THE STRUCTURE AND FUNCTION OF GENÉ'S ORGAN AND ITS ASSOCIATED GLANDS IN TICKS

by

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A Thesis submitted to the
COUNCIL FOR NATIONAL ACADEMIC AWARDS
for the degree of Doctor of Philosophy

Sponsoring Establishment:

School of Biological Sciences and Environmental Health,
Thames Polytechnic, London

Collaborating Establishment:

Wellcome Research Laboratories, (Berkhamsted)

January 1985

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. D.J. Beadle for initiating the project and for giving me every help and encouragement in the work which forms this thesis.

I am also indebted to Dr. R.J. Hart for his supervision and for advice and guidance, and to Dr. R.G. Wilson for his interest in my progress and helpful suggestions and for critically reviewing this thesis. I also thank Dr. C. Potter for many useful discussions, and the staff of the Wellcome Research Laboratories, Berkhamsted for their help and hospitality during my stay as a guest in their laboratories. I am grateful to the Wellcome Foundation Ltd. for providing the use of facilities and for the gift of many materials, and to the S.E.R.C. for providing financial support.

Help with scanning electron microscopy was kindly provided by Dr. J. Beesley and Mr. P. Rowden of the Wellcome Research Laboratories, Beckenham.

My thanks are also due to Mr. R. Lamb for processing the numerous photographs I have taken, and for his technical instruction in microscopy.

Finally I thank all the staff and students of Thames Polytechnic who have contributed to my work, for making my time spent working in the laboratory so pleasant and enjoyable; and my family and friends for their support.

T.F.B.

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Abstract

The objective of the study is to provide information on tick reproduction of use in designing new pest control methods. This is the first study to describe the structure of Gené's organ, the egg-waxing organ in ticks, using electron microscopy. Both ultrastructural and physiological aspects are investigated in two important pest species, Boophilus microplus and Amblyomma variegatum.

The organ performs an essential function in females by coating the eggs with a waterproofing wax layer during oviposition, which prevents desiccation of the embryo, ensuring its viability. It is therefore a potential target for control agents.

The external part of the organ, the 'horns', is an evertable balloon-like cuticular sac which is inflated by fluid pressure and manipulates each egg coating it in wax. The wax passes through pores in the cuticle from the internal lumen. The wax is synthesised by three types of epidermal glands, the tubular, lobular, and accessory glands. The ultrastructure of the gland cells is consistent with their wax secretory function.

Biochemical study using radiolabelled acetate shows that label is incorporated into wax lipids by the glands, and the composition of the lipids extracted from the secretory cells was similar to that of the egg wax. Wax synthesis by Gené's organ was disrupted by precocene treatment, which had a cytotoxic effect on the secretory cells and caused egg desiccation.

The effects of a range of drugs on oviposition was tested; octopamine and certain adrenergic agonists were found to stop oviposition in engorged females in a similar manner to sublethal doses of formamidine acaricides.

Tick muscle has received few previous investigations, so the present study also concerns the ultrastructure and electrophysiology of the retractor muscles controlling the horns and their innervation. Evidence is presented implicating L-glutamate and L-aspartate as excitatory transmitters at the neuromuscular junctions.

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1. Introduction

The ticks are extremely successful obligate parasites of terrestrial vertebrates, and are a group of very ancient ancestry which appears to have changed little over evolutionary time (Hoogstraal, 1978). Their phylogenetics is obscure due to the absence of intermediate forms. The bloodsucking mode of ectoparasitism has made the ticks one of the most important arthropod pests, acting as vectors of many diseases. They transmit pathogenic filaria, protozoa, bacteria, rickettsiae and viruses, and act as reservoirs of diseases in the absence of infected host species due to their ability to pass on pathogens to their offspring transovarially (Burgdorfer and Varma, 1967; Balashov, 1972; Burgdorfer and Brinton, 1975). Ticks surpass all other arthropods in the number and variety of diseases transmitted to domestic animals, and are second only to the mosquitoes in transmitting human disease. Of the 800 or so species of ticks so far described, the ixodid ticks which form the subject of this thesis are among the most commercially important pests of cattle: ticks of the genus Boophilus transmit anaplasmosis and babesiosis to cattle throughout the tropical regions of the world, and the bont tick, Amblyomma variegatum transmits ricketssial disease to cattle in central, eastern and southern Africa.

Although ticks have been known and recognised as pests since at least 800 B.C. (Hoogstraal, 1970-74), it was not until the second half of the 19th century when the world cattle population increased rapidly to feed the human populations of the great industrial centres, that the diseases they transmit and their serious debilitating effect on cattle became a problem. During this period susceptible, exotic breeds of cattle were imported into tick infested areas and exposed unwittingly to tick-borne diseases. Not only do the transmitted diseases, injected in the tick saliva during feeding, cause cattle death or diminished productivity, but the blood loss and opportunity for secondary infection contribute to the animal's malaise, resulting in anaemia and irritation. The tick saliva itself may also cause toxicosis. It was first recognised that the cattle disease known as redwater was transmitted by Boophilus annulatus in 1889; however, the disease itself had been known since 1814. With the realisation that ticks were important vectors of major cattle diseases, such as East Coast Fever, anaplasmosis and heartwater, the first control campaigns were mounted. At first there were considerable cattle losses due to

the lack of effective remedies. The first successful tick control was obtained with the use of arsenical solutions as a dip or spray on cattle in the 1890's. The use of arsenicals enabled the eradication of Boophilus annulatus from most of the United States by 1940, but the development of arsenic resistance in ticks necessitated the development of new acaricides. The rapid acquisition of resistance has needed the continual development of newer, effective acaricides - first the organo-chlorine pesticides, then the organo-phosphates, carbamates and more recently the formamidines. Other factors contributing to the need for new pesticides were the increasingly unacceptable organochlorine residues in meat, milk and by-products, and the environmental impact of acaricide residues. As a result there is an acute need for the development of alternative methods of control, since the genetic plasticity of ticks limits the effective life of an acaricide. Despite this, relatively little is known about the details of tick physiology and anatomy, particularly of the reproductive system, which may be useful in the search for new control methods. There have been few structural studies on ticks using modern electron microscopy techniques, and much of the physiological research has concentrated on the tick feeding mechanism and salivary glands (e.g. Tatchell, 1969; Megaw, 1977; Kaufman and Sauer, 1982; Kaufman, 1984).

Ticks are well known for their high reproductive potential, females of the ixodids Boophilus microplus may lay 2,000 eggs, and in the larger Amblyomma spp up to 20,000 eggs are produced by a single female (Arthur, 1962). The extreme fecundity of the ixodid ticks appears to be an r-selected reproductive strategy (Southwood et al., 1974) in that large numbers of offspring are produced, and associated with this is a high mortality. However, this gives the tick population a capacity for rapid increase in favourable conditions, and in the absence of high mortality, as occurs in cattle husbandry in tropical areas when tick control measures have been ineffective or inadequate. The ixodid ticks are well-adapted to producing large numbers of eggs. Females maximize the amounts of nutrients obtained by attaining a large body size, by concentrating the blood meal by elimination of excess water and ions through salivary gland secretions (Kaufman and Sauer, 1982) and by resuming endocuticle synthesis and expansion during feeding (Hackman and Filishie, 1982). The epicuticle covering non-sclerotized regions of the body cuticle is deeply folded into a herring-bone pattern. Stretching is accomplished by the unfolding

of the epicuticle, and simultaneous growth and stretching of the endocuticle below, allowing accommodation of a large meal. The blood meal is then digested intracellularly (Akov, 1982) and most of the blood-meal protein is then transformed into yolk proteins by the gut and fat body cells (Araman, 1979). The engorged female tick is a virtual "egg factory" which efficiently transforms the maximum amount of its resources into eggs. In B. microplus, egg production was found to average 62% of engorged female weight (Bennett, 1974). In contrast, the Argasid ticks produce smaller numbers of eggs and take smaller blood meals. Life cycle characters contributing to the success of ticks as ectoparasites are the prolonged longevity of the intermediate stages, a reduction of the number of essential host encounters in the life cycle, and a strict coordination between feeding, moulting and reproduction. The life cycle of B. microplus is shown in Fig. 1.1, and the classification of ticks in Fig. 1.2. The engorged female, eggs and newly hatched larvae are the most vulnerable stages in the life cycle of ticks, where the most mortality occurs due to predation (McMurtry, 1984), adverse environmental conditions (Balashov, 1972) or host resistance to feeding (Wikel and Allen, 1982). Tick larvae are slow-moving and especially with field-parasitizing forms such as B. microplus the greatest difficulty they encounter is in finding a host before they die. B. microplus is a one-host tick, so only a single encounter with a host is necessary for a larva to complete the life cycle. The research which forms this thesis is aimed at gaining more information on the basic anatomy and physiology of reproduction, in particular Gené's organ, the egg-waxing organ of ticks. A thorough study of the cytology, physiology and biochemistry may eventually lead to the development of improved control methods.

The genital system in female ixodids (reviewed by Balashov, 1972) consists of an ovary with paired oviducts which join together as a common oviduct or uterus (Fig. 1.3). A short connecting tube leads from the uterus into the vagina which is subspecialised into cervical and vestibular regions. After copulation, prostriate ixodids (ixodes spp.) store the male endospermatophores in the expanded cervical portion of the vagina. Metastriate ixodids (all genera except ixodes) store the endospermatophores in a receptaculum seminis which is formed post-ereodorsally by a sac-like extension of the cervical portion of the vagina. Tubular accessory glands open into the vagina between the cervical and vestibular regions. In feeding ixodids, the epithelium of the vestibular vagina develops into a lobular accessory gland.

Fig. 1.1 The life cycle of Boophilus spp.

1. Larvae hatch from the eggs. Seven to ten days later the larvae climb vegetation and seek a host.

2. They attach to their host and engorge on its blood within three to five days. They then pass into a moult. Two days later nymphs emerge from the moult and take a blood meal lasting from five to six days.

3. The engorged nymphs pass into a two-day moult from which adult males and females emerge. After mating the female completes a large blood meal.

4. The engorged female drops to the ground twenty or more days after attaching as a larva and seeks a suitable place to lay her eggs. 2,000 eggs are laid in a humid niche on the ground.

A. The engorged female.

B. The male.

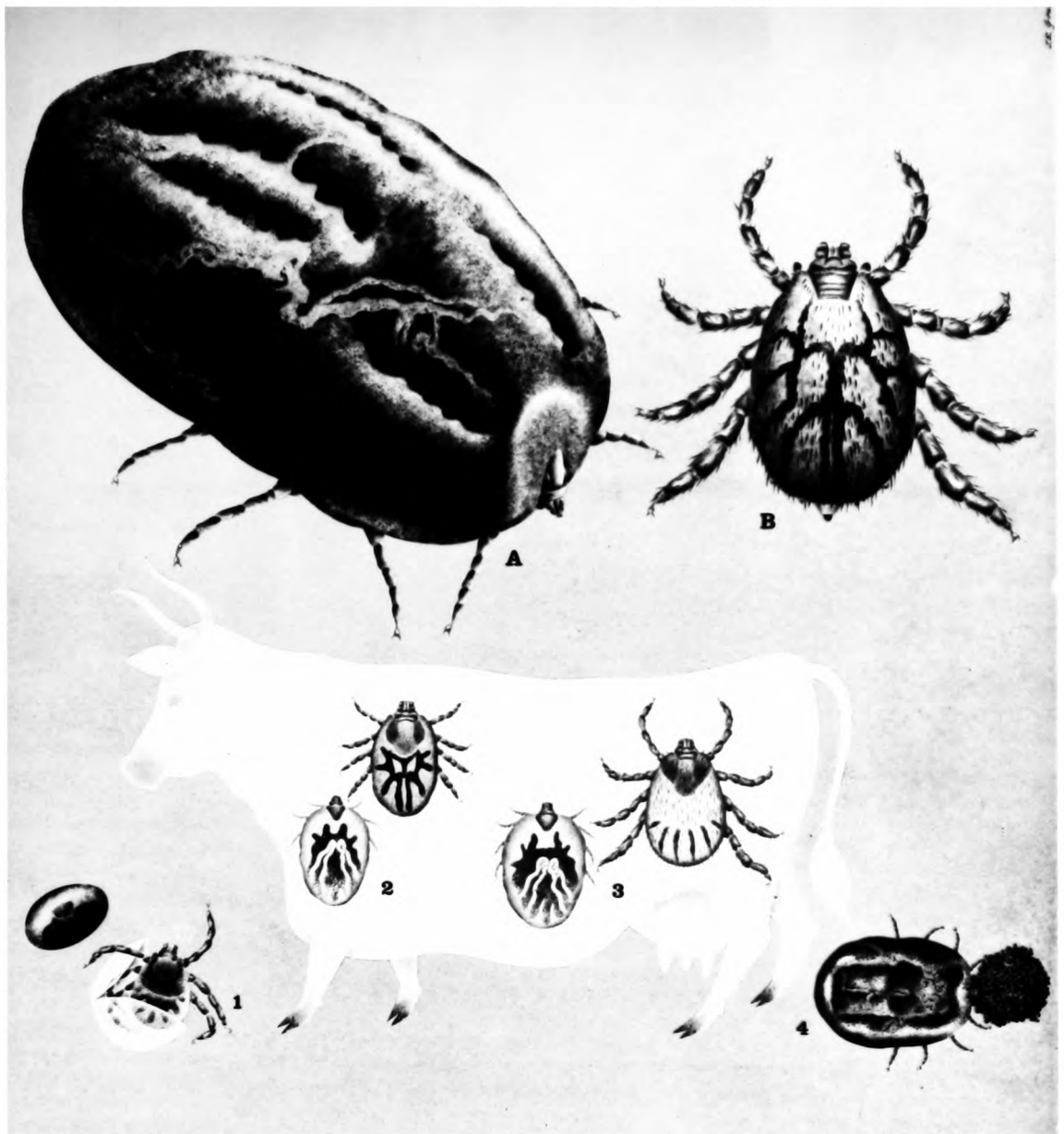
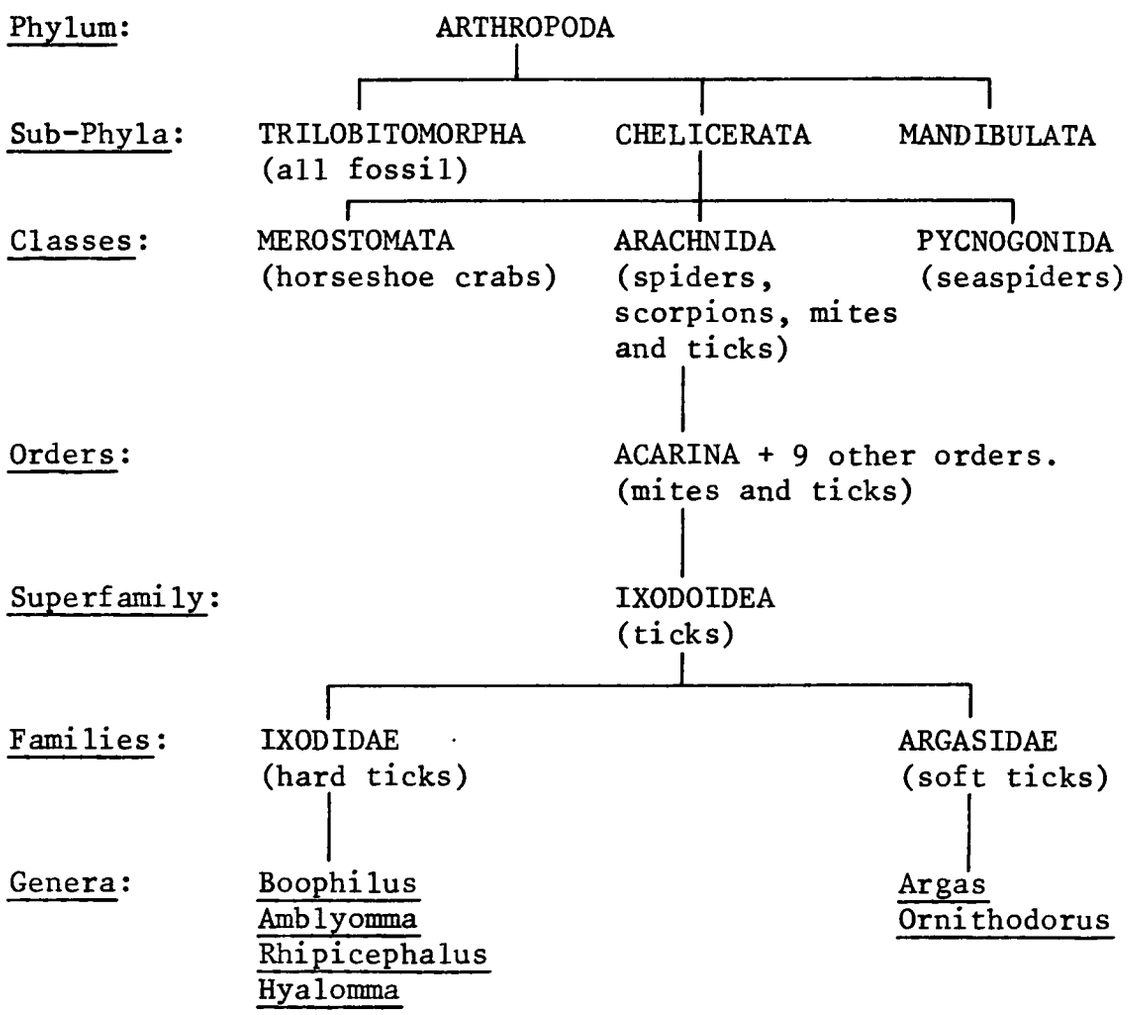

A
B
1
2
3
4

Fig. 1.2 Systematic position of the Ixodidae (Grassé, 1949)



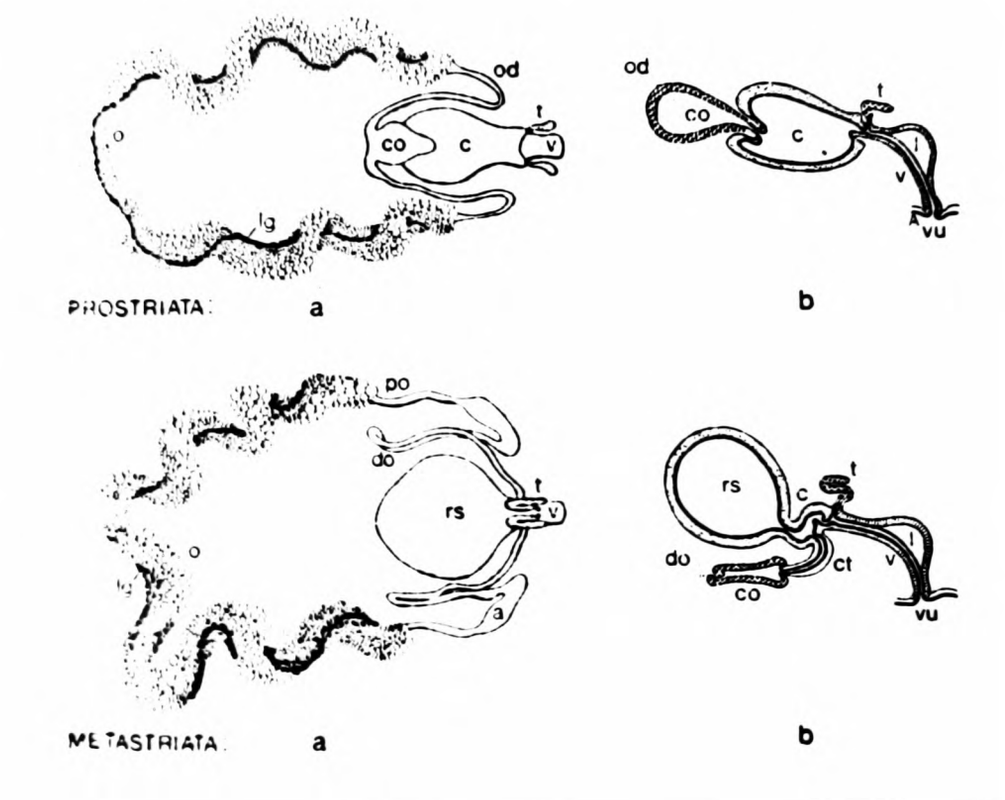


Fig. 1.3 (a) Diagrammatic dorsal and (b) sagittal views of the female genital systems of the Ixodidae-Prostriata and Ixodidae-Metastriata (after Diehl et al., 1982). O, ovary; lg, lateral groove; po, proximal oviduct; do, distal oviduct; a, ampulla; od, oviduct; ct, connecting tube; rs, receptaculum seminis; c, cervical vagina; v, vestibular vagina; t, tubular accessory glands; l, lobular accessory gland; vu, vulva.

In B. microplus, the replete mated female generally begins to lay eggs after a pre-ovipositional period lasting for one or two days, depending on temperature. The female then begins to oviposit, and this period lasts about 10 days at 30°C.

The process of oogenesis has been reviewed by Diehl et al. (1982). The oviduct wall is capable of stretching to accommodate the developed eggs, which move along the oviduct by peristaltic contractions of muscles attached to the outside of the basal lamina. The oogonia first appear in the ovarian primordia of unfed larvae (Balashov, 1972). Oogonial divisions occur during the development of the female tick through its instars. The primary oocytes first appear in fed nymphs, and then enter prophase of the first maturation division. A fold or longitudinal groove running along the surface of the ovary contains the oogonia and primary oocytes in their earliest development stages. The oocytes situated outside the longitudinal groove are more advanced, and as growth continues after the female begins feeding the oocytes protrude into the haemocoel giving the ovary a grape-like appearance. By this stage the oocytes are connected to the ovarian wall solely by a thin stalk, the funicle, of elongated epithelial cells. This differs from the ovary of insects in which developing oocytes are surrounded by a layer of follicular cells. Externally, the basement lamina forms a barrier between the ovarian tissue and the haemolymph. Trachea and tracheoles and a few muscles penetrate between the cells of the ovary. During the stage of cytoplasmic growth, the nucleus, nucleolus and cytoplasm enlarge greatly and the oocyte protrudes from the ovarian wall, and vitellogenesis occurs. Membrane-limited yolk granules appear in the cytoplasm, and the yolk originates from both intra- and extracellular sources. Yolk vesicles derived from the rough endoplasmic reticulum and dictyosomes fuse into larger vacuoles, and material of extracellular origin is taken up by micropinocytosis (Brinton and Oliver, 1971). Between the yolk granules are also glycogen stores and triglyceride-rich lipid inclusions (Diehl et al., 1970). At the end of this development the nuclear membrane and nucleolus disappear, the chromosomes condense into a karyo-sphere and ovulation takes place. The former point of oocyte attachment to the funicle region is visible as a thinned area on the egg shell. The basement lamina and the developing egg-shell forms a mechanical barrier which protects the oocyte to some extent against invading pathogens, however some proteins are able to pass across from the haemolymph to the egg cytoplasm (Lees and Beament, 1948). If properly fertilized,

engorged female ticks begin to ovulate within 1-2 weeks after the start of feeding. The actual process of ovulation has not been observed, but it is assumed that ovulation occurs as the result of increasing pressure exerted by the growing oocyte into the ovarian lumen, or by breakage of the cellular connections of the funicle. Following ovulation, peristaltic movements of the ovary and oviducts transport the eggs towards the lower parts of the oviducts and into the uterus. The mechanism controlling the contraction of muscles involved in the transport of the oocyte is unknown, it may be nervous and/or hormonal control. As ovulation continues, oocytes accumulate in the lumen of the ovary or of the uterus and these regions become greatly distended. In the oviducts, the oocytes are aligned one behind the other. The egg shell becomes progressively hardened during passage through the oviduct, and water uptake occurs until final oocyte volume is attained. Syngamy probably occurs in the lower parts of the oviducts (Balashov, 1972).

The oocytes of the tick ovary do not develop synchronously during vitellogenesis since oocytes of very different developmental stages can be encountered at any one time. This mechanism ensures that the period of vitellogenesis,ovulation and oviposition in the tick is prolonged over several days or weeks. This allows a steady production of eggs, and must be advantageous due to the restricted space for egg storage in the oviducts and the time-consuming requirement for individual handling of eggs during their waterproofing by Gené's organ. This therefore allows the production of large egg batches.

The egg laying females of both the ixodid and argasid ticks are unique among the Acarina in that they possess a glandular organ system, Gené's organ. In Boophilus it consists of a pair of balloon-like horns which have a thin, plicate glistening cuticular surface enclosing a fluid-filled lumen, and a single cell thick secretory epithelium which is a proliferation of the body wall epithelium. In the ixodidae, the organ emerges from between the posterior edge of the capitulum and the anterior margin of the scutum, in the camerostomal fold. It is absent from the male and immature stages and only develops in engorged females prior to oviposition.

The organ was first described by Gené (1848) who noted that when it was pricked with a needle, the eggs deposited near the vagina rapidly shrivelled. Bertkau (1881) also found that eggs prevented

from coming into contact with Gené's organ shrivelled much more rapidly than eggs laid normally, but thought that the horns were a receptaculum seminis. Lees and Beament (1948) showed that the function of Gené's organ in Ixodes ricinus and Ornithodorus moubata is to provide the eggs with a waterproof covering which prevents excessive water loss. They occluded the opening through which the organ is everted and followed the fate of the eggs. It was found that most of the eggs laid with Genés organ free hatched normally within twelve days whereas none of those laid without the intervention of the organ hatched and all were completely shrivelled even in humid conditions. When eggs which were laid without coming into contact with the horns were immediately incubated under paraffin oil, a large proportion underwent normal development and hatched into larvae, indicating that it was a lack of waterproofing that caused shrivelling. Extraction of the egg shells with organic solvents caused desiccation by removing the waxes. They also noted that the presence of a waxy covering on the eggs, applied by Gené's organ, prevented the growth of fungi. The eggs of Ixodes ricinus, however, were found to be already partially waterproofed before contact with Gene's organ. This was shown to be due to an incomplete wax layer being applied during passage through the oviduct. This is correlated with the fact that the ixodids have a lobular accessory gland in the vagina, which is absent from the argasidae. The eggs of the argasids are completely unwaterproofed until they come into contact with Gené's organ.

The morphology of Gené's organ was described briefly by Lees and Beament (1948) in Ornithodorus moubata and in more detail by Arthur (1953) in Ixodes ricinus and by Balashov (1972) in Hyalomma asiaticum. However, no previous investigations using electron microscopy appeared to have been made to discover the fine structure of the organ, and this was the aim of the present study (Booth et al., 1984a) together with a description of oviposition in Boophilus microplus.

Oviposition is a time-consuming process, requiring individual handling of the eggs. In I. ricinus, egg deposition time varies from 3-12 minutes (Lees and Beament, 1948). In Hyalomma marginatum the maximum egg laying rate is one every 1.23 min (Knight et al., 1978). Argasids lay eggs much more infrequently than ixodids (Hafez et al., 1972). Oviposition may easily be disturbed by handling the ticks or by changing illumination (Lees and Beament, 1948) and engorged females actively seek dark, sheltered environments.

During oviposition in B. microplus, when an egg is produced from the vagina by peristalsis, the latter prolapses, pushing the egg between the horns of Gené's organ, which manipulate the egg, covering it in wax. The horns are then retracted beneath the scutum and the egg is deposited on the dorsal surface of the tick by movements of the mouthparts. As the horns of Gené's organ emerge from beneath the scutum they pass directly over two groups of pores on the dorsal surface of the mouthparts, the areae porosae. These porose areas are the external openings of the accessory glands of Gené's organ and were first described by Feldman-Muhsam and Havivi (1960). These glands are present only in female ixodid ticks and in Boophilus microplus they consist of two groups of 80-100 gland cells. Each cell is about 80 µm diameter and the cells are arranged in clusters of about ten cells. Thereis an internal secretory lumen within each cell connected to an individual duct and the two groups of ducts lead to the paired groups of secretory pores in close proximity to Gené's organ. The position of the porose areas in relation to Gené's organ leads to the incorporation of their secretion into the egg wax during oviposition (Atkinson and Binnington, 1973). The fact that maximal development of the accessory glands coincides with the onset of oviposition also suggests that they play a role in egg waxing, although their exact function is unclear. As a preliminary step in determining the function of these glands and their secretion, their fine structure was investigated (Booth et al. 1984b).

Interference with tick mating behaviour may lead to some measure of control and some work has been directed towards this end (Feldman-Muhsam et al., 1970; Gladney and Drummond, 1970; Feldman-Muhsam and Borut, 1971; Gladney, 1971). Oviposition is clearly a vulnerable stage of the tick life cycle which could be a target for control methods, either preventing it altogether or by causing production of non-viable eggs. Little is known, however, about how reproduction is controlled and co-ordinated by hormones and the nervous system in the engorged female. Interference with the production of egg wax by Gené's organ, causing egg desiccation, for example, could be exploited as a control method.

Lees and Beament (1948) made some investigations into the water repellent properties of the extracted lipids from tick egg-waxes. Although the composition of the egg-waxes, produced by Gené's organ, and its associated glands, have been analysed in B. microplus (Gilby,

1957; Cherry, 1969; McCamish *et al.*, 1977) no details of wax lipid synthesis by Gené's organ or by the fat body or lobular glands have hitherto been investigated. In particular the contribution that the different cell types make to the egg wax, and the sites and biochemical pathways of egg wax synthesis are unclear. The wax consists of branched and non-branched chained alkanes, fatty acids and esters, corresponding wax esters, cholesteryl esters and possibly free steroids. The significance of the complex composition is unknown but it is similar to the composition of insect cuticular waxes which are generally considered to have a waterproofing function (Beament, 1964; Jackson and Baker, 1976). In addition to their important function in restricting water loss, cuticular waxes in arthropods also serve as a barrier to the penetration of microorganisms (Koidsumi, 1957), reduce the absorption of pesticides and chemicals from the environment (Nelson and Sukkestad, 1970) and some wax components have been shown to serve as pheromones and kairomones (Howard and Blomquist, 1982). Studies on the biochemistry of insect waxes have used *in vivo* techniques, and the biosynthetic pathways for the major hydrocarbon and ester components have been determined (for review; Blomquist and Jackson, 1979). *In vitro* experiments have shown that the cuticular hydrocarbons in insects are synthesised in the integument from acetate and palmitate (Nelson, 1969) and also in the oenocytes (Diehl, 1973) from where they are transported to the cuticle, passing through the pore canals to the surface (Neville, 1975). One of the aims of the present study was to investigate the lipids in the gland cells of Gené's organ and compare them to those present in the waxes, and to gain some information on the main sites of wax synthesis in the tick and the factors affecting it. In insects, incorporation of labelled acetate into cuticular waxes occurs readily *in vivo*, particularly into the hydrocarbon fraction (Jackson and Baker, 1970; Lambremont *et al.*, 1966; Nelson, 1969; Piek, 1964). This is thought to occur via the elongation-decarboxylation pathway, in which fatty acids are joined together and their carboxyl groups are removed to form alkanes. The incorporation of labelled acetate into egg-wax hydrocarbons and non-polar lipids by Gené's organ was therefore studied in *B. microplus*, both *in vivo* and *in vitro*.

There is increasing evidence of the importance of endocrine and neuroendocrine mechanisms in the reproductive processes of ticks. The roles of neurosecretions, juvenile hormones (JH) and ecdysteroid hormones in insects have been extensively studied (Highnam and Hill, 1977; Handler and Postlethwait, 1978). The juvenile hormones are

involved in the control of reproduction of insects, and are responsible for stimulating and regulating vitellogenesis and ovarian development in the female (for review see Novak, 1974). The role of JH was elucidated by a varietyof ligation, extirpation, and transplantation studies, and also by the deranging effects of exogenous JH and JH analogues on metamorphosis.

The JH's and JH analogues were initially thought to have some excellent possibilities as control agents for arthropod pests, since JH regulates processes which have no endocrinological counterparts in mammals and would thus be of low toxicity to mammals (Bowers et al., 1976). The juvenile hormones prevent insects from maturing and thus must be absent from the last stages of insect metamorphosis for adult development to occur. If JH or one of its analogues is applied to an insect at a time when it should naturally be absent, adult morphogenesis is deranged, resulting in insects with a mosaic of juvenile and adult characters which are unable to feed, mate or reproduce and which soon die (Bowers et al., 1976). The main disadvantage in the potential use of JH as a pesticide is that there is only a short duration of the development period, the metamorphosis from immature to adult, when an excess of JH can upset development. Since field populations contain mixed developmental stages, the use of JH analogue itself is not usually sufficiently effective to provide acceptable control. The immature and adult stages are not controlled by excess JH and are able to continue the life cycle, causing crop damage or disease transmission. Despite this, a highly active JH analogue, methoprene, has been used effectively to control mosquitoes and flies, which are killed as pupae, preventing the emergence of the adults which cause the damage and disease transmission.

JH is a sesquiterpenoid based on farnesol (Fig. 1.4a) and is synthesised and secreted in insects by the corpora allata. Although the latter are quiescent during the ultimate stages of metamorphosis, they reawaken in the adult stage and again secrete JH's, which are necessary for ovarian development (Wigglesworth, 1936). If the corpora allata are removed surgically, the ovaries fail to develop and the insect is sterile (Chen et al., 1962).

Diapause is a condition of arrested activity during which arthropods do not feed, mate or reproduce. The diapause state allows many arthropods to survive periods of climatic stress, such as winter and drought.

Fig. 1.4A: The juvenile hormones, based on farnesol:

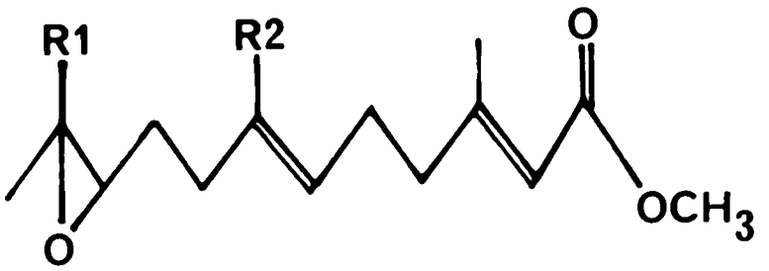
	R1	R2	
JH I	CH_2CH_3	CH_2CH_3	
JHII	CH_3	CH_2CH_3	
JHIII	CH_3	CH_3	Methyl 10,11 epoxy farnesoate

The juvenile hormone mimics:

	R3	R4
ZR 515, 'Altosid'	OCH_3	$OCH(CH_3)_2$
ZR 615	H	$NHCH_2CH_3$

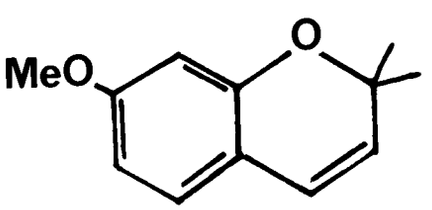
B: The precocenes, compounds with anti-allatal activity (Bowers et al., 1976).

A

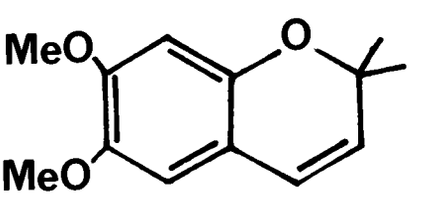


Farnesol

B



Precocene 1



Precocene 2

In certain insects that diapause as larvae, a continuing titre of JH is necessary (Chippendale and Yin, 1973) whereas a lack of JH causes diapause in many adult insects (de Wilde and De Boer, 1961; Bowers and Blickenstaff, 1966). The JH's are therefore able to induce diapause by their presence or absence during different stages. The anti-allatal or anti-juvenile hormones, the Precocenes (Fig. 1.4b), isolated from plants, have shown promise as potential pesticides and proven useful tools in the study of arthropod developmental hormones (Bowers et al., 1976). Precocene treatment in heteropteran insects causes precocious metamorphosis by removing the source of endogenous JH. For example in the cotton stainer, Dysdercus cingulatus, precocene treatment causes sterility by the inhibition of yolk deposition and resorption of basal oocytes (Bowers, 1976; Bowers et al., 1976). Certain insects which diapause through lack of JH are also artificially thrust into diapause. Removal of the corpora allata (allatectomy) has an equivalent effect, and the effects are reversable by treatment with exogenous JH, indicating that Precocene acts by reducing the titre of JH or by stopping JH production. The anti-juvenile hormone properties of precocene have been attributed to a selective toxic effect causing degeneration and atrophy of the corpus allatum, which is visible ultrastructurally in treated insects (Unnithan et al., 1977). The cytotoxicity of precocene in the corpus allatum appears to be due to its metabolism into a reactive epoxide by an enzyme involved in JH synthesis, methyl farnesoate epoxidase, which is present in the corpus allatum (Pratt et al., 1980). The resulting epoxide, an alkylating agent, causes selective cell death in the corpus allatum by forming covalent attachments to cellular macromolecules.

Studies on the involvement of JH in ticks have been mainly concerned with the argasids (Diehl et al., 1982). Part of the aims of the present work were to gain information on a possible involvement of JH in the control of wax production by Gené's organ and in ovarian development of an ixodid species, by using precocene treatment and electron microscopy.

In the argasid Ornithodorus moubata, Aeschlimann (1968) demonstrated that a homogenate of the central nervous system (synganglion) could induce oogenesis and oviposition in unfed virgin females. Ovarian development is also blocked when the central nervous system is ligated from the rest of the body in female A. arboreus (Shanbaky and Khalil, 1975). Haemolymph or synganglion extracts taken from fed-mated females, when injected into the isolated rear body portions, caused the same degree of oocyte development as in the fed-mated controls. Induction of

diapause in this species is also stimulated by a factor released by the synganglion in response to a short-day photoperiod (Khalil and Shanbaky, 1976). This diapause in fed, mated females can be broken by treatment with a JH analogue (Bassal and Roshdy, 1974), providing evidence that the gonadotrophic hormone of ticks is similar to insect JH. Precocene 2 has been found to prevent ovarian development and vitellogenesis in the female argasid Ornithodorus parkeri (Pound and Oliver, 1979) and this blockage was relieved by juvenile hormone application. Juvenile hormone treatment also induces oogenesis in Ornithodorus spp. in the absence of mating (Walton, 1979). Leahy and Booth (1980) demonstrated that precocene caused sterility in the ixodid R. sanguineus and argasid Argas persicus, but JH treatment failed to reverse this at the doses used, and it is not clear from their study whether the sterility was caused by regression of the ovaries or by egg desiccation. These data suggest that ticks may use a hormone similar to JH, and that the counterpart of the corpus allatum is situated in the synganglion and is sensitive to precocene cytotoxicity. The precocene experiment carried out in this study on B. microplus was an attempt to clarify the situation in the ixodidae, and Gené's organ was examined as a possible site for precocene cytotoxicity.

Anatomical studies have so far failed to identify glandular organs that may be responsible for producing JH or ecdysteroid hormones in ticks. The endocrine control centres have been identified in insects, where the moulting hormone (MH), one of the ecdysteroid hormones, induces a larval moult in the presence of high juvenile hormone titre. A pupal moult is induced by MH when the JH titre is low and the adult moult is induced by MH without JH. The neuroendocrine system of these insects (eg. Diptera, Orthoptera) consists of neurosecretory cell centres in the brain with principal axon release sites in the corpora cardiaca, the adjacent corpora allata which synthesise JH and the prothoracic glands which release ecdysone. The anatomy of these structures has permitted ligation, extirpation and re-implantation experiments which have confirmed their roles in the endocrine control of insects. The fusion of the tick central nervous system into the synganglion has made the identification of the endocrine centres in ticks difficult. Ultrastructural evidence has implicated the retrocerebral organ complex in the perineurium of the synganglion as a neurohaemal organ in B. microplus (Binnington, 1983) and no evidence was found of a discrete peripheral neurohaemal organ such as the corpus cardiacum of insects. The lateral organ of B. microplus is rich in smooth endoplasmic reticulum

and seems to be a true endocrine gland, and is possibly analogous to the corpora allata of insects (Binnington, 1981).

As in insects, the moulting hormones in ticks are ecdysteroids and they are also involved in some aspects of reproduction (Solomon et al., 1982). Cox (1960) showed that moulting in ticks was caused by a circulating hormone in the haemolymph by ligation experiments. Transplantation experiments showed that the production of moulting hormone occurred mainly by tissues in the anterior, and was initiated by the synganglion. Treatment with exogenous ecdysteroids in ticks causes "supermoulting" in which the adult stage undergoes an extra moult (Mango et al., 1976). Super moulting was first demonstrated by Wigglesworth (1954) by implantation of nymphal prothoracic glands into adult Cimex or Rhodnius bugs, or by parabiosis with moulting nymphae. Oogenesis in the argasid tick O. moubata begins normally in ecdysteroid fed-mated females, but the developing oocytes are resorbed prior to a peak in ecdysteroid titre which precedes moulting (Mango, 1979). In ixodids, exogenous ecdysteroids also break larval diapause (Wright, 1969). It was found that in argasid ticks, exogenous JH and ecdysone have a competitive effect. When ecdysone fed-mated females were treated with low doses of JH III, supermoulting was induced and the ticks failed to oviposit. If high doses of JH III were used, normal oogenesis and oviposition occurred and there was no supermoulting (Obenchain and Mango, 1980). It appears that ecdysone stimulates the utilization of nutrients in producing a new cuticle before apolysis of the old cuticle, and inhibits the assimilation of nutrients into lipovitellin synthesis, preventing oviposition. Exogenous JH III however inhibits the moulting process and stimulates oogenesis. This behaviour suggests the involvement of ecdysones in the tick's endogenous control of moulting, and the effects of exogenous ecdysones in preventing oviposition may have implications for tick control.

There have been several studies which showed that JH mimics or analogues such as altosid (Fig. 1.4) have toxic effects on ticks, in particular an ovicidal activity not caused by naturally occurring JH's (Solomon et al., 1982). Bassal (1974) showed that treatment of eggs with certain JH mimics caused post-hatch larval mortality in the eggs of Hyalomma dromedarii. Treatment of female B. decoloratus with JH mimics on the day of engorgement resulted in the desiccation of eggs 5 days after the start of oviposition (Solomon and Evans, 1977). Much of the ovicidal activity was lost when desiccation was prevented by incubating

the eggs under paraffin oil (Solomon et al., 1982). Even when several JH mimics were incorporated into the paraffin oil incubation medium, embryogenesis proceeded normally and egg hatching was not significantly lower than that observed when untreated eggs were incubated in pure paraffin oil. Treatment with the JH-mimics seemed to have no effect on the maturation of Gené's organ or its associated glands, but may have caused a change in the egg wax, or stopped its production. An unusual component detected in the UV spectrum of egg wax from ticks treated with the JH-mimic ZR 515 may have been the cause of the ovicidal effects (Solomon et al., 1982). It was concluded that the ovicidal effects of the JH-mimics were probably due to disruption of normal egg wax metabolism rather than stimulation of normal tick endocrine mechanisms.

There is little direct evidence for the role of neural mechanisms in the regulation of reproduction in ticks. It seems obvious that the co-ordination of motor-activities are necessary during oviposition, particularly with the precise manipulation of the eggs by Gené's organ. The nervous system is almost certainly involved in the regulation of the peristaltic contractions of the ovary and oviducts. The pharmacology of oviposition in B. microplus and the electrophysiology of the Gené's organ retractor muscles in A. variegatum were therefore studied in an attempt to gain more information about the innervation of Gené's organ and the oviduct, and the possible neurotransmitters involved. Very little is known about the chemical basis of neurotransmission in the tick nervous system which might be exploitable in the search for new control agents. Most of the chemical control agents now available appear to exert their action by disruption of the nervous system. The pyrethroids were found to disrupt the cockroach nervous system by Lowenstein (1942) and it is now known that synthetic pyrethroids and DDT interfere with nerve-muscle transmission (Narahashi, 1976). The pyrethroids cause depolarisation of nerve cells by altering the gating properties of the voltage-dependent ion channels (Narahashi, 1981; Leake et al., 1980). The organophosphates act by inhibiting the enzyme acetylcholinesterase in insects. This enzyme breaks down the synaptic transmitter, acetylcholine (Metcalf and March, 1949). Its inhibition causes the accumulation of neurotransmitter, prolonging post-synaptic depolarisation, and causes bursts of potentials and neuronal block (Twarog and Roeder, 1957). The mechanisms of action of these compounds have only been discovered after their development as pesticides; however, the exploration of unexploited target sites in the nervous system raises hopes that new specific neurotoxic control agents will be able to be designed. The recently developed

formamidine pesticides, such as chlordimeform, and amitraz (Fig. 1.5) have a more restricted spectrum of activity than the pyrethroids and organophosphates in that they effectively control ticks, but only a few insect pests. The mode of action of the formamidines seems to be based on their action on the octopamine receptor, for which they are an agonist (Hollingworth and Murdoch, 1980; Nathanson and Hunnicutt, 1981). The formamidine chlordimeform has been shown to act as an agonist of octopamine at the locust neuromuscular junction (Evans and Gee, 1980), and metabolism of amitraz in the tick yields a chlordimeform-like compound which is an octopamine analogue (Fig. 1.5).

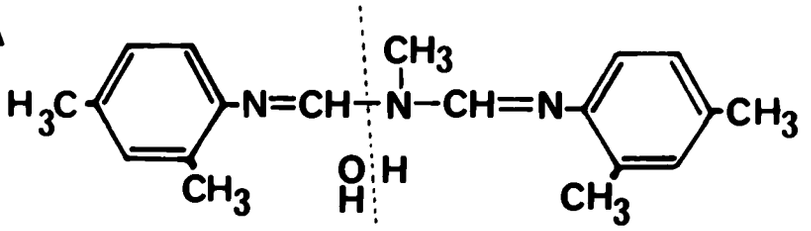
In insects octopamine is involved as a neuro-modulatory transmitter or local neurohormone, modulating the effectiveness of neuromuscular transmission (Evans and O'Shea, 1977, 1978; O'Shea and Evans, 1979; Buchan and Evans, 1980). Octopamine has been shown to act as a circulatory hormone in the crustacea (Evans et al., 1976a,b) and in insects (Candy, 1978; Goosey and Candy, 1980). Octopamine has also been identified as the probable transmitter involved in the production of light from the firefly lantern (Nathanson, 1979; Copeland and Robertson, 1981). Octopamine stimulates the release of lipid from the locust fat body (Orchard et al., 1982; Orchard and Lange, 1983) and acts as a neurotransmitter mediating hormone release in locusts (Orchard and Loughton, 1981). The effects of octopamine in insects are generally those which would be expected if this amine were mediating a similar response to that of adrenaline-noradrenaline in vertebrates.

Octopamine receptors on the locust extensor tibiae muscle mediate a myogenic rhythm in a specialized bundle of muscle fibres, and control the twitch tension and relaxation rate of the extensor tibia muscle. These functions are important in maintaining the insect's posture and walking ability (Evans, 1981). Both chlordimeform and octopamine also excite neurones in the abdominal ganglion of the tobacco hornworm, Manduca sexta (Lund et al., 1979). The sub-lethal effects of chlordimeform are increased motor activity and unco-ordination, which disrupts feeding and mating behaviour. The action of the formamidines on the octopamine receptor may thus be the basis of their effectiveness in controlling certain pests by disrupting normal behaviour (Evans and Gee, 1980).

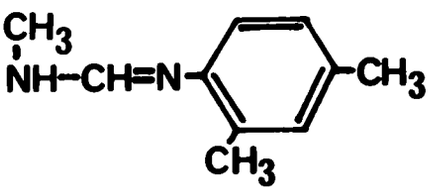
The control of oviposition in the locust is octopaminergic, the myogenic contractions of the oviduct being inhibited by release of octopamine from the oviducal nerve terminals (Lange et al., 1984). In

Fig. 1.5 The structure of formamidines and octopamine: A: amitraz, which is split into two in the tick to form a toxic formamidine B. C; Chlordimeform; D: Octopamine.

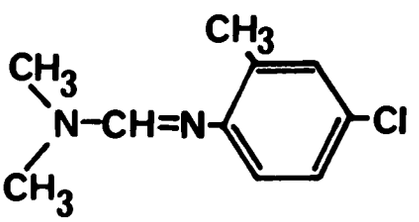
A



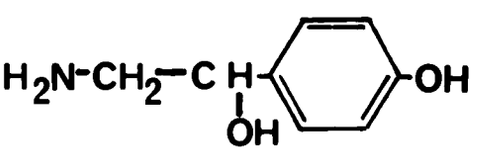
B



C



D



ticks, the presence of catecholamines have been demonstrated (Binnington and Stone, 1977; Megaw, 1977). The general effects of formamidines in ticks include the induction of detachment from the host and significantly, a reduction of oviposition and egg viability (Knowles and Roulston, 1973). One formamidine also increases the spike frequency of a coxal nerve in B. microplus (Binnington and Rice, 1977). A range of catecholaminergic agonists and antagonists were therefore tested on engorged female B. microplus, and compared with the effects of the formamidine amitraz, to investigate the possible involvement of biogenic amines in the control of oviposition in ticks.

The wide range of putative neurotransmitters identified from mammalian studies, including acetylcholine and the amino acids, also occur in the insect nervous system (Leake and Walker, 1980). Evidence for acetylcholine in ticks comes from demonstration of acetylcholinesterase in the central nervous system (Lee and Batham, 1966; Roulston and Wharton, 1967), and the effects of cholinergic agonists such as pilocarpine (Tatchell, 1967). The only evidence for the presence of amino-acid transmitters comes from one study of the tick tibia neuromuscular junction, which showed that L-glutamate is the probable excitatory transmitter (Hart, 1982).

The neuronal terminal at a neuromuscular junction transmits signals from the axon to the muscle cell by releasing a chemical transmitter, which diffuses across the synaptic cleft between nerve and muscle cell membranes. The transmitter substance interacts with the postsynaptic receptor - ionophore complex and thereby opens, transiently, ionophores in the post-synaptic membrane. This results in a measurable current flow across the membrane caused by the electrochemical potential generated by the varying intra- and extra-cellular ionic concentrations. Depending on which ion is moving, and its charge, the current flow can be measured either as a depolarization or a hyper-polarization. The depolarization excites the muscle and activates the contraction mechanism, wheras hyperpolarizing signals reduce the level of depolarization and inhibit contraction (Katz, 1966). Studies on arthropod neuromuscular junctions, using crustacea and insects, show that the skeletal muscles are usually innervated by a mixture of axon types, each axon forming multisynaptic connections with the muscle. There are three main classes of axon type: fast (phasic) axons causing rapid depolarization of the muscle, slow excitators causing tonic contraction and inhibitory axons causing a reduction in tension or amplitude of contraction. The

extensor tibia muscle of the metathoracic leg of the locust has been extensively studied and appears to have a fourth type of axon with a modulatory effect on muscle tone (Hoyle, 1978).

There have been few studies of chelicerate somatic muscle innervation. Multiaxonal and multi-terminal excitatory innervation has been demonstrated in the claw closer muscle of the king crab, Limulus polyphemus, (Hoyle, 1958), and in the pedipalp closer muscle of the scorpion (Gilai and Parnas, 1970). In the leg muscle of the tick Amblyomma maculatum (Chow and Lin, 1972), both depolarizing and hyperpolarizing potentials were recorded. The depolarizing potentials were "fast", while the hyperpolarizing potentials were "slow", lasting up to 100 msec. Hart (1982) recorded both fast and slow depolarizations in the leg muscle of Amblyomma variegatum.

The ultrastructure and position of motor nerve terminals in insects has been reviewed by Osborne (1970; 1975). Each terminal is usually about 15-20 μm in diameter and contains electron lucent vesicles, of diameter 20-60 nm and roughly spherical. There are often larger vesicles in addition to the smaller ones, of 50-100 nm in diameter, which have electron dense cores. All the terminals at neuromuscular junctions are separated from the muscle by a narrow synaptic cleft, but the positions of contact vary, even in different muscles of the same insect (Neal, 1975).

Studies on the role of amino acids in nerve-muscle transmission in insects, using mainly the locust leg muscles and abdominal muscles of dipteran larvae, have identified L-glutamic acid as the excitatory transmitter (Usherwood, 1981). There is some evidence that L-aspartic acid may also be involved at some neuromuscular junctions (Irving and Miller, 1980).

Synapses using amino-acids such as L-glutamic acid or 4-aminobutyric acid as the neurotransmitter could provide a new target site for arthropod control agents. For example, the glutamic acid agonist quisqualic acid is effective in controlling helminth worms, which also use glutamate as the excitatory transmitter at the neuromuscular junction. Avermectin, another pesticide used on mites and fleas is a 4-aminobutyric acid agonist, and also has some effect on ticks (Nolan, 1981). The widespread occurrence of L-glutamic acid as a neurotransmitter responsible for excitatory motor activity at the insect neuromuscular junction and its absence from motor terminals in mammals could be an advantage in

designing pesticides with a low mammalian toxicity.

Although ultrastructural and electrophysiological studies of insect and crustacean muscles are common there have been very few such investigations of acarine muscle. The fine structure of skeletal muscle in Boophilus decoloratus (Beadle, 1973) and the structure of neuromuscular junctions in the leg muscles of Amblyomma variegatum (Hart et al., 1980) have been described and an attempt to identify the neurotransmitters at these junctions has been carried out (Hart et al., 1982; 1984). As in insects and crustacea L-glutamate is the likely transmitter at this site. The structure of tick muscle showed the striations, filament arrangement and T-system typical of arthropod muscle (Hart, 1982). Muscle attachment in the tick resembles the muscle insertions in insects and spider cuticle more closely than those of the mites with which ticks are commonly compared (Beadle, 1973). The present study attempts to extend our knowledge of the musculature of the retractor muscles involved in the control of Gené's organ in ticks, and as a comparison with other arthropods. The horns of Gené's organ are controlled by the retractor muscles, which appear to act against hydrostatic pressure since there are no directly acting muscles capable of causing eversion (Arthur, 1953; Booth et al., 1984), although muscles controlling the raising and lowering of the capitulum may play a part. Besides describing the ultrastructure of the retractor muscles, their innervation and insertions, this study provides further evidence for L-glutamate and L-aspartate as putative excitatory transmitters at tick neuromuscular junctions.

2. MATERIALS AND METHODS

2.1 Microscopy

This section describes the techniques used for the preparation of tick specimens for scanning and transmission electron microscopy and light microscopy in an examination of the ultrastructure and histochemistry of Gené's organ and its associated glands and musculature.

Gené's organ was examined in newly-dropped, fully engorged female Boophilus microplus Canestrini (Paquera strain) originally obtained from South America, and now maintained as a culture at the Wellcome Research Laboratories, Berkhamsted. The ticks were obtained for study on either the day of dropping or the second day after dropping, and their individual stages of development were well synchronised. The structure of Gené's organ was also examined in partially engorged and unengorged female B. microplus, and in unengorged adult females of the sheep tick Ixodes ricinus and the tropical bont tick Amblyomma variegatum (Fabricus) as a comparison to ensure that B. microplus was not atypical of the ixodidae. A. variegatum were originally obtained from Kenya and and are also maintained as a culture, and I. ricinus were obtained from the Ministry of Agriculture.

Oviposition was observed in newly dropped engorged B. microplus, which were maintained in petri dishes on moist filter paper at 30°C. Oviposition began within two days under these conditions. The oviposition process was easily disturbed, and was only observable under dim lighting conditions with a Nikon binocular microscope in ticks kept in warm moist conditions by taping them to the side of a small plastic container immersed in a water bath at 30°C in a dark room. The horns of Gené's organ became visible about a day before onset of oviposition, and were everted and retracted spontaneously but could also be everted by depressing the mouthparts with a pair of fine forceps, and applying gentle pressure to the body of the tick. Gené's organ was dissected and fixed from egg-laying females in both the retracted and everted position prior to and at different stages of oviposition. A circular cut was made in the body wall cuticle around the mouthparts and genitalia, including the first pair of legs using microscissors. The resulting piece of cuticle containing Gené's organ, the accessory glands, mouthparts and oviduct was then washed in 0.1 M phosphate buffer containing 0.3 M sucrose, to remove the gut contents. The tissue was

then either fixed and embedded for sectioning whole, or groups of accessory glands, and the secretory lobes of Gené's organ, observed under a binocular microscope were removed and embedded separately, as it was found that the thick cuticle caused problems with sectioning. In order to obtain specimens fixed with the horns in the everted position, they were artifically everted and held in position by taping the tick between two glass microscope slides. The surface of the horns were de-waxed with a brief wash in chloroform, and the tick was then immersed in glutaraldehyde fixative. Fixative was also injected under the scutum. After a few minutes the horns were fixed sufficiently to remain in their natural positions when the tick was dissected, and the anterior regions were then replaced in fixative, and processed for electron microscopy.

2.1.1 Transmission electron microscopy

For transmission electron microscopy (TEM) tissues were fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde (Karnovsky, 1965) in Millonig's 0.1M phosphate buffer (Millonig, 1961) pH 7.2 containing 0.3M sucrose, for 1 hour. The osmotic pressure of all solutions used in fixation were adjusted to approximate to that of tick haemolymph with the addition of sucrose to give 420 mOs/kg (Hart, 1982). After a wash in sucrose-containing phosphate buffer, post-fixing in 2% osmium tetroxide in the same buffer for 2-3 hours was followed by another wash in buffer and then *en bloc* staining with uranyl acetate in 70% ethanol during dehydration in an ethanol series. All dissections, fixations and dehydrations were carried out at room temperature. 2.5% glutaraldehyde fixation without the paraformaldehyde or *en bloc* uranyl acetate was also used and found to give less dense staining of the specimens with more detail but less contrast. The tissues were embedded in the epoxy resin, araldite (Durcupan, Emscope Ltd.). The ethanol in the tissue was replaced with a resin miscible solvent, propylene oxide, to which increasing amounts of resin were added gradually during agitation in a Taab specimen rotator.

The remaining solvent was allowed to evaporate overnight and the tissue was embedded in fresh resin, moulded into blocks and cured at 60°C for 48 hours.

Ultrathin sections of 50-80 nm were cut on an LKB Ultrotome III, collected on copper grids and stained with lead citrate (Reynolds, 1963), and uranyl acetate, using a grid stainer capable of staining up

to eight specimen grids simultaneously (Forsdyke, 1979). This reduced the amount of stain needed, ensured that each grid received the same reproducable treatment and minimised the risk of section damage or contamination. Formvar plastic films were used on grids for support of particularly fragile sections, but were avoided if possible due to the increased contamination and reduced contrast. Sections were observed in a Jeol JEM 100S electron microscope operated at 60-80 KV for the production of micrographs.

2.1.2 Light microscopy

For light microscopy, 0.5 - 1.0 µm thick sections of resin embedded specimens were stained with toluidine blue or methylene blue-basic fuchsin (Aparicio and Marsden, 1969). The periodic acid-Schiff (PAS) method, using methylene blue as a counter-stain, was used to demonstrate carbohydrates. Sections were mounted in canada balsam on glass slides and observed in a Zeiss light microscope fitted with an Olympus 35mm reflex camera with automatic exposure for taking colour slides or black and white negative photomicrographs. Whole mounts of cells were also photographed using phase-contrast microscopy with transmitted light, or by reflected light, with this set-up.

Some specimens of engorged females were prepared for light microscopy using frozen sections made with a cryostat-mounted microtome. Wholebody sections of engorged B. microplus were possible with these techniques. Ticks were rapidly frozen by immersing them in hexane cooledwith liquid nitrogen to just above melting point (- 95°C) Rapid freezing minimized ice-crystal damage to the tissues. The anterior region of the tick was removed and transferred with a cold scalpel to a Bright Instruments cryostat-mounted rotary retracting microtome at -25°C. The frozen tissue was mounted on the pre-cooled specimen holder using a solution of carboxymethylcellulose polymer as an embedding agent. This was frozen rapidly using arcton cooling spray. Ribbons of 4-6 µm thick sections were transferred to unsubbed glass slides, and allowed to freeze-dry in the cryostat. Slides were then stained with haematoxylin after fixation with formol-calcium solution. Oil red-O and sudan black B stains, to demonstrate lipids, and the PAS stain, were used.

2.1.3 Scanning electron microscopy

For scanning electron microscopy (SEM) specimens at different stages of oviposition with the horns of Gene's organ in everted or retracted positions were surface washed with chloroform to remove superficial wax and debris from the cuticle and rapidly frozen with arcton freezing aerosol. The anterior region of the tick was then cut off with a cold scalpel and fixed in acetone at -25°C for 10 days. After several changes of fresh 100% acetone to ensure dehydration the specimens were removed and the acetone was evaporated in a vacuum chamber at 21°C. Specimens were mounted on stubs using silver dag, sputter coated with carbon and gold, and examined in a Philips 35S microscope. It was found that this treatment preserved the horns of Gene's organ in their natural position. Specimens of eggs laid by precocene-treated ticks, and with the horns of Gene's organ blocked by araldite, were also observed in an ISI SX-30 scanning electron microscope and compared with normally oviposited eggs. It was found that fixation, dehydration and sputter coating of the eggs with a conducting layer was not necessary to obtain a good image. The egg specimens were mounted untreated on aluminium stubs with silver dag. Micrographs were taken within a few minutes of placing the specimens in the microscope, since the vacuum caused gradual collapse of the shells of normally-laid eggs.

The cuticular covering of the horns was stained with ammoniacal silver nitrate after removal of surface waxes with chloroform to demonstrate the non-sclerotized areas lacking a cement layer.

2.1.4 Buffers, reagents and staining solutions

Millonig's Phosphate buffer pH 7.4

Sol. 1 KH_2PO_4 2.74g in 100 ml water.
Sol. 2 NaOH 0.08g in 100 ml water.

Mix 50 ml Sol. 1 plus 30 ml Sol. 2 and dilute to 360 ml with water

Karnovsky's fixative. 2.0g paraformaldehyde; dissolve in 20 ml water, heat at 60-65°C, in a fume cupboard, add a few drops of 1 M NaOH, then add 50 ml phosphate buffer, 10 ml 25% glutaraldehyde and 20 ml water. Add sucrose to make 420 mOs/kg (approx 10g/l).

Osmium tetroxide. 1g dissolved in 50 ml phosphate buffer, add sucrose 0.5g.

Formol Calcium. Dissolve 10ml formaldehyde, 7.5g sucrose, 1g $CaCl_2$ in 50ml 0.1M cacodylate buffer pH 7.4, add 40ml water. Fix cryostat slides for 15 min.

Stains for light microscopy with resin embedded sections

1. Toluidine blue 1% in water with 1% borax. Stain slides for 2 min at 60°C, wash in water, mount in canada balsam (non-permanent in xylene based mountants).

2. Aparicio and Marsden stain

   Sol. 1 Methylene blue 0.1g in 10ml 1% borax.

   Sol. 2 Basic fuchsin 0.2g in 10ml water.

   Stain 2 to 3 min. at 70°C with Sol. 1. Wash in hot tap water, when cool, stain 2 to 5 min. at 21°C with Sol. 2. Wash in water, mount in canada-balsam.

3. Periodic acid-Schiff stain. Treat slides in 1% HIO_4 10 min, rinse in tap water, stain in Schiff's reagent 20 min, then rinse in tap water. Counter stain in methylene blue 2 min. Carbohydrates stain pink.

Stains for ultrathin sections

Sol. 1. Uranyl acetate, 30% in ethanol, stain for 2 to 15 min at 20°C, then wash in water

Sol. 2. Reynolds stain: lead nitrate 1.3g, sodium citrate 1.76g in 30ml water (shake the mixture and let stand). Stain 2 to 3 min, then wash in water.

Stains for frozen sections

Oil Red O, saturated in 70% propanol. Stain 10 min, wash in water, counter stain in haematoxylin 2-3 min, wash in tap water, blue in Scott's solution 3 mins.

Fats stain orange/red, nuclei stain blue.

Scott's Solution. $NaHCO_3$ 2.0g, $Mg(SO_4)_2$ 20g, water 100 ml.

Sudan Black B, saturated in 70% ethanol, stain slides 30 min, rinse in 70% ethanol, counterstain with eosin (30 sec) and haematoxylin (10 min). Wash in tap water. Lipids - black.

Ammoniacal silver nitrate 2ml 28% Ammonia, add 35 ml 5% silver nitrate.

Araldite resin Durcupan Resin ACM.A - 10 ml.
Hardener 964B - 10 ml
Plasticiser D - 0.5 ml
Accelerator 964C - 0.4 ml

All components were mixed together before adding accelerator. Air was removed by placing in a vacuum for 5 min.

TEM processing schedule for tissue specimens

1. Fixation in paraformaldehyde - glutaraldehyde solution 1-2h, 21°C.
2. Rinse in buffer 20 min at 21°C or overnight at 4°C.
3. Fixation in osmium tetroxide, 2-3h, 21°C.
4. Rinse in buffer 20 min.
5. Dehydration in 50% ethanol, 10 min.
6. Stain in uranylacetate in 70% ethanol, 10 min.
7. Dehydrate in 90% ethanol (10 min) and three changes of 100% ethanol (10 min each)
8. Propylene oxide, 10 min.
9. Propylene oxide: resin (1:1) 2h in specimen rotator.
10. Propylene oxide: resin (1:3) 2h in specimen rotator.
11. 100% resin overnight (allowing propylene oxide to evaporate).
12. Embed tissue in fresh resin at 60°C 48h.

2.2 Egg wax lipids

Lipids were extracted from Gene's organ and its associated glands that had been dissected from ovipositing ticks and also from the superficial wax of the eggs by the method of Bligh and Dyer (1959). The main lipid classes were separated by thin-layer chromatography (TLC) and gas-liquid chromatography (GLC).

2.2.1 Extraction of wax lipids

Ten ovipositing B. microplus were dissected and the secretory glands of Gene's organ were removed and homogenised in chloroform: methanol (2:1, 4 ml). A sample of eggs (0.8g) were surface washed in the same extraction medium for 10 min. The homogeniser tube and pestle were rinsed in a further 4 ml of Chloroform:methanol and the washings transferred. Water (1.6 ml) was added to the extracts and then vigorously shaken and centrifuged to give a biphasic system. The lower phase was removed and the upper aqueous methanol phase was re-extracted with chloroform (4 ml). The chloroform phases were combined and evaporated to dryness under nitrogen.

