifugation method. Initially only one specimen was placed in each agar block but the inefficiency of this method will be appreciated in view of the previous comments about variable responses of individual worms to fixation! About 12 pieces of nematode were normally embedded together and even this number was sub-optimum because it necessitated cutting several blocks to obtain a few good sections.

Dehydration was carried out through a graded alcohol or acetone series followed by xylene and propylene oxide before introducing the epoxy resin. Initially the araldite "Durcupan" (Fuka Buchs, Switzerland) was used until TAAB Laboratories introduced their new embedding resin which has a lower viscosity and is readily miscible with alcohol, thus eliminating the necessity for xylene and propylene oxide. However, care must be taken to ensure that the absolute ethanol is water-free either by using anhydrous copper sulphate for instance, or a molecular sieve such as BDH type 13X pellets.

The TAAB resin was freshly made up prior to use. Portions were warmed to 40°C then vigorously shaken with absolute ethanol to provide a six-stage series of increasing concentration from alcohol to pure resin. The agar blocks containing the nematodes were passed up this series, pausing about 30 minutes in each mixture or as long as it took for the agar to sink; presumably this indicated that equilibrium was reached. These embedding mixtures were kept at 40°C but after entering the pure resin the worms were cooled to 25°C overnight and continuously agitated. They were transferred to fresh resin the next morning and polymerised at 60°C for two days. It was noticed that at least a fortnight was required for the blocks to "mature" before sectioning and improved cutting properties were apparent after a post-polymerisation period of several weeks.

Although the TAAB resin is recommended because of its superior embedding qualities, it has the disadvantage of polymerisation inconsistencies. Occasionally the blocks failed to harden completely yet when returned to the oven they melted, which should be impossible after initial polymerisation. Also there was a tendency for the tips of the block to remain soft, particularly when the resin is processed in Beem capsules; TAAB attribute this to a contaminent from the capsule templet and indeed this phenomenon was not encountered when the resin was polymerised in gelatine capsules.

The formulations recommended for hard and soft blocks were found to be unsatisfactory; obviously a hard resin is required for nematode cuticle to be sectioned but the suggested 2:1:1 proportions of resin: DDSA:MNA was far too brittle and the blocks tended to shatter during trimming. After considerable experimentation the combination of 10 parts resin: 7 parts DDSA : 3 parts MNA was

found to have the most satisfactory properties.

Sections were cut with either a 44° diamond knife from Ge-Fe-Ri, Frosinone, Italy or with glass knives made on an LKB Life-Maker Type 7801A, to give pale gold or silver-grey interference colours. They were usually collected on uncoated 50 or 100 micron copper grids but initially wider-aperture grids with formvar films were used. However, the advantages of being able to view a larger area of section were counteracted by the imperfections in the formvar membrane. When not coated, the grids were dipped into a cellotape-chloroform mixture to ensure adherance of sections.

Staining was usually done with lead and/or uranyl acetate, but occasionally 1% potassium permanganate was used (Pease 1964). Karnovsky's (1961) lead hydroxide was tried but better overall contrast was obtained with lead citrate as prepared by Reynolds (1963). A variety of saturated uranyl acetate solutions were sampled including aqueous (Watson, 1958) and 50% ethanol (Gibbons & Grimstone, 1960) but usually increased contrast was obtained with methanol as the solvent. The sections were stained for 30 minutes in this solution but it was necessary to rinse off excess uranyl acetate with methanol afterwards. Equally important was the rinse with 0.02N sodium hydroxide after staining with lead citrate; considerable crystalline debris was present when distilled water alone was used for the wash.

Sections were also mounted on thin glass cover-slips and stained with toluidine blue in 1% borax solution for examination with the light microscope.

Electronmicroscope observations were made with either a Siemens Elmiskopp I, and JEOL Co. Ltd. Jem 7, an AEI EM6 or an EM6B operated at 60, 75, 80 or 100 KV.

Attempts were made to investigate the possibility of large macromolecules being absorbed through the body wall of parasite *Howardula* sp. and *Deladenus siricidicola*. Ferritin was chosen because it is very electron-dense and therefore readily visible with the electronmicroscope. Koch-Light Laboratories 2600t ferritin, crystallised twice from horse serum, was used in a 10% solution made up with Clarke's modified entomological saline. Undamaged living parasites freshly dissected from their hosts were put into the ferritin solution for periods of ½ minute to 10 minutes, increasing the time by one minute stages. Control nematodes were placed for similar periods in Clarke's entomological saline alone and other worms were fixed in Gordon's cacodylate buffered glutaraldehyde and then exposed to the ferritin solution for as long as 10 minutes.

All the nematodes were rinsed in saline then fixed in Gordon's glutaraldehyde for 2-4 hours, washed in cacodylate buffer with sucrose and osmicated in Millonig's phosphate buffered osmium tetroxide for 1-3 hours before dehydrating, embedding and sectioning as described previously.

II. 3. OBSERVATIONS

II. 3.1. The free-living infective *Howardula* sp.

PLATE 4 shows a transverse section through the body of the infective female *Howardula* sp. and the typical nematode arrangement of layers can be seen. The cuticle on the outside surround the hypodermis which is expanded to form cords in the dorsal, ventral and lateral regions thus dividing the somatic musculature into four sectors. The muscle cells abut the hypodermis in the intercordal regions and are platymyarian with a meromyarian arrangement. Usually only 2 muscle cells are seen in each sector but up to 4 may be present, depending on the degree of overlap with the spindle-shaped ends of anterior or posterior cells.

The cuticle is 0.15-0.21μm wide (mean = 0.18μm) and has superficial transverse grooves which can be seen in longitudinal section (PLATE 5). Six layers can frequently be distinguished in glutaraldehyde-fixed larvae (FIGURE 17A and PLATE 5): an outer electron-dense layer which does not appear to be trilaminate; a thin electron-transparent zone which merges with a dense layer below, together they are 15-44 nm wide (mean = 25 nm) and can probably be interpreted as the outer and inner regions of the outer cortex; a granular homologous zone 22-45 nm wide (mean = 36 nm) comprising the inner cortex; a striated layer in the matrix and finally a basal layer about 20 nm wide which may represent a single fibre layer or a basal lamina (PLATE 6) but which is frequently not discernable.

The striated layer completely encircles the body and has a similar appearance whether viewed transversely or longitudinally; usually the striations are perpendicular to the surface but they are

occasionally obliquely orientated. This region is 66-105 nm deep (mean = 89 nm) and has dark electron-dense bands 4nm wide separated by a less dense space 11nm wide. The superficial annulations of the cuticle never extend down to the striated layer and usually only the outer regions of the cortex are involved.

The cuticle is modified in the regions of the lateral fields (PLATE 7) where the cortical layers are expanded to form conspicuous folds. Typically there are 6 ridges and the 2 outer ones are much larger than the others, but variation occurs in the anterior and posterior regions of the nematode where the lateral alae terminate. Dense material is present in the outer ridges which resembles the outer cortex of other regions, otherwise the cuticle of the lateral fields is amorphous except beneath the folds where there are two fibre layers. These fibres are contiguous with the striated zone which is absent in the lateral field and the fibrils in each fibre layer are similarly aligned. The angle at which the fibres of one layer cross the other is about 100°.

The hypodermis is usually very dense and has local thickenings with many half-desmosome attachments to the muscle cells and to the cuticle (PLATE 6). Nuclei and mitochondria are lacking in the intercordal hypodermis which is 29-111nm wide, but rough endoplasmic reticulum and glycogen are frequently seen. In the median and lateral regions (PLATE 7) there are mitochondria, lipid droplets, glycogen, rough endoplasmic reticulum and vesicles containing fibrillar material; nuclei with prominent nucleoli are present in the lateral hypodermal cords only. The membrane which marks the cuticle-hypodermis boundary is frequently convoluted, in contrast to the underlying basal lamina which separates the hypodermis from the somatic musculature. This granular layer is 28-44 nm wide

(mean = 35nm) and it completely surrounds the sarcolemma of the muscle cells.

Unlike other platymyarian muscle cells those of Howardula larvae are not obviously separated into contractile and non-contractile regions (PLATE 6). The sarcoplasmic region if present but apparently confined to a limited area near the neuromuscular process, consequently mitochondria and other organelles of the sarcoplasm are only rarely encountered in sections (PLATE 8). The most prominent feature of the somatic muscle cells are the large myofilaments (23 nm diameter) which probably contain myosin and the smaller myofilaments (4.5nm diameter) which surround them and probably contain actin. The "H" "A" and "I" bands can be recognised by the occurrence of large myofilaments alone (H band), large myofilaments surrounded by the small ones (A band) and small myofilaments alone (I band). "Z" bands or other supporting cytoskeletal fibres have not been observed. The somatic muscle cells are clearly demarked from the pseudocoelomic cavity by the surrounding basal lamina which follows the contours of the sarcolemma.

PLATE 4

OBLIQUE TRANSVERSE SECTION THROUGH AN INFECTIVE FEMALE HOWARDULA sp. IN THE ANTERIOR REGION.

c cuticle
h hypodermal cords
m somatic muscle
a amphid cilia
l lipid
s stylet lumen with dense contents
g guide sheath membranes attached to stylet
p protractor muscles of stylet

PLATE 4

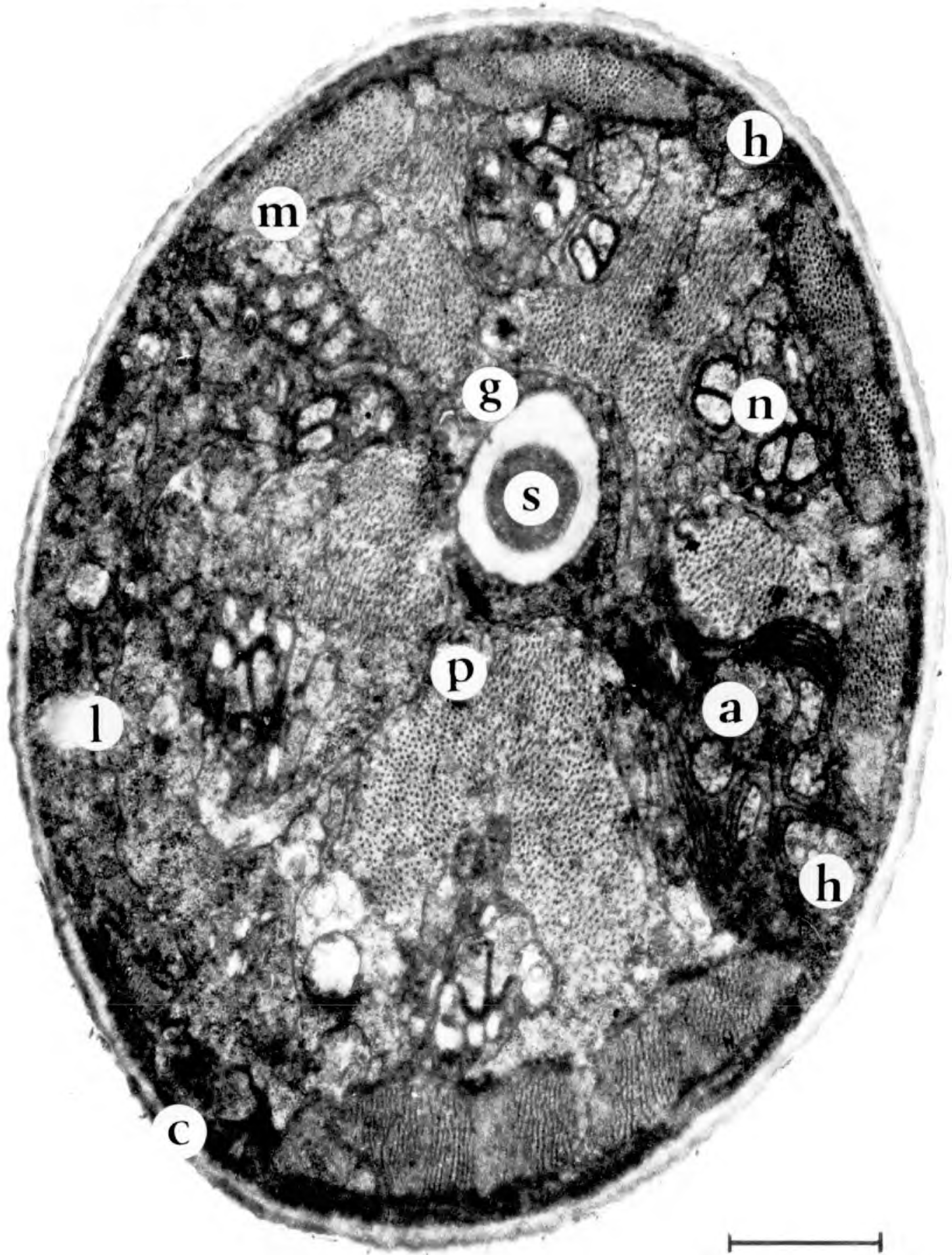


PLATE 5

LONGITUDINAL SECTION THROUGH THE CUTICLE OF INFECTIVE FEMALE HOWARDULA sp.

d dense outer layer
o outer cortex comprising external and internal regions, the latter being more electron dense
i inner cortex
s striated zone
h hypodermis

PLATE 6

TRANSVERSE SECTION THROUGH THE BODY WALL OF INFECTIVE FEMALE HOWARDULA sp.

c cuticle
h hypodermis
b basal lamina
s somatic muscle cell

PLATE 5

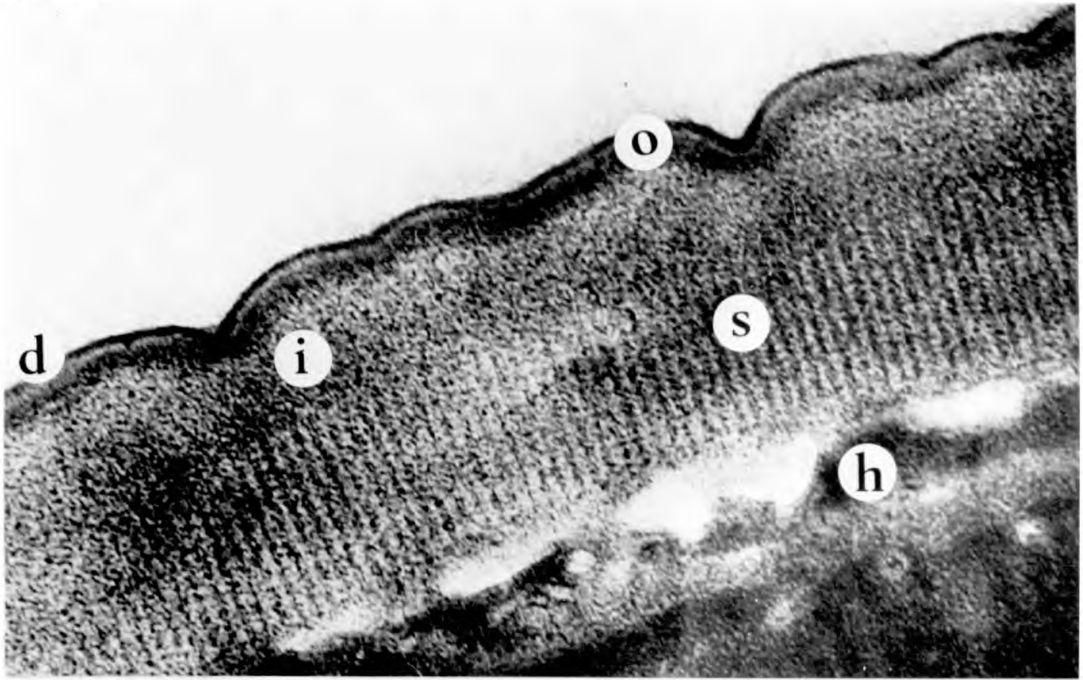


PLATE 6

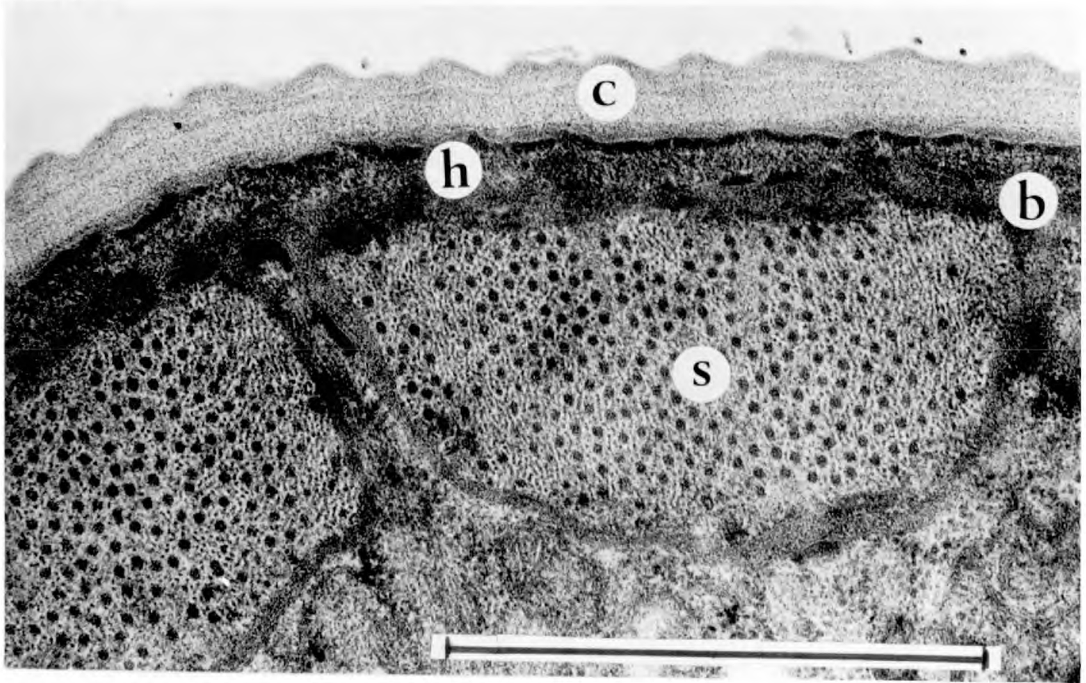


PLATE 7

TRANSVERSE SECTION THROUGH THE BODY WALL OF INFECTIVE FEMALE HOWARDULA sp. IN THE LATERAL FIELD.

L	lateral lines
1, 2	fibrillar layers of cuticle
c	cuticle
m	mitochondria
h	hypodermal cord
l	lipid
n	nucleus with prominent nucleolus

PLATE 7

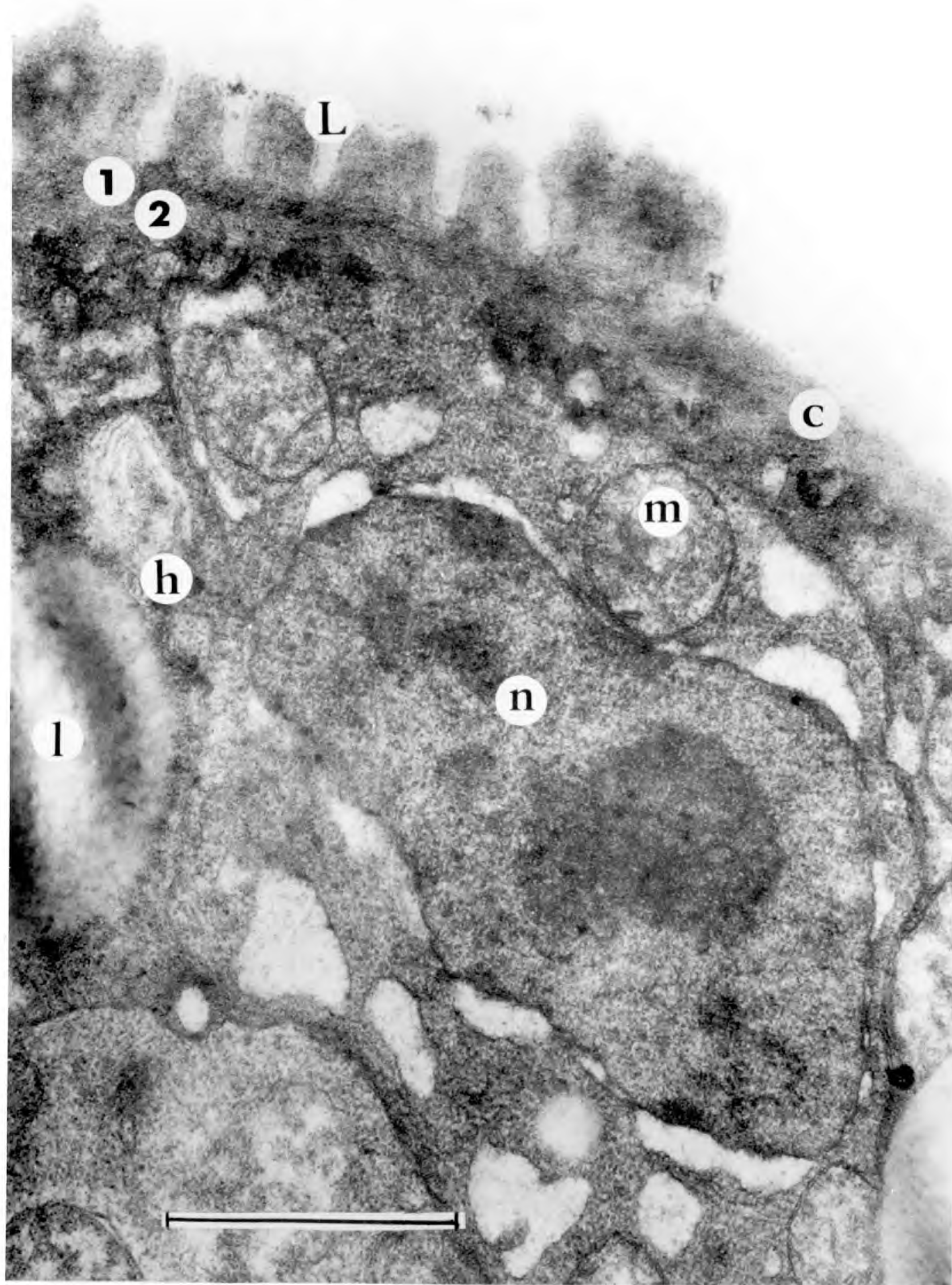
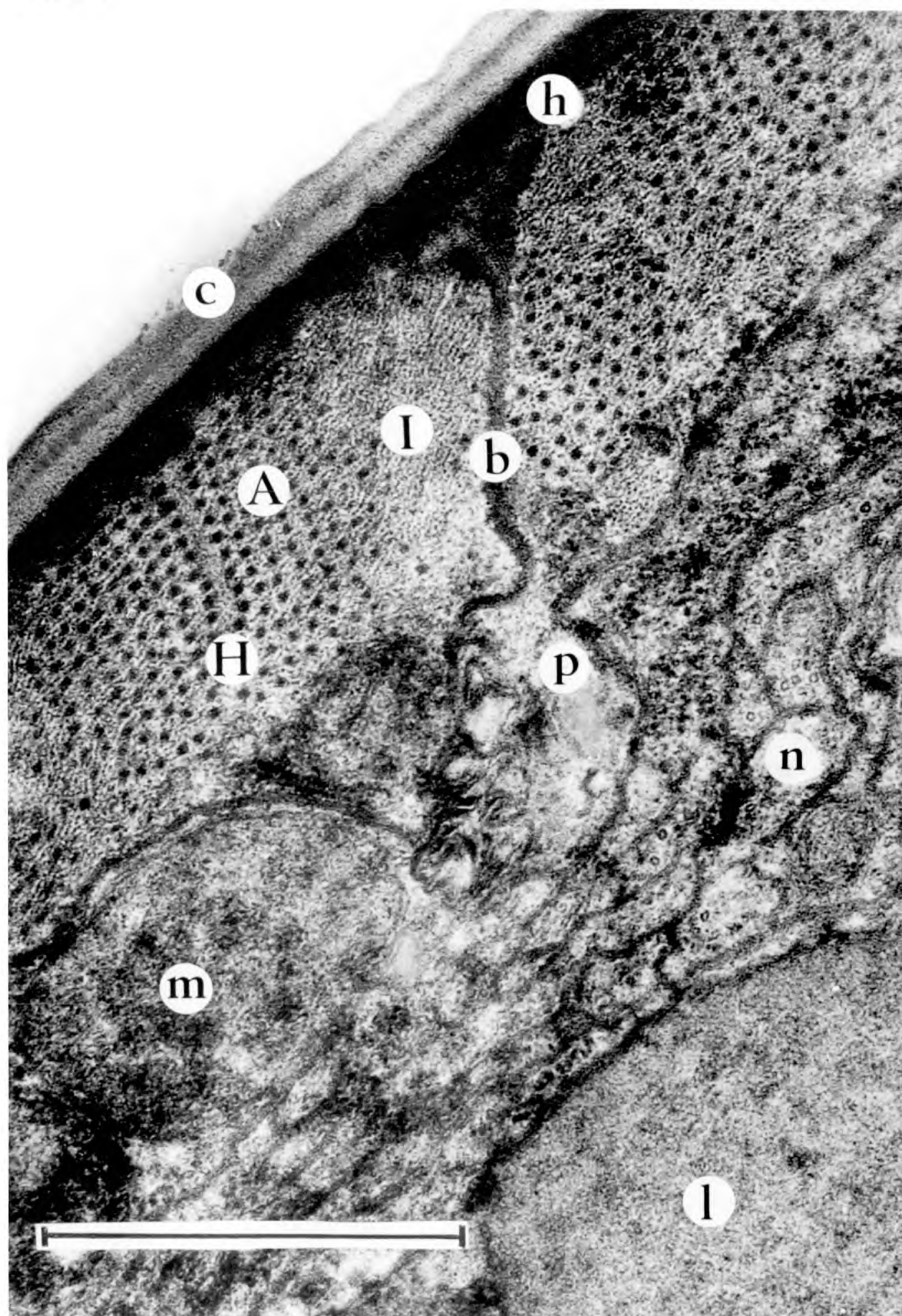


PLATE 8

TRANSVERSE SECTION THROUGH THE BODY WALL OF HOWARDULA sp. INFECTIVE FEMALE IN AN INTERCORDAL REGION.

c	cuticle
h	hypodermis
H, A, I	zones of myofilaments in somatic muscle cell
b	basal lamina
p	neuromuscular process of somatic muscle cell
n	nerve axons
m	mitochondria
l	lipid

PLATE 8



II. 3.2. MOULTING OF THE YOUNG PARASITIC HOWARDULA Sp.

The first obvious sign of moulting at the ultrastructural level is the hypertrophy of the hypodermis, particularly in the intercordal regions. The nuclei enlarge considerably and the nucleoli are much more prominent. The mitochondria are more abundant and there is increased rough endoplasmic reticulum, free ribosomes and glycogen. The hypodermal membrane underlying the cuticle increases its surface area and becomes greatly convoluted. PLATE 9 shows a transverse section through the body of a young moulting parasite and by comparing it with PLATE 4 which illustrates the free living infective larva, the magnitude of the hypodermis hypertrophy will be appreciated.

Interesting concurrent though probably unrelated changes occur to the external surface of the nematode after it has been inside the host for a few hours. Large particles collect on the outside of the cuticle which are quite unlike debris that occasionally adheres to the cuticle of the free living larvae (PLATES 10 and 11 and FIGURE 17B, C, D). They are very electron dense and range from 24-72 nm diameter (mean = 43 nm); initially they are large and sparse but gradually appear to break down and form a frequently uninterrupted layer of fine particles around the periphery of the cuticle. Without exception they are present on every moulting parasitic larva examined, but they have never been seen on the free living larvae.

As moulting proceeds, the hypodermis develops a multitude of vesicles which impart an alveolate appearance and account for the further increase in volume of the hypodermal regions. Most of the vesicles contain filamentous material and they probably arise via

cisternae from rough endoplasmic reticulum or ergastoplasm. PLATE 11 illustrates the abundance of the vesicles in the hypodermis at an advanced stage of moulting and the change will readily be perceived when compared with the condition shown in PLATE 10 at the onset of moulting.

Roughly synchronised with the initiation of vesicle production, the basal layers of the cuticle begin to break down and dense fibrillar material collects below. (PLATE 12 and FIGURE 17B, C). The hypodermis becomes separated from the cuticle by the mass of fibrils but some of its desmosome attachments still adhere. The striated layer of the cuticle persists but is reduced and may contribute to the formation of the fibrillar material. Similarly the cortical layers may also be involved because the cuticle is reduced and the striated layer appears nearer to the surface of the nematode.

Concurrent with these developments a second component is seen in the space between the cuticle and hypodermis and this probably arises from the hypodermis. (PLATES 12 and 13 and FIGURE 17C). It consists of dense granular particles and is usually clearly demarked from the fibrillar material although interconnecting zones sometimes occur where interchange of material is suggested (PLATE 13).

There is some indication that the granular material is released from the vesicles at the surface of the hypodermis and the membrane walls of the empty vesicles fuse with the hypodermal membrane to increase its surface area. This enlarged plasma membrane then forms many finger-like folds which ultimately become the microvilli on the outside of the adult nematode.

The muscle cells also appear to undergo changes during the moult because the contractile region of myofilaments is reduced, with a corresponding increase in the sarcoplasm (PLATE 13). After

considerable deterioration there is a little reformation but the muscle cells of the adult are never as complete as those of the larvae.

Further differentiation of the microvilli is seen in PLATE 14 and the granular component can no longer be distinguished from the fibrous material which underlies the old 4th stage cuticle. Eventually this material disappears and may possibly be absorbed through the microvilli and contribute to the formation of the fibrillar supporting elements which are later observed. An apparently empty space is left between the larval cuticle and the adult microvilli and exsheathment occurs at this stage. (PLATE 15 and FIGURE 17D). The striated layer is no longer visible in the moulted cuticle which has been reduced to about half its original depth.

FIGURE 17 illustrates some of the changes which have been described in this final moult of the female Howardula sp.

FIGURE 17.

DIAGRAM TO SHOW THE CHANGES WHICH OCCUR DURING THE FINAL MOULT OF THE FEMALE *HOWARDULA* sp.

FIG. 17A. The cuticle and hypodermis of the free living fourth stage larva.

OL = outer layer
O = outer region of outer cortex
I = inner region of outer cortex
OC = outer cortex
IC = inner cortex
M = matrix
Sl = striated layer
B = basal lamina
H = hypodermis

FIG. 17B. Onset of moulting - after about 12 hours inside the host. Dense granules (D) appear on the outside of the larval cuticle; the hypodermal membrane (HM) becomes convoluted and separates from the cuticle; vesicles (V) are formed in the hypodermis; there is an increase in rough endoplasmic reticulum, mitochondria, ribosomes and glycogen.

FIG. 17C. Moulting well advanced - after 12-36 hours inside the host. Granular material (G) is released from the vesicles (V) in the hypodermis and mixes with fibrillar material (F) from the old cuticle. The hypodermal membrane forms finger-like projections which will eventually become microvilli.

FIG. 17D. Completion of the moult - after 36-48 hours inside the host and prior to exsheathment. The larval cuticle (C) is separated by an apparently empty space (S) from the microvilli of the adult (MV) and part of it has been resorbed. Fibres (F) support the microvilli and the hypodermis (H) has now attained the adult condition.

FIG.17.

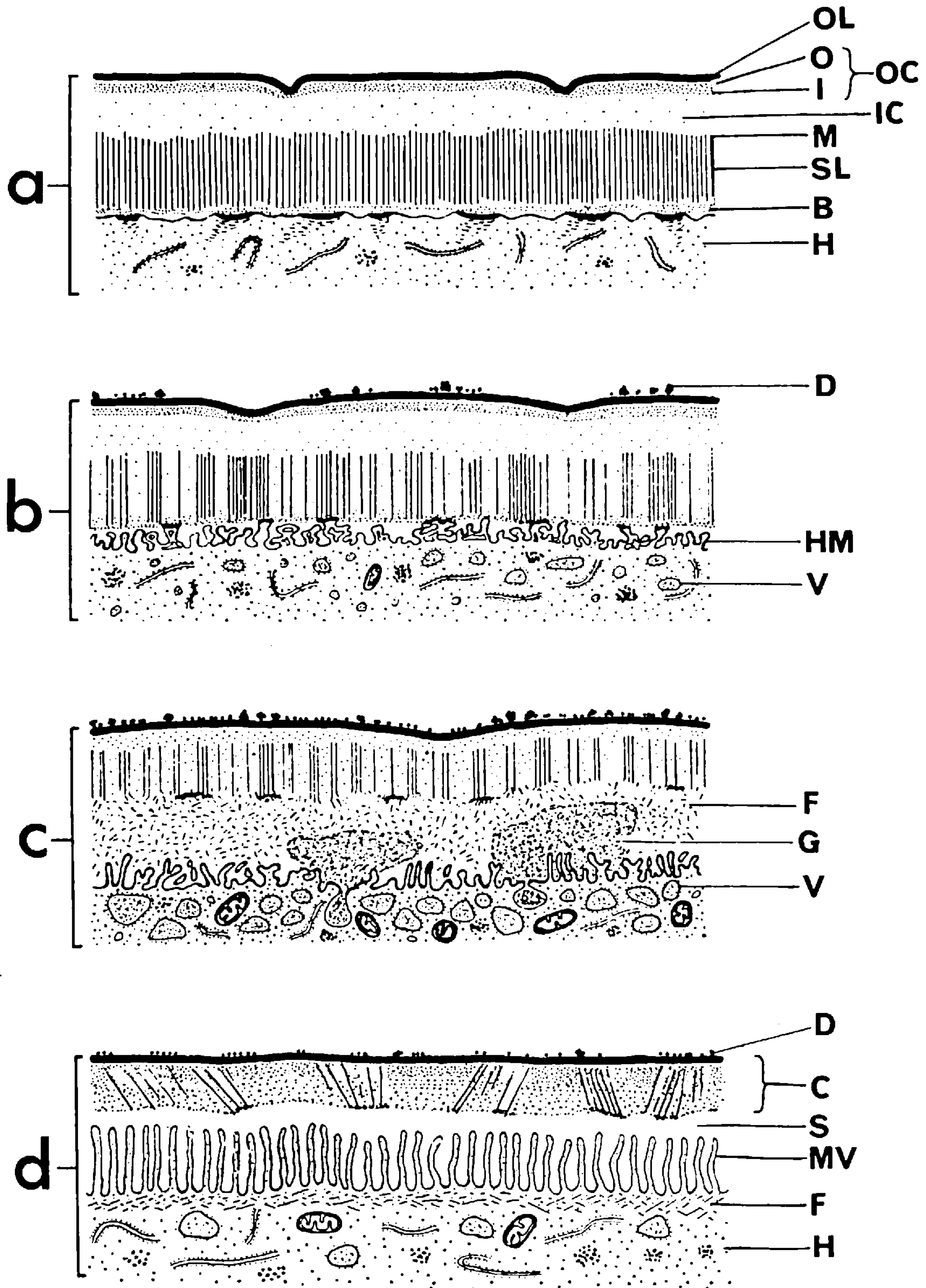


PLATE 9.

TRANSVERSE SECTION THROUGH A MOULTING PARASITIC *HOWARDULA* sp.

H	greatly hypertrophied lateral hypodermal cords
h	median hypodermal cords
s	somatic muscle sectors
n	nucleus of hypodermis
m	microvilli of adult
c	cuticle of fourth stage larva

PLATE 9

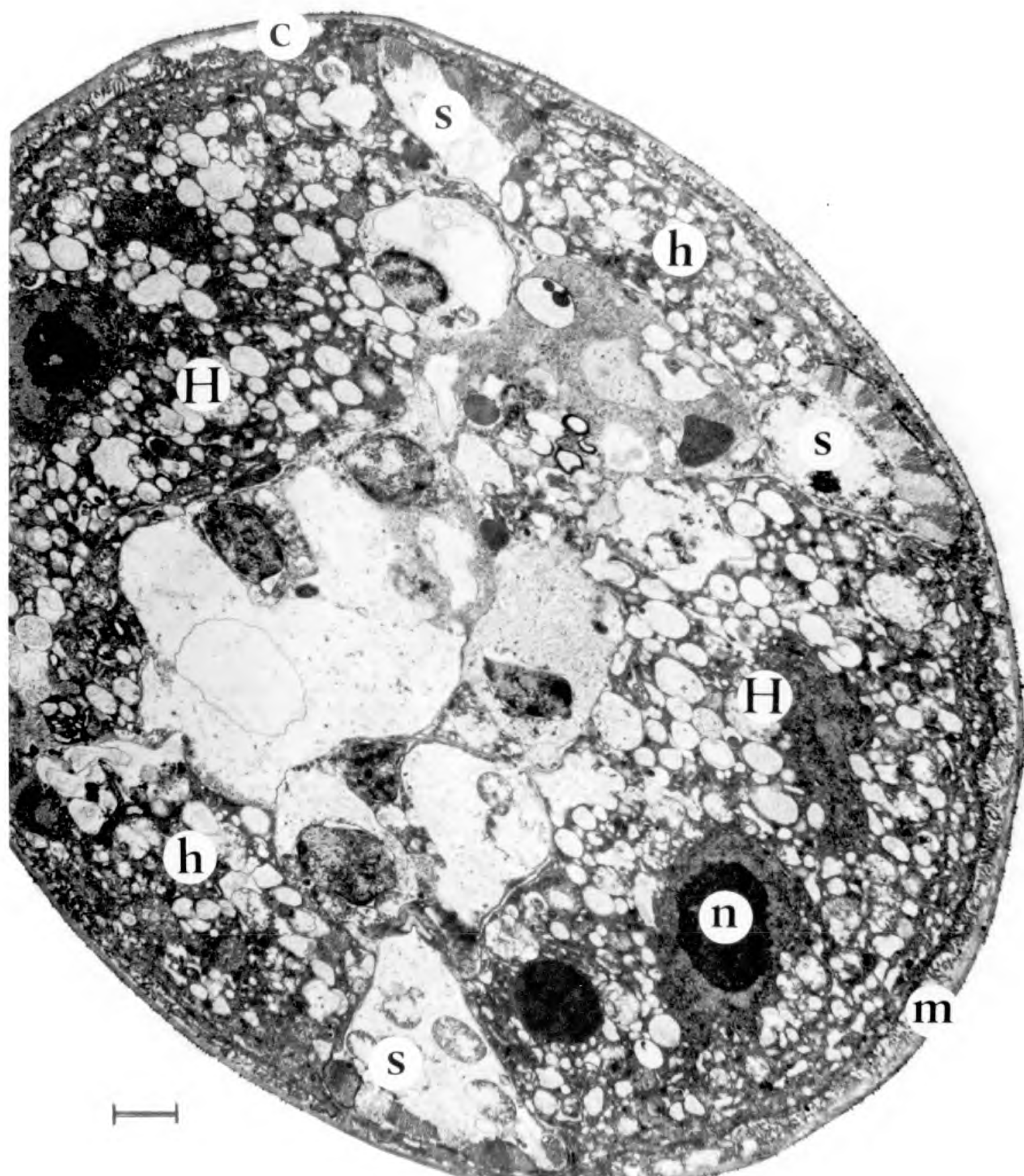


PLATE 10.

