An ultrastructural description of the epidermis and cuticle from Ophryotrocha Cyclops, sp.nov. (Eunicida: Dorvilleidae), a small polychaete recently identified from salmonid aquaculture sites on the south coast of Newfoundland

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AN ULTRASTRUCTURAL DESCRIPTION OF THE EPIDERMIS AND CUTICLE FROM *OPHRYOTROCHA CYCLOPS, SP*.NOV. (EUNICIDA: DORVILLEIDAE), A SMALL POLYCHAETE RECENTLY IDENTIFIED FROM SALMONID AQUACULTURE SITES ON THE SOUTH COAST OF NEWFOUNDLAND

by

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ABSTRACT

Murray, H.M. 2016. An ultrastructural description of the epidermis and cuticle from *Ophryotrocha Cyclops, sp* .nov. (Eunicida: Dorvilleidae), a small polychaete recently identified from salmonid aquaculture sites on the south coast of Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 3161: vi + 12 p.

Epidermal and cuticular ultrastructure of *Ophryotrocha cyclops, a* newly described species of Dorvilleidae was investigated using histology and electron microscopy. The integument consisted of a cuticular and epicuticular layer, an underlying epidermis, and a subepidermal region characterized by cellular and connective tissue components. The epidermis was populated by support cells some of which were observed to be multiciliated. Histochemical staining indicated that the layer was rich in mucopolysaccarides. The support cells were columnar or cuboidal with basal nuclei and an apical cytoplasm containing many membrane-bound vesicles and mitochondria suggesting metabolic activity. The ciliated cells were arranged in tufts and the cilia were of the typical 9 + 2 configuration. Basal structures with striated rootlets anchored the cilia in the cytoplasm. The apical membrane of the epidermal cells was folded into microvilli which extended into the cuticle. Microvilli extended through the cuticle to the epicuticular surface. The cuticle was composed of regular fibrous bands oriented parallel to the apical surface of support cells. The epicuticle appeared non-fibrous and was composed of two layers of differing electron density. This description is one of only a few studies investigating the cellular organization of *Ophryotrocha* sp. epidermis and contributes to our understanding of basic structure and function.

RÉSUMÉ

Murray, H.M. 2016. Description ultrastructurelle de l'épiderme et de la cuticule de *Ophryotrocha Cyclops* sp. nov. (Eunicidé : Dorvilleidae), un petit polychète récemment identifié à des sites de salmoniculture sur la côte sud de Terre-Neuve-et-Labrador. Can. Tech. Rep. Fish. Aquat. Sci. 3161: vi + 12 p.

L'ultrastructure de l'épiderme et de la cuticule de *Ophryotrocha cyclops*, une espèce nouvellement décrite de la famille *Dorvilleidae*, a été évaluée par histologie et microscopie électronique. Le tégument était composé d'une couche de cuticule et d'épicuticule, d'une couche dermale sous-jacente et d'une région sous-dermale caractérisée par des tissus conjonctifs et cellulaires. L'épiderme était constitué de cellules d'appui, dont certaines étaient multiciliées. La coloration histochimique a révélé que la couche était riche en glycosaminoglycanes. Les cellules d'appui étaient columnaires ou cubiques, avec des noyaux gris centraux et un cytoplasme apical contenant de nombreuses vésicules membranaires et mitochondries, ce qui laisse entendre une activité métabolique. Les cellules ciliées étaient disposées en touffes, et les cils affichaient une configuration typique de 9 + 2. Les structures basales comportant des racines striées étaient ancrées dans les cils du cytoplasme. La membrane apicale des cellules épidermiques était pliée en microvillosités, qui se rendaient jusque dans la cuticule. Les microvillosités se prolongeaient au-delà de la cuticule, jusqu'à la surface épicuticulaire. La cuticule était composée de brins fibreux réguliers parallèles à la surface apicale des cellules d'appui. L'épicuticule ne semblait pas fibreuse, et elle était constituée de deux couches aux densités d'électrons différentes. Cette description est l'une des rares études concernant l'organisation cellulaire de l'épiderme de *Ophryotrocha* sp. et contribue à notre compréhension de la structure et de la fonction de base