2.2.2 Separation of alkanes from fatty acids

The lipid extracts were heated for 15 minutes in a dry block at 65°C with 1.0 ml of methanolic sodium hydroxide (0.5M). This resulted in hydrolysis of esters and dissolution of the fatty acids as sodium salts. The alkane fraction was separated from the fatty acid fraction by adding 1ml of hexane to the vials, forming a biphasic system. The vials were shaken and then the two phases were allowed to separate. The hexane phase contained the non-polar alkane fractions, with the fatty acid fraction remaining in the more polar methanolic phase. The hexane phase was then removed and reduced to a small volume for analysis of the alkanes by GLC. The methanolic fractions containing fatty acids were heated for a further 25 minutes at 65°C after addition of 1.25 ml boron trifluoride/methanol reagent (Sigma) in order to methylate the fatty acids thus forming their respective methyl esters allowing their analysis by GLC. Tubes were then cooled and saturated sodium chloride (4 ml) was added. The methyl esters were then extracted by shaking with three (5 ml) portions of petroleum ether. The combined ether extracts were dried over anhydrous sodium sulphate and then taken to a small volume under nitrogen. All four samples: Gené's organ alkane fraction, fatty acid fraction, and the egg wax alkane and fatty

acid fractions were then dissolved in 1 ml hexane for injection onto the GLC column. GLC analysis was carried out using a 3m glass column packed with 10% Sp-2330 on 100/120 Chromosorb W. (Supelco Inc.) as a stationary phase, in a Hewlett-Packard 5730A gas chromatograph, with a hydrogen flame ionisation detector. Helium was used as the moving gas phase at 240 ml/min. The column was temperature programmed from 100-300°C at 8°C/min. The lipids extracted from Gené's organ were compared qualitatively with those of the egg wax, and identified by comparison with the retention times and chain lengths of C19-C30 n-alkane standards and C16-C22 fatty acid (methylester) standards. Peaks were identified using log-linear plots of retention time against chain length for the standards. Peak areas and retention times were determined using a Supergrator integrator system, in order to compare the relative abundance of each component in the different samples. All solvents used were re-distilled grade of purity.

2.2.3 Wax lipid synthesis in Gené's organ

The synthesis of lipids by Gené's organ was studied by following the incorporation of ^{14}C-labelled acetate into wax lipids *in vivo* and *in vitro*. For *in vivo* studies sodium ^{14}C-1-acetate (58 mCi/mmol, Amersham) was injected into ovipositing *B. microplus* adjacent to the scutum (0.02 - 0.16 µCi per tick). The ticks were then attached to the base of petri dishes by their dorsal surfaces using double-sided adhesive tape, and left in the incubator at 30°C for 24h for oviposition. The oviposited eggs were then weighed and the surface wax lipids were extracted by washing for 2 min. in 0.5 ml chloroform:methanol (2:1 v/v). Egg washings were then reduced to a small volume under N_2 and samples were placed in scintillation vials (β-vials) in 'instagel' scintillant (Packard) for determination of β-particle counts per minute in a Nuclear Chicago liquid scintillation counter. Lipids were separated using thin layer chromatography (TLC) on silica gel plates (Kieselgel 60, Merck) developed in hexane or hexane:ether:acetic acid (80:20:1 v/v). The wax extracts were crystallised under N_2 and redissolved in a small volume of hexane for spotting on to TLC plates using micropipettes. Spots on plates were visualized by exposure to I_2 vapour for 10 min, and spots were scraped off into scintillation vials to determine radio-activity. Samples of tissue from Gené's organ, the accessory glands and lobular glands dissected from ^{14}C-1-acetate injected ticks were also extracted for determination and identification of labelled lipids. Spots were identified by comparison with n-alkane and fatty acid

standards run under identical conditions. The residual activity in the eggs after removal of surface wax was also determined.

For *in vitro* studies, ovipositing *B. microplus* were dissected in Shneider's medium (Gibco) and isolated Gené's organs, lobular glands and accessory glands were incubated in small plastic dishes (Falcon, 3cm) containing Liebowitz L15 tissue culture medium (Gibco) with a known quantity of ^{14}C-1-acetate (0.16 µCi) added, for 1½ or 2h at 30°C or 4°C. The tissues were then blotted dry, weighed, and the radiolabelled lipids were extracted and analysed by TLC and liquid scintillation counting as described above. Incorporation of the label into the extracted lipids was expressed as counts per minute per mg fresh weight of tissue, or per mg protein as determined with a Biorad protein assay kit.

The histological sites of ^{14}C-1-acetate uptake and incorporation in the tick were determined by autoradiography of frozen sections of egg laying females injected with ^{14}C-1-acetate. Ticks were injected with 0.16 µCi of ^{14}C-1-acetate and allowed to oviposit for 24h at 30°C. The ticks were then rapidly frozen and sectioned as previously described (Section 2.1.2). Freeze-dried wholebody sections on glass slides were dipped into Ilford K5 emulsion (10g emulsion plus 10g distilled water) at 43°C under dark red safelighting and then dried vertically allowing excess emulsion to run off. The slides were exposed in a light tight box for one to three weeks at -24°C and were then developed in Kodak D19 developer (1:1 dilution with water) for 5 min at 21°C, washed for 2 min. in distilled water, and fixed for 5 min in Ilfofix (1:1 dilution). Control slides included non-labelled tissue, and labelled tissue with the emulsion fogged by exposure to white light. After a final wash the slides were stained in haematoxylin, mounted, observed and photographed as described in Section 2.1.

2.2.4 The effect of precocene on lipid synthesis *in vitro*

The effect of 0.1M precocene 2 (added as a solution dissolved in dimethyl sulphoxide (DMSO)) in the incubation medium on *in vitro* wax lipid synthesis by isolated Gené's organs from labelled acetate was determined in comparison with control (DMSO) incubated Gené's organ. Tissues were incubated at 30°C for 1½h in Liebowitz L15 medium, and lipids were extracted and quantified as described in Section 2.2.3. β-ecdysone, JH III and amitraz treatments were also tried to see if

these substances affected lipid synthesis. Control and precocene-treated Gené's organs were fixed and processed for TEM after incubation *in vitro* as described in Section 2.1.1. to determine any ultrastructural changes caused by precocene 2 treatment and the *in vitro* incubation conditions.

2.3 Pharmacological studies on oviposition

The effects of several drugs, including a range of catecholamine and phenolamine agonists and antagonists and the effects of precocene and juvenile hormone treatment on oviposition in *B. microplus* were studied.

Newly-dropped engorged female *B. microplus* were used for pharmacological studies. Drugs were injected after onset of oviposition using a Burkard microapplicator fitted with a 100 µl glass syringe and a 31 gauge needle (0.25 mm o.d., 0.13 mm i.d.) which was 6mm long. The dimensions of the needle were critical. The short length minimised the dead volume, and the diameter was small enough to avoid causing a debilitating wound but large enough to prevent blockage with tissue. Volumes of 0.2 - 0.8 µl of drug dissolved in distilled water were injected into the haemocoel adjacent to the scutum. Ticks in which the gut became accidentally ruptured were discarded. Drugs were injected at dosesof 25 and 50 µg per tick, in replicates of 4 or 6 ticks. Higher doses of up to 150µg were used in some cases. Control ticks were injected with distilled water. The ticks were then placed ventral side up on double-sided adhesive tape inside petri dishes containing moistened filter paper to maintain high humidity, and placed in an incubator at 30°C. Egg production was monitored by weighing the eggs and the effects on oviposition behaviour were observed under a binocular microscope. Ticks which ceased oviposition were dissected to observe the condition of the ovary and oviducts.

The drugs tested were the catecholamines D,L-dopamine HCl (Sigma) L-noradrenaline (L-arteronol); the catecholaminergic mimic reserpine (which depletes endogenous catecholamines); the α-adrenergic agonists D,L octopamine HCl, D,L synephrine, clonidine, tolazoline, naphazoline, and apomorphine; the β-adrenergic agonist isoprenaline; the α-adrenergic antagonists phentolamine, chlorpromazine and metachlopromide; the β-adrenergic antagonist propranolol; the acetylcholine agonists pilocarpine and arecoline; the catecholamine antagonist haloperidol; the histamine agonists 1-methyl histidine and 3-methyl histidine; the

neurohormone serotonin (5-hydroxy-tryptamine) and the formamidine-like acaricide amitraz (1,-5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene). Drugs were obtained from the Wellcome Research Laboratories and originally supplied by the Sigma Chemical Co., apart from amitraz which was supplied by Boots Ltd.

Precocene 2 (6,7-dimethoxy-2-2-dimethoxychromene) and Juvenile hormone III (10-epoxy-3,7,11-trimethyl-2,6-trans trans dodecadienoic acid, methyl ester) obtained from Sigma were applied topically in dimethylsulphoxide(DMSO) in doses of 0.5 mg/tick (precocene 2) and 5 and 10 µg per tick (JH III) on the dorsal body cuticle, and by injection at doses of 5-25 µg per tick, immediately after dropping from the host but before oviposition. Ticks were treated with combinations of precocene, JH III and β-ecdysone (5-20 µg per tick injected). Specimens of drug treated ticks were dissected, and processed for TEM to determine any ultrastructural changes in Gené's organ.

2.4 Electrophysiology

For ultrastructural studies of Gené's organ retractor muscles, unengorged adult female A. variegatum and adult female B. microplus at different stages were examined. The muscles were either dissected and embedded separately or the wholescutal region was fixed and embedded as described in Section 2.1.1.

Unengorged adult female A. variegatum were used for electrophysiological studies due to the large size and ease of identifying the muscles. Animals were dissected from the dorsal surface in tick saline (200 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, 1.5 mM $MgCl_2$, 3mM $NaHCO_3$, 0.7 mM KH_2PO_4, 370 mOs/kg, pH 6.9, Hart 1982), pinned in a 3cm wax-filled dish, and a cut was made along the posterior and lateral margins of the sclerotised scutal plate. The scutum was folded anteriorly and the gut, salivary glands and heart removed, exposing the synganglion, frontal nerves and retractor muscles (Fig. 2.1). Intracellular recordings were made using microelectrodes of 5-12MΩ resistance. The intracellular electrodes were glass micropipettes filled with 3M KCl, formed using a micro-electrode puller (Scientific and Research Instruments Ltd.) from 1.3 mm diameter glass tubing (Clarke Electromedical). The glass tubing contained a glass fibre in the lumen to facilitate filling. The reference electrode was made from a rod of silver chloride granules fused to a silver wire, and the microelectrode wire also had a coating of silver chloride. Electrodes were positioned with the aid of micromanipulators

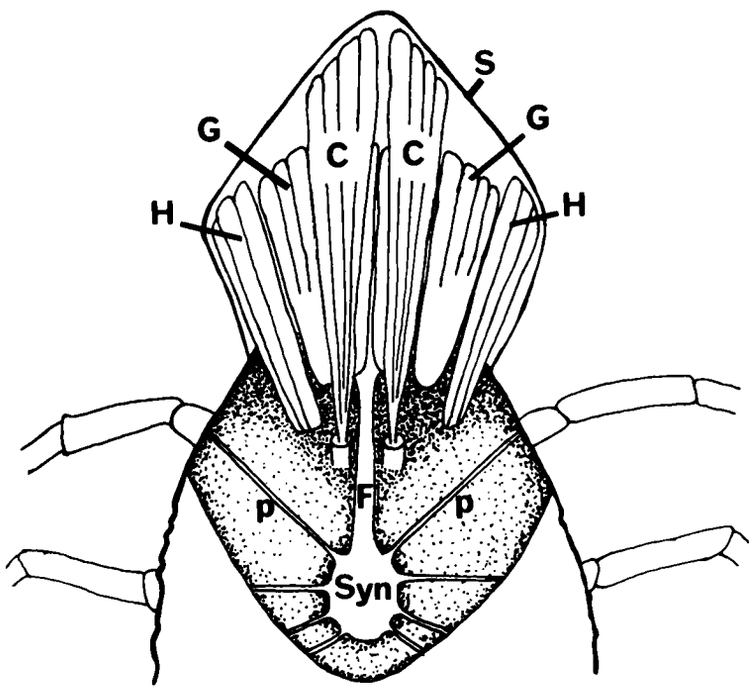


Fig. 2.1. Diagrammatic representation showing a dorsal view of an unengorged adult female Amblyomma variegatum, dissected to expose the Gené's organ retractor muscles (G) for electrophysiology. The hypostomal muscles (H) and cheliceral retractor muscles (C) also insert into the scutum (S), which has been folded anteriorly, exposing the synganglion (Syn) with the pedal nerves (p) and frontal nerves (F) extending outwards.

(Micro Instruments Ltd.) and the preparation was viewed with a Leitz dissecting microscope at a magnification up to 160x with fibre optics illumination. Membrane potentials were recorded via a high impedance probe connected to an oscilloscope (Tektronix 5111) for display. The output was also recorded onto magnetic tape (Racal Store 4) and pen recorder (Gould Brush 220). Events displayed on the oscilloscope screen could also be photographed using a polaroid camera. Resting and evoked potentials were measured with a Winston 1090 amplifier and bridge unit. The amplifier had the facilities for offsetting capacitance in the circuit and continuous checking of the electrode impedance and voltage calibration. The frequency response of the pen recorder was 100 Hz but magnetic tape records could be replayed into the pen recorder at a reduced speed to boost the response by a factor of four if required. Evoked potentials were recorded from the muscles by electrical stimulation of the frontal nerves with silver wire electrodes using 1 ms D.C. pulses of 1-3 V from a CFP 8128 stimulator which also triggered the oscilloscope sweep.

Candidate neurotransmitters were applied to the muscle by pressure ejection from a low resistance micropipette containing 0.5M solutions of sodium L-glutamate, sodium L-aspartate, acetylcholine, 4-aminobutyric acid and D,L-octopamine in tick saline, using a Picospritzer II pressure system which had previously been calibrated. The response of the muscle membrane potential to the application of neurotransmitters was recorded simultaneously. All chemicals were of Analar quality from BDH chemicals.

The effects of bath-applied neurotransmitters at concentrations of 10^{-3}M and 10^{-4}M on the spontaneous and evoked potentials recorded from the muscles was also determined.

3. RESULTS

3.1 The structure of Gene's organ and its associated glands

3.1.1 The oviposition process

In B. microplus the external portion of Gené's organ consists of a pair of balloon-like horns which have a thin, plicate, glistening cuticular surface when everted from beneath the scutum in an ovipositing tick (Fig. 3.1a). The horns emerge from between the scutum and capitulum (mouthparts) and completely cover the latter when everted.

During oviposition the capitulum is retracted close to the body wall and the horns are extended towards the vagina (Figs. 3.1b and 3.2). The vagina prolapses as the egg is produced by peristaltic contractions, forming an extended tube or ovipositor that deposits the egg between the horns (Fig. 3.1b). The egg is then manipulated by the horns becoming coated with a superficial waxy layer which spreads from the horns over the eggs, vulva and whole anterior of the tick (Fig. 3.1a). The eggs are manipulated by repeated pulsations of the horns against the newly oviposited egg which is held between the horns and the prolapsed vagina. The egg is gradually rotated around by this action, which takes up to 30 seconds, so that a large part of its surface comes into contact with the horns. The horns are then retracted beneath the scutum and the egg is then deposited on the dorsal surface of the tick by movements of the mouthparts towards the anterior. The whole sequence takes less than a minute and is repeated for each egg. The eggs accumulate at the anterior dorsum of the ovipositing tick, and their waxy covering makes them adhere together in a dense cluster (Fig. 3.36a), often completely covering the mouthparts and genitalia.

The glandular epithelium of Gené's organ remains inside the body wall during eversion (Fig. 3.2) and consists of tubular folds of a single layer of columnar cells (Fig. 3.3a) enclosing a lumen containing the secretion (Fig. 3.4). The tubular folds of cells are separated from the horn cuticle by the lumen contents (Figs. 3.2, 3.10). The horns are pulled inside-out during retraction through the narrow slit in the camerostome, folding in on themselves many times (Figs. 3.2, 3.12, 3.21).

Fig. 3.1 a. Light micrograph showing the everted horns of Gené's organ (H) of an ovipositing female B. microplus. The retracted mouthparts can be seen just below the everted horns which are extended towards the vagina (V). A glistening waxy secretion can be seen, covering the whole anterior region of the tick, and the oviposited eggs (E) adhere together in a cluster on the dorsal surface.

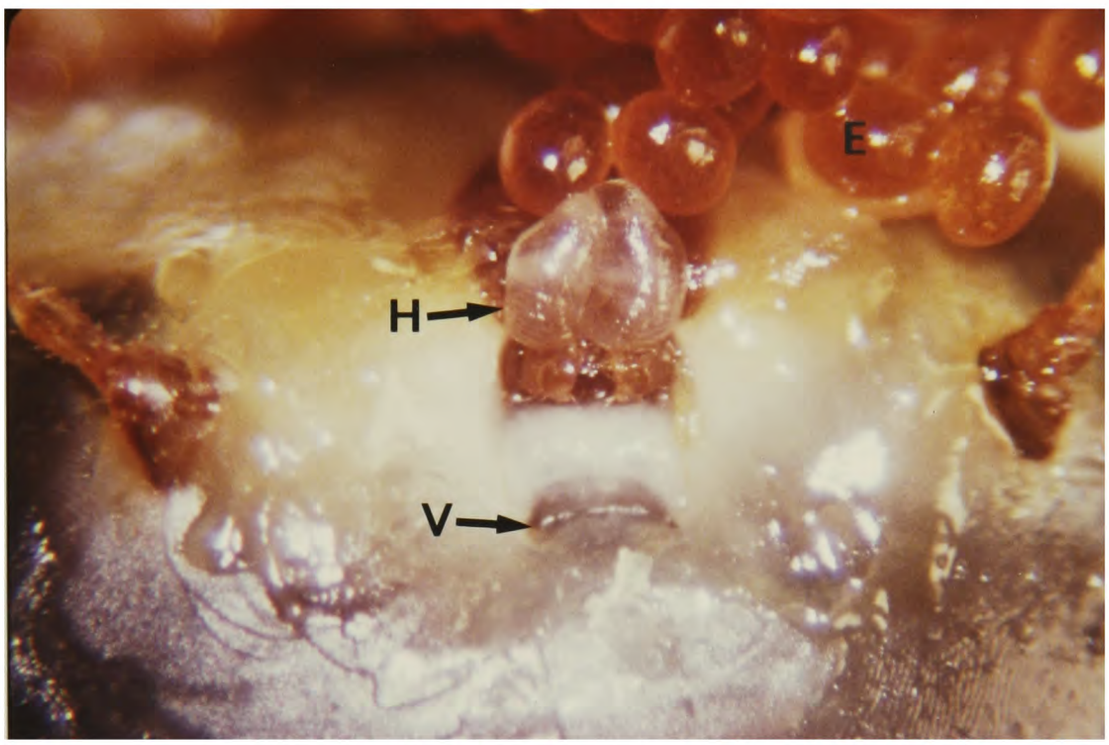
x 35

b. Diagrammatic drawing showing the sequence of events during oviposition in B. microplus.

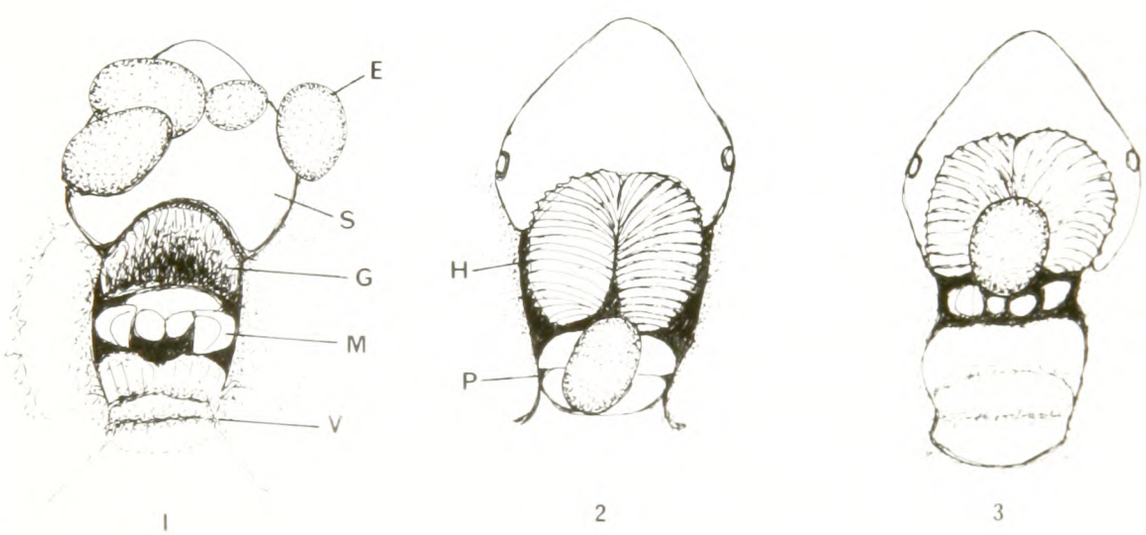
1. The retracted horns of Gené's organ (G) lie immediately above the mouthparts (M) which are retracted in the camerostomal fold, bringing the vagina (V) in close proximity to Gené's organ. Gené's organ lies beneath the scutal plate (S) on which eggs (E) are deposited.

2. The prolapsed vagina (P) pushes the egg towards the simultaneously everted horns (H), the mouthparts being depressed to bring the everted horns in contact with the egg. The egg is manipulated in a rolling fashion by pulsations of the horns.

3. The vagina retracts, leaving the egg adhering to the horns. The horns then retract and the mouthparts are raised, depositing the egg on the scutum.



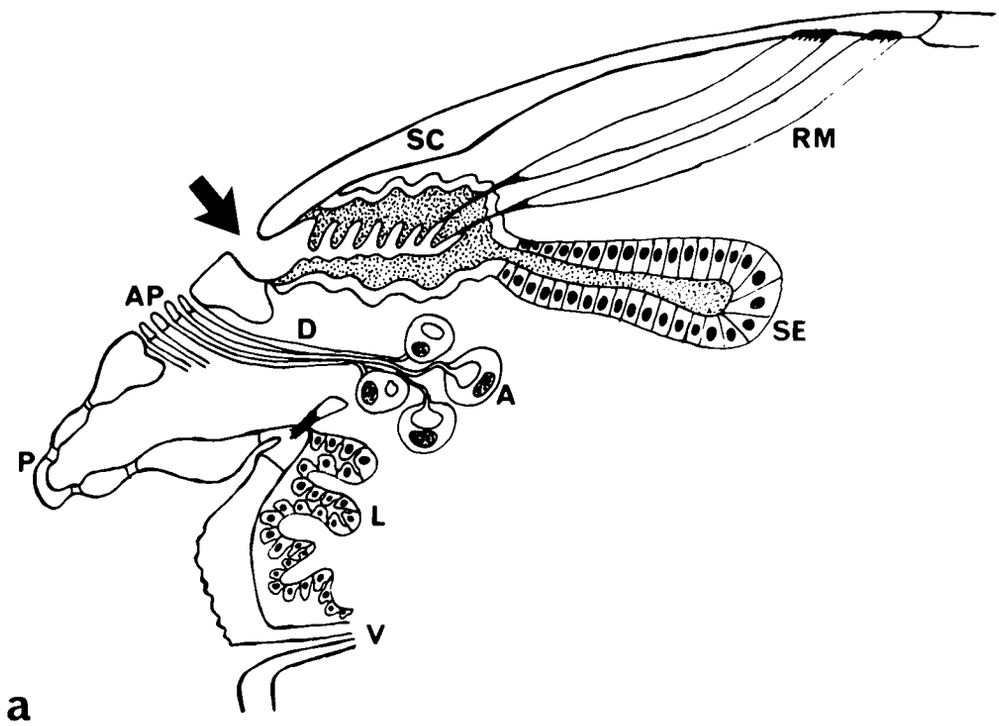
a



b

Fig. 3.2 a. Generalised drawing showing the relative positions of the mouthparts, Gené's organ and oviduct of a newly dropped engorged female B. microplus in sagittal section, before commencement of oviposition. The mouthparts and palps (P) are perpendicular to the scutum (SC) and the horns of Gené's organ remain retracted within the camerostomal fold at the articulation between mouthparts and scutum (arrow). The secretory epithelium of Gené's organ (SE) encloses a lumen (shaded area) which is continuous with the thin cuticle enclosing the horns. The accessory glands (A) situated in the haemocoel to either side of the secretory glands of Gené's organ are connected to the areae porosae (AP) on the tectum of the capitulum by ducts (D). The lobed gland cells (L) also a continuation of the body wall epithelium, stretch from Gené's organ to the oviduct and enclose a sub-cuticular lumen containing the secretion. V - vagina.

b. Similar drawing showing an ovipositing tick with Gené's organ everted. During the pre-ovipositional period the mouthparts are retracted posteriorly beneath the scutum and are angled ventrally towards the vagina. This widens the gap between capitulum and scutum allowing the horns of Gené's organ to evert and protrude ventrally to meet the vulva, which is now open and is closer to the mouthparts and faces dorsally towards the horns. During oviposition the horn cuticle (HC) turns inside out as the organ everts, and is retracted by retractor muscles (RM) inserting into the outer cuticle by rods (R) which pass through the lumen. Eversion of the horns is accompanied by production of the oocyte from the prolapsed vagina. The ventral surfaces of the horns pass directly over the outlets of the accessory glands at the areae porosae, leading to the incorporation of their secretion in the egg wax which spreads from the horns to the egg shell.



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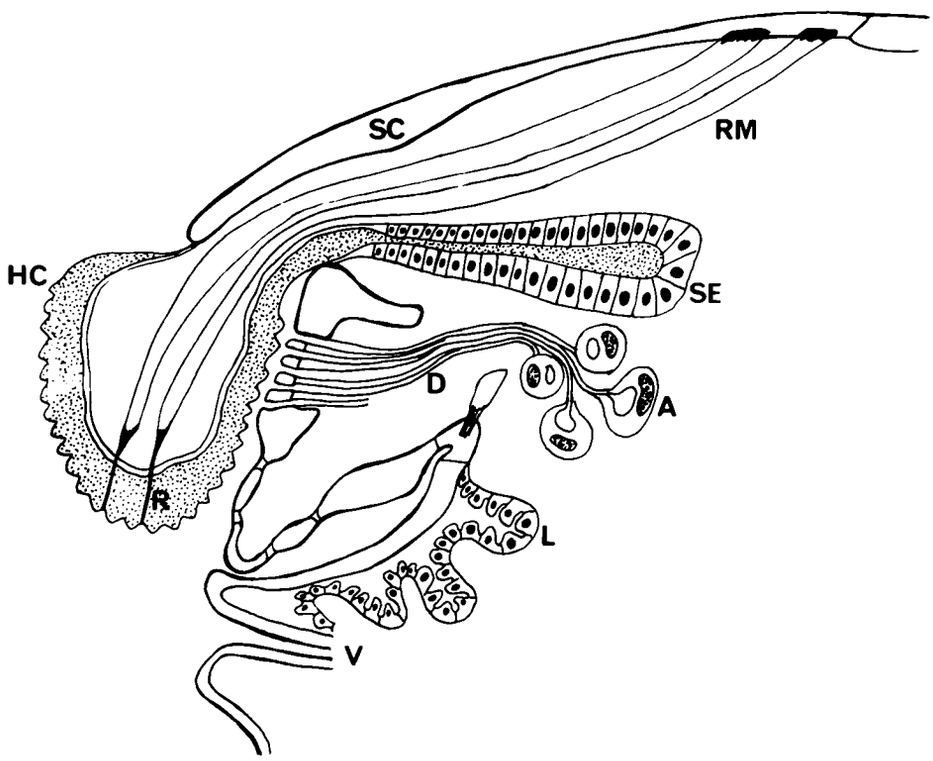


b

3.1.2 Changes during the pre-ovipositional period

After dropping from the host gravid female B. microplus undergo several changes which are visible externally before oviposition commences. After dropping, the mouthparts (capitulum) are visible protruding from the anterior margin of the scutum (Fig. 3.2a). The areae porosae are visible in the light microscope and SEM as two patches of pores on the dorsal surface of the capitulum, posterior to the chelicerae (Fig. 3.5a,b). At this stage the articulation between capitulum and scutal plate is very close, the horns of Gené's organ remaining retracted within this cleft, and the vaginal opening is closed.

Oviposition commences within 2-3 days of dropping but before this occurs the mouthparts are gradually retracted by muscles into a deep fold, the camerostome, which forms beneath the scutum (Fig. 3.6a). The mouthparts are retracted until the palps are level with the anterior margin of the scutum. Associated with this is a deepening of the camerostomal fold, as the tick loses water, and the body wall cuticle becomes less turgid, gaining its characteristic folds along the lines of the dorso-ventral muscle insertions (Fig. 3.36). The retraction of the mouthparts enlarges the gap between the scutum and the posterior margin of the capitulum allowing the horns to evert and retract, passing over the dorsal surface of the mouthparts (Figs. 3.5a,c; 3.6b). This process is shown diagrammatically in Fig. 3.2. The retraction of the mouthparts and the deepening of the camerostomal fold also brings the vaginal opening into closer proximity with the horns when they are everted and oviposition movements begin when the vaginal opening enlarges and begins muscular contractions. The lumen of the oviduct at this stage is full of mature eggs.

3.1.3 The ultrastructure of Gené's organ

In longitudinal sections of ovipositing ticks with the horns everted it can be seen that the ventral surfaces of the horns which come into contact with the eggs, pass directly over the porose areas (Figs. 3.5e). In transverse section, with the horns retracted, it can be seen that each porose area lies immediately ventral to one of the horns, so that the exits of the pores face the cuticular covering of Gené's organ (Fig. 3.4b). Each pore is connected to a duct, and the groups of ducts, clustered together, lead away posteriorly from each porose area (Fig. 3.5d). The ducts connect to groups of acinar glands having the appearance of

bunches of grapes, situated in the haemocoel on either side of the tubular secretory glands of Gené's organ (Fig. 3.5f).

In SEM's of specimens with the horns everted it can be seen that the median anterior and ventral regions of the horns are covered with fine, cone-like cuticular setae about 1 μm long (Figs. 3.5c, 3.7a, 3.7b). These are much smaller than setae found elsewhere on the body surface. They are also visible in sections of the horns. The horn cuticle is composed of two layers, a thin dense-staining outer one about 50 nm across and a thicker, 0.15 μm, less dense inner layer (Fig. 3.8). The inner layer is often incomplete. Distributed evenly over the whole surface of the horns, and particularly in the regions where the cuticle is folded into pleats (Fig. 3.5c) are circular pits about 0.6 μm across and 2-4 μm apart. The pits are visible as slight indentations in the cuticle which can be seen in the SEM (Fig. 3.7b). The pits can also be resolved in the light microscope. In the TEM, it can be seen that each pit is lined with pore canals about 50 nm in diameter with electron dense walls which penetrate the outer cuticle, and extend as tube-like filaments through the inner layer of the epicuticle (Figs. 3.8 and 3.9). The pore canals, or wax canals, cannot be resolved in the light microscope. However, treatment of the horns with chloroform followed by staining with ammoniacal silver nitrate caused deposits of reduced silver around the pits, and also on the cone-like setae. The lumen contents enclosed by the epicuticle of the horns have a granular appearance in TEMs of ticks at or near to oviposition (Figs. 3.8, 3.10b), but this is less prominent in unengorged ticks (Fig. 3.10a). This material stains densely with methylene blue in plastic sections (Fig. 3.4).

In the horns the epithelium underlying the lumen is thin and reduced to a strip of cytoplasm (Fig. 3.8). Further inwards, towards the tubular glands, the epithelial cells increase in size and subcellular organelles become more evident (Figs. 3.10b and 3.11a). Here the inner layer of cuticle lines the apical surface of the cells, which have elaborate laterally folded intercellular junctions, and the basal plasma membranes are infolded, forming extracellular spaces beneath the basement lamina. There are few lipid-like inclusions and ER in these cells. The lumen contents of the horns in this region is thus held between two layers of cuticle (Fig. 3.10b). The inner cuticle layer does not extend into the tubular glands, and the epithelial cells in this region have a microvillarborder which has direct access to the lumen (Figs. 3.4a, 3.10a, 3.13b). It appears that the horns are thus formed by separation of the

outer and inner cuticular layers of the integument, since in unengorged females areas can be found where separation of these layers is incomplete.

The horns are almost fully developed in the unengorged adult female B. microplus (Fig. 3.10a) and A. variegatum (Fig. 3.11a) although the mouthparts are not retracted and they are not evertable. The secretory epithelium however is not well developed in unengorged females and the secretory cells are small and contain few secretory vesicles (Figs. 3.10a, 3.11b, 3.12). As engorgement proceeds, the secretory cells grow and develop into large secretory cells which are columnar in shape and are 50 μm or more deep, with extensive glycogen reserves and secretory vesicles (Figs. 3.4a, 3.13-3.16). There is a large, central nucleus and the cytoplasm contains organised arrays of rough endoplasmic reticulum (Fig. 3.15a), Golgi bodies (Fig. 3.14b), numerous mitochondria, large inclusion droplets and areas containing a granular material (Figs. 3.13b, 3.15a). The granular material consists of arrays of glycogen α-particles 140 nm in diameter distributed singly and in concentrated arrays throughout the cell (Figs. 3.13b, 3.15a). These areas have a strong periodic-acid Schiff-reaction consistent with them being composed of carbohydrate (Fig. 3.4a). The apical surface of the secretory cells has a well developed microvillar border (Figs. 3.13b, 3.14a, 3.16) which extends into deep intercellular crypts. The microvilli appear to contain tubular elements of smooth membrane, which may be an extension of smooth-membrane reticulum (Figs. 3.14b, 3.16). At the intercellular borders between adjacent cells the plasma membranes are joined by septate junctions (Fig. 3.15b). The cells have a prominent basement lamina and this basal membrane has extensive infoldings forming extracellular spaces (Figs. 3.13a, 3.16). The material in the lumen of the glands is amorphous and appears less granular in TEMs than that in the horns. In some sections there are crystal-like lipid inclusions in some of the secretory vesicles (Figs. 3.16, 3.17). The general organisation of the secretory cells of the tubular glands of Gene's organ is shown in Fig. 3.18.

3.1.4 Muscle insertions of Gené's organ

The general organisation of the muscles involved in the movements of the horns, capitulum and chelicerae is shown diagrammatically in Figs. 2.1 and 3.19a. In longitudinal sections it can be seen that the retractor muscles of Gené's organ insert into the horns at their anterior end, and the scutum at their posterior end (Fig. 3.19b). In transverse sections with the horns retracted it can be seen that the muscles which

raise the capitulum, the capitulum extensors, insert into the posterior of the dorsal capitulum adjacent to the bifurcation of Gené's organ into its two horns (Fig. 3.20).

The retractor muscles insert into a series of long cuticular rods or apodemes at their anterior end, which can be seen to be continuous with the epicuticle of the horns of Gené's organin horizontal light microscope sections (Fig. 3.21). The organisation is shown diagrammatically in Fig. 3.22.

The cuticular rods pass through the lumen of Gené's organ and are composed of several cuticular fibres bound together. The attachment to the muscles is via tendon cells containing longitudinally orientated microtubules (Fig. 3.23). The attachment to the scutum at the posterior end of the muscles is similar to other skeletal muscle insertions of the tick (Beadle, 1973) in that the apodemes are much shorter ingrowths of the cuticle (Fig. 3.24a). The myoepithelial junction is characterized by a complex system of plicate desmosomes which form an interdigitating layer between the muscle and tendon cell (Fig. 24). The junction is strengthened by the occurrence of hemidesmosomes on either side of the intercellular gap. The tendon cells are densely packed with microtubules which connect the desmosomes of the myoepithelial junction to those lining the plasma membrane where it contacts the cuticular rods (Figs. 3.24b, 3.25).

3.1.5 Ultrastructure of the muscles and motor terminals

The retractor muscles are striated and have the same general structure as other arthropod muscles (Pringle, 1972). The structure is similar in both A. variegatum and B. microplus. The nuclei are restricted to the periphery of the fibres as are the majority of the mitochondria (Fig. 3.26). The muscle is surrounded by a basement matrix and plasma membrane. The fibres consist of thick and thin filaments divided into sarcomeres by Z-discs. The sarcomere length varies from 1.6 μm to 2.4 μm and A and I bands are present (Fig. 3.26a). Some fibres can be seen to pass through gaps in the Z-discs (Fig. 3.26b). The muscles also contain an internal tubular sarcoplasmic reticulum. Electron micrographs show the muscles to be innervated by numerous terminals and several axons can be seen to form synapses with the muscle fibres. The neural lamella of the axon is continuous with the basement matrix of the muscle cell. The nerve axons are surrounded by glial cell wrappings but are separated from them by invagination of the basement lamella. The axon terminals on the

retractor muscles contain two distinct populations of agranular synaptic vesicles. The most common are the clear-centred, oval type having an average size of 49.1 nm (s.e.m. 5.0 nm) by 34.5 nm (s.e.m. 2.8 nm) in B. microplus (Fig. 3.27) and 49.4 nm (s.e.m. 1.4 nm) by 28.2 (s.e.m. 2.8 nm) in A. variegatum (Fig. 3.28a). Some terminals also contain smaller quantities of dense-cored vesicles, often aggregated together (Figs. 3.27a, 3.28a). These are round in section with an average diameter of 100 nm (s.e.m. 27 nm) in B. microplus and 106 nm (s.e.m. 16 nm) in A. variegatum. The terminals also contain mitochondria. The axon terminal membranes also have thickened, electron dense projections on the pre-synaptic side opposite a similar density on the muscle plasma membrane (Figs. 3.27b and 3.28b). These membranes are separated by a 20-30 nm synaptic cleft.

3.1.6 Ultrastructure of the accessory glands of Gené's organ

The duct cell which connects each accessory gland cell to one of the pores in the areae porosae consists of a narrow tube surrounded by sheath cells (Fig. 3.29a). The duct cell lines the pore in the cuticle and forms a simple junction with the cuticle. The internal lumen (the duct) which is lined with intermittent microvilli is about 7 µm in diameter. The cytoplasm of the duct contains numerous longitudinal microtubules, transversely sectioned in the plane of Fig. 3.29a. No branches or joins in the ducts have been observed in sections and it appears that each duct connects a single gland cell to one of the pores. The accessory gland cells are about 80 µm in diameter and are joined together in clusters of 10-15 cells, each cell having a central lumen about 20-30 µm across, which connects with the duct, and a large lateral nucleus, both visible in the light microscope (Figs. 3.29b, 3.32). The general organisation of the accessory glands is shown diagrammatically in Fig. 3.30.

The internal lumen of the gland cells has a microvillar border (Fig. 3.29c). The cells contain many mitochondria which are most concentrated around the apical border of the cell. Numerous small membrane-bound vesicles are associated with Golgi bodies (Fig. 3.31a) and the small vesicles are often aggregated around much larger membrane-bound vesicles, 0.5 - 1.6 µm in diameter. Many of the larger vesicles are aggregated closely around the apical border of the cell (Fig. 3.29c) and some can be seen to be fusing with the apical plasma membrane and may be undergoing exocytosis (Fig. 3.31b). The contents of the vesicles are extracted during tissue processing suggesting that they contain lipid. The lack of staining with osmium also suggests that the lipids are mainly saturated.

The cytoplasm of these cells also contains large arrays of smooth endoplasmic reticulum which may be in tubular form (Fig. 3.31c) or separated into isolated vesicles (Figs. 3.29c, 3.33). Rough endoplasmic reticulum is also present and there are many small ribosome-like particles (Fig. 3.31a). Glycogen α-particles are distributed singly in the cytoplasm and in large concentrated arrays around the basal border of the cells, and also around the nucleus (Fig. 3.33). These areas of the cell have a strong periodic acid-Schiff reaction consistent with their being composed of carbohydrate (Fig. 3.32).

The basal plasma membrane of the accessory gland cells is infolded beneath the basal lamina forming extracellular spaces (Fig. 3.31c). Adjacent cells have a continuous basal lamina and their plasma membranes are separated by an intercellular space being joined intermittently at gap junctions (Fig. 3.34a).

The duct cell passes through the gland cell to connect with the lumen (Fig. 3.31d) and there does not appear to be any specialized membrane junctions between these cells. The duct cells are bound together by narrow processes of sheath cells that fold around them, binding them into clusters (Fig. 3.34b). As the ducts pass along the inside of the capitulum integument, they are separated from the cuticle by body wall epithelial cells (Fig. 3.34b), until they reach the porose areas where they pass through the epithelium and connect with the pores in the cuticle. Here the central lumen of the ducts connects with the outside.

3.1.7 Tubular and lobular accessory glands of the oviduct

Both the tubular and lobular accessory glands appear to be involved in producing a secretion involved with the eggs. However, neither the ultrastructure of these glands in the ixodidae, nor their histology at the light microscope level in B. microplus, have previously been investigated.

The tubular accessory glands of B. microplus open into the oviduct, and in transverse section it can be seen that they consist of gland cells packed with spherical secretory vesicles (Fig. 3.35a). These vesicles are surrounded by rough endoplasmic reticulum, and are probably protein since they stain green with Aparicio and Marsden stain. The lobed cells consist of a proliferation of the body wall epithelium around the oviduct and opening of the vagina, forming a glandular epithelium with a subcuticular lumen (Figs. 3.2, 3.35b). The gland is thus analogous

to Gené's organ; however there are no wax canals in the cuticle of the oviduct as in the horns of Gené's organ. The lobular cells have extensive rough ER and lipid vesicles and an apical microvillar border (Fig. 3.35c).

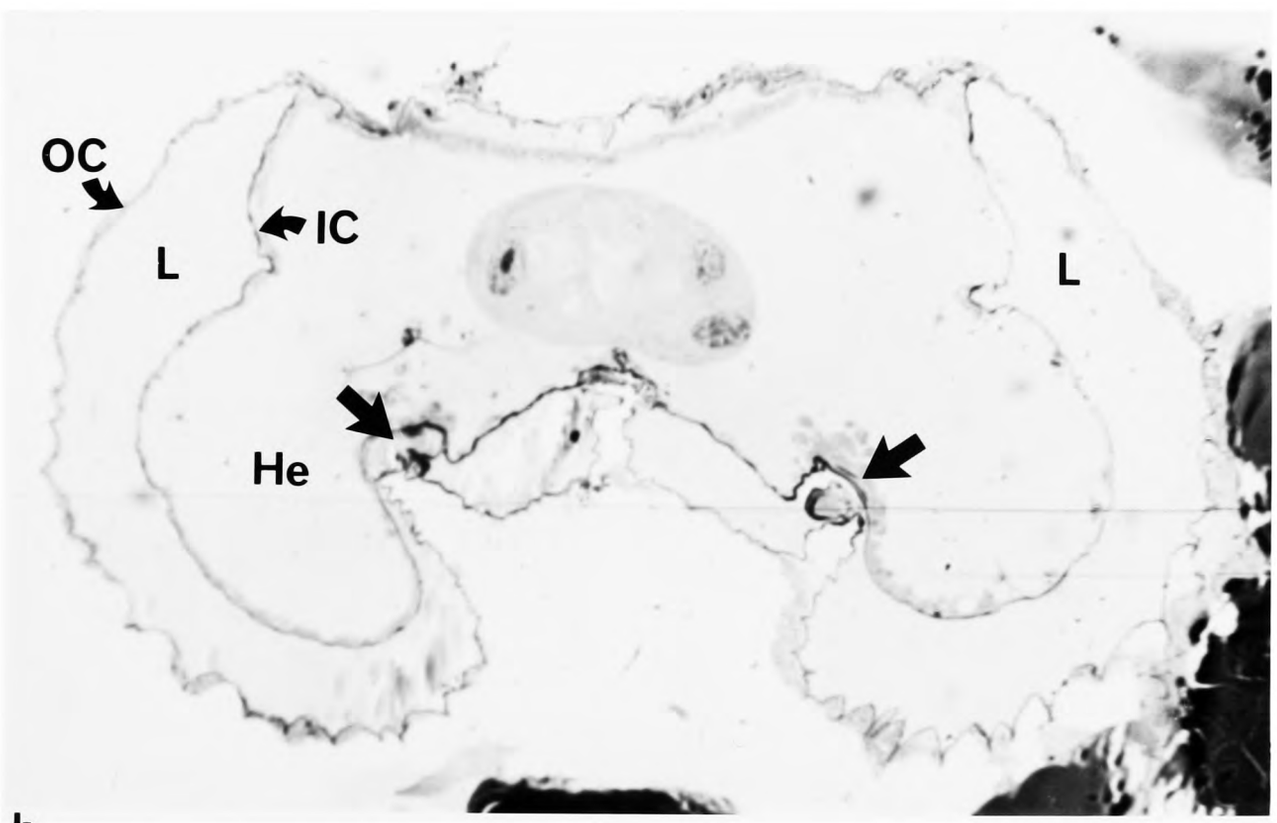
Fig. 3.3 a. Light micrograph showing two lobes of the tubular secretory epithelium of Gené's organ dissected from an ovipositing B. microplus.

x 50

b. Light micrograph showing a transverse section through the everted horns of Gené's organ, showing the inner cuticular layer (IC) with its reduced epithelial cells, the outer cuticle (OC) enclosing the lumen (L) and the inner region containing haemolymph (He). The positions of the retractor muscle insertions are also visible in transverse section (arrows). The inner and outer cuticular layers are continuous on the dorsal surface of the horns. Thus the lumen containing the secretion is only on the ventral side of the horns, which come into contact with the eggs.

x 230.

a



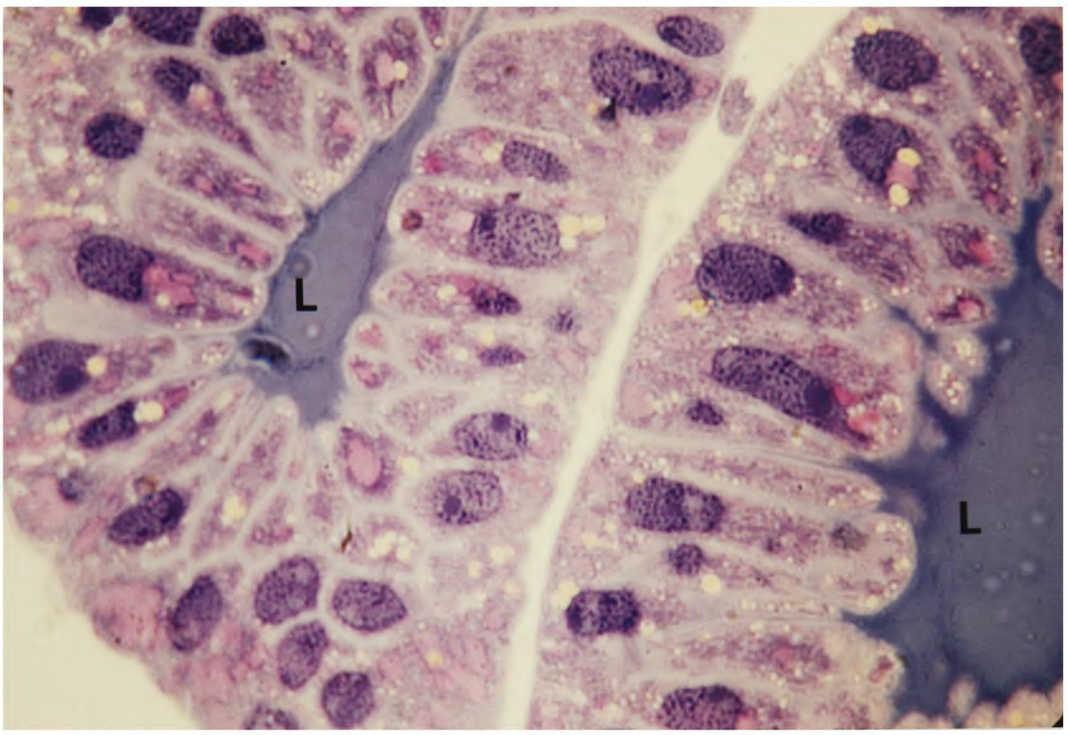
b

Fig. 3.4 a. Photomicrograph of a section through the tubular gland of Gené's organ showing the columnar epithelial cells surrounding the lumen (L) which stains densely with methylene blue. Areas staining pink correspond to glycogen stores, and the round lipid vesicles of varying sizes are distributed throughout the cells, often adjacent to the prominent nuclei (Methylene-blue Basic fuchsin).

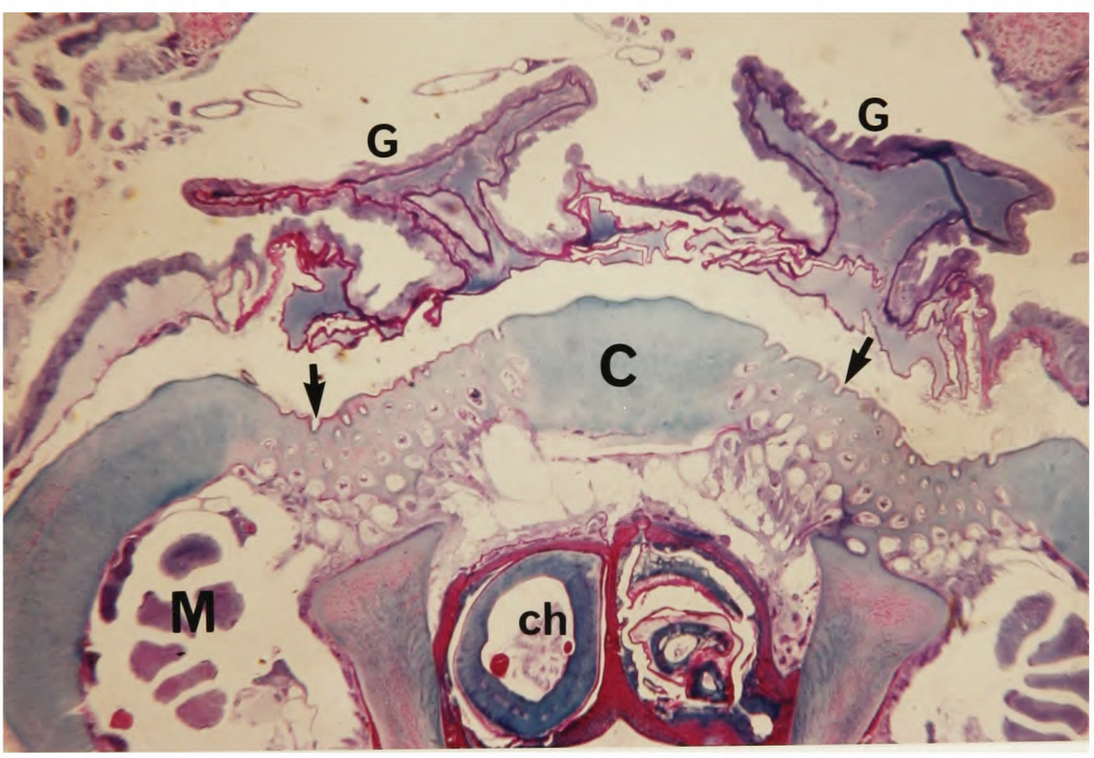
x 700

b. Photomicrograph showing a transverse section of the capitulum (C) and the retracted horns of Gené's organ (G) at the level of the areae porosae, showing the accessory gland ducts exiting through pores in the dorsal surface of the capitulum (arrows). The lumen of Gené's organ, which is stained blue, is visible between the inner and outer cuticular layers which stain red. Within the capitulum can also be seen the palpal muscles (M) and cheliceral shafts (ch). (Methylene-blue Basic fuchsin).

x 280



a



b

Fig. 3.5 a. SEM showing the mouthparts of a newly dropped adult female B. microplus during the pre-ovipositional stage. The areae porosae are visible as two patches of pores (AP) on the dorsal surface of the capitulum, posterior to the palps (P) and chelicerae (CH). The fold between the capitulum and scutum (S) through which the horns of Gené's organ emerge during oviposition is also visible (arrow). x 230.

b. SEM showing detail of the pores of one of the porose areas. The mouth of each duct cell can be seen just within the opening of each pore. x 1700.

c. SEM showing an adult female B. microplus during the early stages of oviposition, fixed with the horns (H) of Gené's organ everted towards the vulva (VU). The horns completely cover the dorsal surface of the retracted mouthparts. x 230.

d. Light micrograph of a sagittal section through one of the porose areas,showing the cluster of ducts (D) passing away posteriorly from the pores towards the accessory gland cells. The pores in the cuticle of the capitulum (C) can be seen in longitudinal section (arrow). x 420.

e. Light micrograph showing a vertical sagittal section through Gené's organ and the mouthparts of an ovipositing female B. microplus with the horns everted. It can be seen that the horns pass directly over the pores of the areae porosae (arrow) during eversion and retraction. HC, horn cuticle; HL, horn lumen; S, scutum; RM, retractor muscles of Gené's organ; P, palp; E, epithelial cells of horns. x 170.

f. Light micrograph showing a whole mount of a cluster of accessory gland cells. The nucleus (N) and lumen (L) of each cell are visible. (Unstained, with phase contrast). x 260.

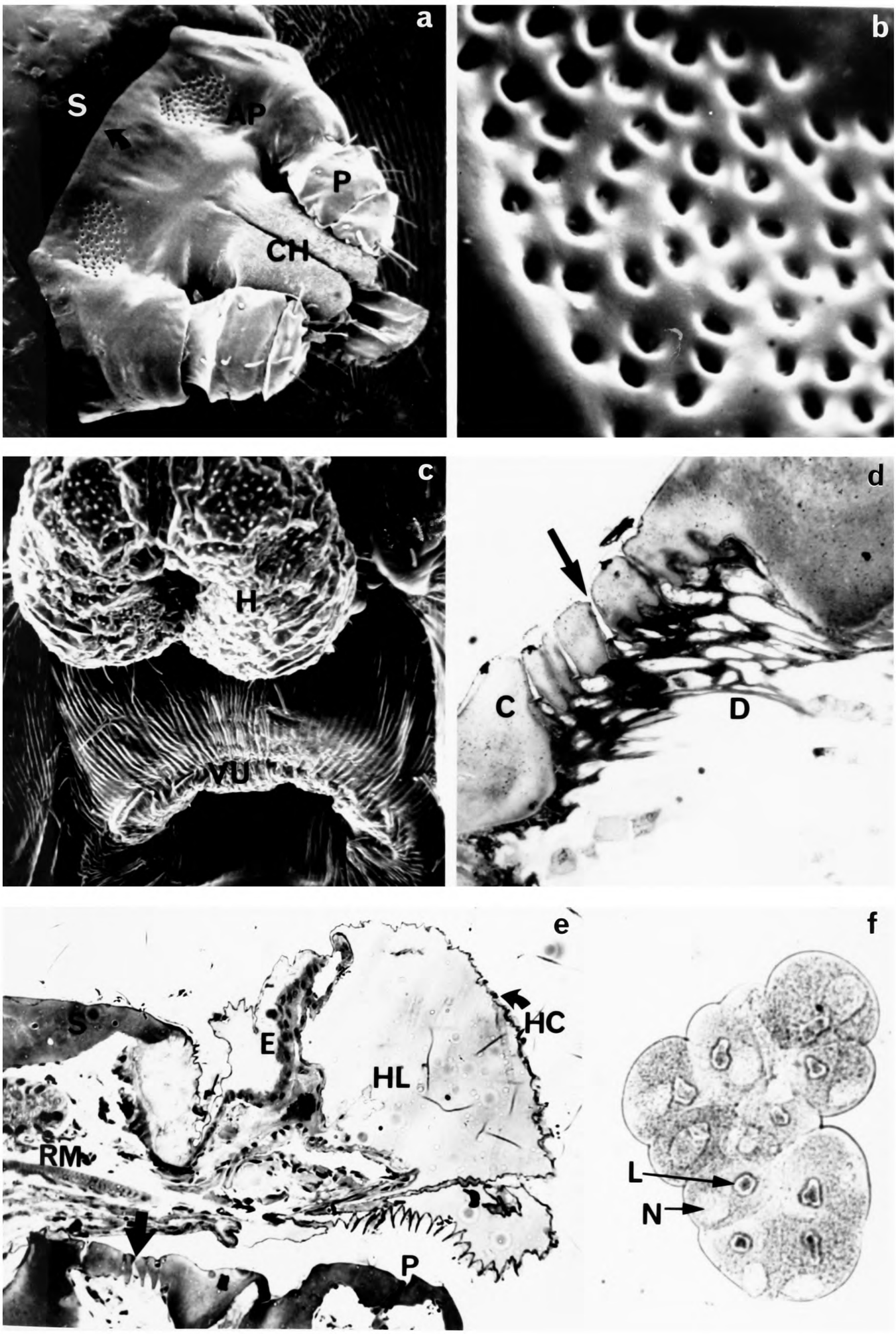
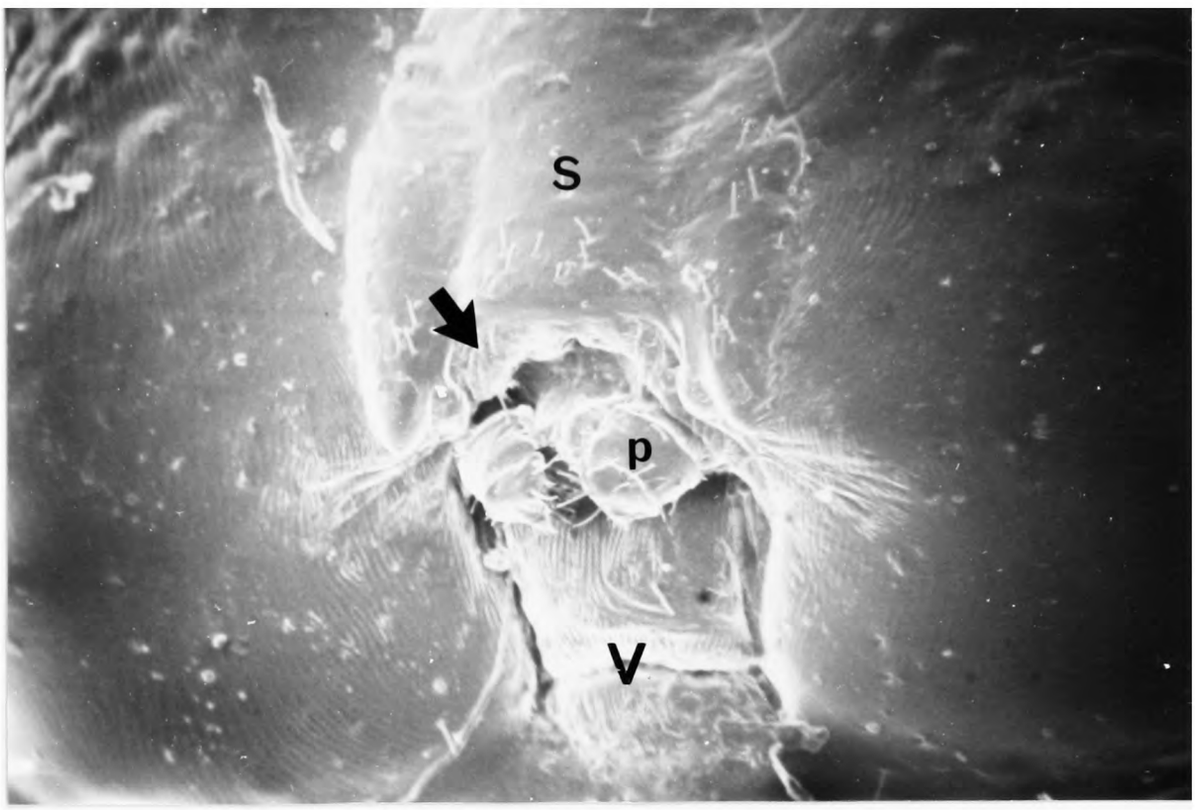
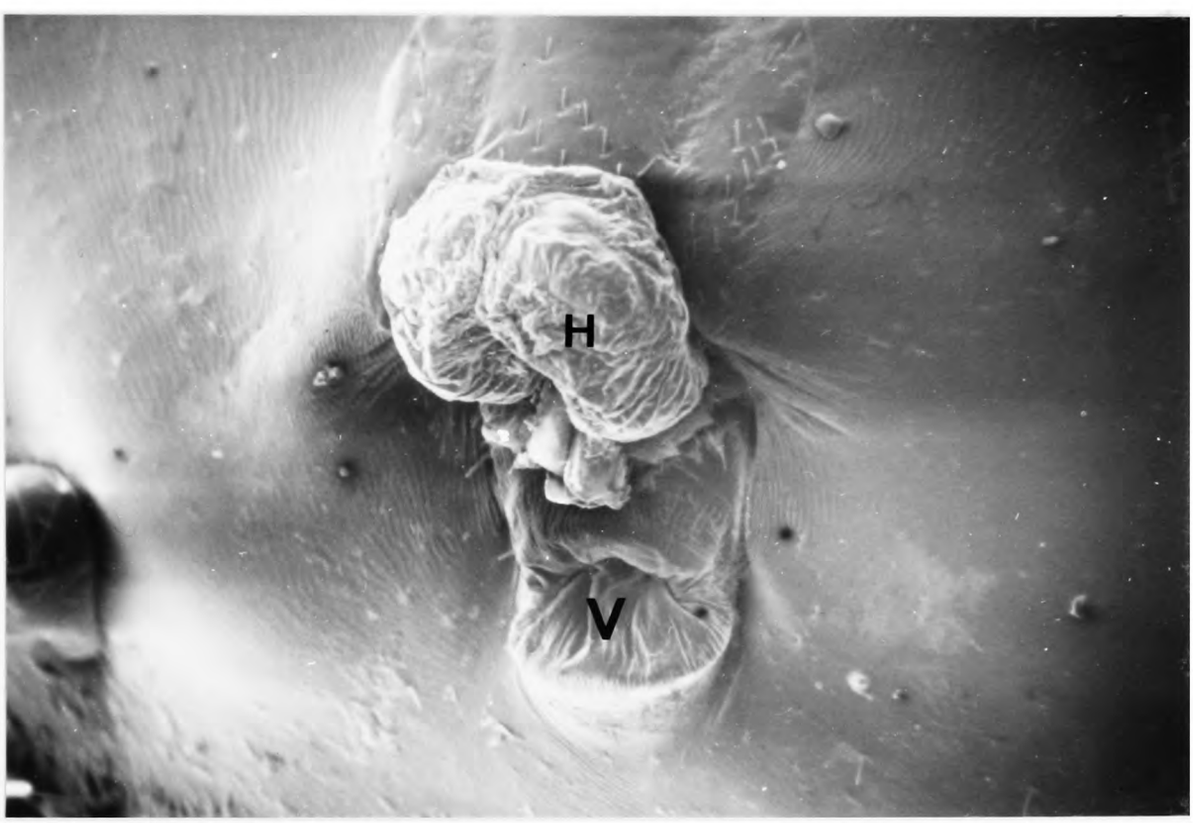

a
S
AP
P
CH
b
c
H
VU
d
C
D
e
S
E
HC
HL
RM
P
f
L
N

Fig. 3.6 a. SEM showing the mouthparts of an ovipositing female B. microplus with Gené's organ retracted in the camerostomal fold (arrow). The mouthparts are withdrawn beneath the scutum (S), with only the palps (P) being visible externally. V, vagina.
x 70.

b. SEM showing the horns (H) of Gené's organ everted towards the partially prolapsed vagina (V).
x 70.



a



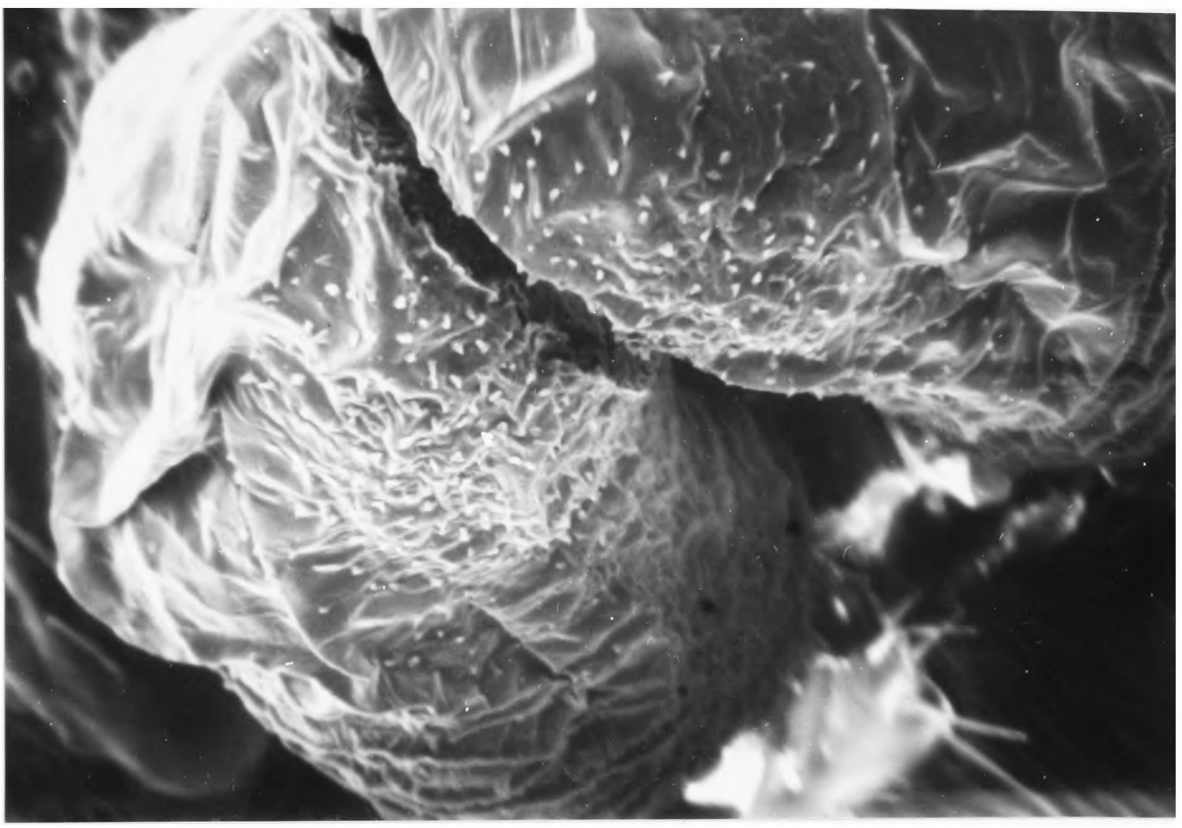
b

3.7 a. SEM showing the anterior and ventral surfaces of the everted horns of Gené's organ covered with numerous 1 μm long setae.