TRANSVERSE SECTION THROUGH THE CUTICLE AND HYPODERMIS OF A MOULTING PARASITIC HOWARDULA sp.

The hypodermal membrane (m) has become more dense ans is convoluted. It is still in contact with the cuticle (c) and dark particles (d) are present on the outside of the nematode. The nucleus (n) has a prominent nucleolus and many ribosomes are visible in the hypodermis (h).

PLATE 11.

TRANSVERSE SECTION, AS PLATE 10 ABOVE, AT A LATER STAGE OF MOULTING.

The hypodermal membrane has formed into microvilli (m) and the cuticle (c) is diffuse. The hypodermis (h) contains many vesicles (v) with fibrillar material. Many dark granules are present on the external surface.

PLATE 10

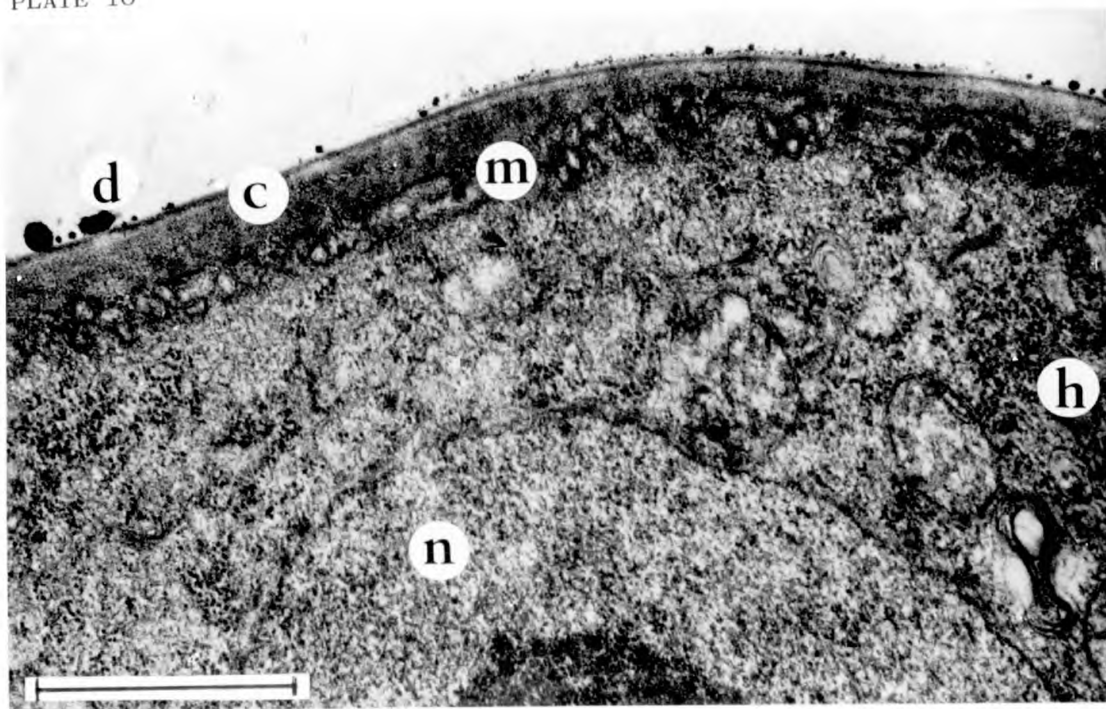


PLATE 11

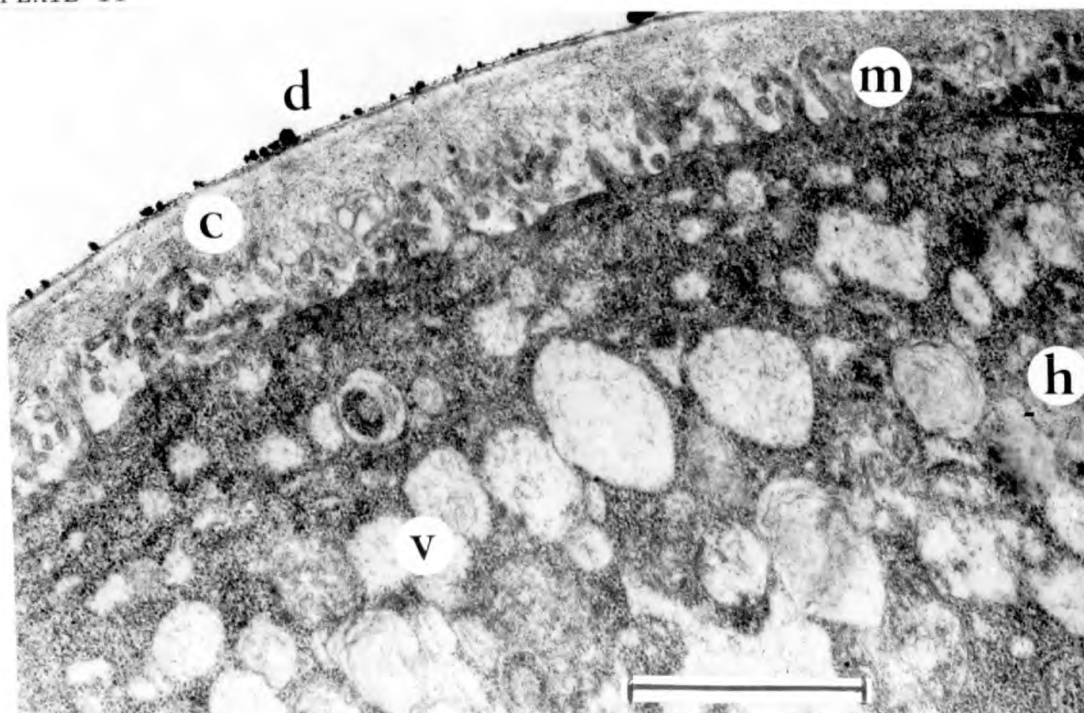


PLATE 12 (opposite) and PLATE 13 (next page).

TRANSVERSE SECTIONS THROUGH THE BODY WALL OF MOULTING PARASITIC *HOWARDULA* sp.

Microvilli (m) are differentiating from the hypodermis (h) and the space below the larval cuticle (L) is filled with fibrous (f) and granular (g) material. The latter appears to arise from the hypodermis at sites marked by asterisks. The double asterisks in PLATE 13 indicate an area where the granular and fibrillar components merge. Arrows mark the dense particles on the external surface and near to the arrow in PLATE 12 the persisting striated layer with desmosome connections can be seen. Many vesicles (v) are present in the hypodermis.

Additional labels:

PLATE 12

s surface of hypodermis.
l lipid.

PLATE 13

d desmosome.
b basal lamina.
s sarcoplasmic region of somatic muscle cell.

PLATE 12

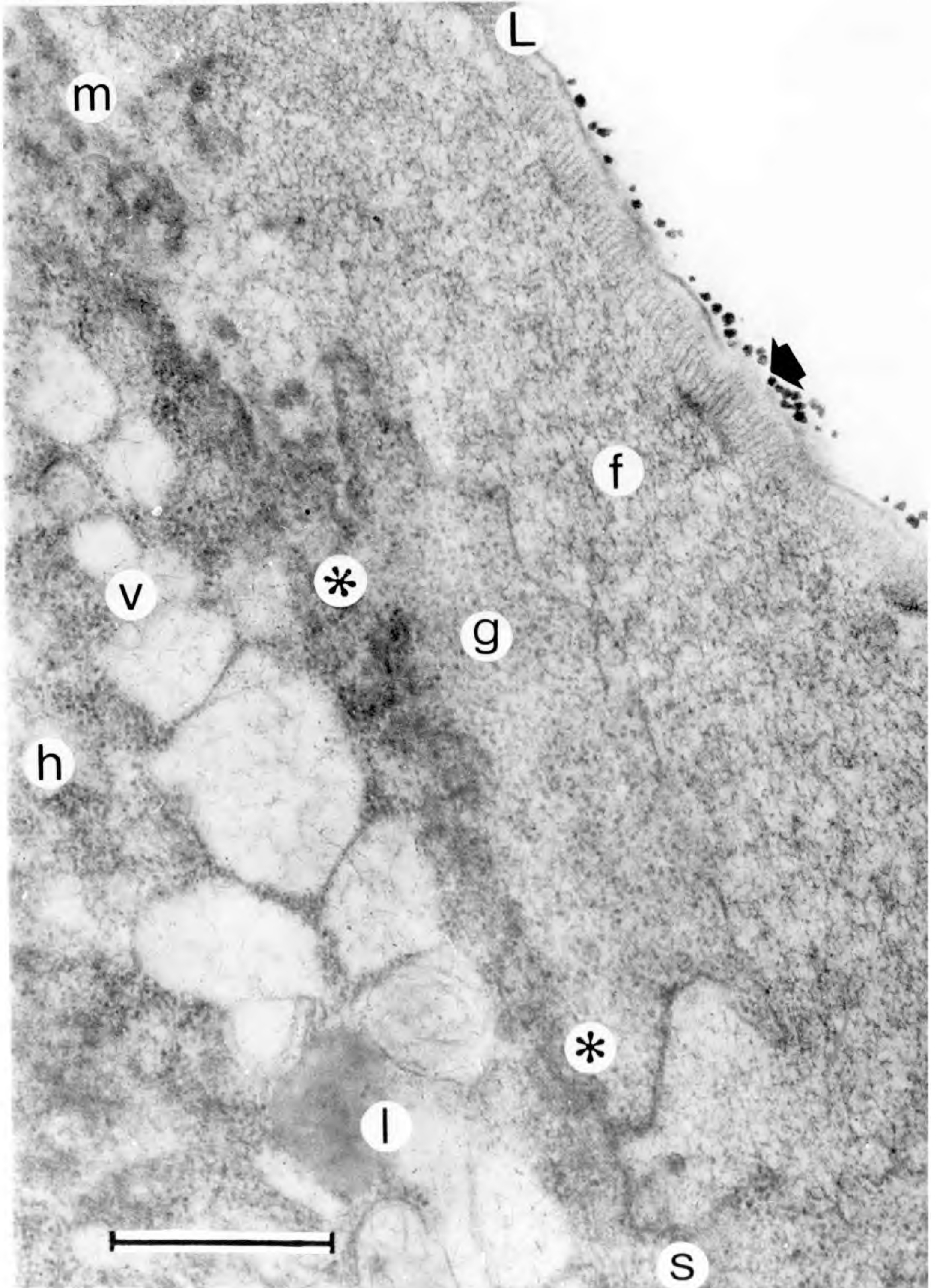


PLATE 13

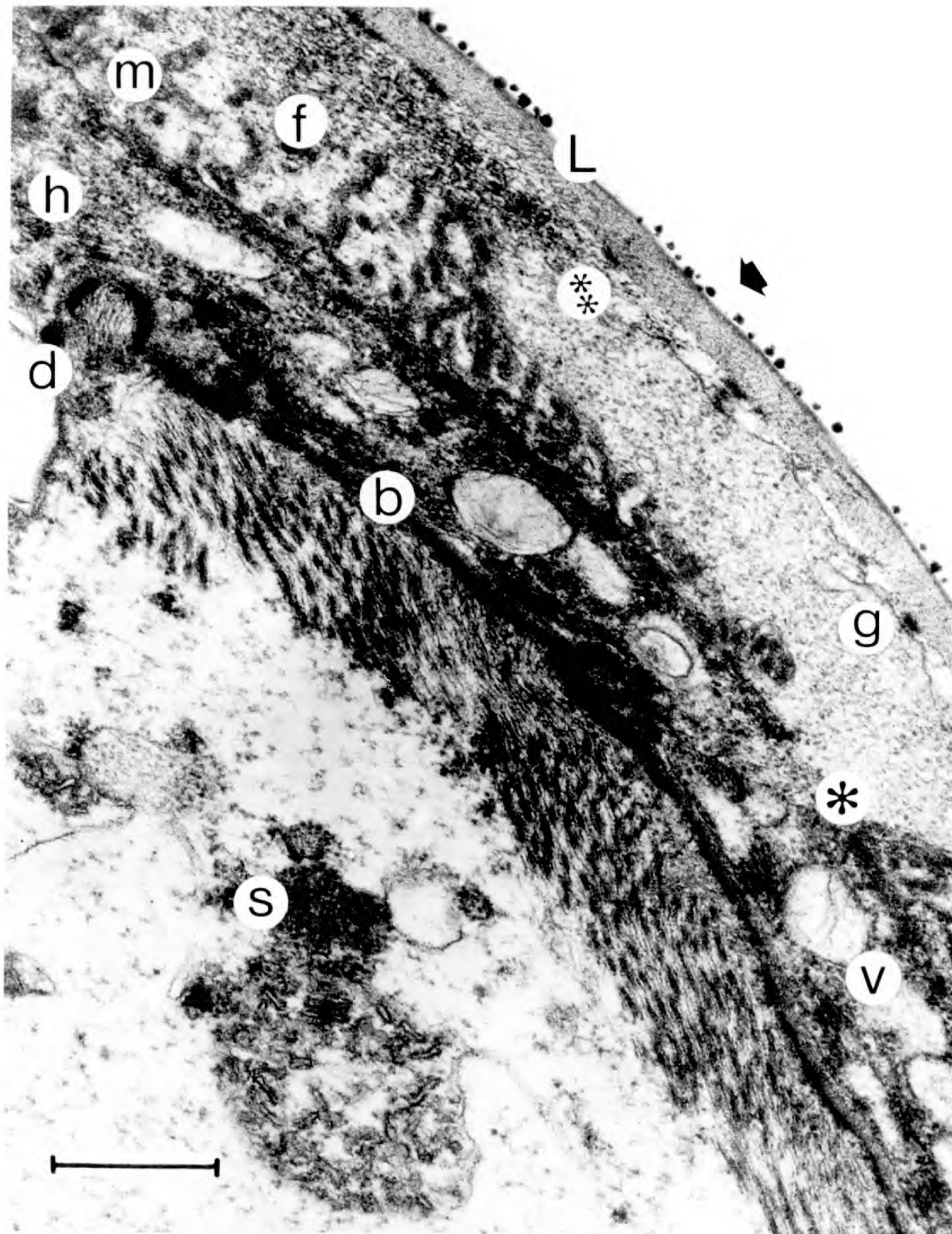


PLATE 14 and PLATE 15

TRANSVERSE SECTION THROUGH THE CUTICLE AND HYPODERMIS OF PARASITIC *HOWARDULA* sp. PRIOR TO ENSHEATHMENT.

c fourth stage larval cuticle

m microvilli of adult

h hypodermis

Fibrillar material (f) shown in PLATE 14 is prolific but disappears prior to ensheathment when an apparently empty space (s) is seen, as in PLATE 15.

PLATE 14

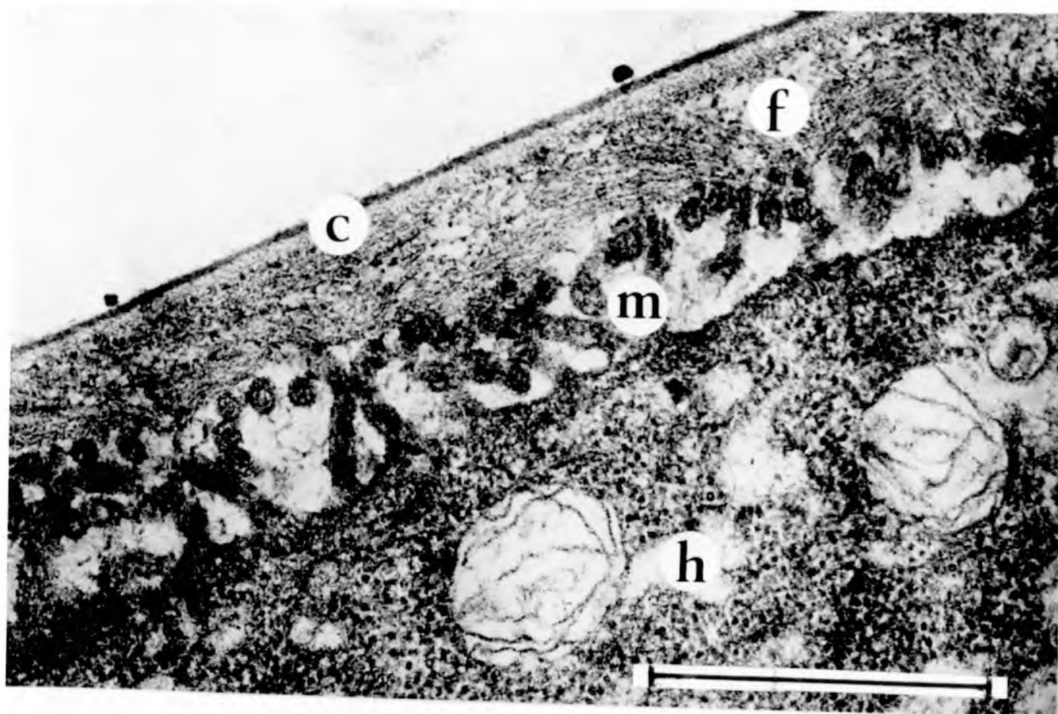
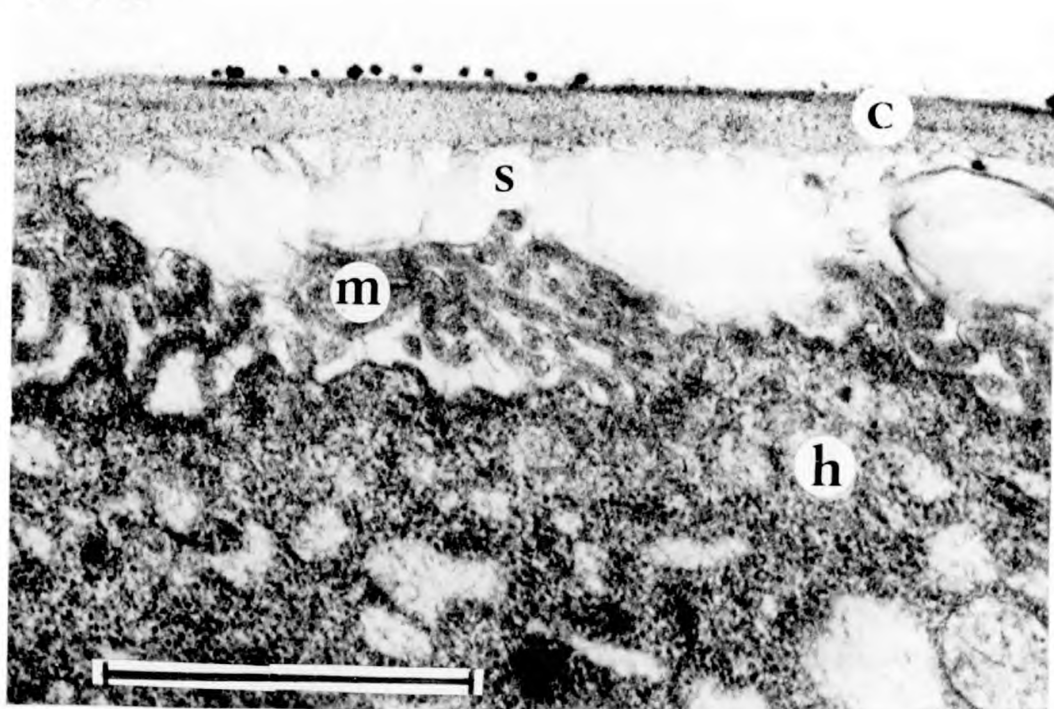


PLATE 15



II. 3.3. THE ADULT PARASITIC *HOWARDULA* Sp.

The proliferation of the hypodermis which occurs during the final moult of the young parasite continues after exsheathment and the nematode grows rapidly until it becomes sexually mature. The hypertrophy of the hypodermis and reduction of the somatic musculature leads to a marked disparity in the organisation of the body wall when seen in section and the obvious modification is apparent when a transverse section of *Howardula* is compared with that of a typical nematode (FIGURE 18A and 18B). The four somatic muscle sectors which are normally very prominent are barely discernable at the light microscope level in *Howardula* Sp., but the hypodermal cords occupy a conspicuous position.

Similarly the lack of cuticle is reflected in transverse sections because, not being able to maintain a high internal hydrostatic pressure, *Howardula* only rarely approximates to a circular section which is characteristic of the nematodes and lateral fields are absent. This is shown in the camera lucida drawings in FIGURE 19 and it can be seen that only in the anterior (FIGURE 19A) and posterior (FIGURE 19D) regions does *Howardula* sp. have a roughly circular form. Considerable distortion occurs along the rest of the body which invariably collapses where the body wall is most narrow, in the region of the muscle cells. The shape attained depends largely on the arrangement of the gonad which coils many times inside *Howardula* sp. but also on the pressure exerted by the internal organs of the host which may produce a very constrained habitat. It is interesting to note that the distortions seen in transverse sections, two of which are illustrated in FIGURE 19B and C, are always symmetrical and the hypodermis is on the outside of the curves.

The external surface of the adult nematode is covered with microvilli which range from 0.8-4 μm in length (mean = 2.5 μm) as shown in PLATES 16-19 and FIGURE 20. It is possible to detect the microvilli with the light microscope but it must be admitted that they were unnoticed until exposed by the electronmicroscope! The microvilli are usually long, thin and straight but frequently arborescent forms occurs with wide stalks and many branches which occasionally anastomose. Others have swollen regions, particularly at the apex or base, which may contain granular material. Only one of the microvilli types is present in a given area and gradation from one group to another has not been observed, but the long straight forms are most abundant at the anterior and posterior ends and microvilli are sparse in the region of the somatic muscle cells. Most microvilli are supported by axial fibres (PLATE 18 inset) which in some areas coalesce below the surface layer to form a loose terminal web-type arrangement (PLATE 17 and FIGURE 20).

The hypodermal cords form the bulk of the body wall and may be 2-20 μm thick (mean = 10 μm), being narrowest in the middle region of the body, where the nematode has its greatest diameter, and in the intercordal regions where it averages 1-2 μm. The lateral hypodermal cords are transversely longer than the dorsal and ventral cords (FIGURE 19) and are probably syncytial although occasionally a limiting membrane is indicated at the junction of the intercordal and cordal hypodermis, near the somatic muscle cells (PLATE 19, asterisks). However these may be desmosome attachments from the muscles. The arrangement of nuclei in the hypodermis suggests that the lateral cords may have originated from a double row of cells and the dorsal and ventral hypodermal cords from a single row.

The hypodermis of the mature female Howardula sp. (PLATES 16-19) contains lipid, milochondria, ribosomes, rough endoplasmic

reticulum, glycogen, lysosomes and myelin figures of phospholipid residues. Also there are many vesicles surrounded by rough endoplasmic reticulum which contain fibrous material as shown in PLATE 18. Frequently prominent swellings are seen on the surface of the nematode (PLATE 19) which appear to contain lipid and some have only a tenuous connection to the nematode and are probably in the process of being sloughed off into the host's haemocoel.

The four somatic muscle sectors may be completely surrounded by the hypodermis of the adjacent cords (PLATE 19). One to four platymyarian muscle cells can be seen in transverse section but frequently they are difficult to distinguish in the middle of the worm because the large myofilaments often have a sparse distribution or are lacking. The apparently disintegrated condition of most muscle cells suggest they are non functional.

After incubation in the Ferritin medium large electron dense particles were visible in the microvilli of Howardula sp. after as little as half a minute. After 1-2 minutes the particles had penetrated the hypodermis and were observed in the cytoplasm between the organelles as well as in the mitochondria and the secretory vesicles and they collected in dense clusters around the lipid droplets. After 5-10 minutes the material was very densely distributed throughout the hypodermis but was not present in the pseudocoelom or the gonads. The animals used in the control experiments did not contain any similar electron dense particles.

However the validity of results obtained after using Ferritin as an absorption-marker have been questioned by Lumsden et al (1970a) since these experiments were performed. The implications of their report and its relevance to the above observations will be discussed

later, but it is now suggested that the electron dense material absorbed by *Howardula* was not Ferritin and consequently relevant electromicrographs have not been included in the study.

FIGURE 18.

RECONSTRUCTED DIAGRAMS OF TRANSVERSE SECTIONS THROUGH THREE NEMATODES.

FIG. 18A. A typical nematode showing 4 prominent muscle sectors separated by 4 inconspicuous hypodermal cords.

FIG. 18B. *HOWARDULA* sp. showing reduced musculature and hypertrophied hypodermis.

FIG. 18C. *DELADENUS SIRICIDICOLA* showing reduced musculature and enlarged lateral hypodermal cords.

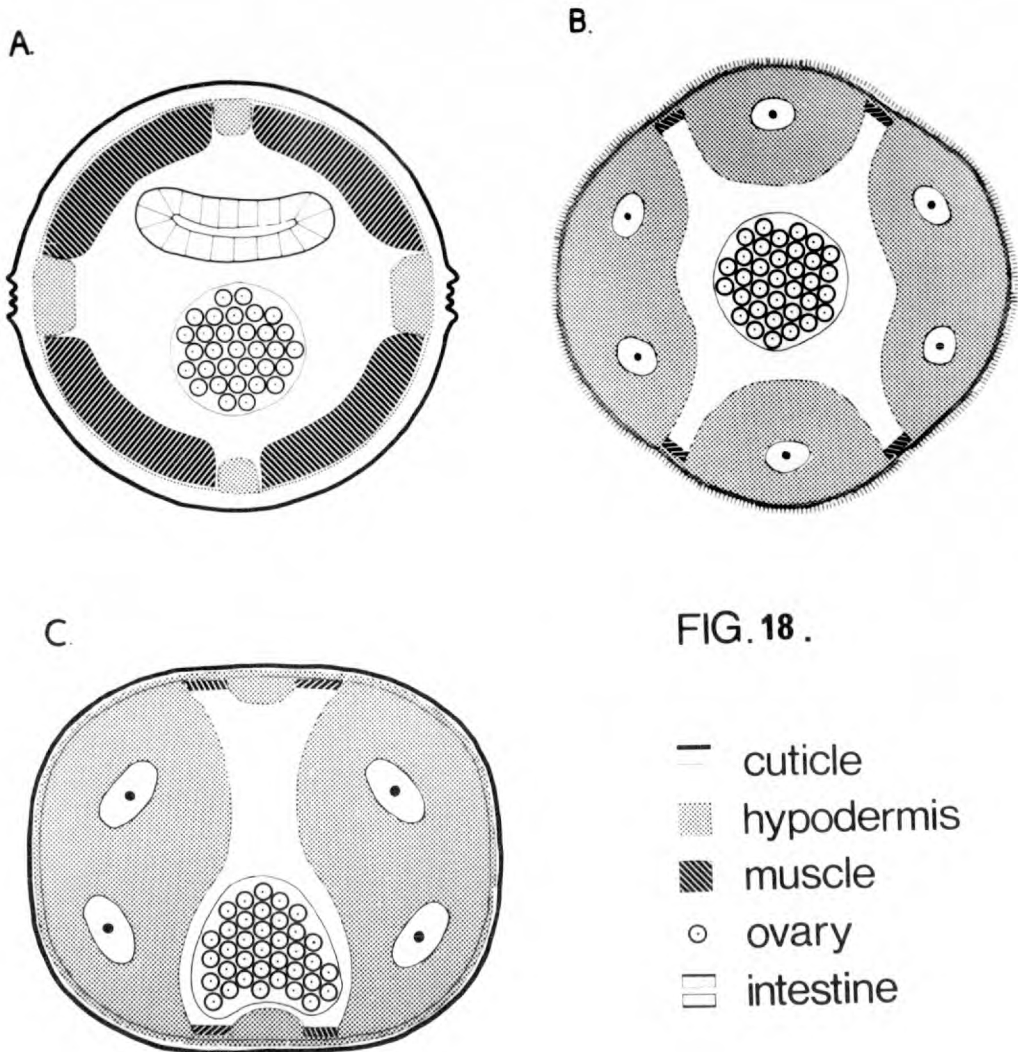


FIG. 18.

FIGURE 19.

TRANSVERSE SECTIONS THROUGH MATURE PARASITIC HOWARDULA.

FIG. 19A.	Anterior region.
FIG. 19B.	Mid region showing a commonly occurring distortion.
FIG. 19C.	Mid region with a typical distortion.
FIG. 19D.	Posterior end in the vulval region.

o	ovary
u	uterus
e	egg
v	vulva
c	cells representing the gut
s	somatic musculature
h	hypodermal cords
p	pseudocoelom
m	microvilli

FIG.19.

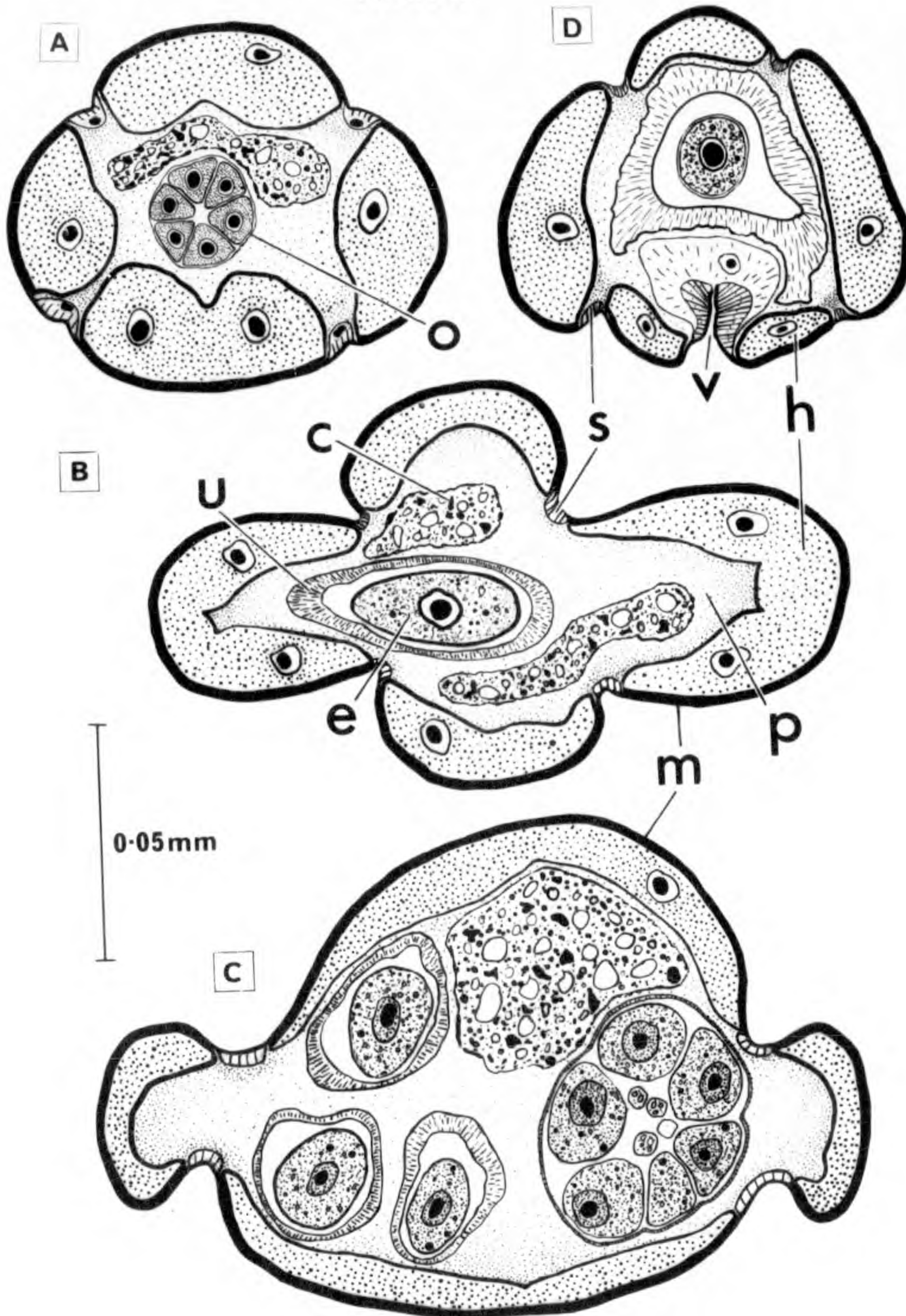


PLATE 16

TRANSFERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC *HOWARDULA* sp. IN THE INTERCORDAL REGION.

m	microvilli
h	hypodermis
l	lipid
w	whorls of membranes, probably phospholipid residues
p	pseudocoelom
o	oviduct

PLATE 16

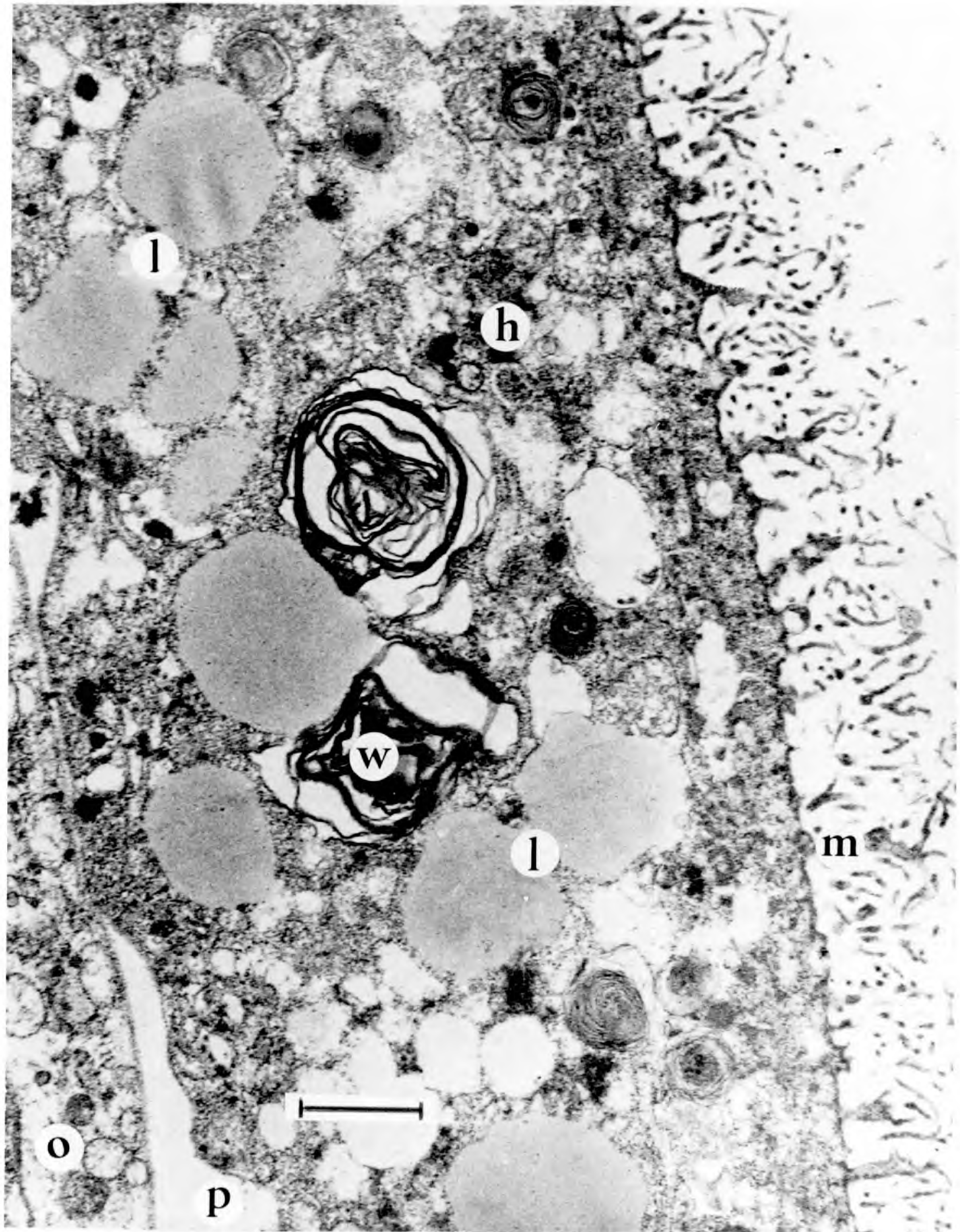


PLATE 17

TRANSVERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC *HOWARDULA* sp. IN THE INTERCORDAL REGION.