INTRODUCTION

The integument (epidermis and associated cuticle) of the polychaete is known to be an important interface with the surrounding environment and as such is thought to be the morphological region whereby some species interact with the habitat in which they live (Menon and Arp, 1993; Menon et al., 2003; Hausen, 2005; Ito et al. 2011) The ecological role that this group of marine worms may play in the many challenging environments in which it is found has stimulated an interest in understanding the physiology and functional anatomy of its many species (Akesson, 1970; Tsutsumi, 1990; Paavo et al., 2000; Mastrodonato et al., 2006; Papaspyrou et al., 2007; Paxton and Akesson, 2007; Dean, 2008; Paxton and Davey, 2010; Murray et al. 2012).

Hamoutene et al. (2013) demonstrated that opportunistic polychaete complexes have a role as indicators of benthic habitat condition at finfish aquaculture sites in Newfoundland. Preliminary investigations had indicated that the worms associated with these complexes belonged to the polychaete genus *Ophryotrocha* (Gerhard Pohle and H. Wiklund, personal communication). Recently, Salvo et al. (2014) was able to demonstrate that these polychaetes were a new species of the above genus, i.e. *Ophryotrocha cyclops* sp. nov.

Members of the genus *Ophryotrocha*, Claparede & Meeznikow, 1869, Family Dorvilleidae are known to live in a range of habitats and are considered as a stress tolerant and opportunistic species commonly found at high densities in organically enriched environments i.e. polluted harbours, aquaculture sites, deep-sea sediments and whale falls (Paxton and Davey, 2010; Murray et al., 2012; Salvo et al. 2014; Levin et al. 2003; Thornhill et al., 2009; Wicklund et al., 1984). To begin developing an understanding of the biology of these worms and their possible role in the ecology of the benthos below aquaculture sites, Murray et al. (2012) investigated their epidermal histology and mucus histochemistry. The purpose of the present paper is to expand on the above study by providing ultrastructural detail of the epidermal and cuticular organization of the integument of *Ophryotrocha cyclops* sp. nov.

MATERIALS AND METHODS

COLLECTION

Worms were collected from beneath active commercial Atlantic salmon cage sites located in Hermitage Bay, Newfoundland (Latitude*: 47.616° N,* Longitude: -*55.831°W)* during June 2012. Samples were taken from hard bottom substrates at an approximate depth of 70 m and a mean temperature of 2 ^{0}C. Polychaetes were sampled using a weighted modified egg net (mesh size 500 um) with an oval opening measuring 45 cm by 30 cm and reinforced with metal tubing. The net was towed along the bottom to collect groups of individuals. An underwater video camera (Shark Marine Technologies, 550 TV lines of resolution with 2

external 150 watt lights) was attached to the metal tubing frame in order to verify the presence of worm complexes and increase the probability of obtaining a usable sample.

SAMPLE PROCESSING

Worms sampled from the OPCs were immediately rinsed in sea water of an appropriate temperature and then chemically fixed for both histology/histochemistry (10 % neutral buffered formalin for 24 to 48hrs at 4◦C) and electron microscopy (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C for 24 hrs).

HISTOLOGY AND HISTOCHEMISTRY

Ten individual worms were chosen for histological and mucus histochemical analysis and were dehydrated through ethanol series, cleared in two changes of xylene, and infiltrated and embedded in paraffin for both longitudinal and transverse sections. Six to eight micron thick sections were cut serially using a rotary microtome (Leica, RM2265) to ensure all sections were captured for each individual. Section ribbons were then placed on uncoated glass slides, dried overnight at 37°C, and stored at room temperature. Tissues were stained with Alcian blue (AB) pH 2.5 and Periodic Acid Schiff's reagent (PAS) (Bancroft and Cook, 1984). Staining was performed in batches using a Leica, Auto Stainer XL and tissue sections were subsequently examined using a Zeiss Axio Imager-A1 compound microscope with attached AxioCam HRc camera and associated software. Image plates were created using Photoshop Elements 11.0.