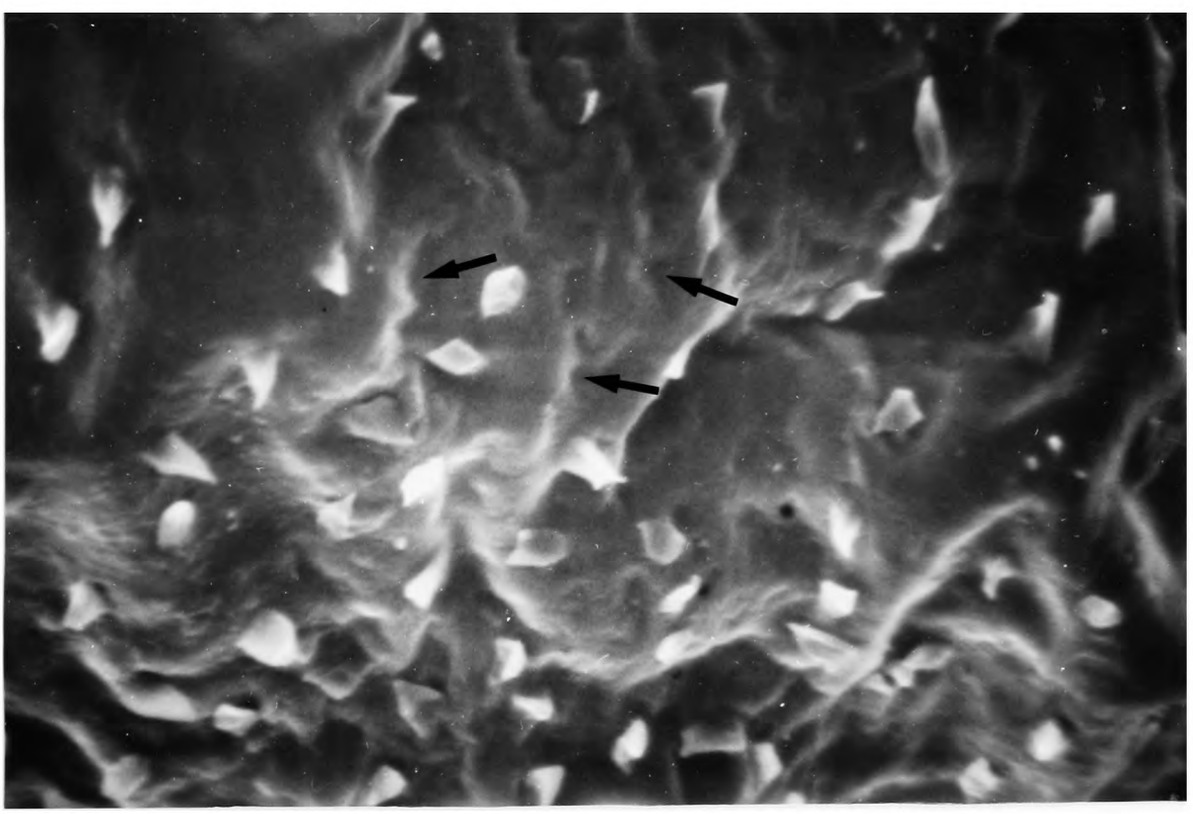
x 300.

b. SEM showing detail of the cuticular setae of the horns and also numerous dimples of 'pits' in the surface of the cuticle (arrows) which can be seen to be lined with pore canals in TEMs (Fig. 3.8)

x 950.



a



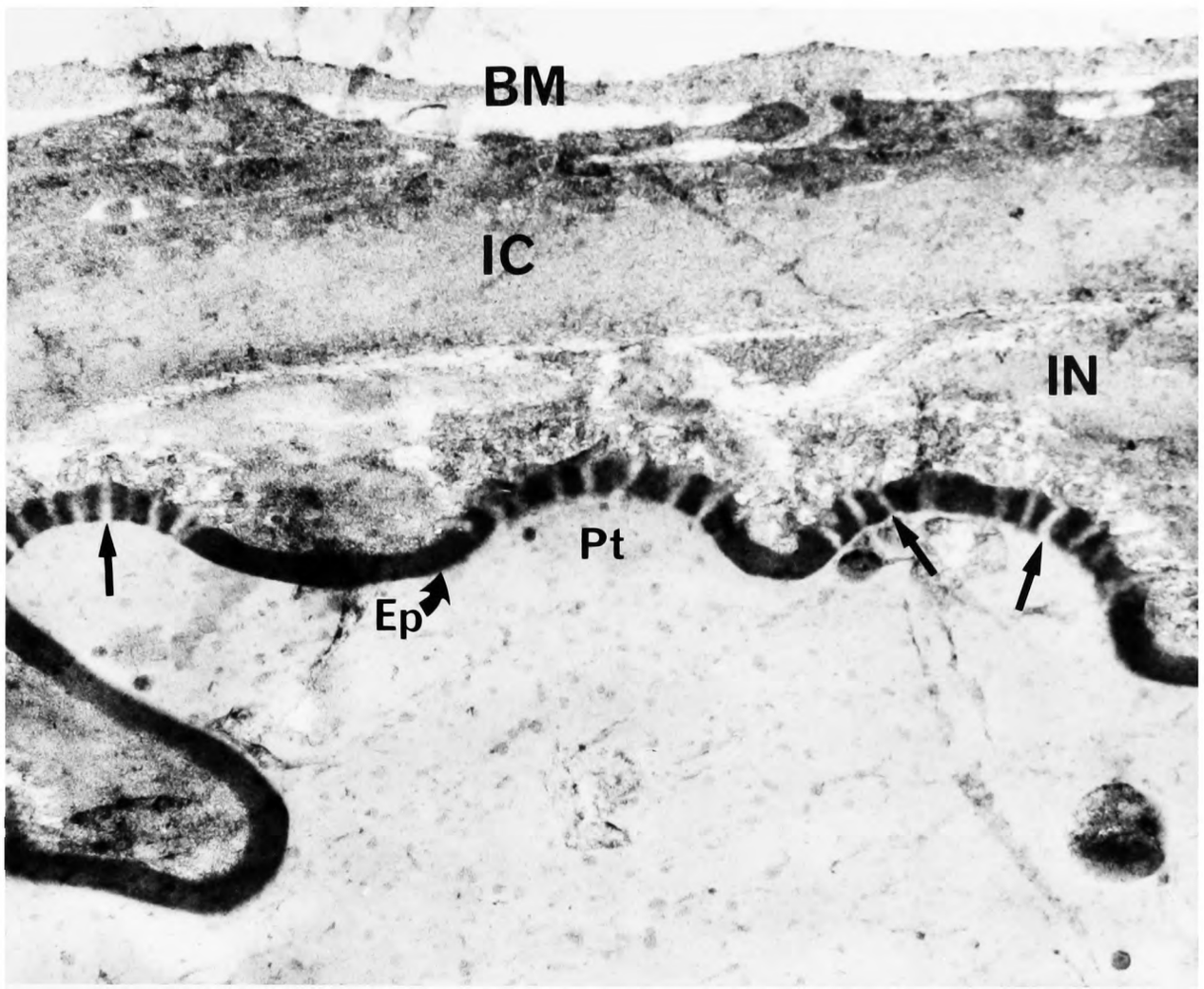
b

Fig. 3.8 a. TEM of a section through the horns of Gené's organ showing the pits (Pt) in the outer epicuticle (Ep) containing pore canals (arrows) which connect the lumen contents to the outside. There is a thin layer of cytoplasm between the basement membrane (BM) and inner cuticle (IC). The epicuticle has an indistinct less dense inner layer (IN) immediately beneath it, through which the wax canals pass.

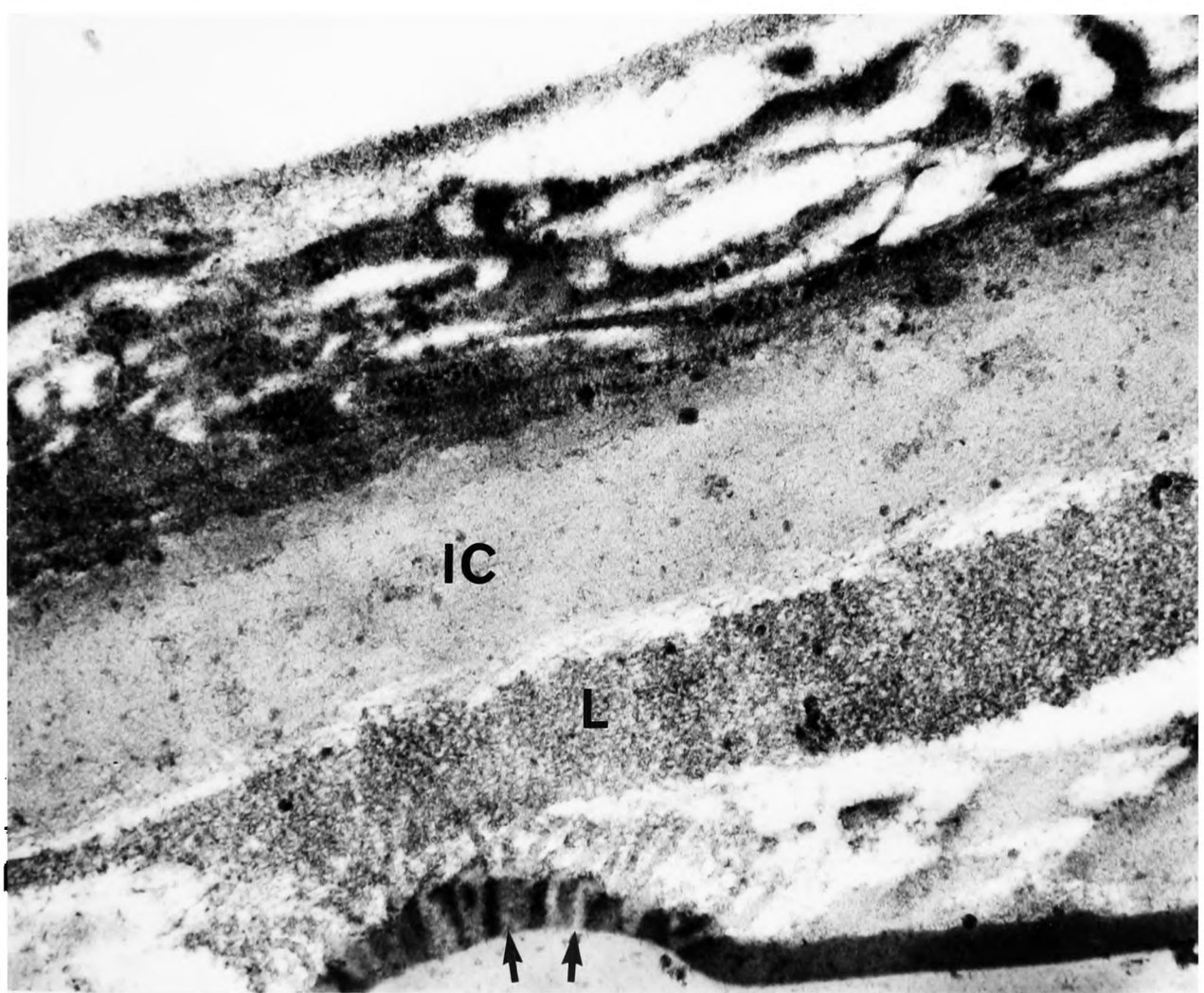
x 35,000

b. Section showing the connection of the lumen contents (L) with the outside via the pore canals in the epicuticle (arrows). The secretory material has a granular appearance which may be due to precipitation during tissue processing.

x 41,400



a



b

Fig. 3.9 a. TEMs showing detail of the pore canals lining the pits (Pt) in the epicuticle (Ep). The pore canals are lined by narrow tubular filaments (arrows) seen in longitudinal section (B. microplus).

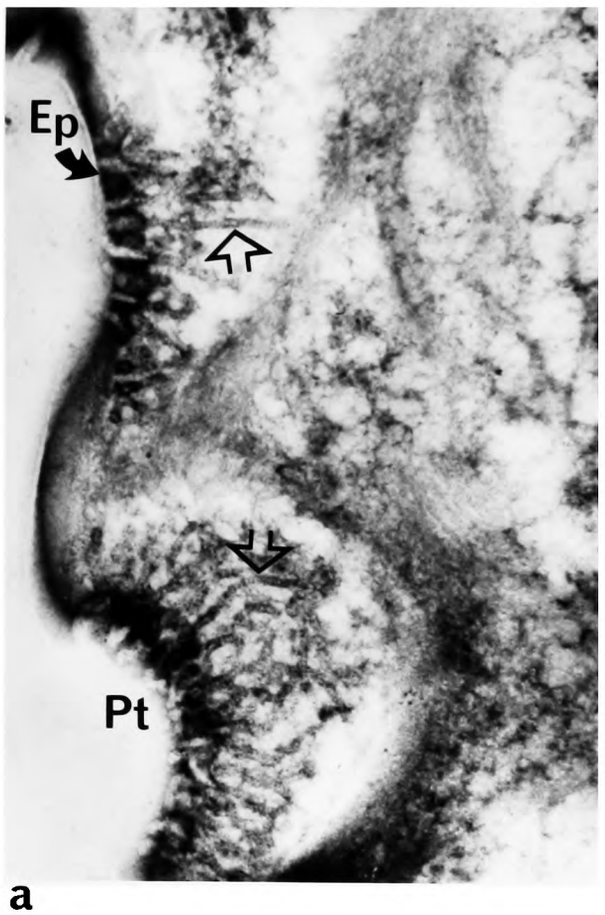
x 38,000

b. Horizontal section through a pit (arrow) in the epicuticle of the horns of B. microplus, showing the pore canals as tubes in transverse section.

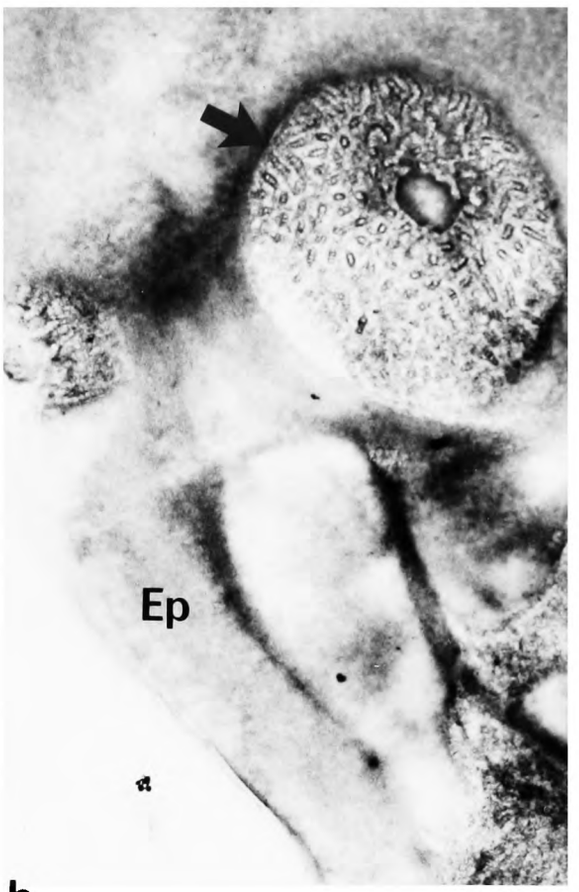
x 32,000

c. Section showing the outer cuticular layer (C) of the horns of A. variegatum, with the same pits (Pt) lined with pore canals connecting the lumen (L) to the outside, as are found in B.microplus. The lumen contents are also granular in appearance.

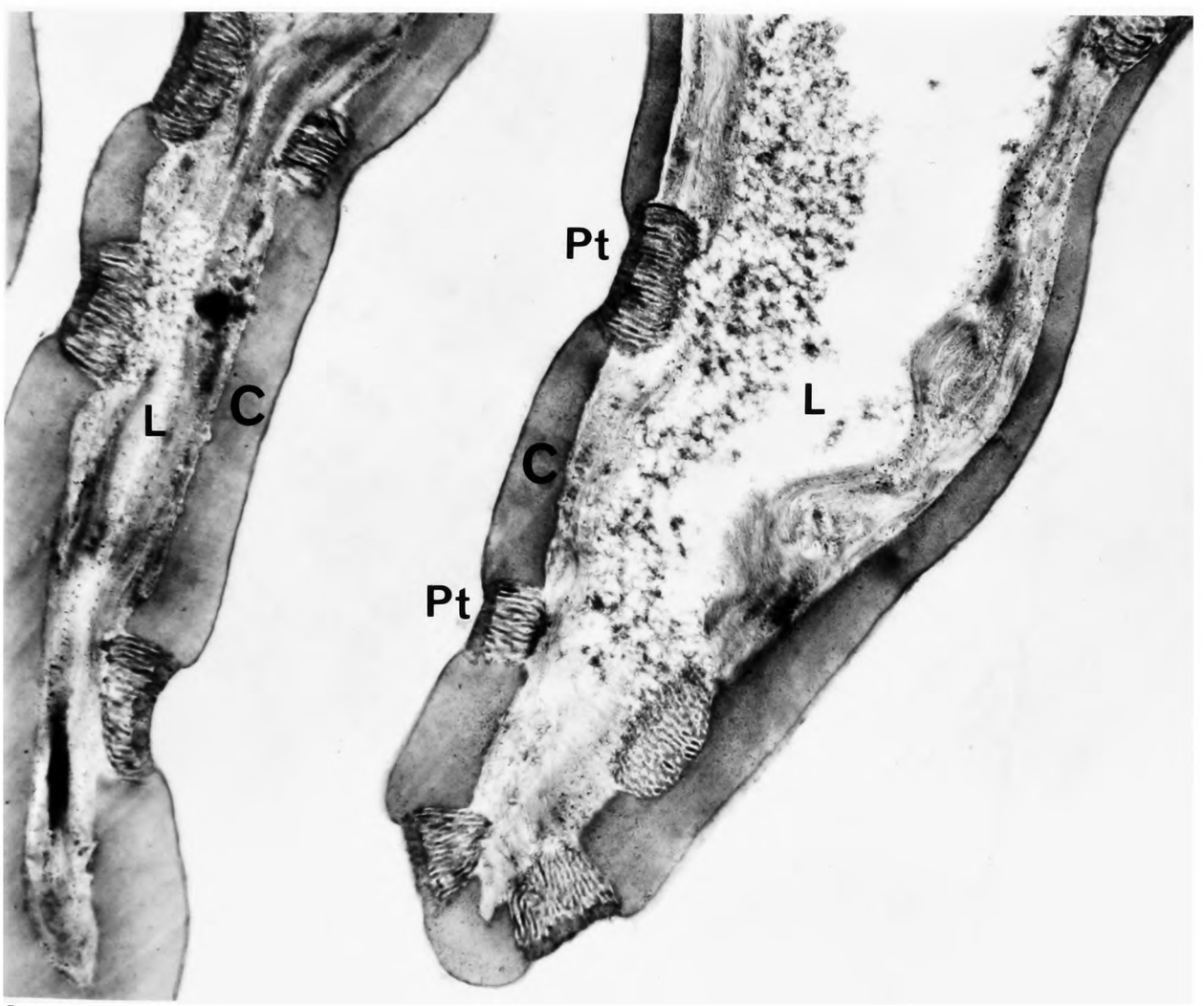
x 16,000



a



b



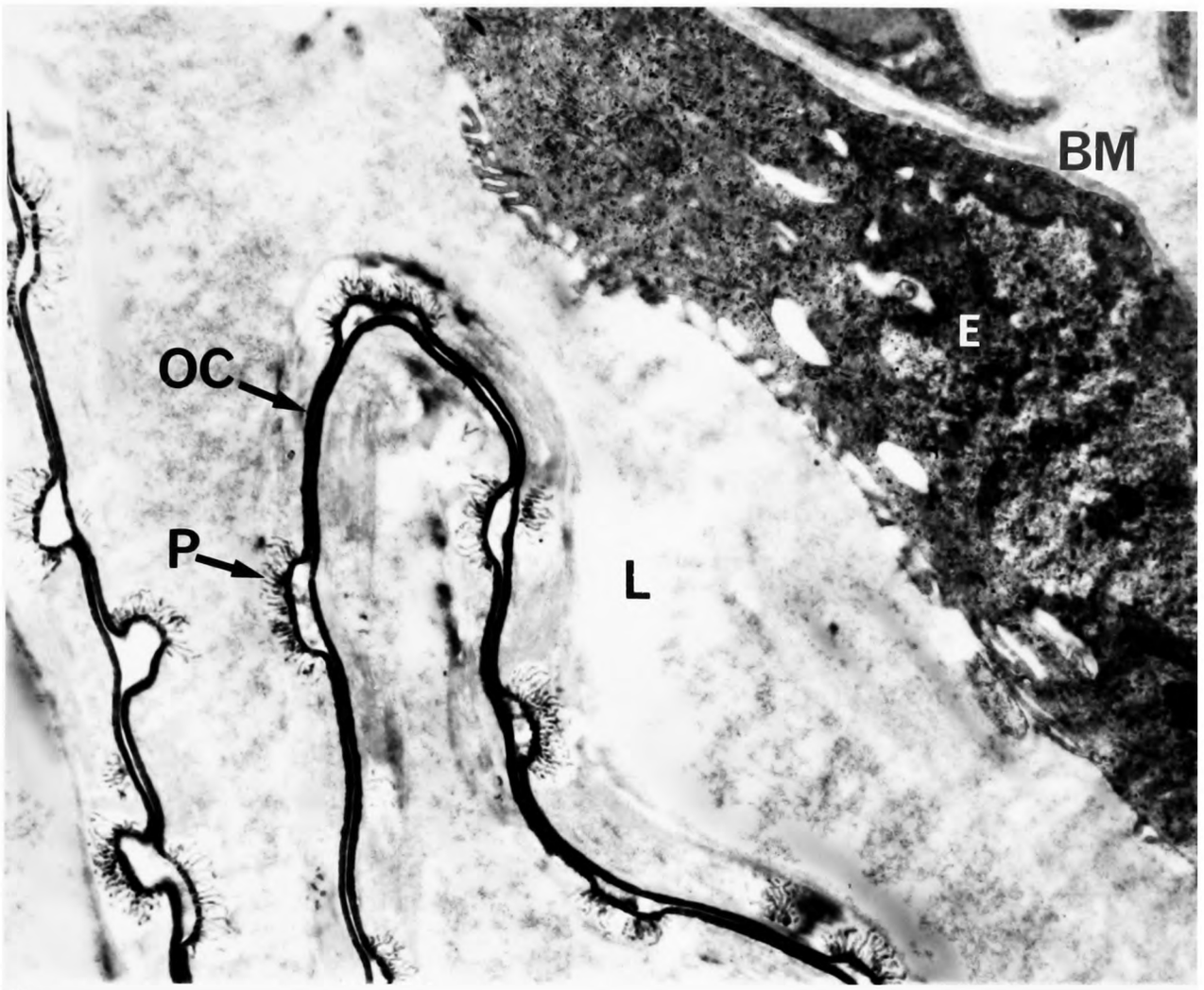
c

Fig. 3.10 a. TEM showing a section of the retracted horns of Gené's organ in an unengorged female B. microplus showing the folded outer cuticular layer of the horns (OC) and the lumen (L) which contains little secretion and is not distended. The glandular epithelial cells (E) are relatively poorly developed and are small in comparison with fully engorged females. The pore canals (P) are already developed at this stage. BM, basement membrane.

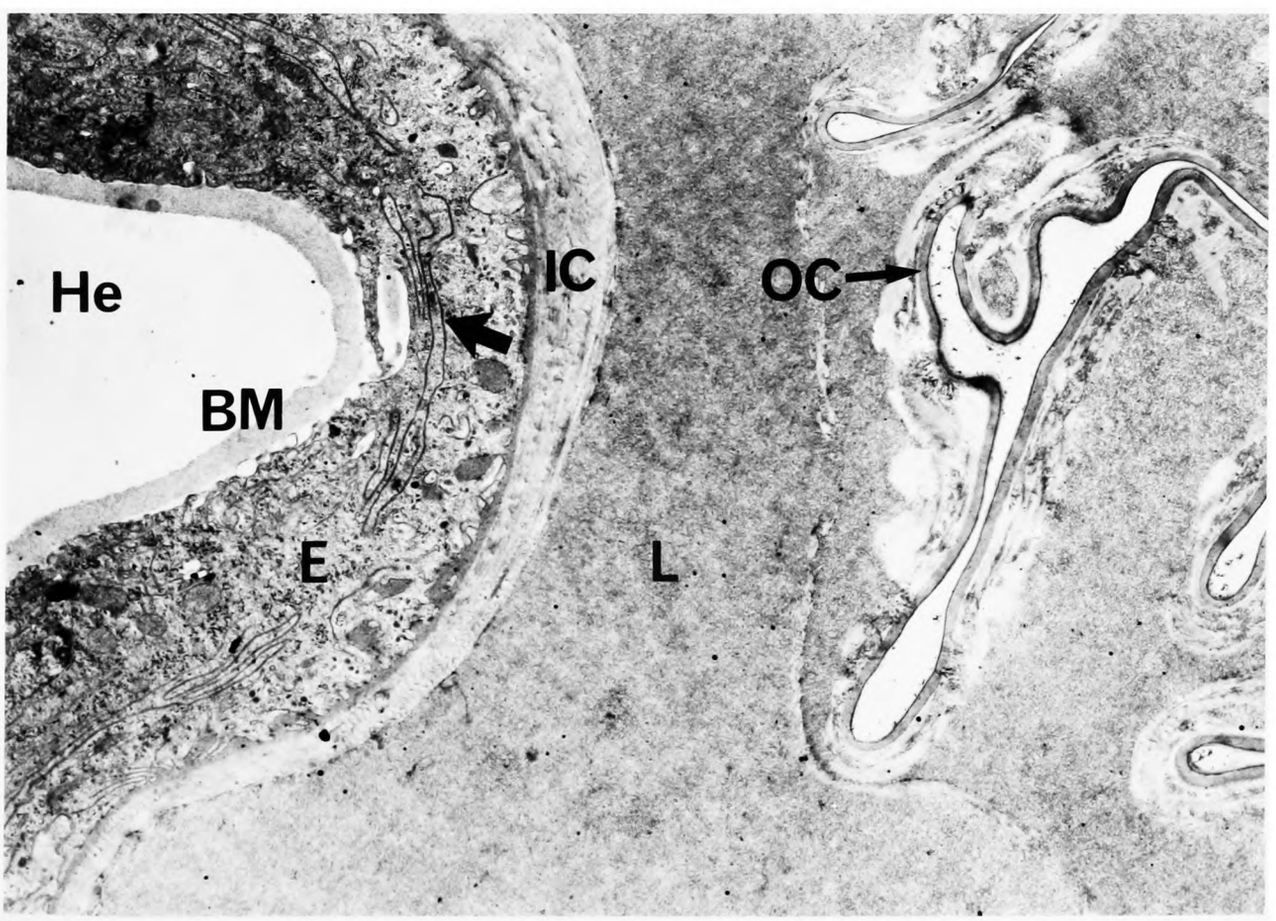
x 18,900

b. The retracted horns of Gené's organ in a section from a fully engorged, ovipositing B. microplus near the tubular glands. The epithelial cells here have developed an inner cuticular layer (IC) in addition to the outer epicuticle (OC) containing pore canals. The epithelium in this region is not glandular, and the lumen (L) functions as a storage reservoir for the secretion. The lateral cell membranes are extensively folded back on themselves (arrow). He, haemolymph; BM, basement membrane.

x 7,000



a



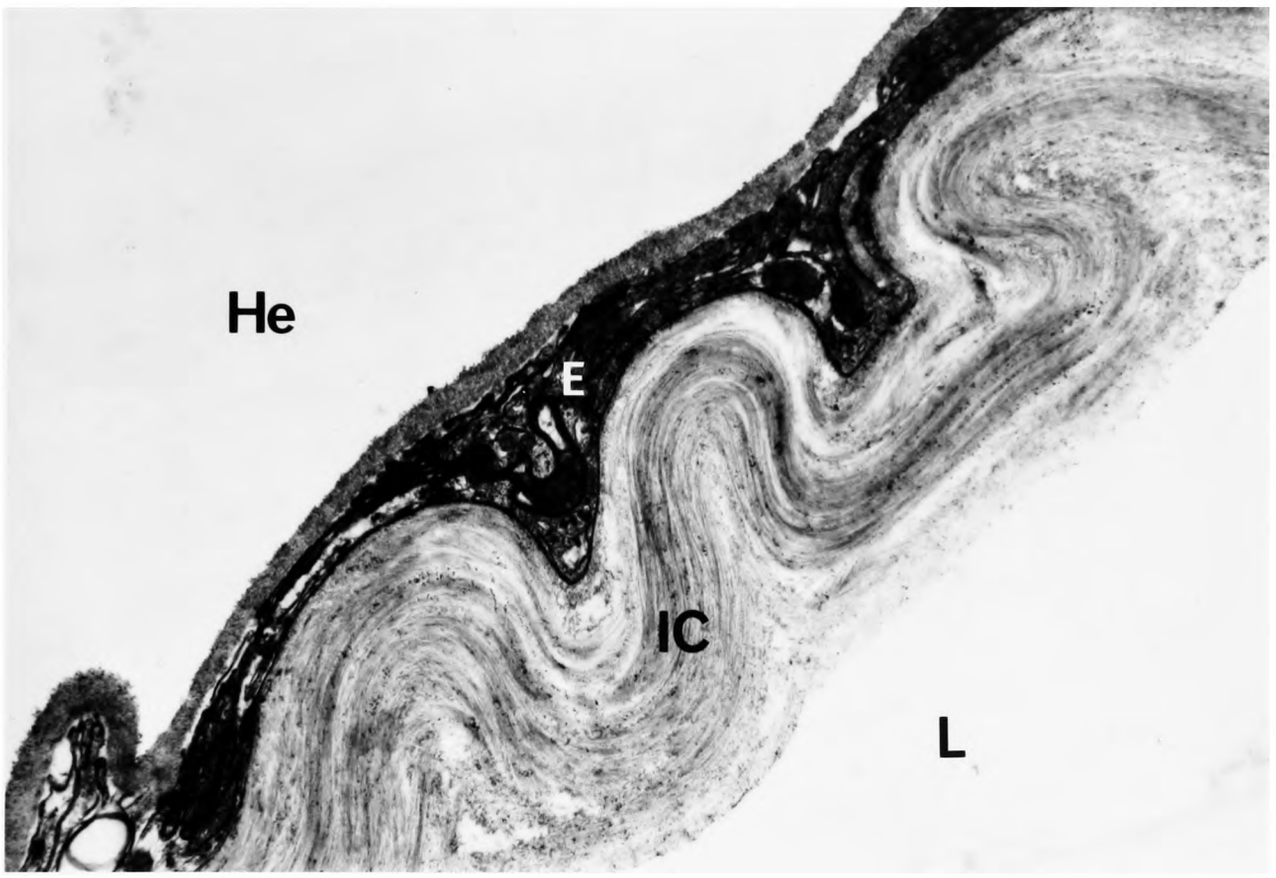
b

Fig. 3.11 a. Section showing the epithelial cells of the horns of Gene's organ in an unengorged female A. variegatum, showing the thin, poorly developed epithelial cells (E) and the well developed inner cuticular layer (IC) in this region, lining the lumen of the horns (L). He, haemolymph.

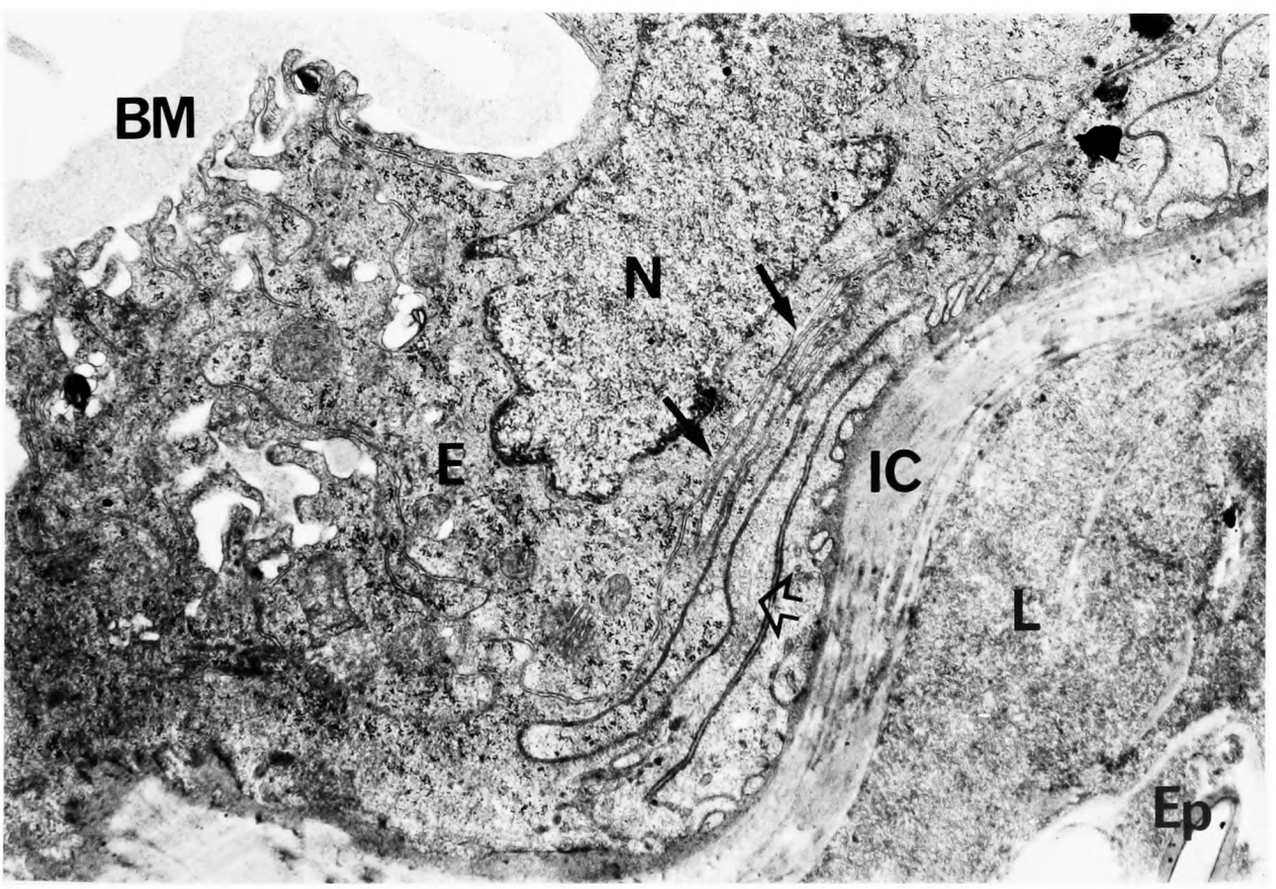
x 10,600

b. The epithelial cells of the horns of Gene's organ in an ovipositing B. microplus, showing the lumen (L) inner cuticular layer (IC) and epithelial cells (E) which perform a structural rather than a secretory function, and have elaborate, folded inter-cellular borders, joined by septate desmosomes (dense arrows). Ep - epicuticle of horns; BM - basement membrane; N - nucleus. The cells also contain many microtubules running laterally, difficult to see at this magnification (clear arrow). The apical and basal membranes are also extensively infolded to form invaginations.

x 13,300.



a

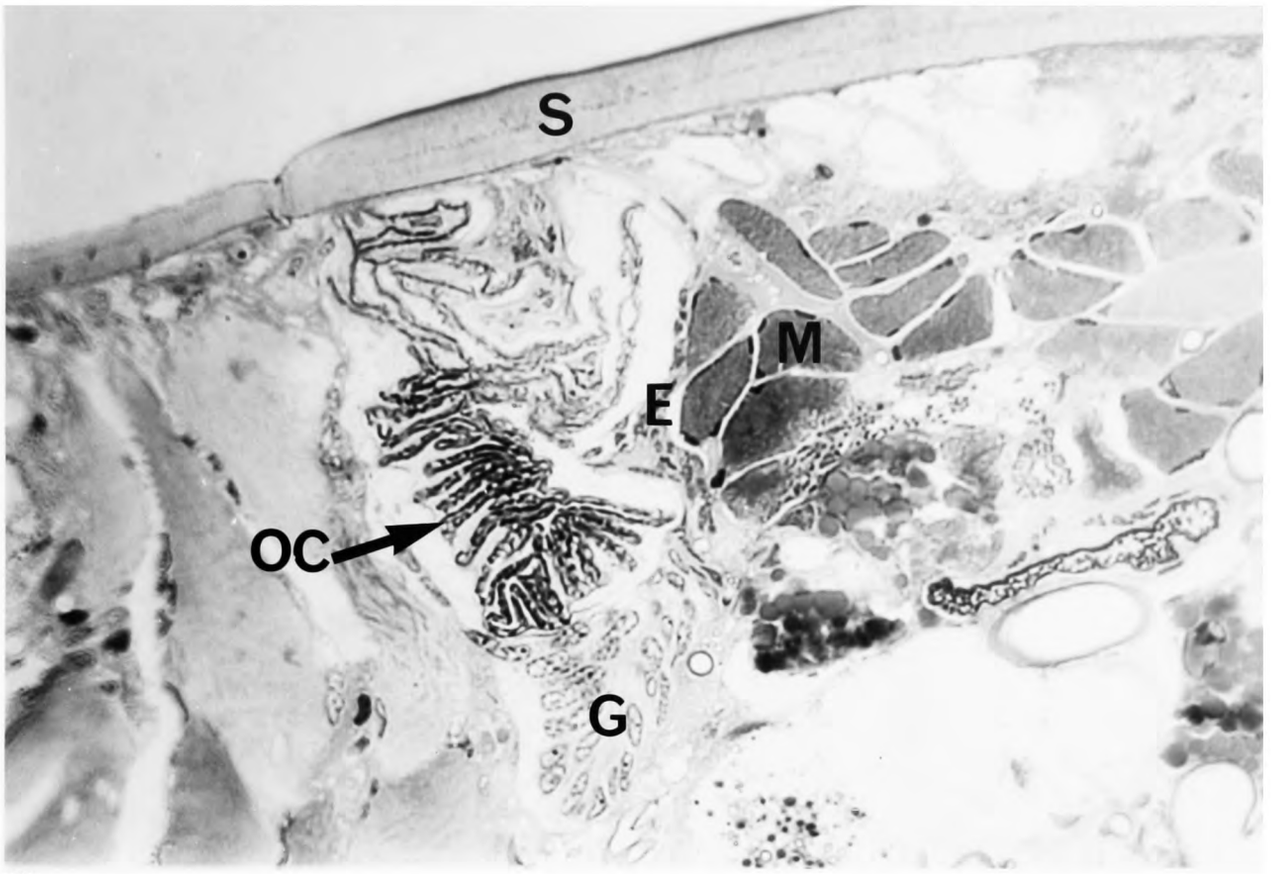


b

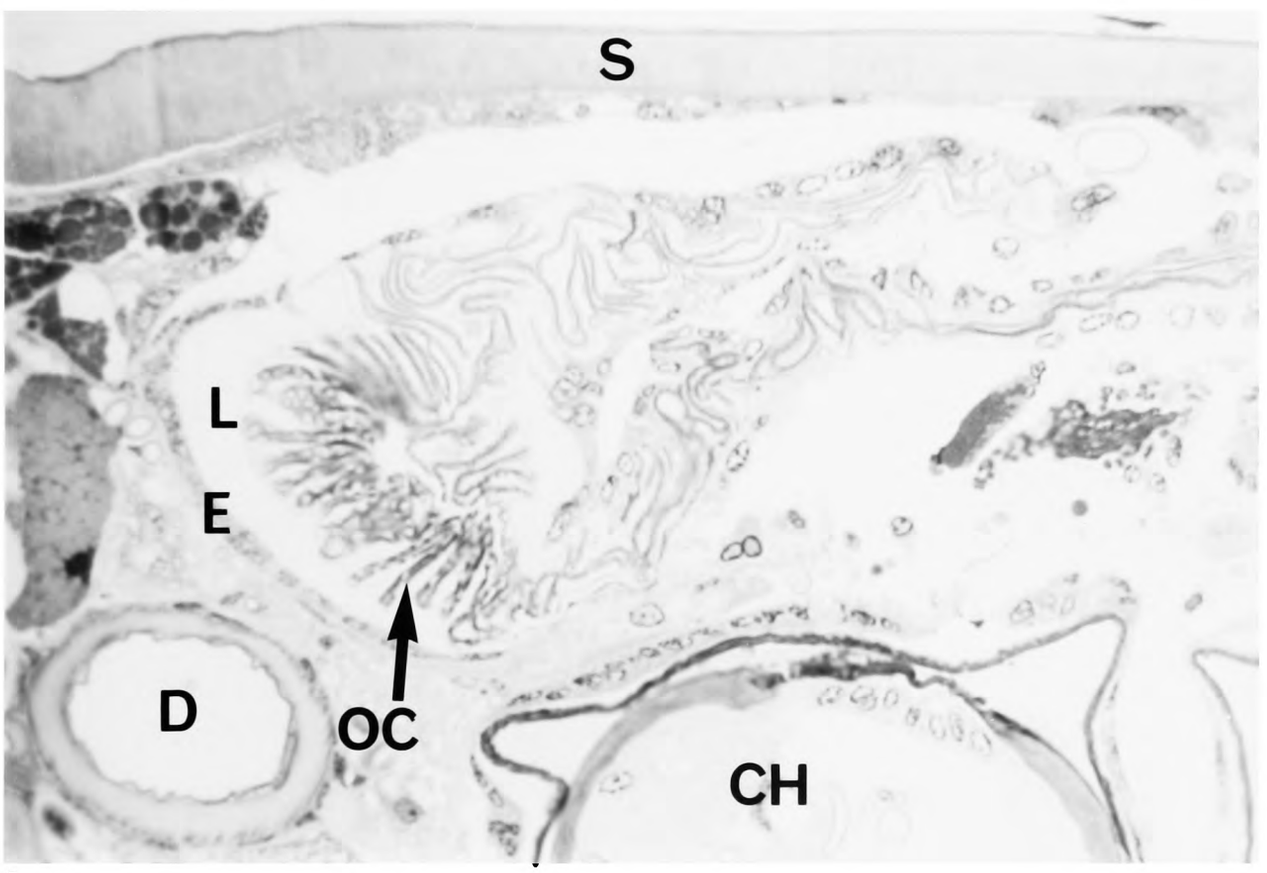
Fig. 3.12 a. Light micrograph showing a retracted horn of Gené's organ in an unengorged adult female B. microplus in transverse section. The outer cuticle of the horns (OC) containing the secretory pits, is highly folded and collapsed in on itself and is surrounded by a very thin layer of epithelial cells (E). The secretory glands (G) are continuous with the horn epithelium but are poorly developed at this stage. The scutal retractor muscles (M) with their dark staining peripheral nuclei can be seen in transverse section. S, cuticle of scutum.

b. Section through a partially engorged female B. microplus, showing one of the horns of Gené's organ, sectioned in a slightly different plane to that of section a. The thin epithelial cells (E) are stretched to accommodate the lumen contents (L) causing the retracted horns to be more distended, with the folded outer cuticle (OC) in the centre. The epithelial cells of the horn are also larger than in section a. S, scutum; D, salivary duct; CH, cheliceral shaft.

a and b x 700.



a



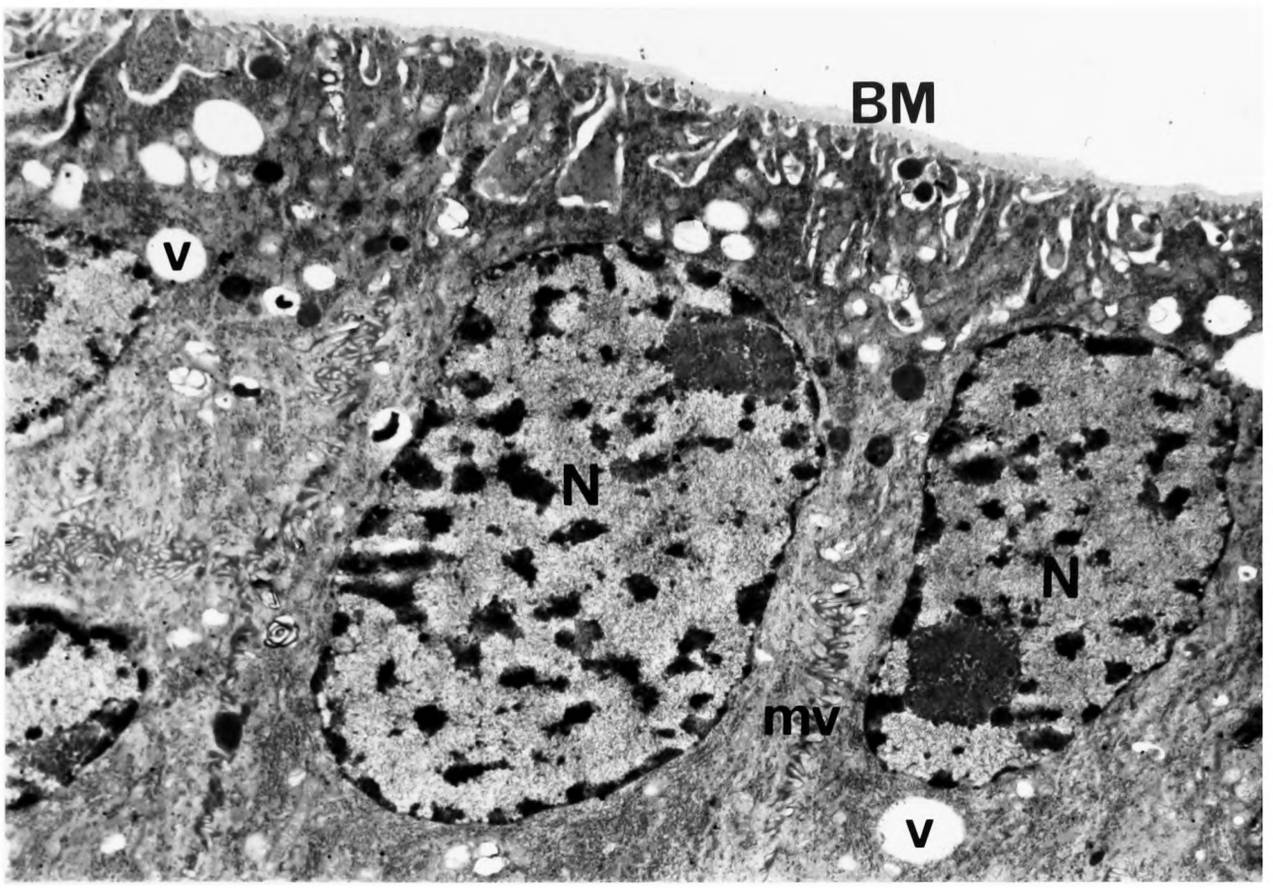
b

Fig. 3.13 a. TEM showing the basal region of three of the secretory cells of the tubular glands of Gené's organ which form the columnar epithelium, in an ovipositing B. microplus. BM, basement membrane; N, nucleus; V, secretory vesicles probably of lipoidal nature; MV, microvillar border of the apical surface of the cells which is folded into intercellular crypts.

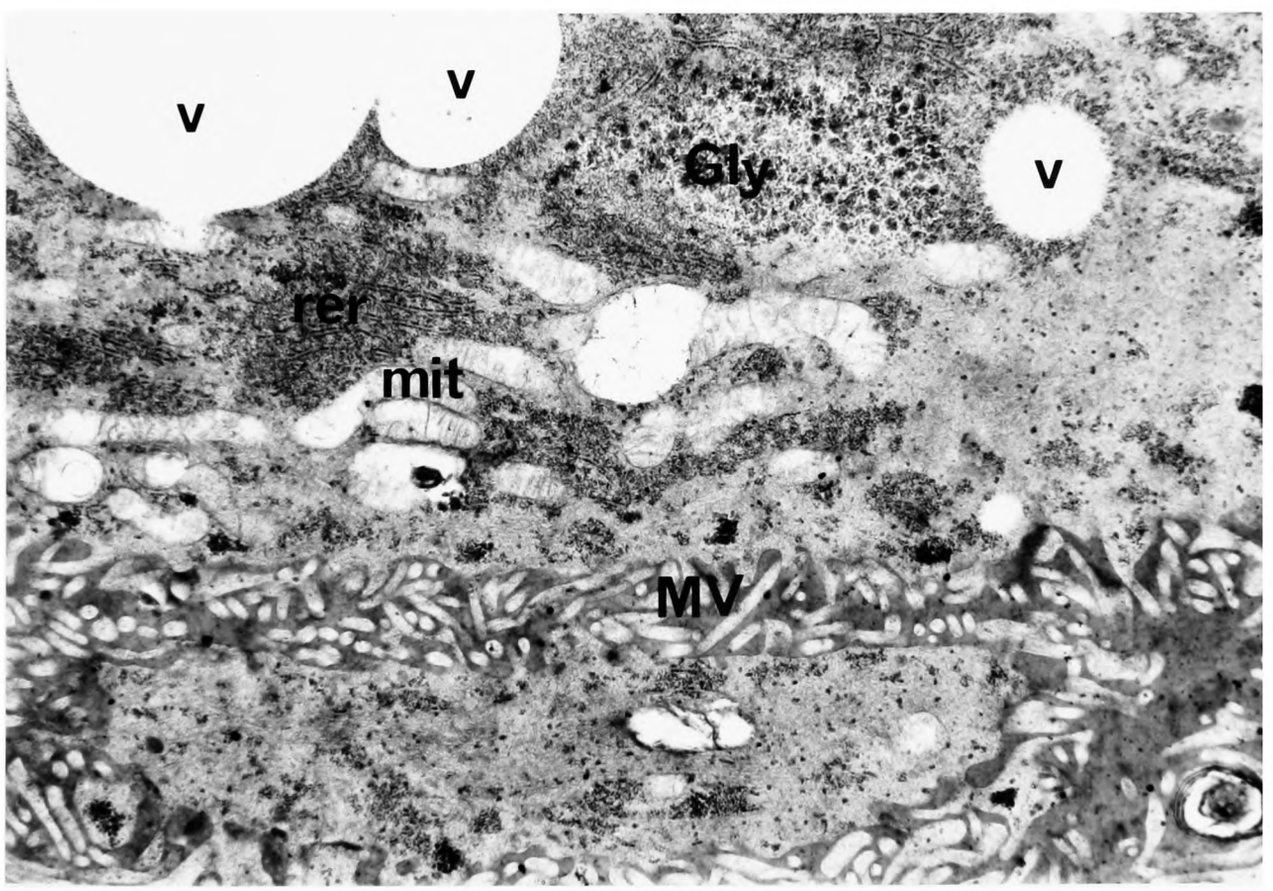
x 5,300

b. TEM showing an apical surface of a secretory cell of Gené's organ, from an ovipositing B. microplus. V, lipid vesicles; Gly, glycogen particles; mit, mitochondrion; rer, rough endoplasmic reticulum; MV, microvillar border.

x 13,300



a



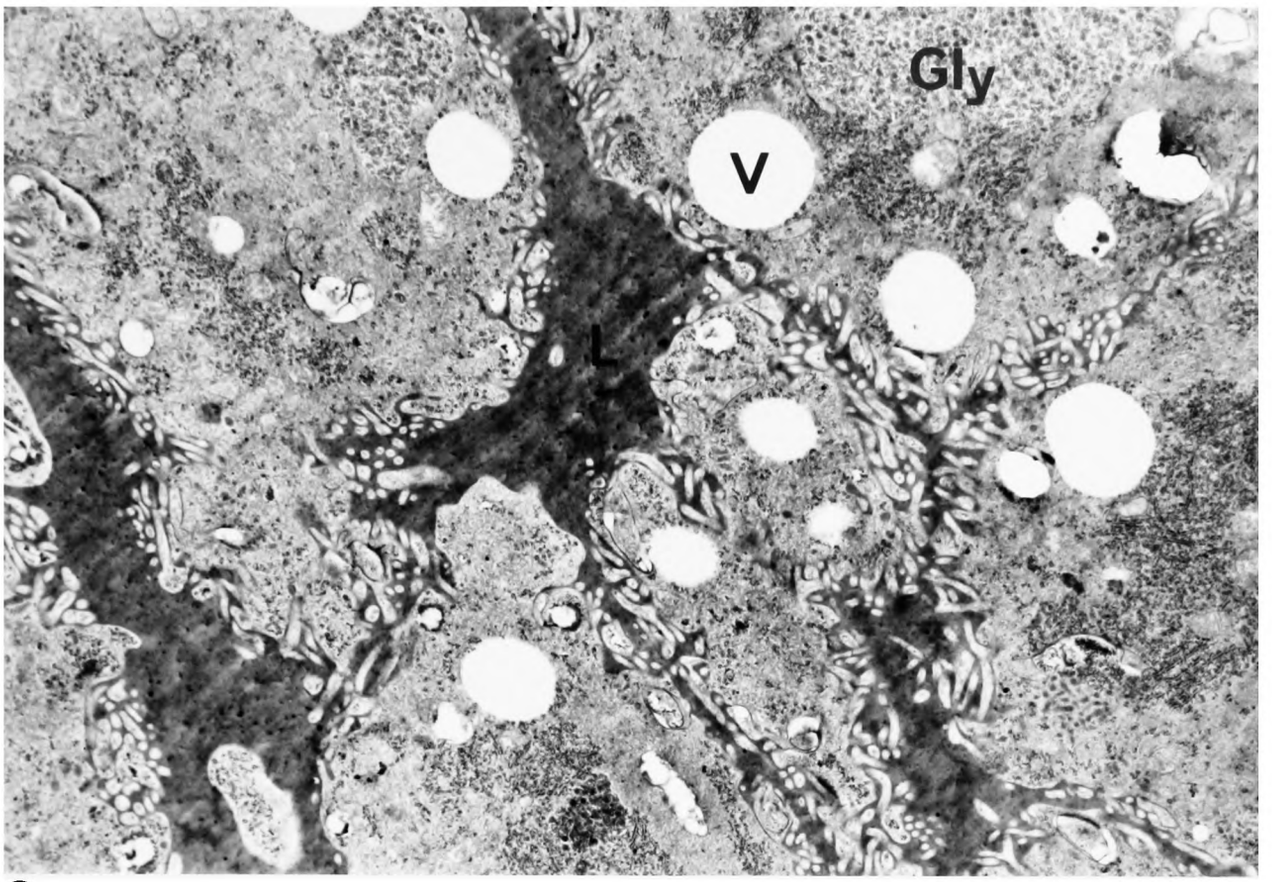
b

Fig. 3.14 a. Transverse section through the apex of the secretory cells of Gené's organ, showing the lumen contents (L), lipid vesicles (V) and extensive glycogen stores (Gly) in an ovipositing B. microplus.

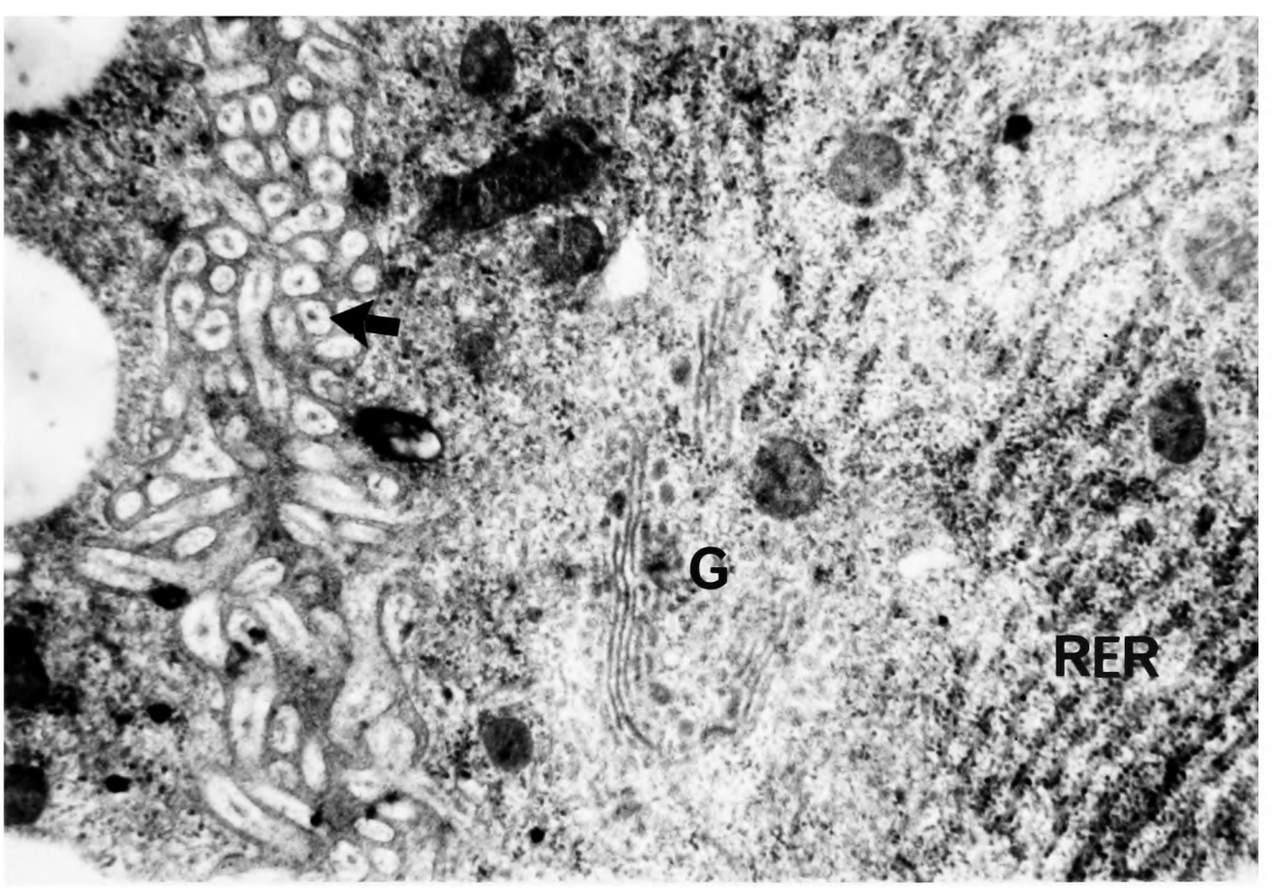
x 8,000

b. TEM showing the apical surface of one of the secretory cells of Gené's organ, showing the microvilli on the apical surface containing internal tubular membranous projections, here seen within each villus in transverse section (arrow). G, Golgi dictyosome; RER, rough endoplasmic reticulum.

x 34,000



a



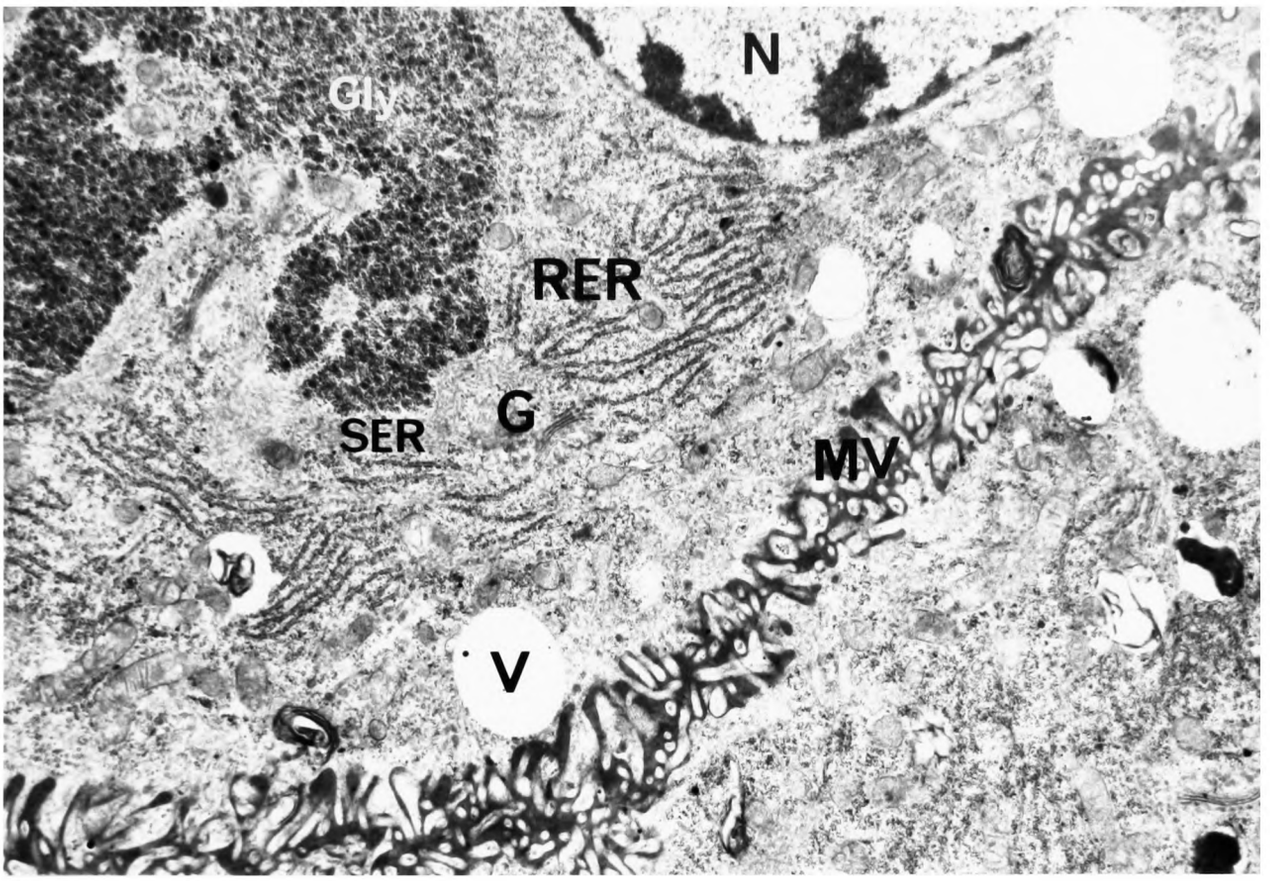
b

Fig. 3.15 a. TEM showing the distribution of various organelles in the Gené's organ secretory epithelium in the early stages of oviposition in B. microplus. N, nucleus; Gly, glycogen particles; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; G, Golgi body; V, lipid vesicles; MV, microvillar border.

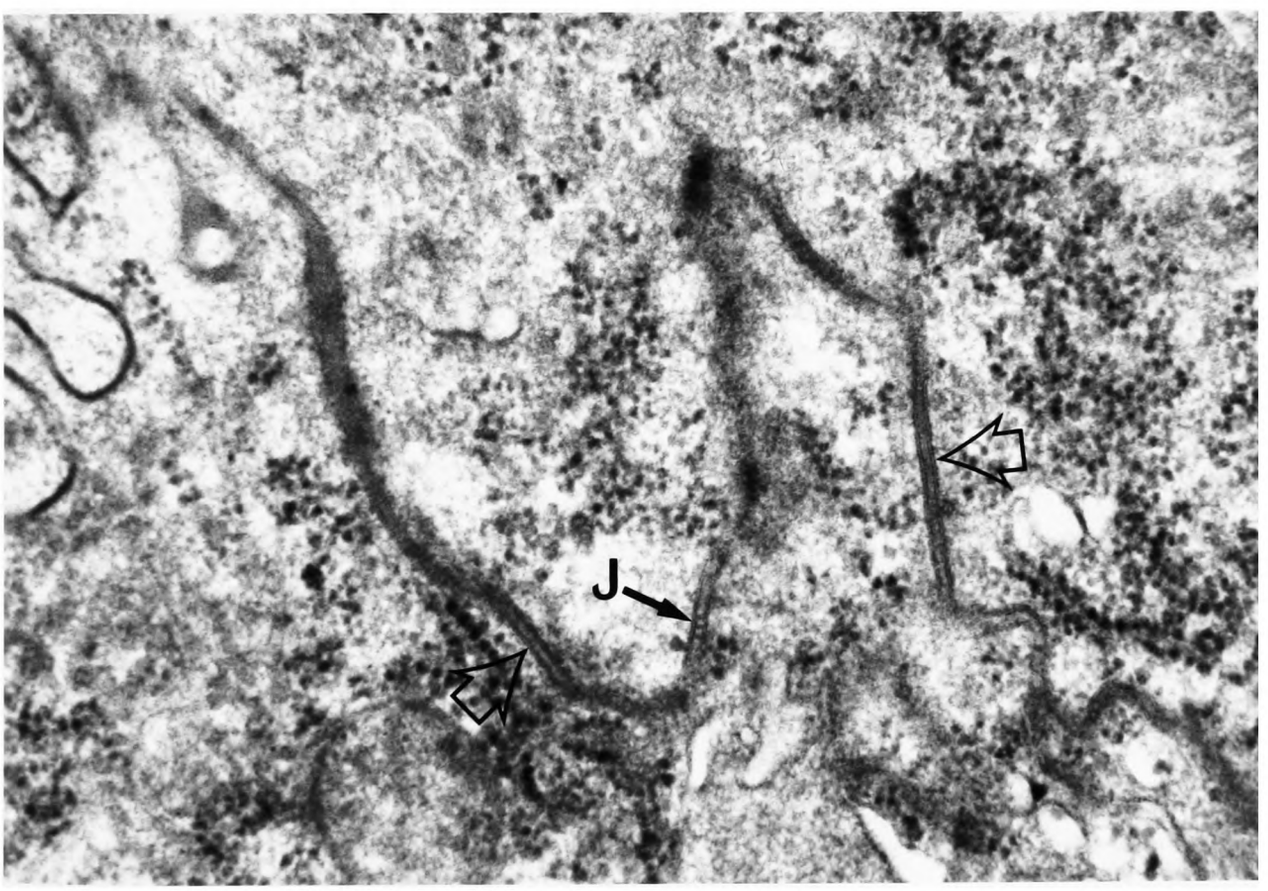
x 13,300.

b. TEM showing the intercellular connections between the lateral plasma membranes of adjacent cells in the secretory epithelium of Gené's organ. The double unit-membrane structure of the cell junctions can be seen (arrows) and septate bridges (J) between the membranes can be seen where the plane of the section cuts the junction at the correct angle.

x 66,500.



a



b

Fig. 3.16 TEM showing the microvillar crypts (MV) penetrating very near to the basal border of the cells (BM) of the Gené's organ secretory epithelium. The basal plasma membrane is infolded, forming extracellular spaces (arrows). In addition to the usual lipid vesicles (V) strange crystal-like vesicles (CV) are visible, which are probably also lipoidal in nature. The ramifications of the smooth endoplasmic reticulum (SER) into the microvilli can be clearly seen.

x 15,100.