M	microvilli
f	fibrillar layer or terminal web
h	hypodermis
m	mitochondria
w	whorls of membranes, probably phospholipid residues
l	lipid
n	nucleus
p	pseudocoelom

PLATE 17

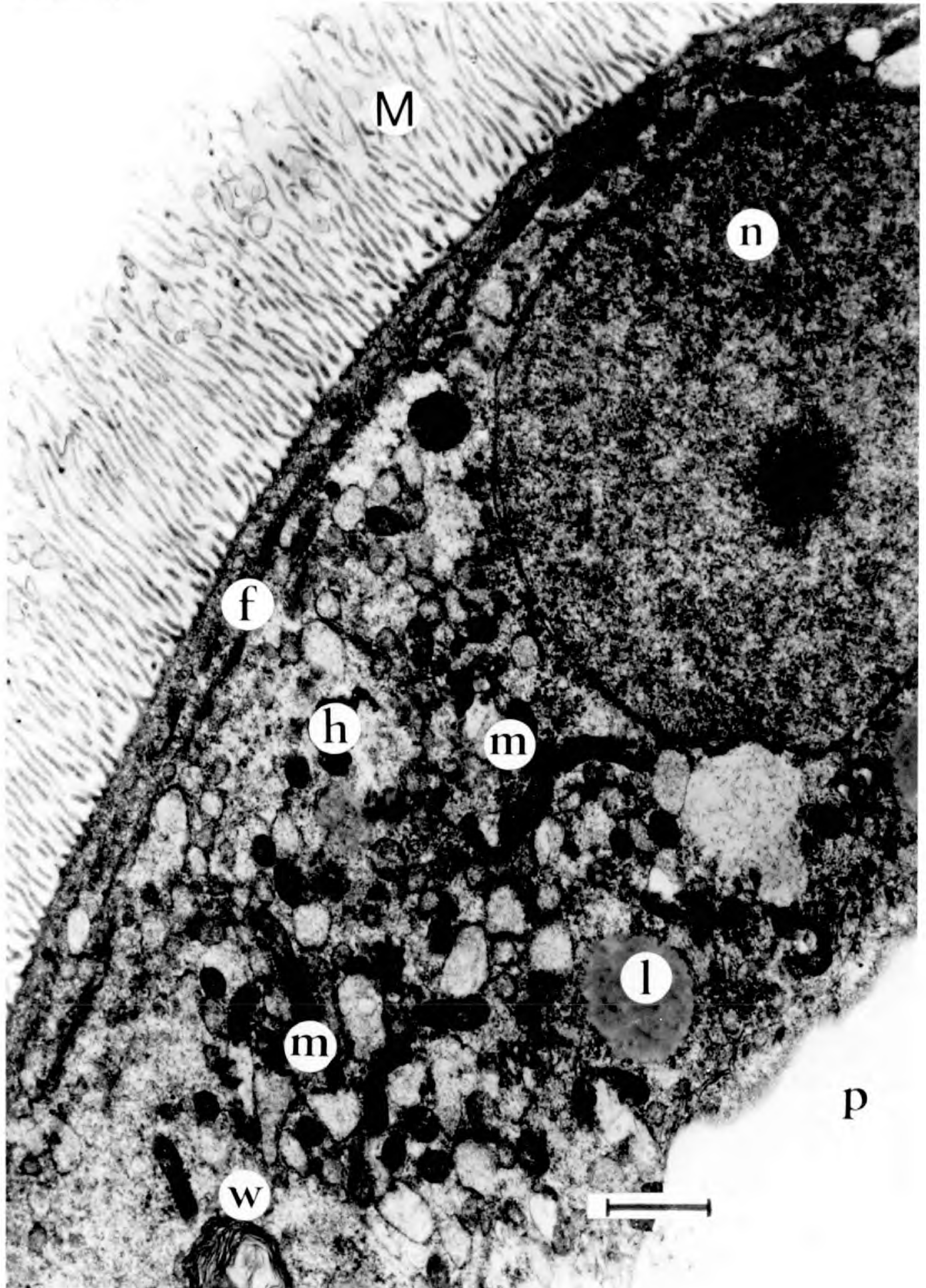


PLATE 18

TRANSVERSE SECTION THROUGH THE BODY WALL OF MATURE PARASITIC <u>HOWARDULA</u> sp. AT A HIGHER MAGNIFICATION.

v	microvilli
s	swollen base of microvilli
r	rough endoplasmic reticulum forming cisternae and fibrous vesicles
m	mitochondrion
h	hypodermis
p	pseudocoelom

INSET. T.S. microvilli. Arrows indicate sections where the central core of fibres (probably microtubles) are visible.

PLATE 18

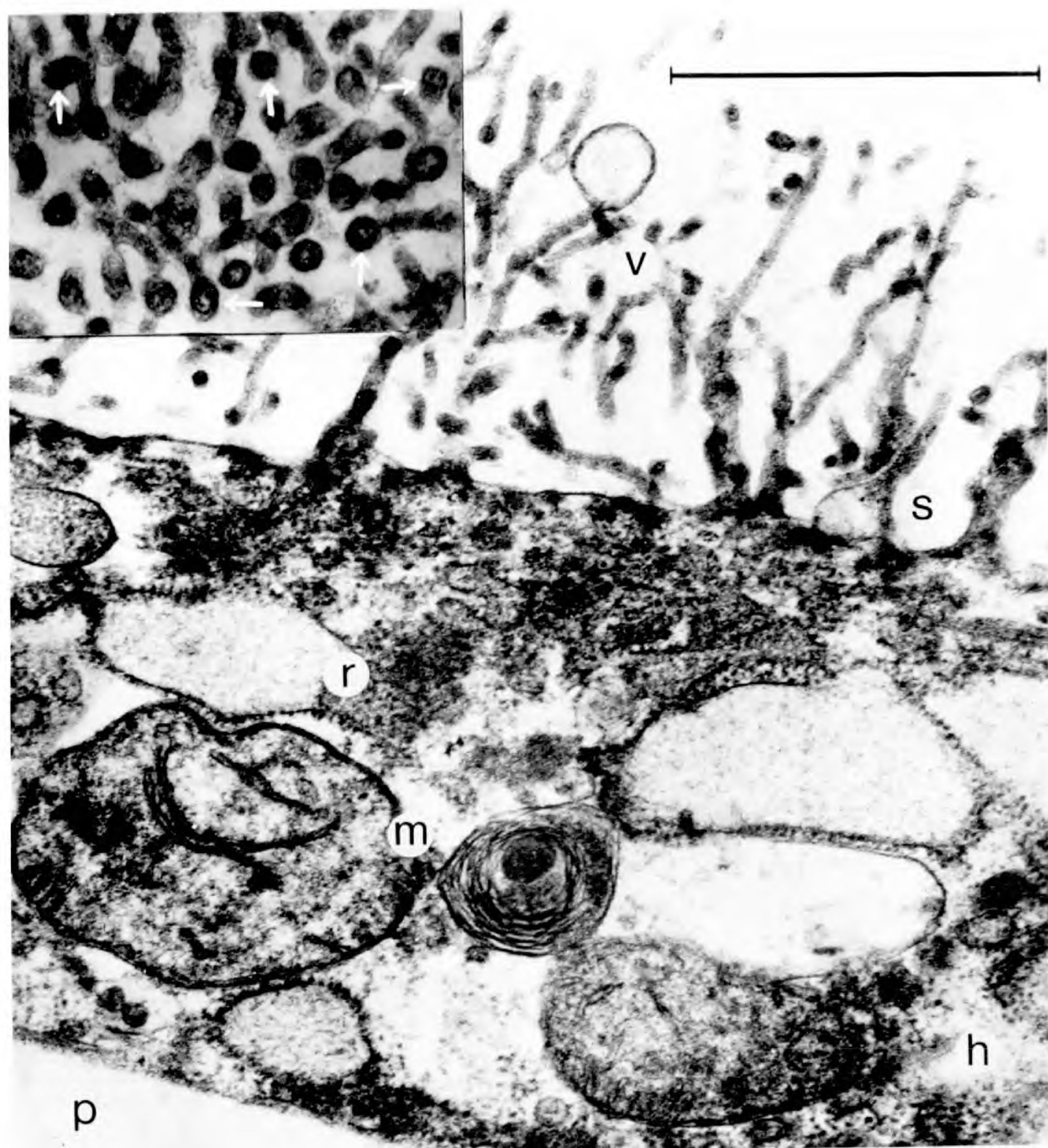


PLATE 19.

OBLIQUE TRANSVERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC *HOWARDULA* sp IN THE REGION OF A SOMATIC MUSCLE CORD.

v	microvilli
l	lipid
L	probable lipid extrusion from surface of nematode
h	hypodermis
m	somatic muscle cell
s	sarcoplasmic region of muscle cell
d	desmosome attachment
p	pseudocoelom

Arrows on the surface indicate electron dense membranes, either tight junctions of adjacent hypodermal cells or desmosome attachments from the muscles.

PLATE 19

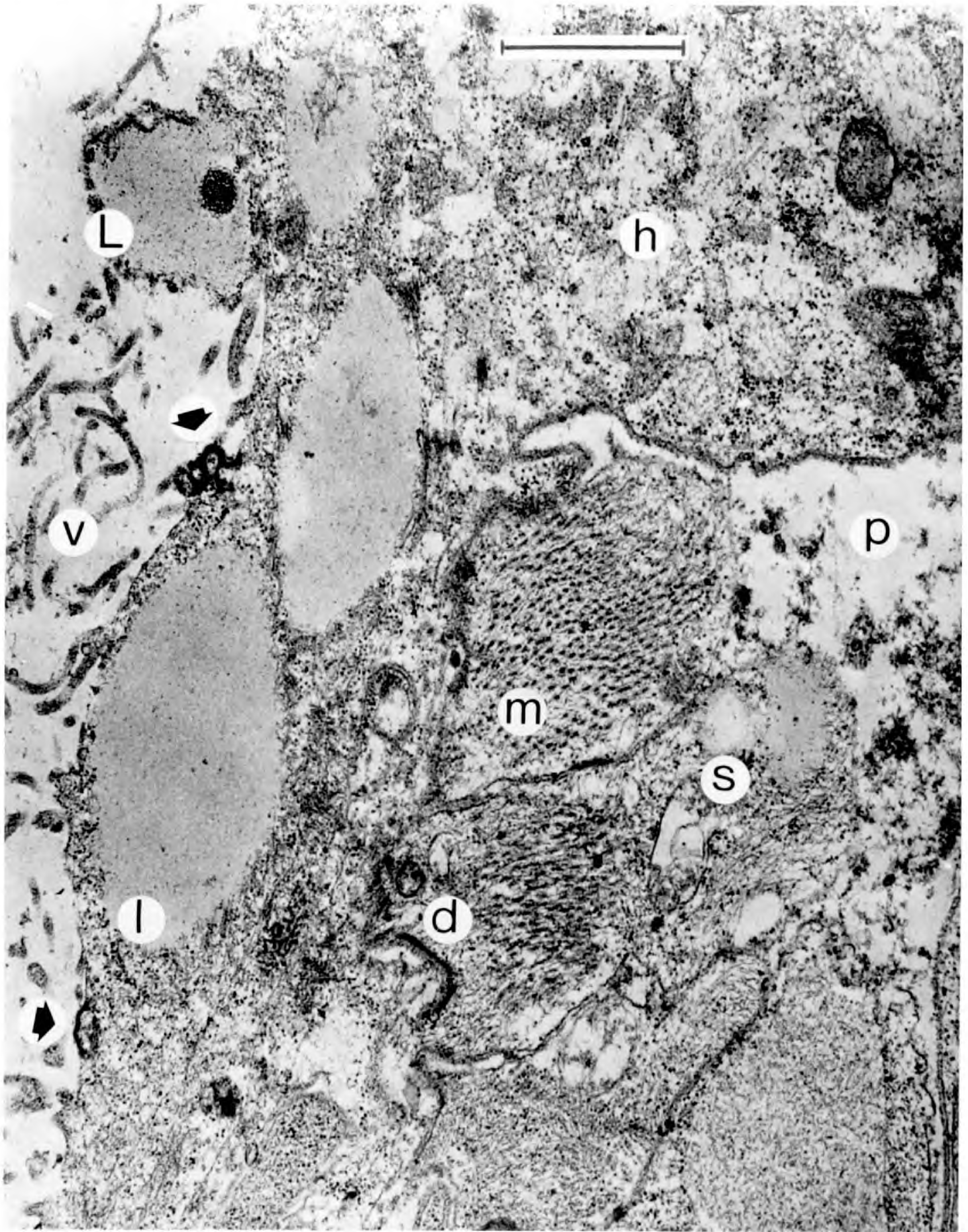


FIGURE 20.

RECONSTRUCTED DIAGRAM OF THE BODY WALL OF ADULT PARASITIC HOWARDULA.

1, 2, 3 show the different kinds of microvilli observed on the surface.

mv	microvilli
fl	fibre layer - terminal web
v	vesicles
l	lipid
b	basal lamina
s	somatic muscle
m	mitochondria
p	pseudocoelom
er	endoplasmic reticulum

Whorls of phospholipid and glycogen are also included and a nucleus is shown in the lateral hypodermal cord.

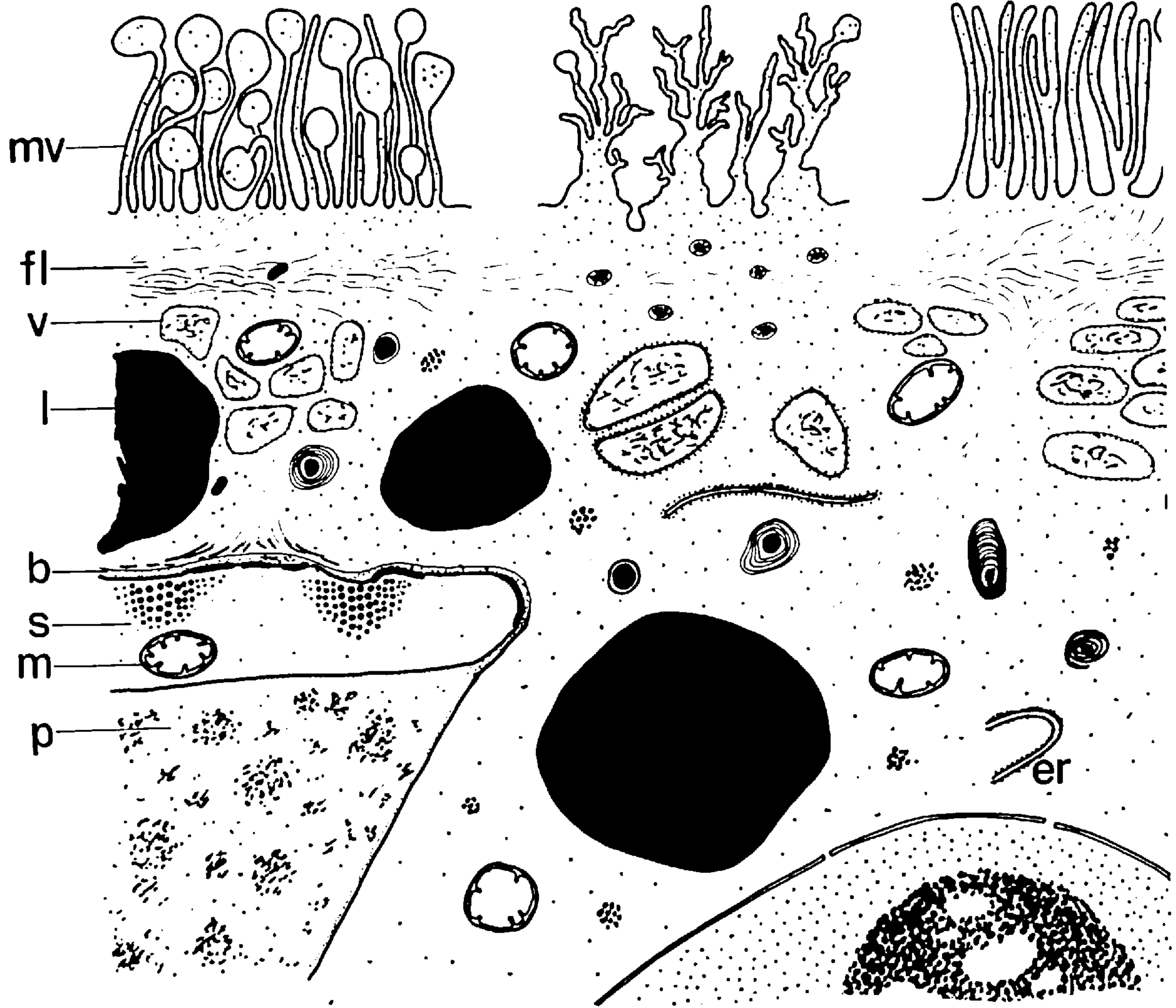


FIG.20.
1
2
3
mv
fl
v
l
b
s
m
p
er

II 3.4. THE MYCETOPHAGOUS ADULT FEMALE DELADENUS SIRICIDICOLA

A transverse section of the adult female mycetophagous Deladenus siricidicola shows the same basic arrangement of the body layers as illustrated for the typical nematode in FIGURE 18A. There is a cuticle with prominent lateral fields, four small hypodermal cords and four conspicuous muscle sectors each containing 3-5 platymyarian cells.

The cuticle is 0.36-0.45μm wide (mean = 0.41μm) and has many superficial transverse annulations. It is limited externally by a narrow electron dense layer about 8 nm wide (PLATE 20). The cortex comprises two regions; a narrow dense outer cortex and a wider amorphous inner cortex which merges imperceptibly with the matrix. The salient feature of the cuticle is the striated layer which has a mean width of 0.17μm and surrounds the body in a radial orientation. The dense rod like structures have a greater periodicity when viewed transversely; in longitudinal section they appear closer together (mean interval = 11.2 nm) and are nearly as wide as the distance between them (mean bar width = 10.8 nm). When the cuticle is sectioned obliquely as in PLATE 21, the palisade formation is replaced by a variety of geometric patterns including lattice, punctuation and herringbone, but they are never randomly arranged. Below the striated zone is a single fibre layer which is in close proximity with the hypodermis because there does not appear to be a basal lamella.

In the lateral fields the cuticle is folded to form up to 18 ridges which are usually of equal size (PLATE 22). The cuticle in this region is often 2 or 3 times thicker than in other parts

(mean width = 1μm) and much of the increased width is due to four prominent zones of fibrous material, as shown in PLATE 23. The inner most fibrous zone appears to be a continuation of the basal fibre layer of the rest of the cuticle but the other three zones are not found elsewhere and may be formed from the striated layer which is absent in the lateral field. The first two fibre layers (numbered in centripetal order) closely follow the contours of the lateral folds but the third and fourth layers are concentric with the hypodermis. It is interesting that there is no fluid-filled layer or vacuolated region in D. siricidicola as has been reported in the lateral field of several other nematodes.

The hypodermis in the lateral cords contains nuclei, rough endoplasmic reticulum, glycogen, mitochondria and some lipid, in contrast to the intercordial hypodermis which shows little structure other than irregular masses of amorphous electron dense material.

The somatic muscle cells are platymyarian with a meromyarian arrangement and have frequent desmosome attachments to the hypodermis and cuticle. Each cell is largely composed of large and small myofilaments with little sarcoplasm and few neuromuscular processes. The muscles are separated from the pseudocoelomic cavity by a thin basal lamella.

The body wall of the free living infective Deladenus siricidicola has only been briefly examined but it appears to be almost identical to that of the mycetophagous female described here.

PLATE 20.

LONGITUDINAL SECTION THROUGH THE BODY WALL OF ADULT FEMALE MYCETOPHAGOUS DELADENUS SIRICIDICOLA.

d	dense outer layer of cuticle
c	cortex region of cuticle
h	hypodermis
s	striated zone
m	somatic muscle

PLATE 21.

OBLIQUE SECTION THROUGH THE CUTICLE OF ADULT FEMALE MYCETOPHAGOUS DELADENUS SIRICIDICOLA.

Labels as above. The striated zone has a herring-bone pattern, in contrast to the palisade arrangement seen in longitudinal section.

PLATE 20

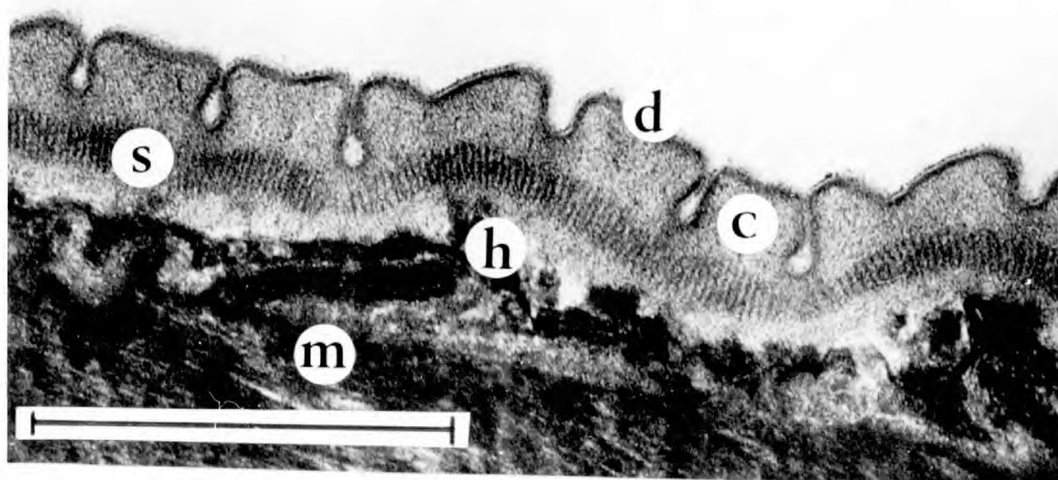


PLATE 21

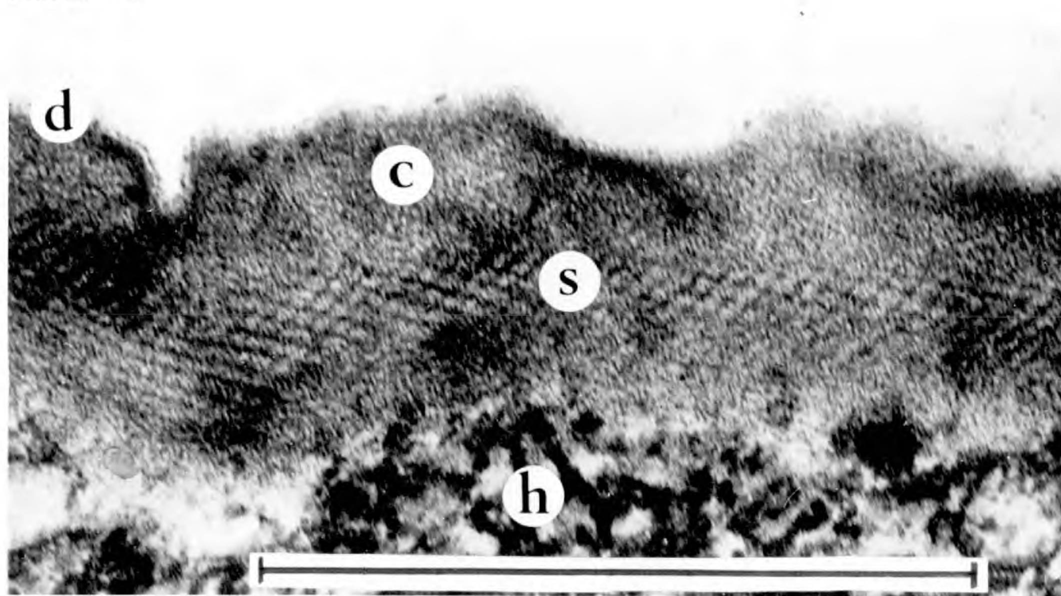


PLATE 22 and PLATE 23.

TRANSVERSE SECTION THROUGH ADULT FEMALE MYCETOPHAGOUS *DELADENUS SIRICIDICOLA* IN THE LATERAL FIELD

Asterisks indicate the extent of the lateral lines in PLATE 22. The striated zone is contiguous with four fibrillar layers (f) which are marked 1 - 4 in PLATE 23.

h	hypodermis
d	dense outer layer of cuticle
c	cortex

PLATE 22

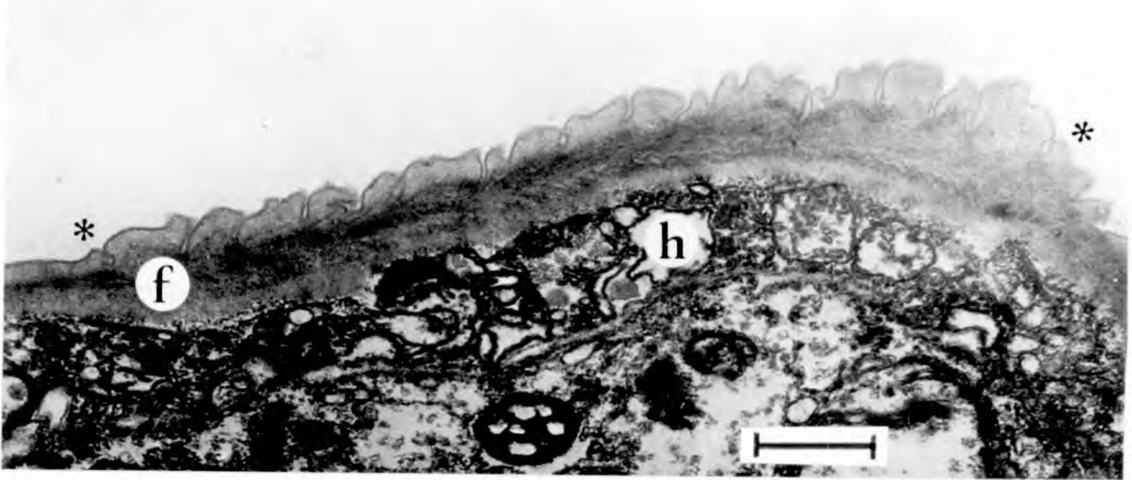
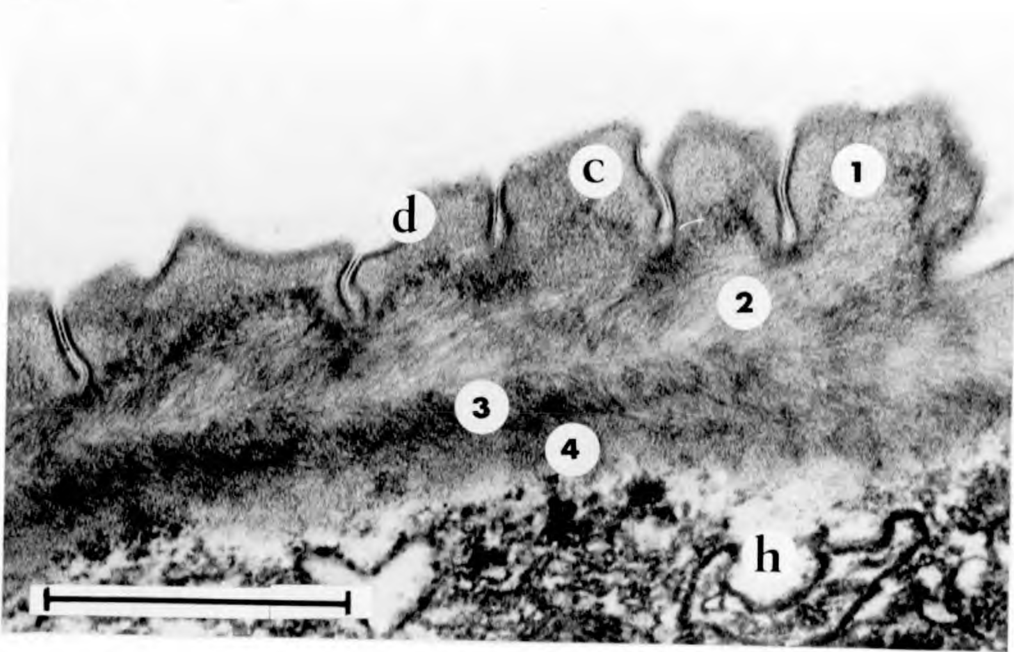


PLATE 23



II 3.5. THE YOUNG PARASITIC DELADENUS SIRICIDICOLA

The young parasitic Deladenus siricidicola which were examined had been inside the host for about three months and had increased in length from 1 mm up to 5 mm, having attained nearly one third their size at maturity. There was a similar increase in width which was mainly due to hypertrophy of the lateral hypodermal cords as shown in the diagram FIGURE 18C, and it is interesting that the hypodermis of the dorsal and ventral cords showed little enlargement.

When the body wall is observed through a light microscope it appears to be surrounded by a normal cuticle which is marked at regular intervals by deeply penetrating dark striations. The electron microscope shows these cuticular striations to be pits of developing microvilli as shown in PLATES 24-26; a thin layer of granular material covers the opening of the pits and surrounds the body to maintain a superficially even surface. The "cuticle" is not stratified and shows little differentiation between the pits of microvilli except for occasional groups of long fibres which have electron dense connections to the surface membrane (PLATES 25 and 26) and are probably desmosome attachments from the fibre layers and somatic muscle cells below. The microvilli may be formed by the gradual subsidence of the surface layer at each side of a fibrous stand (PLATE 25) presumably because the fibres offer some resistance to deformation. Gradually the finger-like projections are isolated as the surrounding tissue recedes and the original level of the surface is indicated by the persistent granular layer as shown in PLATE 27 and FIGURE 21. However this may only account for the origin of the longest microvilli which have a

prominent fibrous core; the young parasitic D. siricidicola has microvilli of varying dimensions and some are very narrow and lack supporting fibres. The majority of microvilli are formed in deep invaginations of the surface membrane and their random orientation indicates that they arise from all sides of the concavity (PLATES 25 and 26). As the nematode continues to grow, the surface layer will be stretched out and the microvilli gradually exposed to the exterior.

The external membrane of the "cuticle" frequently has small fibres projecting at right angles from its inner surface particularly in the regions where it is deeply invaginated (PLATE 26 inset). At the base of these depressions there is some indication that coated vesicles are budded off because they are often found in close proximity and they are surrounded by material which resembles the fibrous lining of the invaginated membrane. The coating is soon lost and the vesicles appear to pass down through the "cuticle" into the hypodermis, where similar vesicles are abundant (PLATE 26). A similar method of vesicle formation is indicated at a later stage of development in PLATE 27 inset where pinocytosis could be occurring at the base of the microvilli.

The "cuticle" is not separated from the hypodermis by a membrane and consequently cannot correctly be termed a cuticle. It is usually loosely demarked from the hypodermis by a band of scattered fibres but there are frequent areas where the two layers are in obvious communication (PLATE 25, asterisk). However the outer layer is clearly distinguished from the rest of the hypodermis because of its lack of organelles.

The hypodermis is packed with lyosomes, phospho-lipid residues, mitochondria, glycogen, lipid droplets, smooth and rough

endoplasmic reticulum which occurs as folded membranes, cisternae or vesicles of differing sizes. Usually two nuclei are present in transverse sections through the lateral hypodermal cords but none have been seen in the dorsal and ventral cords. Fibres may be clustered loosely into two or sometimes three concentric layers below the "cuticle" in the hypodermal cords but this formation is replaced by an oblique orientation in the intercordal regions where the fibres are seen to pass from the basal lamina around the somatic muscle cells to just below the "cuticle" (PLATES 24 and 25 and FIGURE 21).

There are four somatic muscle sectors in each transverse section which have a meromyarian arrangement of two to three cells in each quadrant and they lie much nearer to the mid line of the dorsal and ventral planes than in other nematodes FIGURE 18C). The muscle cells are platymyarian with a reduced contractile region composed of sparse clusters of thick and thin myofilaments with no obvious division into H, A and I regions or Z bands and cytoskeletal elements (PLATE 25). The sarcoplasmic region occupies over half the cell and contains a nucleus and occasional mitochondria but few other organelles. Each muscle sector is surrounded by a basal lamina.

FIGURE 21 illustrates the salient features of the body wall of the young parasitic D. siricidicola and shows two stages in the formation of microvilli.

PLATE 24.

OBLIQUE TRANSVERSE SECTION THROUGH THE BODY OF A YOUNG PARASITIC DELADENUS SIRICIDICOLA AT THE JUNCTION OF THE LATERAL HYPODERMAL CORD AND SOMATIC MUSCULATURE.

The "cuticle" (c) is traversed by narrow fibrous strands (arrow) which have desmosome attachments to the surface. Invaginations of the external layer (e) lead to the formation of microvilli (v) and a granular layer (g) marks the position of the original surface.

Lipid is present (l) in the hypodermis (h) as well as dense fibres (f), multivesicular bodies (M), mitochondria (m), primary lysosomes (p) and many vesicles.

A basal lamina (b) separates the hypodermis from the somatic muscle cells (s) and part of the pseudocoelom (P) is visible.

PLATE 24

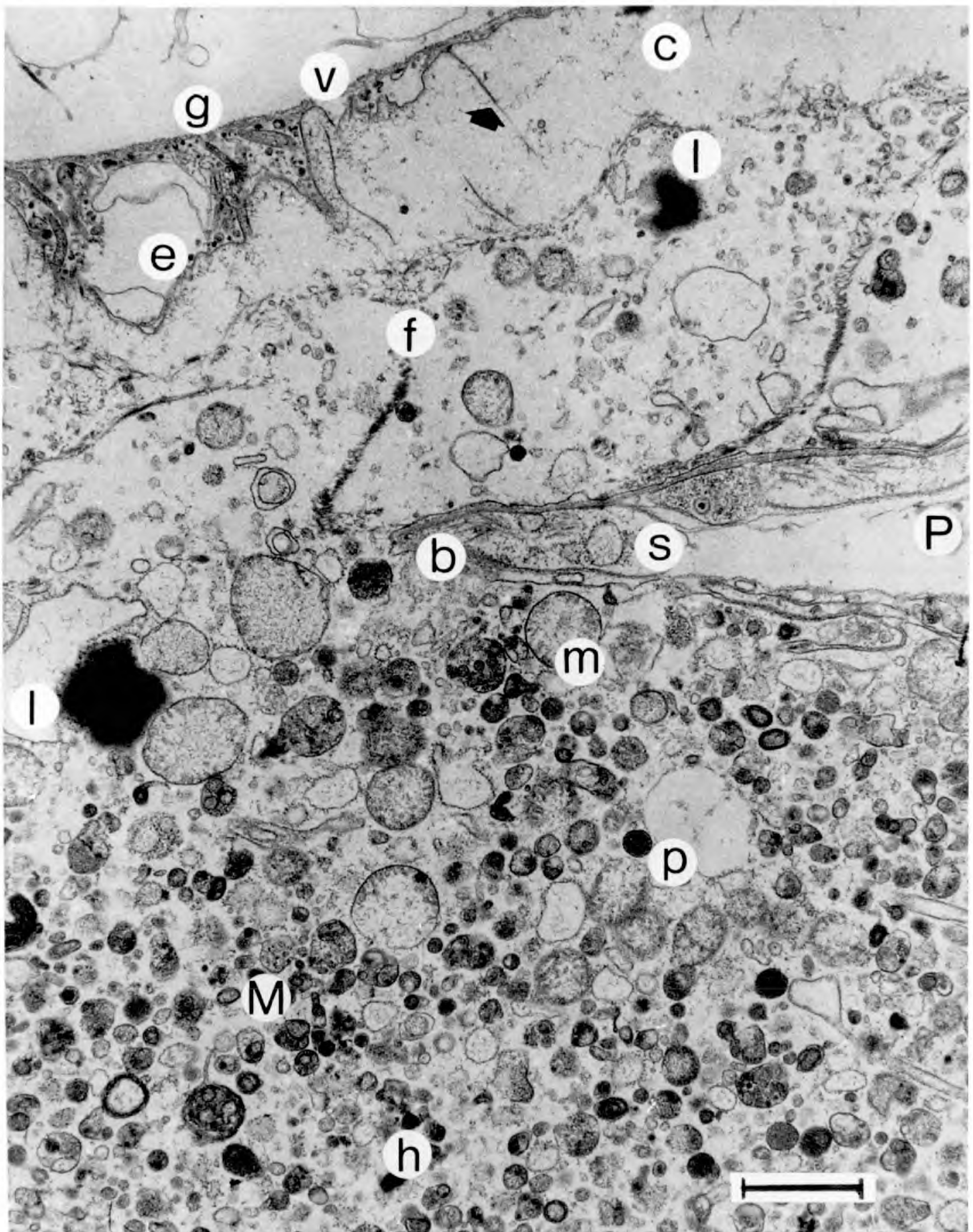


PLATE 25.

TRANSVERSE SECTION THROUGH THE BODY WALL OF A YOUNG PARASITIC *DELADENUS SIRICIDICOLA* IN THE INTERCORDAL REGION.

Pits of microvilli (v) are present in the "cuticle" (c) and a granular layer of material (g) is visible along the surface.

Microvilli appear to form around electron dense material (arrow) which may be contiguous with the fibres (f) of the hypodermis.

Multivesiculate bodies (M), endoplasmic reticulum cisternae (e) and mitochondria (m) are also present in the hypodermis which appears to be incontinuous with the "cuticle" at the place marked by an asterisk.

The basal lamina (b) surrounds the somatic muscle cells (s) which have few myofilaments, and the pseudocoelom is seen at the bottom of the electronmicrograph.