ELECTRON MICROSCOPY

Five individual worms were randomly chosen for ultrastructural analysis and were processed at the Electron Microscopy Facility, Faculty of Medicine, Memorial University of Newfoundland. Briefly, following fixation samples were divided into three relative segments (anterior, mid and posterior), transferred to fresh 0.1M sodium cacodylate buffer pH 7.2 for 5 minutes, osmicated in 1% Osmium tetroxide in the same buffer for 30 minutes at room temperature and then dehydrated through ethanol series (70% ethanol 2 X 15 minutes; 95% ethanol 2 X 15 minutes; absolute ethanol 2 X 20 minutes; absolute acetone 2 X 20 minutes). Subsequently, tissues segments were infiltrated with EPON 812 resin (50:50 acetones: resin for 60 minutes; pure resin 2 X 60 minutes) and then embedded in BEEM capsules in fresh resin and polymerized over night at 80 ^{o}C.

Embedded tissues were trimmed with glass knives and 1 µm sections were cut, placed on glass slides and stained with Toluidine blue. Areas of interest were located and ultrathin sections (70-90 nm) were cut using a Diatome diamond knife and a Reichert ultracut S ultramicrotome. The sections were placed on uncoated 300 mesh copper grids and contrasted on drops of 3% uranyl acetate

followed by lead citrate. Stained grids were viewed on a JEOL 1200 electron microscope with an operating voltage of 80 Kv. Images were recorded digitally using a Maxim DL digital camera with associated software. Figure plates were produced using Adobe Photoshop Elements 11.

RESULTS

The general integument of *Ophryotrocha cyclops* sp. nov. consisted of three histologically and histochemically distinct layers (Figure 1 (a)). These included a cuticle with underlying epidermis, followed by a subepidermal zone characterized by connective tissue infiltrated with cellular components and a variety of fibers that reacted strongly with the PAS reagent (Figure. 1(a)). The epidermis was composed of two primary cell types: non-ciliated epidermal support cells and groups of multiciliated cells (Figure 1 (b)). This general organization of the epidermis was found to be consistent along the entire body including the parapodia and typically exhibited an apparent monolayered configuration (Figure 1(b)). The cells populating this zone and the cuticle, stained strongly with PAS and Alcian blue, indicating high mucopolysaccaride content (Figure 1(a)). Connective tissue associated with the adjacent subepidermal region also stained strongly with Alcian blue (Figure 1 (a)). Deeper layers containing fibrous connective tissue stained primarily PAS positive (Figure 1 (a)).
The non-ciliated support cells were columnar or cuboidal in shape and oriented toward the apical surface with a basal or centrally located oval nucleus with a prominent nucleolus (Figure 1 (b)). The apical portion of the support cells was densely populated by a variety of membrane bound vesicle types (Figure 1 (c)). Some of these vesicles were clear but others were observed to contain either electron dense or flocculent material and were frequently associated with dilated rough endoplasmic reticulum (Figure 1 (c)).

The multiciliated cells were observed to occur singly or in tufts or groups (Figure 1 (b)). The apical portion of the ciliated cells contained numerous mitochondria (Figure 1 (d)). Evidence of the rootlet structure was present within the apical cytoplasm but it was difficult to distinguish detail of the anterior and vertical substructures (Figure 1 (d)). Basal bodies were observed to extend through the cuticle and epicuticle giving rise to the ciliary shafts which come together forming a ciliary tuft at the epidermal surface (Figure 1 (d)). In cross section the cilia exhibited the classical 9 + 2 microtubule configuration (Figure 1 (d))