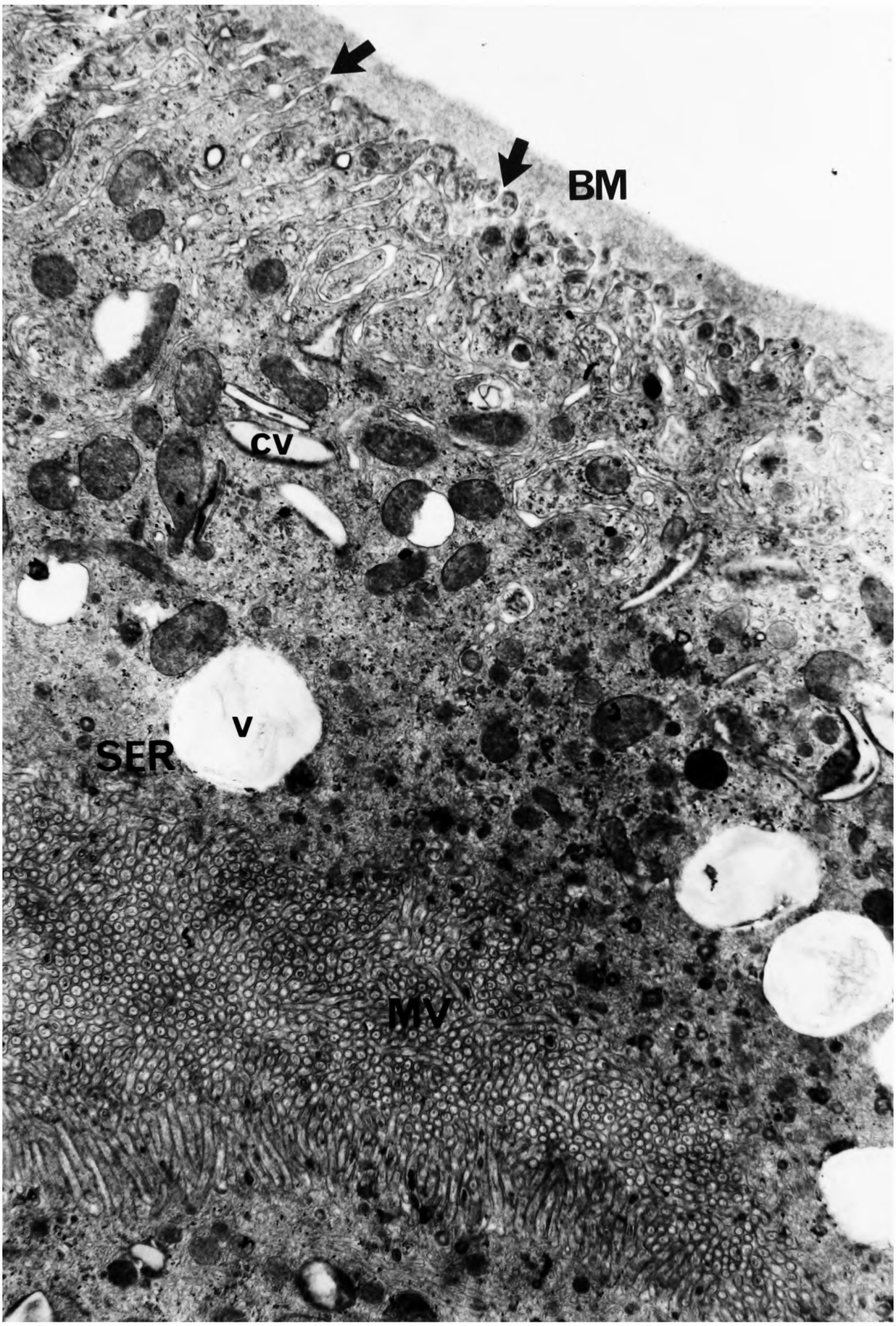

BM
CV
V
SER
MV

Fig. 3.17 TEM showing detail of the microvillar border in the intercellular crypts of Gené's organ showing the projection of the smooth membrane elements into the villi. SER, smooth endoplasmic reticulum.

x 19,600.

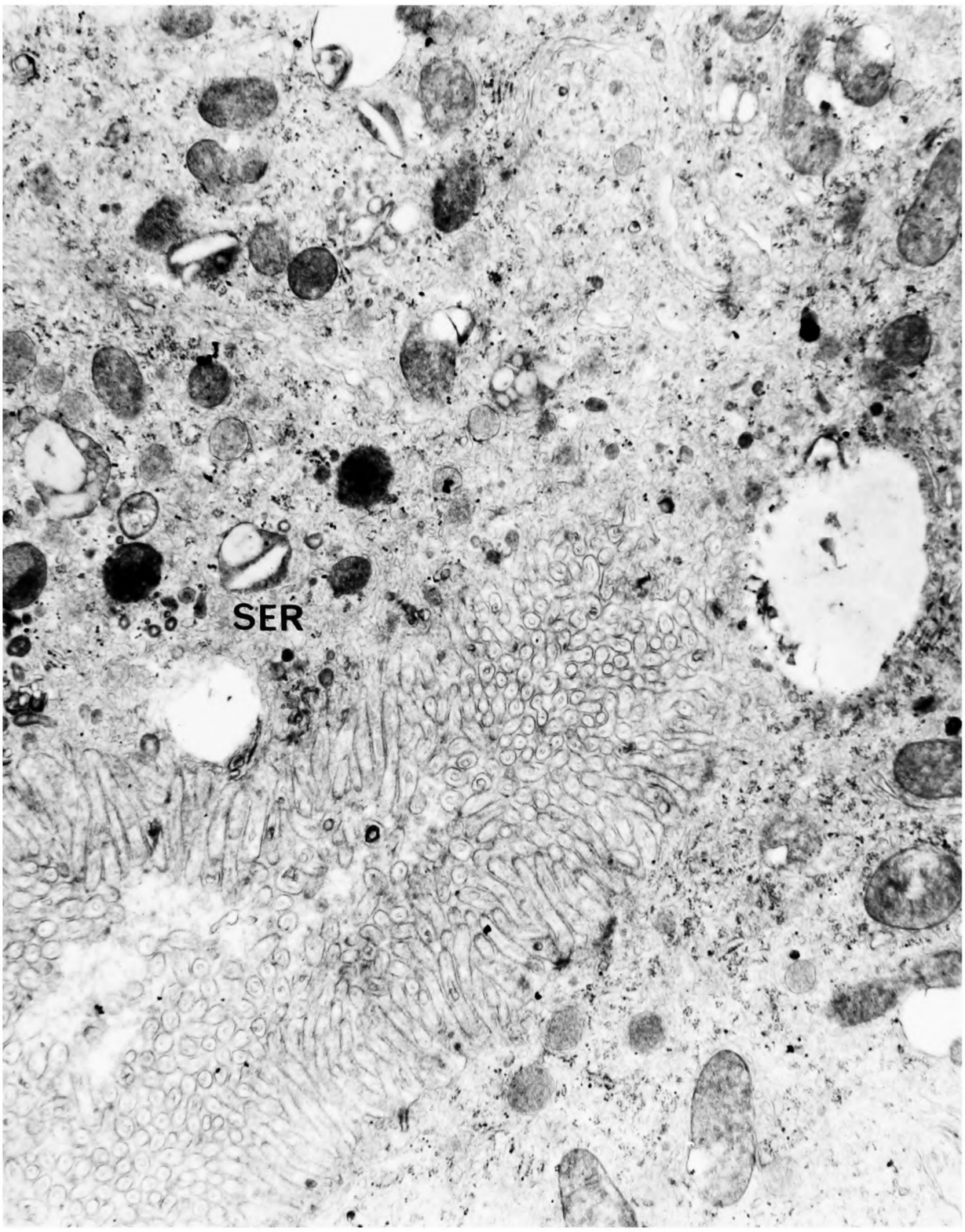

SER

Fig. 3.18 Generalised diagram showing the distinctive features of the glandular epithelium of Gené's organ. BM, basement membrane; Gly, glycogen stores; ER, endoplasmic reticulum; V, secretory vesicles; L, lumen containing budding vesicles of cytoplasm. G, Golgi body.

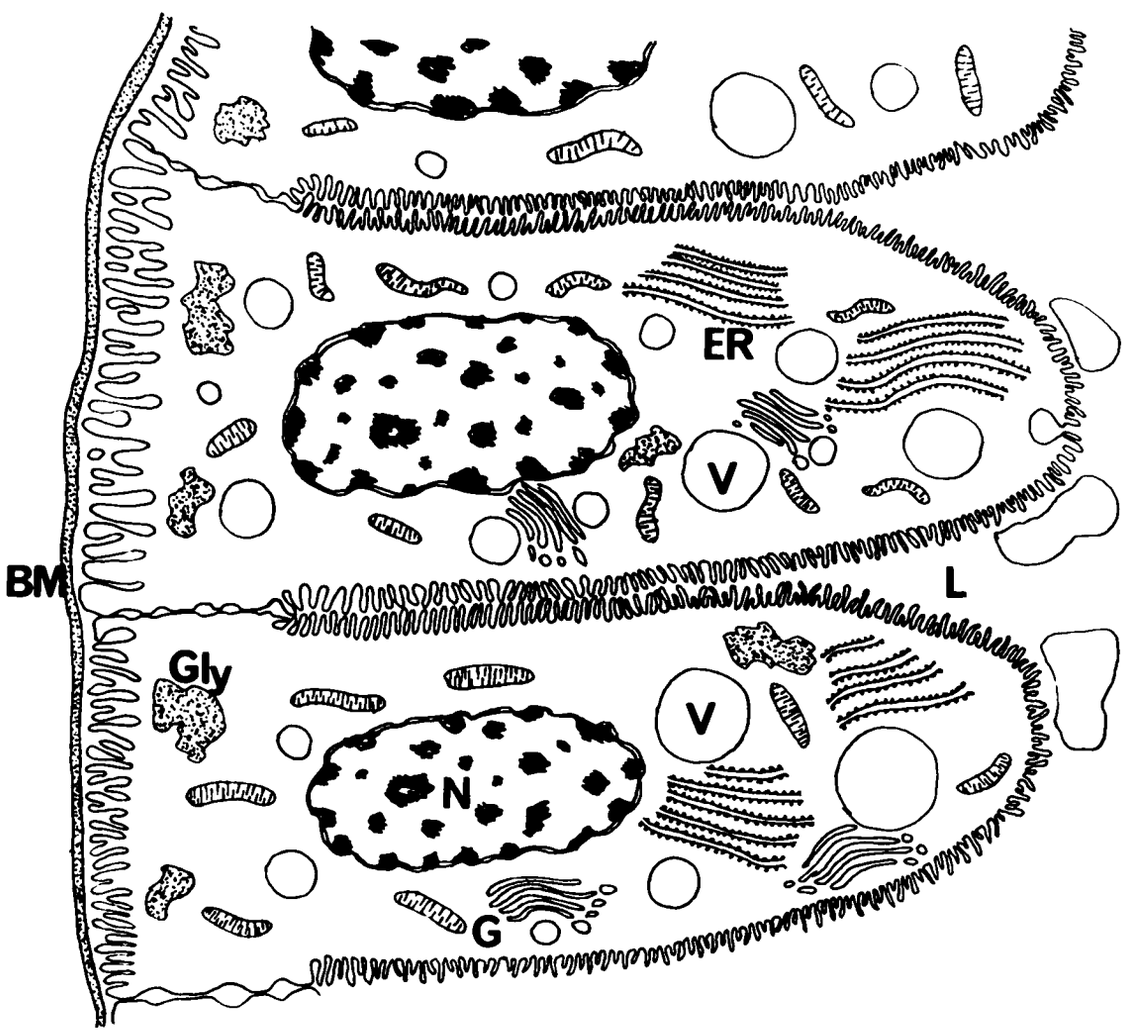
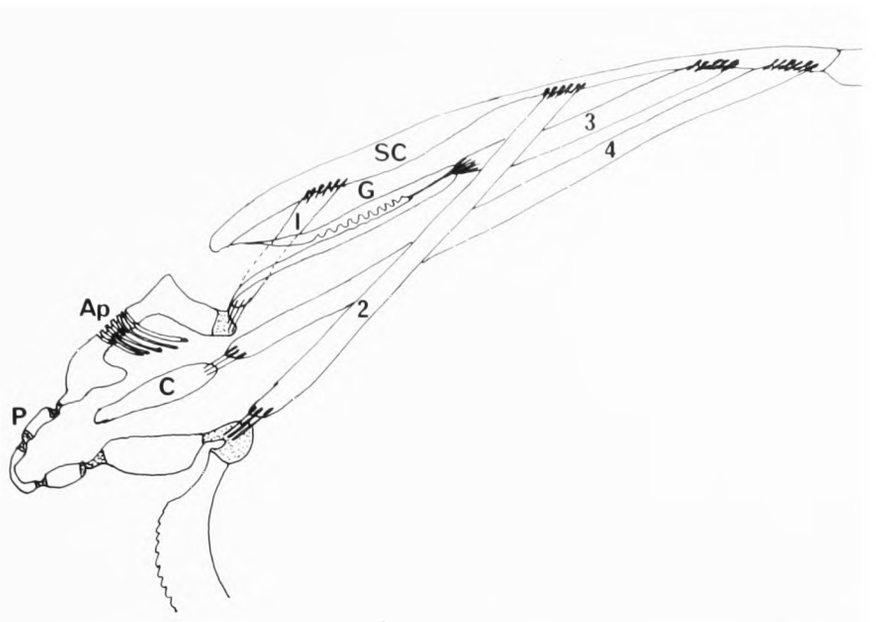

ER
V
BM
L
Gly
V
N
G

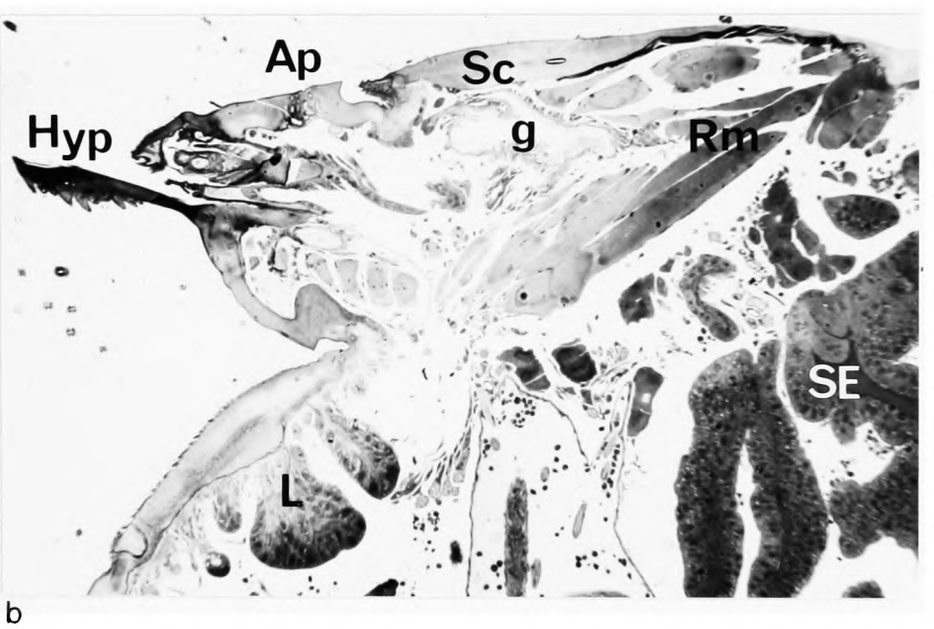
Fig. 3.19 a. Diagram showing the musculature of the mouth-parts and the horns of Gené's organ of an engorged female B. microplus in vertical sagittal section. SC, scutum; G, Gené's organ; Ap, areae porosae; P, palp; C, chelicerae; 1, hypostome extensor muscles; 2, hypostome depressor muscles; 3, Gené's organ retractor muscles; 4, cheliceral retractor muscles.

b. Light micrograph showing a typical sagittal section of an engorged female B. microplus, used in determining the muscle anatomy. Sc, scutum; Hyp, hypostome; Ap, areae porosae; g, Gené's organ (horns only); Rm, retractor muscles; SE, secretory epithelium of Gené's organ; L, lobular glands.

x 80



a



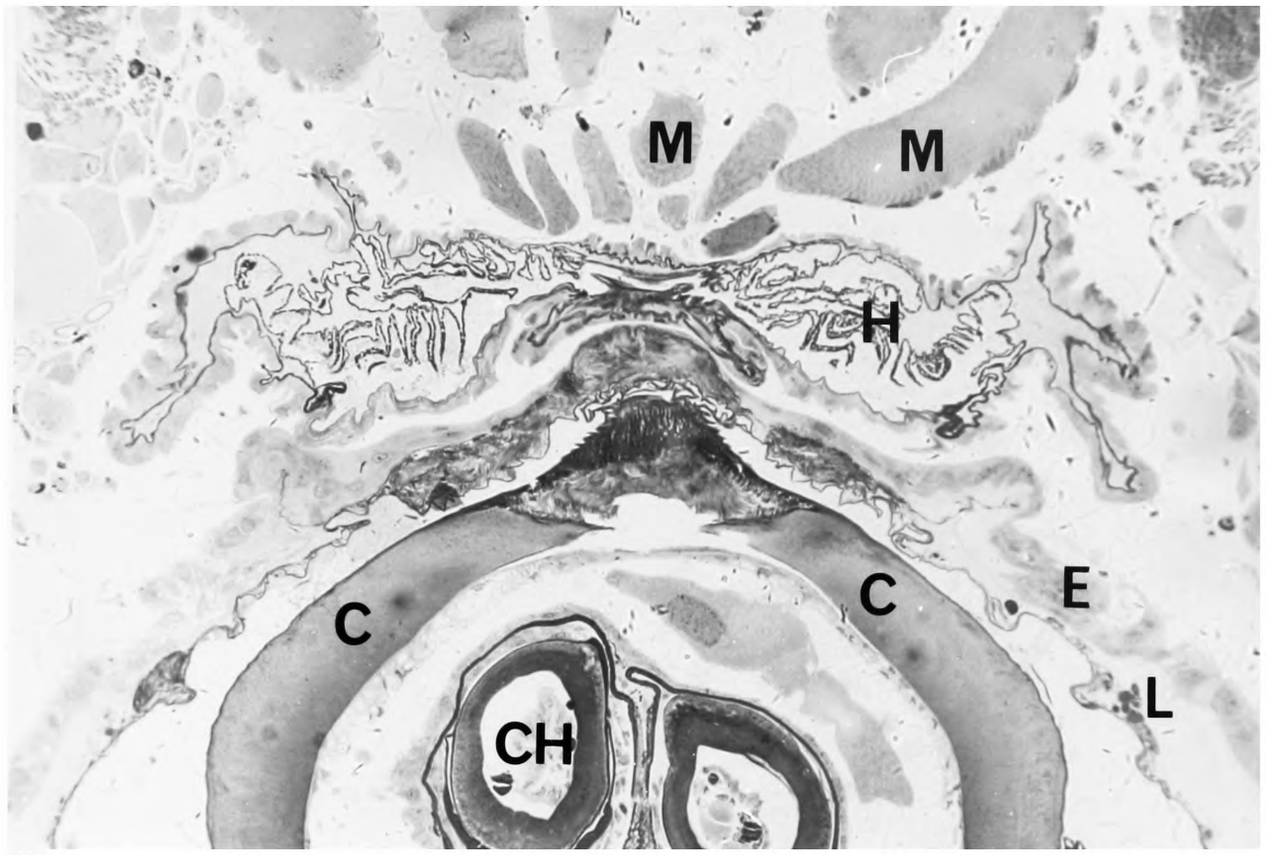
b

Fig. 3.20 a. Light micrograph showing a transverse section through the horns of Gené's organ of an ovipositing B. microplus section immediately anterior to the hypostome extensor muscles (M) showing the retracted horns of Gené's organ (H) before they bifurcate into two lobes. The thin epithelial layer (E) around the articulation of the cuticle of the mouthparts (C) has a very thin layer of cuticle separating the lumen of the lobed gland cells from the outside. CH, chelicerae.

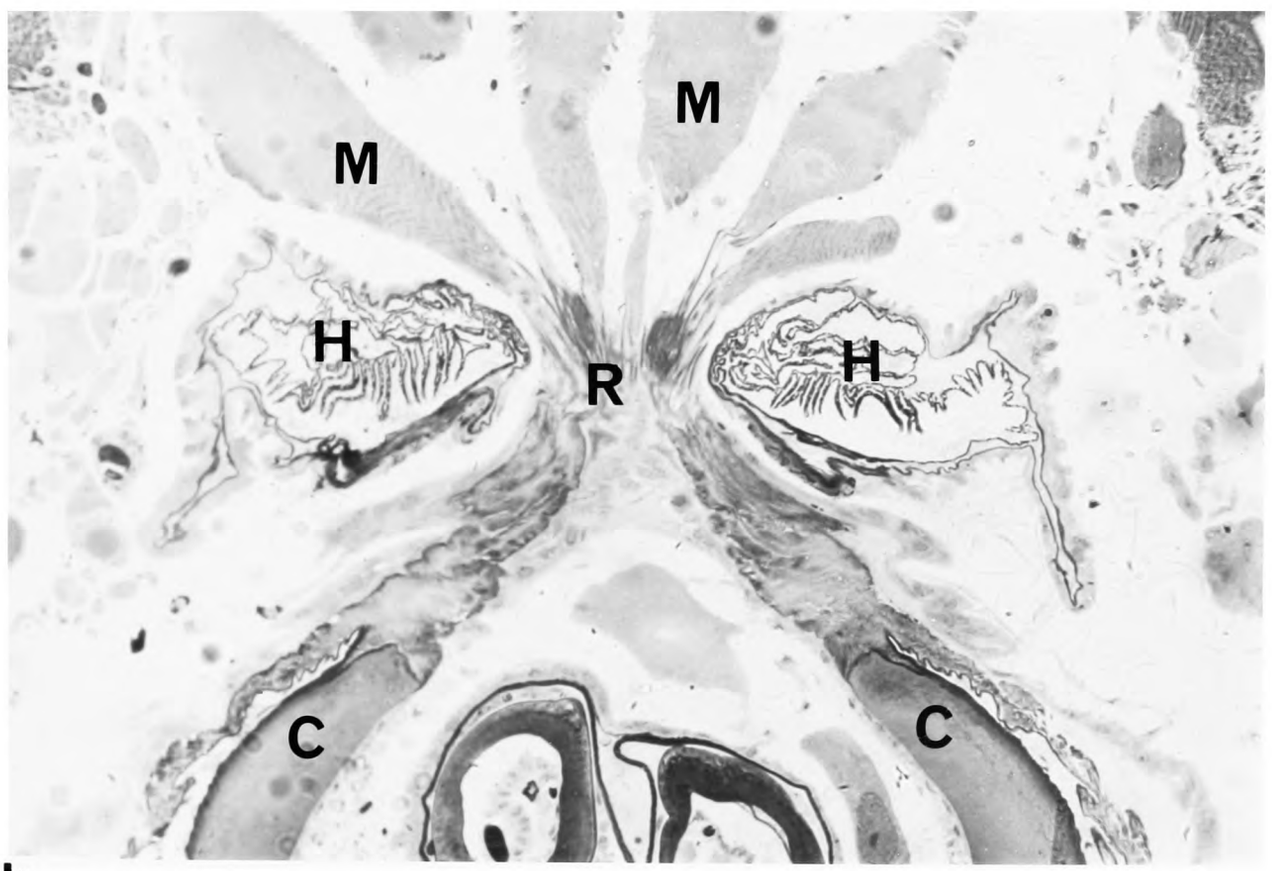
x280.

b. Section in the same plane showing the insertion of the extensor muscles (M) into the dorsal surface of the cuticle of the mouthparts (C) via rods (R). The retracted horns (H) are on either side of the muscle insertions, and the pits in the thin infolded epicuticle of each horn can be seen.

x 280.



a



b

Fig. 3.21 Photomicrograph of a longitudinal section of a retracted horn of Gené's organ in B. microplus, showing the retractor muscles (M) inserting into the horn cuticle (H) by the thin cuticular rods (R). E, epithelial cells of Gené's organ; C, cuticle of mouth-parts; L, lumen of horns. The pits in the outer horn cuticle are also visible.

x 350.

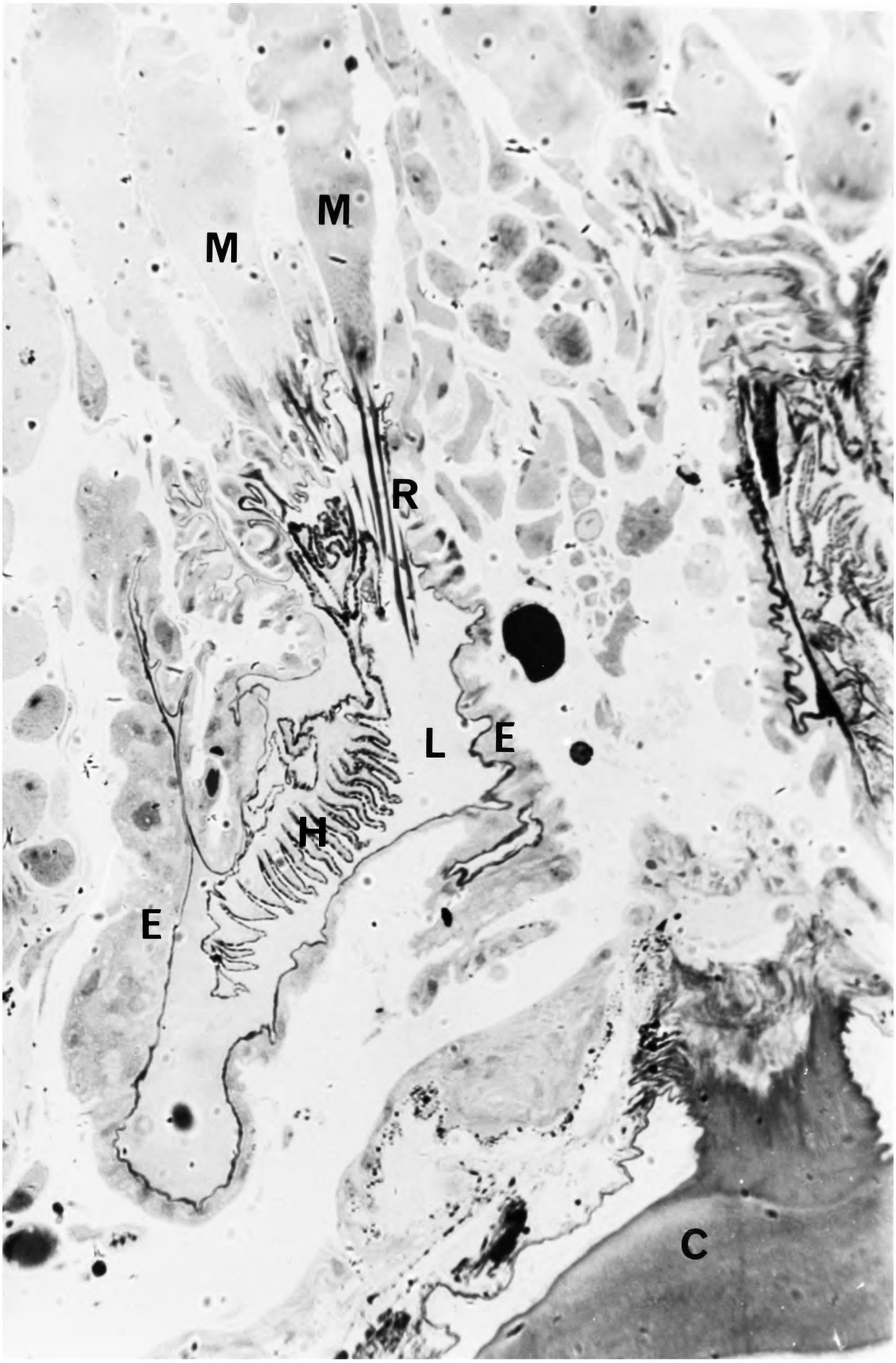

M
M
R
L
E
H
E
C

Fig. 3.22 Diagram showing the organisation of the retractor muscle insertions in the horns of Gené's organ. RM, retractor muscles; R, cuticular rod; IC, inner cuticle; C, outer cuticle of horns; L, lumen of horns; E, epithelial cells of horns; DC, dorsum of capitulum; D, accessory gland ducts.

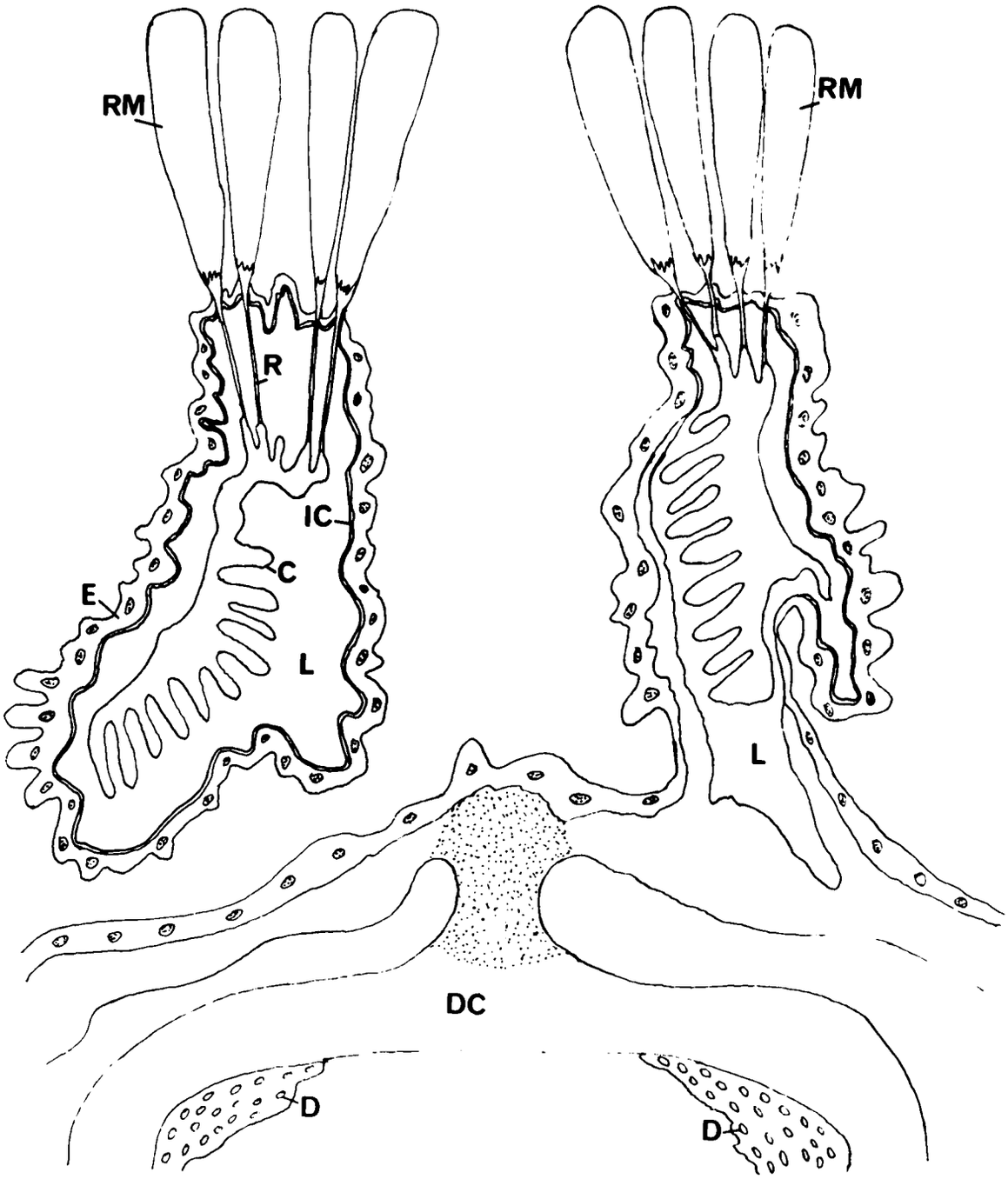
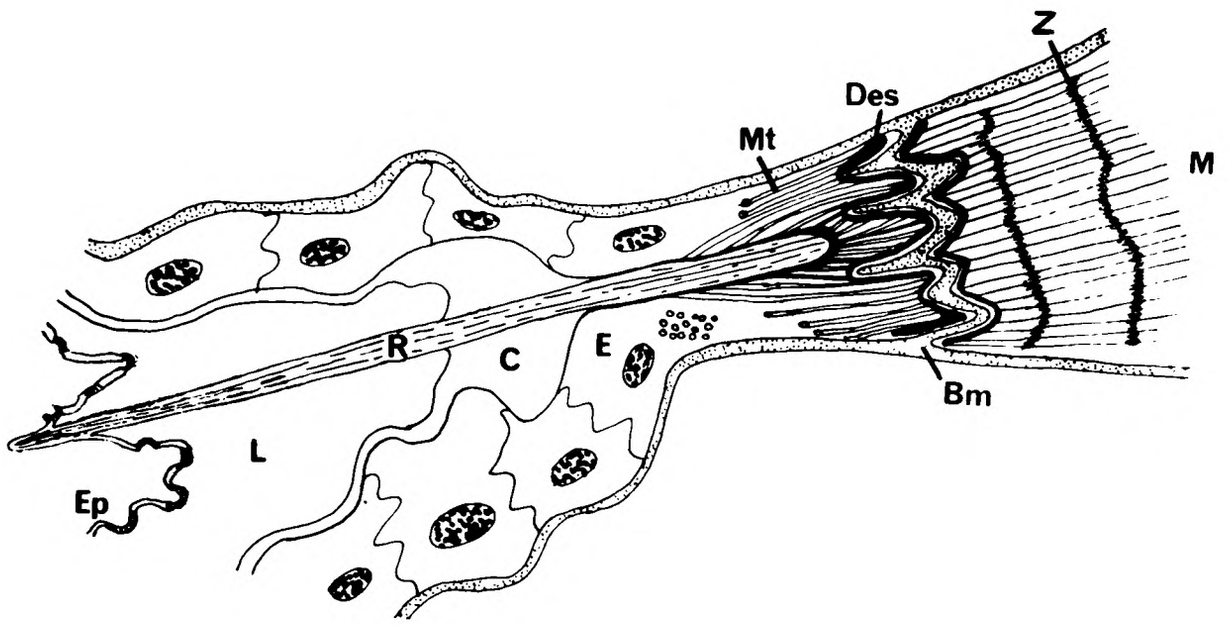

RM
RM
R
IC
C
E
L
L
DC
D
D

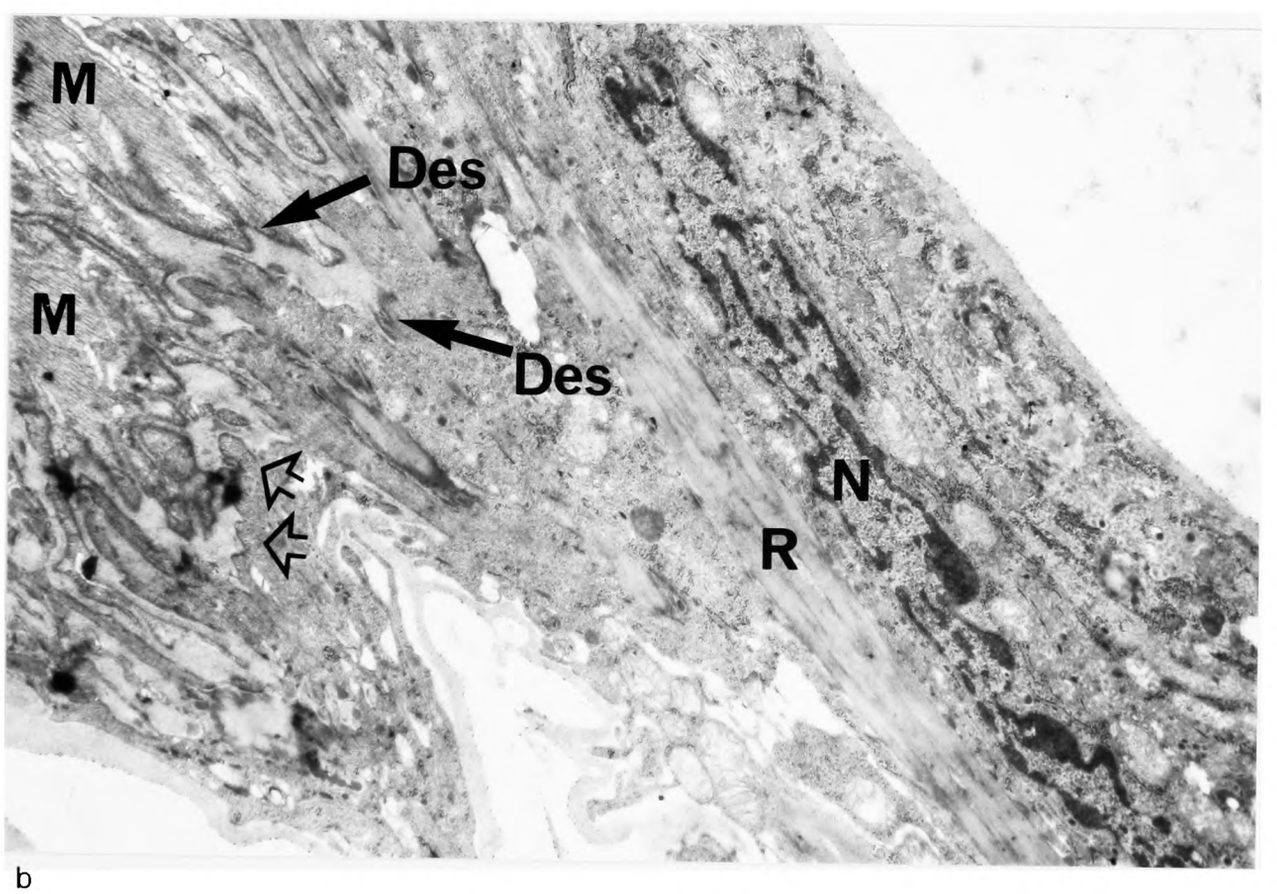
Fig. 3.23 a. Diagrammatic drawing showing the ultrastructural organisation of the rectractor muscle insertions in the horns of Gené's organ. The muscle cell (M) is connected to a tendon cell containing microtubules (Mt) by desmosomes (Des). The microtubules attach to the cuticular rod (R) which passes through the horn lumen (L) and is continuous with the outer epicuticle (Ep). E, epithelial cell; C, inner cuticular layer; Bm, basement matrix.

b. Electron micrograph showing the retractor muscle insertion into a cuticular rod (R) of Gené's organ, composed of cuticular fibres. M, muscle fibres; N, nucleus of tendon cell. Desmosomes (Des) characterize the junction between tendon cell and muscle cell. The tendon cell contains microtubules, often transversely sectioned (clear arrows).

x 10,600.



a



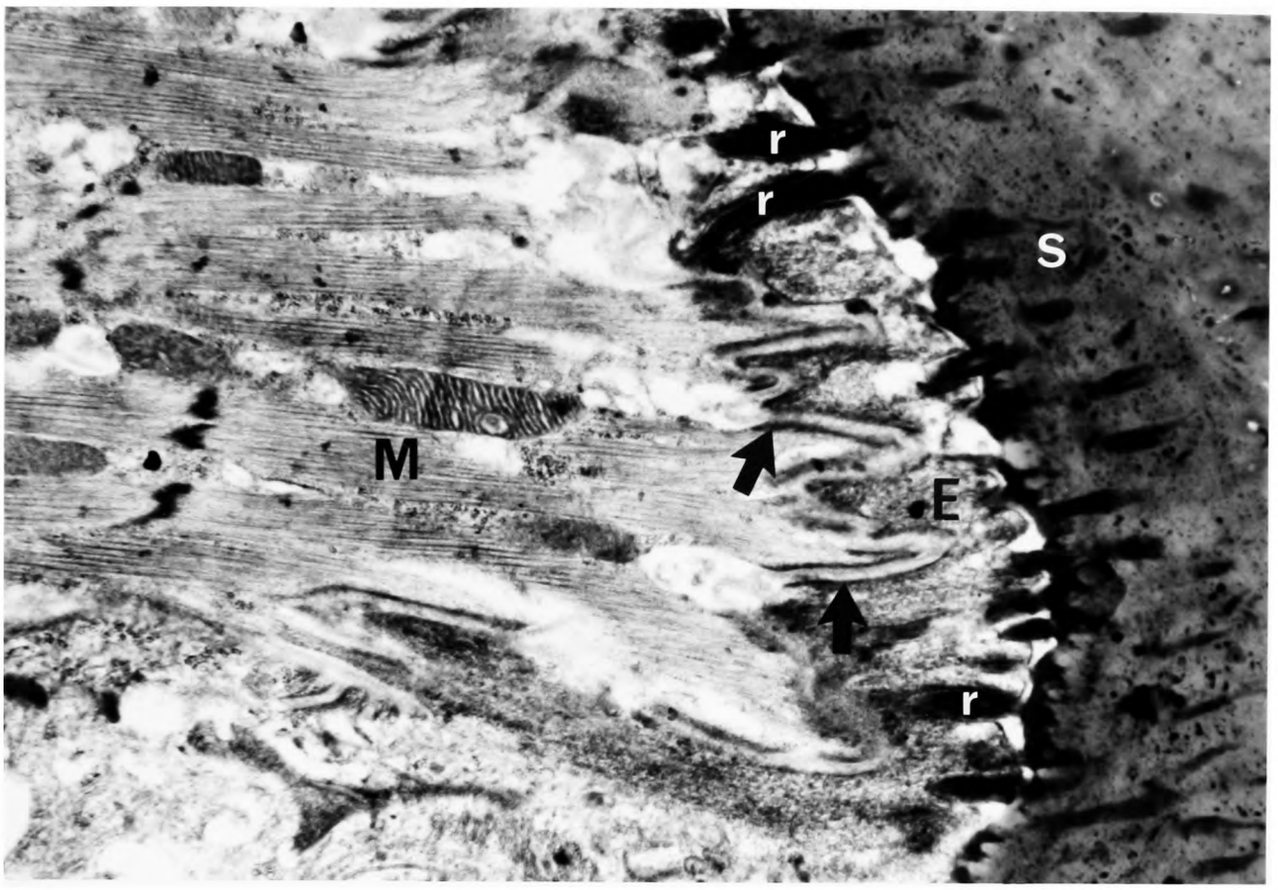
b

Fig. 3.24 a. TEM showing the insertion of a retractor muscle (M) into the scutum (S) in longitudinal section. The muscle interdigitates with the epithelial cell (E) which is linked to the cuticle by means of extracellular rods (r). Within the epithelial cells microtubules, longitudinally sectioned, can faintly be seen (B. microplus). Desmosomes join the muscle with the epithelial cells (arrows).

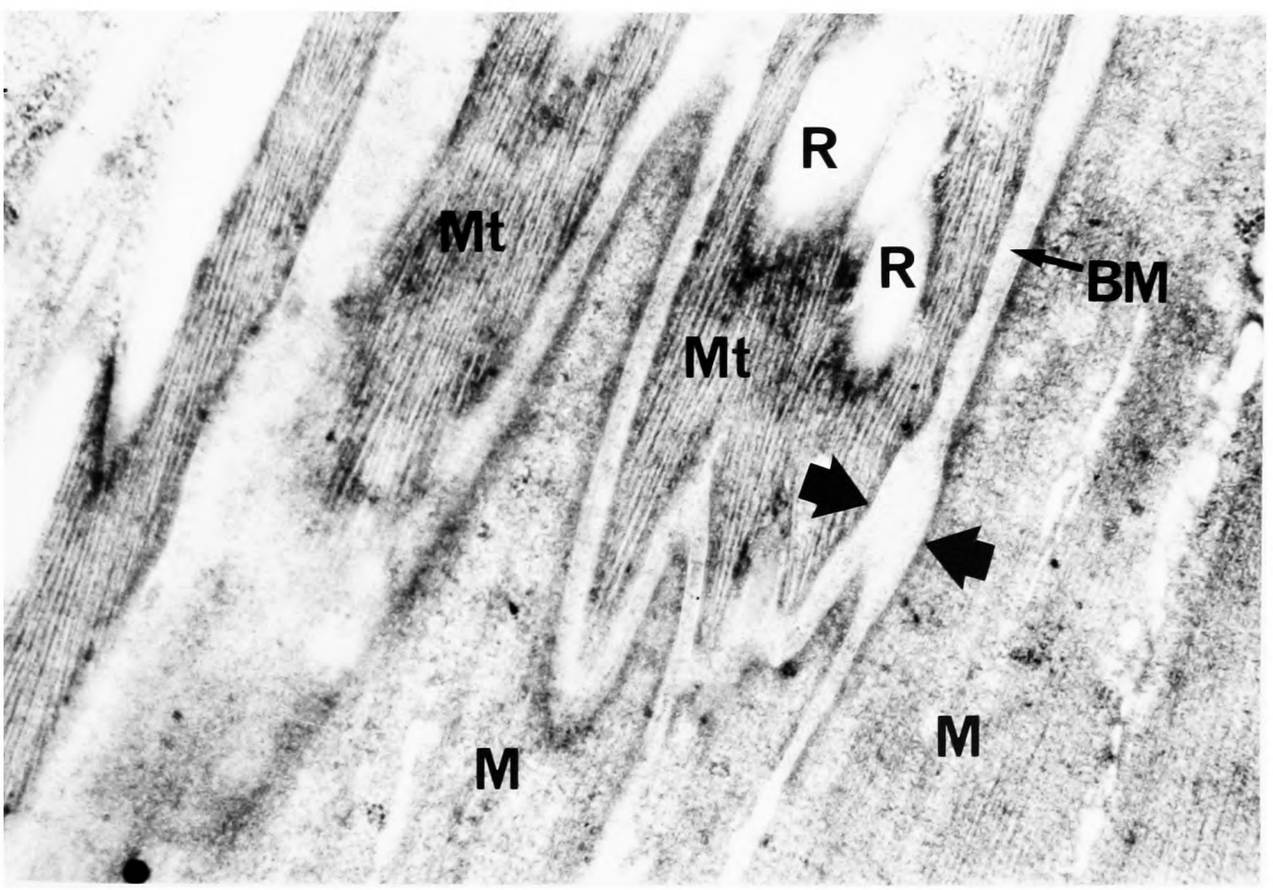
x 10,600.

b. Longitudinal section showing detail of the muscle cell/tendon cell junction in the horns of Gené's organ. The cuticular rod fibres (R) are connected to the muscle cell (M) by microbubules (Mt). The cell-cell junctions are characterised by interdigitating desmosomes (arrows) on either side of the basement matrix (BM).

x 43,200 (B. microplus)



a



b

Fig. 3.25 TEM showing a transverse section of a retractor muscle and its insertion into the horns, dissected from A. variegatum. The interdigitating desmosomes which connect the muscle cell (M) to the epithelial cells via the basement matrix (BM) have dense-staining areas at the microtubule attachment points (arrows). The cuticular rods (r) also have dense staining areas on their periphery. The microtubules in the epithelial cells appear as circular tubes in transverse section (clear arrow).

x 27,000.

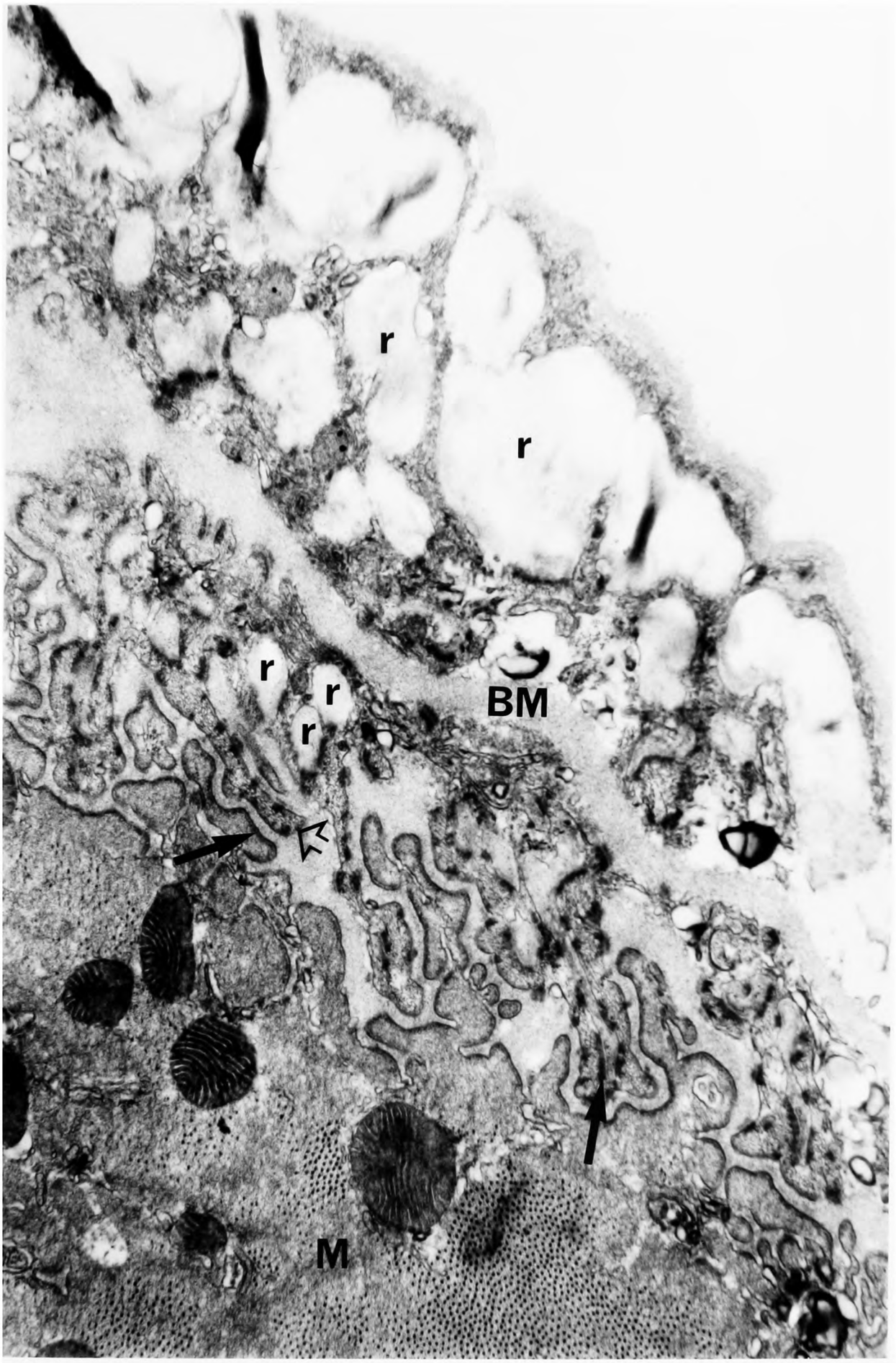
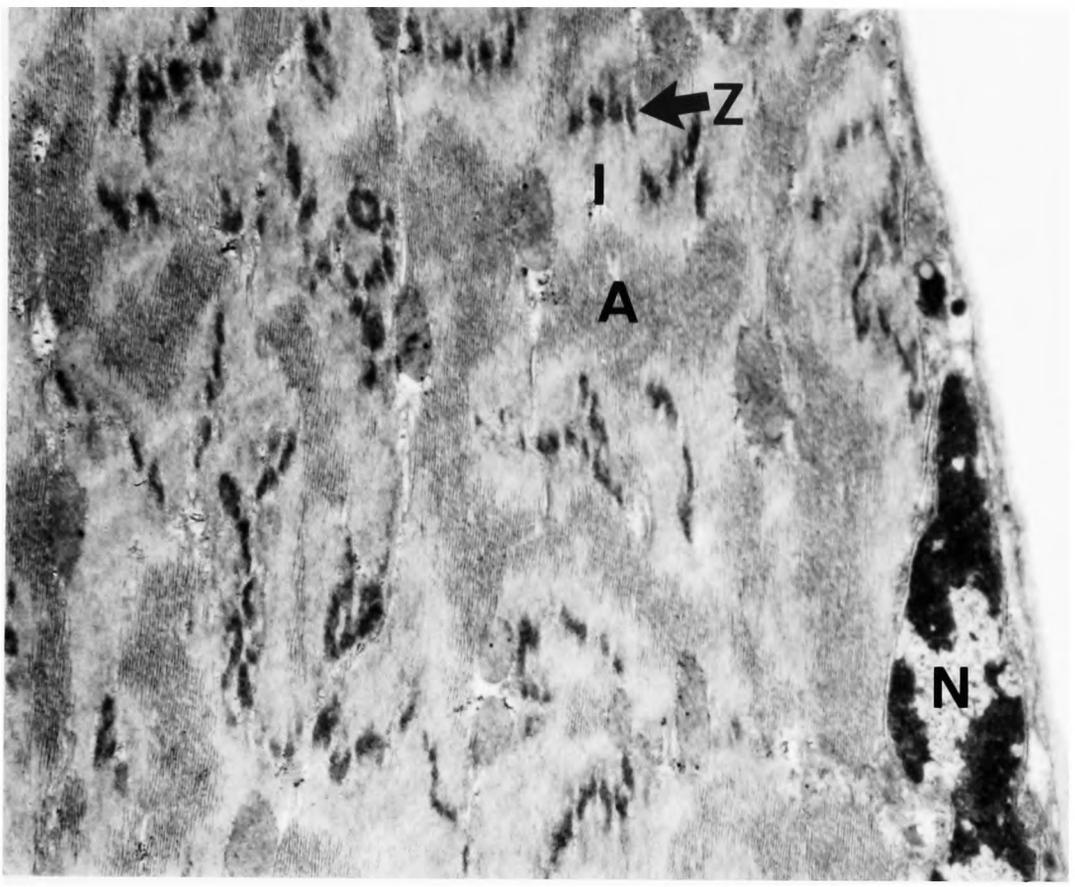

r
r
r
r
r
BM
M

Fig. 3.26 a. TEM of a longitudinal section of a retractor muscle of A. variegatum showing a nucleus (N) and muscle filaments with Z-bands (Z), A-bands (A) and I-bands (I). he, haemolymph.

x 11,200.

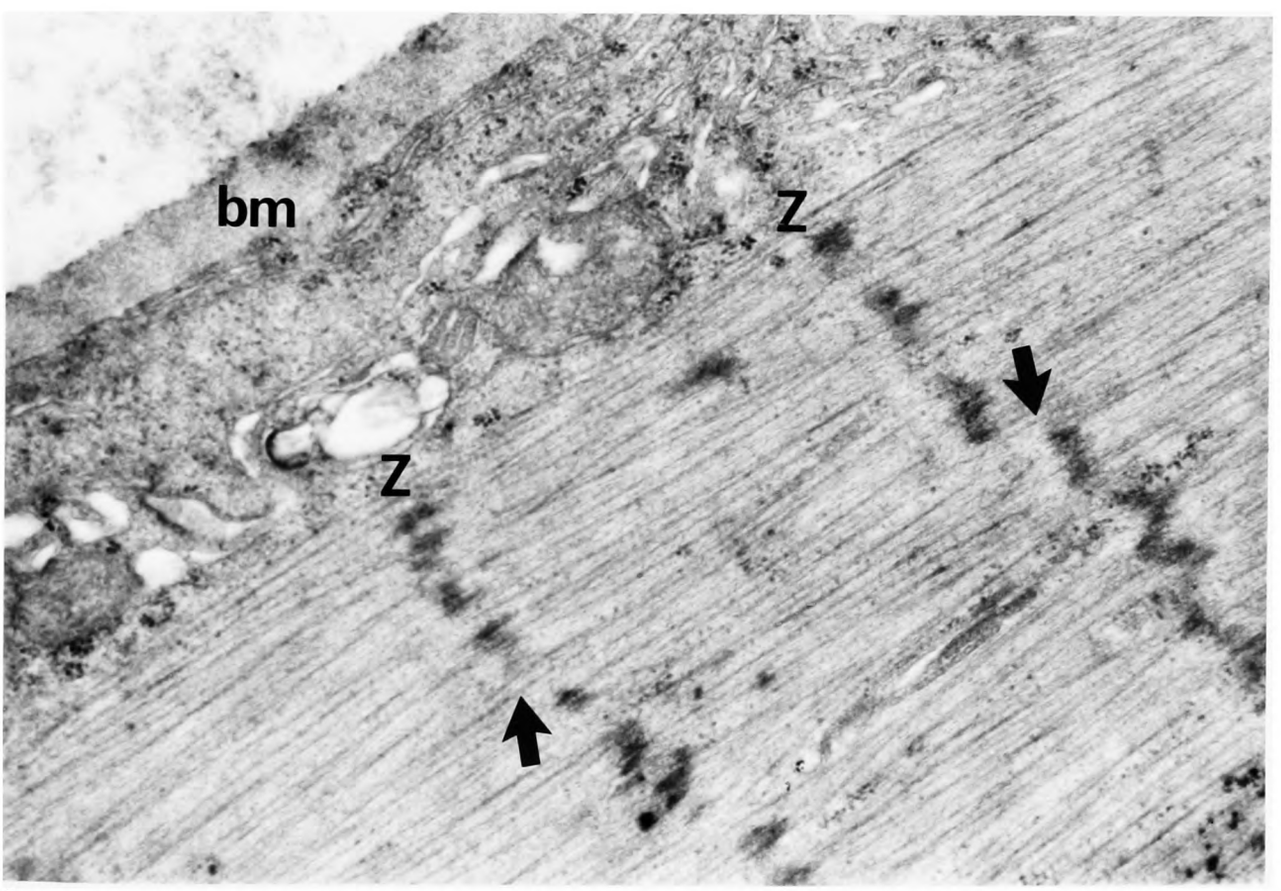
b. Longitudinal section of a retractor muscle of B. microplus shows that some of the muscle fibres pass through the Z-bands (arrows). (bm) - basement matrix.

x 28,000.



he

a



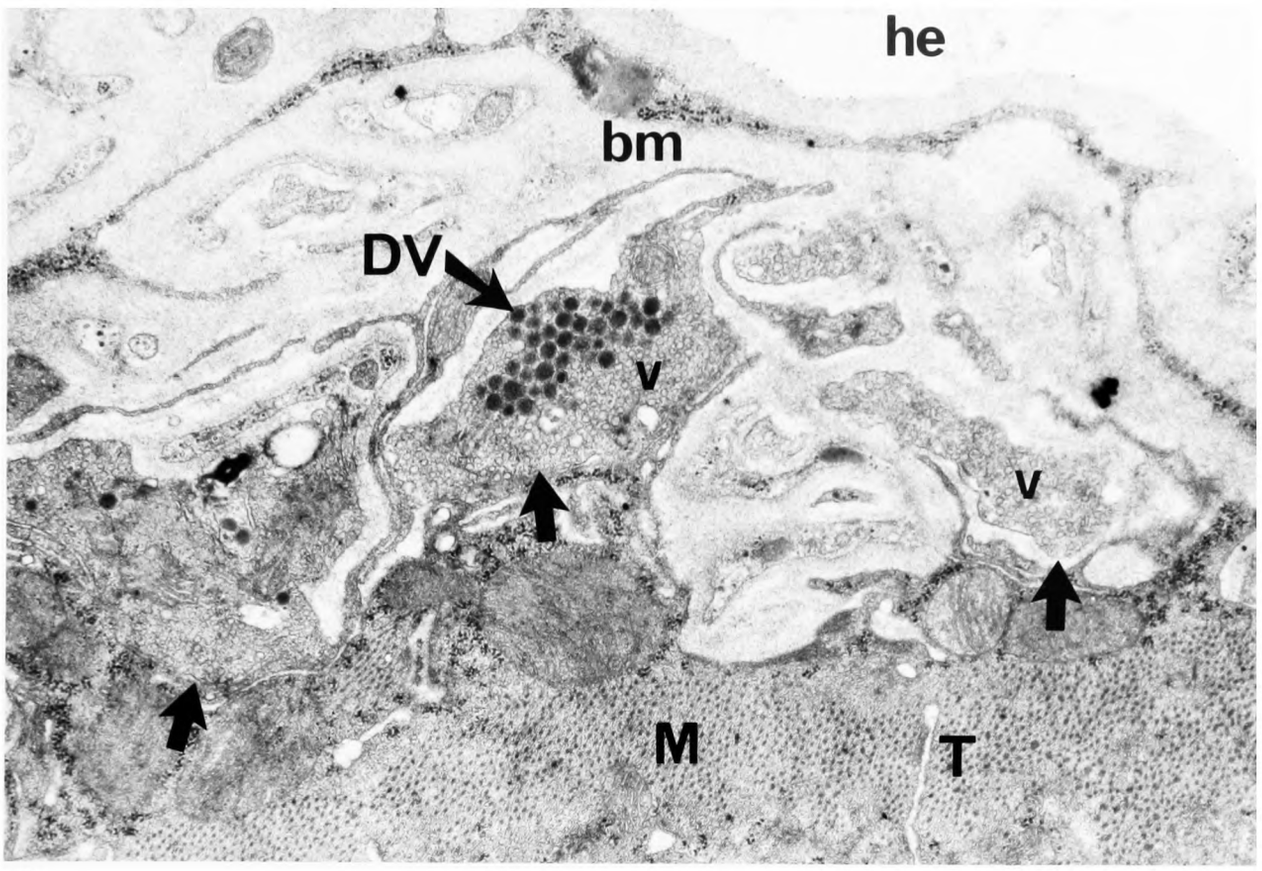
b

Fig. 3.27 a. Transverse section showing a nerve terminal innervating a retractor muscle (M) of B. microplus. The terminal contains both small clear vesicles (V) and dense-cored vesicles (DV). It can be seen that the innervation is multi-terminal (arrows). he, haemolymph; bm, basement matrix; M, muscle cell; T, part of the invaginating transverse tubular system.

