THE ULTRASTRUCTURE AND HISTOLOGY OF THE DEFENSIVE EPIDERMAL GLANDS OF SOME MARINE PULMONATES

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SHIRLEY CLARE PINCHUCK

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ABSTRACT

Histology and electron microscopy were used to describe and compare the structure of the dorso-lateral pedal defensive glands of three species of marine Basommatophora, *Siphonaria capensis, S. serrata* and *S. gigas*. All three species possessed multi-cellular glands that were larger and most abundant in *S. capensis*. In *S. capensis* and *S. serrata,* defensive glands were composed of two types (type I and II) of large secretory cells filled with product and some irregularly shaped support cells that surrounded a central lumen. The product of both cell types was produced by organelles confined to the bases of the cells. The entire gland was surrounded by a well developed layer of smooth muscle and collagen. Type I cells stained positively for neutral and sulphated mucins, and at the transmission electron microscope level the product had a reticulated appearance. By contrast type II gland cells stained very positively for acidic mucins and the secretory product was formed as large granular vesicles. The product from both types of cell, which appeared to be secreted by holocrine secretion, mixed in the lumen of the duct. Individuals of *Siphonaria gigas* had two types of lateral pedal glands, a large multi-cellular type and a tubular unicellular gland. The multi-cellular glands, which were surrounded by poorly developed muscle, contained one type of gland cell that stained for neutral and sulphated mucins only, as well as some support cells. The tubular glands contained a heterogeneous product that stained very positively for neutral and sulphated mucins.

In addition two species of shell-less marine Systellommatophorans, *Onchidella capensis* and *O. hildae,* were examined. Onchidellids also posses large marginal, multi-cellular, epidermal glands that produce a repugnatorial secretion. Like the multi-cellular epidermal glands of siphonariids, those of onchidellids are surrounded by layers of smooth muscle. The muscular capsule was particularly well developed in both species of onchidellid, but more so in *O. hildae*. In addition, this study has shown that unlike siphonariids, muscle fibres run between the gland cells of *O. capensis* and *O. hildae*. Unlike siphonariids, onchidellids have a layer of epithelial cells lining the lumen of the gland. The well developed muscle layer and the strands of muscle running between the different gland cells indicates that the glands can be constricted to forcibly propel their secretions along the length of the duct and away from the body of the animal. Based on their product, glands of *O. capensis* were comprised of five different types of secretory cell and *O. hildae* only four. Histological and histochemical staining of the glands of showed that the secretory product is largely made up of acidic mucopolysaccharides and neutral and sulphated mucins.

A single species from the order Eupulmonata, *Trimusculus costatus*, was examined and the glands were very different to the species from the siphonariids and onchidellids. *Trimusculus costatus* does not have large multi-cellular glands encapsulated in a well developed muscle layer, but based on their cell contents, three different types of large unicellular gland cell can be recognised. The glands of *T. costatus* gave positive results for acid, neutral and sulphated mucins, but negative results for carboxylated mucin. It is possible that the mucus secreted by *T. costatus* is also an anti-bacterial agent and whilst not totally eliminating bacteria may prevent the accumulation of epibionts on these sedentary limpets. The acidic or sulphated nature of the secretions may help in this role.

The defensive mucous secretions of *Siphonaria* and *Onchidella* contain polypropionate derivatives, whilst the active ingredients of *Trimusculus* mucus have been identified as labdane diterpenes, similar to those produced by opisthobranchs. The structure of the glands thought to produce these repugnatorial secretions is very different, with the glands of *T. costatus* resembling those of the opisthobranchs.

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PREFACE

This work is divided into three investigations:

- Structure and histology of the lateral pedal epidermis and glands of three species of *Siphonaria*.
- Structure and histology of the lateral pedal epidermis and glands of two species of *Onchidella*.
- Structure and histology of the lateral pedal glands of *Trimusculus costatus* (Trimusculidae).

Some of the work from the first investigation has been published in the Journal of Molluscan Studies:

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I would like to dedicate this work to the memory of my brother, Peter Haselhurst, who died tragically earlier this year, after a long illness.