The cuticle and associated epicuticle overlying the epidermal cells was found to be approximately 2.5 µm in total thickness the external surface of which was occasionally overlaid by layers of vesicular bodies (Figure. 2 (a)). The epicuticle appeared to be made up of two layers both of which were more electron dense then the underlying portion of the cuticle (Figure 2 (d)). The cuticle measured approximately 2 µm in thickness (without the epicuticle) and was observed to be generally organized in tight bands of fibers running perpendicular to the microvilli

but parallel to the cells apical surface (Figure 2 (a), 2 (b), and 2 (c)). The zone between the collagen bands was observed to contain a very fine granular matrix into which the bands appeared to be embedded (Figure 2 (b), and 2 (c)). The cuticle was also noted to frequently be infiltrated by irregular membrane bound vesicles containing flocculent material (Figure 2 (a), and 2 (c)).
The apical surface of the non-ciliated support cells was also characterized by microvilli which were direct extensions of the apical membrane and appeared to insert into the adjacent cuticle, acting as an anchor point between the epidermal cells and the cuticle itself (Figure 2 (b)). The cuticle was not typically observed to sit flat on the apical membrane of the epidermal cells but was characteristically separated by a defined space between itself and the adjacent cuticle (Figure 2 (b)). Occasionally, microvilli were observed to extend all the way through the cuticle penetrating the epicuticle and projecting onto the surface of the integument (Figure 2(d)).

DISCUSSION

Ophryotrocha cyclops sp. nov. (Eunicida: Dorvilleidae) was collected and described from aquaculture sites in Newfoundland and whale bones in Greenland initiating an interest in understanding more about the general biology of this group in the Northwest Atlantic and its role specifically in aquaculture impacted environments (Salvo et al. 2014). It is thought that the distribution of complexes or aggregations of these worms below farm sites may act as indicators of benthic impact (Hamoutene et al., 2013). Murray et al. (2012) originated a study looking at the epidermis of this worm with a specific focus on the cells involved in mucus production. The present study was designed to expand on Murray et al., (2012) with a more detailed investigation of the ultrastructure of the epidermis and cuticle.

The general structure of the epidermis of polychaetes is well conserved across groups but may vary depending on the environment in which the worm is found. The main structural characteristics have been reviewed repeatedly (Hausen, 2005; Westheide and Rieger, 1978). However, with the exception of a few studies focused on the ultrastructure of specialized sensory organs (Rhode, 1989; Schlawny et al., 1991) no studies have investigated the detailed epidermal and cuticle ultrastructure of species from the genus *Ophryotrocha*.

The non-ciliated epidermal support cells of *Ophryotrocha cyclops sp. nov.* contain many of the characteristic features exhibited by other small free-living polychaetes. Hausen (2005) reviewed many of the general polychaete epidermal support cell characteristics, indicating that different cell morphologies may be present even within single specimens suggesting different physiological specializations. As described in the present study, the primary epidermal support cells of *O. cyclops* were similar along the entire body and were characterized by an elongate shape with an oval euchromatic nucleus and prominent nucleolus

situated in either a basal or central configuration. The apical cytoplasm of these cells was shown to react strongly with PAS/Alcian blue reagent indicating the presence of mucopolysaccarides or mucoproteins. This observation along with the presence of numerous membrane bound vesicles and dilated rough endoplasmic reticulum in the apical cytoplasm suggests significant metabolic activity that may be interpreted as involved in both endocytotic and exocytotic (secretory) events. Rhode (1989) demonstrated ultrastructural evidence of exocytotic activity in association with the apical membrane of supporting cells of *O. puerilis*. Hausen (2005) indicated in his review of polychaete epidermal structure that it was not unusual for epidermal cells to secrete many different substances including those of protein and mucopolysaccaride origin. The function of these various non-glandular secretions in Polychaetes is not yet clear. However, the intense metabolic activity evident in these cells in *O. cyclops* is interesting and while likely not phylogenetically important, may be physiologically significant.