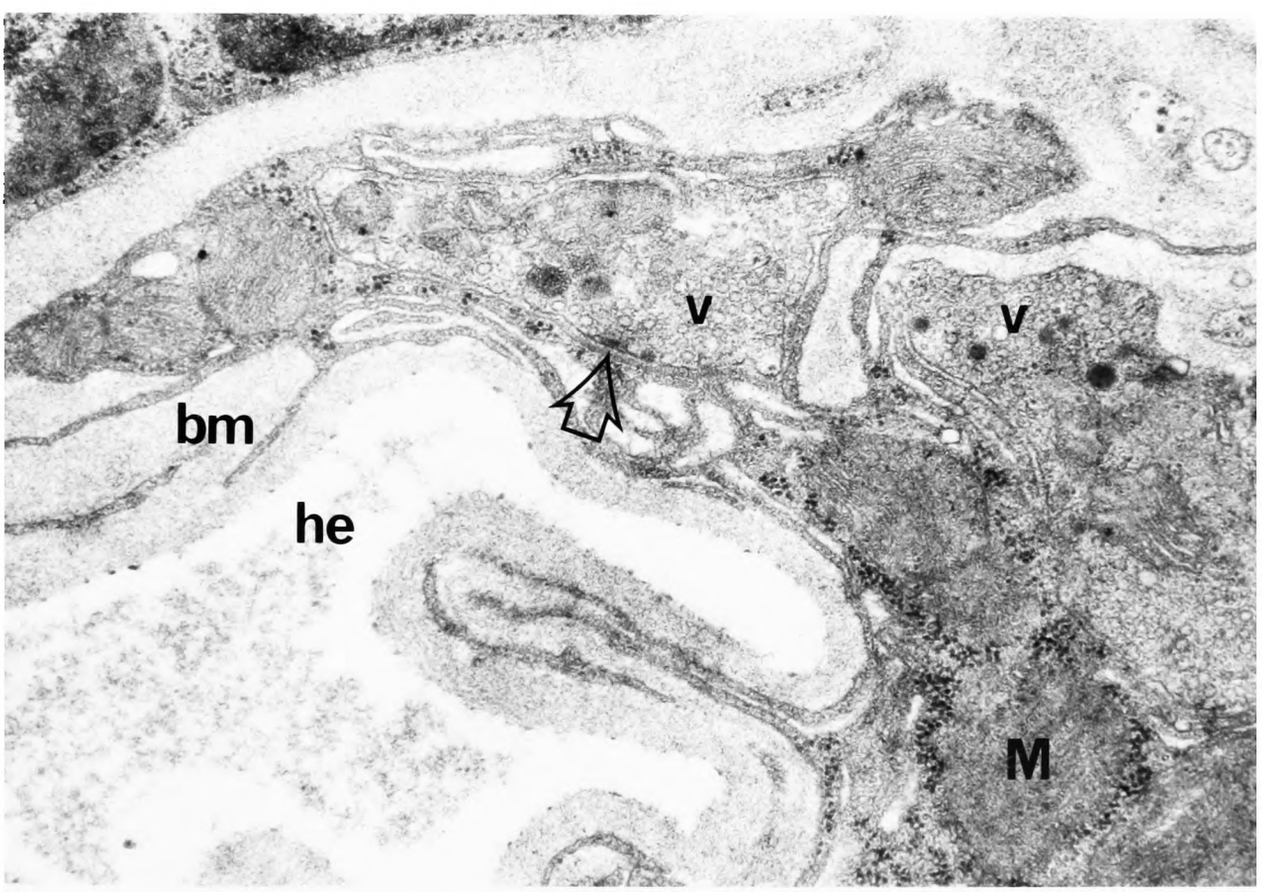
x 19,000

b. A neuromuscular junction in the retractor muscle of B. microplus. The synapse is characterized by membrane density and the presence of vesicles (V) which tend to be aggregated close to a dense staining pre-synaptic projection (arrow). The terminal also has access to the haemolymph (he) by the basement matrix (bm). The muscle cell (M) is characterized by abundant glycogen particles.

x 34,200.



a

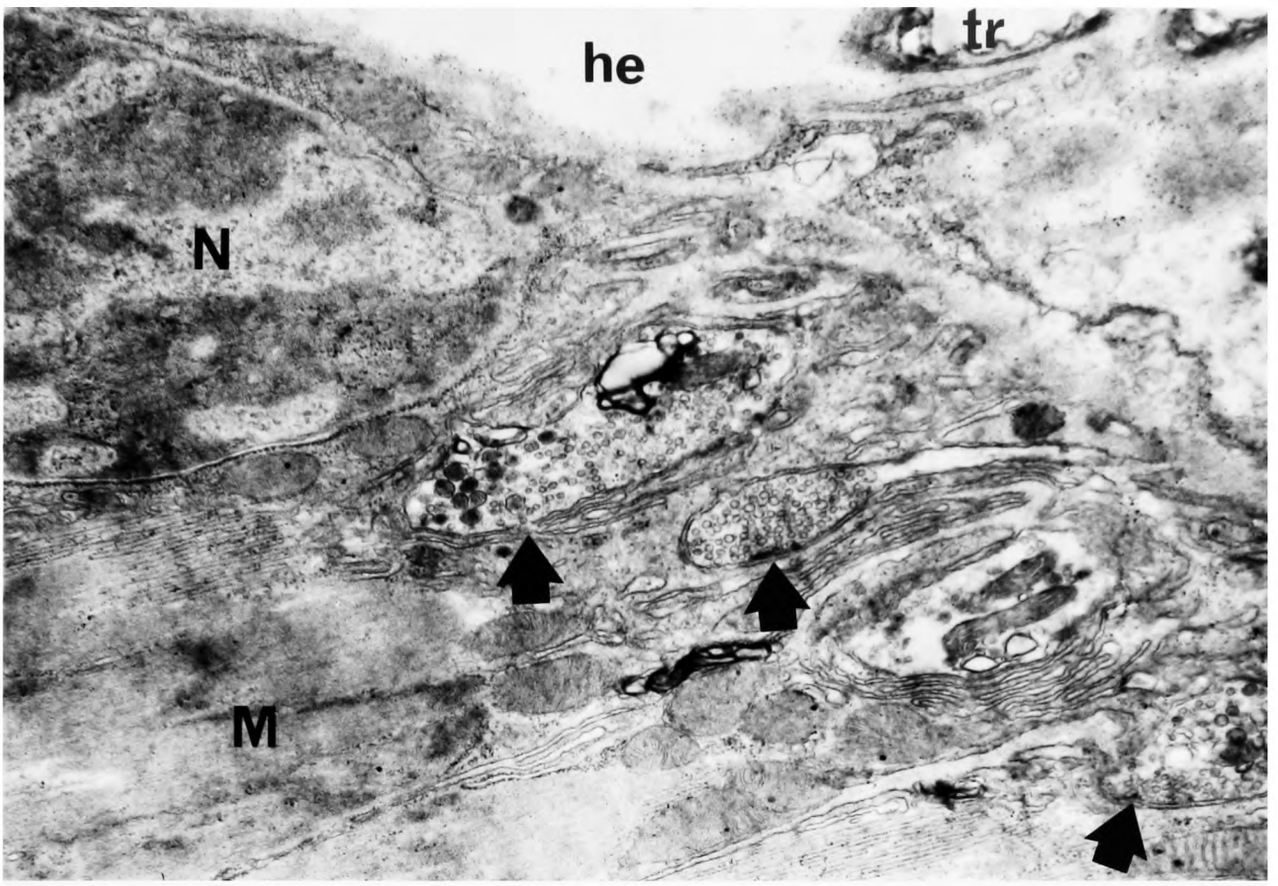


b

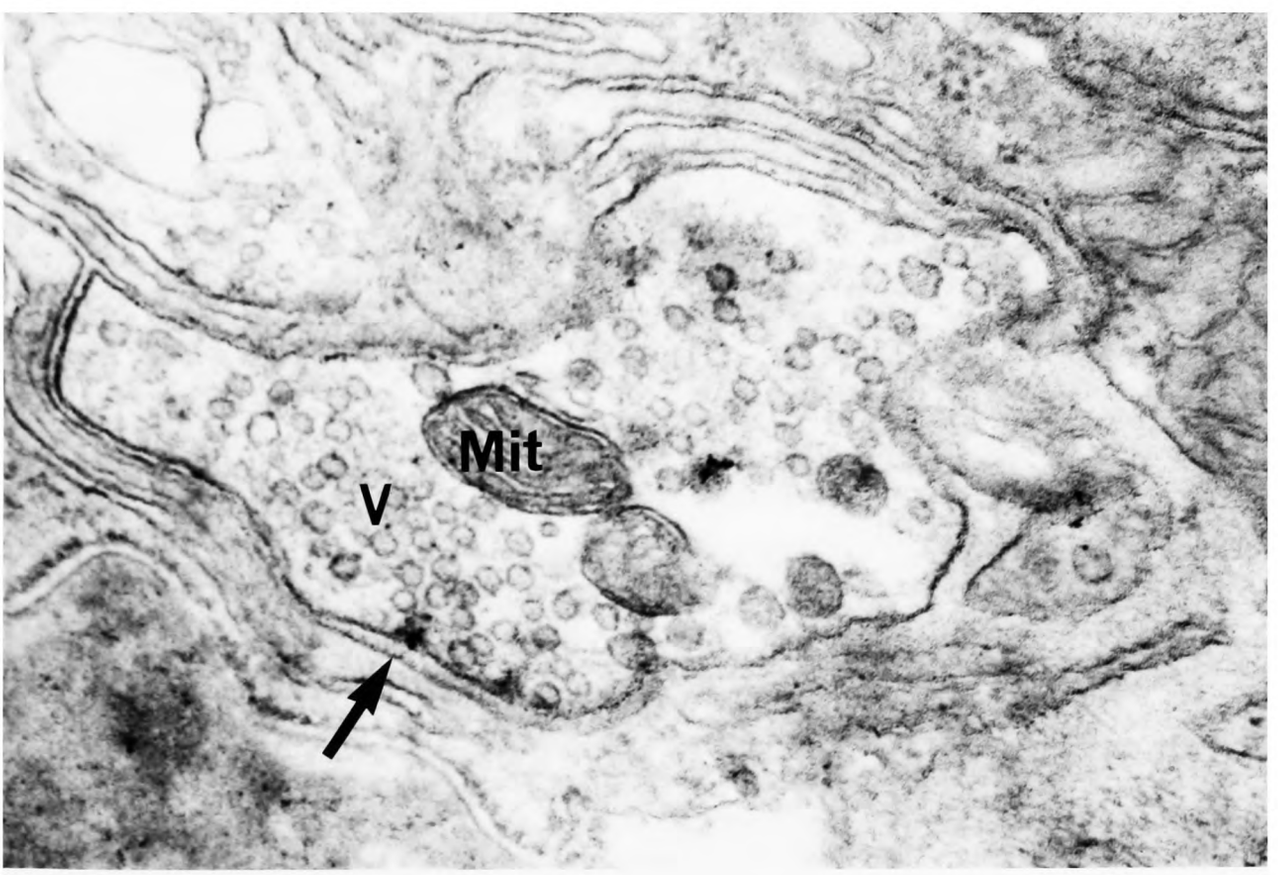
Fig. 3.28 Multi-terminal innervation in A. variegatum retractor muscles. Section a shows three terminals (arrows) innervating a retractor muscle (M), which contains both clear and dense-cored vesicles. N, nucleus; he, haemolymph; tr, tracheole supplying muscle.

Section b shows detail of a synapse (arrow) in which the clear vesicles (V) are aggregated around a dense pre-synaptic projection of the membrane. The terminals also contain mitochondria (Mit).

a x 26,600
b x 75,600



a



b

Fig. 3.29 Structure of Accessory glands in B. microplus.

a. TEM showing a transverse section of the accessory gland duct cells (D) bound together by the processes of sheath cells (Sh). The lumen (L) of each duct is lined with intermittent microvilli. The cytoplasm of the duct contains many longitudinal microtubules transversely sectioned in this plane (arrow).

x 41,400.

b. Light micrograph showing two accessory gland cells in section. The connection between the duct (D) and the lumen (L) of one cell can be seen. Numerous secretory vesicles are grouped around the lumen of each cell. N, nucleus.

x, 600.

c. TEM showing the nucleus (N) and basal and apical surfaces of an accessory gland cell. The apical border is lined with microvilli (MV) and the cell contains large vesicles (V) and glycogen particle arrays (Gly).

x 2,900.

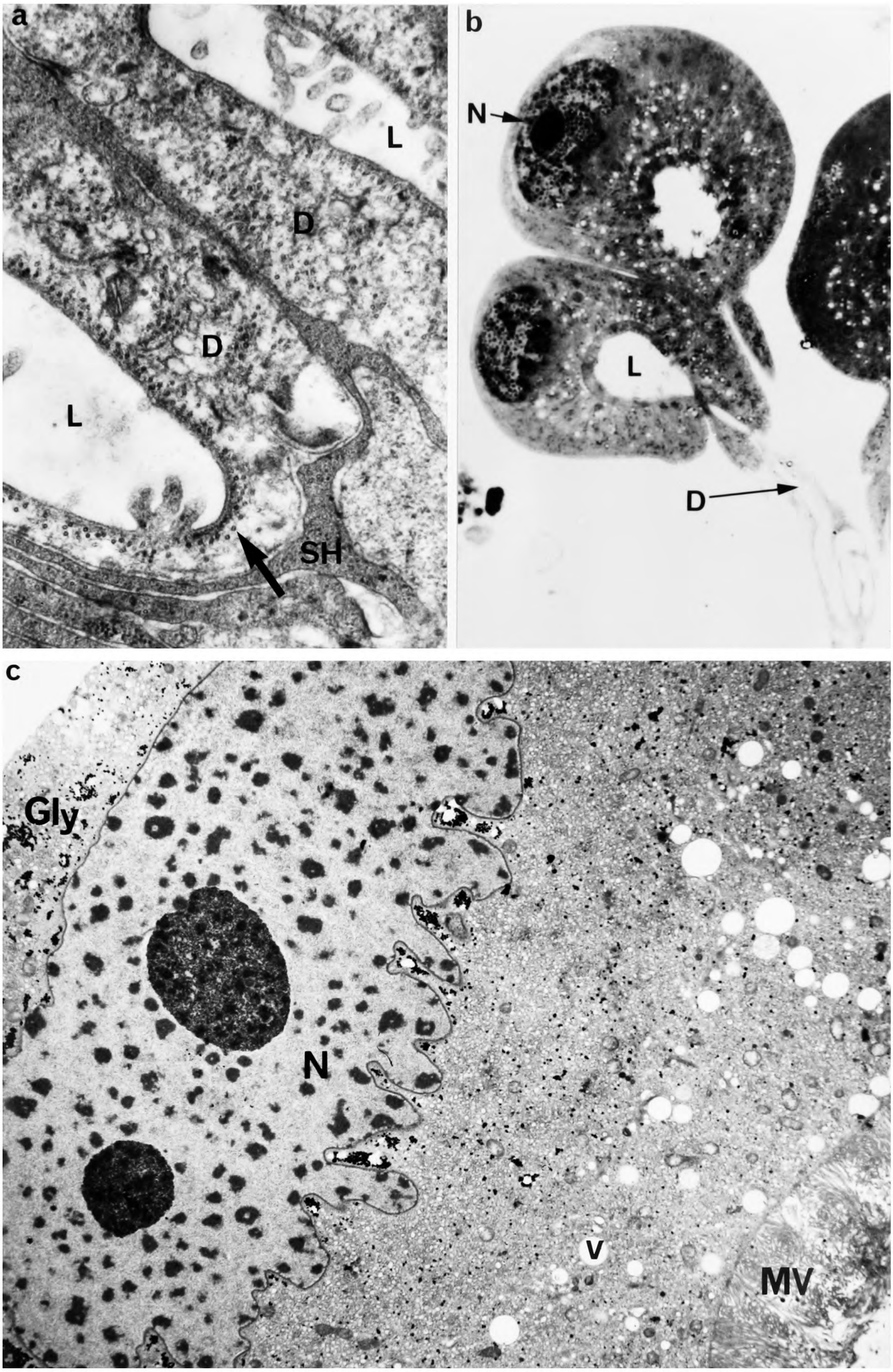

a
L
D
D
L
SH
b
N
L
D
c
Gly
N
V
MV

Fig. 3.30 Diagrammatic representation of the organisation of the accessory glands showing the typical distribution of secretory vesicles around the internal lumen of each gland cell which connects to an individual duct cell. Sheath cells surround each duct and bind the ducts of adjacent cells together.

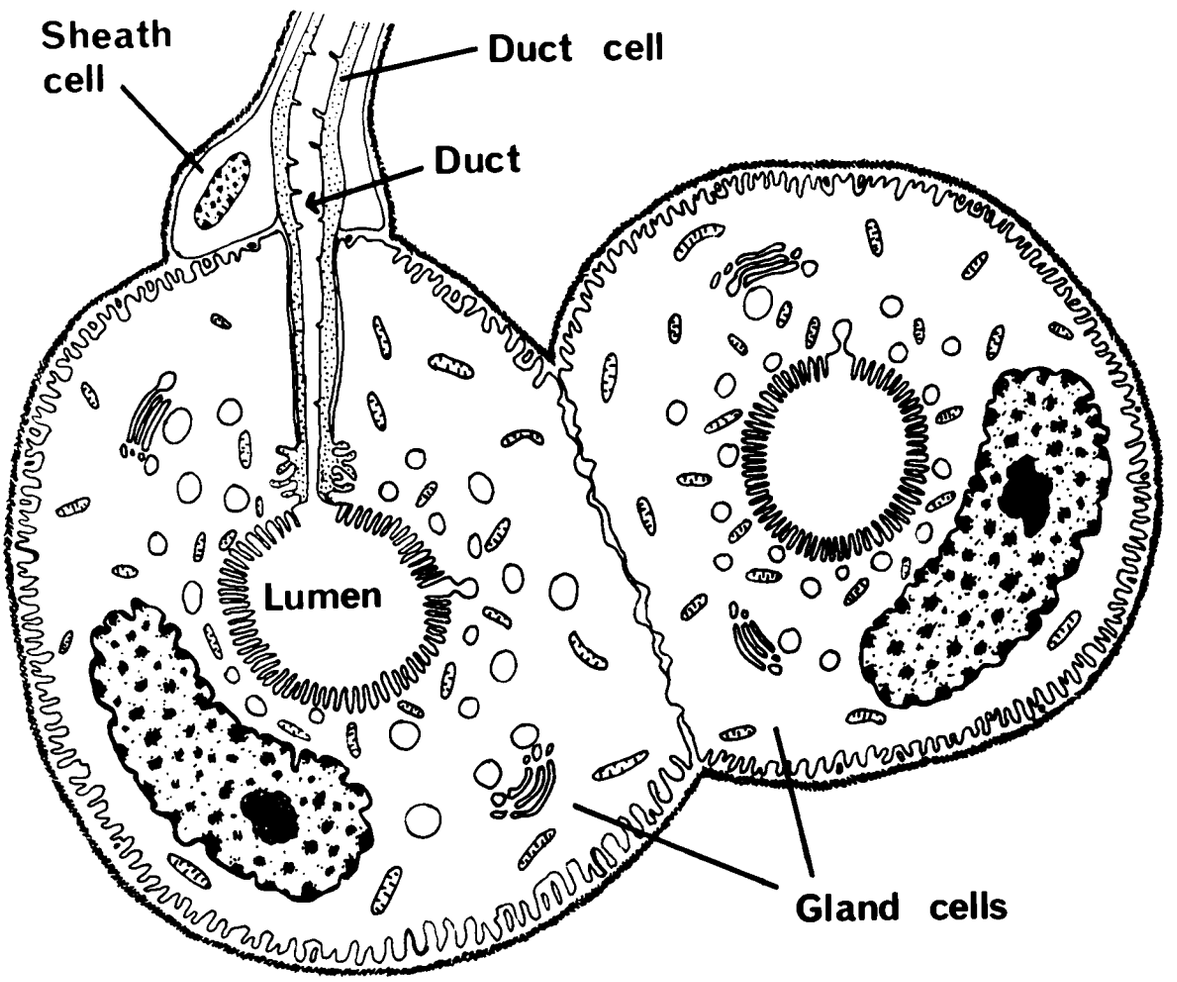

Sheath cell
Duct cell
Duct
Lumen
Gland cells

Fig. 3.31

a. TEM showing tubular smooth endoplasmic reticulum (SER) and isolated smooth membrane vesicles in an accessory gland cell. Golgi dictyosomes (G) are associated with small budding membrane-bound vesicles which appear to be enlarging to form secretory vesicles (V). Mit, mitochondrion; Gly, glycogen particle.

x 38,100.

b. The apical border of an accessory gland cell, showing microvillar border (MV) and secretory vesicles which appear to be undergoing exocytosis into the internal lumen (arrow).

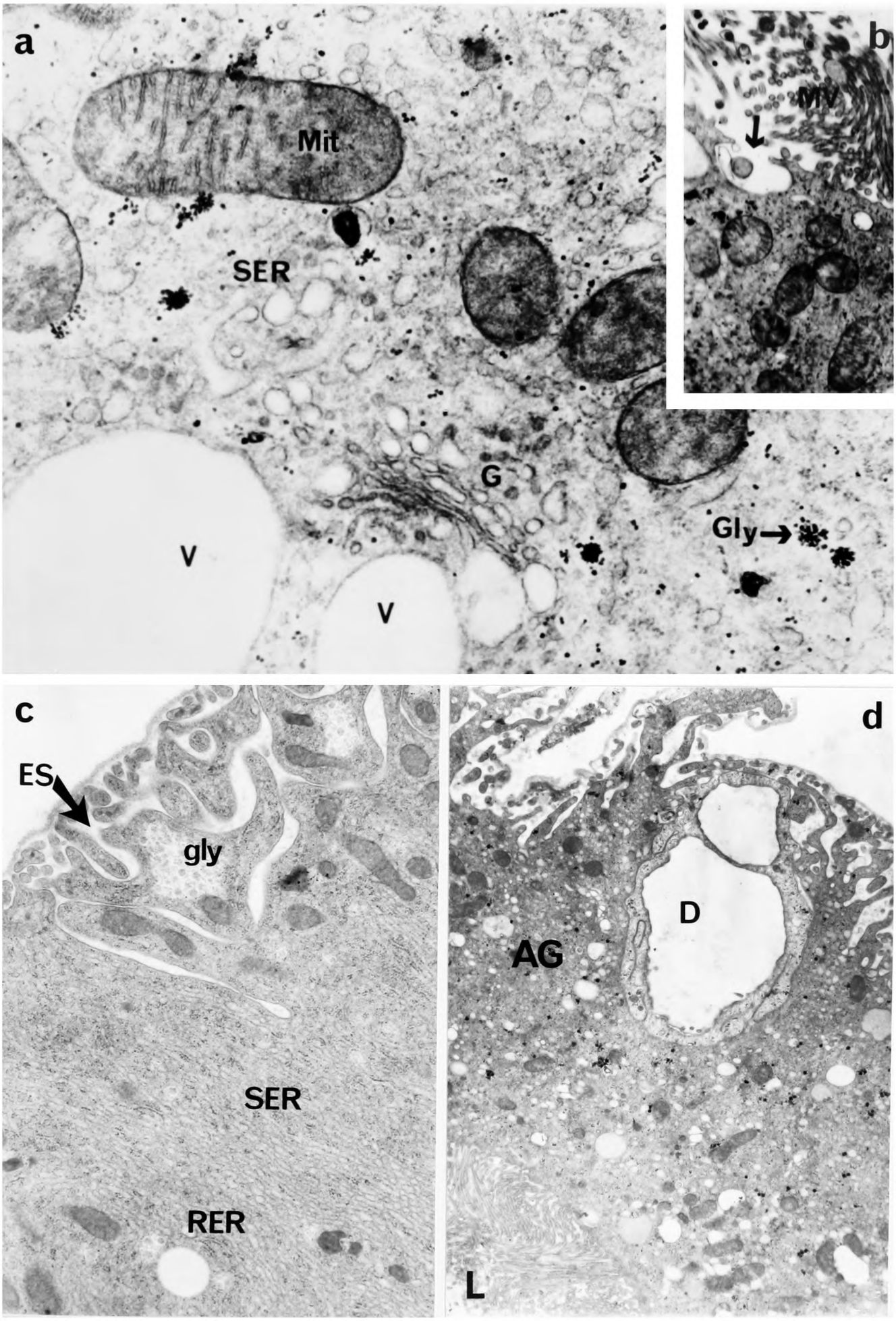
x 11,200.

c. Arrays of smooth endoplasmic reticulum (SER) in the basal region of an accessory gland cell. Rough endoplasmic reticulum (RER) is also present and is continuous with the SER. ES, extracellular space beneath basement membrane. Gly, glycogen particles.

x 13,300.

d. The duct cell (D) passes through the accessory gland cell (AG) on its way to join up with the secretory lumen (L).

x 5,900.


a
Mit
SER
G
V
V
Gly
b
MV
c
ES
gly
SER
RER
d
D
AG
L

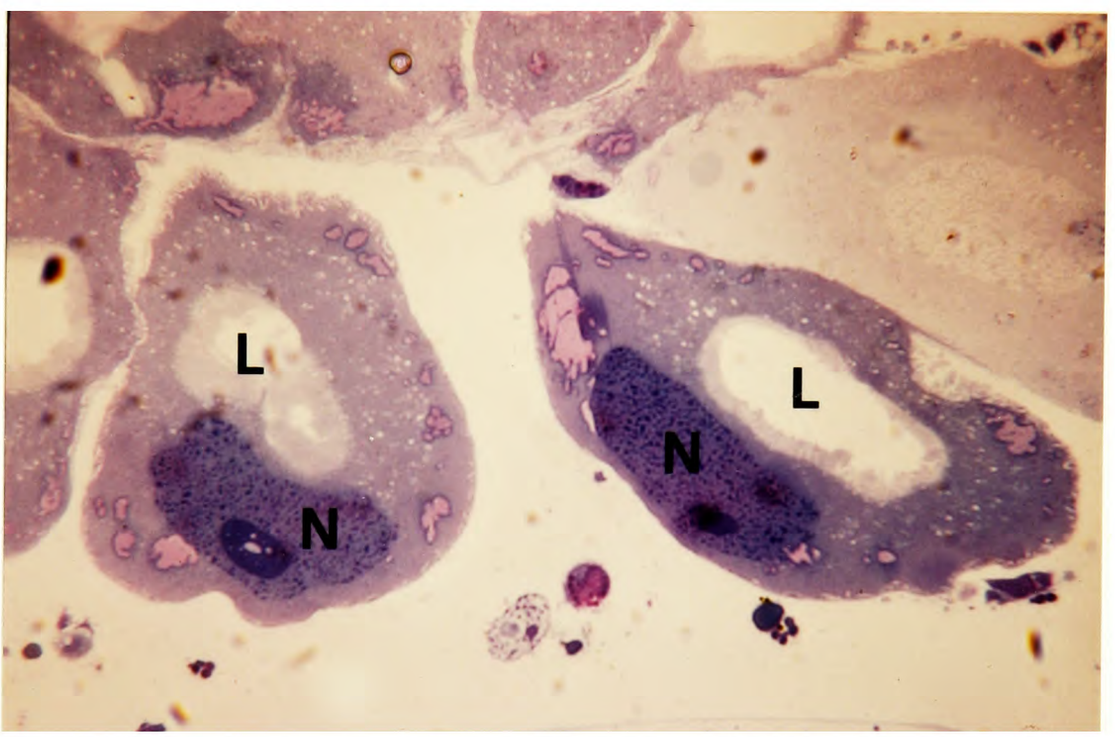


Fig. 3.32 Light micrograph showing two accessory gland cells in section, demonstrating the extensive pink-staining glycogen stores present in these cells. L, lumen of accessory gland; N, nuclei.

x 700.

Fig. 3.33 Smooth endoplasmic reticulum (SER) in tubular form, near the nucleus (N) of an accessory gland cell. Extensive glycogen particle arrays (Gly) are also present, oftensituated in invaginations of the nuclear membrane.

x 27,000.

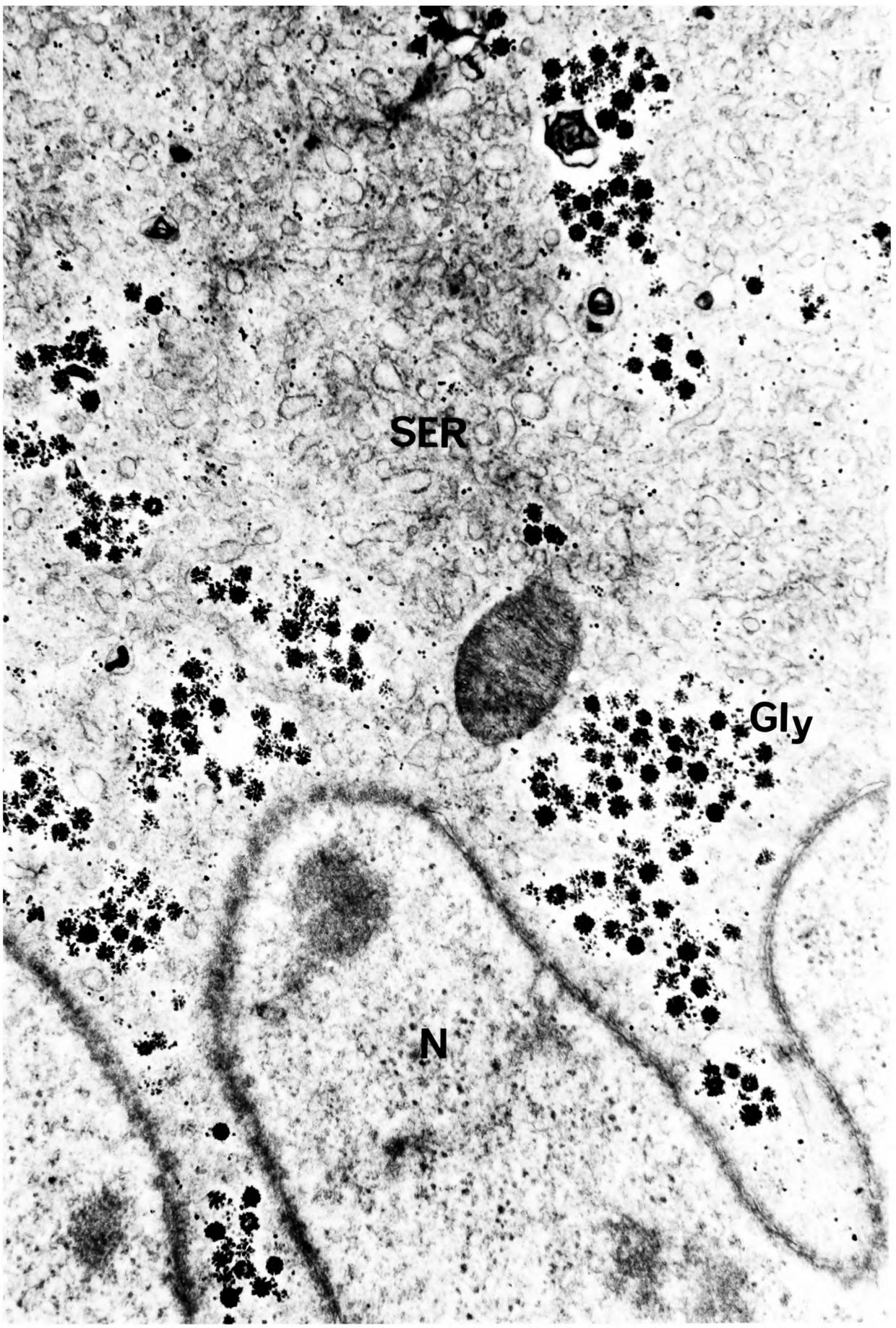
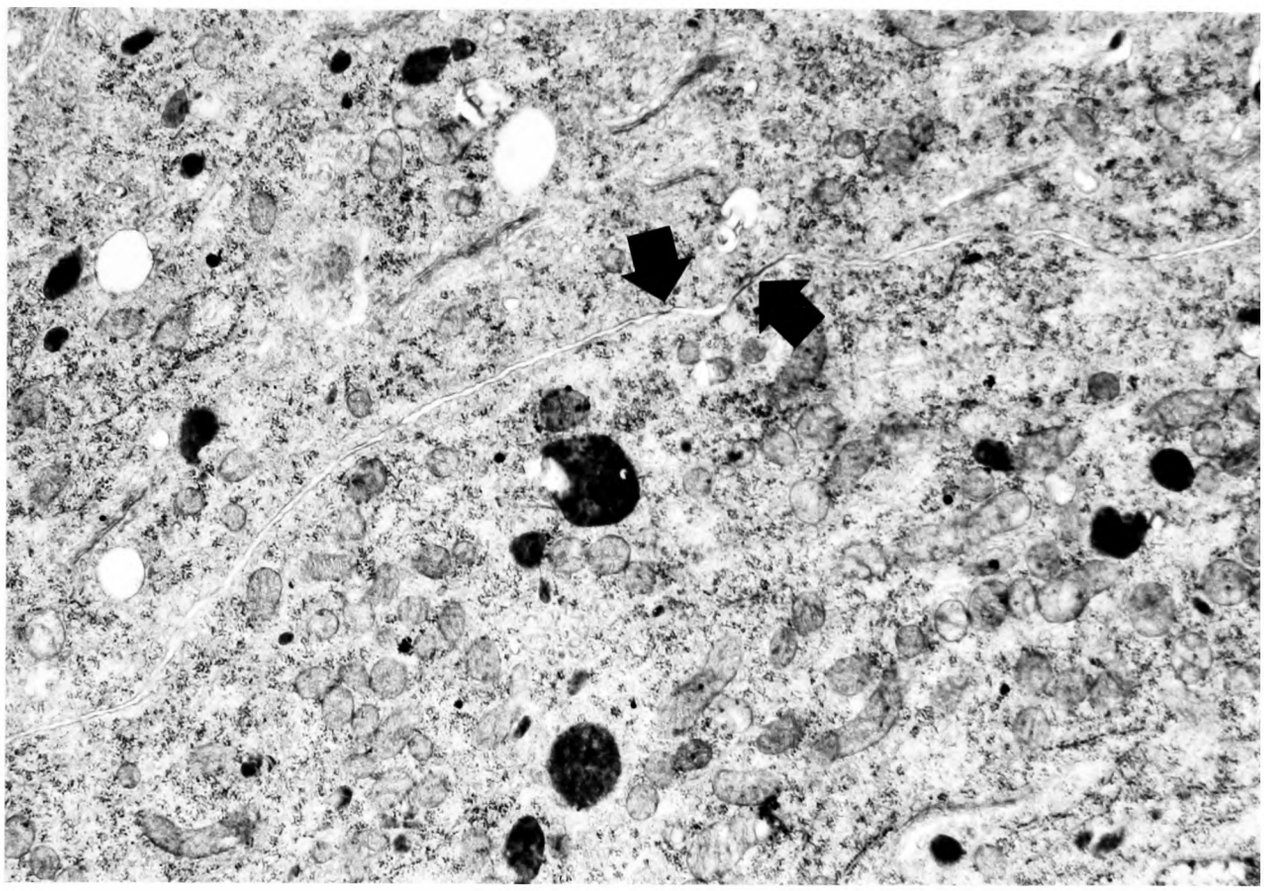

SER
Gly
N

Fig. 3.34 a. TEM showing the cell borders between two accessory gland cells. The plasma membranes are joined intermittently at tight junctions (arrows).

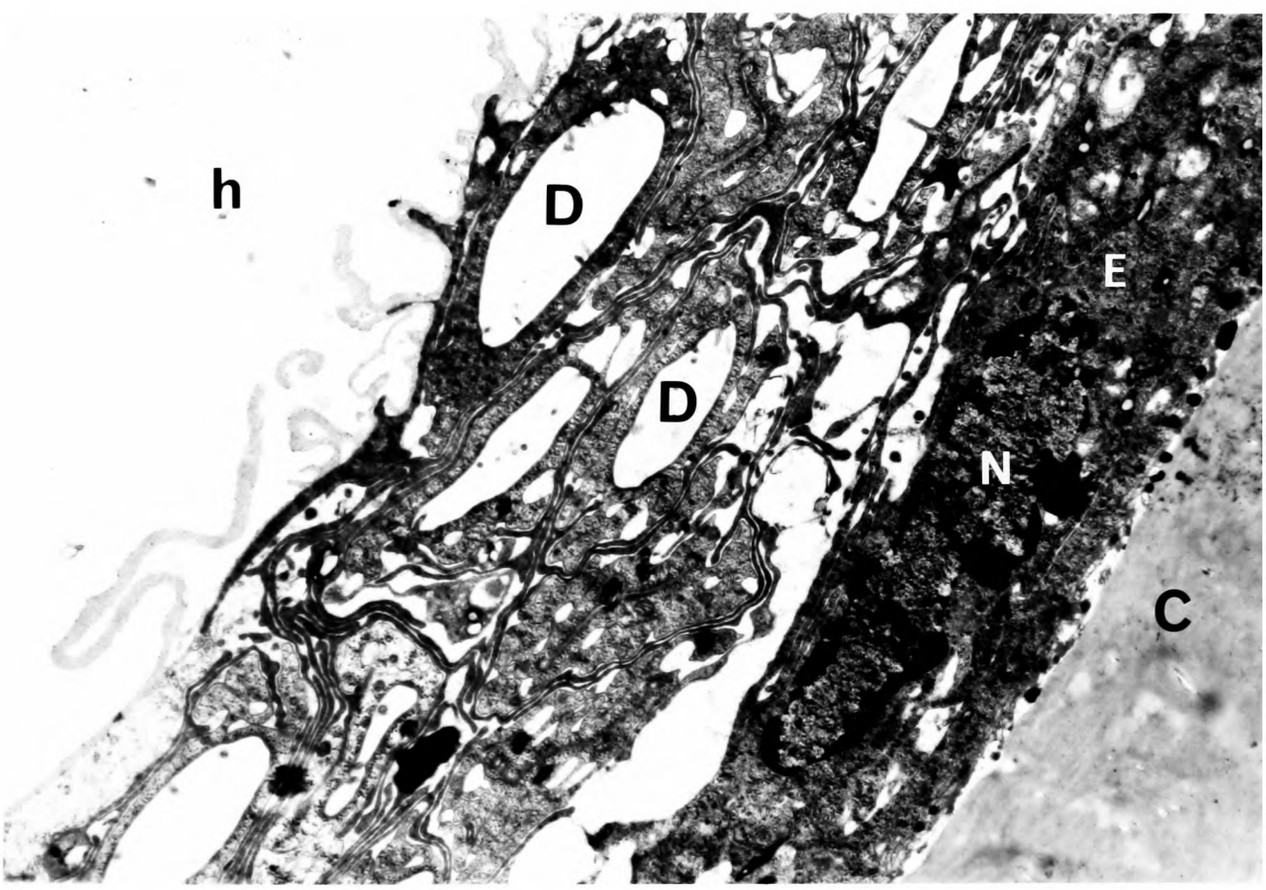
x 11,300.

b. TEM showing clusters of accessory gland ducts (D) bound together by sheath cell processes, and attached to the dorsal cuticle of the capitulum (C) by epithelial cells (E). N, nucleus of epithelial cells; h, haemolymph.

x 8,000.



a



b

Fig. 3.35 a. Light micrograph showing a transverse section of the tubular accessory glands of the oviduct, showing numerous small secretory granules, and larger secretion granules at the apical poles of the cells near the lumen (L). There is a thin muscular sheath around the glands (M). N, nucleus.

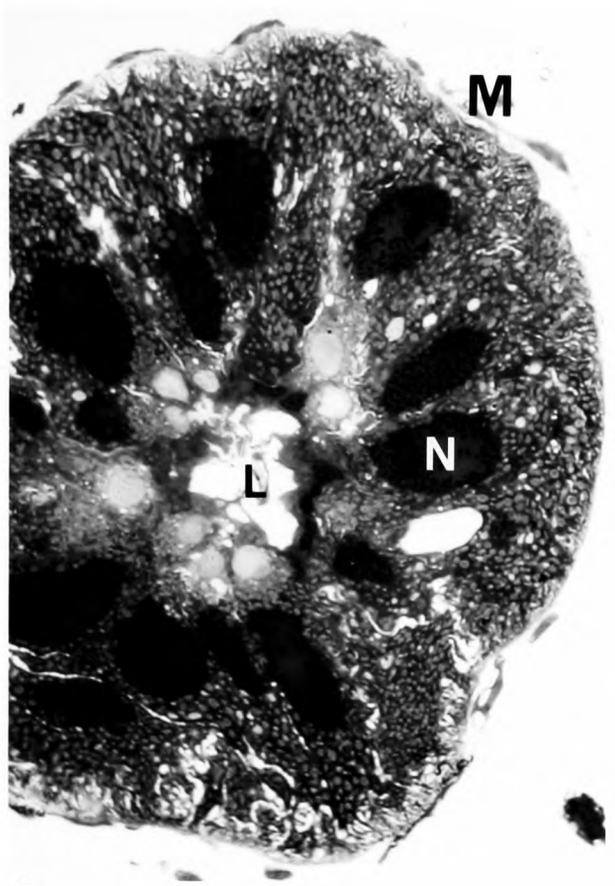
x 500.

b. Light micrograph showing a section of the lobular accessory glands of a female B. microplus, which enclose a lumen (L) beneath the cuticle of the oviduct and vulva. The cells have much larger, more rounded nuclei and wider intercellular crypts than in the glands of Gené's organ. He, haemolymph.

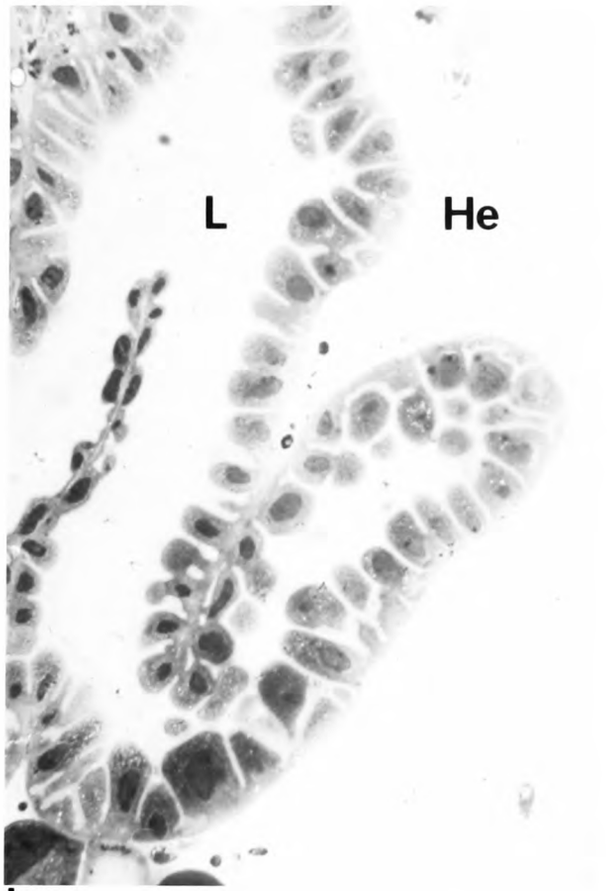
x 200.

c. TEM of a section of the lobular gland in an ovipositing B. microplus. The cells share many of the ultrastructural characteristics of the glands of Gené's organ, having lipid vesicles (V) and abundant endoplasmic reticulum (ER) with a microvillar border (MV) lining the lumen (L). N, nucleus.

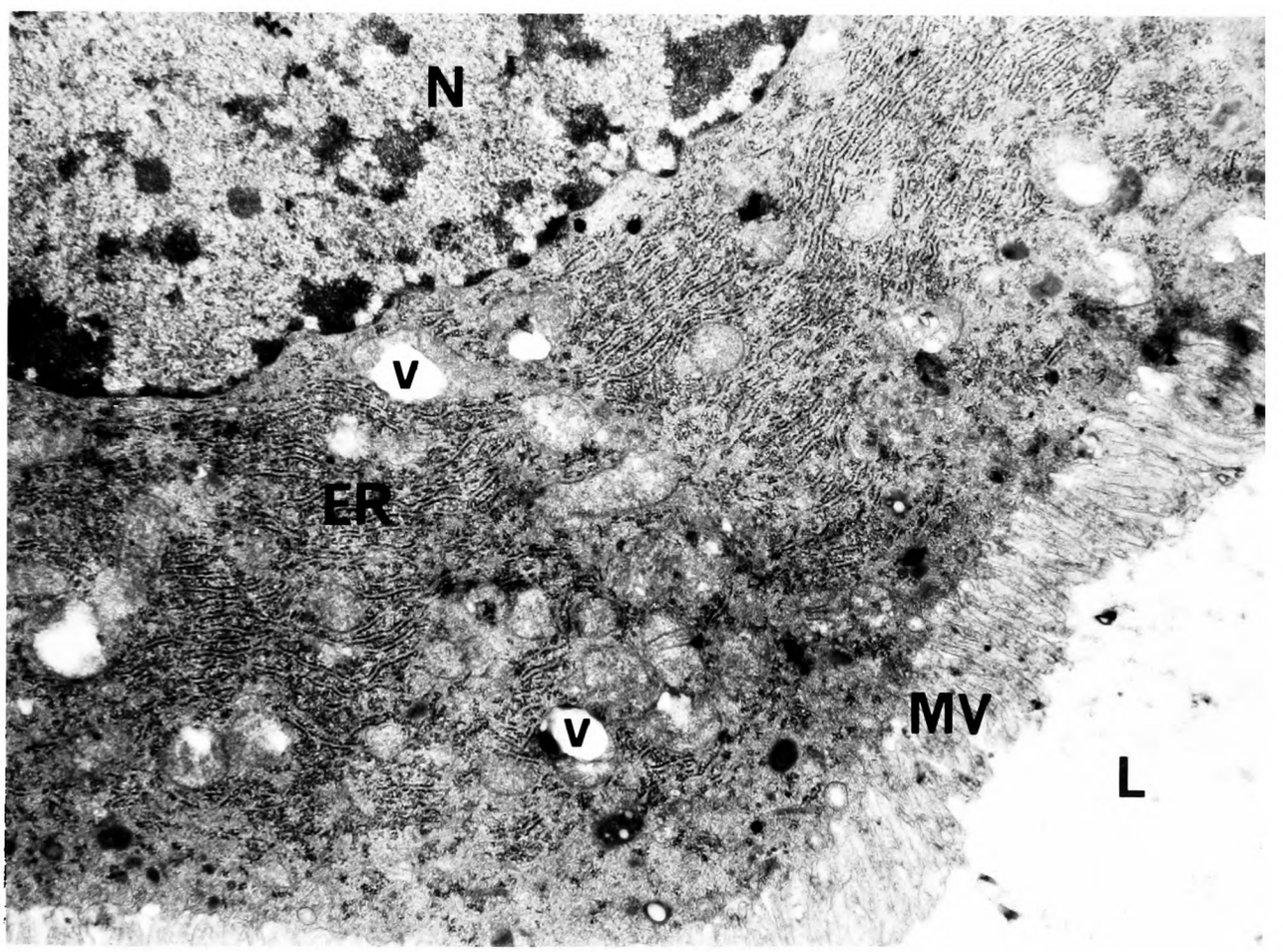
x 14,000.



a



b



c

3.2 Pharmacology

3.2.1 In vivo effects of drugs on oviposition

Octopamine, reserpine and the α-adrenergic agonists clonidine, naphazoline and tolazoline all prevented oviposition after injection into previously ovipositing females (Table 3.1). The acetylcholine agonists pilocarpine and arecoline were also effective in blocking oviposition. In addition to blocking or severely inhibiting egg laying at doses of 25 and 50 µg per tick, the drugs clonidine, octopamine, tolazoline and naphazoline caused an accumulation of abnormal egg wax around the mouthparts, which had a greenish yellow, creamy appearance. An identical effect is seen in amitraz-treated ticks at a sublethal dose of 10 µg per tick. At all doses tested, mortality was no higher than the controls, and the symptoms persisted, ticks which stopped laying eggs did not return to normal oviposition.

Synephrine, the N-methylated analogue of octopamine, and apomorphine, both α-adrenergic agonists, had no effect on oviposition at doses of 25 and 50 µg per tick. Similarly, the catecholamines dopamine and noradrenaline, and the dopamine agonist haloperidol, also had no significant effect on egg output, even when the dose of noradrenaline was increased to 150 µg per tick (Table 3.1). The α-adrenergic antagonists phentolamine, metaclopromide and chlorpromazine did not inhibit oviposition, nor did the histamine agonists 1-Methyl and 3-Methyl histidine, at doses of 25 and 50 µg per tick. Serotonin (5-hydroxytryptamine) also had no effects on oviposition at doses of 25 or 50 µg per tick. No mortality was observed with the drugs which did not prevent normal oviposition either.

Treatment with precocene 2 both topically in DMSO and by injection (25 µg/tick) caused a reduced output of eggs, which became dehydrated and shrivelled rapidly after oviposition, and were non-viable (Fig. 3.36 a,b). The control treated ticks produced normal eggs which hatched after incubation. In the SEM it can be seen that the surface of the shrivelled eggs laid by precocene-treated ticks (Fig. 3.37) lacks a superficial waxy layer present on the normal eggs (Fig. 3.38). The shrivelled eggs are characteristically darkened in colour, and do not adhere together in a cluster like normally oviposited eggs.

The eversion and retraction movements of Gené's organ appeared to be normal and co-ordinated with egg production in the precocene-treated ticks. Co-treatment with juvenile hormone III or β-ecdysone did not

Table 3.1 Effects of drugs injected into ovipositing female B. microplus

Drug	Normal action	Effect on oviposition		Dose µg/Tick
Clonidine	α-adrenergic agonist	Blocking of oviposition,	Abnormal wax	25,50
Octopamine	natural transmitter	"	"	25,50
Tolazoline	α-adrenergic agonist	"	"	25,50
Pilocarpine	acetylcholine agonist	"	"	25,50
Arecoline	acetylcholine agonist	"	-	50
Naphazoline	α-adrenergic agonist	"	-	50
Reserpine	depletes adrenergic terminals	"	-	50
Amitraz	acaricide/octopamine agonist	"	Abnormal wax	10
Apomorphine	α-adrenergic agonist	Normal oviposition		25,50
Synephrine	α-adrenergic agonist	"		25,50
Haloperidol	dopamine agonist	"		25,50
Phentolamine	α-adrenergic antagonist	"		25,50,100
Metaclopromide	α-adrenergic antagonist	"		25,50
Chlorpromazine	α-adrenergic antagonist	"		25,50
Noradrenaline	catecholamine; transmitter	"		25,50,100,150
Dopamine	catecholamine; transmitter	"		25,50
Isoprenaline	β-adrenergic agonist	"		25,50
1-Methyl histidine	Histamine agonist	"		25,50
3-Methyl histidine	Histamine agonist	"		25,50
5-hydroxytryptamine	Neurohormone (serotonin)	"		25,50
Propranolol	β-adrenergic antagonist	"		25,50

Table 3.2 The effect of precocene treatment of newly-dropped adult female *B. microplus* on oviposition and egg viability

Treatment	Effect
Precocene 2, 5µg/Tick, injected.	Normal eggs oviposited.
Precocene 2, 25µg/Tick, injected.	Shrivelled eggs oviposited, onset of oviposition delayed.
Precocene 2, 0.5 mg/Tick, topical application.	"
Precocene 2, 0.5mg + JH III, 5µg, topical application.	"
Precocene 2, 25 µg + ecdysone, 20 µg injected	"
JH III, 10µg/Tick, topical application	Normal eggs
DMSO Control	Normal eggs
β-Ecdysone, 4 µg injected	Normal eggs
β-Ecdysone, 20 µg injected	Normal eggs

Table 3.3 The effect of 0.1M precocene 2 on the incorporation of ^{14}C-1-acetate into wax lipids in excised Gene's organ

Treatment	Labelled lipid, CPM/mg tissue	
Control (DMSO) 1½h at 30°C	1327 ± 501	(n=9)
Precocene 0.1M 1½h at 30°C	265 ± 108	(n=4)
Control (DMSO) 1½h at 4°C	384 ± 35	(n=4)

Incubation medium contained 851 ± 327 cpm/100µl. of ^{14}C-1-acetate.

Table 3.4 The effects of octopamine, JH III, ecdysone and amitraz on in vitro lipid synthesis of Gene's organ

Treatment	Labelled lipid, CPM/mg protein
Octopamine 250µg/ml	62,745 ± 12,246 (n=5)
Juvenile hormone III 25µg/ml	34,422 ± 4,554 (n=5)
β-Ecdysone 25µg/ml	49,024 ± 12,362 (n=5)
Amitraz 25µg/ml	38,862 ± 8,831 (n=4)
Control	49,355 ± 16,103 (n=5)

reverse the effect of precocene in causing egg shrivelling (Table 3.2). Ecdysone treatment on its own at doses of 4 and 20 µg per tick or Juvenile hormone III at 10 µg per tick resulted in normal oviposition.

The ovaries of precocene treated and control ticks, dosed on the day after dropping, were dissected after six days for comparison. The mean weight of precocene treated ovaries was 26.8 mg (s.e.m. 1.8mg, n=3) and that of control ticks 19.4 mg (s.e.m. 2.0 mg, n=3). The eggs developing in the ovary of precocene treated ticks, and mature eggs in the oviduct, appeared normal.

The incorporation of labelled acetate into the lipid fraction in isolated Gene's organs incubated in vitro was affected by presence of precocene in the incubation medium (Table 3.3). Precocene caused a reduction in the amount of label present in the extracted lipids, expressed as counts per mg of fresh tissue. The synthesis of lipids from acetate was also markedly reduced when the incubations were carried out at 4°C instead of 30°C. Addition of octopamine, JH, ecdysone and amitraz had no significant effect on ^{14}C-1-acetate incorporation into lipids by Gené's organ (Table 3.4) at the doses used.

3.2.2 The effect of precocene on the ultrastructure of Gené's organ

Electron micrographs show the effects of precocene 2 on the ultrastructure of the secretory cells of Gené's organ (Figs. 3.39, 3.40, 3.41). The secretory cells from ticks treated with precocene contain fewer large lipid vesicles, and the rough endoplasmic reticulum is disjointed and the cisternae are widened by vacuole-like swellings (Figs. 3.39, 3.40).

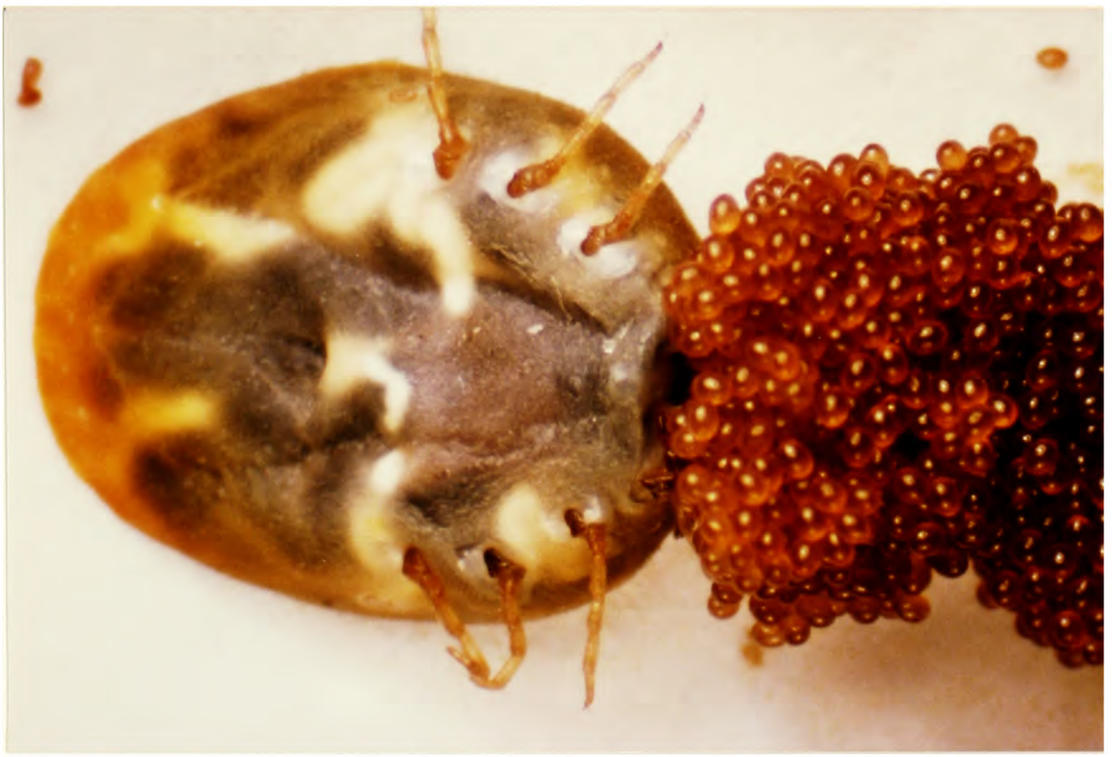
The precocene treated cells also have less rough endoplasmic reticulum and smooth reticulum is more in evidence (Fig. 3.40). There are also autophagic vacuoles which appear to contain broken down organelles or other cellular debris (Figs. 3.39, 3.40b, 3.41a). The microvillar border is also less well organised and does not show the normal regular brush border arrangement (Fig. 3.40b). This is in contrast to the ultrastructure of the control treated ticks, where Gené's organ shows its normal organisation of parallel stacks of rough endoplasmic reticulum with well-organised cisternae (Fig. 3.41b). Precocene treatment also appears to cause degeneration of the infoldings of the basal plasma membrane (Figs. 3.39, 3.40a) so that the extracellular spaces beneath the basal lamina are disjointed and irregular.

Fig. 3.36

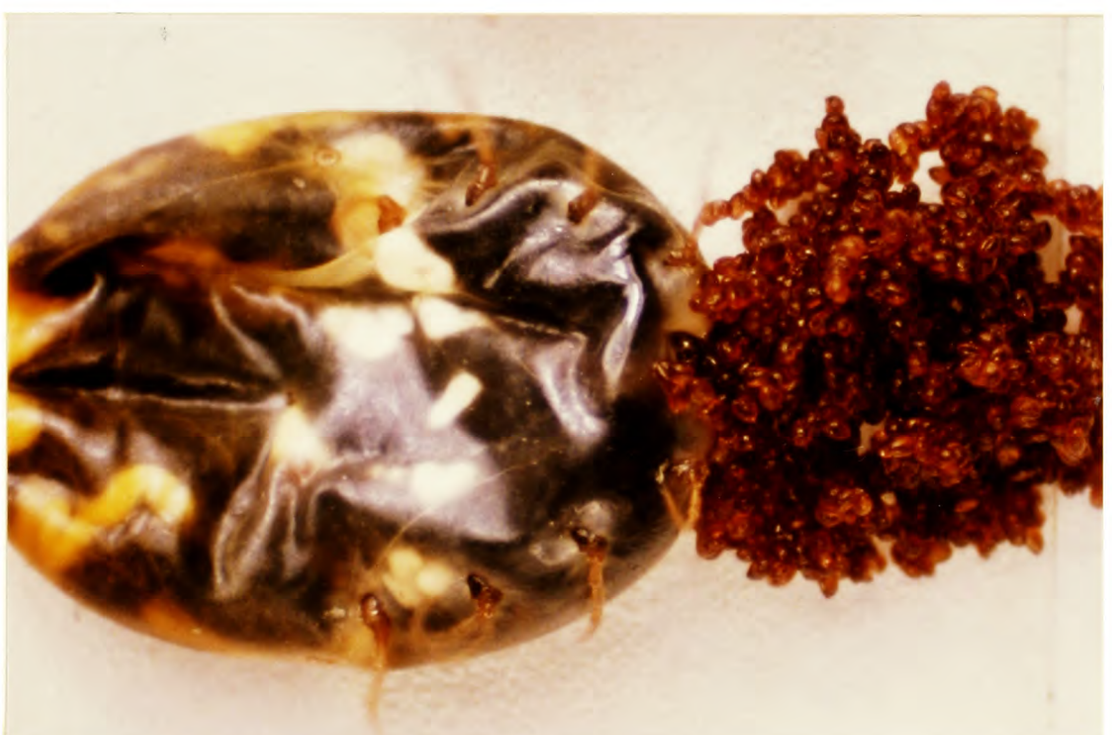
a. An ovipositing female B. microplus, 24 h after a control injection of DMSO, producing a normal cluster of eggs which adhere together due to their waxy coating. The tick was attached ventral surface-upwards on double-sided adhesive tape so that oviposition could be observed and photographed with a dissecting microscope.

b. A similar ovipositing female which had been treated with a 25 µg injection of precocene 2. Oviposition has proceeded normally but the eggs are all severely shrivelled and desiccated. The eggs fail to adhere in a normal cluster, due to the absence of a wax layer on their surface.

a and b x7



a

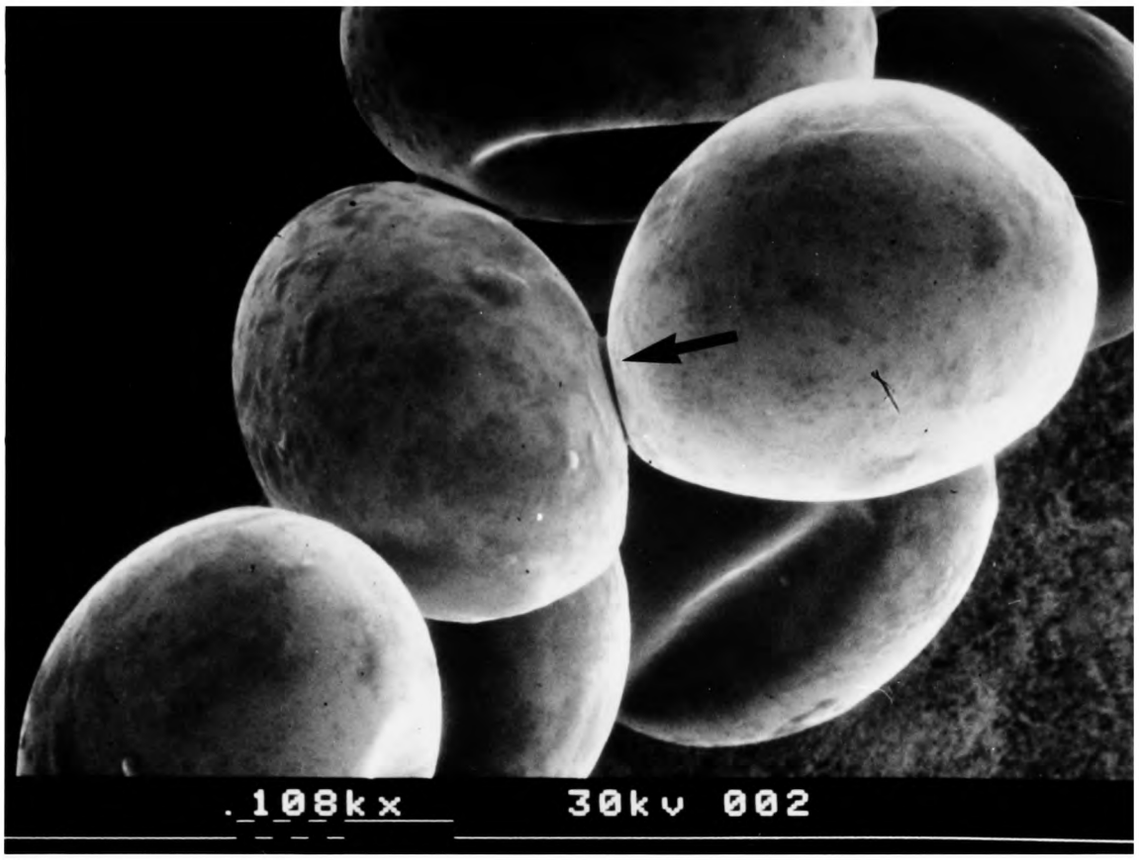


b

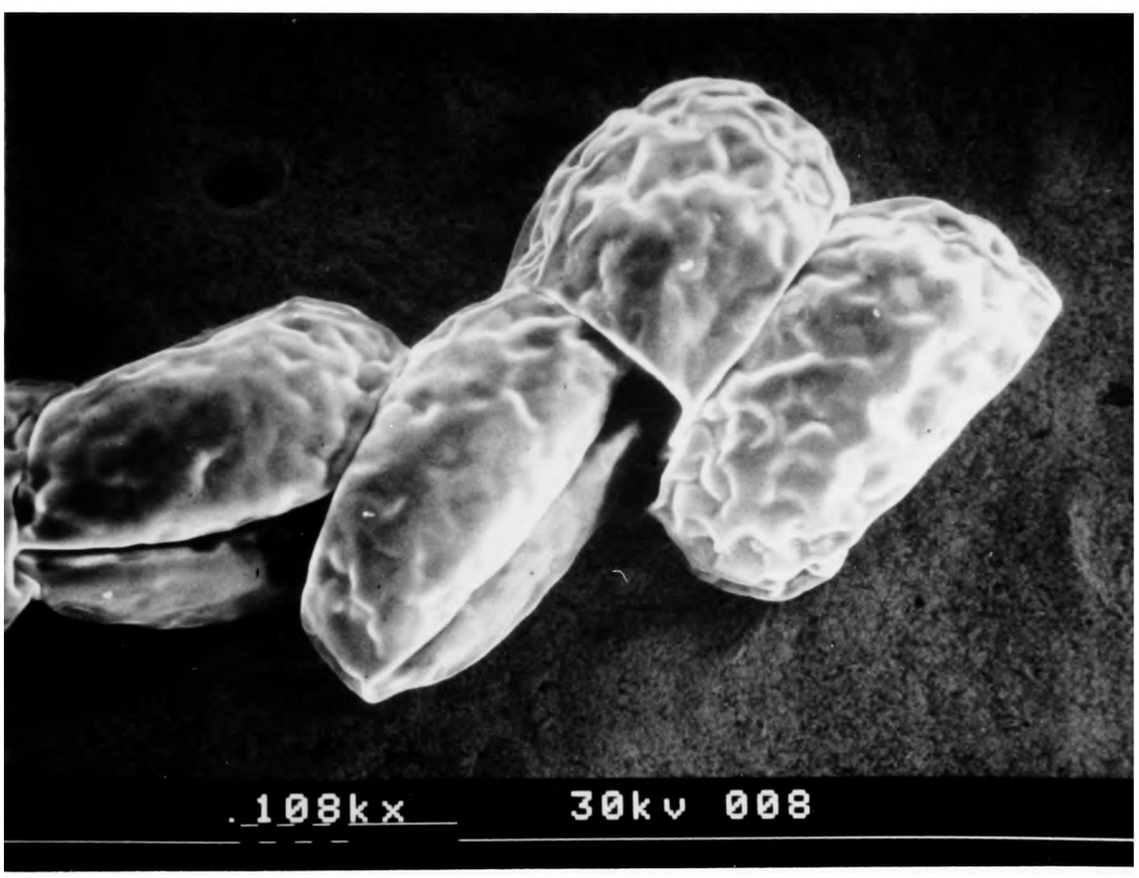
Fig. 3.37 a SEM showing part of a cluster of eggs from a control-treated B. microplus showing that the eggs have a superficial waxy layer. The slight collapse of two of the eggs is due to the evacuation of the air in the microscope. The wax can be seen forming a meniscus between two eggs (arrow).

b SEM showing a group of eggs from a precocene treated tick, showing the shrivelled and collapsed surface of the egg shells. The eggs are also characteristically curled in on themselves and are brittle and do not adhere together strongly.

a and b x 130.



a

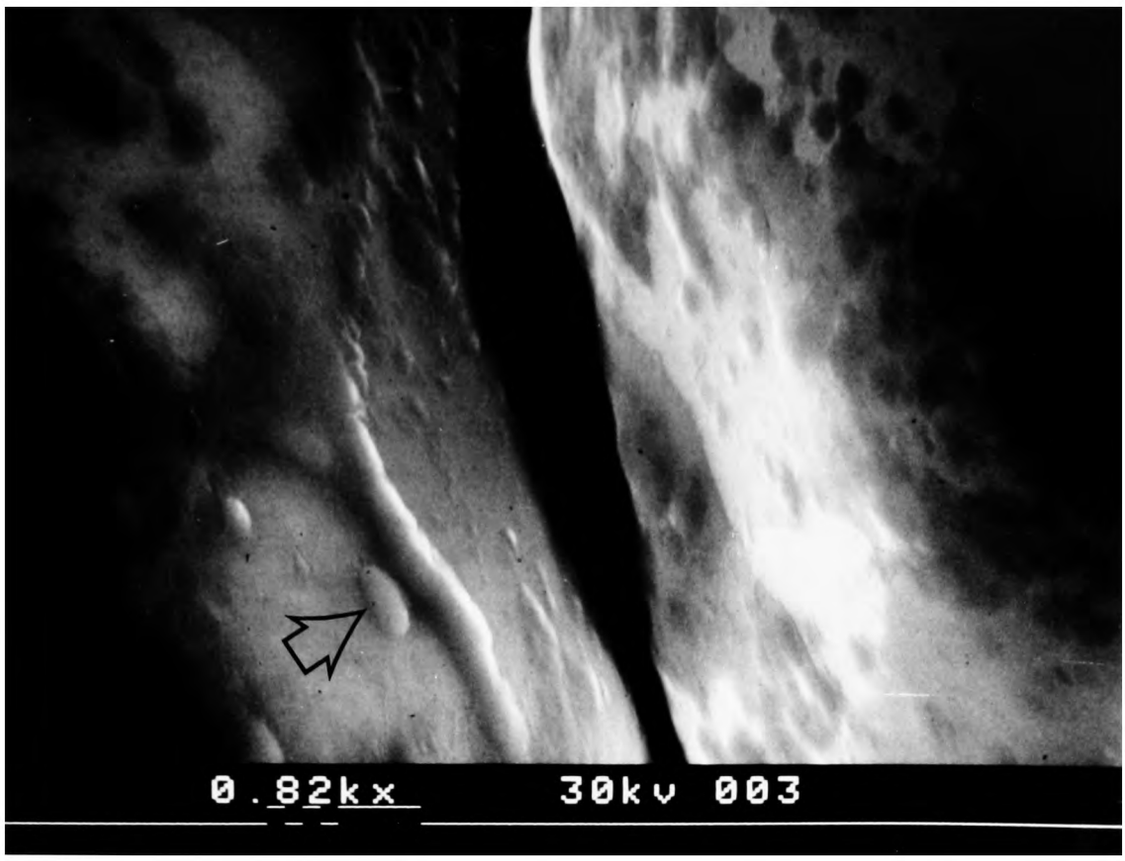


b

Fig. 3.38 a SEM showing the surface of two normal eggs at their point of contact. The surface wax forms irregular droplets on the surface (arrow) and forms a meniscus between the eggs holding them together.

b The surface of a shrivelled egg laid by a precocene treated tick, showing the dimpled surface of the egg shell which is smooth and completely devoid of superficial wax.

a and b x 820.