Chapter 1. General Introduction

“The molluscs are a distinctive and individual phylum without any close resemblance or phylogenetic affinity to any other living group, except in so far as they have retained a number of features indicative of their flatworm ancestry.” (Barnes *et al.,* 1993). Molluscs occupy all major habitats and include representatives of all known feeding types (Barnes *et al*., 1993). They are all soft-bodied unsegmented creatures, although the body can be divided into three parts: a foot, head and a visceral mass containing the gut and other organs. The body is covered by the mantle, which in most molluscs is responsible for laying down the shell and which overhangs the body to create a cavity, the mantle cavity. Another distinguishing feature of most Mollusca are the gills, which are housed in the mantle cavity and are covered in cilia which drive water over their surface. This makes them different from most other animals which cannot generate their own currents of water over the gills (Branch, 1981). The skin or epidermis is another important feature of the Mollusca (Trueman and Clarke, 1988).

The thousands of mollusc taxa living on the land or in water represent only the surviving peaks of evolution; they are the comparatively few successful units of the mass of forms that have evolved (Fretter and Peake, 1978). Molluscs are the second largest animal phylum and the success of the group is probably not due to any particular anatomical or ecological feature but rather due to the extreme plasticity and adaptability of the basic molluscan body plan (Barnes *et al*., 1993). This plasticity can be illustrated by the variation displayed in the function of any single molluscan structure (e.g. the shell, besides being protective, may serve as a flotation device, a burrowing organ, or an endoskeletal plate) and by the multiplicity of the structures which have been adapted to serve a function (*e.g.* food catching organs include ciliated tentacles or palps, greatly enlarged gills, sucker bearing arms, radular teeth *etc*.) (Barnes *et al.*, 1993). The basic molluscan morphology is that of a flatworm with two distinctive additional features: the radula and the dorsal shell (Barnes *et al.*, 1993).

The Gastropoda is the largest class within the Mollusca (Branch, 1981), and has the second largest number of species after the arthropod Class Insecta, with the minimum estimate of extant species at around 40,000 and a maximum of 150,000 (Ponder and Lindberg, 2008). All gastropods, including those without shells, have a large foot, a head with sensory tentacles and eyes, and a visceral mass that contains the gut, blood system, reproductive organs and kidney. The most characteristic feature of all gastropods is that the visceral mass is not symmetrical but has undergone torsion i.e. twisted into a spire, although there are some slug-like forms that have become almost symmetrical (Ponder and Lindberg, 2008).

Gastropoda were divided into three sub-classes: Prosobranchia (the winkles, whelks and limpets), Opisthobranchia (the sea slugs and nudibranchs) and the Pulmonata (snails, slugs and false limpets). More recently Ponder and Lindberg (1997) divided the Gastropoda into two major groups: subclass Eogastropoda, comprising the Patellogastropoda, and the remaining gastropods into the subclass Orthogastropoda. Both the opisthobranchs (the sea slugs and nudibranchs) and the pulmonates (snails, slugs and false limpets) are included in the Orthogastropoda (see Figure 1.1) and are grouped together to form the Heterobranchia.

The class Pulmonata – the land snails, slugs and false limpets – have lost all signs of gills and the roof of the mantle is richly supplied with blood vessels and acts as a lung (hence the name Pulmonata) enabling them to breath air. The classification of the Pulmonata according to Haszprunar & Huber (1990, cited in Ponder and Lindberg, 2008): superfamily groupings follow Hubendick (1978, cited in Ponder and Lindberg, 2008; Stanisic, 1998) who divided the pulmonates into three orders: the Systellommatophora, the Basommatophora and the Eupulmonata (Figure 1.2). Most species in these three orders are terrestrial or freshwater but there are some marine taxa in each. The Order Basommatophora contains numerous limpet-like species that are grouped in the Siphonariidae. The Systellommatophora contain the Onchidellidae and the Eupulmonata the Trimusculidae. These are extremely common to the intertidal regions of warm, temperate to tropical shores, and are most abundant in the Indo-Pacific, especially within the Southern Hemisphere (Hodgson, 1999). The siphonariids can be recognised by the siphon on the right side of the foot which leads into the lung-like mantle cavity. Like true limpets (to which they are only distantly related) *Siphonaria* homes to a fixed scar on a rock in the intertidal zone, to which it returns after feeding (Hodgson, 1999). The Siphonariida*e* (Basommatophorans) and Trimusculidae (Eupulmonates) have a limpet form of morphology, whereas the Onchidellidae (Systellommatophorans) are shell-less and more slug like in appearance.