The presence of microvilli on the apical surface of the epidermal cells is well known to be a characteristic feature of the polychaete epidermis and have been described in many classes of Annelid including polychaetes and oligochaetes [Gustavsson and Erseus, 2000; De Wit et al., 2011; Vodopyanov et al., 2013). Some proposed functions of microvilli include a role in the organization of the collagen fibers of the cuticle during development, absorption of nutrients, excretion and respiration [as discussed in Gustavsson and Erseus, 2000; De Wit et al., 2011; Gustavsson, 2001). Within the present study, microvilli were shown to penetrate and weave through the fibrous network making up the cuticle in *O. cyclops*. They were frequently observed to extend all the way through to the epicuticle and onto the outer surface. This configuration suggests a possible role as an anchoring mechanism for the cuticle itself and was in agreement with the general plan for the polychaete epidermis and cuticle as outlined in the review by (Hausen, 2005). Little is currently known about the true function of epidermal microvilli in annelids and it seems that by far the majority of work has focused on oligochaetes. Gustavsson and Erseus (2000) discussed much of the literature speculation on epidermal microvillar function. The microvilli observed on the surface of cells from *O. cyclops* were typical and inserted directly into the cuticle. The vesicle-like structures observed to be occasionally distributed throughout the cuticle are likely sections through microvilli extending to the epicuticular surface. In the present study, microvilli were also noted to be associated with layers of vesicles on the epicuticular surface, many of which appeared to be associated with the swollen tips of penetrating microvilli. In his review of the structure of polychaete epidermal support cells, Hausen (2005) noted that the tips of microvilli penetrating the cuticle in some polychaetes could be somewhat inflated and lay on the surface of the epicuticle. He also noted that the tips were often surrounded by several membrane bound vesicles which he equated to the epicuticular projections observed in other groups.

Additionally, it is well known that microvilli have a significant role in increasing the apical surface area in epidermal cells and it has been suggested that this increase in surface area may be associated with the uptake of organics and other possible dissolved nutrients within the environment (Westheide and Rieger, 1978; Stephens, 1963; Gomme, 2001). Based on what is known about the enriched environments in which *Ophryotrocha sp*. are commonly found, epidermal cells might have a role in up taking and utilizing dissolved organic nutrients. Work is ongoing to determine the potential for this mechanism in *O. cyclops* and related species from similar environments.

The cuticle of *O. cyclops* was noted to be composed of dense fibers that were organized into bands running perpendicular to the apical microvilli. The structure of the cuticle in Annelids (polychaetes and oligochaetes) and its relationship with the underlying epidermal cells has been investigated extensively (Hausen, 2005; Gustavsson and Erseus, 2000; Vodopyanov et al., 2013; Gustavsson, 2001; Anton-Erxleben, 1981; Riser, 1988; Sjolin and Gustavsson, 2008). Generally the cuticle can be divided into two zones i.e. the basal zone and the overlying epicuticular zone. The basal zone is typically composed of a thin matrix of collagen fibrils embedded in a granular matrix consisting of mucopolysaccarides plus other protein components whereas the epicuticle is normally thought of as nonfibrous or collagen-free (see review by (Hausen, 2005). In *O. cyclops* the basal cuticle was relatively thick and was composed of a network of fibers and in some cases distinct bands running parallel to the apical membrane of the underlying epidermal cells. The bands appeared to be embedded in a granular matrix. The adjacent epicuticle was divided into two layers of differing electron density. The outer layer, being the most electron dense, was also found to be associated with a distinct glycocalax. This basic configuration was similar to that described for other Annelid groups including the oligochaetes (Gustavssson, 2001; Sjolin and Gustavsson, 2008).

Multi-ciliated cells are common in many species of Annelid including polychaetes. Hausen (2005) in a review of the structure of the polychaete epidermis indicated that epidermal support cells could be designated as multi-ciliated or non-ciliated. He also noted that cilia typically lack accessory centriols, but in most cases have two striated rootlets where one projects deeply into the cell while the other runs parallel to the apical cell membrane. In a further review of the evolution and ecology of *Ophryotrocha* (Dorvilleidae, Eunicida), Thornhill et al (2009) indicated that most species of this genus are ciliated having clear bands or tufts of cilia. It was also noted that the retention of cilia in this group may be related to its small size and that they may have a locomotory function. Ciliated cells have also been described in other polychaete groups where they have been found to be associated with various sense organs (nuchal organs or dorsal sense organs) or feeding palps (Schlawny et al. 1991; Purschke, 1990; Jelsing, 2003; Worsaae, 2003; Dauer, et al., 2003). The multiciliated cells in *O. cyclops* occurred in bands or tufts. The cilia passed through the cuticle where the basal body was firmly rooted. Striated root structures were evident in the apical cystoplasm and