PLATE 25

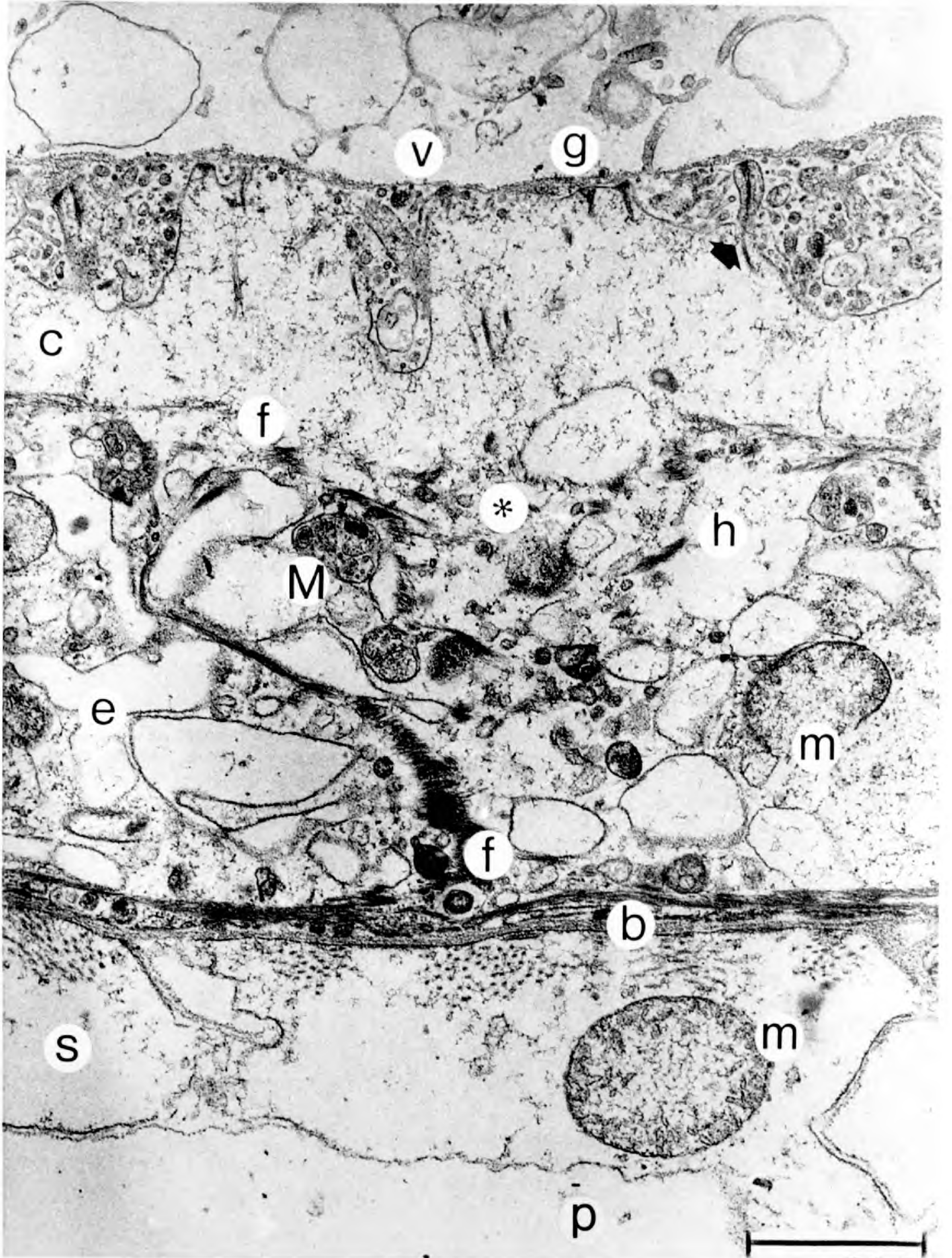


PLATE 26.

TRANSVERSE SECTION THROUGH THE "CUTICLE" AND HYPODERMIS OF A YOUNG DEVELOPING PARASITIC *DELADENUS SIRICIDICOLA*.

s	external surface of nematode
i	invaginations of surface membrane
v	many small coated vesicles in this region
f	fibrillar layer
w	whorls of membranes
m	mitochondrion
h	hypodermis

INSET: Invagination of surface membrane at a higher magnification. The large arrow indicates the fibrous nature of the inner layer of the membrane and the small arrows point to coated vesicles which may have been formed pinocytotically.

PLATE 26

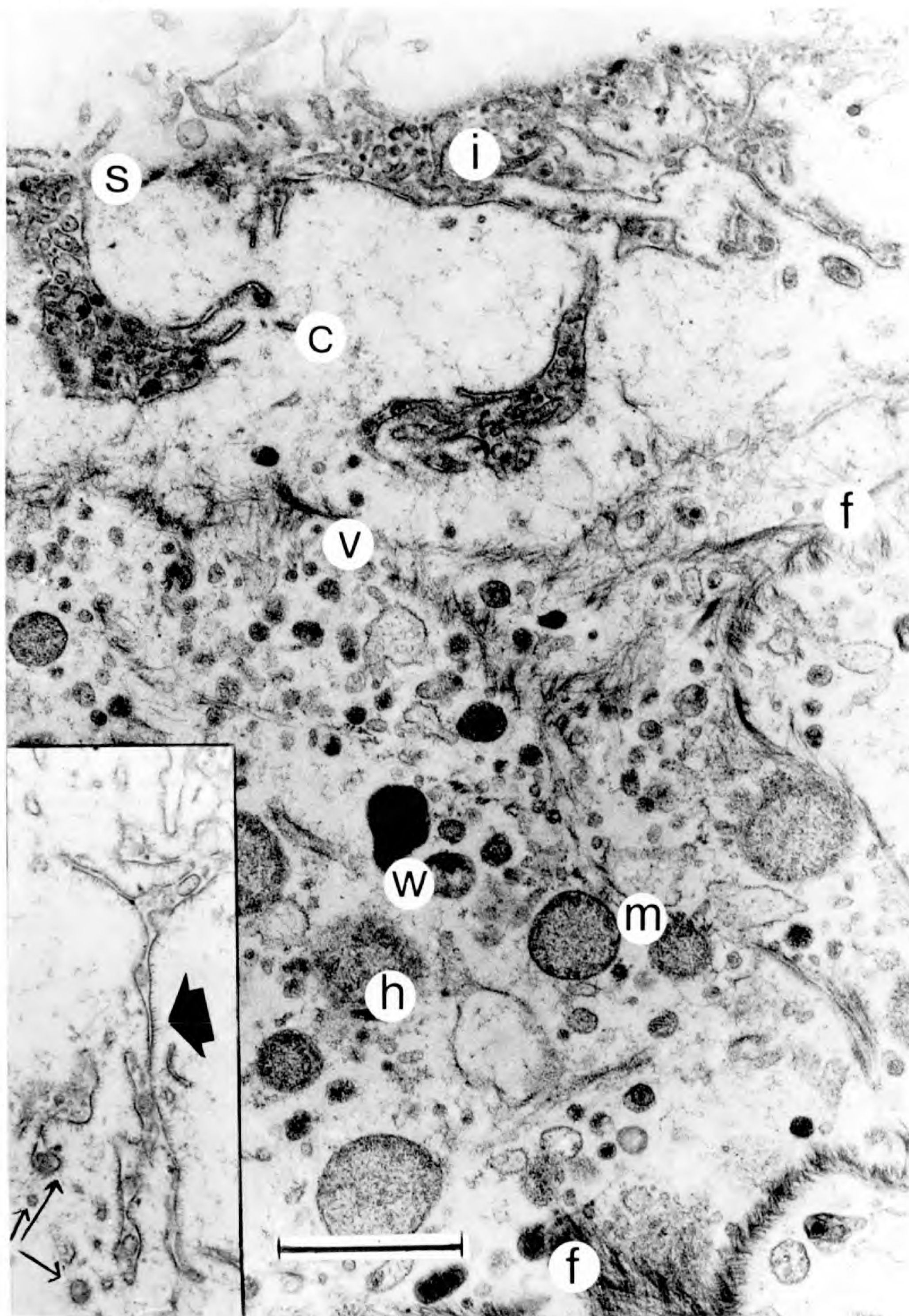


PLATE 27.

LONGITUDINAL SECTION THROUGH THE BODY WALL OF A YOUNG PARASITIC *DELADENUS SIRICIDICOLA* SHOWING A LATER STAGE OF MICROVILLI DEVELOPMENT.

v	microvillus
g	granular material marking the original level of the "cuticle"
h	hypodermis
m	mitochondrion
w	whorls of membranes

INSETS 1 - 3 suggest the sequence of pinocytotic vesicle formation.

PLATE 27

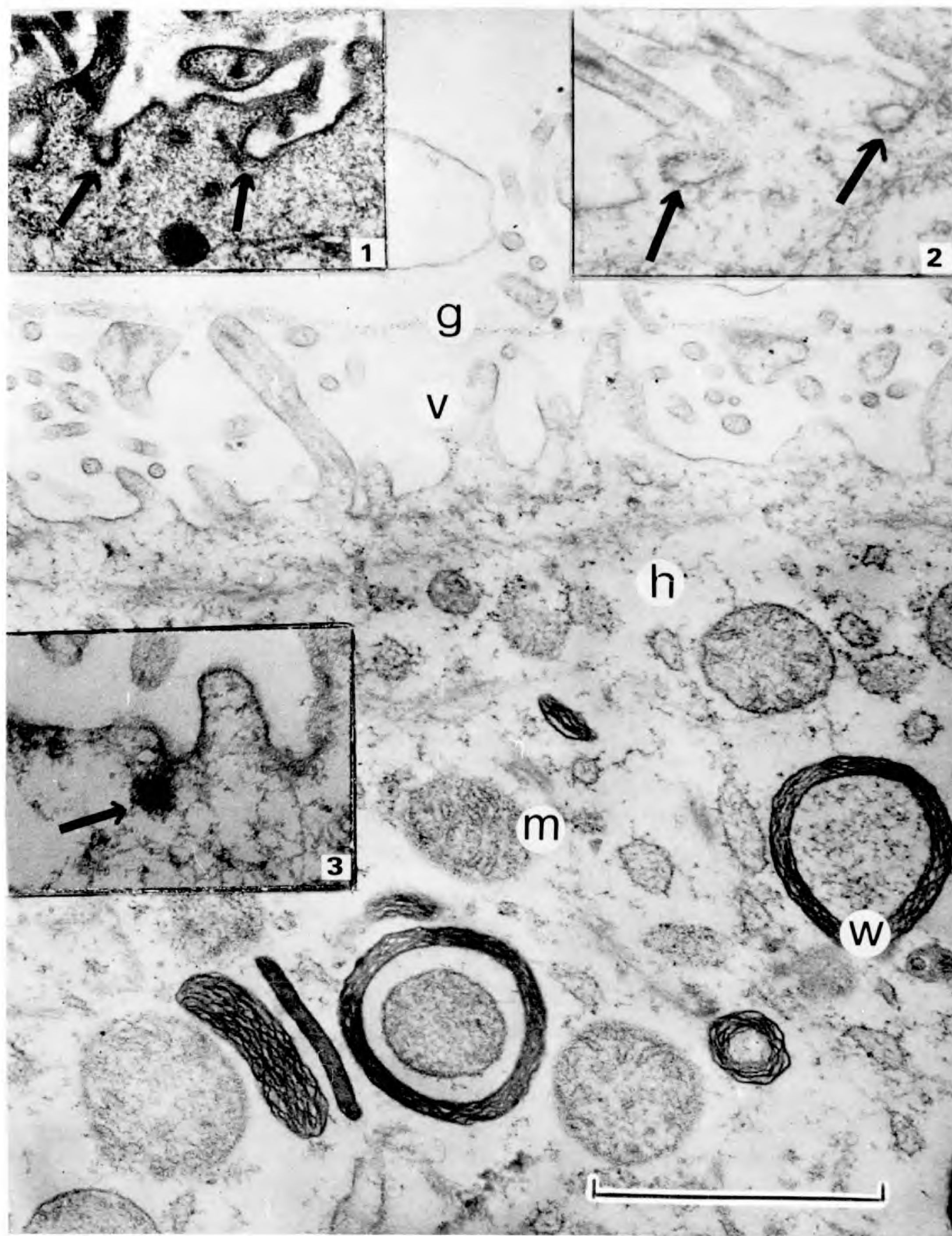


FIGURE 21.

RECONSTRUCTED DIAGRAM OF THE BODY WALL OF THE IMMATURE PARASITIC *DELADENUS SIRICIDICOLA* AT THE JUNCTION OF MUSCLE AND HYPODERMAL CORDS.

Cuticle invagination to form microvilli along the surface is shown in region 1 & 2. Fibrous strands (f) persist as the cores of microvilli and a layer of granular material remains on the surface.

fl	fibre layer - terminal web
v	vesicles - probably formed pinocytotically
er	endoplasmic reticulum
gl	glycogen
b	basal lamina
s	somatic muscle cell
m	mitochondria
p	pseudocoelom
pl	phospholipid

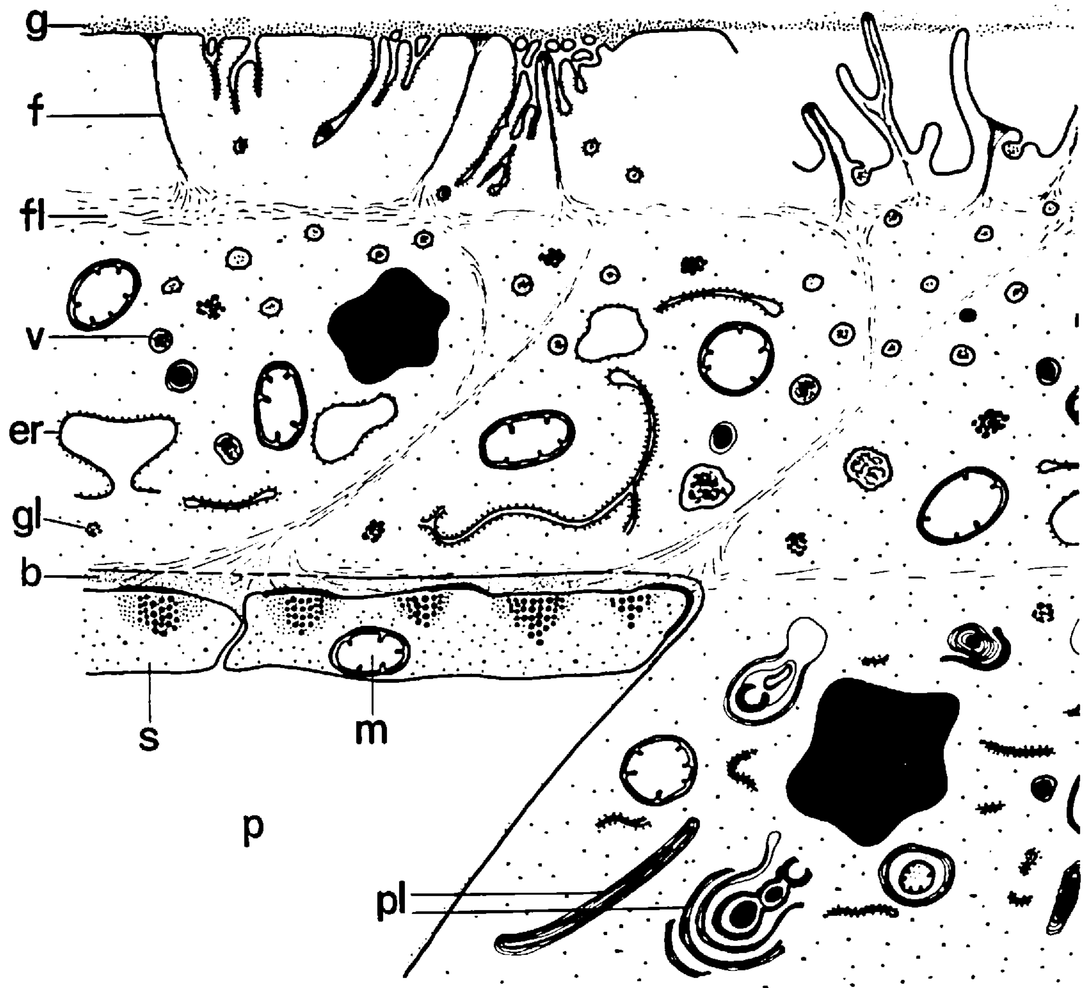


FIG.21.
1
2
g
f
fl
v
er
gl
b
s
m
p
pl

II 3.6. THE MATURE PARASITIC DELADENUS SIRICIDICOLA

The female Deladenus siricidicola may grow as long as 25 mm before it reaches sexual maturity and releases larvae ovoviviparously into the host's haemocoel. A transverse section of the parasite at this stage shows the same basic arrangement of layers as illustrated for the developing female D. siricidicola in FIGURE 18C, and it closely resembles D. wilsoni which is shown in FIGURE 23A. There is considerable hypertrophy of the hypodermis in the lateral cords which are adjacent to the four reduced sectors of muscle cells. Unlike the young parasitic D. siricidicola it can be seen with the light microscope that the mature nematode does not possess a cuticle and that microvilli are present in an intricate tangled array along the irregular undulations of the surface; these are illustrated at higher magnification in the electronmicrographs PLATES 28, 29 and 31.

The microvilli are 1-4 μm long and seldom occur as straight fingerlike projections; usually they bifurcate and have several branches which may anastomose at intervals along their length or fuse at their extremities. An electron dense fibrillar core is frequently a prominent feature of D. siricidicola microvilli and is particularly obvious at the apices which are dilated and contain darkly staining amorphous material (PLATE 31 and FIGURE 22). There is some indication that this is released by the breakdown of the tips and forms the zone of granular material which overlies the microvillous layer. The granular zone is made up of two layers, the inner being slightly more electron dense

than the outer layer and it resembles the material which was observed in reduced quantity on the outside of the developing parasite. Although the granular layer is present in all mature parasites there are occasional patches which lack this covering (PLATE 29) and it is interesting that the microvilli in these regions do not have prominent fibrillar cores or enlarged tips with electron dense contents.

The hypodermis is packed with irregularly shaped mitochondria, vesicles containing fibrillar material, rough and smooth endoplasmic reticulum, Golgi bodies, lyosomes, phospholipid residues (PLATE 30), glycogen and lipid droplets which are frequently near the surface. There are also many strands of fine fibres which form a concentric layer 5-10 below the microvillous layer when viewed transversely and thicker, more electron-dense fibres occur particularly near the somatic muscle cells but also interspersed throughout the hypodermis, with conspicuous aggregations just below the microvilli. These dense fibres appear to give rise to the fibrous cores of the microvilli and thence to the granular external layer (PLATE 31 and FIGURE 22). Large nuclei are present in the lateral hypodermal cords and they often have a stellate or irregular shape. Invariably they are surrounded by highly vacuolated cytoplasm which is packed with rough endoplasmic reticulum, lacunane, cisternae and small vesicles (PLATE 29).

The somatic muscles have a meromyarian arrangement with 2-4 platymyarian cells in each of four muscle sectors. The sparse myofilaments occur in scattered clusters and there are no cytoskeletal structures or Z bands. The sarcoplasm apparently

lacks mitochondria and other organelles and the general appearance indicates further deterioration from the condition observed in the developing parasites. A narrow basal lamina surrounds the somatic muscle cells and separates them from the hypodermis and the pseudocoelom.

Mature D. siricidicola parasites absorbed electron dense particles through the body wall after being incubated in a saline solution of Ferritin for two minutes. After five minutes the particles had travelled about half way across the hypodermis which was packed with dense aggregations after ten minutes incubation.

No similar electron dense particles were present in the control nematodes.

PLATE 28.

OBLIQUE LONGITUDINAL SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC DELADENUS SIRICIDICOLA.

m microvilli
g granular material on surface
h hypodermis
f strands of fibres
l lipid
s somatic musculature

PLATE 28

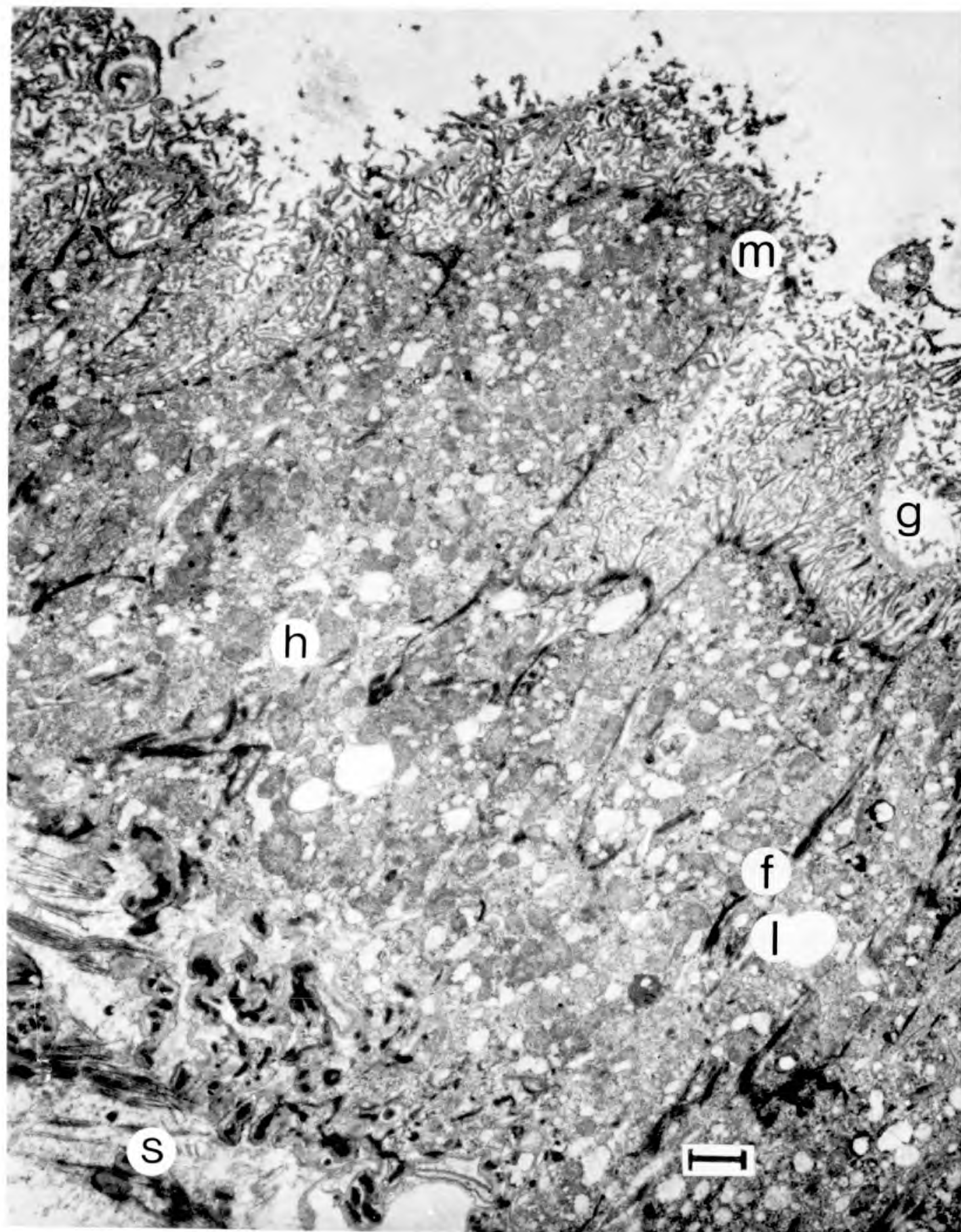


PLATE 29.

TRANSVERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC DELADENUS SIRICIDICOLA IN THE LATERAL HYPODERMAL CORD REGION.

m	microvilli
l	lipid
h	hypodermis
f	fibre layer
v	vesicular region
n	nucleus
p	pseudocoelom

PLATE 29

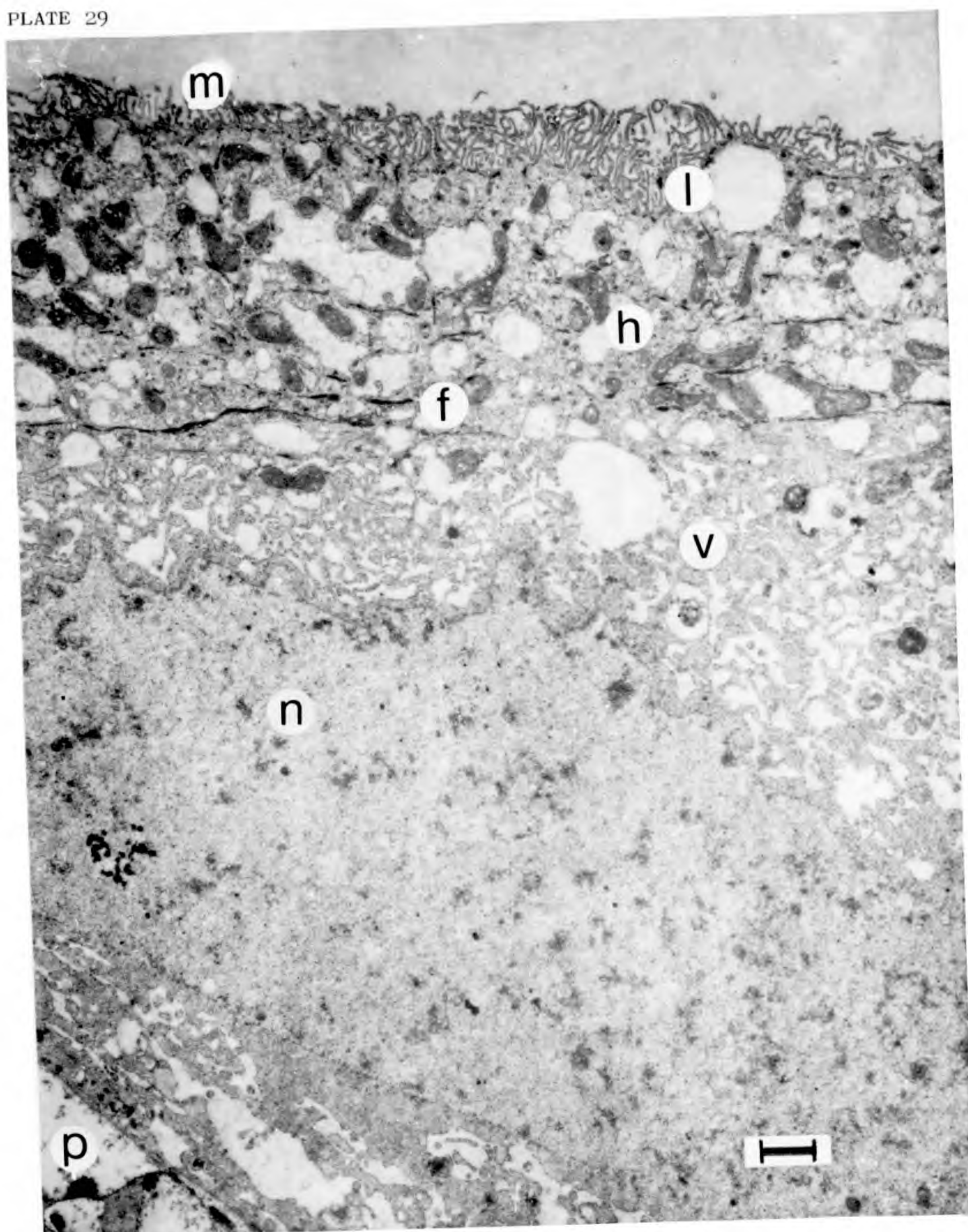


PLATE 30.

THE HYPODERMIS OF A MATURE PARASITIC *DELADENUS SIRICIDICOLA* INDICATING LYSOSOMAL ACTIVITY.

l	primary lysosomes
m	multivesiculate bodies
w	whorls of membranes, probably phospholipid or lipo protein and representing lysosomal residues
g	golgi apparatus

PLATE 30

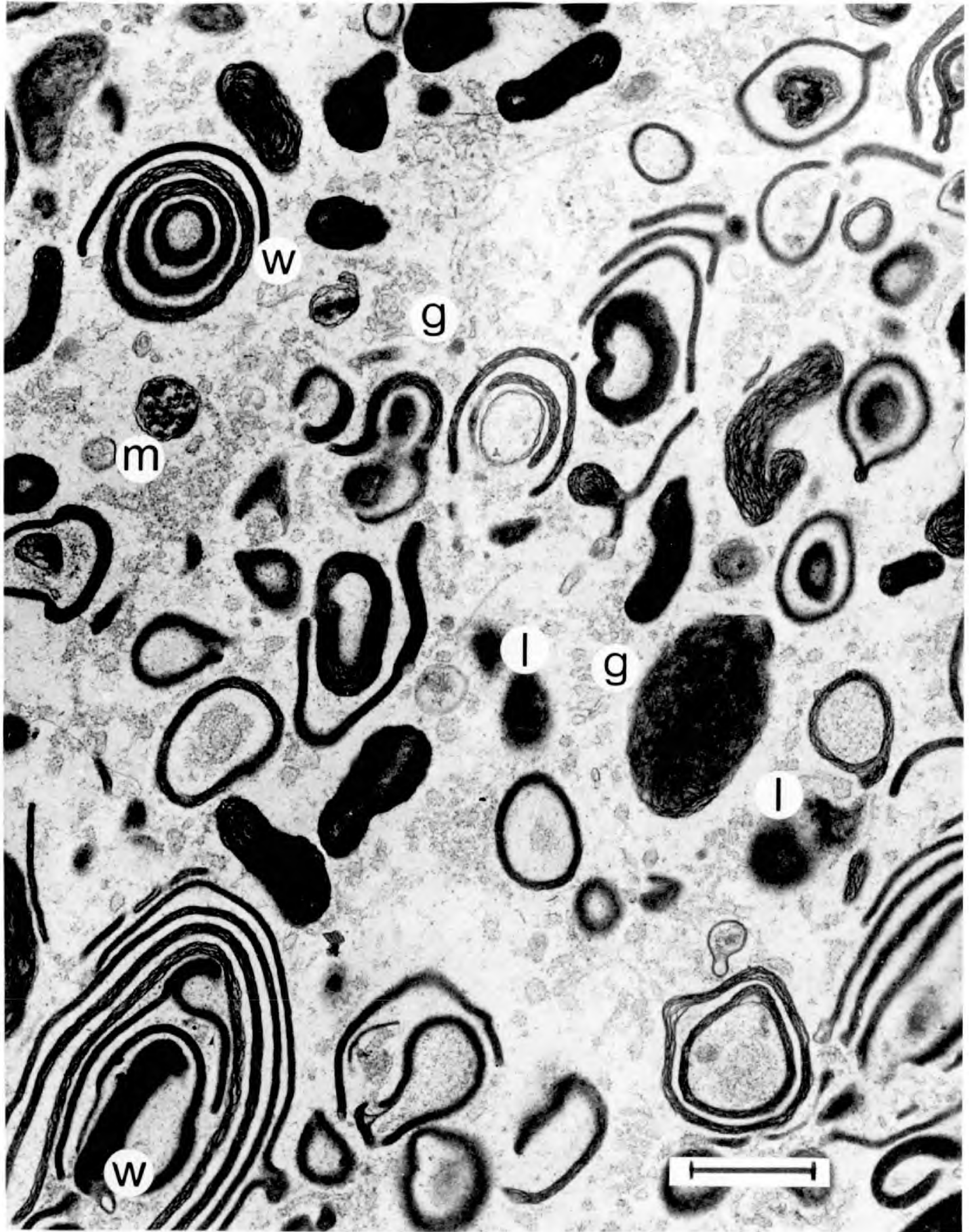


PLATE 31.

TRANSVERSE SECTION OF THE MICROVILLOUS LAYER OF A MATURE PARASITIC DELADENUS SIRICIDICOLA.

1, 2	layers of granular material
e	enlarged tips of microvilli
d	dense core of microvillus
m	microvilli
f	fibrillar material
h	hypodermis

Asterisks indicate regions where the microvilli tips are breaking down to release dense contents which may have originated from the fibrous bundles of the hypodermis.

PLATE 31

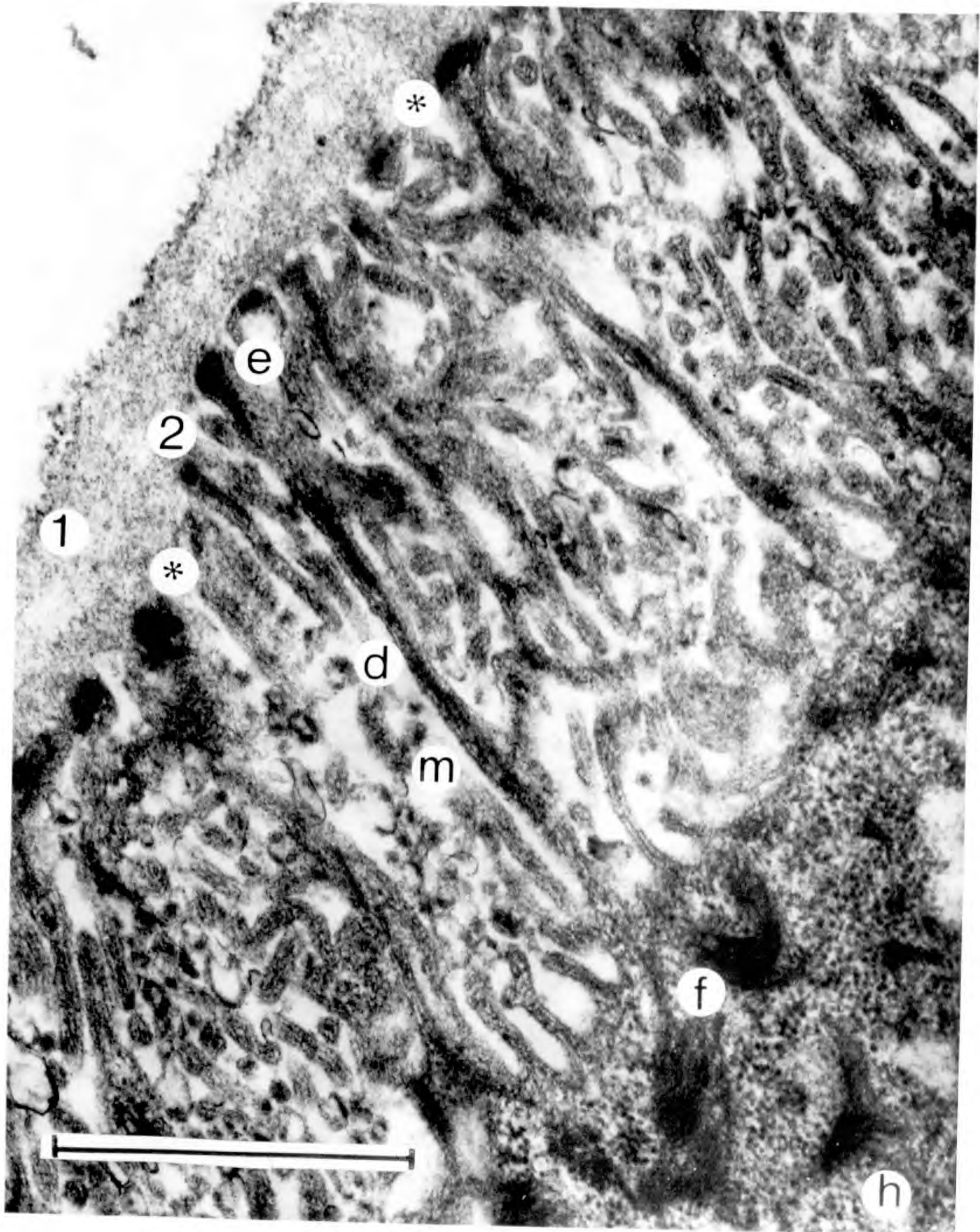


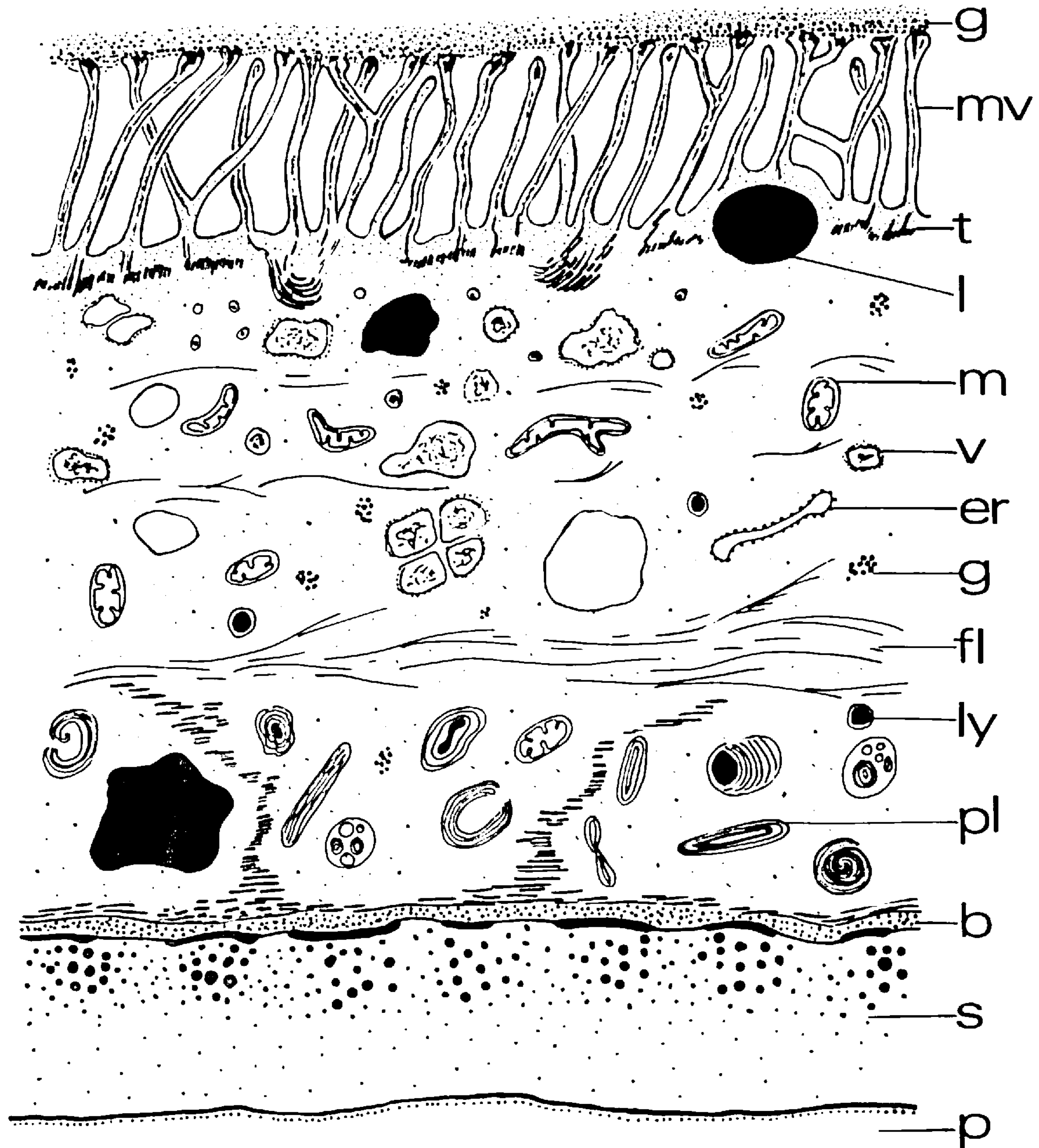
FIGURE 22.