0.82kx
30kv 003

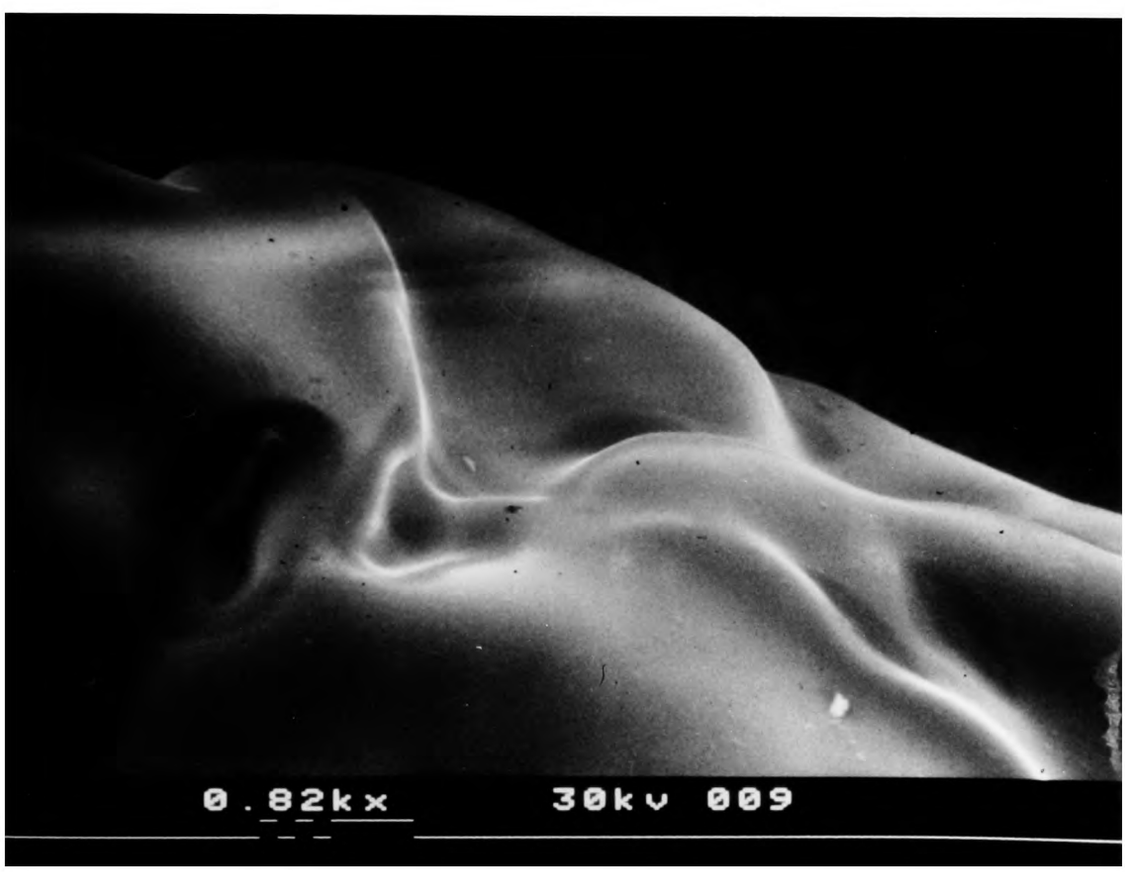

0.82kx
30kv 009

Fig. 3.39 TEM showing a section of a precocene treated secretory gland of Gené's organ. The cell exhibits an unusually rounded nucleus (N) and the endoplasmic reticulum (ER) is disjointed into short sections containing vacuole-like distentions in the cisternae. The microvillar border (MV) is less well defined and the basal border (bm) contains few infoldings of the basal plasma membrane. There are few lipid vesicles, but autophagic vacuoles containing broken down organelles are evident (arrows). Large glycogen arrays are also absent.

x 12,600.

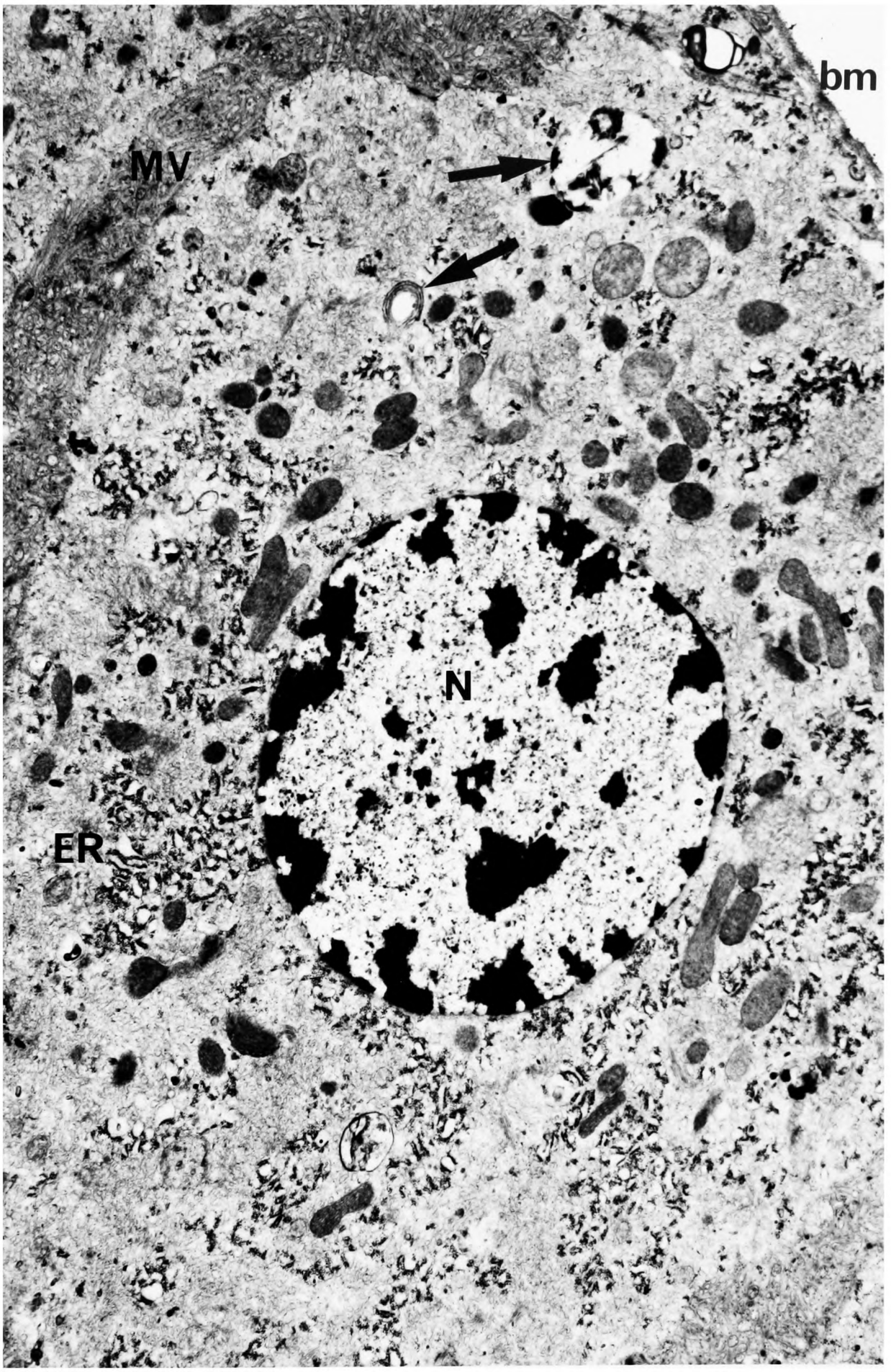
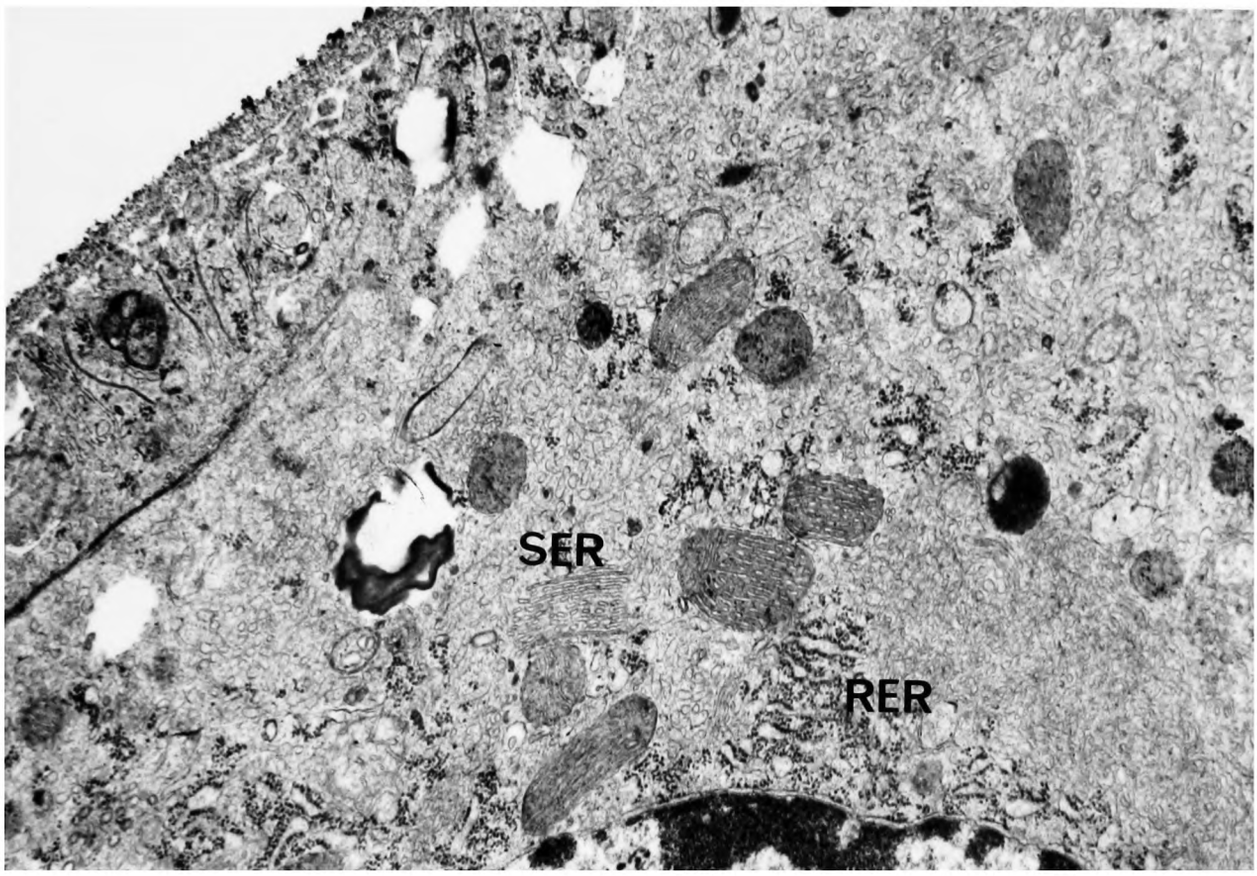

bm
MV
N
ER

Fig. 3.40 a TEM showing detail of the disrupted basal border of a Gené's organ gland cell in a precocene treated tick. The cell contains an increased amount of smooth membrane reticulum, often in the form of stacks (SER) in addition to the disrupted rough ER (RER).

x 19,000

b Detail of the apical border of a precocene-treated Gené's organ gland cell, showing extensive SER and disrupted RER. The microvillar border (MV) is disrupted and indistinct. av, autophagic vacuole.

x 13,300.


SER
RER

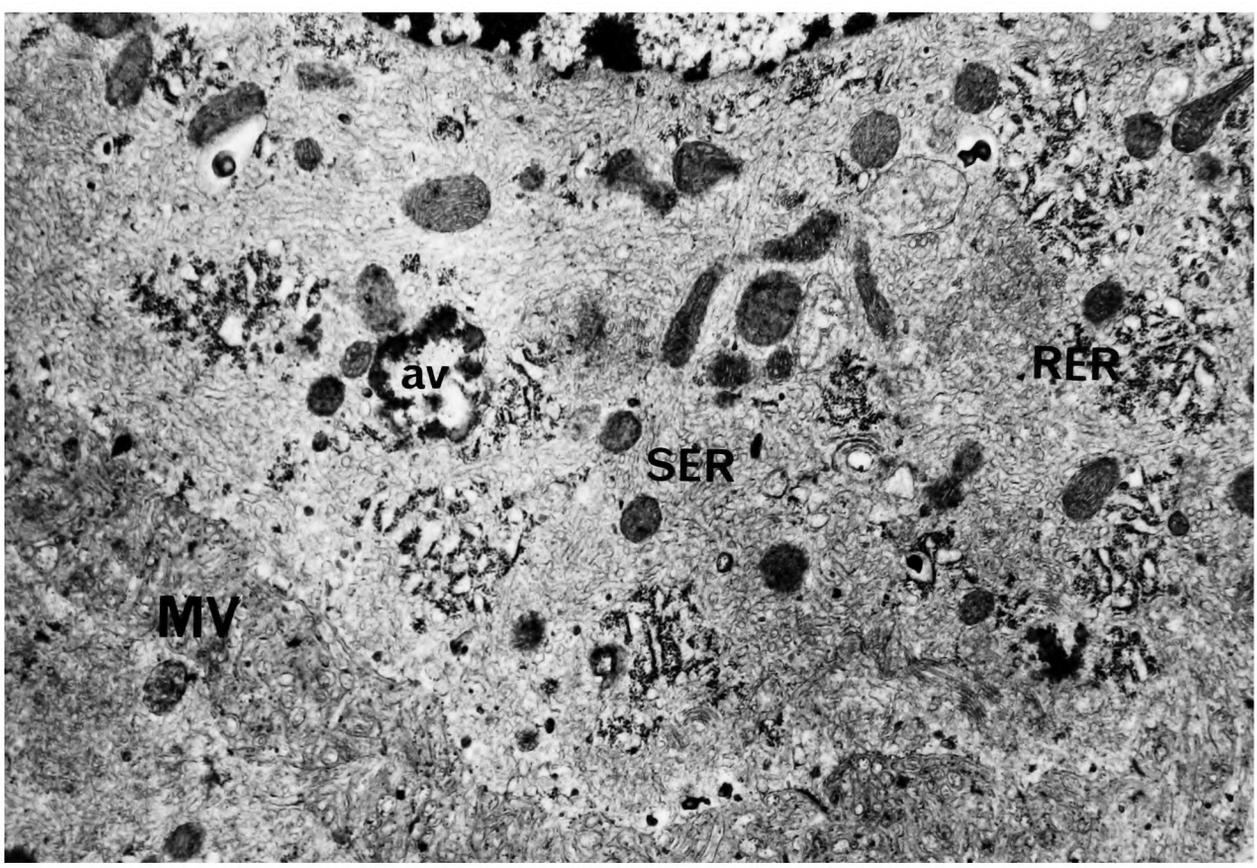
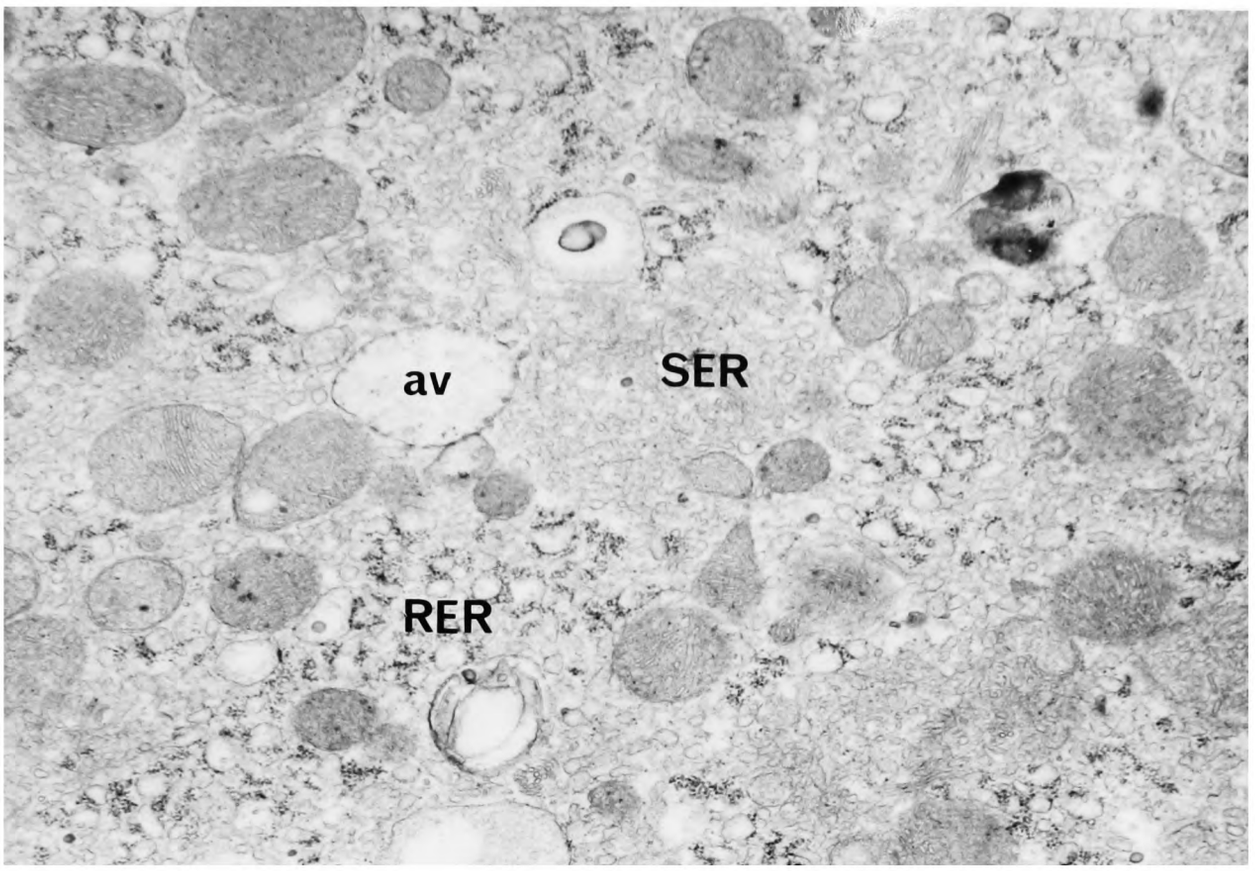
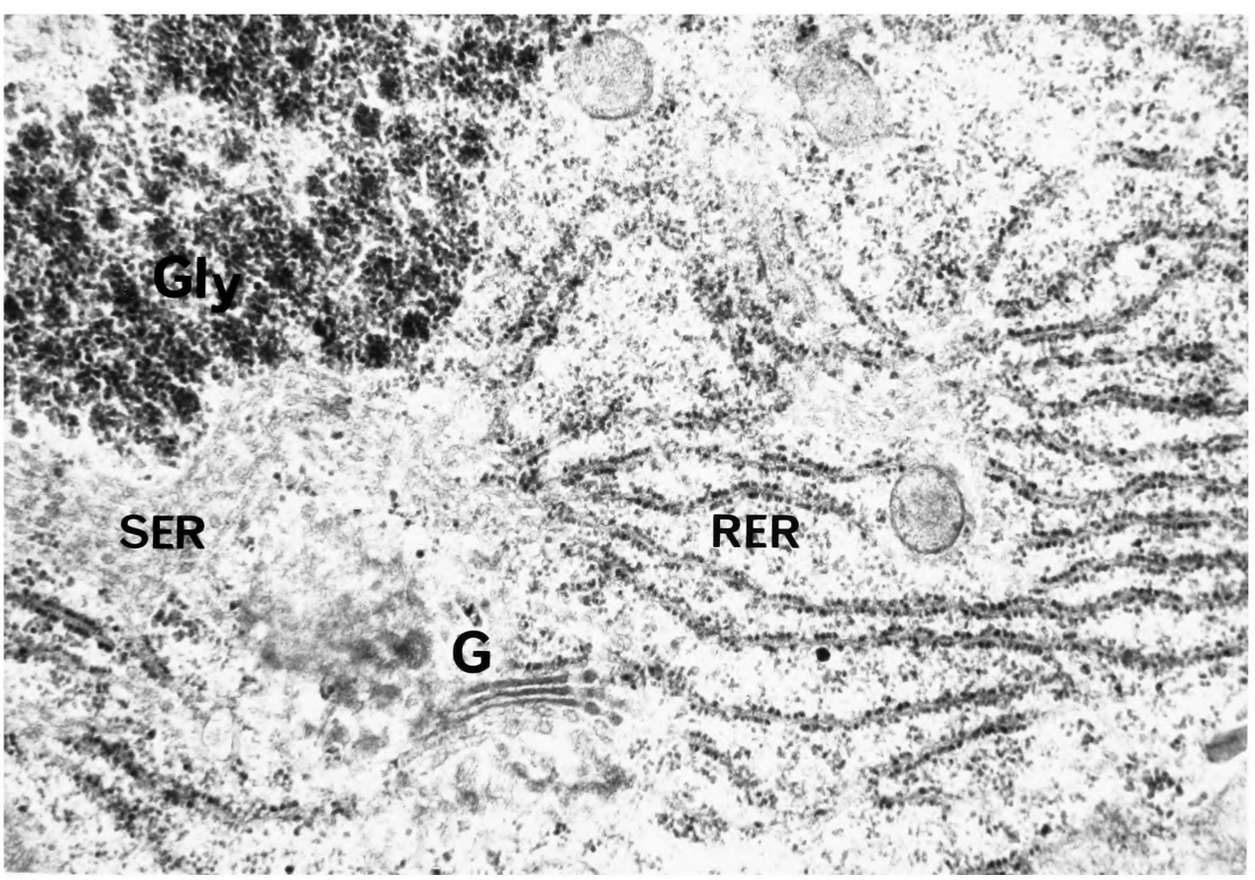

av
RER
SER
MV

Fig. 3.41 Comparison of the ultrastructure of precocene-treated (a) and control-treated (b) gland cells of Gene's organ. The precocene treated cell contains disrupted rough endoplasmic reticulum (RER) in the form of rounded vesicles, extensive SER and autophagic vacuoles (av). The normal ultrastructure (section b) as a contrast has normal parallel stacks of RER with evenly spaced cisternae, extensive glycogen reserves (Gly) and Golgi dictyosomes (G).

a. x 19,000

b. x 10,600


av
SER
RER


Gly
SER
RER
G

3.2.3 Ultrastructure of clonidine and octopamine treated Gené's organs

The drugs clonidine and octopamine, which are both α-adrenergic agonists, are highly active in blocking oviposition, and also cause an abnormal secretion of wax, in ovipositing B. microplus. For this reason it was decided to investigate whether these drugs had an effect on the wax secretory cells by investigating their ultrastructure in treated specimens. Sections of the secretory cells of Gené's organ from clonidine and octopamine treated ticks show budding lobes of cytoplasm on the apical surface of the gland cells, and in the lumen (Fig. 3.42). There are many crystal-like inclusions in the secretory vesicles (Fig. 3.42b) and vacuoles containing glycogen particles. Some cells appear to be degenerating, with the cytoplasm containing many small membranous vesicles, and mitochondria wrapped in membrane whorls (Fig. 3.43a). Some of the degenerating cells contain extensive glycogen stores (Fig. 3.43b).

The accessory glands from the drug-treated ticks also showed differences in ultrastructure. In some cells the basal border appeared to be disrupted (Fig. 3.44a) with the normal infoldings of the basal plasma membrane formed into a series of vesicles beneath the basement lamina rather than an extracellular space as in normal cells. The apical surface at the secretory lumen was also characterized by an increased number of secretory vesicles clustered around the microvillar border (Fig. 3.44b).

Fig. 3.42

a. Light micrograph showing a section of the secretory gland of Gené's organ dissected from a clonidine treated engorged female B. microplus. The lumen (L) contains light staining buds of cytoplasm near the apical border of the cells.

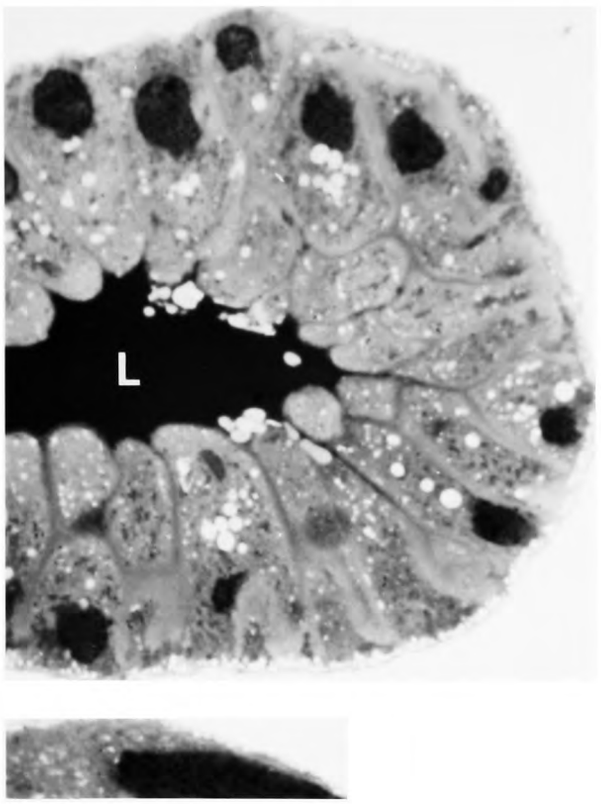
x 500.

b. TEM showing details of a gland cell from a clonidine treated tick. There are many unusual vesicles containing crystal-like inclusions (X) and autophagic vacuoles, one of which appears to contain glycogen particles (arrow).

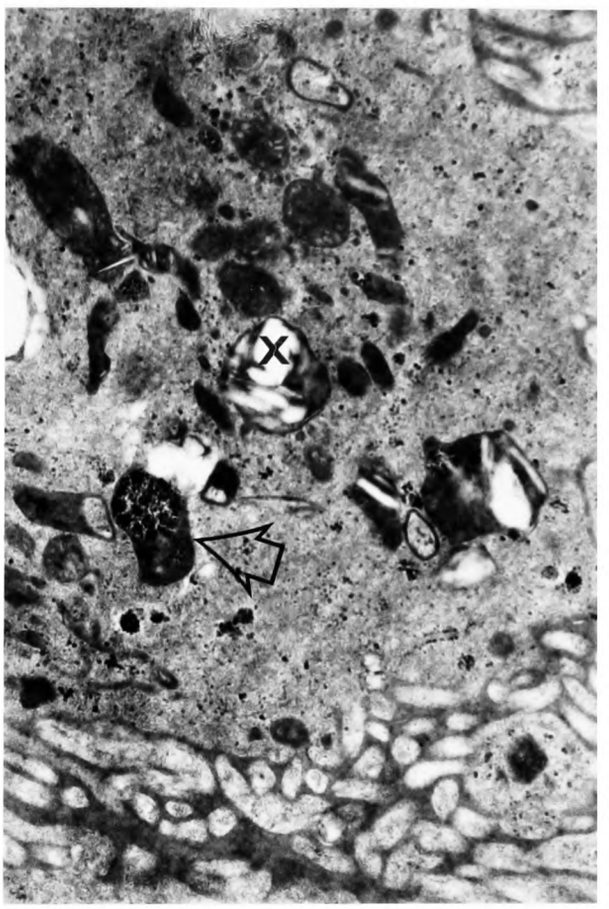
x 9,200.

c. TEM showing cytoplasmic blebs (Cy) in the lumen (L) which appear to be membrane-bound and contain material of amorphous appearance. Their significance is unknown but they are absent in control treated glands. V, lipid vesicles; Mv, microvillar border.

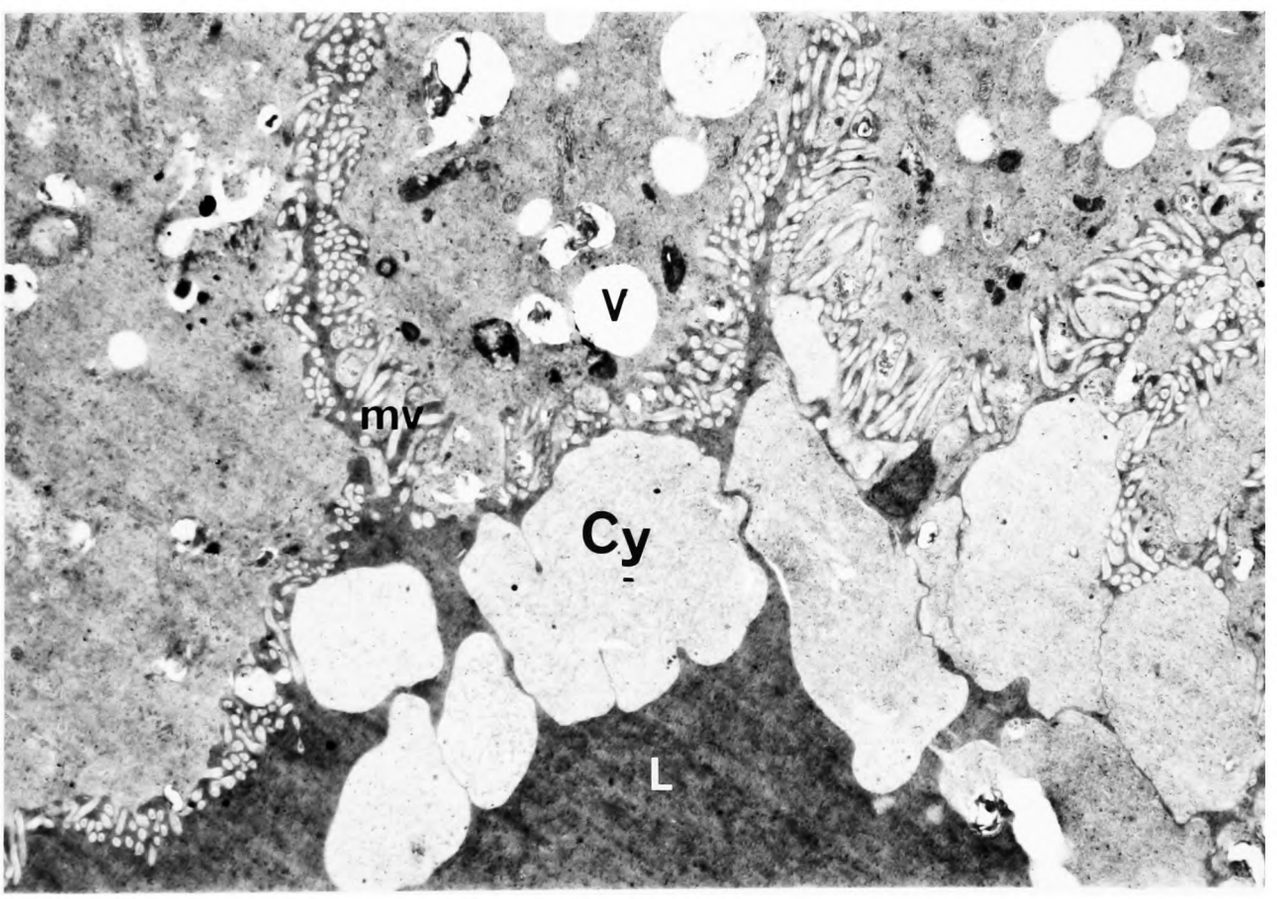
x 8,000.



a



b



c

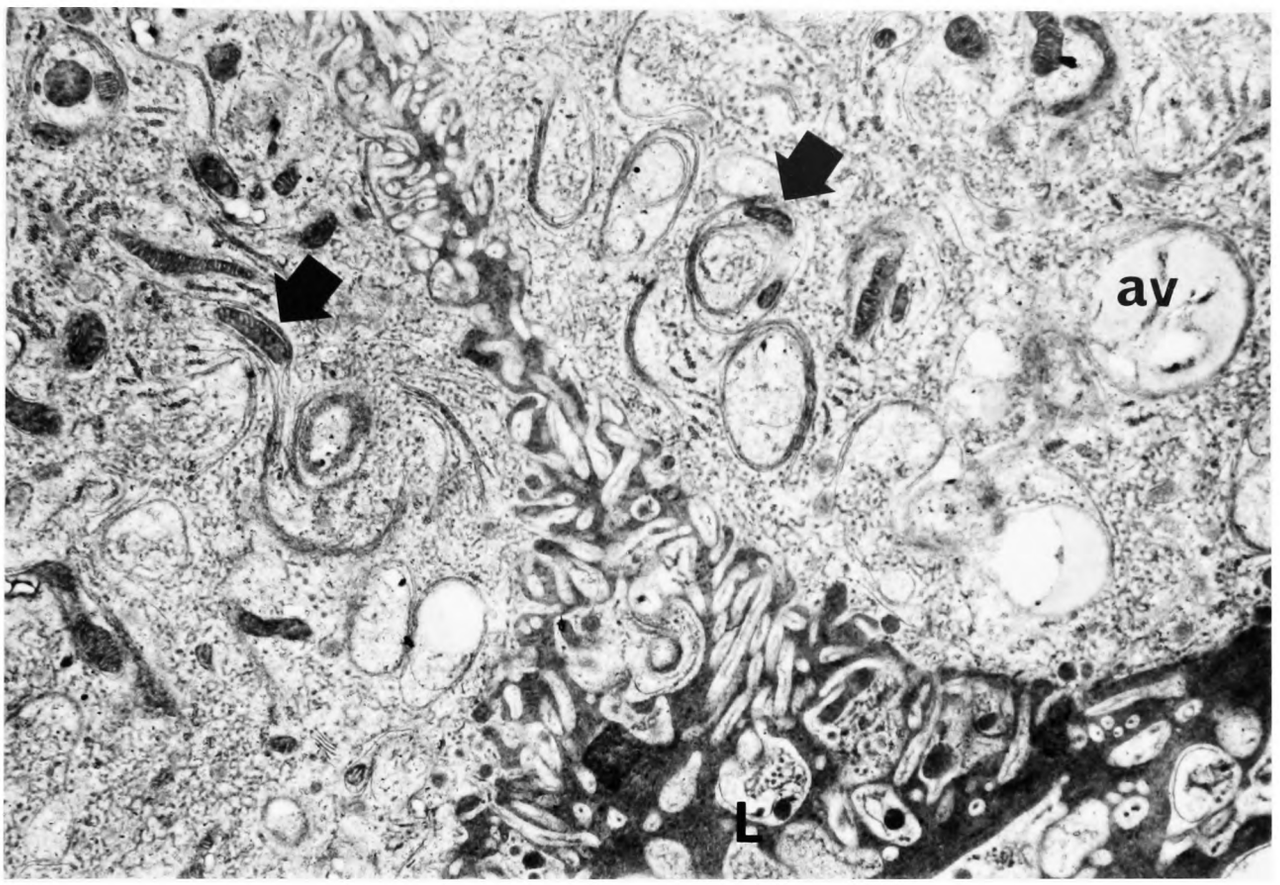
Fig. 3.43

a. The apical border of Gené's organ gland cells from a clonidine-treated tick. The cells contain unusual membrane whorls, which are often wrapped around mitochondria (arrows). The cytoplasm appears diffuse and contains disorganised smooth membranes. The lumen (L) contains much cellular debris. The membrane whorls appear to be involved in forming autophagic vacuoles (av).

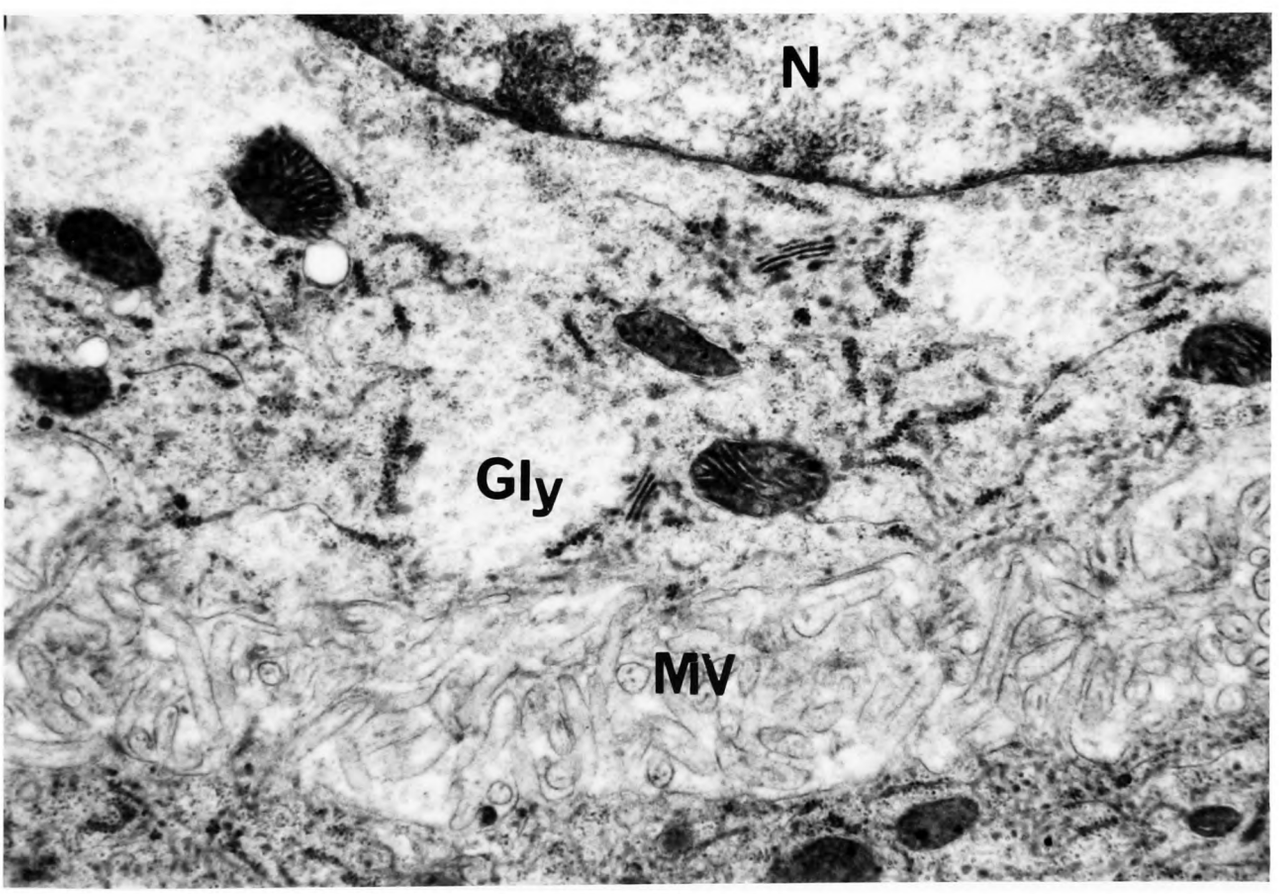
x 13,300.

b. A clonidine-treated Gené's organ gland cell, showing a diffuse cytoplasm with disorganised ER and extensive glycogen particles (Gly). MV, microvillar border; N, nucleus.

x 13,300.



a



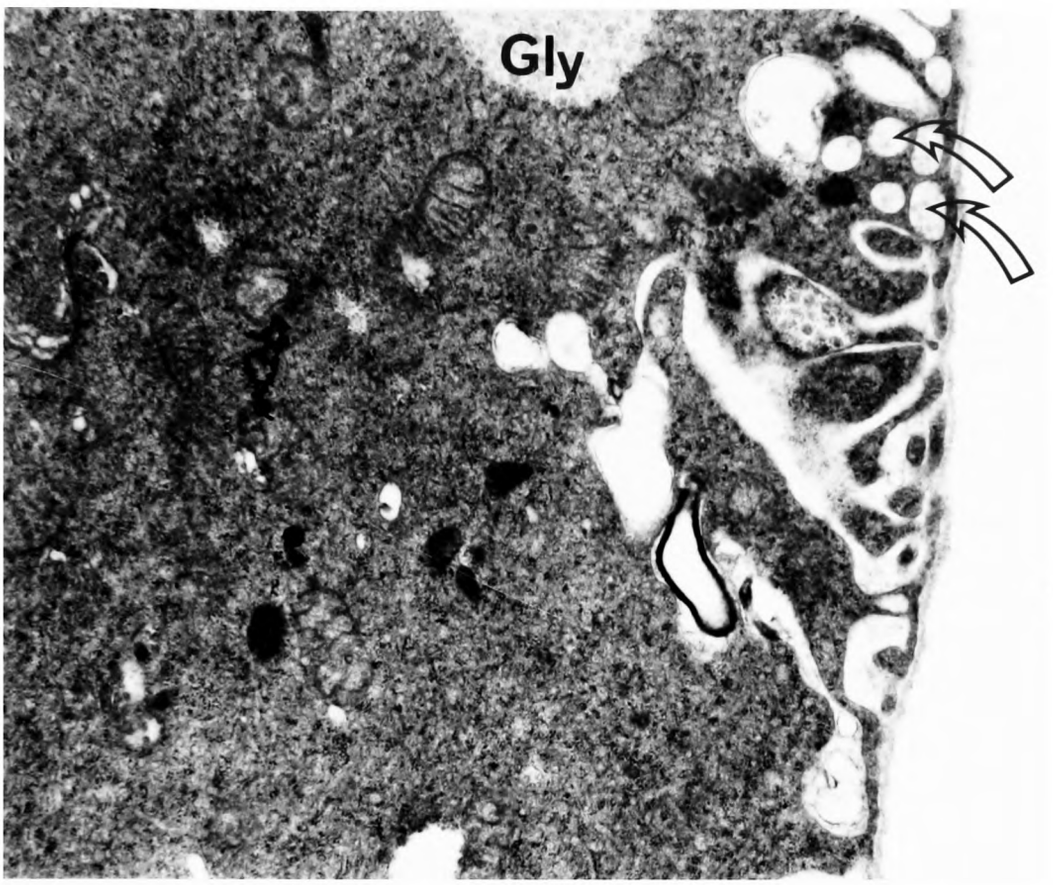
b

Fig. 3.44 TEMs showing the changes in the ultrastructure of the accessory glands of Gené's organ after clonidine treatment.

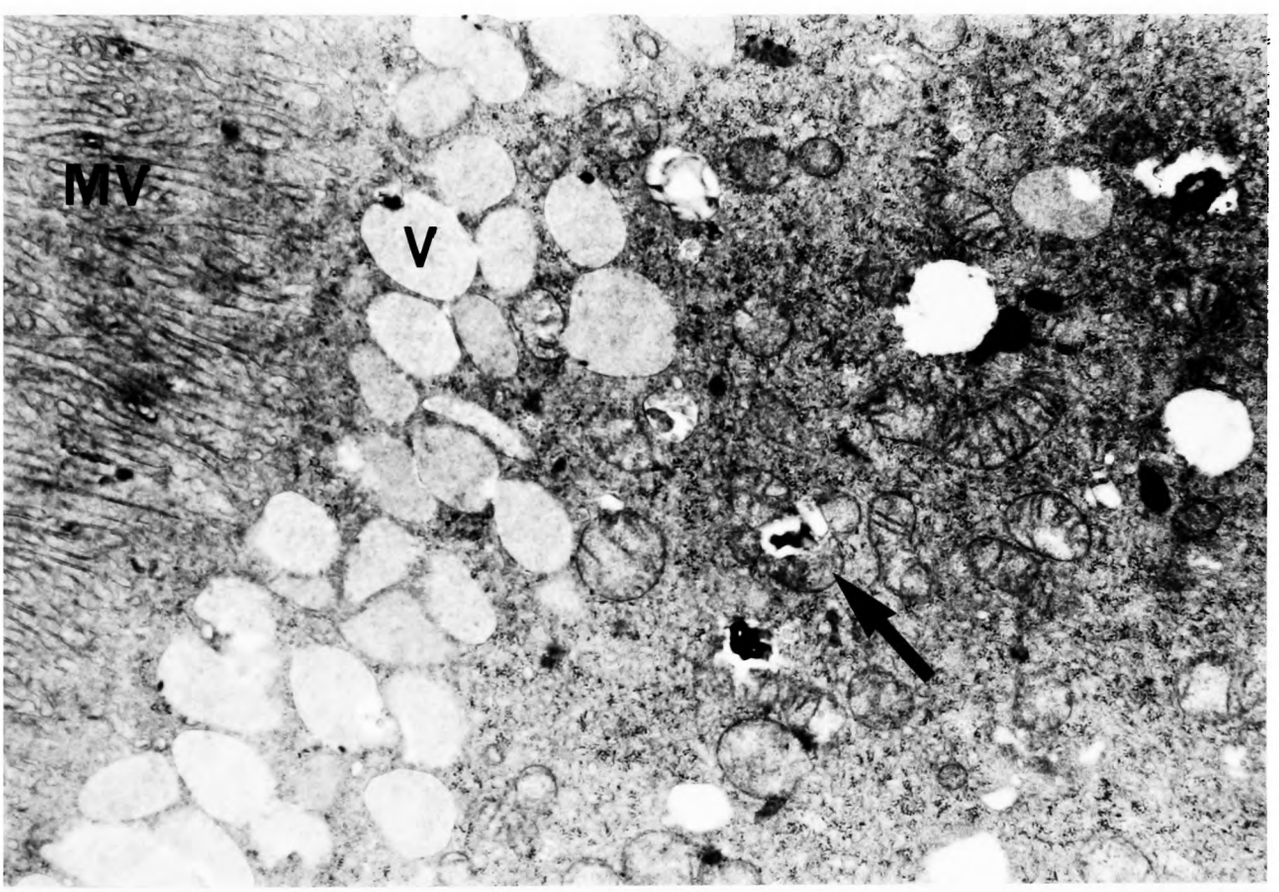
a. The basal border of the accessory glands contains abnormal basal infoldings of the plasma membrane, forming rounded vesicles (arrows). The cytoplasm is very dense, obscuring detail and there are glycogen stores (Gly)

b. The apical border has an increased number of secretory vesicles (V) accumulated beneath the microvilli (MV). Mitochondria can be seen undergoing autophagy (arrow).

a and b x 13,300.



a



b

3.3 Lipids

3.3.1 Wax lipid synthesis by Gené's organ

Ticks injected with 0.02 - 0.16 µCi of ^{14}C-1-acetate produced normal clusters of eggs. The extracted non-polar lipids washed from the surface of the oviposited eggs were found to contain radioactivity (Table 3.5). There was an approximate linear relationship between the amount of label injected and the amount detected in the egg waxes, expressed as acetate incorporation per fresh weight of eggs extracted, 24h after injection (Fig. 3.45). It was found that the majority of the radioactivity incorporated into the eggs was in the lipid fraction of the surface waxes. Two batches of eggs were homogenised and added to scintillation vials after surface washing in chloroform:methanol, and the residual activity of the eggs was 210 and 332 cpm, while that in the extracted lipids was 2538 and 939 cpm respectively.

Dissection of a ^{14}C-1-acetate (0.16 µCi) injected tick after 24h of oviposition revealed that the lobed gland cells, accessory glands and Gene's organ all contained radioactive lipids extractable by chloroform:methanol (Table 3.6).

Table 3.6 Distribution of ^{14}C in lipids in a ^{14}C-1-acetate injected tick

		CPM/extracted lipids	
Wt. Eggs laid	22mg	2933	
lobed gland cells		882	170,000 CPM were injected
accessory glands		716	
Gené's organ glands		1186	
Haemolymph (5µl)		820	(aqueous fraction)
	Total	6537	= 3.8% of ingested.

Dissected lobed gland cells, accessory gland cells and Gené's organ gland cells also incorporated ^{14}C-1-acetate into lipids when incubated *in vitro* at 30°C in Liebowitz L15 medium (Table 3.7).

The extracted lipids from the egg wax of ^{14}C-1-acetate injected ticks, and the lipids extracted from dissected glands incubated *in vitro* with the label were separated for comparison by TLC (Fig. 3.46). The radioactivity of the TLC spots was also determined by liquid scintillation counting for comparison (Table 3.8). Lipids from both Gené's organ gland cells and the extracted egg waxes were separable into eight spots which coincided in RF value. The accessory glands and lobed cells also had coinciding fractions, but spots 2,5,6 and 7 were missing in both extracts.

Table 3.5 Weights of eggs laid by ^{14}C-1-acetate injected ticks, and incorporation of the label into surface wax lipids

Dose ^{14}-C-1-acetate per tick	Wt eggs laid in 24h/mg.	^{14}C, CPM extracted lipids
0.02 µCi	22	126
	26	185
	15	59
	20	63
0.04 µCi	14	56
	24	372
	19	557
	7	372
	15	301
0.06 µCi	24	2538
	19	939
	17	1685
	19	806

Background = 33 cpm.

Table 3.7 Incorporation of ^{14}C-1-acetate into nonpolar lipid by glands in vitro

Tissue	Wt/mg.	^{14}C Lipids, CPM	CPM/mg tissue	Incubation Medium CPM/mg.
Lobed cells	0.8	2932	2345	1044
Lobed cells	0.8	5939	4751	805
Accessory glands	0.9	3129	2816	1021
Gené's organ	2.0	18769	9384	3066
Gené's organ	3.6	9763	2711	780

Spot no. 1 was the most radiactive in all extracts, and coincided with the RF values of N-nonadecane (C19) and n-octacosanol (C28) standards. Hexadecanoic acid (C-16), (palmitic acid), had an RF value of about 0.30 so it is probable that spots 4,5 and 6 are long-chain fatty acids. Most of the detected radioactivity was therefore in the alkane fraction.

Table 3.8 Radioactivity of lipid fractions separated by TLC, and analyses by scintillation counting of spots from Fig. 3.3.2 (figures in counts per minute of ^{14}C)

		Egg Wax	Accessory glands	Lobed glands	Gene's organ
Spot no.	RF	(W)	(A)	(L)	(G)
1	0.95	3,000	56	47	615
2	0.82	182	-	-	40
3	0.68	85	29	10	90
4	0.37	49	31	24	51
5	0.31	144	-	-	14
6	0.27	71	-	-	20
7	0.08	249	-	-	59
8	0.0	48	29	29	55

Background ; 15 CPM

Standards: nonadecane RF = 0.95

palmitic acid RF = 0.30

This was confirmed by fractionating a ^{14}C radiolabelled egg wax extract in hexane:methanol. The hexane fraction consisting of the alkanes contained 3159 CPM whereas the methanol fraction containing the more polar fatty acids and alcohols contained 469 CPM of ^{14}C.

Fig. 3.45 Graph showing the relationship between injected dose of Na ^{14}C-1-acetate and its incorporation into the nonpolar egg wax lipids in 24h in ovipositing B. microplus (Data from Table 3.5).

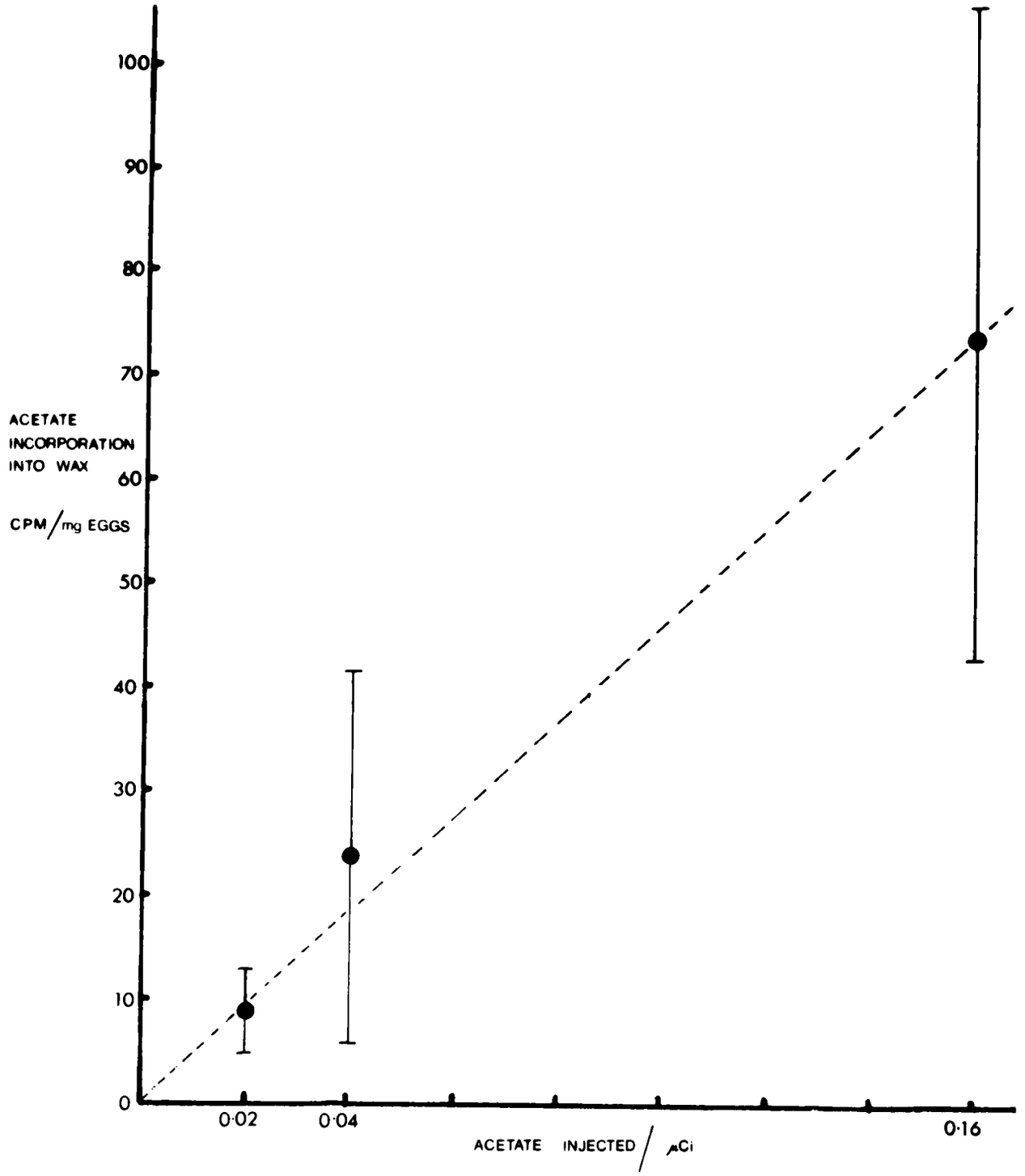
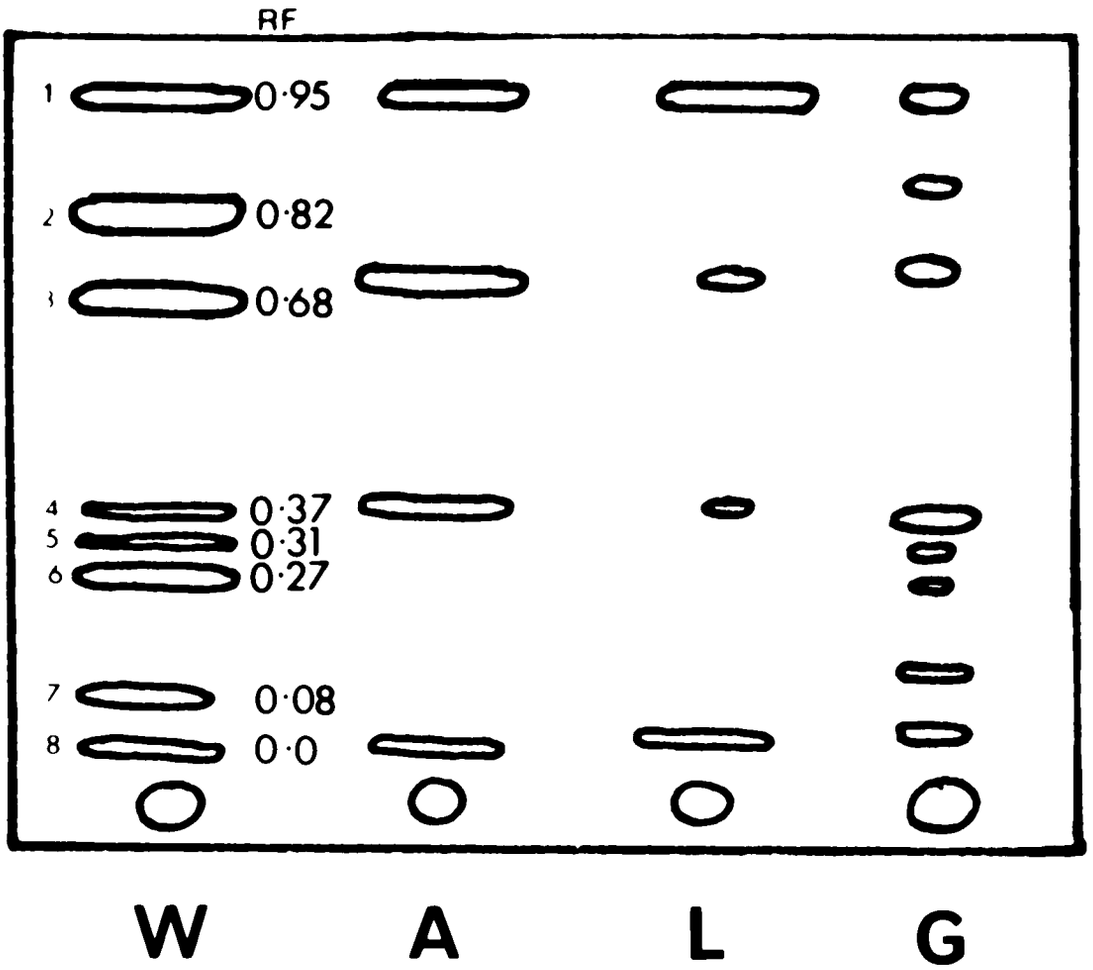

100
90
80
70
60
50
40
30
20
10
0
ACETATE
INCORPORATION
INTO WAX
CPM/mg EGGS
0·02
0·04
0·16
ACETATE INJECTED / μCi

Fig. 3.46 TLC separation of nonpolar lipids extracted from the egg wax (W) accessory glands (A) lobed glands (L) and Gené's organ (G) from ^{14}C-1-acetate injected ovipositing B. microplus. The spots were analysed for radioactivity (Table 3.3.2). Spots 2,5, 6 and 7 were absent from the accessory gland and lobed gland extracts.


RF
1
0·95
2
0·82
3
0·68
4
0·37
5
0·31
6
0·27
7
0·08
8
0·0
W
A
L
G

3.3.2 Autoradiographs

The secretory epithelium of Gené's organ, the accessory glands, lobed cells, gut and muscle cells are recognizable in wholebody cryostat sections of rapidly frozen material (Fig. 3.47a). The large secretory vesicles in Gené's organ are prominent. Autoradiographs of frozen sections from ^{14}C-1-acetate injected ticks show that both the accessory glands (Fig. 3.47b) and Gené's organ gland cells (Fig. 3.47c) take up the label, and the silver grains are most numerous over non-nuclear regions of the cells. The lumen of Gené's organ also contained dense silver grains, whereas the accessory gland lumen had few grains over it.

3.3.3 GLC analysis of lipids from Gené's organ and the egg wax

Alkanes

The alkane fraction from Gené's organ essentially matches that of the egg wax in composition (Fig. 3.48a,b) with the major peaks having similar retention times, with few exceptions. The components, however, differ quantitatively (Table 3.9). Compounds were tentatively identified using semilog plots of chain length against retention time, using the retention times of nC-18 - nC-24 standards (Fig. 3.48c). There were over 60 peaks many of which were not identified. The major differences were that peaks 12, 13 and 14 were abundant in the Gené's organ extract, but were very low in the egg wax. These peaks probably correspond to nC-24 to nC-26 alkanes. Peak 20, corresponding to nC-35 was the most abundant component in the egg wax (34%) whereas it was only 2.9% of the Gené's organalkanes. Also compounds of ECL C-29 and C-31 formed 1.9% and 1.3% of the Gené's organ alkanes, respectively, but were almost absent in the egg wax.

Fatty acid composition

The composition of the methylesters obtained by derivatization of the fatty acid extracts from Gené's organ and the egg wax were similar (Fig. 3.49; Table 3.10). The major peaks were identified as C16-C24 methylesters by comparison of the retention times against standards (Fig. 3.49c,d). Peak 6 corresponded with an unsaturated methylester, C-18:1 (oleic acid methylester). This component was abundant in the Gené's organ extract but was low in the egg wax. Peak 7, tentatively identified as C-18:2 by retention time, was more abundant in Gené's organ than in the egg wax extract. The other components appeared to be mainly saturated.

Fig. 3.47 a. Wholebody cryostat section from a rapidly frozen ovipositing B. microplus showing the Gené's organ secretory epithelium (SE), distended gut (G), lobed glands (L), accessory glands (AG) and muscles (M). The lipid vesicles in Gené's organ are easily visible since they do not stain in haematoxylin.

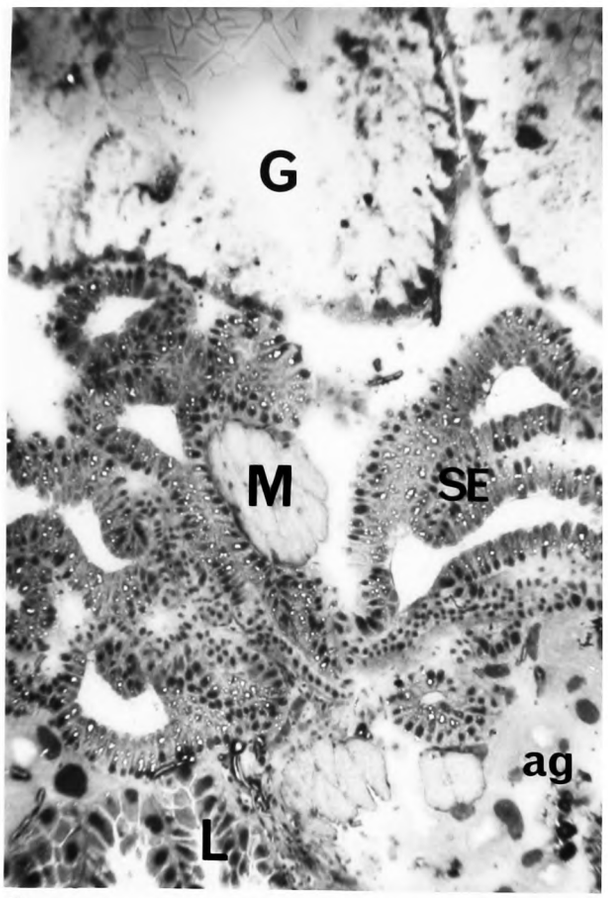
x 80.

b. Cryostat autoradiograph section showing an accessory gland (AG) from a ^{14}C-1-acetate injected ovipositing B. microplus. Silver grains are most concentrated over the cell cytoplasm and lumen (L) and are less prominent over the nucleus (N).