configured generally as indicated in (Hausen, 2005). The ciliated bands in other species of *Ophryotrocha* are thought to be associated with swimming activity and/or the generation of water currents over the body to facilitate access to fresh seawater for gas exchange among other uses including sensory (Westheide and Rieger, 1978; Rhode, 1989; Schlawny et al. 1991).

CONCLUSIONS

In conclusion, the general micro-anatomy and ultrastructure of the integument from *O. cyclops* is similar to that described from other related species but this study adds new information to our understanding of the structure of the cuticle and underlying cells of this group. The functional dynamic of the interaction between the cuticle and the underlying epidermal cells is interesting from a developmental perspective and should be further explored. Also the nature of the metabolic activity associated with the epidermal cells suggests an important physiological role and may be significant in uptake and processing of dissolved organics from the environment. This maybe an adaptation to living in environments with high organic loads like below aquaculture sites. Further work is necessary to develop a complete understanding of the role these animals play.

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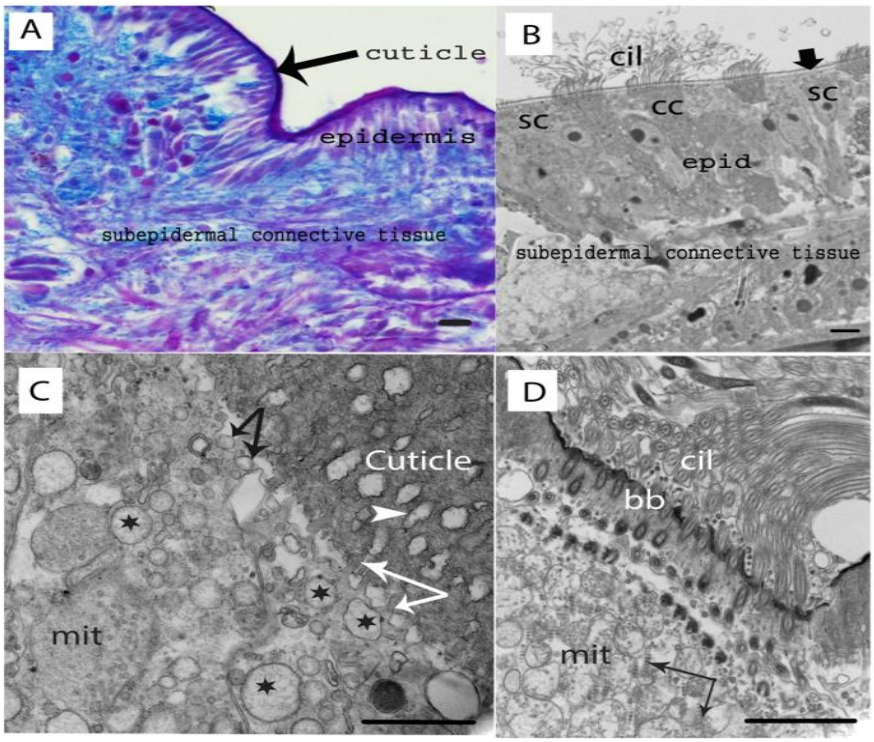