RECONSTRUCTED DIAGRAM OF THE BODY WALL OF THE MATURE PARASITIC *DELADENUS SIRICIDICOLA* IN THE INTERCORDAL REGION.

g	granular layer
mv	microvilli
t	thick fibres
l	lipid
m	mitochondria
v	vesicles
er	endoplasmic reticulum
gl	glycogen
fl	fibriller layer
ly	lysosomes
pl	phospholipid lysosomal residues
b	basal lamina
s	somatic muscle cell
p	pseudocoelom

(Multivesiculate bodies are drawn but not labelled)

FIG.22.



II 3.7. THE MATURE PARASITIC DELADENUS WILSONI

The mature parasitic Deladenus wilsoni is indistinguishable from D. siricidicola at the light microscope level when obtained from the same host species (Bedding, 1968). Both are long (3-25 mm), thin (0.1-0.5 mm), flaccid, thread-like nematodes, often yellowish or greenish in colour and occasionally transversely beaded. A transverse section of D. wilsoni (FIGURE 23A) shows the same lack of cuticle, hypertrophy of the lateral hypodermal cords and reduced somatic muscle sectors as D. siricidicola, but when viewed with the electronmicroscope it can be seen that the external surface of each nematode is usually easily distinguishable.

PLATE 32 shows a low power electronmicrograph of a transverse section through the body wall of a mature parasitic D. wilsoni at the junction of the somatic muscle and hypodermal cords. Microvilli with large balloon like tips can be seen on the outside of the hypodermis which contains two or three prominent layers of fibres. The microvilli arise in clusters along an irregularly undulating surface; they vary in length from 2-8μm and some are much wider than others but usually their stalks do not branch or anastomose (PLATE 33). Many of the enlarged tips of the microvilli contain flocculent fibrillar material and there is some indication that adjacent apices fuse to form very large vesicles (PLATE 35 asterisks) which release their contents into the haemocoel of the host. Alternatively stalkless vesicles containing electron dense material may arise directly from the surface of the hypodermis by a budding-off process (PLATES 34

and 35).

A particularly interesting phenomena of the surface of the adult parasitic D. wilsoni is the conglomeration of electron-dense fibrillar material which collects at the base of the microvilli (PLATES 33-36 and FIGURE 24). The origin of these darkly staining fibres is not clear because although dense material is seen in secretory vesicles throughout the hypodermis, and predominantly near to the surface (PLATES 32-36) they more closely resemble the clusters of thick granulated fibres which occur in layers both near to the somatic muscle cells and just below the surface (PLATES 33 and 36). It may be that the secretory vesicles release their contents into the hypodermis and later this material aggregates at the surface. There is evidence that the electron dense material which lies near to the bases of the microvilli passes out of the hypodermis and becomes dispersed and more granular as it moves towards the host's haemocoel (PLATES 34-36 and FIGURE 24). Occasionally this granular material occurs in great abundance outside the nematode and when so, it appears to be produced not only directly from the microvilli which are largely obliterated in the process (PLATE 36).

Equally abstruse are the large membranous whorls found near the surface of the microvilli in the regions which have unusually large amounts of external granular material (PLATE 36). They resemble phospholipid residues but the usual intermediate forms which normally indicate phospholipid production from lysosomal activity have never been seen. Also the membranous whorls have granular and often crystalline centres and the membranes appear to be disintegrating. There is some suggestion that these whorls

are expelled from the surface of the nematode and also contribute to the external granular material.

Other structures observed in the hypodermis are mitichondria, smooth and rough endoplasmic reticulum, lipid droplets, glycogen and many vacuoles and vesicles of differing sizes. Prominent irregular-shaped nuclei are present in the lateral hypodermal cords (FIGURE 23A) and these are surrounded by an increased amount of rough endoplasmic reticulum and secretory vesicles.

The somatic muscles are present in four reduced sectors near to the mid-dorsal and mid-ventral regions and a meromyarian arrangement is indicated by two to three cells in each sector. The muscle cells are platymyarian with prominent desmosome attachments to the basal lamina and the myofilaments occupy about one third of the cell; they are composed of both thick and thin elements and there is some indication of 'H', 'A' and 'I' bands but there are no cytoskeletal elements. The sarcoplasm contains a nucleus and a few mitochondria and the sarcolemma is surrounded by a basal lamina which separates the somatic musculature from the hypodermis and the pseudocoelom.

A diagram to illustrate the salient features of the mature D. wilsoni is shown in FIGURE 24.

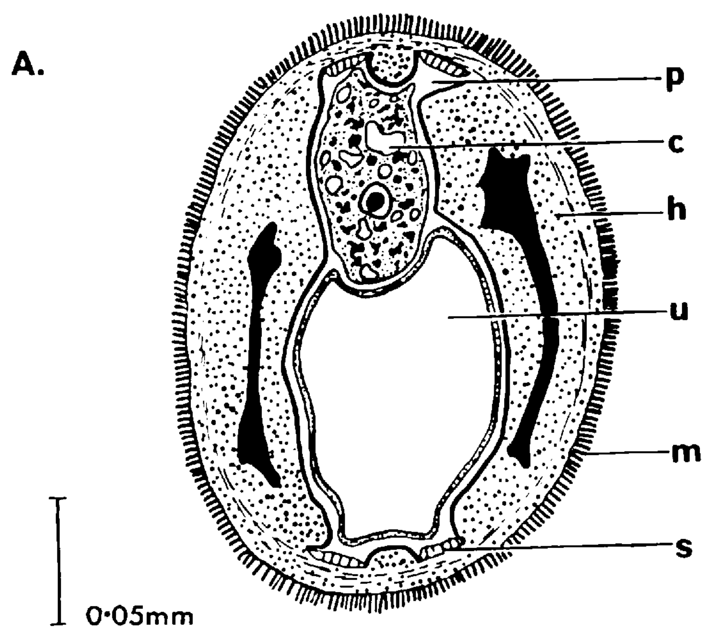
FIGURE 23.

FIG. 23A. TRANSVERSE SECTION THROUGH A MATURE PARASITIC DELADENUS WILSONI.

FIG. 23B. TRANSVERSE SECTION THROUGH A MATURE PARASITIC CONTORTYLENCHUS.

p	pseudocoelom
c	cells representing the gut
h	hypodermis
u	uterus
m	microvilli
s	somatic muscle cells
o	ovary

FIG.23.



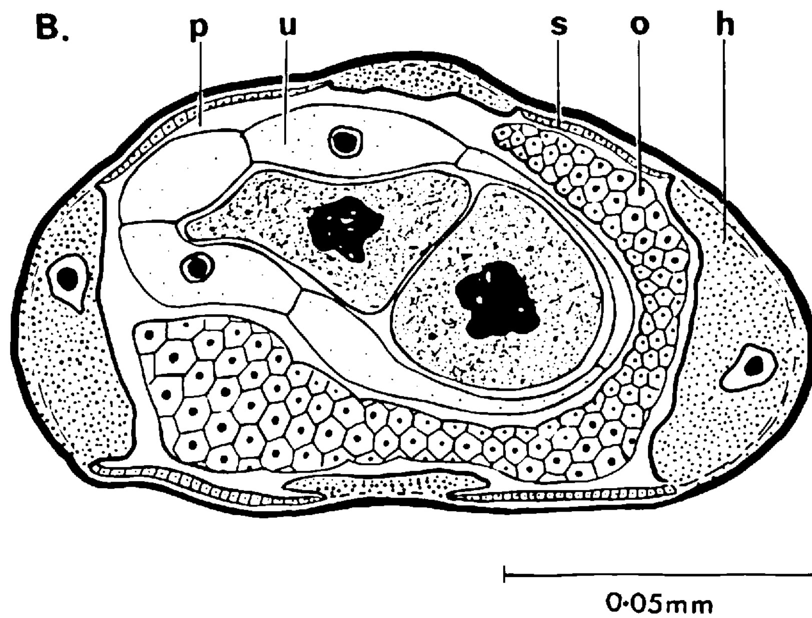


PLATE 32

TRANSVERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC *DELADENUS WILSONI* AT A JUNCTION OF MUSCLE AND HYPODERMAL CORDS.

v	microvilli
h	hypodermis
f	fibre layer
d	desmosome
l	lipid
m	somatic muscle cell
s	sarcoplasmic region of muscle cell
p	pseudocoelom

PLATE 32

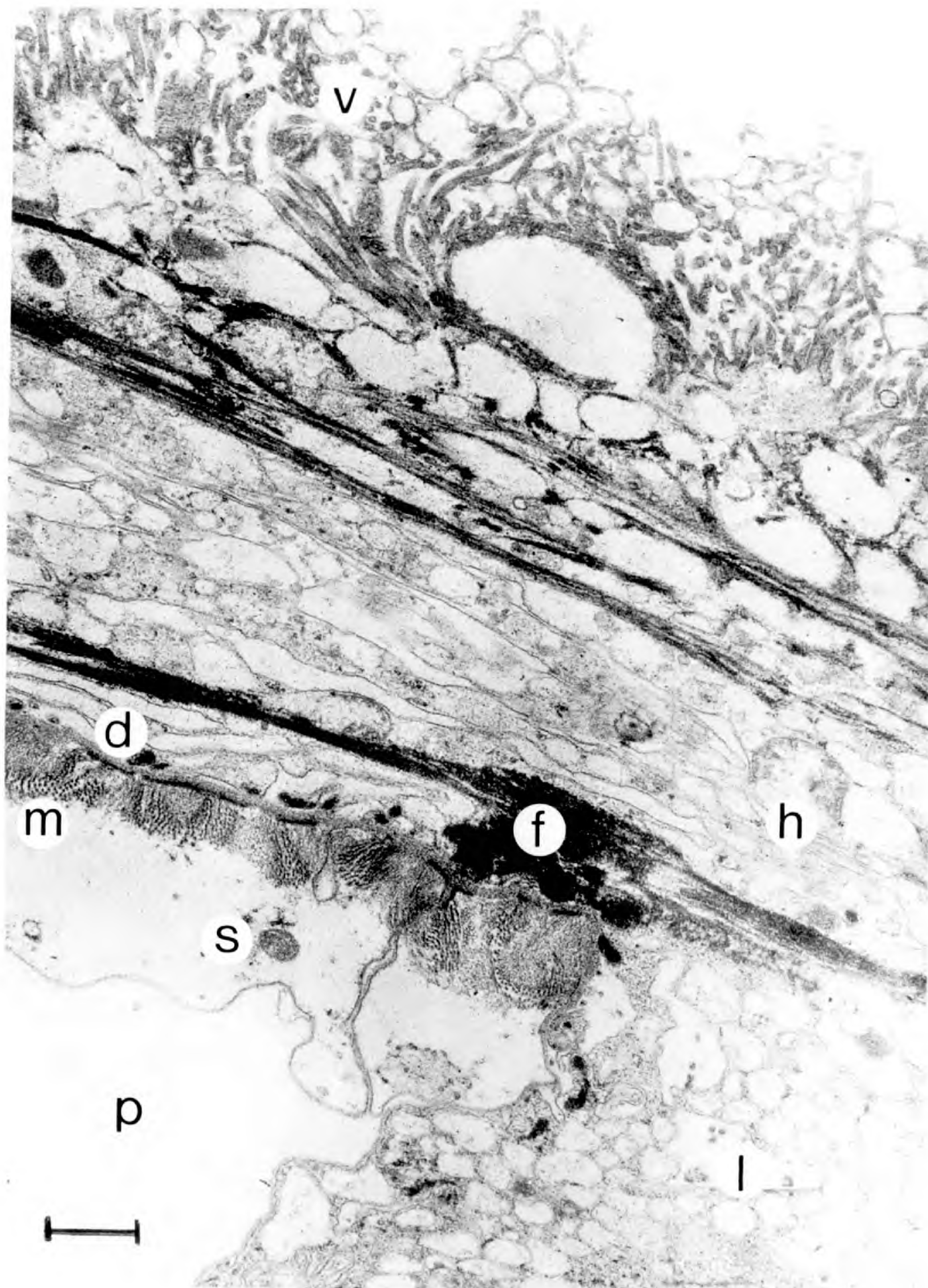


PLATE 33.

SECTION THROUGH THE SURFACE OF A MATURE PARASITIC DELADENUS WILSONI

e	extended tip of microvillus
b	breakdown of microvilli
m	microvilli
g	granular material probably released by microvilli
h	hypodermis
f	fibre layer
v	secretory vesicles with dense contents
p	possibly pinocytotic vesicles .

Arrows indicate dense fibrillar material which may be leaving the nematode.

PLATE 33

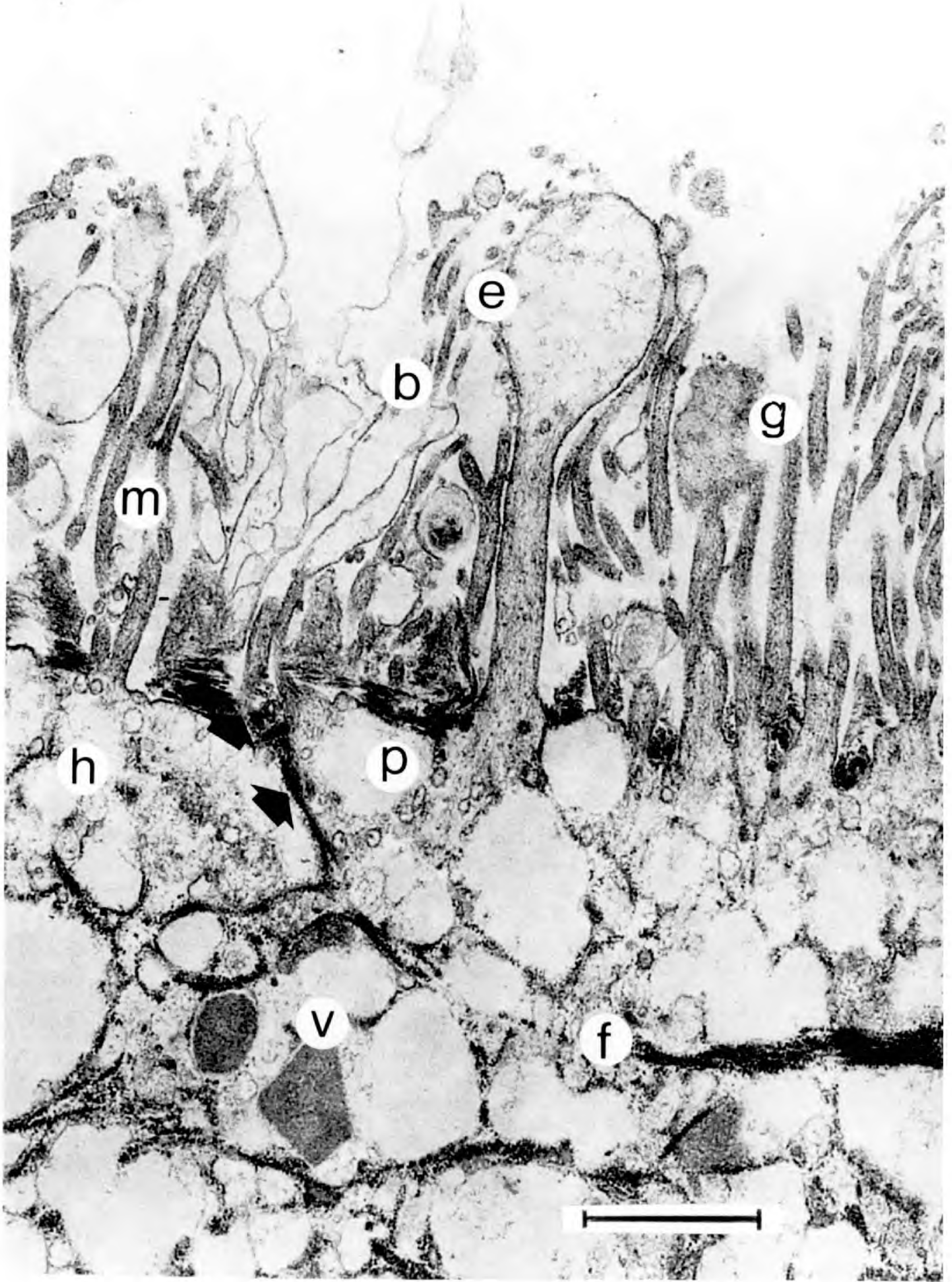


PLATE 34.

SECTION THROUGH THE SURFACE OF A MATURE PARASITIC *DELADENUS SIRICIDICOLA*.

e	enlarged tip of microvillus
s	surface of hypodermis
d	dense material both inside and outside the nematode
v	many small vesicles in this region
f	fibrillar layer
h	hypodermis

PLATE 34

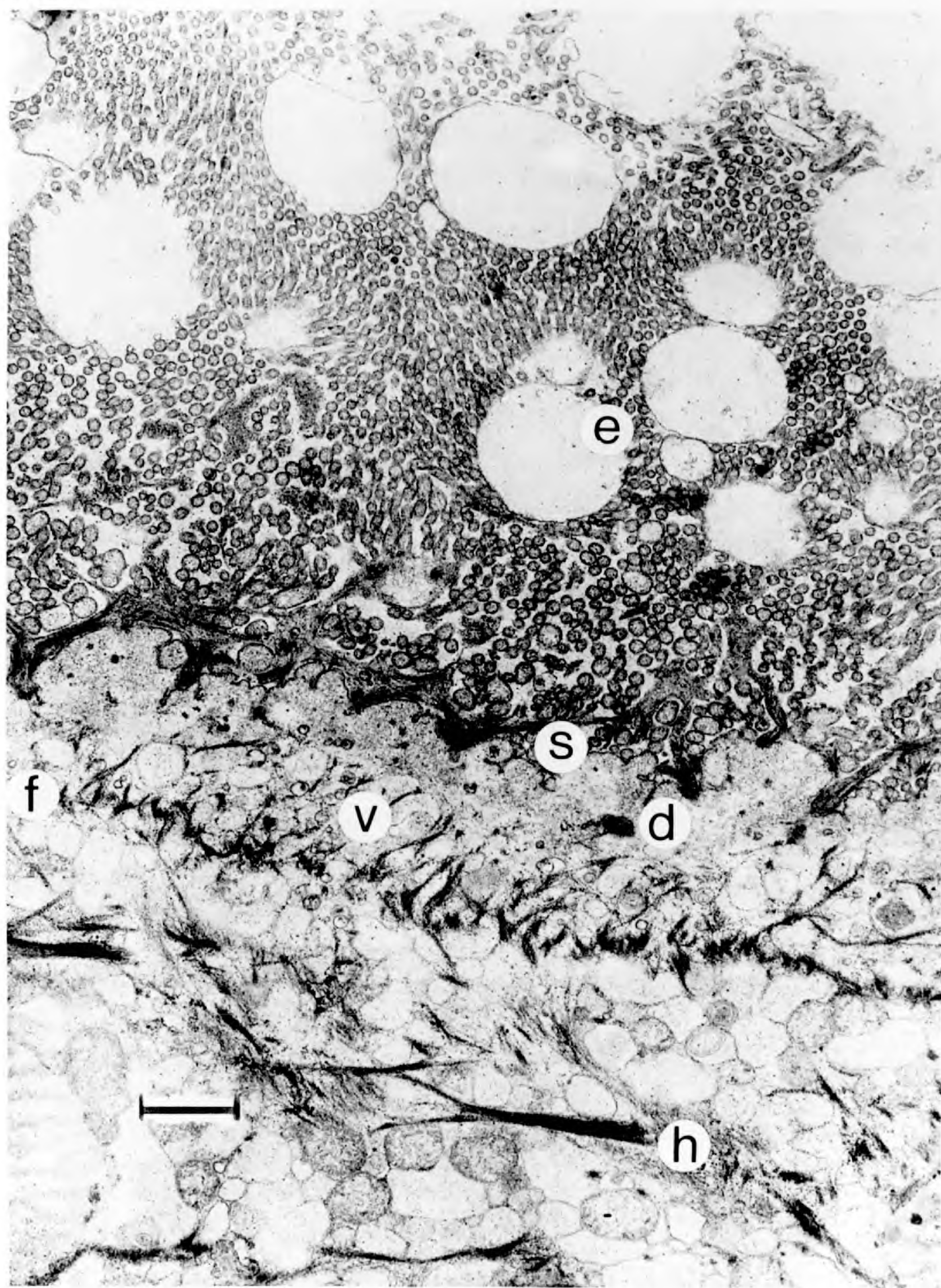


PLATE 35.

TRANSVERSE SECTION THROUGH THE SURFACE OF A MATURE PARASITIC *DELADENUS WILSONI*.

m	microvilli
s	swollen tips of microvilli
d	dense material both inside and outside the nematode
l	lipid
v	vesicle

Asterisks indicate two microvilli contributing to the fibre containing swelling.

PLATE 35

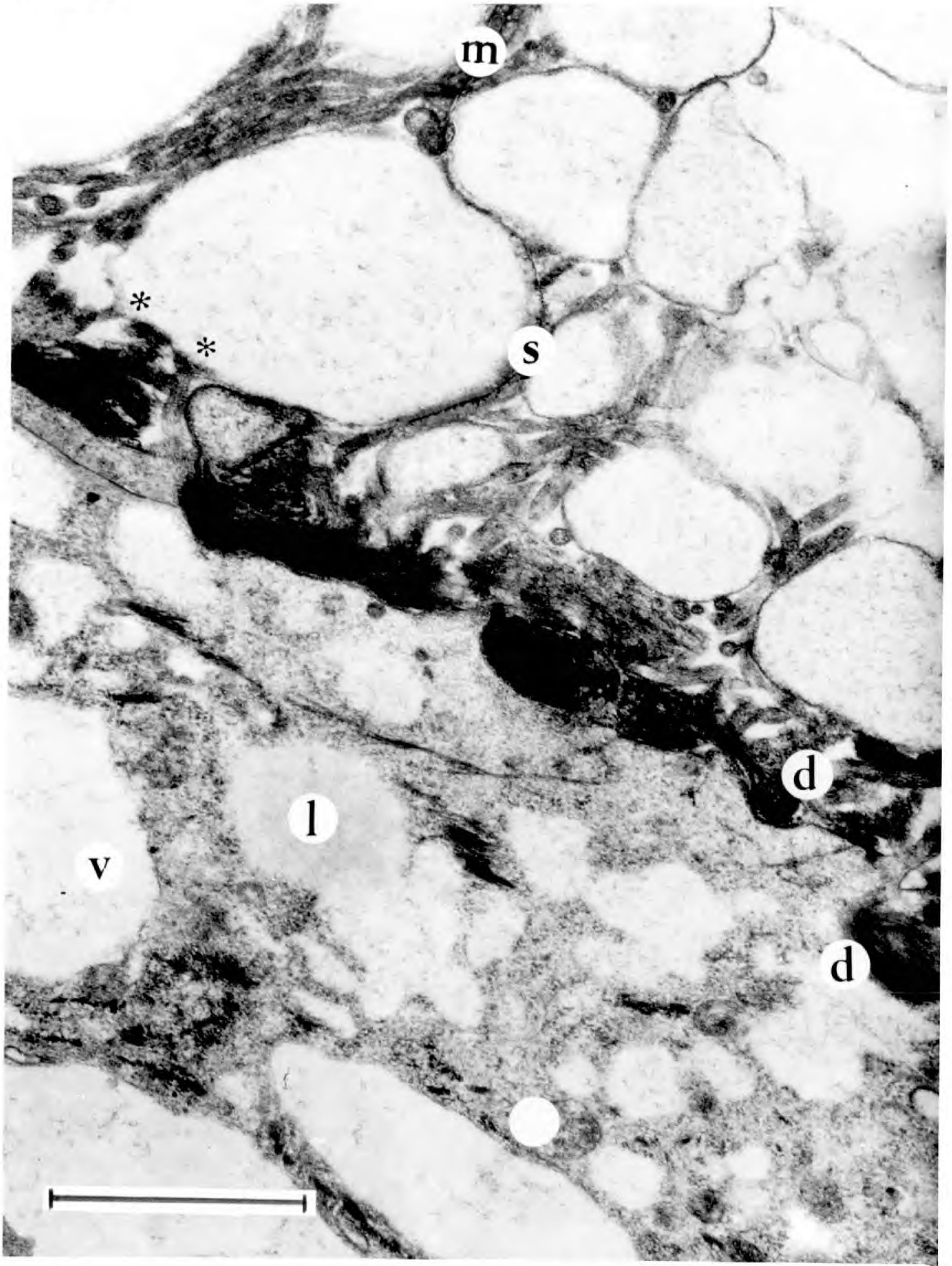


PLATE 36.

TRANSVERSE SECTION THROUGH THE SURFACE OF A MATURE PARASITIC DELADENUS WILSONI.

d	dense granular material
v	coated vesicles
h	hypodermis
w	whorls of membranes with crystalline centres
s	surface of hypodermis
m	microvilli
g	granular material outside the nematode

Asterisks mark the sites where granular material may be seen being extruded from the hypodermis.

PLATE 36

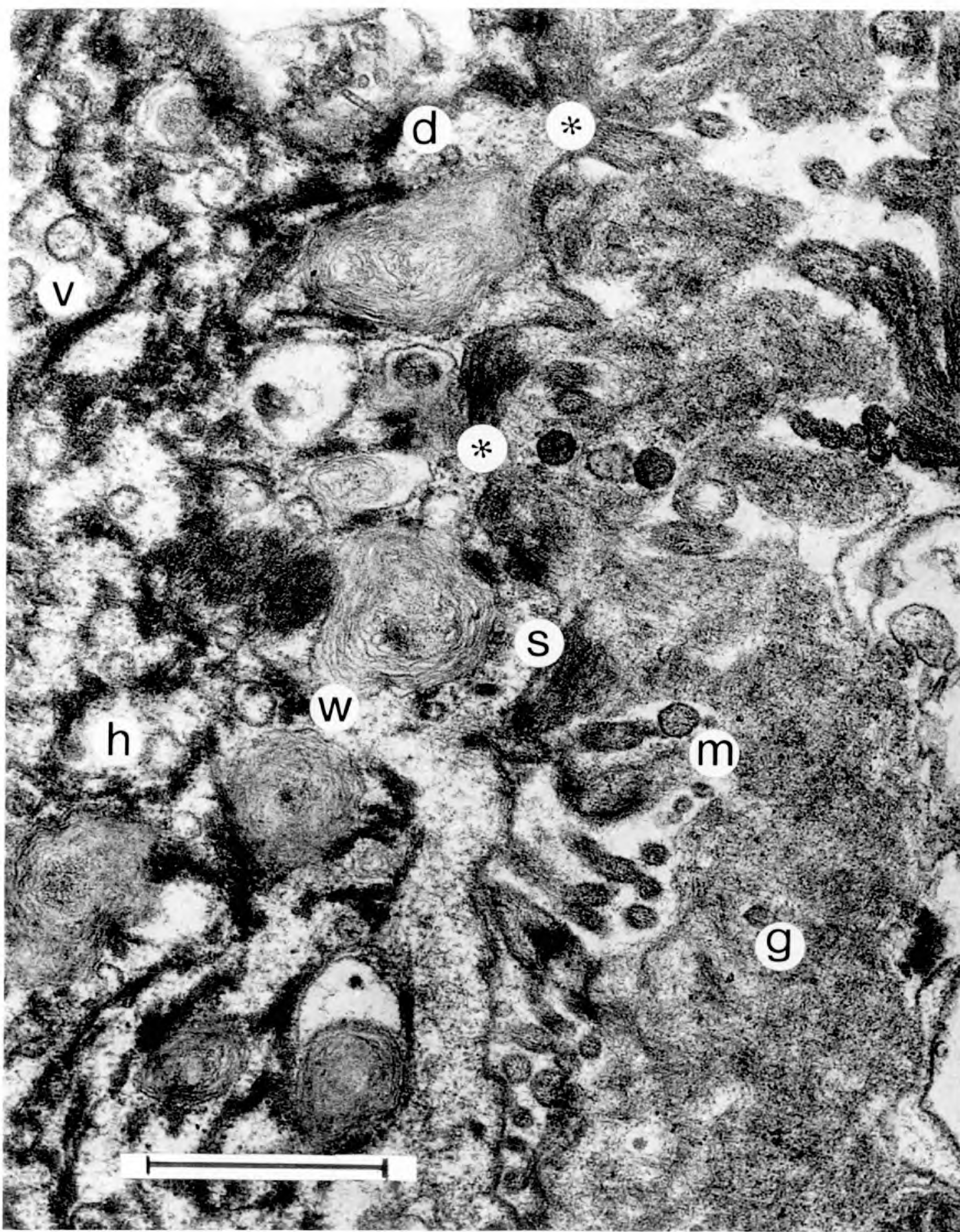
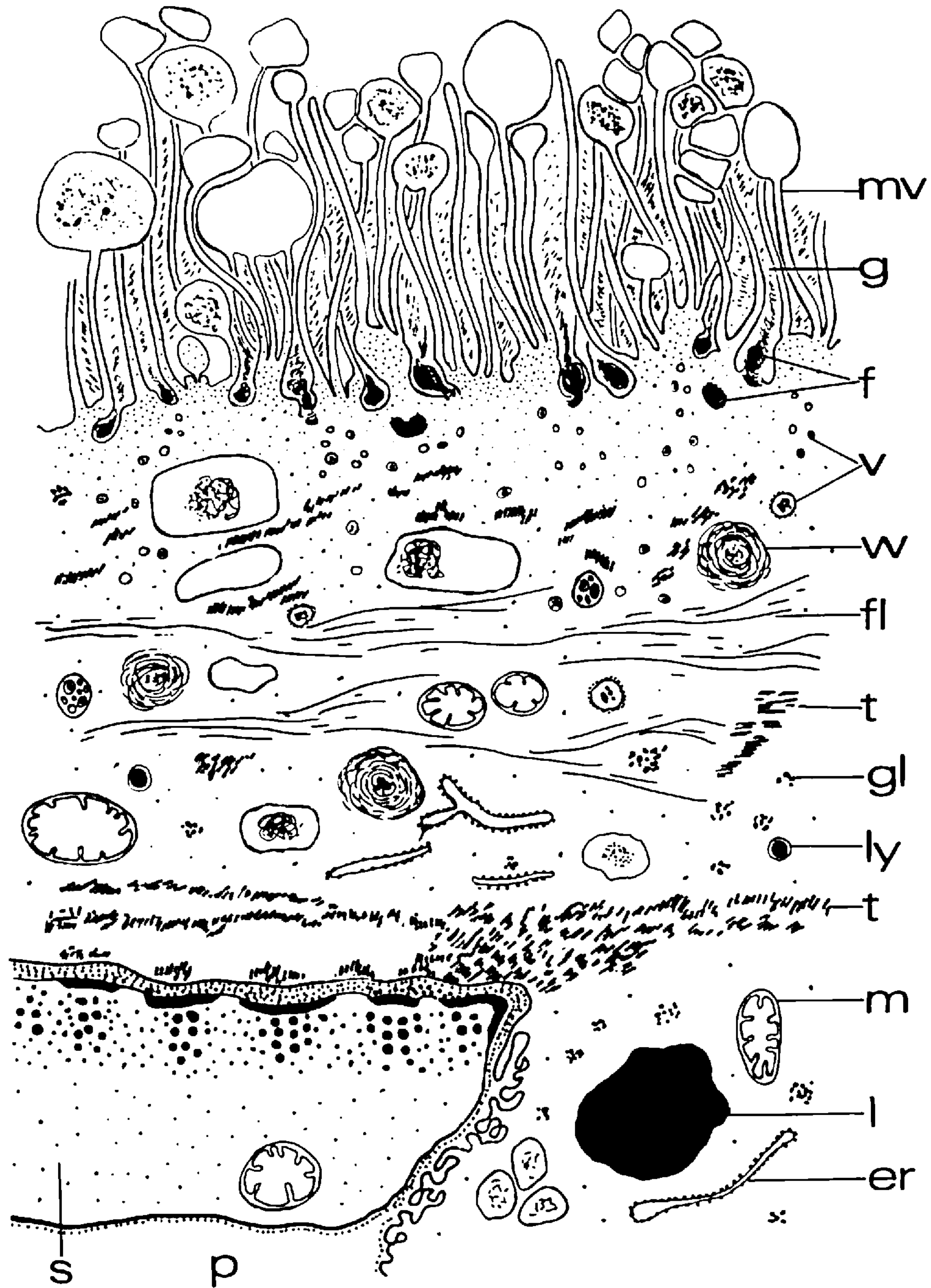


FIGURE 24.

RECONSTRUCTED DIAGRAM THROUGH THE BODY WALL OF THE MATURE PARASITIC *DELADENUS WILSONI* IN THE INTERCORDAL REGION.

mv	microvilli
g	granular material
f	fibrous bundles
v	vesicles
w	whorls of phospholipid
fl	fibre layer
t	thick fibres
gl	glycogen
ly	lysosomes
m	mitochondria
l	lipid
er	endoplasmic reticulum
s	somatic muscle cell
p	pseudocoelom

FIG.24.



II 3.8. THE MATURE PARASITIC CONTORTYLENCHUS

The mature parasitic *Contortylenchus* sp. resembles *Howardula* and the two *Deladenus* species in lacking the typical filariform shape but in several other respects it is quite different. *Contortylenchus* is turgid and soon bursts when taken from its bark-boring beetle host and put into 0.75% entomological saline (Al-Rabiai, 1970). The stylet persists in an anterior position and although the oesophagus is degenerate, the intestine is present together with slight traces of a rectum and anus. The outer covering resembles a cuticle in being smooth, translucent and stratified, and microvilli are not present.

However a transverse section shows that the mature parasitic *Contortylenchus* sp. does not have the usual nematode distribution of hypodermis and somatic musculature and that it is similar to *Howardula* and *Deladenus* in having enlarged hypodermal cords (FIGURE 23B). The hypodermis shows considerable hypertrophy in the laterial cords but little in the median regions where the somatic muscle cells form extensive sectors at each side of the dorsal and ventral hypodermal cords. *Contortylenchus* is usually a round or oval shape when seen in transverse section but distortion occurs according to the degree of pressure exerted by the host's abdominal contents and to the arrangements of the nematode's internal organs. Regions inside the nematode which are not filled with gonad tend to collapse in older specimens and a variety of shapes is assumed, but the common one has three external folds. Simple diagrams to illustrate the probable sequence of fold development are shown in FIGURE 25B, 1-111) and further comments regarding

these three ridges will be included in the discussion section. (Attention is drawn to the fact that unlike the other transverse sections in this study, FIGURE 25B, 1-111 are not camera lucida drawings and the last two should have a more rounded outline).

After examining the mature parasitic Contortylenchus with the electron microscope it was obvious that the body wall, while not possessing microvilli, was nevertheless unlike that of 'normal' nematodes (PLATE 37). The hypodermis is continuous with the outer cuticle-like layer of the body and although it is superficially demarked by an irregular zone of concentric fibres, these fibres form an incomplete layer. Also there is no hypodermal membrane present, other than that which surrounds the outer surface and consequently it is deduced that a cuticle is lacking. However, the intercordal hypodermis and the outer region of the hypodermal cords have several features which are characteristic of cuticle such as the stratification and frequent lack of organelles as shown in PLATES 38 & 39 and FIGURE 25A.

Five layers are usually visible between the external surface and the somatic muscle cells; together these form a region 0.68-1.15μm wide (mean = 0.83μm). On the outside is an electron dense region which is sometimes resolved into the three components characteristic of a unit membrane. Below the surface membrane is a granular layer followed by and overlapping a fibrillar layer, then a broad vaculated region which is traversed by a column of dense fibres; finally there is a band of fine granular material which probably represents a basal lamina. The fourth layer, in centripetal order, is widest and most conspicuous and has the greatest variety of components. Frequently it contains vesicles

which encompass fibrillar material and also large vacuoles may be present and these either contain electron opaque material or they may be empty. In longitudinal section (PLATE 39 and FIGURE 25A) dense prominent columns of fibres traverse this fourth layer and they are frequently orientated perpendicularly to the surface and have fibres radiating from opposite ends of each column which often penetrate the adjacent layers 5 and 3. Depending on whether the perpendicular or the radiating components of these fibre columns have been encountered, transverse section show either 1 or 2 concentric fibre layers or obliquely orientated fibres across the fourth layer. The presence of a single inner layer of fibres in most transverse sections indicates that the radiation of fibres from the base of the columns is greater than at the upper end.

In addition to vesicles, vacuoles and columns of fibres, the fourth layer of the body wall often contains glycogen, occasional mitochondria, rough endoplasmic reticulum and small membrane-bound vesicles with dense contents, which provides further evidence that the outer region of the body wall of *Contortylenchus* is not a cuticle. The amorphous granular fifth layer, or basal lamina, merges imperceptibly with the fourth layer and it provides a surface for the attachment of many half desmosomes from the somatic muscle cells (PLATES 38 and 39).

The hypodermis of the median and lateral cords is continuous with the outer layer and contains nuclei, rough endoplasmic reticulum, mitochondria, lipid droplets, glycogen and many secretory vesicles.

The somatic musculature is divided into four sectors of platymyarian cells which have a meromyarian arrangement of up

to six cells in each quarter. Both thick and thin myofilaments are present and these are tightly packed together at the base of the cell and organised into 'H', 'A' and 'I' bands. The sarcoplasmis region contains a nucleus, endoplasmic reticulum, glycogen and several large mitochondria which have numerous spirally arranged cristae. The somatic muscle cells of the parasitic *Contortylenchus* differ from those of *Howardula* and *Deladenus* in appearing to be very well developed.

A reconstruction of the body wall of *Contortylenchus* is illustrated in FIGURE 25A.

PLATE 37.

TRANSVERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC *CONTORTYLENCHUS* sp.

h	hypodermis
s	somatic muscle cell
p	pseudocoelom
o	oviduct
f	fibrillar layer
d	deep folds of the surface layers

PLATE 37

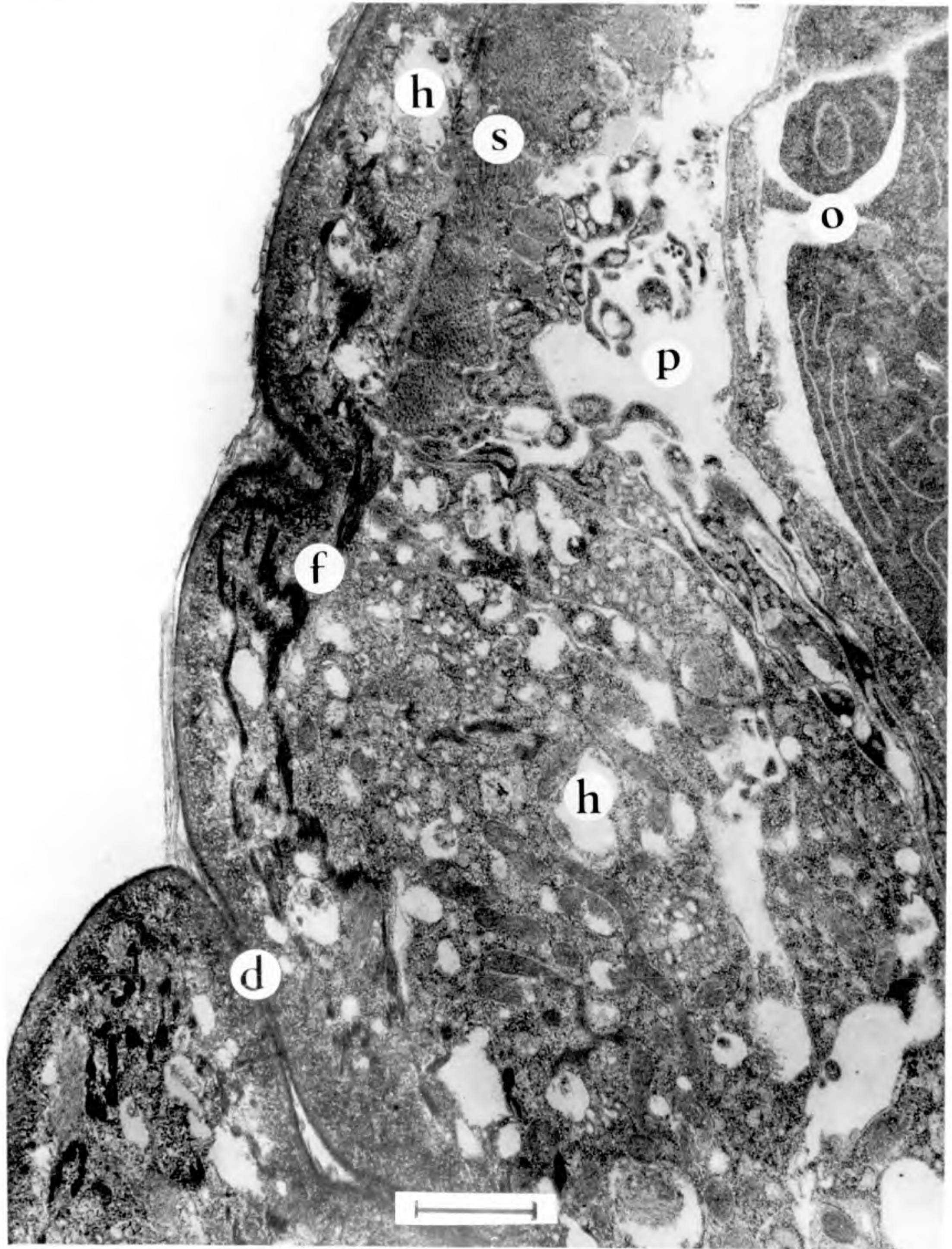


PLATE 38.

TRANSVERSE SECTION THROUGH THE BODY WALL OF A MATURE PARASITIC CONTORTYLENCHUS sp.

m	membranous debris outside the nematode
o	outer layer of "cuticle"
g	granular layer
f	fibres
v	vesiculate region
b	basal lamina
d	desmosome
s	somatic muscle cell

PLATE 39.

OBLIQUE LONGITUDINAL SECTION THROUGH THE BODY WALL OFA MATURE PARASITIC CONTORTYLENCHUS sp.

Labelling as in PLATE 38 above plus:

c	column of fibrous material
p	pseudocoelom

PLATE 38

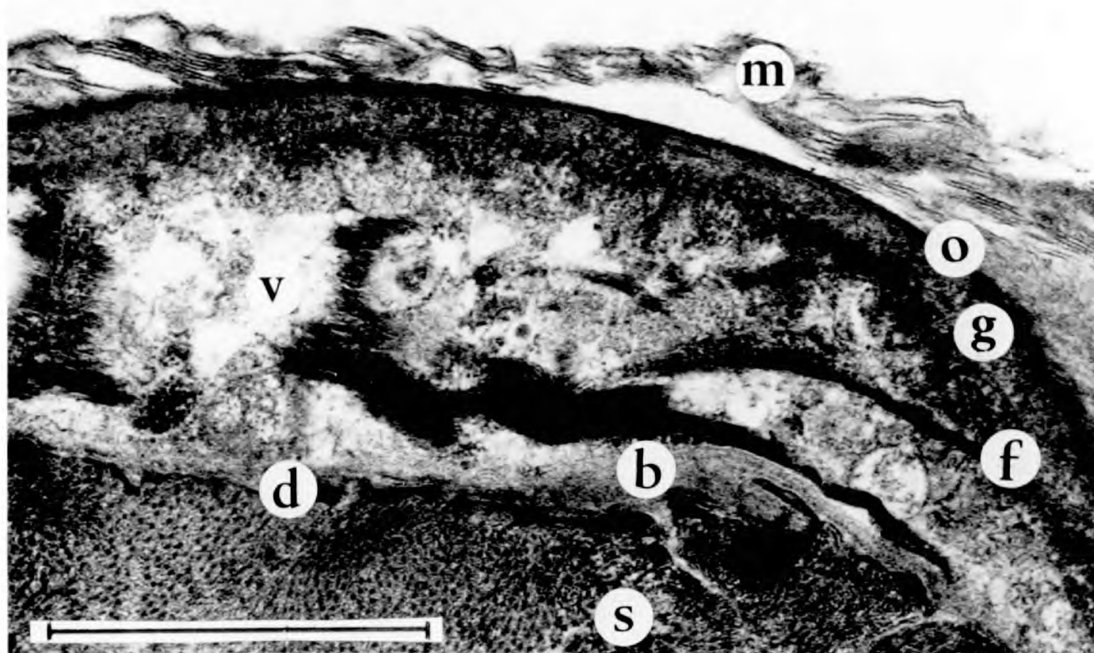


PLATE 39

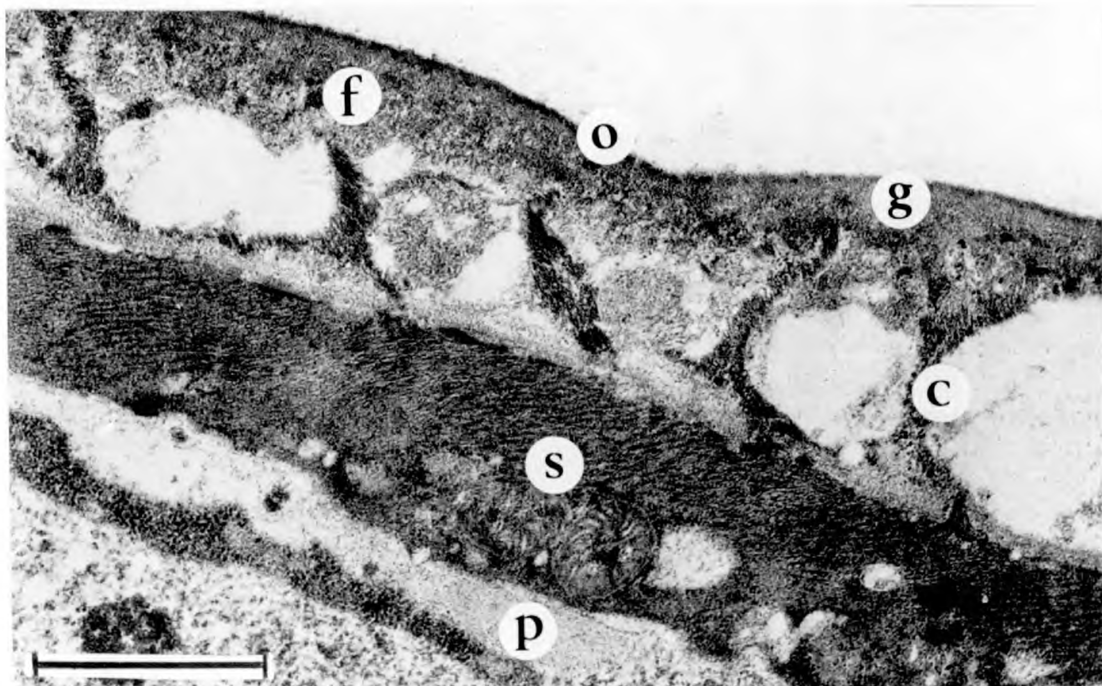


FIGURE 25.

FIG. 25A. RECONSTRUCTED DIAGRAM THROUGH THE BODY WALL OF THE MATURE PARASITIC CONTORTYLENCHUS IN THE INTERCORDAL REGION.

1-5 indicates the regions of the "cuticle".

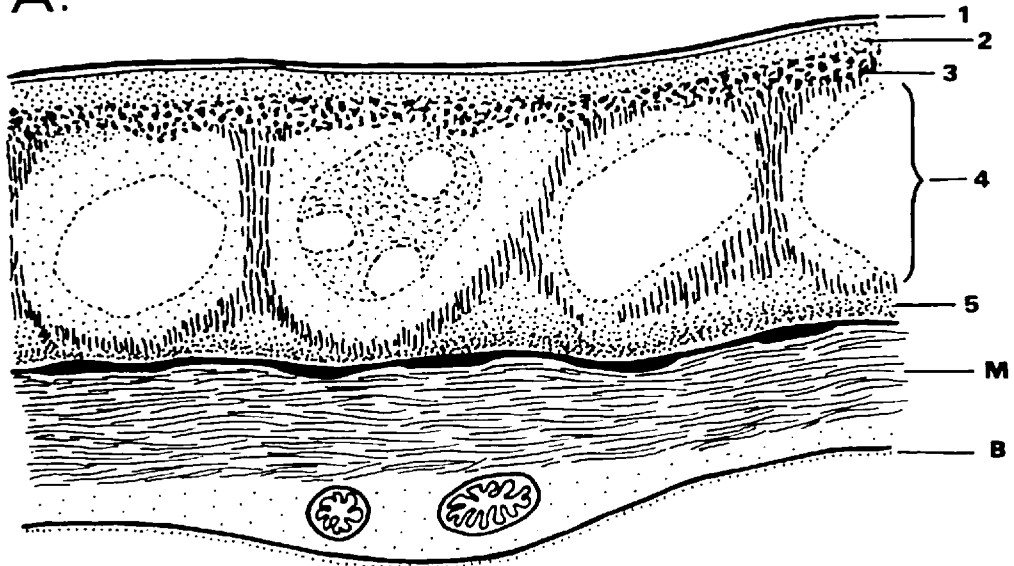
1 dense surface layer
2 granular layer
3 fibrillar layer
4 vacuolated region traversed by dense fibres and containing endoplasmic reticulum and mitochondria
5 basal lamina
m somatic muscle cell
b basal lamina

FIG. 25B. POSSIBLE ORIGIN OF THE THREE BODY WALL FOLDS IN CONTORTYLENCHUS

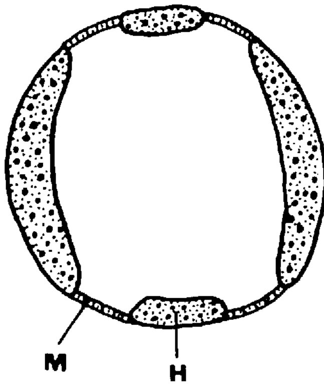
i Normal transverse section through *Contortylenchus* showing hypodermal cords (h) and somatic muscle cells (m).
ii Slight distortion of the body wall due to pressure inside the host haemocoel.
iii Fully distorted, showing 3 folds.

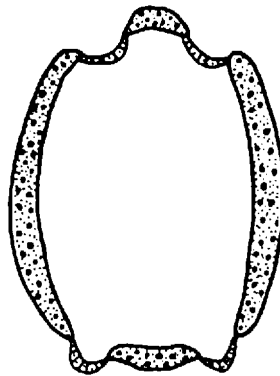
FIG.25.

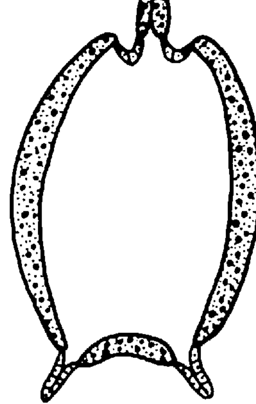
A.



B. i. ii. iii.







II 4. DISCUSSION

It had been anticipated that the body wall of the aberrant parasitic stages of the nematodes chosen for this study would be different from all other members of the phylum so far examined because of the suspected nutritional role of the external surface; these nemas do not have a mouth but they obviously feed. In view of the extent of the structural modifications encountered and the fact that such functional adaptations may be inferred from many of the descriptions of the 120 known Tylenchid parasites of insects, it is curious that the Sphaerularids have escaped the attention of nematologists interested in feeding mechanisms. Presumably the common fallacy that the Tylenchids are all plant parasitic nematodes may explain why the entomophilic species have been overlooked in the current literature on nematode nutrition. Alternatively the fact that most references to parenteral feeding occur in taxonomic papers in the 50 years prior to the 1930's, and more recently in German papers which are difficult to obtain, probably accounts for the neglect of this small but fascinating group of nematodes.

Hypertrophy of the hypodermis and reduction of somatic musculature described in the mature parasitic *Howardula*, *Deladenus* and *Contortylenchus* had been shown as long ago as the last century in *Bradynema* rigidum (von Siebold 1836) zur Strassen 1892 but the significance of this modification had been overlooked. In a well illustrated paper, zur Strassen (1892) drew transverse sections of *B. rigidum* which are almost identical to *Howardula* sp. (FIGURE 18 and 19) and in addition his observations of the surface layer with its

"Stabchenschicht der Cuticula" leave little doubt that B. rigidum also has a layer of microvilli on the outside of the body wall. It was fortunate that Dr. W.R. Nickle (U.S.D.A., Beltsville, Maryland) was able to lend me a permanent preparation of this nematode and as it is up to seven times larger than Howardula sp. I had little difficulty in confirming with the light microscope that B. rigidum has indeed got an outer layer of microvilli.

Several of the early taxonomists to describe nematode parasites of insects drew attention to the cuticular distension and loss of rigidity that occurs as soon as the infective larva becomes a parasite and it was commonly stated that in the absence of a mouth, nutritive fluids enter through the general body surface by "diffusion". Although there was no experimental evidence, this phenomenon was so widely accepted that when Goodey described Tylenchinema oscinellae in 1930 (now Howardula oscinellae (Goodey 1930) Wachek 1955) he had to point out that "as this adult female retains a stylet, it is quite probable that it continues to take in food via the alimentary canal, at any rate for the greater part of its growth, if not for the whole of its parasitic existence". However Wachek (1955) in his major contribution "Die Entoparasitischen Tylenchiden" assumes that most of these entomophilic nematodes feed endosmotically ("Osmotischer Nahrungsaufnahme") inside the body cavity of the host, as does Ruhm (1956).

Warren (1941) attempted to explain the nature of the morphogenesis of the body wall in Acarinicola terribilis (Howardula terribilis (Warren 1941) Wachek 1955) and wrote that "the body of the original nematode is reduced to a swollen sausage-shaped sac .... to be regarded as the cortical layer of mesoderm enclosing all the

organs of the original worm, while the true ectoderm has disappeared. There is no cuticle whatsoever." This is a fair appraisal in view of the present findings but it is not really surprising that much of the early observations have been disregarded because they are often discredited by accompanying examples of the authors incompetence.

Assuming from the ultrastructural observations that the body wall of Howardula, Deladenus and possibly Contortylenchus is adapted for a nutritional function, it is relevant to discuss the transport mechanisms which may operate and the possible lines of investigation which could elucidate them, with particular reference to the functional morphology of known absorptive surfaces such as the tegument of cestodes and acanthocephala, and the intestinal epithelium of nematodes, trematodes and mammals where microvilli are involved.

MICROVILLI FORMATION AND MOULTING

The microvillous surface described in Howardula sp. and Deladenus siricidicola appear to develop in entirely different ways. Both have a "normal" nematode cuticle at the onset of parasitism and Deladenus unlike Howardula, probably produces a modified cuticle during its final moult; this is subsequently slowly eroded by invagination of the surface membrane to form microvilli. During this investigation Dr. R.A. Bedding had no evidence that exsheathment occurred during the parasitic stage of D. siricidicola as it does in Howardula but because of the present findings he has made further investigations (personal communication) and has established that a moult occurs a few days after the infective female penetrates a host. The specimens I examined had been inside the

insect larva for 2-3 months and consequently had sufficient time to modify their immediate post-ecdysis structure. However it seems likely that an amorphous cuticle-like structure was first produced at the final moult and this slowly developed pits of microvilli which were described in the young parasite (PLATES 24-27) and which eventually give rise to the microvillous surface of the adult. In contrast, Howardula shows no indication of forming a cuticle in the final moult. The hypodermal membrane proliferates into finger-like folds which become microvilli in a process lasting two days, while in Deladenus it probably takes 6 months to form a complete layer of microvilli. The truncated life cycle of Howardula presumably necessitates a very rapid formation of an efficient absorptive surface because it only has about two weeks in which to grow, mature and reproduce before the insect is ready to expel the nematode larvae. Deladenus has at least 9 months for the same development and it grows very slowly in comparison with Howardula. It probably relies on stored nutrients until the first few microvilli are functional and gradually increases its food uptake until a complete layer of microvilli is formed, when maximum growth and egg production occurs after several months.

It is relevant to point out that little work has yet been done on the development of microvilli in other animals (Bonneville and Weinstock, 1970) and little is known about their formation on an external surface, but the morphological complexity of the fully differentiated striated or brush border of intestinal cells is now well documented. The comprehensive papers of Ito (1965), Mukherjee and Williams (1967) and Cardell et al., (1967) on mammalian intestine include many references to other studies and in all examples the microvilli are straight finger-like projections of similar length, width and distribution. Usually they contain a central core of dense filaments which can often be seen as "rootlets"

below the apical surface where they merge to form a fibrillar layer, the terminal web. Laguens and Briones (1965) observed that the filaments in the microvilli of human intestinal epithelium are tubular in section and they suggested that all the "filaments" are actually microtubules. This has since been shown by other workers in a variety of animals including Jenkins and Erasmus (1969) in the gut microvilli of the nematode *Metastrongylus* and Jha and Smyth (1969) in the microtriches on the outside of a cestode. Improved fixation and thinner sections of *Howardula* will undoubtedly show the microvillous filaments (PLATE 18 inset) are tubular but there is no indication that this is the condition in *Deladenus*.

It is interesting that the three nematodes with external microvilli are unusual in having regions of irregular, branching and anastomosing forms and the arborescent microvilli of *Howardula* are reminiscent of the polypoid type described by Knoth (1968) in the syncytial trophoblast of a young human embryo. His electronmicrographs show that the syncytium is remarkably like the hypodermis of *Howardula* and the microvilli are first rod-like but become branched, clubbed and polypoid with increasing gestation, particularly along the smoother flat portions of the surface. It may be significant that these two examples of atypical microvilli both occur on an 'external' surface and on a syncytium.

Bonneville and Weinstock (1970) outlined the formation of microvilli which occurs during metamorphosis of *Xenopus* in undifferentiated basal cells below the tadpole's gut epithelium. Many coated vesicles containing fine filamentous material aggregate beneath the surface where the microvilli are destined to form. These vesicles appear to fuse with the pre-existing apical plasma membrane which is thus expanded and a filamentous coat forms on the outside

from the liberated contents of the vesicles. Curved or straight bundles of fine filaments collect near the surface, extending inwards from the membrane, and form a mould around which the microvilli are constructed. Further fusion of the surface-forming vesicles which lie parallel to the bundles of filaments produces "moats" around filamentous cores so that the microvilli gradually elongate. Overton and Shoup (1964) and Overton (1965) suggest a comparable morphogenetic role for the core during the formation of microvilli in chick and mouse intestine and it seems likely that the strands of fibres described in the "cuticle" of the developing parasitic D. siricidicola (PLATE 24 and FIGURE 21) have a similar function. However no such templets have been observed in the formation of microvilli in Howardula which probably accounts for their apparent lack of rigidity and haphazard arrangement (PLATES 14 and 15 and not as illustrated in the simplified diagram FIGURE 17D), in marked contrast to the vertical rod-like appearance of the developing microvilli in Xenopus. The fibrillar core of the microvillus of the mature Howardula is presumably added after ecdysis and is probably associated with the terminal web formation which is not seen during the moult. Because the parasitic Howardula are variable in size, results obtained from measuring developing microvilli are not conclusive, but there appears to be an increase in length and a decrease in width of the microvilli after ecdysis. Xenopus has no noticeable change in microvilli dimensions which remain the same as when first formed, but there are several records of a second morphogenetic stage occurring (e.g. Brown, 1962) when microvilli increase in length while their volumes remain constant and their diameters markedly decrease. Overton et al., (1965) reported changes in the cores of hamster intestinal

microvilli and speculated that this second phase of elongation might be due to a variation in the conformation of filament molecular structure.

In view of the work of Bonneville and Weinstock (1970) on Xenopus, it seems probable that the coated vesicles described at the base of the "cuticular" invagination of the immature D. siricidicola (PLATES 26 and 27) are not pinocytotic vesicles which fuse with the plasmalemma to increase the area available for microvilli formation; in the absence of experimental evidence, one can only speculate. The abundance of vesicles containing filamentous material in the hypodermis of the moulting L_4 Howardula (PLATES 11-15 and FIGURE 17) suggests that they have a similar role, and the contents of the vesicles probably contribute to the glycocalyx. If these vesicles function primarily in the formation of microvilli it may seem surprising that they persist in the mature adult (PLATES 16-18) until one is familiar with the elegant work of Ito and Revel (1966). They demonstrated by autoradiography studies on cat intestinal epithelia that there is a constant renewal of the surface coat in cells with a fully differentiated brush border and although vesicles are not as yet identified as participants in this process, it seems reasonable to suppose that this is why they persist in the mature Howardula considering the established role of surface-forming vesicles in Xenopus.

The changes described during the first stages of the moult in Howardula sp. are similar to those described in a variety of "normal" nematodes (e.g. Bird and Bird, 1969; Davey, 1965) where the nuclei and nucleoli of the hypodermis enlarge, mitochondria multiply and

the cytoplasm becomes vacuolated. The subsequent changes leading to the loosening of the old cuticle and its separation from the hypodermis resemble those illustrated for Nippostrongylus brasiliensis (Lee, 1970a) and Nematospiroides dubius (Bonner et al., 1970) Samoiloff and Pasternak (1968) do not report vacuolation of the hypodermis during the moult of Panagrellus silusiae and it may be that this nematode is different in other respects because Samoiloff (1970) observed that the new cuticle is laid down within the existing cuticle and not below the limiting hypodermal membrane as in Nippostrongylus, Nematospiroides and Hemicycliophora arenaria (Johnson et al., 1970). Thus in Panagrellus it would seem that the cuticle is an extra cellular secretion - a current controversial topic to which Bonner et al., (1970) in particular contributed evidence to the contrary. The difficulty in obtaining adequate fixation of the hypodermal membrane has probably led to this conclusion; Samoiloff (personal communication) maintains that a membrane is not always present between the hypodermis and the cuticle and Bonner et al., show that it has frequent interruptions, but the excellent electronmicrographs of Lee (1970a) leave one in no doubt that the hypodermal membrane is a continuous, discrete structure in Nippostrongylus; in this case the new cuticle is intra-hypodermal. The condition observed in Howardula sp. (PLATE 13) in which there appear to be regions where the hypodermis is in direct communication with the space below the larval cuticle, resembles that of Nematospiroides (Bonner et al., 1970) but they may equally well be artifacts due to poor fixation.

After the separation of the old cuticle from the hypodermis, a second plasmalemma forms below the hypodermal membrane. This is destined to be the cuticle-hypodermis boundary of the adult nematode

in Nippostrongylus and Nematospiroides and the new cuticle is formed between these two regions. A second membrane is never developed in Howardula and this is the first indication of a different procedure occurring, concurrent with the formation of larger and much more numerous vesicles than are seen in the hypodermis of "normal" moulting nematodes. Lee (1970a) suggests that these vesicles contain collagen precursers which have been synthesized from amino acids by the polyribosomes of the rough endoplasmic reticulum. The vesicles fuse with the new hypodermal membrane; their contents are released into the region where the cuticle is developing and it is suggested that the collagen precursers form tropocollagen which becomes orientated in the new cuticle. A similar movement of vesicles occur in Nematospiroides and resembles the observations reported in Howardula. Presumably the vesicles do not contain collagen precursers in this cuticle-lacking parasite; it is likely that they are more concerned with adding membrane units to produce the necessary vast increase of the surface plasmalemma (hence larger and more numerous vesicles) and with the release of glycocalyx material which ultimately surrounds the microvilli. Obviously histochemical tests are required to identify the vesicular contents of the hypodermis in the final moult of Howardula.

Most nematodes produce finger-like folds of the hypodermal membrane as the new cuticle develops, to provide the increase in surface area which is apparent after ecdysis, consequently microvilli formation could be a simple extension of this process. The last areas of contact between the proliferating hypodermal membrane and the old cuticle appear to be the persisting half desmosomes in Nippostrongylus, Nematospiroides and Howardula (FIGURE 17D).

The degree of involvement of the old cuticle in moulting nematodes is variable because there are several reports that no resorption occurs but this is not the case in Meloidogyne javanica (Bird and Rogers, 1965), Ascaris lumbricoides egg moult (Thust, 1968) and probably in Howardula. It has been suggested that resorption of the old cuticle enables nematodes to utilise what would otherwise be wasted material, but Lee (1970a) offers the interesting explanation that ecdysis in the confined environment of Meloidogyne (inside the plant) and Ascaris (inside the egg shell) would be facilitated by a reduced, easily stretched and ruptured cuticle. This certainly applies to Howardula which lacks the necessary musculature for escaping from a rigid container. Cuticle absorption also occurs in the ectoparasitic Hemicycliophora arenaria (Johnson et al., 1970) and although space is unrestricted for this nematode, a reduction in cuticle thickness could be an advantage because of the cumbersome extra sheath which is present in this group. Material is often observed in transit between the hypodermal membrane and the old cuticle but without evidence to show the direction of movement, it is difficult to distinguish between cuticle resorption and hypodermal secretion. Degradation of the old cuticle occurs in Aphelenchus avenae and Hirschmaniella gracilis (Johnson et al., 1970) and in Howardula sp. which may give an erroneous impression of progressive resorption, but discrepancies between the width of the L_4 cuticle and that of the sheath prior to ecdysis suggest that some resorption occurs in Howardula. The striated layer appears to break down during the final moult and there is some indication that it produces the fibrillar component of the dense material observed in the subcuticular space (PLATES 12, 13 and FIGURE 17C). It would be interesting to elucidate the nature of this material, its

relationship to the hypodermis and the dense granules with which it merges. As the electron dense material disappears from the subcuticular space prior to ecdysis, it may have been resorbed by the hypodermis for nutritional purposes.

NUTRITIONAL CONSIDERATIONS

With reference to cestodes, but equally applicable to *Howardula*, *Deladenus* and *Contortylenchus*, Smyth (1969) points out that the absence of an alimentary canal is of considerable physiological importance because the external surface of the body must serve not only as a protective covering but also as a metabolically active layer through which secretions can be transported, nutrients absorbed and waste eliminated. Using the ultrastructural observations recorded in the present study and relating them to similar observations in a variety of absorptive and secretory cells, it is possible to speculate on the types of processes that may be involved in the metabolism of these nematodes.

"How things get into cells" was the title of a paper by Holter (1961) and is a relevant topic which has been more recently discussed with reference to parasites, by Read (1966) and Smyth (1969). The five ways in which material is currently thought to enter cells are (1) through pores, (2) by diffusion, (3) by facilitated or mediated transport, (4) by active transport, (5) by pinocytosis, and as there are well established experimental techniques to investigate each of these mechanisms it is planned that future work on *Howardula* and *Deladenus* will proceed along these lines. The membrane-pore theory of nutrient uptake is now largely discredited because it is difficult to envisage the

usefulness of such a non-selective inflow of material, but mainly because the electron microscope does not show pores in the surface plasmalemma which would have been detected because pores are commonly seen in the nuclear envelope. However, on a much larger scale, macro-pores or pore canals are involved in nutrient uptake in some animals such as Acanthocephela as indicated by Wright and Lumsden (1969) but these merely serve to increase the surface area for absorption by other methods because they end blindly. Pore canals have not been observed in the nematodes of the present study but they have been recorded in the cuticle of other nematodes (e.g., Mermis, Lee, 1970b), although a nutritional role has not been suggested. The ferritin uptake experiment was designed to investigate the feasibility of pinocytosis occurring in Deladenus and Howardula because this method has been successfully used to demonstrate the formation of pinocytotic vesicles in several animals, including the gut cells of trematodes (Dike, 1969). Rothman (1967) found that ferritin entered Hymenolepis diminuta not by pinocytosis but by transmembranosis - the apparent passage of particulate material directly across a membranous interface, but Lumsden et al., (1970a) were unable to repeat Rothman's experiments with ferritin, Thorotrast and Pelikan Ink. Although they were able to verify that electron-dense material had entered the cestodes incubated by Rothman, a comparison with the appearance of each colloid (viewed separately with the electron-microscope) demonstrated conclusively that Rothman's was not the material it had been claimed to be! They were unable to explain the origin of Rothman's electron-dense particles but pointed out that it could be due to impurities because free ions of heavy metals are known to pass readily through the tegument plasmalemma and which they demonstrated with Ag^{++}. The significance of using

5 X crystallised ferritin was not apparent so the only obtainable 2 X crystallised ferritin was used for the incubation of Deladenus and Howardula. In retrospect it seems likely that cadmium or other positively-charged bivalent heavy metal ions were present, which would explain the aggregation of electron-dense particles inside the microvilli and hypodermis of the experimental nematodes and not in the controls! It is planned to repeat these experiments when 5 X crystallised ferritin is available, in order to clarify the role of the coated vesicles frequently seen connected to the plasma membrane at the base of the microvilli in Deladenus. Pinocytosis may occur in D. siricidicola and D. wilsoni but it is not suggested as a means of nutrient uptake in Howardula. Horse radish peroxidase could equally well be used for a protein marker-tracer as demonstrated by Friend and Farquhar (1967) to show pinocytosis and the formation of coated vesicles in the vas deferens epithelia of rats. Similarly a fibrillar protein (amyloid) was recently used by Shirahama and Cohen (1970) with a mouse peritoneal macrophages and it is particularly interesting that a hemidesmosome-like structure appeared to be involved in the formation of pinicytotic vesicles which produced a 'bristle' effect along the inner lining of the surface membrane similar to that described in Deladenus (PLATE 26 inset). Several authors have described the accumulation of dense material under the surface membrane when extra cellular microfibrils are present and hemidesmosomes are widely accepted as an attachment device to extra cellular elements; the amyloid became fixed to the surface because as the membrane invaginated the fibres became vertically aligned and when the vesicle was budded off, the fibres radiated inwards from the perimeter. The external coating of the vesicles was shown to be formed from the

desmosome-type fibres which first aggregated on the inner surface of the membrane where pinocytosis was destined to occur. A similar process of coated vesicle formation may well operate in Deladenus and if material has to be absorbed onto the surface prior to initiation of pinocytosis, this method of nutrient uptake is probably much more selective than critics have suggested.

Passive diffusion, facilitated or mediated diffusion and active transport are three other ways in which nutrients could enter the hypodermis of these nematodes. Lipid-soluble substances and water-soluble vitamins have been shown to enter intestinal cells following the simple laws of diffusion and the process may be quantitatively described by the Fick equation. (Wilson, 1962) but it would be difficult to estimate one of the necessary components of this formula - the surface area - of Deladenus and Howardula because of the irregularity of the microvilli.

Facilitated diffusion differs from passive diffusion because the process is accelerated by a specific structural relationship between the surface membrane and the diffusing material, although there is no direct expenditure of energy involved and it cannot operate against an electrochemical gradient. Active transport involves processes in which a substance moves across the membrane against a concentration gradient and it requires a source of energy from metabolism, as can be demonstrated by the fact that it is inhibited in the presence of chemicals known to block energy-producing pathways. Like facilitated diffusion, active transport involves a specific structural relationship between the translocating material and the surface membrane and also shows stereospecificity which involves competitive inhibitions by chemically similar compounds. Read (1966) points out that more is known about the processes involved in the absorption

of low molecular weight organic compounds by tapeworms than is known of any group of invertebrate metazoans, parasitic or free living. Amino acids, sugars, purines and pyrimidines have been demonstrated to enter the Hymenolepis tegument and more recently Arme and Read (1968) have added fatty acids to this list. A frequently used experimental technique is to incubate live worms in a KRT solution containing a radio active labelled compound and it is proposed to use these methods to investigate the suggested nutritional function of the body wall of Deladenus and Howardula.

It is now well established that particular regions of a cell surface are concerned with the uptake of a specific compound this may be due to morphological or physiological properties such as the involvement of a 'carrier'. For example, there are at least four qualitatively different loci for amino acids absorption in Hymenolepis diminuta (Read et al., 1963). A possible explanation for membrane specificity was suggested by Bonneville and Weinstock (1970) after they had observed the sequence of development of surface forming vesicles from golgi apparatus. The latter could control selective absorption at the cell surface by assembling specific preformed units of membrane which move to the surface as vesicles and become incorporated into the apical plasmalemma by fusion. This would account for the variety of loci involved in nutrient abosorption and their frequency distribution along the surface.

Although a considerable amount of work has been done in the last 15 years in attempts to elucidate the structure of the surface plasmalemma (Robards, 1970) there is no agreed acceptable structure. Yamamoto (1963) drew attention to the fact that the unit membrane

is wider along the surface of the cell and around the golgi vesicles and multivesiculate bodies than in other membranous regions, and this was noticed in Howardula where the mitochondria and endoplasmic reticulum membranes are narrower than the ones which surround the microvilli. Sjostrand (1967, Sjostrand and Barajas, 1968) disagrees with the traditional concept of a lipoprotein bilayer 'Unit' membrane because frequently a globular structure is observed, as Bonner et al., (1970) showed for the hypodermal membrane of Nematospiroides. From observations on mitochondria, Sjostrand theorised a globular lipid structure separated by a protein matrix and gave evidence to show that the typical triple-layered structure seen in many electronmicrographs is due to artifacts from dehydration techniques which displace the lipid component. The tripartite structure frequently shown on the outside of nematodes was very rarely discernable in Contortylenchus, Howardula and Deladenus but unless one is certain that the tissues are well fixed and free from artifacts it is meaningless to enter into the argument!

The glycocalyx or external layer of granular/fibrillar material reported in Howardula and Deladenus has been demonstrated on a wide variety of absorptive surfaces such as the outside of cestodes and Acanthocephela (Lee, 1966b) and on intestinal epithelia. It is thought to have a significant role in the uptake of nutrients and is an integral part of the cell membrane rather than a loose covering "knap" or "fuzz" (Ito, 1965). Mukherjee and Williams (1967) showed that some of the filaments of the microvillus core in the intestine of mice appear to pass across the plasma membrane to form projections on the outside of the microvilli and further evidence that the surface coat is a functional

entity of each individual cell was suggested by the variation in the thickness of this layer from one cell to the next. Wright (1963b) found that the intestinal microvilli of Capillaria hepatica are covered with filaments that arise at the cell surface and radiate outwards in rows of 4-8 filaments aligned together. He discovered they were diastase-fast PAS-positive and did not stain with Alcian blue, hence a non acid mucopolysaccharide or mucoprotein was probably present. Moog and Wenger (1952) suggested that fibrous mucopolysaccharides or mucoproteins may function as skeletal substrated for the alkaline phosphates enzymes which occur in the brush border of vertebrate intestinal cells and von Brand (1966) pointed out that phosphatase activity often marks the sites where absorption can be expected to occur. It will be interesting to find out the nature of the granular layer which is particularly abundant in Deladenus siricidicola and D. wilsoni but one must realise that an absorptive function is not the only role which has been suggested. Monne (1959) proposed that the outer covering of trematodes could be used to protect the parasites by inhibiting the digestive enzymes of the host. Crompton (1963) supported this idea but he thought that the host's enzymic action was resisted rather than inhibited by the acid mucopolysaccharide which he found on the Acanthocephalan Polymorphus minutus. Lyons (1969) made the same comments regarding the mucoprotein and acid mucopolysaccharide which she detected around the external microvilli of Gyrocotyle urna but suggested that an alternative function could be the binding of enzymes to be used in membrane (contact) digestion (Taylor and Thomas, 1968). However, Lumsden et al., (1970b) in their report about the

cytoarchitectural and cytochemical features of tapeworm surfaces drew attention to the fact that the physiological significance of the glycocalyx has yet to be clearly elucidated.

The release of the glycocalyx material from the surface of Howardula and the two Deladenus species is probably quite different in each nematode. The glycocalyx of Howardula is very slight and frequently absent (PLATES 16 and 17) as has been reported occasionally for other microvillous surfaces such as the rat vas deferens (Friend and Farquhar, 1967), the gut of Nippostrongylus infective larva (Lee, 1968) and Dirofilaria immitis (Lee and Miller, 1969). In Howardula it could be produced by the filamentous core material transversing the microvilli plasmalemma (Mukherjee and Williams, 1967) or it may be released from the surface-forming vesicles, but there is no clear evidence to support either idea. There is a strong indication in D. siricidicola that the electron-dense material which is present in the hypodermis and microvilli (PLATE 31 and FIGURE 22) may extrude from the apex by a break down of the surface membrane at the tips of the microvilli. The granular material would then spread vertically and laterally; it appears to become less dense as it moves away from the surface and frequently two distinct layers are formed. Jennings and Colam (1970) described what could be a similar process in the gut of Pontonema when microvilli break down during the release of secretion, because their plate IIIa shows a long dense strand of material passing up into a microvillus from the depths of the cytoplasm. A similar phenomenon was reported by Erasmus and Ohman (1965) in the adhesive organs of Cyathocotyle where gland cell secretions accumulate in lacuna-like spaces under

the surface and they pass up the stalks of the microvilli to emerge from the tips to the exterior.