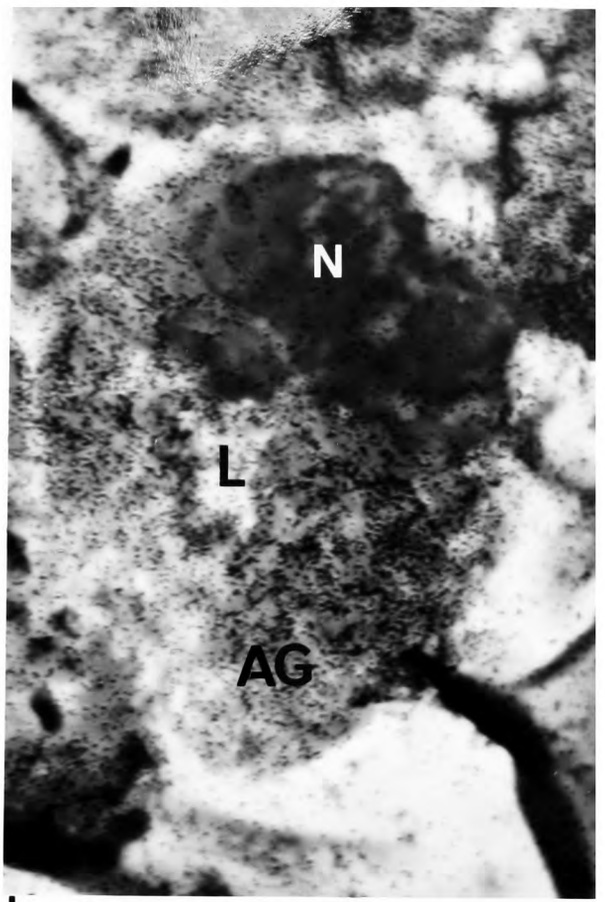
x 500.

c. LM showing a cryostat sectioned autoradiograph of the secretory epithelium of Gené's organ in a radiolabelled acetate-injected tick. Most of the silver grains are concentrated over the secretory cells and the gland lumen (L). There are few background grains on the section.

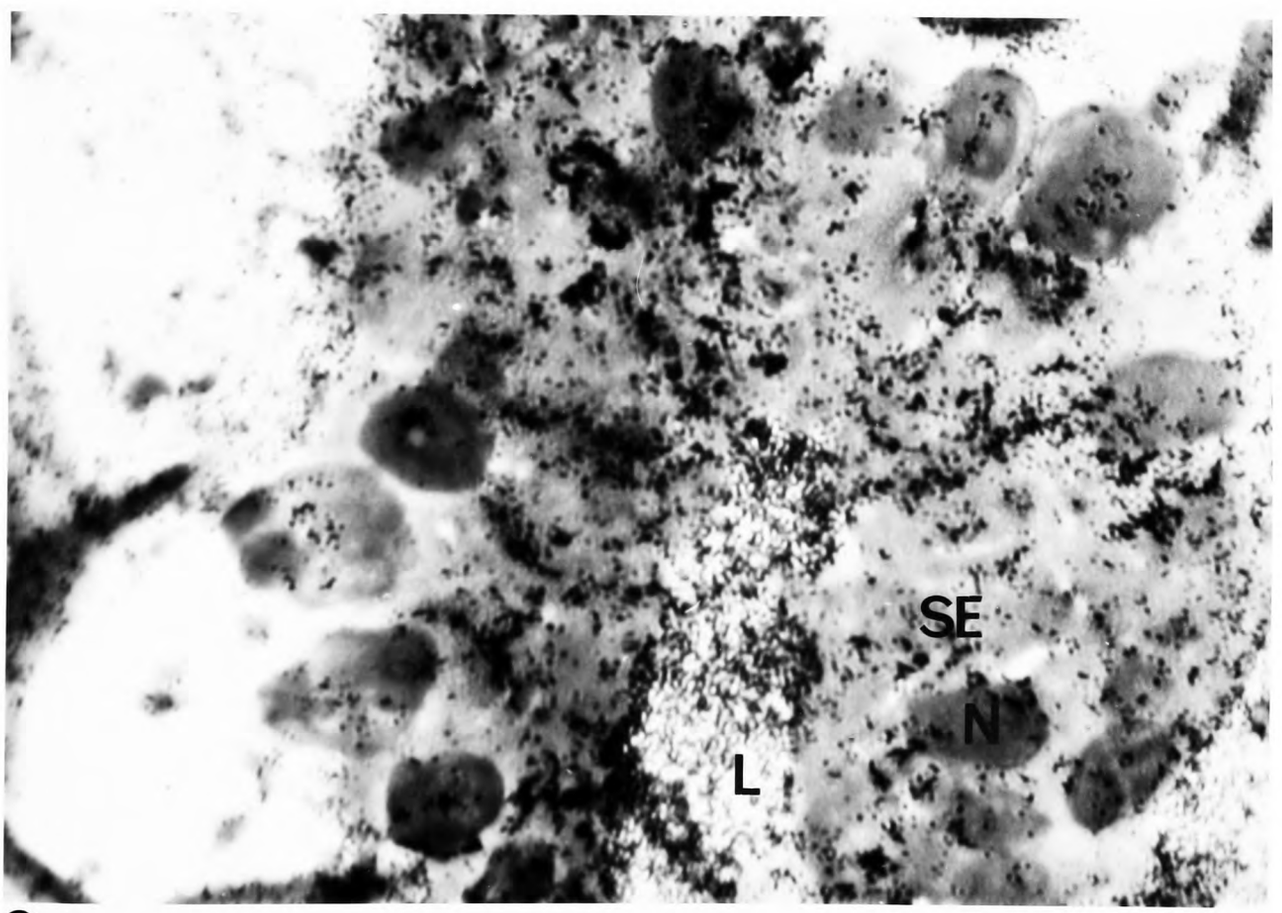
x 700.



a



b



c

Table 3.9 Composition of Alkanes, and equivalent chain lengths

			% composition	
Peak no.	ECL	Structure	Gené's organ	Egg wax
1	18	n-C18	4.3	3.2
2	19	n-C19	2.4	1.7
3	20	n-C20	2.5	1.7
4	21		2.7	1.0
5	21.3		3.1	2.3
6	22	n-C22	2.6	1.6
7	22.6		4.1	2.2
8	23	n-C23	8.0	3.0
9	23.4	n-C24	2.6	1.3
10	23.7		1.3	1.2
11	24		0.7	0.8
12	24.7		5.3	1.2
13	25		31.0	0.8
14	26		7.6	0.3
15	29		1.9	-
16	31		1.3	-
17	32		1.2	12.8
18	33		3.9	13.2
19	34		1.7	11.5
20	35		2.9	34.4
21	36		0.4	-
22	37		0.8	1.2
23	38		1.3	0.9

Only peaks above 0.3% included.

Table 3.10 Composition of methylesters of fatty acids

Peak no.	ECL	Structure	Relative abundance Gené's organ	Egg wax
1	16.0	C16:0	4.2	7.3
2	16.5		2.7	5.6
3	16.9		1.7	2.1
4	17.1	C17:0	1.3	1.9
5	18.0	C18:0	5.8	7.9
6	18.2	C18:1	14.9	1.5
7	18.8	C18:2	4.8	0.5
8	19.7	C20:0	1.3	3.3
9	21.0	C21:0	-	-
10	22.0	C22:0	2.3	2.0
11	23.0	C23:0	-	-
12	24.0	C24:0	1.7	1.9

Equivalent chain lengths calculated from plots of log chain length vs retention time.

Fig. 3.48 GLC analysis of the alkane fractions extracted from the egg wax (a) and Gené's organ (b) and the nC_{18}-nC_{30} alkane standards (c) for comparison, (from ovipositing B. microplus). Major peaks are labelled as to their probable chain lengths.

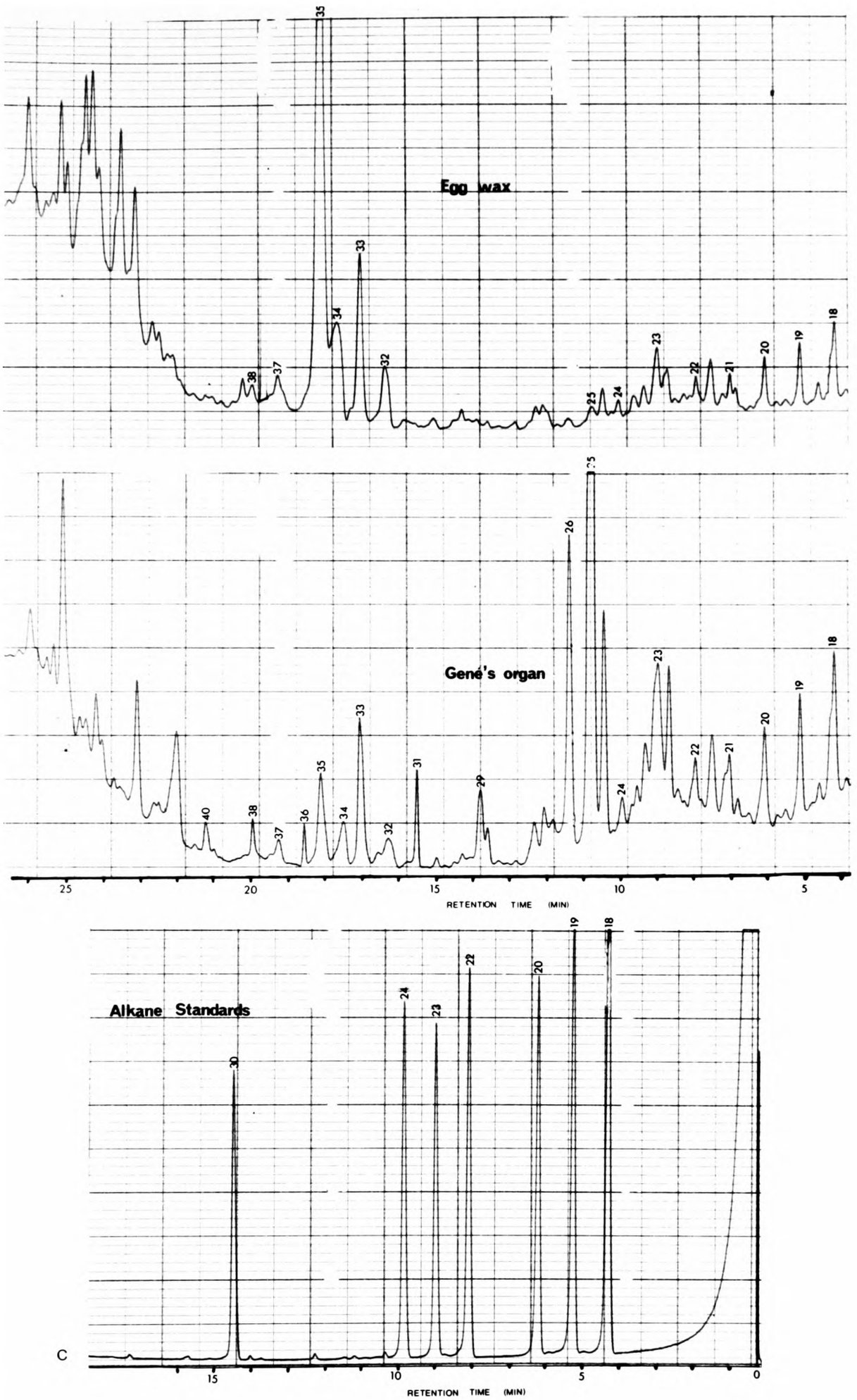
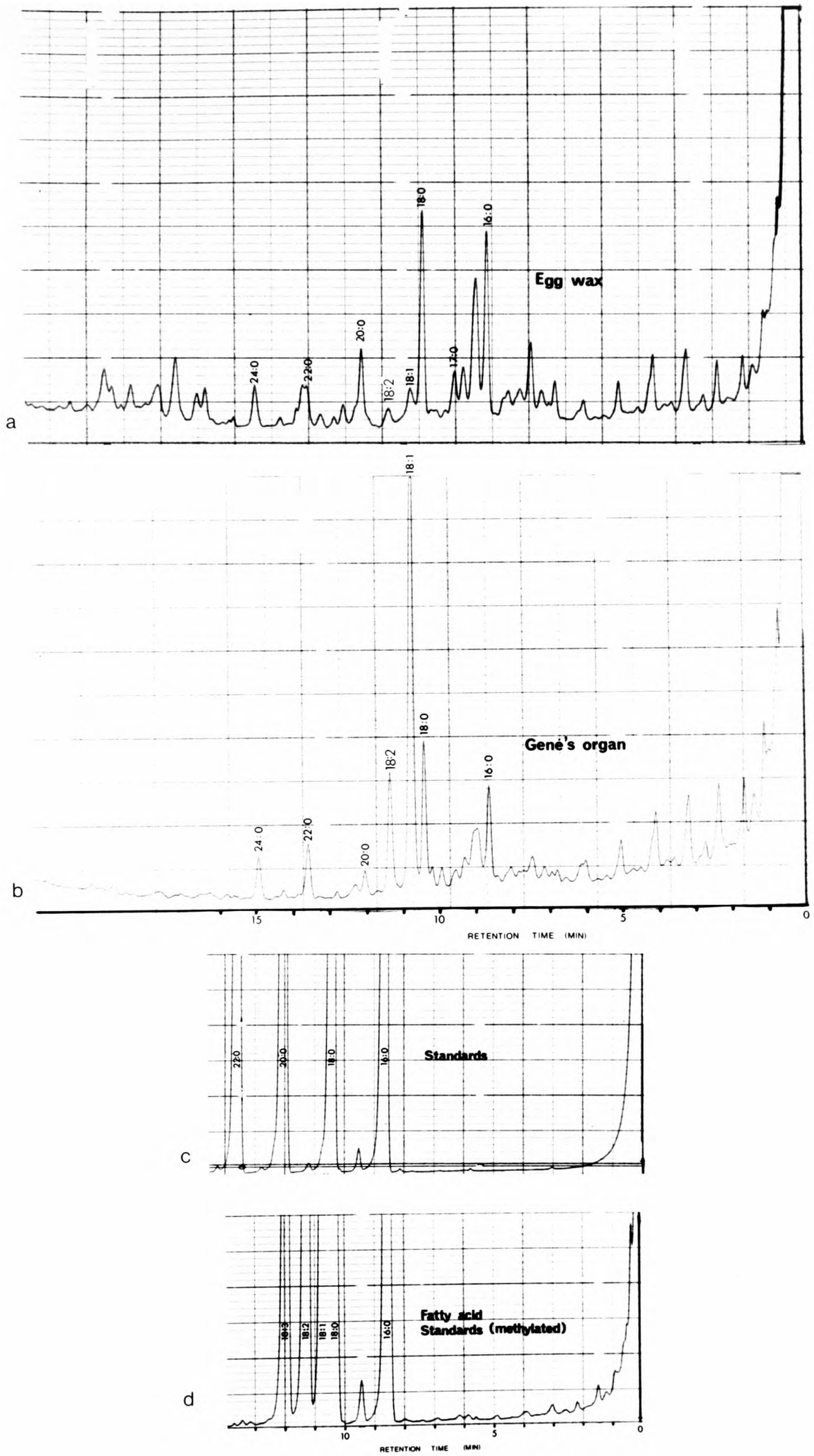

a
Egg wax
35
33
34
32
38
37
25
24
23
22
21
20
19
18
b
Gené's organ
26
25
40
38
37
36
35
34
33
32
31
29
24
23
22
21
20
19
18
25
20
15
10
5
RETENTION TIME (MIN)
c
Alkane Standards
30
24
23
22
20
19
18
15
10
5
0
RETENTION TIME (MIN)

Fig. 3.49 GLC analysis of fatty acid methylesters derived from the fatty acid fractions extracted from the eggwax (a) and Gené's organ (b) in ovipositing B. microplus. Methylester standards, run for comparison, are shown in (c) and (d).


a
Egg wax
24:0
22:0
20:0
18:2
18:1
17:0
18:0
16:0
b
Gene's organ
24:0
22:0
20:0
18:2
18:1
18:0
16:0
15
10
5
0
RETENTION TIME (MIN)
c
Standards
22:0
20:0
18:0
16:0
d
Fatty acid
Standards (methylated)
20:3
18:2
18:1
18:0
16:0
10
5
0
RETENTION TIME (MIN)

3.4 Electrophysiology

The resting potentials of freshly dissected retractor muscles of A. variegatum varied between -50mV and -70mV (mean -59.1mV, s.e.m. 7.7mV, n=14). Spontaneous excitatory post-synaptic potentials (epsps) were frequently encountered in the muscles (Fig. 3.50a). These were depolarising potentials of 10mV to 35mV in amplitude with a rise time of 5-10mS and recovery time of about 50mS. There was also evidence of very small epsps of 2-3 mV with a significantly faster rise time. In some muscles, no spontaneous epsps were observed, but regular fluctuations of the resting potential occurred, of 2-3 mV in amplitude and about 1s periodicity (Fig. 3.51d). The spontaneous events did not usually overshoot zero potential. Neurally evoked epsps obtained from stimulation of the frontal nerves had a rise time of about 10 mS (Figs. 3.50b, 3.51a), and an amplitude that varied according to the intensity of the stimulus (Fig. 3.50b). A single stimulus often gave rise to several spikes being recorded from a single muscle, or a compound potential (Figs. 3.51b, 3.51c). There was also a threshold of stimulus below which no epsp was obtained.

Bath-applied L-glutamate and L-aspartate at 10^{-3}M eliminated spontaneous epsps recorded from the retractor muscles, and caused a depolarization of 10-15 mV which persisted until the bath medium was replaced with fresh saline (Figs. 3.50c, 3.51e). These amino acids also eliminated neurally evoked potentials at 10^{-3}M (Figs. 3.52d, 3.52e). No effect was observed with octopamine, acetylcholine or gamma-amino butyric acid at 10^{-3}M on either spontaneous or evoked potentials. Pressure ejection of L-glutamate and L-aspartate onto the muscle resulted in dose-dependent depolarisation (Figs. 3.50d, 3.52a). The speed of recovery of resting potential and the amplitude of response depended on the position of the micropipette used for pressure ejection and its closeness to the muscle being recorded, as well as the amount of glutamate or aspartate ejected onto the muscle.

Repetitive application of glutamate resulted in desensitisation and eventual hyperpolarisation which lasted 2-3 seconds in some cases, with normal sensitivity to glutamate returning after normal resting potential was regained (Fig. 3.52c). Repeated application of aspartate, to the retractor muscle by pressure ejection, however, did not show the same effect, and each application resulted in an increase in depolarisation and no evidence of desensitization was found (Fig. 3.52b).

Fig. 3.50

A. Spontaneous e.p.s.ps. recorded from the Gené's organ retractor muscles of A. variegatum.

B. Neurally evoked potentials recorded in the retractor muscles upon stimulation of the frontal nerves. An increased stimulus results in an increased response. Arrow-stimulus artefact.

C. The effect of 10^{-3}M glutamate (bath applied) in eliminating spontaneous e.p.s.ps. recorded from a retractor muscle. Arrow-moment of glutamate application.

D. Depolarising responses of the retractor muscle membrane potential to pressure-ejected glutamic acid. The resting potential was about -65 mV. Increasing the amount of glutamate ejected onto the muscle increased the transient depolarization - the arrows represent successively larger doses of 2,4, 6,8 and 10 mS duration. Maximum depolarisation achievable was about 18 mV.

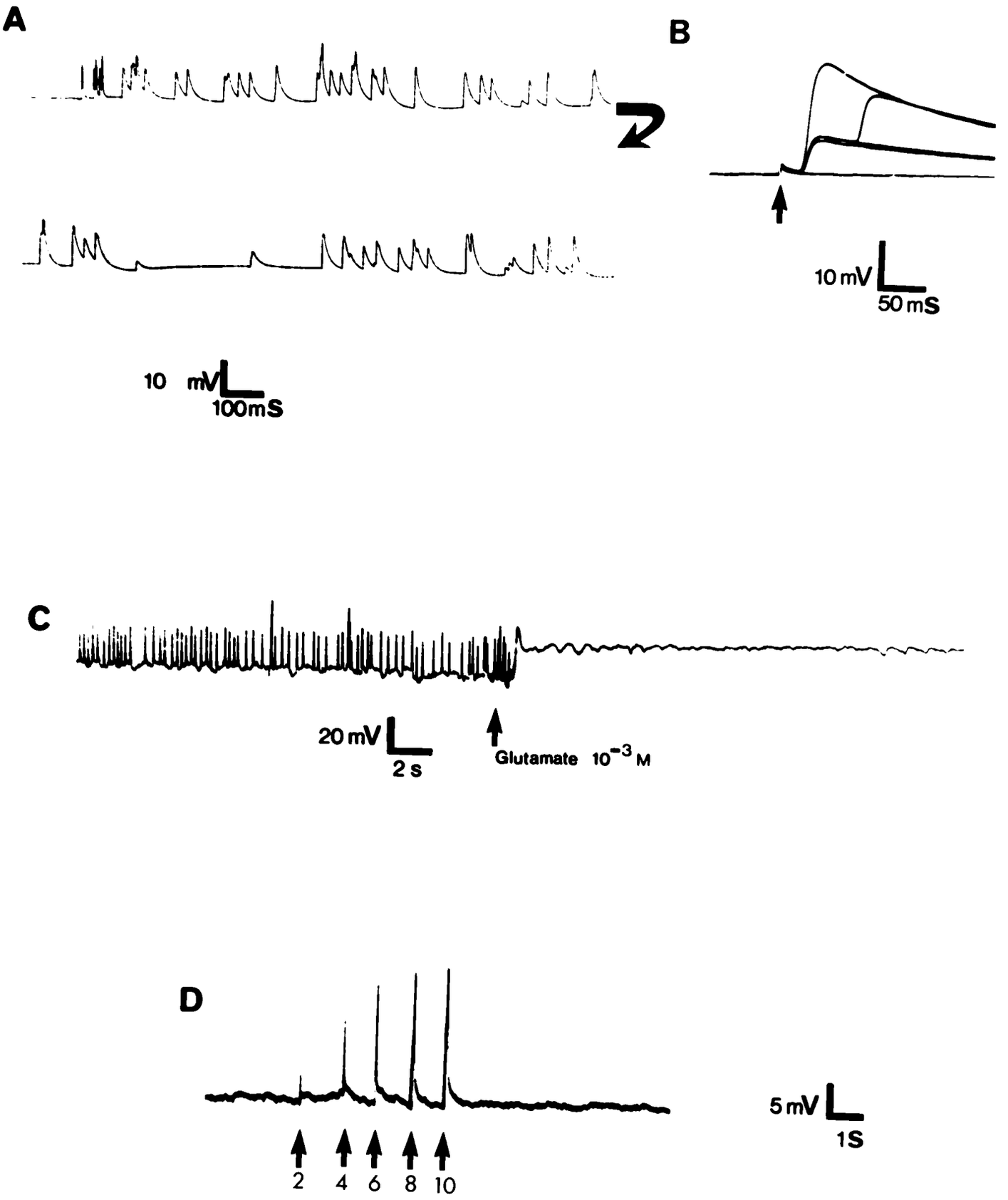

A
B
10 mV
100 mS
10 mV
50 mS
C
20 mV
2 s
Glutamate 10^{-3} M
D
2
4
6
8
10
5 mV
1S

Fig. 3.51 A. A single neurally evoked potential recorded in a retractor muscle. Arrow-stimulus artefact.

B,C. Compound potentials recorded from retractor muscles upon stimulation of the frontal nerves. Arrows-stimulus artefacts.

D. Resting potential fluctuations recorded from a retractor muscle.

E. The effect of 10^{-3}M aspartic acid (bath applied) in eliminating spontaneous e.p.s.ps. recorded intracellularly in a retractor muscle. Arrows-moment of aspartate application. The apparent increase in activity at the time of application is due to an increase in mains noise (50 Hz) caused by the pipette.

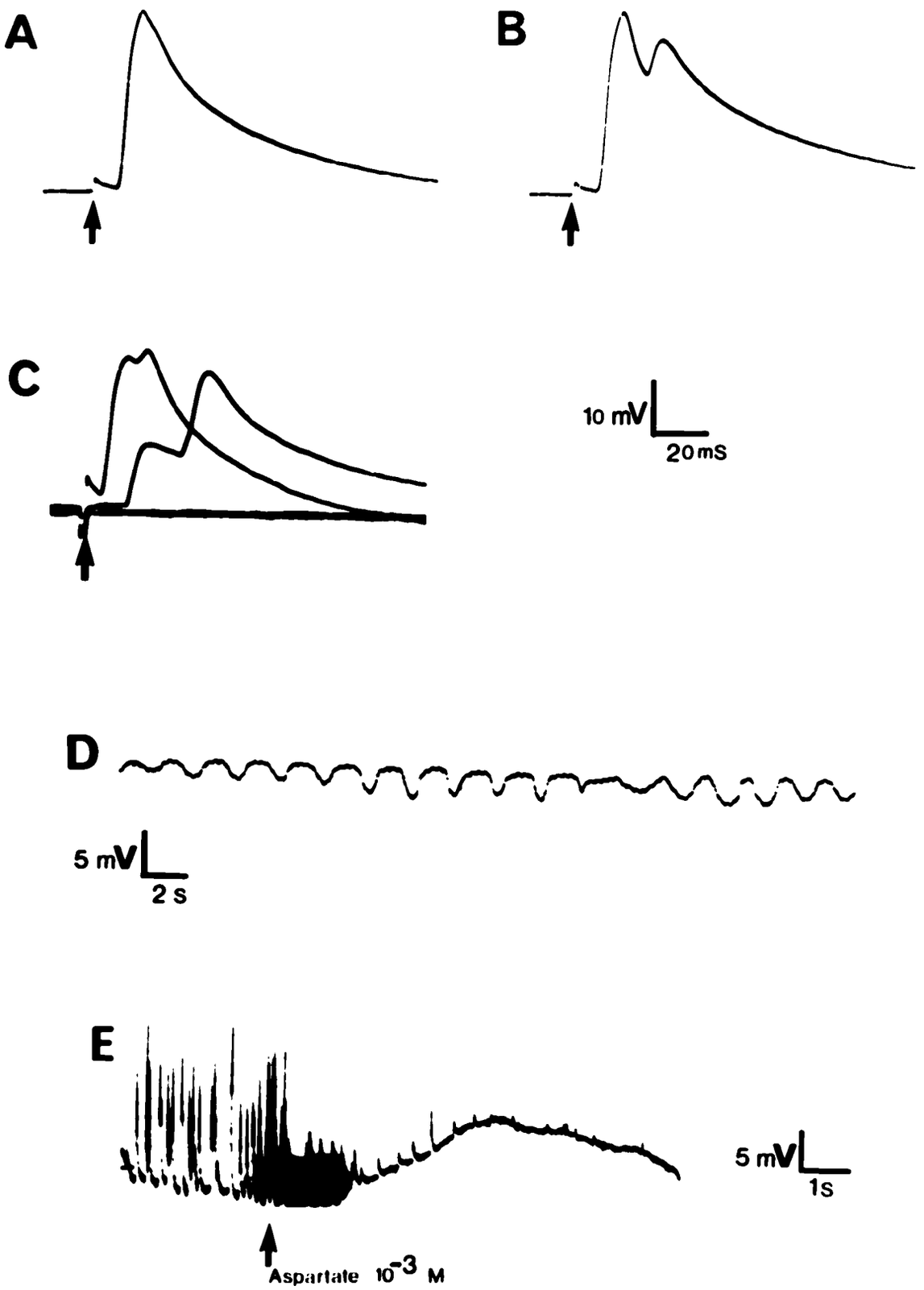

A
B
C
10 mV
20 ms
D
5 mV
2 s
E
5 mV
1s
Aspartate 10-3 M

Fig. 3.52

a. Responses of the retractor muscle membrane potential (about -65mV) to pressure ejected aspartic acid (arrows). A series of linearly increasing doses (2,3,4,5 mS) was recorded showing successively increasing depolarizations. Maximum depolarization was about 20 mV.

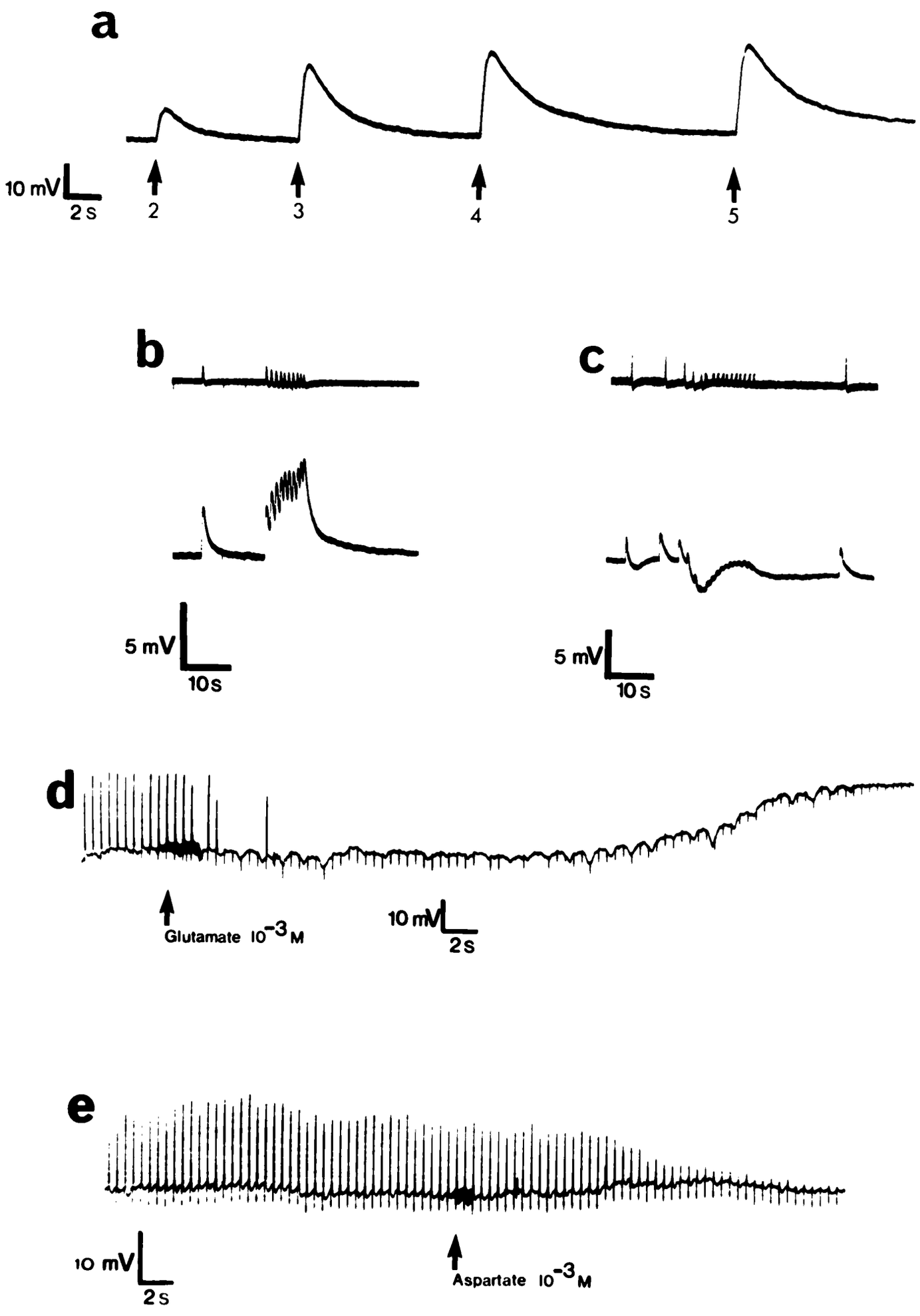
b & c. Upper trace: ejection of ligand; lower trace: membrane potential recorded intracellularly in the retractor muscle.

b. The effect of rapid repetitive aspartate application on to the retractor muscles is successively larger depolarizations.

c. Rapid repetitive ejection of glutamate causes desensitization and hyperpolarization, but normal sensitivity to glutamate returns within a few seconds.

d. The effect of 10^{-3}M glutamate in eliminating neurally evoked e.p.s.ps. recorded from a retractor muscle. Downward deflection, stimulus artefact; upward deflection, depolarizing response.

e. Aspartate also eliminates the neurally evoked potentials at 10^{-3}M.


a
10 mV
2 s
2
3
4
5
b
5 mV
10 s
c
5 mV
10 s
d
Glutamate 10^{-3} M
10 mV
2 s
e
10 mV
2 s
Aspartate 10^{-3} M

Discussion

The present study is the first to describe the ultrastructure of Gené's organ at the electron microscope level, and has revealed many aspects that could not be seen with the light microscope. The structure has also been revealed in two ixodid species not previously described with light microscopy. The egg laying process in B. microplus, one of the most commercially important pest species, has also been described in greater detail.

The structure of Gené's organ in B. microplus is essentially similar to that described in O. moubata (Lees and Beament, 1948), I. ricinus (Arthur, 1953) and H. asiaticum (Balashov, 1972) and consists of a pair of horns that can be everted and retracted, and a glandular secretory epithelium. The egg laying process in B. microplus is also similar to that described in other species, with only minor differences.

Lees and Beament (1948) showed that there were many small areas which stained with ammoniacal silver nitrate on the outer cuticle of the horns, and in light microscope sections they interpreted these indentations in the cuticle as pore canals. The present study has demonstrated that the pore canals described by Lees and Beament are in fact pits in the cuticle, lined with pore canals of 50nm in diameter. The dark areas observed after staining with ammoniacal silver nitrate were assumed to be areas which lacked a protective cement layer allowing the polyphenols in the underlying cuticle to reduce the silver reagent. The present study has shown that these areas are actually small setae on the surface of the cuticle, not previously described, which appear to be devoid of a protective cement layer, rather than representing the distribution of pore canals as suggested by Lees and Beament (1948). The actual pore canals are too small to be resolved in the light microscope. Although the setae are about 1 µm long, they are difficult to see with a binocular microscope unless stained with ammoniacal silver nitrate after de-waxing, and are seldom seen in longitudinal section, due to the low probability of cutting one longitudinally at the correct point. Study with the SEM has made it much easier to determine the form and distribution of these setae, and the pits. The distribution of setae corresponds with the areas of the horns which come into contact with the eggs. The TEM has shown that each pit is lined with pore canals, which appear as filamentous tubes linking the lumen contents of the horns with the outside. These pore canals probably function as

wax canals as in the cuticles of other terrestrial arthropods. The setae may have a function in gripping the eggs as they are manipulated by the horns during oviposition. The egg must adhere to Gené's organ more readily than the prolapsed vagina if transfer is to occur. This is achieved by the presence of the sticky wax layer on the horns but may also be aided by the presence of setae.

Egg clusters adhere together very strongly due to the wax covering and this in itself probably helps to control water loss. The setae might also facilitate the spreading of wax over the egg as it is manipulated. During oviposition not all of the egg surface comes into contact with Gené's organ so that the spreading properties of the wax are important in ensuring an even covering.

It is likely that the wax secretion passes from the lumen of Gené's organthrough the pore canals which line the pits on the cuticular surface. In insects (Neville, 1975) and ticks (Hackman, 1982) the superficial waxes of the body wall cuticle are thought to pass through the pore canals in a similar manner, before the pores are blocked by the deposition of a protective cement layer. The outer cuticle of Gené's organ is very thin so the short length of the pore canals will provide little resistance to the passage of secretion in comparison with the thick body wall cuticle.

This study has shown that although the cuticle of the horns appears to have an outer cement layer, since it does not stain intensely with ammoniacal silver nitrate except on the setae, the pore canals themselves appear to be open and able to allow wax secretion to pass through them as demonstrated in the TEM. Thus the pore canals remain open for continuous secretion of wax. The thinness of the outer cuticle also allows the horns to fold into many pleats during retraction.

The mechanism of eversion of the horns of Gené's organ appears to be hydrodynamic, with simultaneous contraction of the capitulum retractors and relaxation of the horn retractors. There is no intrinsic musculature capable of causing eversion but the dorso-ventral muscles may be important in contracting to cause haemolymph to flow into the horns during eversion.

Simultaneous relaxation of the organ's retractor muscles causes the horns to turn inside out and pass through the slit in the camerostomal fold which is widened by the retraction of the capitulum. The

internal pressure during eversion would be transferred to the lumen contents and this would tend to force the secretion through the pore canals on to the external surface of the horns. The Gené's organ retractor muscles control the pulsating movements of the horns during oviposition by thus acting against fluid pressure of the haemolymph. The fact that gentle pressure on the tick causes eversion of the horns when the mouthparts are depressed provides evidence of the hydrodynamic mechanism. A very localised flow of haemolymph would be needed to cause the horns of Gené's organ to evert and inflate, together with a simultaneous relaxation of the muscles. Thus contraction of the muscles causes retraction of the horns. The powerful dorso-ventral muscles can be seen pulsating during oviposition and this provides further evidence for the hydrodynamic mechanism. However, it may be that the haemolymph is already at a high enough pressure above atmospheric to account for eversion by relaxation of the retractor muscles and lowering of the mouthparts alone. If an incision is made in a fully-engorged female haemolymph pours out and the gut ruptures, indicating that there is considerable internal pressure. The activity of the dorso-ventral muscles is probably more important in the later stages of oviposition when body turgor is much reduced. Extension of the walking legs in ticks similarly involves fluid pressure, but the mechanism is slightly different (Hart, 1982). Fluid pressure has also been suggested in the leg extension of arachnids (Anderson and Prestwick, 1975) and mandibulate arthropods (Manton, 1958; 1977), since in these animals only the leg flexor muscles are well developed and dehydrated animals are unable to extend their legs. The hydrostatic pressure of the haemolymph therefore keeps the legs extended unless flexed by muscle action. The pheromone gland of the adult male scorpion fly consists of evertable cuticular vesicles situated intertergally on the abdomen, which like the horns of Gené's organ have only retractor muscles. Eversion of the gland during pheromone release is similarly achieved by a flow of haemolymph brought about by contraction of the abdominal intersegmental musculature and simultaneous relaxation of the gland retractors (Crossley and Waterhouse, 1969). Fluid pressure in general is clearly a useful method available to arthropods for the extension of appendages, made possible by the stiff exoskeleton. It also reduces the complexity of the musculature needed for control and allows the possibility for inflatable appendages such as the horns of Gené's organ.

The results of this study have confirmed that the wax secretion

is produced in the glandular epithelium that is an extension of the body wall epithelium, using both ultrastructural and biochemical techniques. The ultrastructure of these cells is consistent with a secretory function. The complex microvillar border, arrays of endoplasmic reticulum and well developed Golgi bodies are typical of secretory cells. They contain numerous large secretory droplets whose contents are extracted during tissue processing suggesting that they contained lipids. There are also many smaller vesicles aggregated around the Golgi bodies. The smooth endoplasmic reticulum is not prominent in normal cells; however, there are tubular membranous projections inside the microvilli on the apical border of the cells. These membranes are smooth, like the SER, and are characteristic of cells which secrete lipids (Fawcett, 1981). The gland cells of Gené's organ also contain extensive stores of glycogen, in large arrays of α-particles throughout the cytoplasm. These particles appear ultrastructurally similar to those found in many other tissues, particularly in vertebrate liver cells where they are associated with SER (Fawcett, 1981).

This study has also shown that the accessory glands are also involved in wax synthesis and secretion, in addition to producing the anti-oxidant suggested by Atkinson and Binnington (1973). The accessory glands of Gené's organ have not previously been investigated using electron microscopy, nor has their histology at the light microscope level been adequatelystudied, which are both essential in confirming the nature of their contribution to the egg wax secretion, and correlating their structure with their function.

The accessory glands of Gené's organ have a structure in common with other types of integumentary glands found in terrestrial arthropods, being most similar to the Class 3 type of gland described by Noirot and Quennedey (1974). Each gland cell, containing numerous membrane-bound vesicles and mitochondria, has its own internal secretory lumen, lined with microvilli and a separate duct. Each duct cell connects to an individual secretory pore in the cuticle. The duct is not lined with epicuticle, except where it passes through the body wall cuticle, and in these respects the glands are very similar to those secreting pheromones in Atta sp. (Bazire-Benazet and Zylberberg, 1979). In other Class 3 arthropod integumentary glands, the secretory cells are in close contact with the cuticle, as in the epidermal glands of the termite soldier (Sbrenna and Leis, 1983), cockroach (Brossut and Sreng, 1980)

and locust (Sbrenna and Sbrenna Micciarelli, 1980). These cells differ from the tick accessory glands in that the duct cell is a relatively short structure, whereas in the accessory glands it may be 2mm long.

The accessory glands are associated only with female ixodid ticks and are absent from the Argasidae (Feldman-Muhsam and Havivi, 1960). Feldman-Muhsam (1963) suggested that the areae porosae produced a lubricant for the horns of Gené's organ during oviposition. However, Atkinson and Binnington (1973) found that in B. microplus blocking the pores by electrocautery in newly dropped gravid females had no effect on oviposition or subsequent hatching of the eggs and found evidence that the secretion from the pores exerted an antioxidant effect on certain unsaturated steroid components in the egg wax. Blocking of the pores in adult female B. microplus before commencement of oviposition with rapid hardening resin, araldite, also resulted in normal eggs being laid which were as viable as eggs laid by ticks with their areae porosae unblocked (Booth et al., 1984b). The porose area secretion therefore does not appear to be essential for laying viable eggs, at least under laboratory conditions. It is clear from the lipid studies that the accessory glands do synthesise some of the components of the egg wax. The presence of a separate organ system, the accessory glands, in close relationship with Gené's organ implies however that they have a separate and distinct function related to egg-waxing, and that there is probably a division of labour between the tubular glands of Gené's organ and the accessory glands in producing the egg wax secretion.

The ultrastructure of the accessory glands is characteristic of cells that synthesise lipids. They contain striking amounts of smooth endoplasmic reticulum and many Golgi dictyosomes with small budding membrane-bound vesicles. The appearance of the smooth-membrane reticulum varies between different specimens, and may be in tubular form, in smooth membrane sheets, or as isolated vesicles. This may reflect a different developmental stage of the glands. Many larger vesicles appear to accumulate near the apical border of the cell and undergo exocytosis into the lumen. These vesicles also probably contained lipid since they are electron opaque in thin sections. These characteristics are often associated with lipid synthesis in other invertebrate tissues such as the oenocytes of insects (Locke, 1969) which are known to synthesise the paraffin components of cuticular wax in the locust (Diehl, 1973) and similar cells that are involved in

hydrocarbon synthesis in the cockroach integument (Nelson, 1969; Nelson et al., 1971).

In all of the exocrine glands that have been studied, the product is synthesised in the ER, is transported in small intermediate membrane-bound vesicles to the Golgi cisternae, or directly to a large secretory vacuole at the cells secretory surface. The secretory vacuole is limited by a membrane of Golgi origin which is capable of fusing with the plasmalemma during exocytosis (Fawcett, 1981). The secretory vesicles are able to coalesce with the cell membrane thereby releasing the contents outside the cell. This process appears to occur with the accessory glands of Gené's organ, but in the secretory gland of Gené's organ it is unclear whether the large lipid droplets have a direct secretory function or whether they are involved in storage only.

The results from the lipid synthesis experiments show that the Gené's organ secretory epithelium, the accessory glands and the lobular glands of the oviduct are able to synthesise lipids from radiolabelled acetate. The labelled lipids are mostly nonpolar hydrocarbons and wax esters and this suggests that alkane synthesis occurs by the elongation of fatty acids followed by decarboxylation as in insects (Major and Blomquist, 1978; Chu and Blomquist, 1980). The ultrastructure of all three of these glandular tissues is consistent with their lipid synthesising abilities. The autoradiography experiment showed that both the accessory glands and Gené's organ took up the radiolabelled acetate, and in the case of Gené's organ the label seemed to be concentrated around the apical border of the cells near the lumen. Thin-layer chromatography showed that there were identical components synthesised in Gené's organ corresponding with those in the egg wax. Several spots from both Genés organ and the egg wax, separated by TLC after extraction from ^{14}C-1-acetate injected specimens, had identical RF values and contained labelled lipids. These lipids were tentatively identified as long chain fatty acids and hydrocarbons. The composition of the nonpolar egg wax lipids of B. microplus are well known (McCamish et al., 1977) although it is not clear which components originate from Gené's organ itself or are added by the accessory glands during manipulation of the eggs by the horns or are applied to the egg during passage along the oviduct. The tubular accessory glands of the oviduct contain proteinaceous secretory granules, and abundant endoplasmic reticulum confirming the supposition by Lees and Beament (1948) that these glands produce a protein secretion.

Comparison of the lipids of Gené's organ with those of the egg wax by GLC showed that the compositions were for the most part identical. The data for hydrcarbons shows that there are more of the longer-chained alkanes in the egg wax than in Gené's organ, which would be expected if chain elongation of alkanes is occurring in Gené's organ. The identification of the peaks in this study was not confirmed by mass spectrometry, so it is difficult to compare the results for the egg wax with the study of McCamish et al. (1977). Also a different extraction, purification and derivatization procedure was followed. Steroids are present in the egg wax of B. microplus in much larger quantities than is found in the superficial waxes of other arthropods (Cherry, 1969) and this may be related to the high dietary intake of cholesterol in ticks (Cherry, 1976). The function of these steroids in the egg wax is unknown though it is unlikely that they contribute merely to the waterproofing of the egg since this can be achieved by the far less complex long chain alkanes, alcohols and esters to which the tick has access. It is possible that these steroids satisfy some as yet unknown embryonic requirement by penetrating the egg membrane (McCamish et al., 1977). The ultrastructure of the accessory gland cells with their large quantities of smooth endoplasmic reticulum may be a more likely source of the steroids than the secretory cells of Gené's organ which contain less smooth ER. Smooth endoplasmic reticulum is a characteristic feature of steroid synthesising tissues in vertebrates such as the testicular interstitial cells (Christensen, 1965) and the corpus luteum (Bjersing, 1967). Also the secretory cells of Gené's organ contain plentiful rough endoplasmic reticulum and cytochemical studies of the organ suggest that it produces lipoprotein in addition to free lipids (Lees and Beament, 1948; Chinery, 1965). The results from the lipid studies also indicate that the secretory cells of Gené's organ produce large quantities of free lipids and that much of this is hydrocarbon. The present study did not however analyse the egg wax or secretory cells for steroids, and further biochemical study is needed to confirm the origin of the steroids in the egg wax.

The preliminary study of the effects of a range of biogenic amines and their agonists and antagonists on oviposition in B. microplus was aimed at gaining more information on the possibility that octopamine receptors may be involved in the control of oviposition, and to see whether the octopamine agonists had a similar oviposition blocking effect as the formamidines in B. microplus.

The results from the pharmacological tests indicate that an adrenergic system may be involved in the control of oviposition in the cattle tick. This study has shown that the secretory cells of Gené's organ contain no direct innervation. It is therefore probable that the drug effects are due to interferance with the central nervous system, or peripheral nerves such as the oviducal nerves. The natural transmitter amine octopamine, and the α-adrenergic agonists clonidine, tolazoline and naphazoline all caused cessation of oviposition and the accumulation of a wax deposit around the horns of Gené's organ. The drug reserpine, which is known to deplete endogenous catecholamines, and the formamidine acaricide amitraz, which is known to be an agonist of octopamine receptors (Evans and Gee, 1980) both had similar oviposition blocking effects. Gené's organ appears to behave normally in ticks treated with these drugs, and there seems to be no adverse effects on wax synthesis. The fact that the ovaries and oviducts also appear normal in these treated ticks suggests that oviposition is blocked by a direct or indirect effect in preventing the contractions of the oviduct during oviposition.

Although octopamine was powerful in inhibiting oviposition, synephrine, the N-methylated analogue of octopamine, and apomorphine, both α-adrenergic agonists, had no effect on oviposition at the doses used. This is unusual since synephrine consistently appears to be more potent in stimulating octopamine receptors in the firefly and locust (Evans, 1982). The differences in effect of the different adrenergic agonists on oviposition may be explainable in part by differences in accessibility to the receptor, or inactivation at different rates, or it may be that the supposed octopamine receptors in ticks differ from those of insects.

A characteristic of all octopamine receptors studied in insects, crustaceans and molluscs is that they are blocked by α-adrenergic blocking agents such as phentolamine in preference to β-adrenergic agents such as propranolol (Evans, 1982). Both phentolamine and propranolol were found to be ineffective in blocking oviposition in B. microplus in this study. The α-adrenergic blockers metaclopromide and chlorpromazine were similar to phentolamine in having no effect on oviposition in B. microplus. The situation needs clarification by cotreating ovipositing ticks with octopamine and the various α- and β-blocking agents, to see if they prevent the effects of octopamine and the α-adrenergic agonists in stopping oviposition.

In the locust leg extensor muscle all the octopamine receptors studied are selectively activated by α-adrenergic agonists such as clonidine and naphazoline in preference to β-adrenergic agonists such as isoprenaline (Evans, 1981). The fact that isoprenaline was inactive on oviposition whilst clonidine and tolazoline were effective in blocking oviposition at the same doses therefore supports the theory that their activity is mediated by octopamine receptors. Characteristically, octopamine receptors respond maximally to the phenolamines which have a single hydroxyl group on the aromatic ring, rather than to the catecholamines which have two hydroxyl groups (Evans, 1982). The results from this study are also consistent with this, since the catecholamines dopamine and noradrenaline had no effect on blocking oviposition, even at high doses. Similarly haloperidol, a dopamine agonist was ineffective in blocking oviposition. This gives further support to the theory that the drugs affecting oviposition are mediated through octopamine receptors. It is probable that the main effect would be on the oviducal muscles, inhibiting peristaltic contraction, thus explaining the presence of mature eggs in the oviducts of ticks in which oviposition was blocked by the drugs. The acetylcholine agonists arecoline and pilocarpine were also effective in blocking oviposition. It is impossible to determine whether the drugs are acting centrally or peripherally from whole-body studies. It seems probable that the cholinergic drugs act on the central nervous system, while the adrenergic agonists may be acting peripherally on the oviduct. The presence of octopamine receptors on the Gené's organ secretory glands cannot be ruled out, since several of the α-adrenergic agonists, notably clonidine and tolazoline, seemed to stimulate the egg wax secretion, although this may have been a symptom of oviposition blockage rather than a direct effect.

Further studies are needed to investigate the nature and location of octopamine receptors in ticks and their pharmacology, in order to explain the whole body drug effects. This is particularly important in view of the possibility of using the octopamine receptor as a target for acaricides. The formamidines cause hyper-activity and detachment from the host in *B. microplus* (Stone *et al.*, 1974; Atkinson and Knowles, 1974), and the formamidine chlordimeform is an agonist of neuromodulatory octopamine receptors on locust leg muscle (Evans, 1982). The lethal and behaviour-disrupting activity of the formamidines is thought to be at least partially due to their action on peripheral octopamine receptors (Evans and Gee, 1980). Formamidines have also been shown to

block neuromuscular conduction (Yamamoto and Fukami, 1976) and increase neural activity (Beeman and Matsumura, 1974) in insects, and these actions may involve other types of receptor. The study of biogenic amine-sensitive adenylate cyclases may be a potentially useful tool for studying octopamine receptors in ticks and their pharmacology, and for determining the location of sites in the tick affected by both drugs and acaricides which may be mediated by these receptors.

It is now well established in vertebrates that cyclic AMP is an intracellular second messenger for the action of several circulating hormones (Robison et al., 1965). Formed from ATP by activation of the membrane-bound enzyme adenylate cyclase, cyclic AMP is the first link in the chain of intracellular biochemical reactions that result in the expression of hormone action (Nathanson, 1977, for review).

Hormone-sensitive adenylate cyclases have also been detected in insects and in some cases their functions have been described (Lingle et al., 1981; Bodnaryk, 1982). Many biogenic amine-sensitive adenylate cyclases have been identified in specific insect tissues, such as brain, optic lobes and non-innervated organs such as the fat body which responds to hormone circulating in the haemolymph. The pharmacological properties of membrane suspensions from these tissues have been identified (Bodnaryk, 1982). For example, a highly active and specific octopamine sensitive adenylate cyclase has been detected in the light organ of the firefly Photuris. Octopamine elicits a flash of light when released from the nerves innervating the organ, and the response is mediated by a large increase in the synthesis of cyclic AMP in the lantern (Copeland and Robertson, 1981). This system has proved valuable in investigating the pharmacology of the octopamine receptor in insects. Hormone-sensitive adenyl cyclases have been studied to a lesser degree in ticks. A variety of biogenic amines, of which dopamine is the most potent, stimulate salivary fluid secretion in ixodid ticks and this is mediated by changes in intracellular cyclic AMP levels brought about by adenyl-cyclase (Kaufman and Sauer, 1982). In view of this, further studies investigating octopamine-sensitive adenyl cyclases in ticks could be performed to determine the sites of octopamine receptors, their pharmacology and their involvement in the physiology of reproductive processes. These experiments would also be valuable in identifying sites that are potentially exploitable as targets for acaricides.

The present study also investigated the possible involvement of

juvenile hormones in the functioning of Gené's organ in ovipositing B. microplus by using precocene as an anti-juvenile hormone agent. Reproductive processes in insects such as vitellogenesis are under the control of juvenile hormone, but there is more limited evidence for its involvement in the reproduction of ticks.

Leahy and Booth (1980) demonstrated that precocene exposure in the ixodid R. sanguineus caused female sterility, but did not mention possible mechanisms whereby sterility was caused. In their experiment, very large topically applied doses were used, whereas in this study it was demonstrated that injected doses of 25 µg of precocene were effective in causing sterility in adult B. microplus. In this study it was also shown that precocene interferes with Gené's organ and that sterility is caused by a lack of waterproofing wax on the eggs. Precocene treatment appears to interfere directly with lipid secretion by the secretory cells of Gené's organ, since near normal numbers of eggs are laid, but shrivelling indicates that the eggs are inadequately waterproofed by Gené's organ. In the SEM it can be seen that eggs from precocene treated ticks have no superficial wax layer. The egg clusters failed to adhere together like normally laid eggs. No deposits of egg wax were visible around the mouthparts and Gené's organ, as occurs in untreated ticks. The normal oviposition movements and behaviour of precocene-treated ticks were unaffected, giving further evidence that the desiccation was caused by a lack of wax production or secretion, rather than by preventing the movements of the horns of Gené's organ.

The effect of precocene was not reversed by treatment with juvenile hormone III, at least at the doses used. This suggests that precocene directly affects the secretory epithelium of Gené's organ, rather than by indirect action to prevent JH production. However this does not rule out a rôle for JH in the reproduction of B. microplus, since the doses of JH may have been too low or inappropriate, or may have been rapidly metabolised. Pound and Oliver (1979) found that precocene inhibited maturation of the ovaries in an argasid species of tick, and this was reversed to some extent by co-treatment with JH III. The evidence from this study indicates that precocene treatment in the ixodid B. microplus does not affect the development of the ovaries, but reduced the oviposition rate, resulting in the weight of the ovaries of precocene treated females 6 days after dropping being significantly higher than in normal ticks. This was correlated with the fact that fewer eggs were

laid by the precocene-treated ticks. The development of the ovary thus appeared to be unaffected by precocene.

The results from the radiotracer experiments also suggest that precocene inhibits the normal function of the secretory epithelium of Gené's organ in synthesising lipids, so resulting in the lack of a waterproofing wax layer on the eggs. The incorporation of radio-labelled acetate into nonpolar wax lipids by Gené's organ in vitro was reduced in the presence of precocene. The ultrastructure of precocene treated Gené's organ showed signs of damage and abnormality. This was manifested in disorganisation of the rough ER, an increase in autophagy, an increase in smooth membrane reticulum, and fewer lipid vesicles were present. Precocene also has a destructive effect on the ultrastructure of the insect corpus allatum (Unnithan et al., 1977). Precocene is metabolised in the insect corpora allata to form a reactive epoxide (Pratt et al., 1980). It is suspected that precocene is oxidised by an enzyme involved in JH synthesis, methylfarnesoate epoxidase, the resulting epoxide forming covalent attachments to cellular macromolecules resulting in cytotoxicity. It is probable that cytotoxicity in Gené's organ also occurs in a similar manner to that in the corpora allata of insects. Solomon and Evans (1977) have reported that certain JH-mimics cause desiccation of eggs laid by treated female B. decoloratus. Much of the ovicidal activity was lost when the desiccation was prevented by incubating the eggs under paraffin oil (Solomon et al., 1982). It was concluded, however, that the ovicidal effects of JH-mimics were probably due to disruption of egg wax synthesis, rather than stimulation of normal tick endocrine mechanisms, since Gené's organ developed normally, and this is probably also the case with precocene treatment.

The possible involvement of the juvenile hormones in the reproduction of ixodid ticks needs to be further investigated to see if there is a fundamental difference between the ixodids and argasids. There is some evidence that JH is involved in the argasidae. From this study it appears that precocene has a toxic effect on Gené's organ, resulting in non-viable eggs, but it is unclear whether this is a direct effect or whether precocene also affects oviposition by a general disruption of endocrinological events in the ixodid tick.

There have been many studies of the ultrastructure of insect and crustacean muscle but very few of acarine muscle. The present study has improved our knowledge of tick musculature and compared the retractor muscles with the previously studied leg muscles. The ultra-

structure of the retractor muscles investigated in this study resembles that of other skeletal muscles studied in the ixodid ticks B. decoloratus (Beadle, 1973) and A. variegatum (Hart et al., 1980). The perforated Z-discs are also found in other tick muscles (Hart, 1982). The penetration of the z-discs by the contractile filaments may be a structural adaptation allowing an increased shortening of the muscle during contraction, an adaptation also present in the blow-fly larva (Osborne, 1967). The cuticular insertions of the retractor muscles follow a characteristic pattern of arthropod muscles whose contraction results in longitudinal pulling (Caveney, 1969; Smith et al., 1969; Beadle, 1973). The longitudinal microtubules of the tendon cells transmit the tension from the retractor muscles to the scutal plate and the cuticular rods of the horns.

This study has also described the neuromuscular junctions of the retractor muscles in B. microplus. The only previous study showed that the neuromuscular junctions in the leg muscle of A. variegatum followed the same general pattern as in other arthropods. The neuromuscular junctions of the retractor muscles are also similar to those in the tick leg muscle (Hart et al., 1980) and other arthropod skeletal muscles. The innervation is multiterminal, as in the leg muscles of the locust (Usherwood, 1972; 1974). The synaptic vesicles are similarto those characteristic of the nerve terminals of insects and crustaceans (Osborne, 1975; Atwood and Johnston, 1968). Electron-lucent vesicles, dense projections of the presynaptic membrane, and thickened opposed membranes of the synaptic cleft are characteristic of chemically transmitting nerve-muscle junctions in other arthropods (Osborne, 1975). The retractor muscle terminals also contain numerous dense-core vesicles similar to those found in the tick neuromuscular junction (Hart et al., 1980). This suggests that the nerve terminals have a neurosecretory function in addition to the motor input. The dense core vesicles described are identical in appearance to those present in neurosecretory cells of the tick central nervous system (Binnington, 1983). Neurosecretory terminals have also been found in locust skeletal muscle (Hoyle et al., 1974). They may be responsible for releasing a modulatory factor onto the muscle, such as for the maintenance of muscle tone (Hoyle, 1974). These type of terminals have a modulatory effect on the leg muscle of the locust and have been identified as octopaminergic (Evans, 1982). Alternatively the dense cored vesicles may serve a trophic function (Finlayson, 1975).

It is now well established that L-glutamate is the probable

excitatory transmitter at insect and crustacean neuromuscular junctions but there have been few parallel studies in ticks. The aim of this study was to augment the information available from a previous study of the leg neuromuscular junction in A. variegatum. No previous attempts appear to have been made to confirm glutamate as the neuromuscular transmitter in B. microplus. This study has shown that both L-glutamate and L-aspartate cause depolarisations when applied to the retractor muscles, and that octopamine, acetylcholine and gamma-aminobutyric acid had no effect at the same doses. This makes L-glutamate and L-aspartate likely candidates for the chemical transmitter at the retractor muscle synapses. Glutamate and aspartate also abolish the evoked and spontaneous epsps at a concentration of 10^{-3}M, providing evidence that the receptors on the muscle cell membrane are blocked by an excess of these ligands. The effect could be explained by a loss of ionic driving force due to depolarization of the membrane potential, or changes in muscle membrane conductance or desensitization of the receptors.

The membrane of the retractor muscles was desensitized by repetitive application of glutamate but not by application of aspartate at a similar rate, so this only explains the loss of epsps in the case of glutamate.

Irving and Miller (1980) found evidence for two different receptor populations in the body wall muscle of Musca larvae; glutamate abolished the 'fast' epsps (having a latency of about 1mS and a rise time of less than 1mS) and aspartate abolished the epsps produced by the 'slow' motor axon (having a latency of about 2mS and a rise time of about 3mS). They proposed that the fast axon released glutamate and the slow axon, aspartate. However, Robinson (1981) suggests that these effects might be due to the involvement of extrajunctional receptors, since glutamate was found to reduce both fast and slow excitatory post-synaptic currents whereas aspartate had no effect. The epsps recorded from the retractor muscles appear to be intermediate between the 'fast' and 'slow' potentials recorded from other arthropod skeletal muscles (Hoyle, 1955, 1978), having a rise time of about 1 mS. Hart (1982) detected both fast and slow potentials in the tick leg muscle, which combine to form a compound epsp. There was no evidence of this in the retractor muscles, since all potentials recorded had approximately the same time course. It is believed that the transmitter at the locust fast neuromuscular junction is L-glutamate (Usherwood and Cull-Candy, 1975; Usherwood, 1977). There are also numerous reports of the action of aspartate on arthropod muscles (Constanti and Nistri, 1978, 1979; Crawford and

McBurney, 1976, 1977a,b). Although the action of aspartate has been assumed to be due to effects on the L-glutamate receptor, it is equally possible that separate aspartate receptor populations exist on the muscle. Hart (1982) found that both aspartate and glutamate had similar effects on the tick leg muscle, causing depolarization and abolishing both fast and slow neurally evoked epsps, and that the dose responses for the two agonists were similar. Aspartate and glutamate are both potent agents in depolarising Gené's organ retractor muscle membranes, but further work needs to be done to confirm the nature of the natural chemical transmitter at tick neuromuscular junctions.

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6.2 THE ULTRASTRUCTURE OF GENÉ'S ORGAN IN THE CATTLE TICK *BOOPHILUS MICROPLUS* CANESTRINI

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INTRODUCTION

The egg-laying females of both the ixodid and argasid ticks are unique among the Acarina in that they possess a glandular organ system, Gené's organ, specialized for oviposition. No other groups are known to possess this organ. In *Boophilus* it consists of a pair of balloon-like horns which have a thin, plicate, glistening, cuticular surface enclosing a fluid-filled lumen, and a single cell thick secretory epithelium. The latter is a proliferation of the body wall epithelium. In the Ixodidae, the organ emerges from between the posterior edge of the capitulum and the anterior margin of the scutum, in the camerostomal fold. It is absent from the male and immature stages and only develops in engorged females prior to oviposition.

The organ was first described by Gené (1848) who noted that when it was pricked with a needle the eggs deposited near the vagina rapidly shrivelled. Bertkau (1881) also found that eggs prevented from coming into contact with Gené's organ shrivelled much more rapidly than eggs laid normally. Lees & Beament (1948) showed that the function of Gené's organ in *Ixodes ricinus* and *Ornithodoros moubata* was to provide the eggs with a waterproof covering which prevents excessive water loss. They occluded the opening through which the organ is everted and followed the fate of the eggs. It was found that most of the eggs laid with Gené's organ free hatched normally within twelve days, whereas none of those laid without the intervention of the organ hatched, and all were completely shrivelled even in humid conditions. They also noted that the presence of a waxy covering on the eggs, applied by Gené's organ, prevented the growth of fungi. The eggs of *Ixodes ricinus*, however, were found to be already partially waterproofed before contact with Gené's organ. This was due to an incomplete wax layer being applied during the passage through the oviduct.

The morphology of Gené's organ was described briefly by Lees & Beament (1948) in *Ornithodorus moubata* and in more detail by Arthur (1953) in *Ixodes ricinus* and by Balashov (1968) in *Hyalomma asiaticum*. However, no previous investigations using electron microscopy appeared to have been made to discover the fine structure of the organ. This was the aim of the present study.

MATERIALS AND METHODS

Newly-dropped, fully engorged female *Boophilus microplus* (Paquera strain) were obtained from a culture maintained at The Wellcome Research Laboratories, Berkhamsted. They were maintained in Petri dishes on moist filter paper at 30°C. Oviposition began within two days under these conditions. The oviposition process was observed in ticks kept in warm, moist and under dim lighting conditions, with a binocular microscope.

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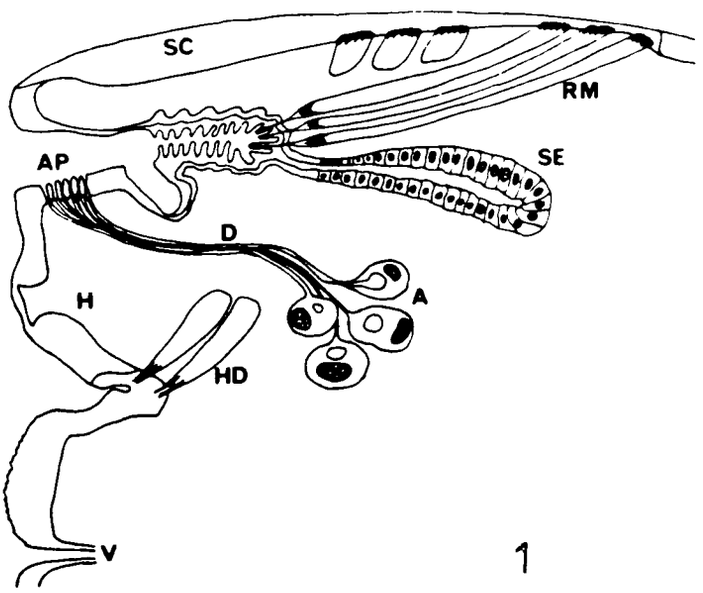
Gené's organ was dissected and then fixed from egg-laying females in both the retracted and everted positions prior to and at different stages of oviposition.