Figure 1 Epidermis of *Ophryotrocha cyclops sp. nov.* (a). Alcian Blue, (pH 2.5)/PAS staining of longitudinal secions of *O. cyclops*. sampled from beneath Atlantic salmon aquaculture sites located on the south coast of Newfoundland. Note differential staining of the epidermal layer and the subepidermal connective tissue layer. Scale bar,1mm (b). Detail of cells populating the epidermal zone. Note distribution and orientation of ciliated and nonciliated support cells. SC, nonciliated support cell; CC, ciliated support cell; CiL, cilia tufts; epid, epidermal layer. Arrow head indicates the cuticle. Scale bar, 1mm (c). Ultrastructural detail of the apical cytoplasm and adjacent cuticle of a nonciliated support cell. Cut, cuticle; mit, mitochondria. White arrows indicate microvilli and associated spaces. Stars indicate dilated membrane bound vesicles containing flocculent material. Note also small vesicles adjacent to the apical membrane (back arrows) Scale bar, (d). Apical cytoplasm of a ciliated support cell showing cilia (Cil) with basal body (BB) cuticle (Cu) insertion point. Note striated rootlet structure (arrow). Mitochondria, Mit. Scale bar, 2.0 µm.

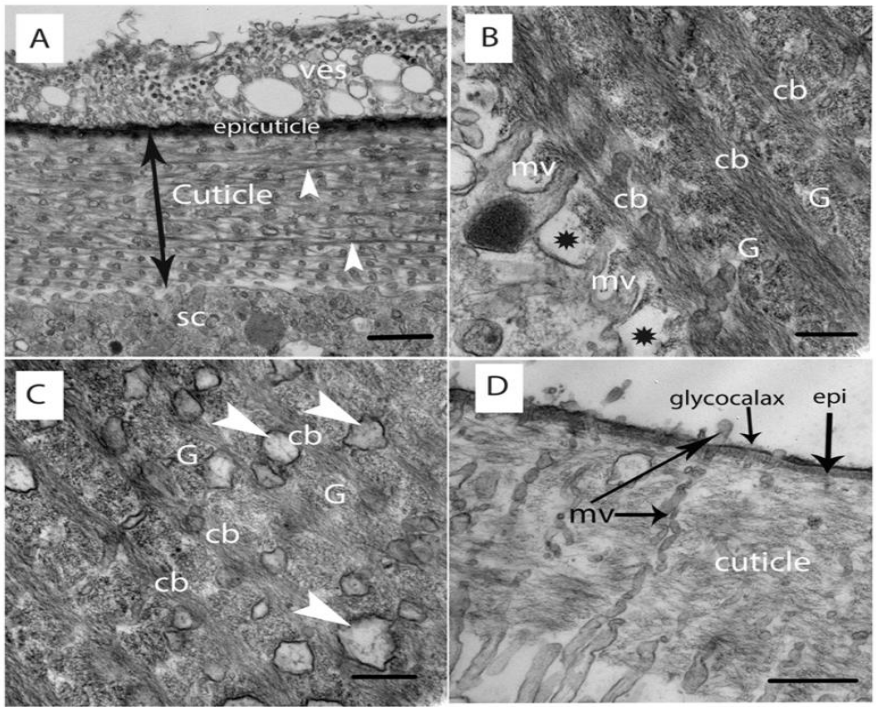

Figure 2. Cuticle structure. (a). Cuticle and epicuticle showing overall thickness above the apical membrane of a nonciliated support cell (SC) (total width to epicuticle was 2.5 µm). Note the layer of vesicular bodies (ves) external to the cuticle. White arrowheads indicate bands of fibers. Scale bar, 2 µm. (b). Detail of the cuticle showing the structure of the thick bands of fine fibers (cb) embedded within granular matrix (G) and the insertion of microvilli (mv) from the support cell into the fibrous cuticle. Stars indicate the space between the apical membrane of the support cell and the adjacent cuticle. Scale bar, 0.25 um. (c) Fine detail of cuticle showing fiber bands (cb) in granular matrix (G). note also the irregular shaped vesicles embedded in the cuticle (white arrowheads). Scale bar, 0.25 um. (d) Cuticle showing microvilli (mv) extending all the way to the epicuticular surface . Note epicuticle (epi) with electron dense outer layer and associated glycocalax (arrow). Scale bar, 5 um.