The secretory process in Deladenus wilsoni appears to be more complicated because there are probably three different exudates (PLATES 35 and 36). The glycocalyx material which is particularly abundant appears to arise as very dense fibres in the distal region of the hypodermis and collects in surface invaginations prior to release between the microvilli. There is a striking resemblance between this process and the one which Jenkins and Erasmus (1969) described in the gut of Metastrongylus and indeed between the gross morphology of the surface of D. wilsoni and the intestinal epithelium of Metastrongylus, as confirmed by Jenkins (personal communication). The characteristic feature of Metastrongylus are the dilated tips of the microvilli which have been shown to contain secretory granules and these are released into the gut lumen for digestive purposes by a budding off process. Kurosomi (1961) calls this type of secretion, where part of the cell is lost, "microapocrine" and there is a strong circumstantial evidence (PLATES 33-35) that this is the way in which Deladenus wilsoni releases material from the hypodermis. Histochemical tests could reveal the nature of the secretion (excretion) being produced and until this information is available one can only speculate that enzymes are being ejected for extracellular digestion in D. wilsoni, but there is no suggestion that a similar process occurs in D. siricidicola or Howardula. The latter surfaces have a strong morphological resemblance to the tegument of cestodes and if this is reflected in a similar physiological behaviour, then extra-

corporeal digestion would not occur in *D. siricidicola* and *Howardula,* because cestodes differ from trematodes and nematodes in this respect (Read 1968).

A similar method of releasing secretions in *D. wilsoni* could be by the extrusion and disconnection of "blebs" from the surface (PLATE 35). There are several reports of this phenomenon occurring in the literature, including the intestine of *Ascaris* (Sheffield, 1964) and *Pontonema* (Jennings and Colam, 1970) and the external surface of *Proteocephalus* (Threadgold, 1965) and *Ligula* plerocercaid (Charles and Orr, 1968)

Little incontrovertible evidence has been produced in these reports to demonstrate whether secretion or excretion is occurring during the release of cytoplasmic bulges from the surface, and the situation is further complicated in *D. wilsoni* by the numerous whorls of membranous material which are apparently expelled at the bases of the microvilli (PLATE 36). They appear to be in the process of disintegration and probably contribute to the excessive granulation, not unlike glycocalyx material, which is present above the surface of such regions. A similar external mass was reported by Charles and Orr (1968), in the plerocercoid of *Ligula* which was not present in the plerocercoid of *Schistocephalus,* and they suggested that it might be related to the marked host-response provoked by *Ligula,* unlike *Schistocephalus* where there is no host response and where granular material was not observed. Whether the substance is produced by the host, or by the parasite in reaction to the host, is unknown. Other than a change in gonad development (Zondag, 1969) there is no information about host reaction to *Deladenus* infections but it would be worthwhile investigating this possibility because the two species occur in different hosts and it

might explain why D. wilsoni usually has a massive granular coating while D. siricidicola only has a thin layer. Whatever the cause or function of this dense material, ultrastructural studies indicate that it is produced by the parasite in each case.

The appearance of the membranous whorls in the distal hypodermis of D. wilsoni resembles phospholipid or lipoprotein which is characteristic of lysosome-activity residues. These structures are known to be present in the majority of cells (Daems et al., 1969) and have been observed in D. siricidicola (PLATE 30) and Howardula (PLATE 16). The nature of their release, as indicated in D. wilsoni, has been referred to as "cellular defaecation" which describes their function in removing indigestible breakdown products from the cytoplasm. Nematodes such as Ascaris (Sheffield, 1964) and Metastrongylus (Jenkins and Erasmus, 1969) have been shown to extrude these "myelin figures" into the gut lumen from where they will pass out of the body during defaecation; it is interesting that D. wilsoni, not having a gut, simply passes them directly to the exterior. Autolysosomes are abundant in Deladenus and Howardula but it is not yet possible to distinguish between autolysosomes which break down aging cellular organelles and heterolysosomes which digests extracellular material that has been taken up by the cell, but the presence of multivesiculate bodies (MVB's) suggests that both types occur. Friend and Farquhar (1967) demonstrated the relationship between lysosomes, MVB's and Golgi apparatus during protein metabolism in a series of experiments using Horse radish peroxidase, thiamine pyrophosphatase and acid phosphatase as markers for protein uptake, golgi membranes and lysosomes respectively. Pinocytotic uptake of Horse radish peroxide occurred and coated vesicles were formed which then migrated inwards.

becoming smoothed surfaced as they did so, and eventually fusing with MVB's. Concomitantly there was an increase in bristle coated vesicles being budded off from the Golgi lamellae and these "primary" lysosomes migrated to the MVB's and fused with them; consequently enzymes and substrate were mixed and digestion could occur. There was also some evidence that the golgi-produced primary lysosomes could migrate directly to the surface to release their contents, in which case both extra and intracellular digestion was facilitated. The change from fibrillar coated to smooth surfaced vesicles during pinocytosis is reminiscent of the vesicles described at the base of the pits of microvilli in the young developing D. siricidicola and together with the abundance of MVB's in the hypodermis may suggest that pinocytosis and intracellular digestion may be occurring. It was stated earlier that these coated vesicles could be engaged in surface-membrane formation but it is equally plausible that during the phase before the microvilli have fully differentiated, the young parasite could temporarily absorb nutrients by pinocytosis. There is no indication of this occurring in the adult D. siricidicola or in Howardula but it may be a subsidiary mechanism in D. wilsoni.

It is particularly interesting that lipid accumulates with increasing age of these nematodes and in Howardula there is a possibility that it may be sloughed off at the surface(PLATE 13), where prominent swellings of what looks like lipid are frequently seen protruding from the body. Generally it has been assumed that nematodes rely to a great extent on lipids as energy reserves, in contrast to cestodes where lipids are thought to represent waste products of metabolism (Lee, 1966b; Smyth, 1969). If this is so, then it would seem that Howardula and probably Deladenus have more

in common with cestodes which have been shown to accumulate lipids in the older proglottids and get rid of it through the excretory system or possibly via the tegument (Smyth, 1947). However, in view of the work of Ginger and Fairburn (1966a, b), Arme and Read (1968) and others, a more complex idea of cestode lipid metabolism is developing; similarly it is realised that not all nematodes conform to the same utilisation of stored fat as the free living aerobic non-feeding infective larvae. The oxygen tension of insect haemolymph could determine lipid metabolism in entomophilic nematodes but little is known about the subject although Mordue (personal communication) suggests that it is very low. The respiratory pigment, haemoglobin, has been discovered in several nematodes (e.g. Davenport, 1949) and considering the greenish colouration often observed in *Howardula* and in *Deladenus* (Bedding, 1968) one wonders if the green respiratory pigment Chlorocruorin could be present in these animals? The use of a microspectrophotometer would quickly determine if this were so.

Contortylenchus sp. is surprisingly different from *Deladenus* and *Howardula* considering that it also is thought to lack a mouth (Al-Rabiai 1970) and presumably must rely on the body wall for nutrient absorption. On first inspection with the electronmicroscope it appeared to have a normal cuticle but closer examination showed this to be an uninterrupted continuation of the hypodermis. Although the distal region of the hypodermis has a modified layered appearance, the presence of organelles such as mitochondria and secretory vesicles confirm that it cannot be regarded as a cuticle because, while being metabolically active (Anya, 1966a, b), nematode cuticle does not contain these structures. However, it has no

obvious ultrastructural features to suggest how uptake of materials could occur. There are no microvilli or pore canals although the surface has deep folds in some regions (PLATE 37); there is no glycocalyx but what appear to be loose scattered membranes are sometimes present externally and there are no obvious coated vesicles to suggest pinocytotic mechanisms. All of which indicate that vital dyes or other means should be used to establish with certainty whether or not nutrients can enter through what was originally the stoma. It seems more than likely that the oral entrance is closed because Al-Rabiai spent three years closely observing Contortylenchus and could find no evidence of a mouth. Thus it would appear that nutrients enter by passive diffusion, facilitated diffusion or active transport, none of which necessitates obvious morphological features although they presumably involve biochemical structural adaptations.

From the ultrastructural observations the hypodermis of Contortylenchus shows that unlike Deladenus and Howardula it probably retains many of the processes of cuticle synthesis after the final moult. Presumably some of the properties which stabilise and confer resistance to the cuticle of nematodes (see Lee, 1966b) will have been lost and work is at present in progress to attempt to elucidate the histochemical composition of Contortylenchus "cuticle". Lee (1965a) in discussing cuticle permeability reported that glucose will pass through cuticle when the muscles and hypodermis have been scraped away, which would suggest that the hypodermis and not the cuticle is responsible for selective permeability.

Al-Rabiai (1970) described three prominent ridges extending from the body of Contortylenchus when viewed transversely and she

suggested that if feeding is occurring through the body wall it must be at the base of the ridges in the dorsal and ventral regions, where the hypodermis is very thin. However, it seems more likely that absorption would be reduced in the thin regions, because this is where it overlies the four muscle cords and has a reduction in mitochondria and other organelles; the thick median and ventral hypodermal cords which have many lysosomes, MVB's, ribosomes and membrane-bound vesicles are probably the more active regions metabolically. The three ridges are probably not permanent features of Contortylenchus morphology because they were only rarely encountered and always in specimens which appeared to have collapsed. FIGURE 25B (1-111) illustrates how the body wall could have been distorted, and the fact that most of Al-Rabiai's observations were made on parasites sectioned in situ (inside the beetle host where they are moulded to fit the shape of the space available) probably accounts for the unusual contours she described.

The somatic musculature of Contortylenchus is very well developed compared with the reduced, myofilament-depleted muscle cells of Howardula and Deladenus. This is reflected in the movements of Contortylenchus which unlike Howardula and Deladenus is able to bend its extremities quite vigorously, although it cannot locomote.

Although Contortylenchus does not show the same degree of structural adaptations to a nutritional function as the two Deladenus species and Howardula, nevertheless its body wall is considerably different to that of "normal" nematodes. It will be interesting to find out if the cuticle of the free living female Contortylenchus is comparable to the free living Deladenus and Howardula which give no indication of the metamorphosis which occurs at the final moult. The ultrastructural difference of the body wall in the larval and

adult females which have been reported in this study reflect the contrasting requirements of the two stages. Locomotion and resistance to fluctuating environmental conditions is of prime importance to the free living infective nematodes whose job it is to locate a host. Consequently it was found that the free living *Deladenus siricidicola* and *Howardula* sp. have a well developed thick, resistant cuticle and a prominent robust somatic musculature. The changes which were observed at the onset of parasitism are considered to be elegant examples of adaptations to a different mode of life where locomotion is no longer necessary, but an efficient method of nutrition and elimination of waste is crucial. These requirements are reflected in the drastic reduction of somatic musculature and loss of cuticle which facilitates hypertrophy of the hypodermis to increase the area available for maximum interchange of the materials involved in metabolism.

Comments on the possible evolutionary implications of these observations will be given in the General Discussion, where relevant information from Section I can be included.

SUMMARY - Section II

1. The body wall ultrastructure of free living female entomophilic tylenchids *Howardula* sp. and *Deladenus siricidicola* is described and compared with that of other nematodes. Each has a typical cuticle, hypodermis and meromyarian, platymyarian muscles. The cuticles are composed of six layers; an outer electron dense zone, inner and outer cortex, matrix encompassing a striated region and finally a single basal layer of fibres. In the lateral fields the striated layer is modified to form two fibre layers in *Howardula* and four in *D. siricidicola.*

2. Ultrastructural studies on the body wall of mature parasitic Howardula show that it is unlike that of other nematodes so far examined. A cuticle is lacking, the musculature is degenerated and the external surface is covered with microvilli.

3. Events of the moult and exsheathment described in Section I are elucidated and the hypodermis of the young parasitic female *Howardula* is shown to form microvilli instead of producing a new cuticle. Parts of the cortex, striated and basal layers appear to break down into a fibrillar component in the space which forms below the old cuticle. The hypodermis hypertrophies and develops vesicles which appear to release granular material at the surface of the hypodermal membrane, which convolutes and ultimately assumes the form of microvilli. The old cuticle becomes separated, subjacent electrondense material disappears and exsheathment occurs.

4. Ultrastructural examination of parasitic *D. siricidicola* and *D. wilsoni* revealed a similar body wall modification with microvilli replacing cuticle, a hypertrophied hypodermis and reduced musculature. The *Deladenus* species differ from *Howardula* in having abundant electron dense material internally and externally, and they can readily be distinguished from each other by the shape of the microvilli.

5. The final moult of D. siricidicola is quite unlike that of Howardula because another cuticle is initially developed, although there is no indication of layering as in a cuticle of the free living mycetophagous female. Subsequent erosion of the cuticle by invagination of the surface layer eventually leads to the formation of microvilli.

6. The ultrastructure of the body wall of Contortylenchus sp. is described and in contrast to the previous entomophilic tylenchs, it does not possess microvilli. A layered outer region is present but it is shown to be in continuation with the hypodermis, contains organelles and cannot be regarded as a normal cuticle. Unlike Howardula and Deladenus, Contortylenchus may have a minute stomal aperture.

7. Ultrastructural observations on the four different nematodes are compared and contrasted throughout the discussion section which also includes; a review of literature where a nutritional role for the body of parasitic allantonematids is inferred; the formation of microvilli in other animals; current ideas on uptake of nutrients by parenteral feeding parasites as well as absorptive gut cells of vertebrates and some of the techniques which could be used to investigate its occurrence in these nematodes.

Comments on the evolutionary implications of apparently unique characteristics of allantonematids are included in the General Discussion, together with relevant data from Section I.

GENERAL DISCUSSION

The discovery of microvilli on the outside of *Howardula* sp. *H. benigna*, *Deladenus siricidicola*, *D. wilsoni* and *Bradynema rigidum* is convincing circumstantial evidence supporting the statements of Wachek (1955), Ruhm (1956) and earlier workers, that the sac-like insect-parasitising Tylenchs feed through the "cuticle". The description of the new *Howardula* life cycle and the comparison with related species, together with the observation that at least five entomophilic nematodes lack a cuticle, enables speculation on phylogenetic relationships and evolution of the sphaerularids.

Wulker (1923) suggested that terrestrial nematodes evolved from marine ancestors via fresh water and Chitwood (1970), on the basis that Tylenchida and Rhabditida have fewer cells, proposed that these orders are the most primitive, with rhabditids closest to at least one kind of ancestral form. Wachek (1955) also pointed out that the organisation of tylenchs is primitive and attempted to outline the evolution of plant and insect parasitic forms from free living nematodes. To account for the morphological and biological variation among entomophilic tylenchs as observed in the different body shapes, cuticle flaccidity, stylet form and life cycles, Wachek postulated that these nematodes originated from the ancestral plant parasitic tylenchids at a very early stage; the cuticle was very plastic allowing for distortion, and a stylet had not started to evolve. *Bradynema* would have been the first genus to become isolated by parasitising insects, and in the constant environment of the host's haemocoel subsequent modification would be reduced to a minimum; consequently the stylet-lacking flaccid-bodied *Bradynema* is a "preserved" primitive

form. At a later stage when the ancestral plant parasitic nematodes were developing a stylet (by the inward sloughing of the cuticularised stomatal lumen) and the cuticle was less elastic Howardula become isolated; the simple thin walled tube-shaped stylet being retained. When the plant parasitic ancestors developed a more perfect stylet and the cuticle become less distensible, the bean-shaped entomophilic nematodes such as Contortylenchus originated. The cuticle become more rigid, at which stage the forms with anterior and posterior cones not included in the central body expansion were isolated (e.g. Bovienema) and as the cuticle hardened and muscle attachment sites were developed at the onset of true plant parsitism, the more highly developed Sphaerularids (e.g. Sphaerularia, Heterotylenchus) diverged from the ancestral stock. Because of the hardened cuticle, the gonad of phylogenetically more recent insect parasites could no longer proliferate; Wachek explains that the uterus prolapse in Sphaerularids and the incorporation of a second reproductive phase into the life cycle of allantonematids (e.g. Heterotylenchus, Parasitotylenchus and Fergusobia) are mechanisms which have been evolved to compensate for the enforced gonad reduction.

It is difficult to accept that this postulation was seriously intended, unless Wachek has been completely misrepresented in translation. Ultrastructural observations on infective female Howardula sp. clearly show that the larvae have a normal cuticle and a stylet, as can be deduced from light microscopy, and if ontogeny recapitulates phylogeny Howardula and the other Sphaerularids have evolved from stylet-bearing cuticle-covered.

nematodes. It is inconceivable that allantonematid adults lacking a stylet could be said never to have evolved the structure when a stylet is known to be present in the larvae.

In contrast to Wachek's theory it seems more probable that Howardula and Bradynema have reached a higher degree of specialisation than all the allantonematids because it has been shown that these nematodes are unique in having evolved a cuticleless, microvilli-covered hypodermis. They can only be regarded as primitive in so much as they probably represent the oldest parasitic relationship with insects and presumably diverged from the tylenchid ancestral stock at a very early stage, as was suggested by Wachek but for very different reasons. Contortylenchus with its lack of cuticle, but no microvilli, may represent a phylogenetically younger relationship and probably the bean-shaped Allantonema and Bovienema have reached a similar level of evolution. The cuticle of sphaerularids and heterogonic tylenchs may be less adapted for a nutritional role. Compensation in the former by a prolapsed absorptive uterus and in the latter, by addition of a second generation to each cycle producing per os feeding parasitic larvae to supplement the subsequent adult nutritional level, would enable these groups to attain the fecundity of microvillous nematodes. It would be interesting to examine the body wall ultrastructure of sphaerularids and Heterotylenchus, Parasitylenchus and Fergusobia, and also to check on the fecundity of the two reproducing stages of the latter genera, for comparison with the nematodes in the present study.

Ruhm (1950) pointed out that tylenchs tend towards adult parasitism while aphelenchs are mainly parasitic as larvae and

that both have developed independently, as did Fuchs (1938), However, Wachek (1955) thought there was no doubt that larval parasitism is a preliminary stage in the evolution of adult parasitism and suggested that Tylenchoidea, with Chondronema the only larvae-parasitising genus, were phylogenetically older. A parallel situation occurs in Mermithoidea where mermithids have free living adults and parasitic larvae while Tetradonematids parasitise in both stages; in the absence of direct evidence to the contrary, it would seem more reasonable to suppose that larval and adult parasitism evolved separately.

Stammer (1934) and Currie (1937) attempted to explain the origin of entomophilic tylenchids; the latter suggested that the initial contact was accidental when plant parasitising nematodes encountered mining insects such as onion flies, frit flies, gall-forming insects and bark boring beetles. Stammer thought that nematodes looking for shelter may have entered the gut and eventually the haemocoel of insects, subsequently becoming modified for 'osmotic' feeding.

The life cycles of many tylench obligate parasites of insects is closely adapted to that of the host as was shown in Contortylenchus (Al-Rabibi, 1970) and Howardula sp., Entomophilic nematodes have to be able to overwinter if the insect host only has one generation a year, and as anabiosis has not been demonstrated in these nematodes, they frequently survive inside the insect. Many nemas are able to live in the biotope for several weeks, during which time they moult and mature until the appropriate host stage is available for parasitism. These infective females are characterised by a well developed stylet and increased activity necessary for locating the males and maggots

which may have dispersed in the interval. Nematodes which parasitise hosts with rapid life cycles have a short period in which to mature, necessitated by the brief developmental stages of the insect, and consequently the number of moults in the external environment is frequently reduced and the nematodes have decreased locomotory ability. Wachek (1955) pointed out that the phylogenetically young sphaerularid parasites moult twice before leaving the host then undergo two more moults before recommencing the parasitic cycle; older parasites lose a moult first in the male nematodes and then in the female and subsequent reduction in moults leads to two or one in the second generation of the Heterotylenchus life cycle. As exsheathment was not mentioned by Wachek and other authors it can probably be assumed that this term was mistakenly synonomized with "moulting". Observations on Howardula sp. show that the actual number of moults in the life cycle, generally accepted as four in the nematodes, is unaltered but the number of exsheathments is reduced to three in the female cycle and two in the male. The difficulty in distinguishing the triple nature of the male Howardula sp. sheath which was confirmed by Mr. J.J. Hesling (personal communication) probably accounts for the reported single moult in other Howardula free living males. Reduction in exsheathments facilitates truncated external development as does the advanced maturation of parasitic larvae in other species of Howardula; both adaptations permit a brief transmission period which reduces the influence of abiotic factors on the free living nematodes and enhances the incidence of parasitism.

Deladenus Thorne, unlike Howardula sp. and Contortylenchus sp. is a member of the Neotylenchidae but the morphological and

biological features of the entomophilic D. siricidicola and D. wilsoni described by Bedding (1968), are characteristic of the Sphaerularidae whose family diagnoses were amended by Nickle (1967). The mycetophagus free living Deladenus have a knobbed stylet and oesophageal glands identical to the allantonematids Parasitylenchoides Wachek, Protylenchus Wachek and Proparasitylenchus Wachek and also to some species of the genus Allantonema Leukart and Parasitylenchus Micoletzky. Bedding pointed out that such characters may have little phylogenetic importance because they vary so considerably within a simple species of Deladenus, and although he was aware that the Sirex parasites were like Parasitylenchus (Dale, 1967) Bedding placed his two new species in the Neotylenchidae on the assumption that the fungal feeding forms were the more primitive. No other insect parasitising Deladenus species have yet been described but several are known to share a common habitat such as oak galls and the tunnels of bark boring beetles and Saunders and Norris 91961) suggested a phoretic relationship with Scolytus multistriatus. Bedding thought that other Deladenus species could have allantonematid forms parasitic on insects and inferred that Ruhm (1956) may have evidence to this effect when Sphaerulariopsis Wachek (Sphaerularidae) was synonymized with with Stictylus Thorne (Neotylenchidae). However Nickle (1963) rejected Ruhm's synonymy and Sphaerulariopsis is currently included with the Sphaerularids.

The discovery of microvilli on the outside of parasitic Deladenus, as in Howardula and Bradynema, cannot be used to support Nickle's opinion (personal communication) that the neotylench would be better placed with the Sphaerularids, because convergence - where similar forms are produced as a result of exposure to similar

environmental conditions - is a well established phenomenon. In these three genera the nematodes live in the coelomic cavity of an insect, surrounded by haemolymph, and it is reasonable to suspect homeomorphy. The polymorphism of Howardula sp. is further evidence that parasitic adults have little taxonomic significance and the three Bradynema species (Poisson, 1933) and four Howardula (Warren, 1941; Wachek, 1955) as well as many other allantonematid species established solely on the parasitic forms, could usefully be declared species inquirendae.

The morphological characteristics of the free living stages of Deladenus are also known to have little systematic importance and it would seem that biological features may be more useful in separating neotylenchs from Sphaerularids. The dual sexual cycles of Deladenus are reminiscent of the allantonematid Parasitylenchus except that both generations are parasitic in the latter. Heterotylenchus also has two cycles and Nickle (1967) reported the possibility of Allantonema stricklandi and A. muscae being different females from one Heterotylenchus species, such is the sexual dimorphism in this group. Deladenus can be distinguished from Heterotylenchus because contrary to Bedding's original supposition (1967) it does not have a parthenogenetic cycle (Bedding, 1968). Fergusobia with its apparently unique double parasitism of plant and animal (Jones, 1965) where the parthenogenetic females occur in Eucalyptus galls and produce free living nematodes which ultimately parasitise the "gall fly" Fergusonina tillyardi Tonn., is probably nearest to the bionomics of Deladenus. However it is impossible to overlook the significance of the fungal feeding Deladenus siricidicola and D. wilsoni being able to reproduce indefinitely in the external environment, unlike the allantonematids where a precise

alternation of generations exists and each cycle is committed to the next. Furthermore the free living female sphaerularids in the amended diagnoses of Nickle (1967) have a unique small finger-like gonad with few oocytes, a short oviduct and a prominent uterus; these characteristics are observed in the infective Deladenus but the mycetophagus female has a large gonad with many oocytes, and fully developed eggs are present in the uterus. Consequently as Deladenus can be distinguished from the Sphaerularids both morphologically and biologically, it must remain with the neotylenchs at the present time until it can be shown that some of the allantonematids have unlimited, independently reproducing, free-living generations.

The life cycle of the new Howardula and its comparison with that of other related nematodes, together with discovery of five cuticle-lacking, microvilli-covered species from two different families, indicate that convergence has occurred during evolution of insect-haemocoel parasitising Tylenchoidea.

LITERATURE CITED

ANYA, A.O. 1966a. The localisation of RNA in the nematode cuticle. *Nature*, *209*:827.

ANYA, A.O. 1966b. The structure and chemical composition of the nematode cuticle. Observations on some oxyurids and *Ascaris*. *Parasitology*, *56*:179.

AL-RABIAI, S.M.A. 1970. Studies on the biology and life history of the nematodes associated with bark boring beetles, (Scolytidae). Ph.D. thesis, Faculty of Science, London University.

ARME, C. & READ, C.P. 1968. Studies on membrane transport: II The absorption of acetate and butyrate by *Hymenolepis diminuta*. *Biol. Bull. mar. biol. Lab.*, Woods Hole, Mass., *135*:80.

BAILEY, N.T.J. 1959. Statistical methods in biology. The University Press Ltd., 102 Newgate St., London E.C.1.

BECKETT, E.B. & BOOTHROYD, B. 1961. Some observations on the fine structure of the mature larvae of the nematode *Trichinella spiralis*. *Ann. trop. Med. Parasit.*, *55*:116.

BEDDING, R.A. 1967. Parasitic and free living cycles in entomogenous nematodes of the genus *Deladenus*. *Nature*, *214*:174.

BEDDING, R.A. 1968. *Deladenus wilsoni* n.sp. and *D. siricidicola* n.sp. (Neotylenchidae), entomophagous - mycetophagous nematodes parasitic in siricid wood wasps. *Nematologica*, *14*:515.

BIRD, A.F. 1957. Chemical composition of the nematode cuticle. Observations on individual layers and extracts from these layers in *Ascaris lumbricoides* cuticle. *Expl. Parasit.*, *6*:383.

BIRD, A.F. 1964. Embedding and staining small nematodes for electron-microscopy. *Nature*, *203*:1300.

BIRD, A.F. 1968. Changes associated with parasitism in nematodes. III. Ultrastructure of the egg shell, larval cuticle and contents of the sub ventral oesophageal glands in *Meloidogyne javanica*, with some observations on hatching. *J. Parasit.*, *54*:475.

BIRD, A.F. & BIRD, J. 1969. Skeletal structures and integument of Acanthocephala and Nematoda. *Chemical Zoology*, *III*:253.

BIRD, A.F. & ROGERS, G.E. 1965. Ultrastructure of the cuticle and its formation in Meloidogyne javanica. Nematologica, 11:224.

BONNER, T.P., MENEFEE, M.G. & ETGES, F.J. 1970. Ultrastructure of cuticle formation in a parasitic nematode, Nematospiroides dubius. Z. Zellforsch., 104:193.

BONNEVILLE, M. & WEINSTOCK, M. 1970. Brush border development in the intestinal absorptive cells of Xenopus during metamorphosis. J. Cell Biol., 44:151.

BROWN, A.L. 1962. Microvilli of the human jejunal epithelial cell. J. Cell Biol., 12:623.

BOVIEN, P. 1932. On a new nematode, Scatonema wülkeri gen. et spec. n. parasitic in the body cavity of Scatopse fuscipes Meig. (Diptera: Nematocera). Vidensk. Meddr. dansk naturh. Foren., 94:13.

CAMPION, D. 1969. A nematode parasite of the red bollworm Diparopsis castanea Hmps. and the implications of such a parasite as a possible biological control agent. Tropical Science, 10:155.

CARBONELL, L.M. & APITZ, R.C. 1960. Sulfhydryl and Disulfide groups in the cuticle of Ascaris lumbricoides var. suis. Expl. Parasit., 10:263.

CARDELL, R.R., BADENHAUSEN, S. & PORTER, K.R. 1967. Intestinal triglyceride absorption in the rat. J. Cell Biol., 34:123.

CAULFIELD, J.B. 1957. Effects of varying the vehicle for osmium tetroxide in tissue fixation. J. biophys. biochem. Cytol., 3:827.

CHARLES, G.H. & ORR, T.S.C. 1968. Comparative fine structure of the outer tegument of Ligula intestinalis and Schistocephalus solidus. Expl. Parasit., 22:137.

CHITWOOD, B.G. & CHITWOOD, M.B. 1950. An introduction to Nematology. Monumental Printing Co., Baltimore.

CHITWOOD, M.B. 1970. The value of evolutionary trends and relationships in taxonomy. Second Int. Congress of Parasitologists, Wash. 1970.

COBB, N.A. 1921. Howardula benigna. A nema parasite of the cucumber beetle. Science, 54:667.

COLAM, J.B. 1969. Histochemical, biochemical and ultrastructural studies on certain species of nematodes, with special reference to their nutrition. Ph.D. Thesis, University of Leeds.

CROLL, N.A. 1966. Ecology of Parasites. Heinmann Educational Books Ltd., London.

CROMPTON, D.W.T. 1963. Morphological and histological observations on Polymorphus minutus (Goeze 1892) with special reference to the body wall. Parasitology, 53:663.

CURRIE, G.A. 1937. Galls on Eucalyptus trees. A new type of association between flies and nematodes. Proc. Linn. Soc. N.S.W., 62:147.

DAEMS, W.T., WISSE, E. & BREDEROO, P. 1969. Lysosomes in Biology and Pathology, I. Ed. Dingle, J.T. & Fell, H.B., North Holland Publs Amsterdam.

DALE, P.S. 1964. A new species of Cephalobellus (Nematoda: Thelastomatidae) from the larva of Costelytra zelandica (Coleoptera: Melolonthinae). N.Z. Jl Sci., 7(4). .

DALE, P.S. 1967. Nematodes associated with the Pine bark-beetle, Hylastes ater in New Zealand. N.Z. Jl. Sci. 10:222.

DAVENPORT, H.E. 1949. The haemoglobins of Nippostrongylus muris (Yokogawa) and Strongylus spp. Proc. R. Soc. B., 136:271.

DAVEY, K.G. 1965. Moulting in a parasitic nematode, Phocanema decipens. I. Cytological events. Can. J. Zool., 54:997.

DAVEY, K.G. & KAN, S.P. 1967. Endocrine basis for ecdysis in a parasitic nematode. Nature, 214:737.

DICK, T.A. 1970. Observations of the fine structure and development of the cuticle of the mouse pinworms. Syphacia obvelata and Aspicularis tetraptera. Second Int. Congress of Parasitology, Wash. 1970. Abstract J. Parasit., 56:80.

DIKE, S.C. 1969. Acid phosphatase activity and ferritin incorporation in the ceca of digenetic trematodes. J. Parasit., 55:111.

DROPKIN, V.H. 1969. Cellular responses of plants to nematode infections. A. Rev. Phytopath., 17:101.

DUTKY, S.R., THOMPSON, J.V. & CANTWELL, G.E. 1964. A technique for mass propagation of the DD136 nematode. J. Insect Path., 6:417.

ERASMUS, D.A. & OHMAN, C. 1965. Electronmicroscopic studies of gland cells and host-parasite interface of the adhesive organ of Cyathacotyle bushiensis Khan 1962. J. Parasit., 51:761.

FAIRBURN, D. 1960. The physiology and biochemistry of nematodes. 'Nematology, Fundamental and Recent Advances.' Ed. Sasser, J.N. and Jenkins, W.R., Chapel Hill: University N. Carolina Press.

FLEGG, J.J.M. & HOOPER, D.J. 1970. Extraction of free-living stages from soil. Laboratory methods for work with plant and soil nematodes. Ed. Southey, J.F., Minist. Agric. Fish. Fd Tech. Bull. 2, 1970.

FRIEND, D.S. & FARQUHAR, M.G. 1967. Functions of coated vesicles during protein absorption in the rat vas deferens. J. Cell Biol. 35:357.

FUCHS, G. 1938. Neue parasitische und halbparasitische Nematoden bei Borkenkäfern und einige andere Nematoden. Zool. Jahrb. (Syst.) 71:123.

GINGER, C.D. & FAIRBURN, D. 1966a. Lipid metabolism in helminth parasites. I. The lipids of Hymenolepis diminuta (Cestoda). J. Parasit., 52:1086.

GINGER, C.D. & FAIRBURN, D. 1966b. Lipid metabolism in helminth parasites. II. The major origins of lipids of Hymenolepis diminuta (Cestoda). J. Parasit., 52:1097.

GOODEY, T. 1930. On a remarkable new nematode, Tylenchinema oscinellae gen. et sp. n., parasitic in the Frit fly, Oscinella frit L., attacking oats. Phil. Trans. R. Soc., 218:315.

GORDON, A.B., MILLER, L.R. & BENSCH, K.G. 1963. Fixation of tissue culture cells for ultrastructural cytochemistry. Expl. Cell Res. 31:440.

HARRIS, J.E. & CROFTON, H.D. 1957. Structure and function in nematodes: internal pressure and cuticular structure in Ascaris. J. Expl. Biol., 34:116.

HINZ, E. 1963. Elektronenmikroskopische Untersuchungen an Parascaris equorum. Protoplasma, 56:202.

HOLTER, H. 1961. How things get into cells. Scient. Am., Sept. 1961.

HUSSEY, N.W. 1959. Biology of Mushroom Phorids. Mushroom Science, IV: 260.

HUSSEY, N.W. 1960. Identification of Phoridae (Diptera) attacking cultivated mushrooms in Britain. Ann Mag. nat. Hist., iii:599.

HUSSEY, N.W. 1961. Recent work on the control of the Worthing Phorid. Mushroom Growers Assoc. Bull., 144:495.

HUSSEY, N.W. 1964a. Economics in pest control suggested by a study of Phorid flight activity. Mushroom Growers Assoc. Bull. Feb.1965.

HUSSEY, N.W. 1964b. Observations on the association between Bradynema sp. (Nematoda: Allantonematidae) and the mushroom infesting fly Megaselia halterata (Diptera: Phoridae). Proc. XIII Int. Congr. Ent., London, 1964 (1965).

HUSSEY, N.W. 1967. Biological control of mushroom pests - fact and fantasy. Mushroom Growers Assoc. Bull., Spring 1968.

HUSSEY, N.W. & GURNEY, B. 1964. Rearing techniques for mushroom fly. Plant Pathology, 13:38.

INATOMI, S., SAKUMOTO, D., ITANO, K & TANAKA, H. 1963. Studies on the submicroscopic structure of body surface of larval nematodes. Japan J. Parasit., 12:16.

INGLIS, W.G. 1964a. The structure of the nematode cuticle. Proc. Zool. Soc. Lond., 143:465.

INGLIS, W.G. 1964b. The comparative anatomy of the Ascaridoid cuticle (Nematoda). Societé Zoologique de France, 89:317.

ITO, S. 1965. The enteric surface coat on cat intestinal microvilli. J. Cell Biol., 27:475.

ITO, S. & REVEL, J.P. 1966. Autoradiography of intestinal epithelial cells. "Electron Microscopy", 1966, II. Ed. Uyeda, R., Maruzen Co., Ltd., Tokyo. 585.

JASKOSKI, J.B. 1960. Relation of age and fat reserves in swine ascarid eggs. II. Infectivity in the hamster. Expl. Parasit., 10:333.

JENKINS, T. 1969. Electron microscope observations of the body wall of Trichuris suis, Schrank, 1788 (Nematoda: Trichuraidea). I. The cuticle and bacillary band. Z. Parasitenk, 32:374.

JENKINS, T. & ERASMUS, D.A. 1969. The ultrastructure of the intestinal epithelium of Metastrongylus sp. Parasitology, 59:335.

JENNINGS, J.B. & COLAM, J.B. 1970. Gut structure, digestive physiology and food storage in Pontonema vulgaris (Nematoda: Enoplida). J. Zool. Lond., 161:211.

JHA, R.K. & SMYTH, J.D. 1969. Ultrastructure of microtriches in Echinococcus grandulosus. Expl. Parasit., 25:232.

JOHNSON, P.W., VAN GUNDY, S.D. & THOMSON, W.W. 1970. Cuticle formation in Hemicycliophora arenaria, Aphelenchus avenae and Hirschmanniella gracilis. J. Nematol., 2:59.

JONES, F.G.W. 1965. Structure and classification of nematodes. Plant Nematology, 2nd Ed. Tech. Bull., 7. Minist. Agric. Fish. Fd, Lond.

KAN, S.P. & DAVEY, K.G. 1968. Molting in a parasitic nematode Phacanema decipiens. III. The histochemistry of cuticle deposition & protein synthesis. Can. J. Zool., 46:723.

KAY, D. 1967. Techniques for electronmicroscopy. 2nd Ed., Blackwells Scientific Publications, Oxford.

KHAN, M.A. 1957. Sphaerularia bombi Duf. (Nematoda: Allantonematidae) infesting Bumble bees and Sphaerularia hastata sp. nov. infesting bark beetles in Canada. Can. J. Zool., 35:519.

KARNOVSKY, M.J. 1961. Simple methods for "staining with lead" at high pH in electron-microscopy. J. Biophysics Biochem. Cytol., 11:729.

KNOTH, M. 1968. Ultrastructure of chorionic villi from a four-somite human embryo. J. Ultrastruct. Res., 25:423.

KUROSOMI, K. 1961. Electron microscopic analysis of secretion. Int. Rev. Cytol., 11:1.

LAGUENS, R. & BRIONES, M. 1965. The fine structure of the microvillus of columnar epithelial cells of human intestine. Lab. Invest., 14:1616.

LEE, C.C. & MILLER, J.H. 1969. Fine structure of the intestinal epithelium of Dirofilaria immitis and changes occurring after vermicidal treatment with caparsolate sodium. J. Parasit., 55:1035.

LEE, D.L. 1965a. The physiology of nematodes. University Reviews in Biology, Oliver & Boyd, Edinburgh & London.

LEE, D.L. 1965b. The cuticle of adult Nippostrongylus brasiliensis. Parasitology, 55:173.

LEE, D.L. 1966a. An electron microscope study of the body wall of the third stage larva of Nippostrongylus brasiliensis. Parasitology, 56:127.