For transmission electron microscopy, tissues were fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde (Karnovsky 1965) in 0.1 M phosphate buffer, pH 7.1, containing 0.25 M sucrose for 1 hour. Following post-fixing in 0.3% osmium tetroxide for 2–3 h the tissues were stained *en bloc* with uranyl acetate during dehydration in an ethanol series, and embedded in Araldite. Sections were cut on an LKB Ultratome III. One μm sections were stained with methylene blue-basic fuchsin (Aparicio & Marsden 1969), and ultrathin sections were stained with lead citrate for observation in a JEM 100S electron microscope.

For scanning electron microscopy specimens with Gené's organ everted were rapidly frozen with a freezing aerosol, the anterior region cut off and dehydrated in acetone at −25°C. The acetone was then evaporated off in a vacuum before sputter coating with carbon and gold. Specimens were examined in a Philips 35S microscope.

OBSERVATIONS

Gené's organ is a specialized region of the body wall epithelium where the cuticle has become separated from the epithelial cells. The space between them forms a lumen (Fig. 1). During oviposition the capitulum is retracted close to the body wall and the horns are inflated and extended towards the vagina (Figs. 2 and 4). The vagina prolapses as the egg is produced, forming an extended tube that deposits the egg between the horns. The egg is then manipulated by the organ and becomes coated with a superficial wax layer. The organ then retracts, and the egg is deposited on the dorsal surface of the tick by movements of the hypostome. The whole sequence takes less than one minute and is repeated for each egg. The glandular epithelium remains inside the body wall during eversion (Fig. 2). It consists of many tubular folds of cells separated from the horn cuticle by the lumen contents (Figs. 2 and 7). The horns are pulled inside-out during retraction through the narrow slit in the camerostome (Fig. 3), folding in on themselves many times.



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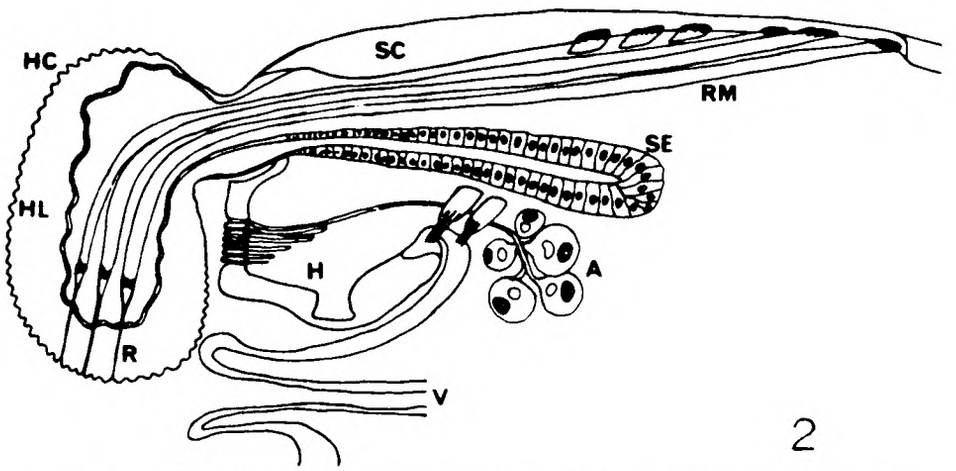


Figs. 1 and 2 – Schematic drawings showing longitudinal sections of an ovipositing female *B. microplus* with Gené's organ retracted (Fig. 1) and everted (Fig. 2). The organ is retracted beneath the scutum (SC) by a group of retractor muscles (RM) although the secretory epithelium of the organ (SE) always remains within the tick. Also shown are the accessory glands (A) with their ducts (D) opening on to the area porosa (AP), the hypostome with the attached hypostome depressor muscles (HD) and the vagina (V). When the organ is everted (Fig. 2) the horns emerge through the opening between the scutum (SC) and the hypostome (H). The horn cuticle (HC) and lumen (HL) are shown and the cuticular rods that attach the retractor muscles (RM) to the organ. Eversion is accompanied by contraction of the hypostome depressor muscles bringing the area porosa and the vagina closer to the everted horns.

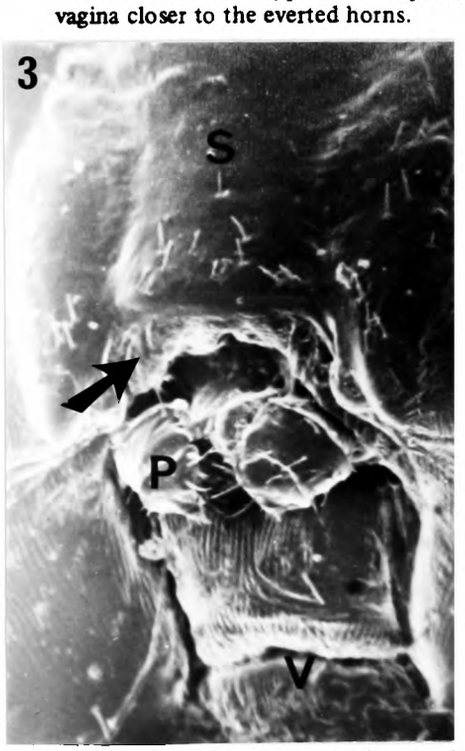
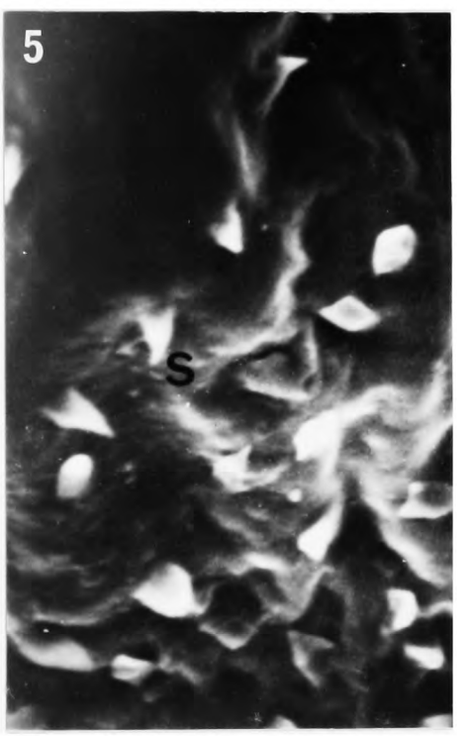


Fig. 3 – Scanning electron micrograph showing the mouthparts of an ovipositing female *B. microplus* with Gené's organ retracted in the camerostomal fold (arrow). S, scutum; P, palps of mouthparts (retracted); V, vagina. × 60.

Fig. 4 – Scanning electron micrograph showing the horns (H) of Gené's organ everted and the partially prolapsed vagina (V). × 60.

When the horns are everted it can be seen that the median anterior and ventral regions are covered with fine, cone-like cuticular setae about 1 μm long (Fig. 5). These are much smaller than the setae found elsewhere on the body surface. The horn cuticle is composed of two layers, a thin dense outer one (about 50 nm across) and a thicker (0.15 μm) less dense inner layer (Fig. 6). The inner layer is often incomplete. Distributed evenly over the whole surface of the horns are circular pits, about 0.6 μm across, and 2–4 μm apart. Each pit is lined with pore canals about 50 nm in diameter with electron dense walls which penetrate the outer cuticle (Fig. 6). The lumen contents enclosed by the cuticle have a granular appearance.



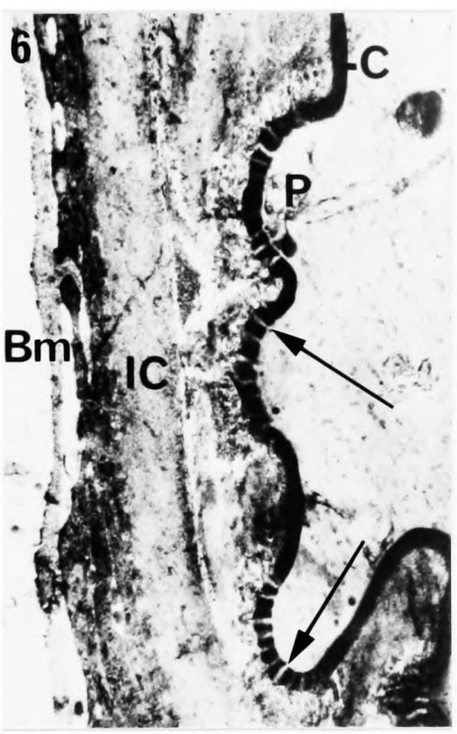


Fig. 5 – Scanning electron micrograph showing a high magnification of the cuticular surface of Gené's organ showing setae (S). × 900.

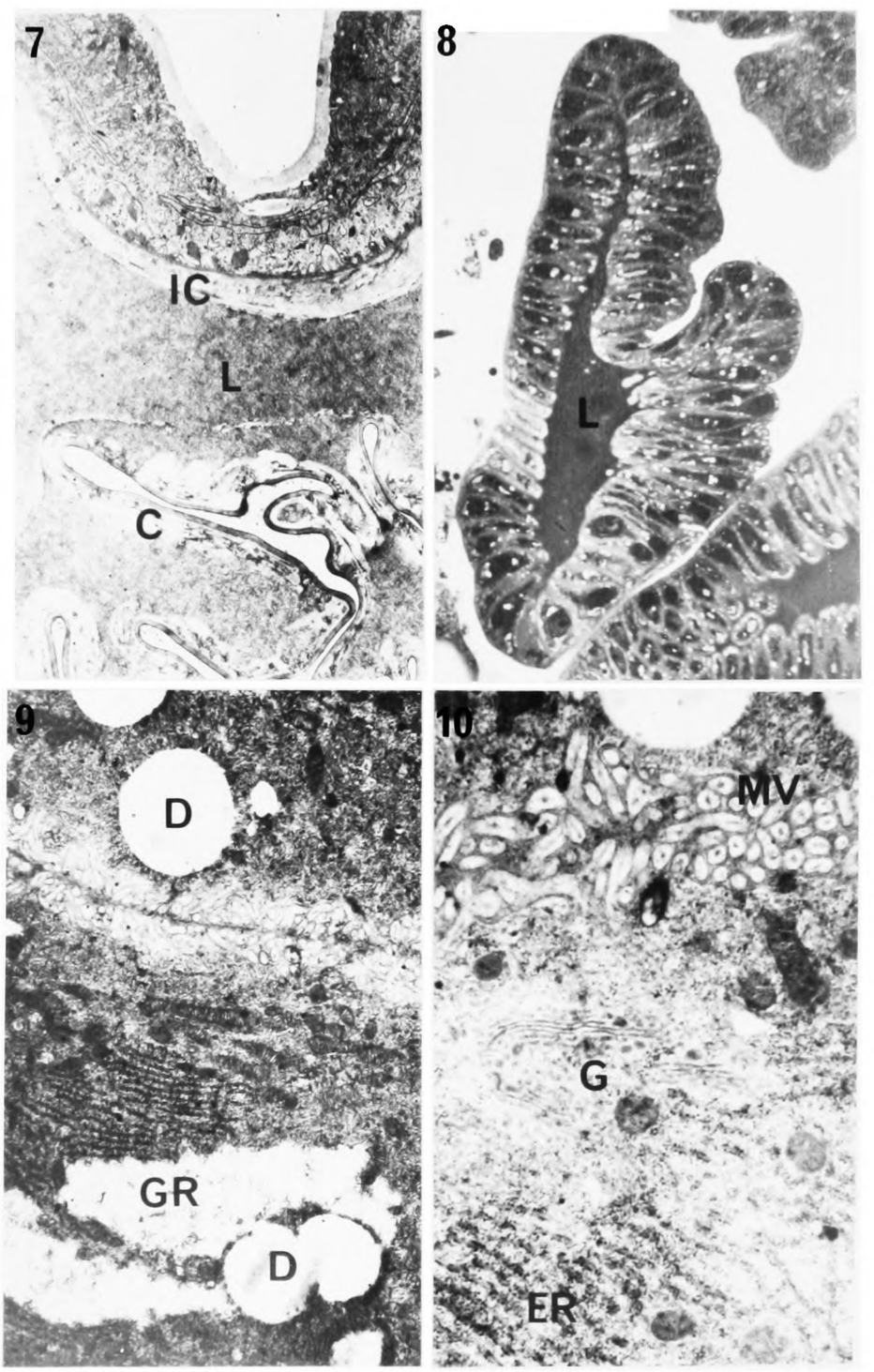
Fig. 6 – Transmission electron micrograph of a section through the horns of Gené's organ showing pits (P) in the outer cuticle (C) lined with pore canals (arrows). There is a thin layer of cytoplasm between the basement membrane (BM) and inner cuticle (IC). × 17 000.

Fig. 7 – Transmission electron micrograph of a section through the retracted horns of Gené's organ near the tubular glands showing the folded outer cuticular layer (C) containing pore canals separated from the inner cuticle (IC) by a lumen (L) containing a granular material. The epithelial cells underlying the inner cuticle are larger than in Fig. 6. × 3900.

Fig. 8 – Photomicrograph of a section through the tubular glands of Gené's organ showing the columnar epithelial cells surrounding the lumen (L). × 240.

Figs. 9 and 10 – Transmission electron micrographs showing sections of the tubular gland of Gené's organ. The cells have a microvillar border (MV) and contain rough endoplasmic reticulum (ER), Golgi bodies (G), numerous mitochondria and large inclusion droplets (D). There are areas containing a granular material (GR).
Fig. 9 – × 7700. Fig. 10 – × 19 800.

▷


7
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In the horns, the epithelium underlying the lumen is thin and reduced to a strip of cytoplasm (Fig. 7). Further inwards, towards the tubular glands, the cells increase in size, and subcellular organelles become more evident. There are elaborate laterally folded intercellular junctions. The basal plasma membranes are infolded and form extracellular spaces beneath the basement lamina. The epithelial cells are lined on their apical surface with a cuticular layer distinct from the outer cuticle of the horns (Fig. 7). In this way the lumen contents in this region are held between two layers of cuticle. The inner cuticular layer does not extend into the tubular glands, so that the epithelial cells in this region have direct access to the lumen.

The glandular cells are columnar in shape and 50 μm or more deep (Fig. 8). There is a large, central nucleus and the cytoplasm contains organized arrays of rough endoplasmic reticulum, Golgi bodies, numerous mitochondria, large inclusion droplets and areas containing a granular material (Figs. 9 and 10). The apical surface has a well-developed microvillar border which extends into deep intercellular crypts. The cells rest upon a prominent basement lamina, and the basal membrane has extensive infolding forming extracellular spaces. The material in the lumen of the glands is amorphous and less granular than that in the horns.

DISCUSSION

The structure of Gené's organ in *B. microplus* is essentially similar to that described in *O. moubata* (Lees & Beament 1948), *I. ricinus* (Arthur 1953), and *H. asiaticum* (Balashov 1968), and consists of a pair of horns that can be everted and retracted, plus a glandular secretory epithelium. The present study has shown that the horns are covered by fine-pointed setae and also by pits that are lined with pore canals. The distribution of the setae corresponds to those areas of the organ that come into contact with the eggs, and they may well have a function in gripping the eggs as they leave the vagina. The egg must adhere to Gené's organ more readily than the vagina if transfer is to occur. This is achieved by the presence of the sticky wax layer on the organ, but may also be aided by the presence of the setae. Egg clusters adhere together very strongly owing to the wax covering, and this in itself probably helps to control water loss. The setae might also facilitate the spreading of wax over the egg as it is manipulated. During oviposition only part of the egg comes into contact with Gené's organ, so the spreading properties of the wax must be important in ensuring an even covering.

It is likely that the wax secretion passes from the lumen of Gené's organ through the pore canals which line the pits on the cuticular surface. The superficial waxes of the body wall cuticle are thought to pass through the pore canals in a similar manner to the cuticle of insects (Neville 1975) and ticks (Hackman 1982) before they are blocked by the deposition of a cement layer. The outer cuticle of Gené's organ is very thin, so the short length of the pore canals will provide little resistance to the passage of secretion in comparison with the thick body wall cuticle. According to Lees & Beament (1948) the cuticle of Gené's organ has no outer cement layer, thus the pore canals remain open for continuous wax secretion. The thinness of the outer cuticle also allows the horns to fold into many pleats during retraction.

The mechanism of eversion of Gené's organ appears to be purely hydrostatic with some involvement of the capitulum retractor muscles. There is no intrinsic musculature capable of causing eversion, but the dorso-ventral muscles may be important in causing an increase in haemolymph pressure by contraction during eversion. Simultaneous relaxation of the organ's retractor muscles would cause the horns to turn inside out and pass through the slit in the camerostomal fold which is widened by the retraction of the capitulum. The high internal

pressure during eversion would be transferred to the lumen contents and this would tend to force the secretion through the pore canals on to the external surface of the horns.

The wax secretion is produced in the glandular epithelium that is an extension of the body wall epithelium. The ultrastructure of these cells is consistent with a secretory function. The complex microvillar border, arrays of endoplasmic reticula, and well-developed Golgi bodies are typical of secretory cells. They contain numerous large secretory droplets whose contents are extracted during tissue processing, suggesting that they contain lipid. Little is known of the chemical composition of the secretion from Gené's organ. Lees & Beament (1948) made some investigations into the water repellent properties of the extracted lipids from tick egg waxes, and more recently the chemical composition of surface egg waxes from *B. microplus* has been determined (Gilby 1957, Cherry 1969a, b, McCamish *et al.* 1977). The wax consists of branched and non-branched chain alkanes, steroids, fatty acids and alcohols, and corresponding wax and cholesteryl esters. The significance of this complex composition is unknown, but it is similar to the composition of insect cuticular waxes. The latter are generally considered to have a waterproofing function (Beament 1964, Jackson & Baker 1970). Further work on the nature of the wax and its mode of production by Gené's organ is currently in progress.

SUMMARY

The ultrastructure of Gené's organ, the egg-waxing organ in ticks, is described in the cattle tick, *Boophilus microplus*. The organ is a single cell epithelium continuous with the body wall and situated in the camerostomal fold between the posterior edge of the capitulum and anterior margin of the scutum. The cuticle of the organ unfolds and inflates into a paired balloon-like structure (the horns) which contain an extracellular lumen. During oviposition the organ receives and manipulates each egg as it is laid, coating it with a waxy layer that prevents desiccation. The tubular glands are continuous with the lumen of the horns and have an ultrastructure consistent with a secretory function. The thin outer cuticle of the horns contains pits lined with 50 nm diameter pore canals. These connect the lumen contents with the outside surface, which is covered with 1 μm long setae. The organ appears to be everted by hydrostatic pressure from the haemolymph, and is retracted by muscles.

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TISSUE & CELL 1984 16 (4) 589–599

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ULTRASTRUCTURE OF THE ACCESSORY GLANDS OF GENE'S ORGAN IN THE CATTLE TICK, *BOOPHILUS*

Key words: Accessory glands, *Boophilus microplus*, egg wax, oviposition, Gene's organ

ABSTRACT. The organization and ultrastructure of the accessory glands of the cattle tick, *Boophilus microplus*, are described. The glands consist of two groups of acinar cells situated on either side of Gene's organ. A single acinus consists of from eight to 12 cells and each cell is connected via an individual duct to pores on the dorsal surface of the mouthparts. The position of these pores is such that the secretion of the accessory glands is incorporated into the egg wax during oviposition. Each gland cell has striking quantities of smooth endoplasmic reticulum and numerous Golgi dictyosomes and appears to produce a secretion that is lipoidal in nature. Each cell secretes into its own individual lumen and is connected to a cuticular pore by a duct cell.

Introduction

Ovipositing adult female ticks possess a unique organ system, Gene's organ, specialized for applying a superficial waxy secretion to the eggs which protects them from excessive water loss and fungal attack and causes the eggs to adhere in a cluster (Lees and Beament, 1948; Arthur, 1953; Chinery, 1965). The ultrastructure of Gene's organ and its associated secretory epithelium has been described by Booth *et al.* (1983) in the cattle tick, *Boophilus microplus*. The external part of the organ consists of a pair of balloon-like horns which emerge from beneath the scutal plate, passing over the dorsal surface of the mouthparts to reach the vagina. When an egg is produced the vagina prolapses, pushing the egg between the horns, which manipulate the egg, covering it with wax. The horns are then retracted beneath the scutum and the egg is deposited on the dorsal surface of the tick by movements of the mouthparts. The wax passes from the lumen within the horns to the outside surface through 50 nm wide pore canals in the thin outer cuticle. The horns are inflated by hydrostatic pressure and retracted by muscles, and this process is repeated for each egg.

As the horns of Gene's organ emerge from beneath the scutum they pass directly over two groups of pores on the dorsal surface of the mouthparts, the areae porosae. These porose areas are the external openings of the accessory glands of Gene's organ and were first described by Feldman-Muhsam and Havivi (1960). These glands are present only in female ixodid ticks, and in *Boophilus microplus* they consist of two groups of 80–100 gland cells. Each cell is about 80 μm in diameter and the cells are arranged in clusters of about ten cells. There is an internal secretory lumen within each cell connected to an individual duct and the two groups of ducts lead to the paired groups of secretory pores in close proximity to Gene's organ. The position of the porose areas in relation to Gene's organ leads to the incorporation of their secretion into the egg wax during oviposition (Atkinson and Binnington, 1973). The fact that maximal development of the acccessory glands coincides with the onset of oviposition also suggests that they play a role in egg waxing, although their exact function is unclear. As a preliminary step in determining

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Received 6 June 1983.
Revised 30 January 1984.

the function of these glands and their secretion, their fine structure has been investigated. It is the results of this study that are reported here.

Materials and Methods

Newly dropped fully engorged adult female *Boophilus microplus* (Paquera strain) were obtained from a culture maintained at the Wellcome Research Laboratories, Berkhamsted. They were maintained in Petri dishes on moist filter paper at 30°C.

The accessory glands were dissected from engorged females by making a circular cut in the body wall cuticle around the mouthparts and genitalia including the first pair of legs using microscissors. The resulting piece of cuticle containing Gene's organ, the accessory glands, mouthparts and oviduct was then washed in 0·1 M phosphate buffer containing 0·3 M sucrose, to remove the gut contents. The tissue was then either fixed and embedded for sectioning whole, or groups of accessory glands, observed under a binocular microscope were removed by severing the ducts and embedded separately. Gravid females were dissected immediately before onset of oviposition after dropping from the host, and at different stages during oviposition.

For transmission electron microscopy (TEM) tissues were fixed in a solution containing 2% paraformaldehyde and 2·5% glutaraldehyde (Karnovsky, 1965) in Millonig's 0·1 M phosphate buffer (Millonig, 1961) pH 7·2 containing 0·3 M sucrose, for 1 hr. After a wash in sucrose buffer, post-fixing in 2% osmium tetroxide in the same buffer for 2–3 hr was followed by another wash in buffer and then *en bloc* staining with uranyl acetate in 70% ethanol during dehydration in ethanol series. Tissues were embedded in Araldite. Sections were cut on an LKB Ultrotome III and 1 μm sections were stained with either methylene blue–basic fuchsin (Aparicio and Marsden, 1969) or by the periodic acid–Schiff (PAS) method followed by methylene blue.

Ultrathin sections were stained with lead citrate (Reynolds, 1963) and uranyl acetate for observation in a JEM 100S electron microscope.

For scanning electron microscopy (SEM) specimens at different stages of oviposition with the horns of Gene's organ in everted or retracted positions were surface washed with

Fig. 1. SEM showing the mouthparts of a newly dropped adult female *B. microplus* during the pre-ovipositional stage. The areae porosae are visible as two patches of pores (AP) on the dorsal surface of the capitulum, posterior to the palps (P) and chelicerae (CH). The fold between the capitulum and scutum (S) through which the horns of Gene's organ emerge during oviposition is also visible (arrow). ×230.

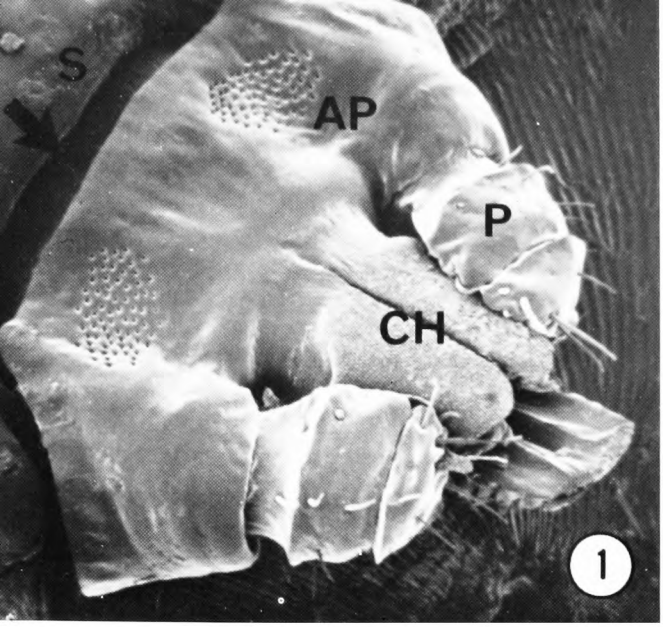
Fig. 2. SEM showing detail of the pores of one of the porose areas. The mouth of each duct cell can be seen just within the opening of each pore. ×1700.

Fig. 3. SEM showing an adult female *B. microplus* during the early stages of oviposition, fixed with the horns (H) of Gene's organ everted towards the vulva (VU). The horns completely cover the dorsal surface of the retracted mouthparts. ×230.

Fig. 5. Light micrograph showing a sagittal section through Gene's organ and the mouthparts of an ovipositing female *B. microplus* fixed with the horns everted. It can be seen that the horns pass directly over the pores of the areae porosae (arrow) during eversion and retraction. HC, horn cuticle; HL, horn lumen; S, scutum; RM, retractor muscle of Gene's organ; P, palp; E, epithelial cells of horns. ×170.

Fig. 6. Light micrograph of a sagittal section through one of the porose areas, showing the cluster of ducts (D) passing away posteriorly from the pores towards the accessory gland cells. The pores in the cuticle of the capitulum (C) can be seen in longitudial section (arrow). ×420.

Fig. 7. Light micrograph showing a whole mount of a cluster of accessory gland cells. The nucleus (N) and lumen (L) of each cell are visible. ×260.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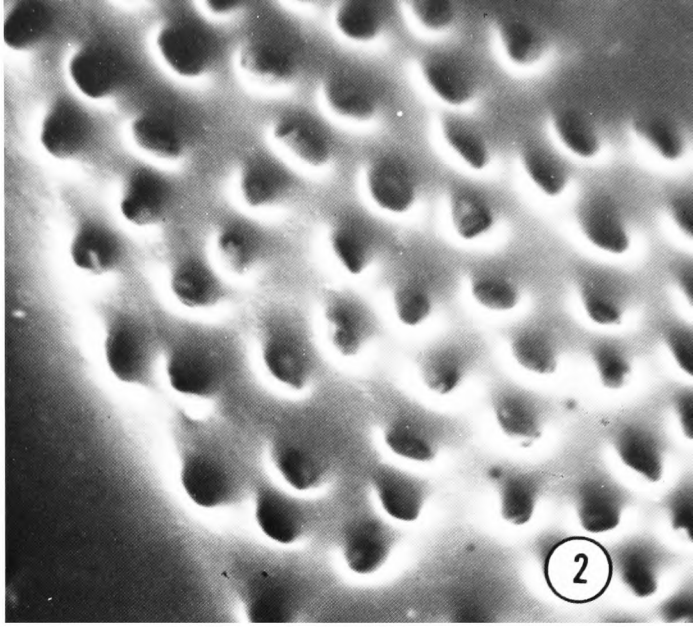
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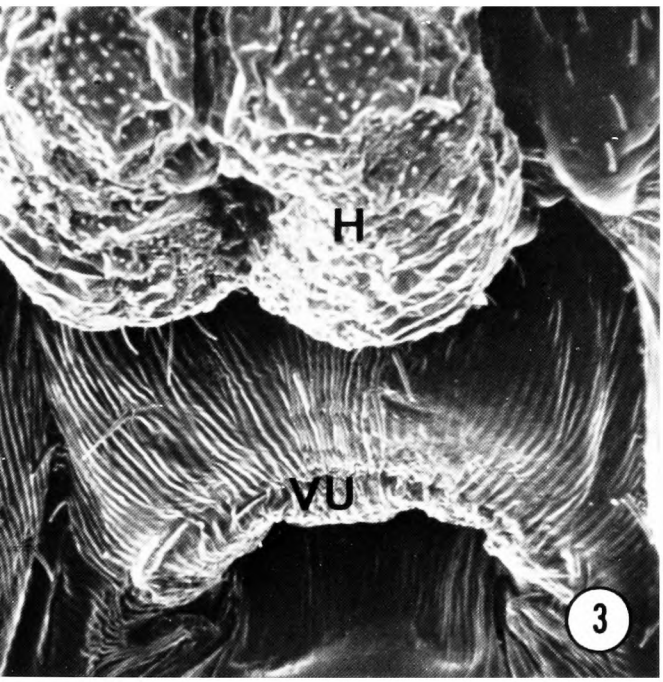
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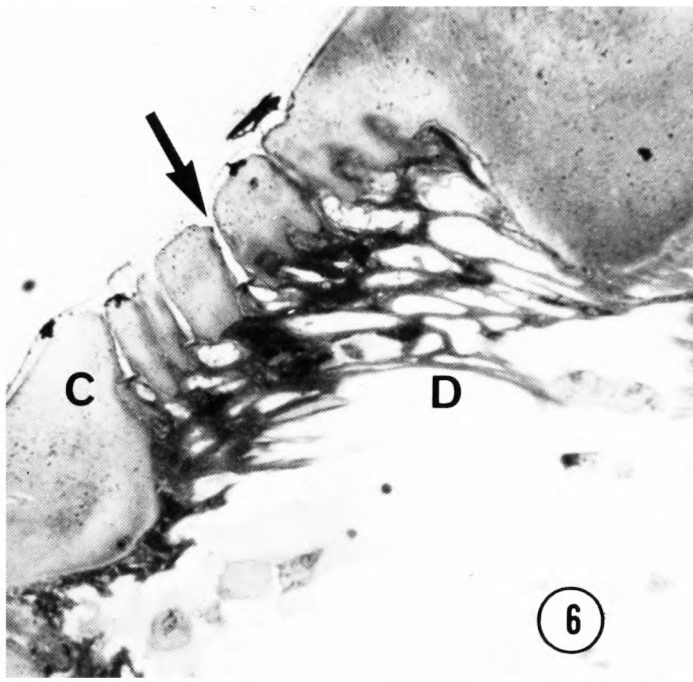
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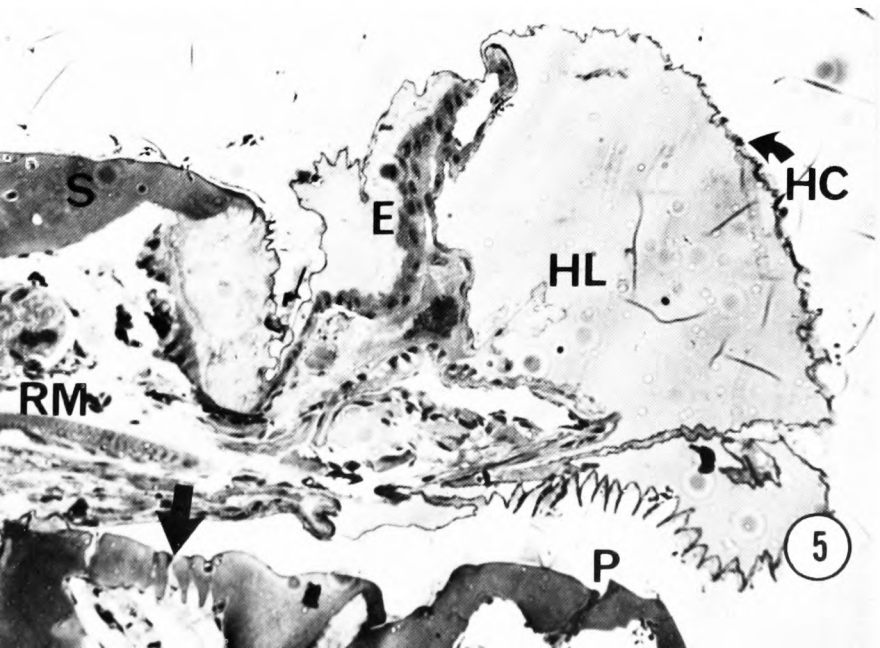
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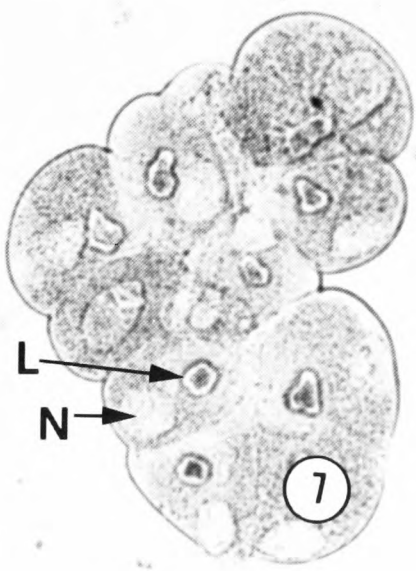
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chloroform to remove superficial wax and debris from the cuticle and rapidly frozen with a freezing aerosol (Arcton). The anterior region of the tick was then cut off with a cold scalpel and fixed in acetone at −25°C for 10 days. After several changes of fresh 100% acetone to ensure dehydration the specimens were removed and the acetone was evaporated in a vacuum chamber at room temperature. Specimens were mounted on stubs, sputter coated with carbon and then gold, and examined in a Philips 35S microscope.

Observations

Gravid female *Boophilus microplus* undergo a single gonotrophic cycle after completion of the final blood meal and dropping from the host, during which up to 2000 eggs are laid. After dropping, the mouthparts (capitulum) are clearly visible protruding from the anterior margin of the scutum, and the *areae porosae* are visible as two patches of pores on the dorsal surface of the capitulum, posterior to the chelicerae (Figs. 1, 2). At this stage the articulation between capitulum and the scutal plate is very close, the horns of Gene's organ remaining retracted within this cleft and the vaginal opening is closed.

Oviposition commences within 2–3 days of dropping but before this occurs the mouthparts are gradually retracted by muscles into a deep fold, the camerostome, which forms beneath the scutum. The retraction of the mouthparts enlarges the gap between the scutum and the posterior margin of the capitulum allowing the horns of Gene's organ to evert and retract, passing over the dorsal surface of the mouthparts (Fig. 3). The retraction of the mouthparts also brings the vagina into closer proximity with the horns when they are everted and oviposition movements begin when the vaginal opening enlarges and begins muscular contractions. This process is shown diagrammatically in Figs. 4a, b.

In longitudinal sections of ovipositing ticks with the horns everted it can be seen that the ventral surfaces of the horns which come into contact with the eggs, pass directly over the porose areas (Fig. 5). Each pore is connected to a duct, and the groups of ducts,

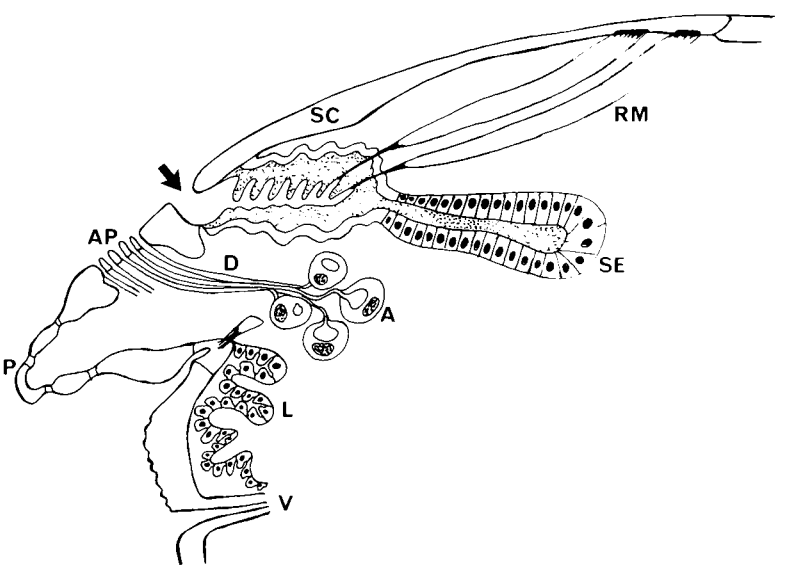


Fig. 4a. Generalized drawing showing the relative positions of the mouthparts, Gene's organ and oviduct of a newly dropped engorged female *B. microplus* in sagittal section, before commencement of oviposition. The mouthparts and palps (P) are perpendicular to the scutum (SC) and the horns of Gene's organ remain retracted within the camerostomal fold at the articulation between mouthparts and scutum (arrow). The secretory epithelium of Gene's organ (SE) encloses a lumen (shaded area) which is continuous with the thin cuticle enclosing the horns. The accessory glands (A) situated in the haemocoel to either side of the secretory glands of Gene's organ are connected to the areae porosae (AP) on the tectum of the capitulum by ducts (D). The lobed gland cells (L) are a proliferation of the body wall epithelium between the mouthparts and vagina (V).

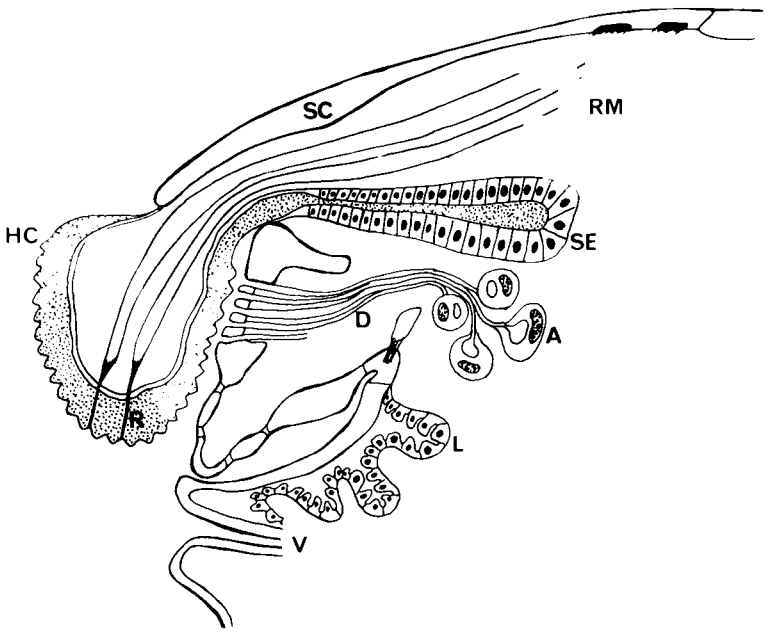
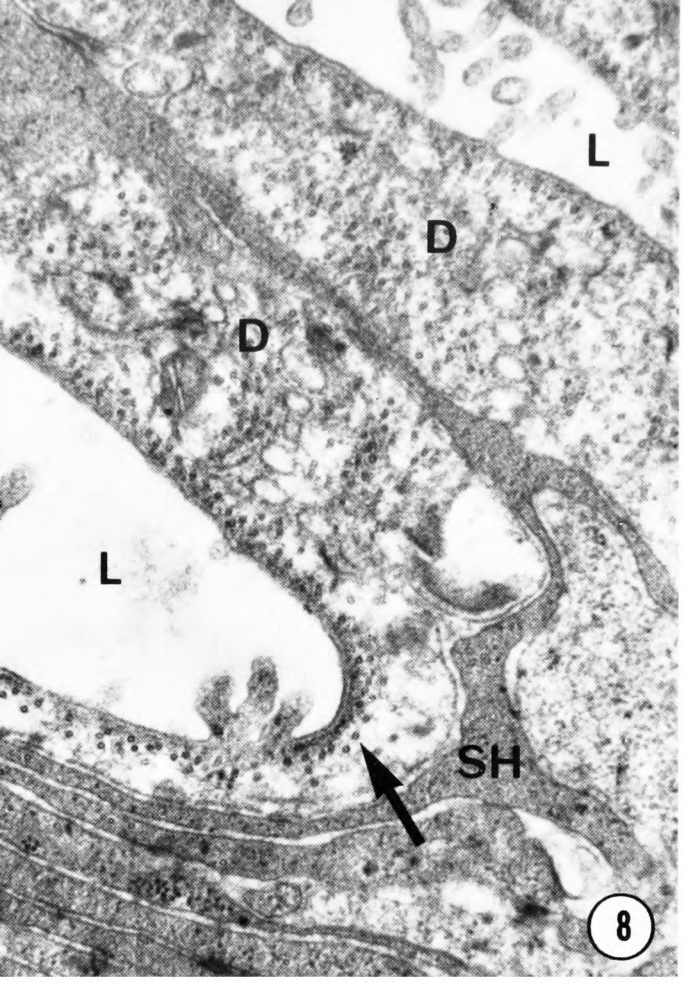


Fig. 4b. Generalized drawing showing an ovipositing tick with Gene's organ everted. During the pre-ovipositional period the mouthparts are retracted posteriorly beneath the scutum and are angled ventrally towards the vagina. This widens the gap between capitulum and scutum allowing the horns of Gene's organ to evert and protrude ventrally to meet the vulva, which is now open and is closer to the mouthparts and faces dorsally towards the horns. During oviposition the horn cuticle (HC) turns inside out as the organ everts, and is retracted by retractor muscles (RM) inserting into the outer cuticle by cuticular rods (R) which pass through the lumen. Eversion of the horns is accompanied by production of the oocyte from the prolapsed vagina. The ventral surfaces of the horns pass directly over the outlets of the accessory glands at the areae porosae, leading to the incorporation of their secretion in the egg wax which spreads from the horns to the egg shell.

clustered together, lead away posteriorly from each porose area (Fig. 6). The ducts connect to groups of acinar glands having the appearance of bunches of grapes, situated in the haemocoel on either side of the tubular secretory glands of Gene's organ (Fig. 7). The duct cell consists of a narrow tube surrounded by sheath cells (Fig. 6). The duct cell lines the pore in the cuticle and forms a simple junction with the cuticle. The internal lumen (the duct) which is lined with intermittent microvilli is about 7 μm in diameter. The cytoplasm of the duct contains numerous longitudinal microtubules transversely sectioned in this plane (Fig. 8). No branches or joins in the ducts have been observed in sections and it appears that each duct connects a single gland cell to one of the pores. The accessory gland cells are about 80 μm in diameter and are joined together in clusters of 10–15 cells, each cell having a central lumen about 20–30 μm across which connects with the duct, and a large lateral nucleus, both visible in the light microscope (Fig. 9). The general organization of the accessory glands is shown diagrammatically in Fig. 10.

The internal lumen of the gland cells has a microvillar border (Fig. 11). The cells contain many mitochondria which are most concentrated around the apical border of the cell. Numerous small membrane-bound vesicles are associated with Golgi bodies (Fig. 12) and the small vesicles are often aggregated around much larger membrane-bound vesicles, 0·5–1·6 μm in diameter. Many of the larger vesicles are aggregated closely around the apical border of the cell (Fig. 11) and some can be seen to be fusing with the apical plasma membrane and may be undergoing exocytosis (Fig. 13). The contents of the vesicles are extracted during tissue processing suggesting that they contain lipid. The cytoplasm of these cells also contains large arrays of smooth endoplasmic reticulum which may be in tubular form (Fig. 14) or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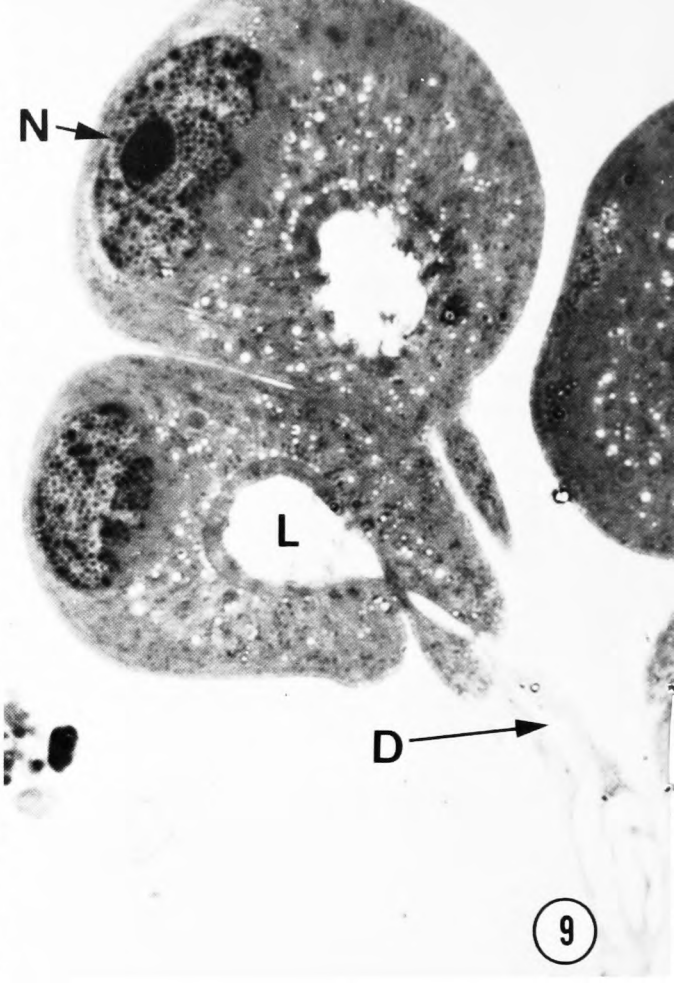
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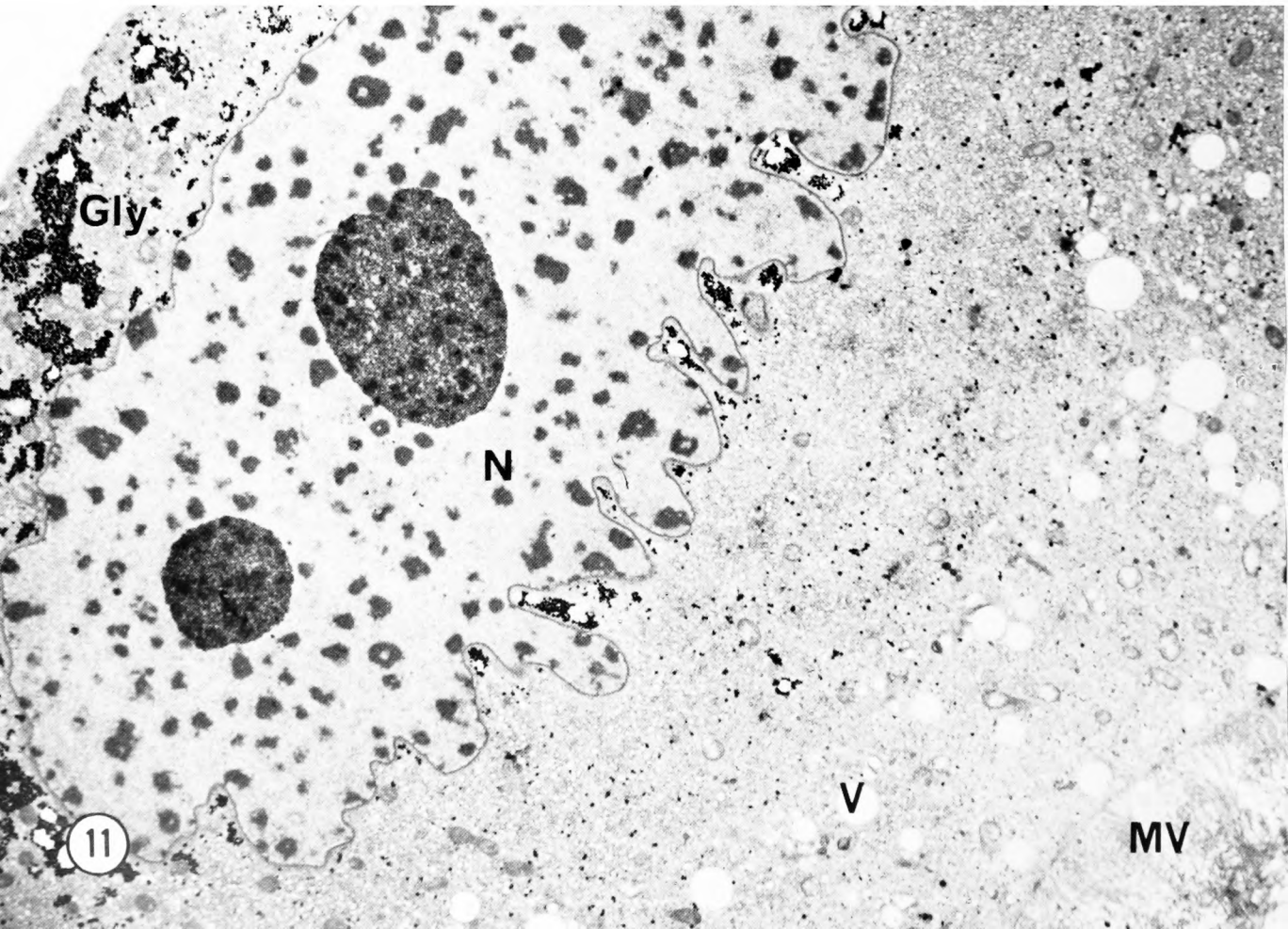
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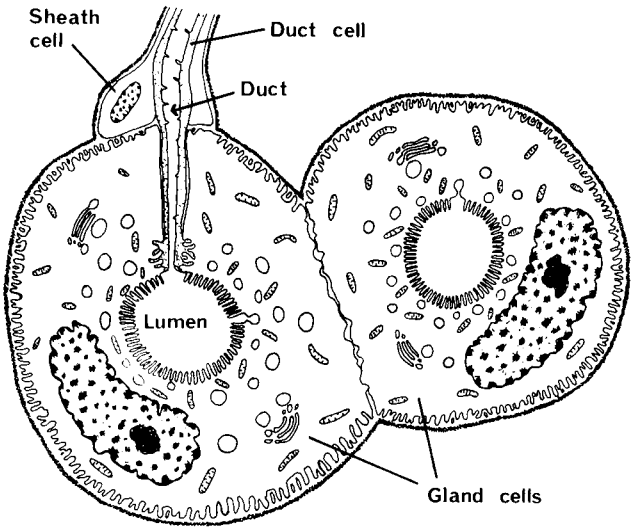


Fig. 10. Diagrammatic representation of the organization of the accessory glands showing the typical distribution of secretory vesicles around the internal lumen of each gland cell which connects to an individual duct cell. Sheath cells surround each duct and bind the ducts of adjacent cells together.

separated into isolated vesicles (Fig. 11). Rough endoplasmic reticulum is also present, and there are many small ribosome-like particles (Fig. 12). Glycogen α-particles are distributed singly in the cytoplasm and in large concentrated arrays around the basal border of the cells (Fig. 11). These areas of the cell have a strong periodic acid–Schiff reaction consistent with them being composed of carbohydrate.

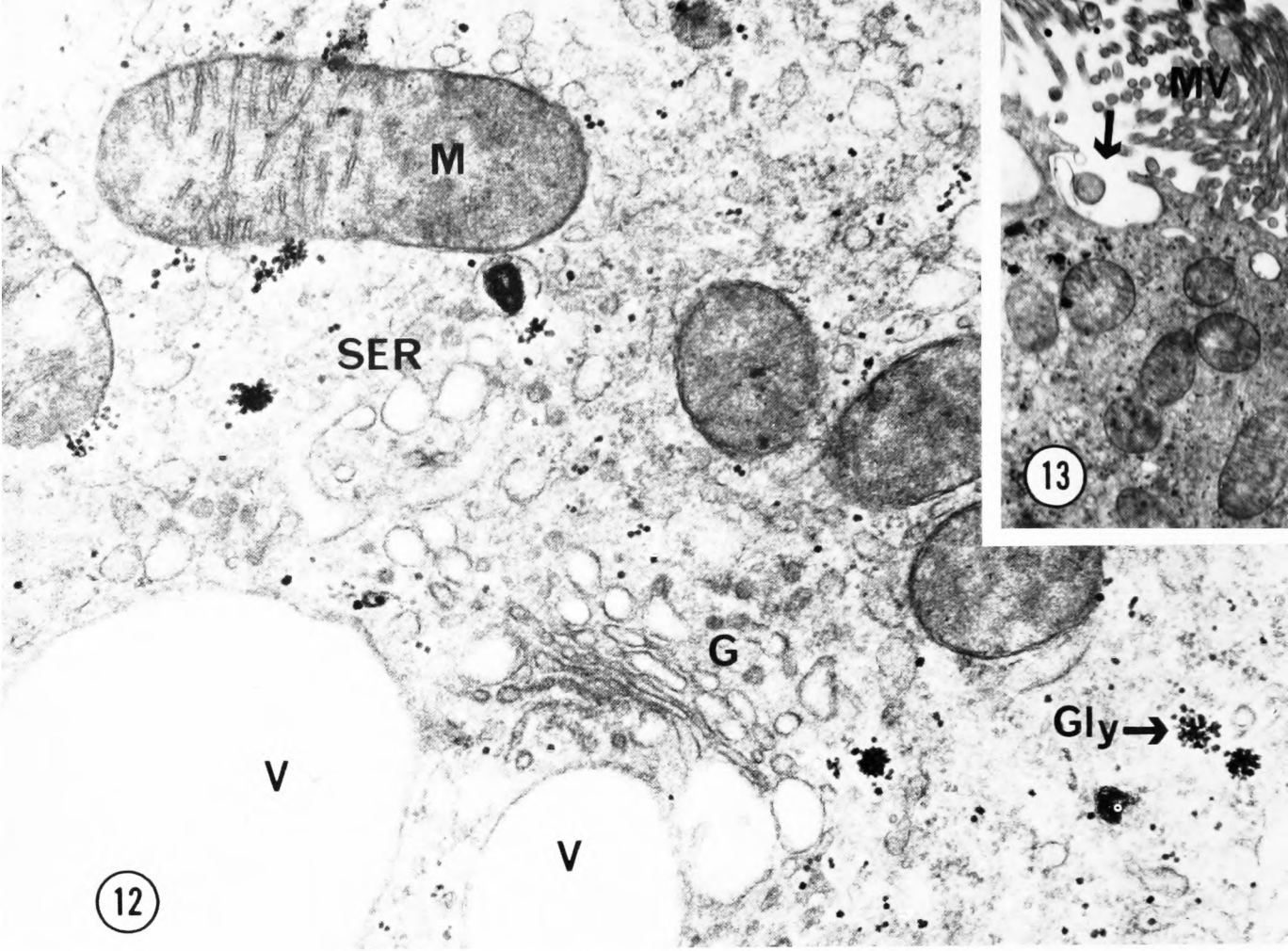
The basal plasma membrane of the accessory gland cells is infolded beneath the basal lamina forming extracellular spaces (Fig. 14). Adjacent cells have a continuous basal lamina and their plasma membranes are separated by an intercellular space being joined intermittently at gap junctions.

The duct cell passes through the gland cell to connect with the lumen (Fig. 15) and there does not appear to be any specialized membrane junctions between these cells. The duct cells are bound together by narrow processes of sheath cells that fold around them, binding them into clusters. As the ducts pass along the inside of the capitulum integment they are separated from the cuticle by body wall epithelial cells until they reach the porose areas, where they pass through the

Fig. 8. TEM showing a transverse section through the accessory gland duct cells (D) bound together by the processes of sheath cells (SH). The lumen (L) of each duct is lined with intermittent microvilli. The cytoplasm of the duct cell contains many longitudinal microtubules (arrow). ×41,000.

Fig. 9. Light micrograph showing two accessory gland cells in section. The connection between the duct (D) and the lumen (L) of one cell can be seen. Numerous secretory vesicles are grouped around the lumen of each cell. N, nucleus. ×600.

Fig. 11. TEM showing the nucleus (N) and basal and apical surfaces of an accessory gland cell. The apical border is lined with microvilli (MV) and the cell contains large vesicles (V) and glycogen particle arrays (Gly). ×2900.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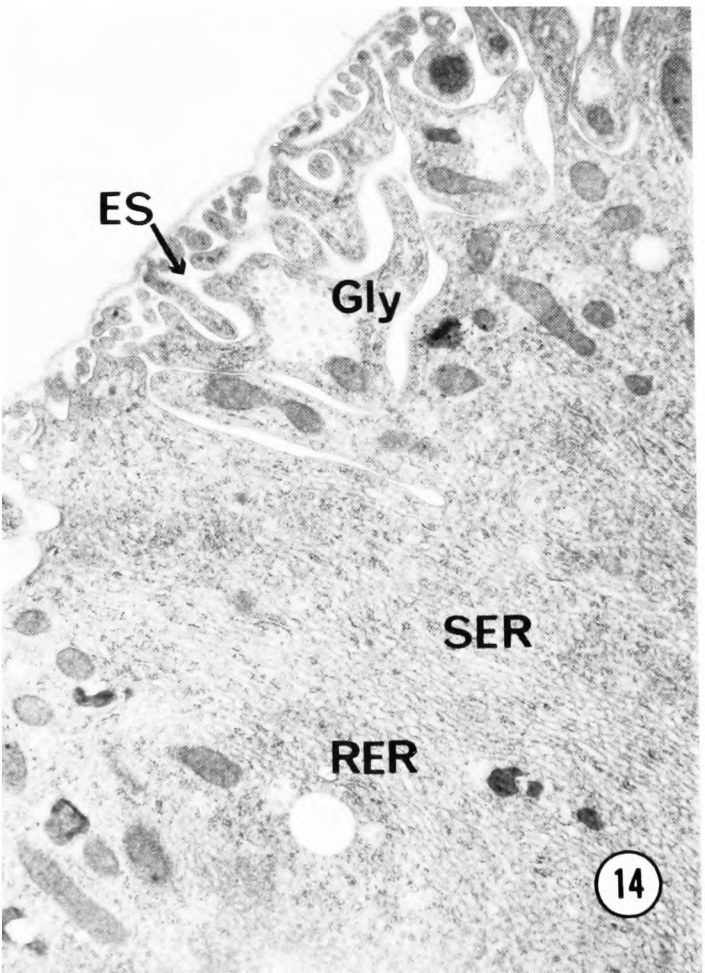
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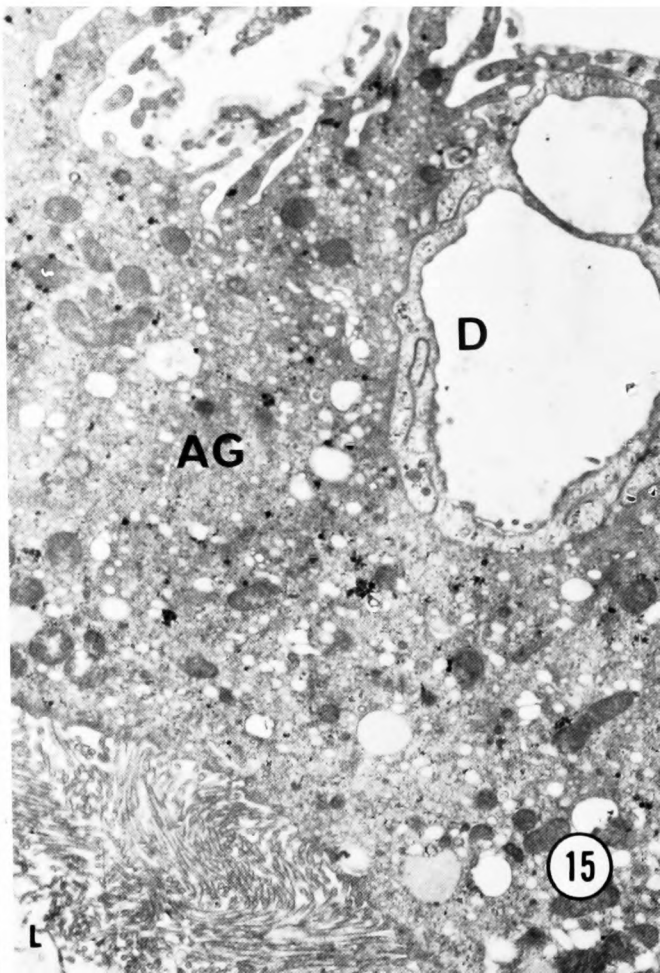
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epithelium and connect with the pores in the cuticle. Here the central lumen of the ducts connects with the outside.

Discussion

The accessory glands of Gene's organ have a structure in common with other types of integumentary glands found in terrestrial arthropods, being most similar to the class 3 type of gland described by Noirot and Quennedey (1974). Each gland cell, containing numerous membrane-bound vesicles and mitochondria, has its own internal secretory lumen, lined with microvilli and a separate duct. Each duct cell connects to an individual secretory pore in the cuticle. The duct is not lined with epicuticle, except where it passes through the body wall cuticle, and in these respects the glands are very similar to those secreting pheromones in *Atta* sp. (Bazire-Benazet and Zylberberg, 1979). In other class 3 arthropod integumentary glands, the secretory cells are in close contact with the cuticle, as in the termite soldier (Sbrenna and Leis, 1983), cockroach (Brossut and Sreng, 1980) and locust (Sbrenna and Sbrenna Micciarelli, 1980). These cells differ from the tick accessory glands in that the duct cell is a relatively short structure, whereas in the accessory glands it may be 2 mm long.

The accessory glands are associated only with female ixodid ticks and absent from the Argasidae (Feldman-Muhsam and Havivi, 1960). Feldman-Muhsam (1963) suggested that the areae porosae produced a lubricant for the horns of Gene's organ during oviposition. However, Atkinson and Binnington (1973) found that in *B. microplus* blocking the pores by electrocautery in newly dropped gravid females had no effect on oviposition or subsequent hatching of the eggs and found evidence that the secretion from the pores exerted an antioxidant effect on certain unsaturated steroid components in the egg wax. We have also blocked the pores in adult female *B. microplus* before commencement of oviposition using rapid hardening resin, Araldite, and found that the eggs were laid normally and were as viable as eggs laid by ticks with their areae porosae unblocked. The porose area secretion therefore does not appear to be essential for laying viable eggs, at least under laboratory conditions and its exact function and chemical identity has yet to be determined. The presence of a separate organ system, the accessory glands, in close relationship with Gene's organ implies, however, that they have a separate and distinct function related to egg-waxing.

The ultrastructure of these glands is characteristic of cells that synthesize lipids. They contain striking amounts of smooth endoplasmic reticulum and many Golgi dictyosomes with small budding membrane-bound vesicles. The appearance of the smooth-membrane reticulum varies between different specimens, and may be in tubular form, in smooth membrane sheets, or as isolated vesicles. This may reflect a different developmental stage of the glands. Many

Fig. 12. TEM showing tubular smooth endoplasmic reticulum (SER) and isolated smooth membrane vesicles in an accessory gland cell. Golgi dictyosomes (G) are associated with small budding membrane-bound vesicles which appear to be enlarging to form secretory vesicles (V). M, mitochondrion; Gly, glycogen particle. ×38,000.

Fig. 13. The apical border of an accessory gland cell, showing microvillar border (MV) and secretory vesicles which appear to be undergoing exocytosis into the internal lumen (arrow). ×11,000.

Fig. 14. Arrays of smooth endoplasmic reticulum (SER) in the basal region of an accessory gland cell. Rough endoplasmic reticulum (RER) is also present and is continuous with the SER. ES, extracellular space beneath basement membrane; Gly, glycogen particles. ×11,000.

Fig. 15. The duct cell (D) passes through the accessory gland cell (AG) on its way to join up with the secretory lumen (L). ×6900.

larger vesicles appear to accumulate near the apical border of the cell and undergo exocytosis into the lumen. These vesicles probably contained lipid since their contents are extracted during tissue processing. These characteristics are often associated with lipid synthesis in other invertebrate tissues such as the oenocytes of insects (Locke, 1969) which are known to synthesize the paraffin components of cuticular wax in the locust (Diehl, 1973) and similar cells that are involved in hydrocarbon synthesis in the cockroach integument (Nelson, 1971).

The composition of the non-polar egg wax lipids of *B. microplus* are well known (McCamish *et al.*, 1977) although it is not clear which components originate from Gene's organ itself or are added by the accessory glands during manipulation of the eggs by the horns or are applied to the egg during passage down the oviduct. Steroids are present in the egg wax of *B. microplus* in much larger quantities than is found in the superficial waxes of other arthropods (Cherry, 1969) and this may be related to the high dietary intake of cholesterol in ticks (Cherry, 1976). The function of these steroids in the egg wax is unknown though it is unlikely that they contribute merely to the waterproofing of the egg since this can be achieved by the far less complex long chain alkanes, alcohols and esters to which the tick has access. It is possible that these steroids satisfy some as yet unknown embryonic requirement by penetrating the egg membrane (McCamish *et al.*, 1977). The ultrastructure of the accessory gland cells with their large quantities of smooth endoplasmic reticulum may be a more likely source of the steroids than the secretory cells of Gene's organ which contain little smooth ER. Smooth endoplasmic reticulum is a characteristic feature of steroid synthesizing tissues in vertebrates such as the testicular interstitial cells (Christensen, 1965) and the corpus luteum (Bjersing, 1967). Also the secretory cells of Gene's organ contain plentiful rough endoplasmic reticulum (Booth *et al.*, 1983) and cytochemical studies of the organ suggest that its main product is lipoprotein rather than free lipids (Lees and Beament, 1948; Chinery, 1965). However, clarification of the contribution made to the egg waxes by the accessory glands must await further biochemical studies.

Acknowledgements

We thank Dr J. Beesley and Mr P. Rowden of the Wellcome Research Laboratories, Beckenham, for their help with scanning electron microscopy, and Mr R. Lamb for photographic assistance. Ticks were kindly supplied by the Wellcome Research Laboratories, Berkhamsted. T. Booth was supported by an SERC studentship.

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