LEE, D.L. 1966b. The structure and composition of the Helminth cuticle. Advances in Parasitology IV. Ed. Ben Dawes, Academic Press, London and New York.

LEE, D.L. 1968. The ultrastructure of the alimentary tract of the skin penetrating larva of Nippostrongylus brasiliensis (Nematoda). J. Zool. Lond., 154:9

LEE, D.L. 1970a. Moulting in Nematodes: The formation of the adult cuticle during the final moult of Nippostrongylus brasiliensis Tissue & Cell, 2:139.

LEE, D.L. 1970b. The ultrastructure of the cuticle of the adult female Mermis nigrescens (Nematoda). J. Zool. Lond., 161:513.

LUFT, J.H. 1956. Permanganate - a new fixative for electronmicroscopy. J. Biophys. Biochem. Cytol., 2:799.

LUMSDEN, R.D., OAKES, J.A. & DIKE, S.C. 1970. Cytoarchitectural and cytochemical features of tapeworm surfaces. Paper submitted 2nd Int. Congr. Parasitology, Wash. 1970. Abstract J. Parasit., 56:217.

LUMSDEN, R.D., THREADGOLD, L.T., OAKS, J.A. & ARME, C. 1970. On the permeability of cestodes to colloids: an evaluation of the transmembranosis hypothesis. Parasitology, 60:185.

LYONS, K.M. 1969. The fine structure of the body wall of Gyrocotyle urna. Z. Parasitenk., 33:95.

LYSAGHT, A.M. 1936. A note on the adult female Anguillilina optini (Sharga), a nematode parasitising. Aptinothrips rufus Gmelin. Parasitology, 28:290.

MALHOTRA, S.K. 1962a. Experiments on fixation for electronmicroscopy. I. Unbuffered osmium tetroxide. Quart. J. Micr. Sci., 103:5.

MALHOTRA, S.K. 1962b. Experiments on fixation for electronmicroscopy. II. Alkalized osmium tetroxide. Quart. J. Micr. Sci., 103:287.

MALHOTRA, S.K. 1963. Experiments on fixation for electronmicroscopy. IV. The sodium veronal buffer. Quart. J. Micr. Sci., 104:155.

MASSEY, C.L. 1962. Life history of Aphelenchulus elongatus Massey, an endoparasite of Ips confusus Le Conte, with a description of the male. J. Insect. Pathol., 4:95.

MILLONIG, G. 1961. Advantages of a phosphate buffer for osmium tetroxide solutions in fixation. J. Appl. Physiol., 32:1637.

MOWNÉ, L. 1960. On the physiological role of the polyphenols in cell and tissue envelopes. Ark. Zool., 13:287.

MOOG, F. & WENGER, E.L. 1952. The occurrence of a neutral mucopolysaccharide at sites of high alkaline phosphatase activity. Am. J. Anat. 90:339.

MORETON, B.D. & JOHN, M.E. 1955. The mushroom Phorid fly epidemic of 1953. Plant Pathology, 4:9.

MUKHERJEE, T.M. & WILLIAMS, A.W. 1967. A comparative study of the ultrastructure of microvilli in the epithelium of small and large intestine of mice. J. Cell Biol., 34:447.

NICKLE, W.R. 1963. Observations on the effect of nematodes on Ips confusus (Le Conte) and other bark beetles. J. Insect Pathol., 5: 386.

NICKLE, W.R, 1967a. On the classification of the insect parasitic nematodes of the Sphaerularidae Lubbock, 1861 (Tylenchoidea: Nematoda). Proc. Helminth. Soc. Wash. 34:72.

NICKLE, W.R. 1967b. Heterotylenchus autumnalis sp. n. (Nematoda: Sphaerularidae), a parasite of the Face Fly, Musca autumnalis de Geer. J. Parasit., 53:398.

NICKLE, W.R. 1970. A taxonomic review of the genera of the Aphelenchoidea (Fuchs, 1937) Thorne, 1949 (Nematoda: Tylenchida). J. Nematol, 2: 375.

NICKLE, W.R. & WOOD, G.W. 1964. Howardula aptini (Sharga 1932) parasitic in Blueberry Thrips in New Brunswick. Can. J. Zool., 42:843.

OLDHAM, J.N. 1933. On Howardula phyllotretae n.sp., a nematode parasite of Flea Beetles (Chrysomelidae: Coleoptera), with some observations on its incidence. J. Helminth, 11:119.

OVERTON, J. 1965. Fine structure of the free cell surface in developing mouse intestinal mucosa. J. Expl. Zool., 159:195.

OVERTON, J., EICHHOLZ, A. & CRANE, R.K. 1965. Studies on the organisation of the brush border in the intestinal epithelial cells. II. Fine structure of fractions of Tris-disrupted hamster brush borders. J. Cell. Biol., 26:693.

OVERTON, J. & SHOUP, J. 1964. Fine structure of cell surface specializations in the maturing duodenal mucosa of the chick. J. Cell Biol., 21:75.

PANTIN, C.F.A. 1946. Notes on microscopical techniques for Zoologists. Cambridge University Press. 76 pp.

PEASE, D.C. 1964. Histological techniques for electronmicroscopy. Academic Press Inc., New York, 381 p.

PETERS, B.G. 1952. Toxicity tests with vinegar eelworm. I. Counting and Culturing. J. Helminth., 26:97.

POINAR, G.O. 1965. The bionomics and parasitic development of Tripius sciarae (Bovien) (Sphaerularidae) a nematode parastic of sciarid flies (Sciaridae: Diptera). Parasitology, 55:559.

POINAR, G.O. 1969a. Diplogasterid nematodes (Diplogasteridae: Rhabditida) and their relationship to insect disease. J. Invert. Pathol., 13: 447.

POINAR, G.O. 1969 b. Praecocilenchus rhaphidophorus n. gen., n. sp. (Nematoda: Aphelenchoidea) parasitising Rhynchophorus bilineatus (Montrouzier)(Coleoptera: Curculianidae) in New Britain. J. Nematol., 1:227.

POINAR, G.O. & GYRISCO, G.G. 1962. Studies on the bionomics of Hexamermis arvalis Poinar and Gyrisco, a mermithid parasite of the Alfalfa weevil, Hypes postica (Gyllenhal). J. Insect Pathol, 4:469

POINAR, G.O. & THOMAS, G.M. 1966. Significance of Achromobacter nematophilus Poinar and Thomas (Acromabacteraceae: Eubacteriales) in the development of the nematode, DD136 (Neoaplectana sp. Steinernematidae). Parasitology, 56:385

POISSON, R. 1933. Trois nouvelles espèces de Nematodes de la cavité Générale d'Hemiptères aquatiques. Ann. Paras., 11:463.

READ, C.P. 1966. Nutrition of intestinal helminthes. 'Biology of Parasites" Ed. Soulsby, E.J.L., Academic Press, New York.

READ, C.P. 1968. Some aspects of nutrition in parasites. Am. Zoologist, 8:139.

READ, C.P., ROTHMAN, A.H. & SIMMONS, J.E. 1963. Studies on membrane transport with special reference to parasite-host integration. Ann. N.Y. Acad. Sci., 113:154.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-/opaque stain in electronmicroscopy. J. Cell Biol., 17:208.

RIDING, I.L. 1970. Microvilli on the outside of a nematode. Nature, 226:179.

ROBARDS, A.W. 1970. "Electronmicroscopy and plant ultrastructure" McGraw-Hill, London.

ROGERS, W.P. 1962. The nature of parasitism. Academic Press, London and New York.

ROGERS, W.P. 1965. The role of leucine aminopeptidase in the moulting of nematode parasites. Comp. Biochem. Physiol., 14:311.

ROGERS, W.P. & SOMMERVILLE, R.I. 1963. The infective stage of nematode parasites and its significance in parasitism. Advances in Parasitology I.Ed. Ben Dawes, Academic Press, London and New York.

ROGGEN, D.R. 1966. On the morphology of Xiphinema index reared on grape fanleaf virus infected grapes. Nematologica, 12:289.

ROGGEN, D.R., RASKI, D.J. & JONES, N.O. 1967. Further electron microscopic observations of Xiphinema index. Nematologica, 13:1.

ROSENBLUTH, J. 1965. Ultrastructural organisation of obliquely striated muscle fibres in Ascaris lumbricoides. J. Cell Biol., 25:495.

ROTHMAN, A.H. 1967. Colloid transport in the cestode Hymenolepis diminuta. Expl. Parasit., 21:133.

RUHM, W. 1956. Die Nematoden der Ipiden. Parasitol. Schriftenreike, 6:1.

SAMOILOFF, M.R. 1970. Ultrastructure of the cuticle and moulting in Panagrellus silusiae (de Man 1913) Goodey 1945. Paper presented 2nd Int. Congr. Parasitology, Wash. Abstract J. Parasitol., 56:299.

SAMOILOFF, M.R. & PASTERNAK, J. 1968. Nematode morphogenesis: fine structure of the cuticle of each stage of the nematode Panagrellus silusiae (de Mann, 1913) Goodey 1945. Can. J. Zool., 46:1019.

SAUNDERS, J.L. & NORRIS, D.M. 1961. Nematode parasites and associates of the smaller European Elm Bark Beetle Scolytus multistriatus Marsham . Ann. Ent. Soc. Am., 54:792.

SEINHORST, J.W. 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. Nematologica, 4:67.

SHARGA, U.S. 1932. A new nematode, Tylenchus aptini n. sp., parasite of Thysanoptera (Insecta: Aptinothrips rufus Gmelin). Parasitology, 24:268.

SHEFFIELD, H.G. 1964. Electronmicroscopic studies on the intestinal epithelium of Ascaris suum. J. Parasit, 50:365.

SHIRAHAMA, T. & COHEN, A.S. 1970. Association of hemidesmosome-like plague and dense coating with the pinocytotic uptake of a heterologous fibrillar protein (Amyloid) by macrophages. J. Ultrastruct. Res., 33:587.

SHUTE, P. & MARYON, M. 1966. Laboratory techniques for the study of Malaria. Churchill, London. 2nd Ed. 112 pp.

SJÖSTRAND, F.S. 1967. The structure of cellular membranes. Protoplasma, 63:248.

SJOSTRAND, F.S. & BARAJAS, L. 1968. Effect of modifications in conformation of protein molecules on structure of mitochondrial membranes. J. Ultrastruct. Res. 25:121.

SMYTH, J.D. 1947. The physiology of tapeworms. Biol. Rev., 22:214.

SMYTH, J.D. 1969. The physiology of cestodes. University Reviews in Biology. Oliver & Boyd, Edinburgh. 279 pp.

STAMMER, H.J. 1934. Die Nematoden als Kommensalen und Parasiten der Insekten. Verh. Deutsch. Zool. Ges., 36:195.

STOFFOLANO, J.G. & NICKLE, W.R. 1966. Nematode parasite (Heterotylenchus sp.) of Face Fly in New York State. J. Econ. Entomol., 59:221.

SWEETMAN. H.L. 1963. Principles of Biological Control, p.85. W.C. Brown, Dubuque, Iowa.

TAYLOR, E.W. & THOMAS, J,N. 1968. Membrane (contact) digestion in three species of tapeworm, Hymenolepis diminuta, Hymenolepis microstoma and Moniezia expansa. Parasitology, 58:535.

THREADGOLD, L.T. 1965. An electron microscope study of the tegument and associated structures of Proteocephalus pollanicoli. Parasitology, 55:467.

THUST, R. 1968. Elektronenmikroskopische Untersuchungen Über den Bau des larvalen Integumentes und zur Hautungsmorphologie von Ascaris lumbricoides. Zool. Anz. 188:411.

von BRAND, T. 1966. Biochemistry of parasites. Academic Press. New York.

VON SIEBOLD, C.H.E. 18 6. Uber die Spermatozoen der Crustacean, Insecten, Gasteropoden, und einiger anderen wirbellosen Thiere. Arch. Anat. Physiol u. Wissensch. Med., 13:53.

WACHEK, F. 1955. Die ento parasitischen Tylenchiden. Parasitol. Schriftenreihe, 3:1.

WARWICK, R.M. & CHIA, F.S. 1969. Assimilation of labelled glucose from sea water by marine nematodes. Nature, 224:720.

WARREN, E. 1941. On the occurrence of nematodes in the haemocoel of certain Gamasid mites. Ann. Natal Mus., 16:79.

WATSON, B.D. 1962. Structure of the body wall, cuticle formation and moulting in the Nematoda. Ph.D. Thesis. University of Cambridge.

WATSON, B.D. 1965. The fine structure of the body wall and the growth of the cuticle in the adult Ascaris lumbricoides. Quart. J. Micr. Sci., 106:83.

WATSON, M. 1958. Staining of tissue sections for electronmicroscopy with heavy metals. J. Biophys. Biochem. Cytol., 4:475.

WELCH, H.E. 1959. Taxonomy life cycle, development and habits of two new species of Allantonematidae (Nematoda) parasitic in Drosophilid flies. Parasitology, 49:83.

WELCH, H.E. 1962. Nematodes as agents for insect control. Proc. Entomol. Soc. Ont., 92:11.

WELCH, H.E. 1963. Nematode infections. Insect Pathology. An Advanced Treatise, 2: Ed. Steinhaus, E.A. Academic Press. New York.

WELCH, H.E. 1965. Entomophilic nematodes. An. Rev. Entomol., 10:275

WILCOXON, F. 1949. Some rapid approximate statistical procedures. American Cyanamid Co., New York.

WILSON, T.H. 1962. Intestinal absorption. W.B. Saunders, London.

WISSE, E. & DAEMS, W.Th. 1968. Electronmicrosopic observations on second stage larvae of potato root eelworm Heterodera rostochiensis. J. Ultrastructure Res., 24:210.

WRIGHT, K.A. 1963a. Cytology of the bacillary bands of the nematode Capillaria hepatica (Bancroft 1893). J. Morph., 112:233.

WRIGHT, K.A. 1963b. The cytology of the intestine of the parasitic nematode Capillaria hepatica (Bancroft, 1893). J. Ultrastruct. Res., 9:143.

WRIGHT, K.A. 1966. Cytoplasmic bridges and muscle systems in some polymyarian nematodes. Can. J. Zool., 44:329.

WRIGHT, K.A. 1968. The fine structure of the cuticle and interchordal hypodermis of the parsitic nematodes Capillaria hepatica and Trichuris myocastoris. Can. J. Zool., 46:173.

WRIGHT, K.A. & HOPE, W.D. 1968. Elaborations of the cuticle of Acanthonchus duplicatus Wieser 1959 (Nematoda: Cyatholaimidae) as revealed by light & electron microscopy. Can. J. Zool., 46:1005.

WRIGHT, K.A. & JONES, N.O. 1965. Some techniques for the orientation & embedding of nematodes for electron microscopy. Nematoligica, 11:125.

WRIGHT, R.D. & LUMSDEN, R.D. 1969. Ultrastructure of the tegumentary pore-canal system of the Acanthocephalan Moniliformis dubius. J. Parasit., 55:993.

WÜLKER, G. 1923. Über Fortpflanzung und Entwicklung von Allantonema und verwandten Nematoden. Curr. Topics Microbiol. Immunol., 5:389.

YAMAMOTO, T. 1963. On the thickness of the unit membrane. J. Cell Biol., 17:413.

ZONDAG, R. 1962. A nematode disease of Sirex noctilio (F.). Interim Research Release: New Zealand Forest Service.

ZONDAG, R. 1969. A nematode infection of Sirex noctilio (F.) in New Zealand. N.Z. Jl. Sci., 12:732.

ZUR STRASSEN, O.K.L. 1892. Bradynema rigidum v. Sieb.. Ztschr. Wissensch. Zool., 54:655.

Appendix
TABLE 1. THE DEVELOPMENT OF INFECTIVE FEMALE NEMATODES FROM LARVAE AT DIFFERENT TEMPERATURES

TIME IN DAYS	TEMPERATURE °C					
	5	10	15	20	25	30
1	0	0	0	0	0	0
2	0	0	0	12.0	7.2	0
3	0	0	6.2	8.9	14.6	0
4	0	0	7.8	16.0	10.6	0
5	0	0	5.0	8.2	8.7	0
6	0	0	8.3	5.1	6.3	0
7	0	0	5.3	-	-	0
8	0	0	6.1	-	-	0

Appendix
TABLE 2. THE DEVELOPMENT OF MALE NEMATODES FROM LARVAE AT DIFFERENT TEMPERATURES

TIME IN DAYS	TEMPERATURE °C					
	5	10	15	20	25	30
1	0	0	0	0	0	0
2	0	0	0	6.0	3.1	0
3	0	0	2.5	2.2	1.9	0
4	0	0	2.9	2.5	1.2	0
5	0	0	5.0	1.6	0.9	0
6	0	0	2.5	1.0	0.5	0
7	0	0	0.9	0.6	-	0
8	0	0	0.3	-	-	0

Appendix
TABLE 3. REPRODUCTION OF THE PARASITE INSIDE YOUNG HOSTS

AGE OF HOST (IN DAYS)		MEAN NO. OF PARASITE EGGS	EGG RANGE PER PARASITE	MEAN NO. OF PARASITE LARVAE	RANGE OF LARVAE PRODUCED PER PARASITE
PUPAE	1	0	0	0	-
	2	0	0	0	-
	3	0	0	0	-
	4	0	0	0	-
	5	7.2	1-19	0	-
	6	14.6	0-46	0	-
	7	60.0	18-97	0	-
	8	149.0	79-112	0	-
	9	174.0	26-243	0	-
	10	178.0	124-232	0	-
	11	196.0	119-312	11.1	0.7-51
FLIES	1	134.0	89-197	9.9	3-37
	2	87.3	69-105	79.5	54-112
	3	93.3	60-125	113.3	72-160

Appendix
TABLE 4. HATCHING OF PARASITIC LARVAE INSIDE THE FLIES

AGE OF FLIES IN DAYS	MEAN EGGS	MEAN FIRST STAGE LARVAE	MEAN SECOND STAGE LARVAE	MEAN TOTAL LARVAE	MEAN TOTAL EGGS AND LARVAE
1	428	128	234	362	790
2	232	542	116	658	890
3	224	278	432	710	934
4	306	528	310	838	1138
5	212	180	464	644	856
6	192	350	552	902	1094
7	290	570	238	808	1098
8	280	516	354	870	1150
9	212	322	580	902	1114
10	304	582	276	858	1162

Appendix
TABLE 5. PARASITE MORPHOMETRICS AND MULTIPLE INFECTIONS

MEAN DIMENSIONS OF PARASITES	NUMBER OF PARASITES PER FLY							
	1	2	3	4	5	6	7	8
Body length	841	732	695	532	553	552	610	432
Body width	124	104	96	82	80	73	78	60
a	6.8	7.2	7.2	6.4	7.0	7.5	8.0	6.5
Distance of vulva from anterior	720	632	545	435	457	476	554	369
V (%)	85	86	84	85	82	86	85	87

Appendix
TABLE 6. DECREASE IN PARASITE FECUNDITY IN MULTIPLE INFECTIONS

Number of Adult ♀ Parasites per Fly	Mean Number of Progeny per Parasite	Log Mean Progeny per Parasite	Range of Progeny per Parasite	Log Range of Progeny per Parasite
1	627	2.797	370-900	2.568-2.954
2	482	2.683	355-730	2.550-2.863
3	357	2.553	213-487	2.238-2.687
4	325	2.512	222-387	2.346-2.587
5	292	2.465	218-362	2.338-2.558
6	221	2.344	166-306	2.220-2.486
7	195	2.290	156-250	2.193-2.398
8	154	2.187	151-157	2.179-2.196
9	173	2.238	152-195	2.182-2.290
10	-	-	-	-
11	-	-	-	-
12	139	2.143	-	-
13	57	1.756	-	-

Appendix
TABLE 7. % PARASITISM OF FLIES COLLECTED AT VARIOUS MUSHROOM FARMS FOR 3 YEARS

MONTH	1968		1969		1970	
	No. of observations	Mean % Parasitism	No. of observations	Mean % Parasitism	No. of observations	Mean % Parasitism
JANUARY	3	48	2	23	1	16
FEBRUARY	2	30	1	14	-	-
MARCH	1	2	3	9	1	7
APRIL	1	0	1	1	-	-
MAY	1	3	1	2	1	3
JUNE	2	10	1	4	1	16
JULY	1	21	1	5	1	41
AUGUST	1	26	1	29	1	63
SEPTEMBER	3	63	3	27	-	-
OCTOBER	3	77	3	72	-	-
NOVEMBER	1	68	3	57	-	-
DECEMBER	1	32	2	28	-	-

Appendix
TABLE 8. NUMBER OF EGGS LAID BY CLEAN AND PARASITISED FLIES

EGGS LAID BY CLEAN FLIES						EGGS LAID BY PARASITISED FLIES									
30	0	17	3	0	18	0	0	0	18	0	38	0	5	1	13
29	11	1	18	26	0	12	0	15	0	5	5	13	0	27	4
0	30	7	23	3	10	1	0	0	0	0	0	0	0	9	7
15	10	6	13	11	31	0	2	0	0	9	0	4	0	0	4
0	12	59	24	10	0	12	11	5	0	2	0	0	0	1	8
28	34	21	24	20	25	8	6	20	0	4	7	0	6	8	0
11	4	7	0	11	10	0	0	5	0	0	0	0	10	0	9
41	0	10	16	3	26	0	9	2	0	4	0	0	1	7	0

Appendix
TABLE 9. MULTIPLE PARASITISM AND HOST EGG PRODUCTION

	NUMBER OF ADULT PARASITES PER FLY												
	1	2	3	4	5	6	7	8	9	10	11	12	13
MEAN NO. EGGS/FLY	19.2	9.9	7.5	5.4	4.3	4.0	3.5	0.8	1.5	0	0	0	0
EGG RANGE	0-38	0-33	0-23	0-16	0-13	0-17	0-11	0-4	0-6	0	0	0	0
NO. OBSV.	14	16	9	9	10	4	8	5	8	5	2	3	2
% REDUCTION IN FLIES EGGS	23	60	70	78	83	84	86	97	94	100	100	100	100

Appendix
TABLE 10. THE INCIDENCE OF PARASITISM IN FLY BREEDING CAGES

SOURCE OF FLIES	WORTHING, SUSSEX									
GENERATION	-	1	2	3	4	5	6	7	8	9
% PARASITISM	8.5	20	64	95	0	0	-	-	-	-
SOURCE OF FLIES	DIDCOT, OXON									
GENERATION	-	1	2	3	4	5	6	7	8	9
% PARASITISM	3.7	17	53	93	0	0	-	-	-	-
SOURCE OF FLIES	ANDOVER, HANTS									
GENERATION	-	1	2	3	4	5	6	7	8	9
% PARASITISM	6.5	43	71	91	57	95	0	0	0	0

Appendix TABLE 11. THE KNOWN SPECIES OF THE GENUS *HOWARDULA*

SPECIES	*HOWARDULA BENIGNA* (Type species)			*HOWARDULA ACARINORUM*			*HOWARDULA AORONYMPHIUM*			*HOWARDULA APTINI*		
AUTHOR	COBB, 1921			WACHEK, 1955			WELCH, 1959			(SHARGA, 1932) WACHEK, 1955		
STAGE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE
LENGTH	-	(0.54)	3.5-5.0	0.38-0.43	0.46-0.51	1.20-2.21	0.36-0.47	0.42-0.48	1.28-3.00	0.22-0.27	0.24-0.27	0.18-0.25
WIDTH	-	(20)	-	0.0[illegible]-0.02	0.02-0.02	0.07-0.11	0.01-0.02	0.01-0.01	0.15-0.16	0.01-0.01	0.01-0.01	0.04-0.08
a.	-	-	21	-	-	-	28-34	31-35	8.4 -9.3	27-29	27-34	3.0 -4.8
c.	-	-	-	-	-	-	7.0 -8.7	-	-	7-9	13	-
V% or T%	-	-	(91)	-	(83)	-	53-66	79-83	vulva not visible	-	84	85
STYLET	none	present	present but obscure	none	12-13 µm		not visible	14-15µ	stylet not visible	none	11-12µm	11-12µm
SPICULE	present	-	-	11-13 µm			Left = 19µm Right= 21µm			10µm		
GUBERNACULUM	present			3-5 µm			none			5µm		
CAUDAL ALAE	narrow & transparent			long and narrow.			none			Peloderan		
HOSTS	*Diabrotica vittata*, *D. trivittata* and *D. 12-punctata* (COLEOPTERA)			*Parasitus fucorum* and *Poecilochirus necrophori* (ACARINA)			*Drosophila phalerata* and *D. kuntzei* (DIPTERA)			*Kliniella vaccinii*, *Taeniothrips vacinophilus* and *Aptinothrips rufus*. (THYSANOPTERA)		
LIFE CYCLE NOTES	The parasites viviporously produce 10,000-20,000 larvae which exit via genitalia or rectum at L_3 stage. One moult outside host gives rise to mature males and females which copulate and the female enters a host.			Larvae leave host via rectum and mature in 4-6 days; copulate then infective female enters host and produces 150-280 progeny oviporous. Some parasites are encapsulated. Anus of female parasite is prominent.			Infective female moults inside the host. Development of male larvae inside host is more advanced than female. Free living stages moult and mate then female enters a new host. Some parasites are encapsulated.			The nematodes mature inside the host and leave it as fully developed free living forms. Copulation occurs in the gall of the Thrips and the female enters a new host.		

Means given in parenthesis; - data not recorded.

Measurements given in mm unless stated otherwise.

OTHER SPECIES

Howardula dubius (Christie, 1938) Nickle 1965. Host unknown.
Only the free living female is known L = 0.096-1.07, a = 29-40, V% = 81-90 (88), Stylet = 9µm.

Howardula cuneifer (Warren, 1941) Wachek, 1955. Host = *Cosmolaelaps cuneifer* (Acarina)
Only the parasitic female and larvae are known.
Parasitic female = L = 0.300, W = 0.025.

Howardula claviger (Warren, 1941) Wachek, 1955. Hosts = *Cosmolaelaps Hypoaspis* sp. (Acarina)
Only the parasitic female and larvae are known.
Parasitic female is "smaller" (?) than *H. cuneifer*.

Appendix TABLE II continued.

SPECIES	*HOWARDULA OSCINELLA*			*HOWARDULA PHYLLOTRETAE*			*HOWARDULA* (?) Sp.		
AUTHOR	(GOODEY, 1930) WACHEK, 1955			OLDHAM, 1933					
STAGE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE	MALE	FREE LIVING FEMALE	PARASITIC FEMALE
LENGTH	0.55-0.65	0.50-0.6	1.6	not seen	not seen	0.83-2.2	0.34-0.39	0.36-0.48	0.37-1.33
WIDTH	0.01-0.02	0.01-002	0.13			0.06-0.17	0.01-0.02	0.01-0.02	0.06-0.20
a.	36-37	33-36	-			10-16	21-24	21-30	3-11
c.	-	-				-	10-13	9-11	-
V% or T%		-					52-59	81-83	76-96
STYLET	none	20-21µm	present		23-24µm	present	none	12-18µm	none
SPICULE	11µm						10.6-12.2µm		
GUBERNACULUM	3µm						none		
CAUDAL ALAE	narrow						Peloderan		
HOSTS	*Oscinella frit* (DIPTERA)			*Phyllotreta undulata* and three other species (COLEOPTERA)			*Megaselia halterata* (DIPTERA)		
LIFE CYCLE NOTES	The parasitic femaledoes not moult inside the host and is viviparous. the parasitic larvae show sexual differentiation and leave the host via anus. Copulation occurs and female enters another host. Three host – parasitic life cycles occur in 1 year, with highest incidence of parasite in Spring.			The free living stages were not found and could not be obtained by culturing. Probably a lengthy period of external development is required because the host has one generation per year. Exit via host vulva.			Larvae moult once inside the host and are sexually differentiated. Females moult two cuticles in the free living stage and males moult three cuticles. Copulation occurs and the female enters a host where the final moult occurs.		

Howardula terribilis (Warren, 1941) Wachek, 1955. Host = *Euryparasitus terribilis* (Acarina)
Only the parasitic female and larvae are known
Parasitic female = L 0.300, W 0.110. (2 Specimens!)

Howardula hirsutus (Warren, 1941) Wachek, 1955. Host = *Haemagamasus hirsutus* (Acarina)
Only the parasitic female and larvae are known
No measurements given for parasitic female (1 Specimen!)

APPENDIX 12

PARASITE FECUNDITY AND MULTIPLE INFECTIONS:

Calculation of best fit regression line.

$$b = \frac{xy - \frac{(\ x)(\ y)}{n}}{x^2 - \frac{(\ x)^2}{n}}$$

where n (observations) = 7, x (sum parasites/fly) = 28 and y (sum $\log_{10}$ mean offspring) = 17.644, then $b = -0.0817$

a (intercept on the y axis) = $\bar{y} - b\bar{x}$

where $\bar{y}$ = 2.521 and x = 4, then $a = 2.848$

y (intercept for the value of x = 7) = 2 + bx therefore

$y = 2.276$.

After plotting the best fit regression line from the values of a and y, the reduction in offspring with increasing numbers of adult parasites was found by calculating the ratio between any two observations along the line. For example, the regression line intercepts for the occurrence of one and two parasites per fly give reading of:-

Log_{10} for 1 parasite/fly = 2.766, antilog = 2320

Log_{10} for 2 parasites/fly = 2.685, antilog = 1920

The ratio $P_2 : P_1 = \frac{1920}{2320} = 0.829$

(See FIGURE 13)

It was found that for every additional parasite, the progeny of each adult female decreased by a factor of 0.829. The absolute reduction of progeny can be calculated from

$$p = a + bP_n$$

Where a = $\log_{10}$ progeny when no parasitism (i.e. $P_n = 0$) = 2.848

b = regression coefficient indicating a decreased progeny ratio of 0.829

P_n = number of adult parasites per fly.

APPENDIX 13

EFFECT ON PARASITE ON HOST EGG PRODUCTION:

Test for significance

Number of 'clean' flies,	n_1	= 48
Number of parasitised flies,	n_2	= 80
Mean eggs laid by 'clean' flies,	$\bar{x}_1$	= 14.8
Mean eggs laid by parasitised flies,	$\bar{x}_2$	= 4.2
Variance of eggs laid by 'clean' flies,	s_1	= 162.83
Variance of eggs laid by parasitised flies,	s_1	= 44.37

When samples have approximately equal variances the results can be substituted in 'Student's $\underline{t}$ test where:-

$$t_{\text{degrees of freedom}} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

but in this case the variance of eggs laid by unparasitised flies is much greater than that of parasitised specimens and consequently it is necessary to calculate a modification for the degrees of freedom (f = 126) using the special procedure outlined by Bailey (1959) for samples with unequal variances:-

$$\text{modified degrees of freedom, } f = \frac{1}{\frac{u^2}{n_1 - 1} + \frac{(1 - u)^2}{n_2 - 1}}$$

Where

$$u = \frac{\frac{s_1^2}{n_1}}{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}} = \frac{\frac{162.83}{48}}{\frac{162.83}{48} + \frac{44.37}{80}} = \frac{3.39}{4.545} = \underline{\underline{0.7459}}$$

and is substituted in the above formula so that

$$f = \frac{1}{(0.7459)^2 + (1-0.7459)^2} = \frac{1}{0.118 + 0.0645} = \underline{\underline{13.10}}$$

Applying the modified degrees of freedom (f=13) in 'Student's' $\underline{t}$ test:

$$t_{13} = \frac{14.8 - 4.2}{\sqrt{\frac{162.8}{48} + \frac{44.37}{80}}} \quad \frac{10.6}{3.945} = \underline{\underline{5.34}}$$

Using 'Student's' $\underline{t}$ - Distribution Table it was found that the probability of observing a value for $\underline{t}$ with 13 degrees of freedom, greater in absolute value than 4.221, is 0.001. The calculation showed that $t_{13} = 5.34$ and therefore being greater than 4.221, the probability of obtaining this result by chance is less than one in a thousand. Consequently the Null Hypothesis that parasitised and unparasitised flies lay the same number of eggs must be rejected.

APPENDIX 14.

(*Reprinted from Nature, Vol.* 226, *No.* 5241, *pp.* 179–180, *April* 11, 1970)

MICROVILLI ON THE OUTSIDE OF A NEMATODE

By

IRENE L. RIDING

Microvilli on the Outside of a Nematode

THE females of insect parasitic nematodes of the order Tylenchida are bizarre in form and live in the haemocoels of their hosts[1-3]. After the infective larva enters the haemocoel it usually moults, grows rapidly and becomes sexually mature. Its feeding apparatus quickly degenerates, the mouth and anus often disappear and if the intestine persists it loses its connexion with the oesophagus[4] when food must presumably be absorbed through the body wall. In keeping with this function the external surface of one of these parasites was found to be quite unlike that of nematodes so far described.

The nematode studied, *Bradynema* sp., was an undescribed parasite of the mushroom pest *Megaselia halterata* (Diptera, Phoridae)[5,6]. The adult female reproduces inside the haemocoel of the fly and second stage larvae leave the host during oviposition or defaecation. The larvae rapidly moult in the mushroom compost and develop into adult males and infective females within 48 h at 25° C. After copulation the female enters a second or third instar maggot where it grows and lays eggs in about 7 days.

Flies were dissected in entomological saline and the worms transferred to either 2·5 per cent glutaraldehyde in cacodylate buffer or osmium tetroxide buffered according to Millonig or Zetterquist. The adult female parasites were 1 mm long and each was cut into three or four pieces and left in the fixative for 2 to 24 h. Those fixed in glutaraldehyde were rinsed in cacodylate buffer and post fixed in either Millonig's or Zetterquist's osmium tetroxide for a further 1 to 4 h. They were rinsed in the appropriate buffer, orientated in agar[7], dehydrated in alcohols and slowly transferred to pure epoxy resin through six mixtures of increasing concentration. They were left overnight at 25° C before being transferred to fresh resin for polymerization at 60° C for two days. Pale gold sections were cut with glass knives on a Reichert ultramicrotome, mounted on uncoated copper grids and examined with a Jem 7 electron microscope at 80 kV.

Fig. 1 shows microvilli on the outside of the worm which cover the entire surface. The density, shape and size of microvilli differ according to their position and the age of the worm; some are simple finger-like projections, others have enlarged tips and several have branches which may

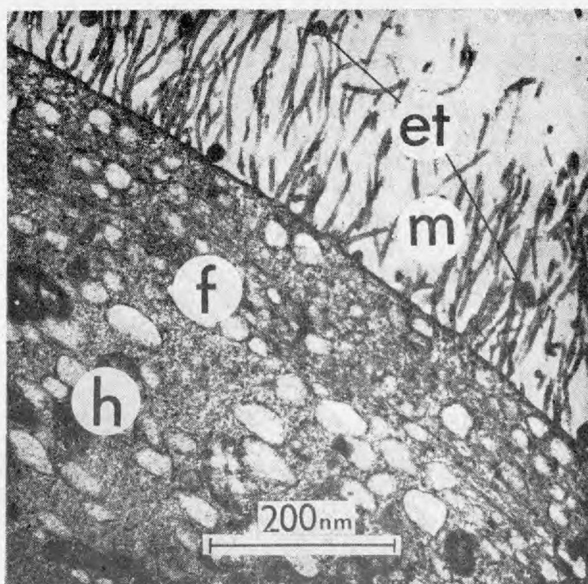


Fig. 1. Transverse section of the body wall surface showing microvilli (*m*) on the outside, some with enlarged tips (*et*) and supported by fibres(*f*) below in the hypodermis (*h*). The nematode was fixed for 3 h in Zetterquist's osmium tetroxide and stained with uranyl acetate and lead citrate.

anastomose with adjacent microvilli. Scattered fibres occur below the surface together with endoplasmic reticulum, lipid droplets, membranous whorls presumably of phospholipid, mitochondria, ribosomes and occasional nuclei, which suggest that the cuticle is absent and this region is the hypodermis. Two to eight cells in each complete transverse section contain what appear to be sparse myofilaments and probably represent degenerate muscle cells.

The body wall of nematodes is composed of three main regions: cuticle, hypodermis and muscle[8-10]. The cuticle is outermost and is composed of several different layers, but apparently does not absorb food as does the tegument of other parasitic helminths. The apparent lack of cuticle in the mouthless, gutless nematode parasite of *Megaselia*

and its possession of microvilli suggest that nutrients pass through the body wall. The outer covering of this worm has more in common with a tapeworm tegument than has a normal nematode body wall.

The adult female parasite of *Deladenus siricidicola*[11,12] from the haemocoel of the wood wasp *Sirex noctilio* has scattered clusters of microvilli, but I am not yet certain that its cuticle is entirely absent.

I thank Dr Donald L. Lee, Houghton Poultry Research Institute, for help, Dr N. W. Hussey, Glasshouse Crops Research Institute, for supplying infected *Megaselia halterata* and Mr F. Wilson of the CSIRO Sirex Unit for supplying *Deladenus siricidicola*.

IRENE L. RIDING

Imperial College of Science and Technology,
Department of Zoology and Applied Entomology,
Prince Consort Road, London SW7.

Received January 13, 1970.

1 Nickle, W. R., *Proc. Helminth. Soc. Wash.*, 34, 72 (1967).
2 Welch, H. E., in *Insect Pathology* (edit. by Steinhaus, E. A.), 363 (Academic Press, New York, 1963).
3 Welch, H. E., *A. Rev. Ent.*, 10 (1965).
4 Poinar, G. O., and Doncaster, C. C., *Nematologica*, 11, 73 (1965).
5 Hussey, N. W., *Mushr. Sci.*, 4, 260 (1959).
6 Hussey, N. W., *Proc. Thirteenth Intern. Congr. Ent.*, London, 1964, 752 (1965).
7 Wright, K. A., and Jones, N. O., *Nematologica*, 11, 125 (1965).
8 Lee, D. L., *Parasitology*, 56, 127 (1966).
9 Watson, B. D., *Quart. J. Microsc. Sci.*, 106, 83 (1965).
10 Wisse, E., and Daems, W. Th., *J. Ultrastruct. Res.*, 24, 210 (1968).
11 Bedding, R. A., *Nature*, 214, 174 (1967).
12 Bedding, R. A., *Nematologica*, 14, 515 (1968).