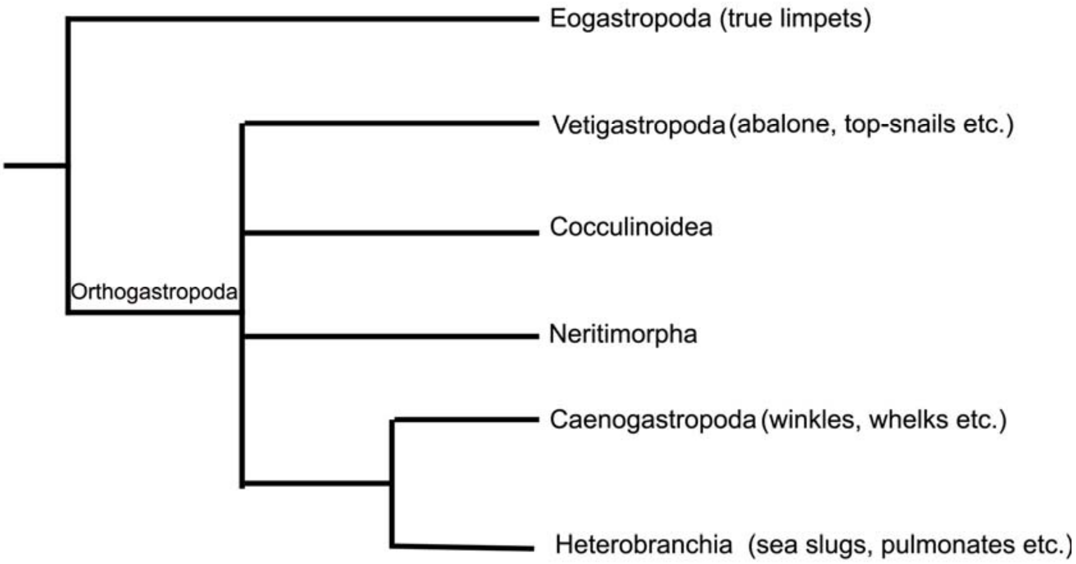

Figure 1.1. Gastropod higher classification (redrawn from Ponder and Lindberg, 1997)

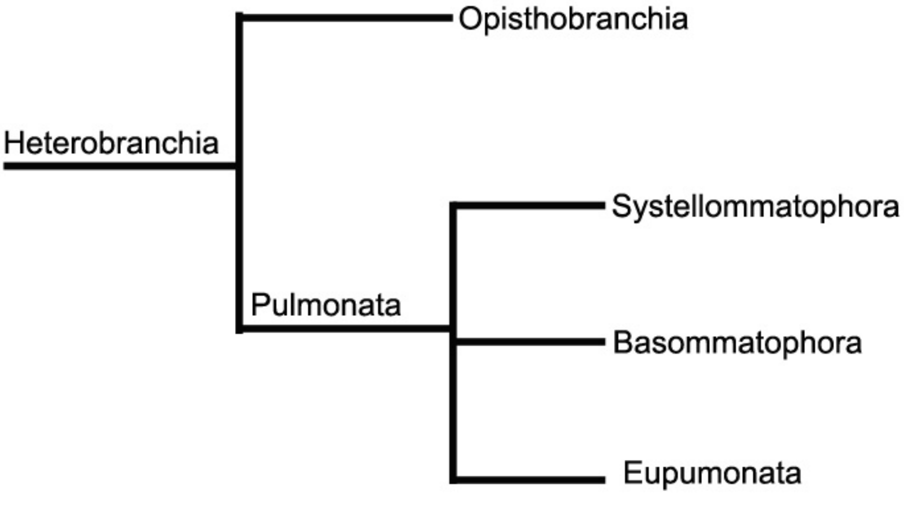

Figure 1.2. Heterobranch classification (redrawn according to Stanisic, 1998, cited in Smith and Stanisic, 1998)

A distinctive feature of the gastropods is the epidermis. Bubel (1984) described the epidermis as that body part that protects a mollusc against environmental conditions whilst, at the same time, informing the animal about the external world in order that it may survive. The gastropod epidermis functions not only as a tegument (the protection boundary between internal and external) but also plays a role in osmoregulation, respiration, locomotion, perception, formation and regeneration of the shell (Zylstra, 1972). These functions are localised in specific regions of the epidermis and it is therefore logical to assume that the structure of the epidermis displays specialised features in the area that possesses specialised functions (Zylstra, 1972). The molluscan epidermis has two functions which are not normally associated with skin – it can participate in transport of particulate matter *e.g.* food, faeces etc. in some regions of the body and it is involved in much of the locomotary activity (Bubel, 1984). These functions are carried out by the epidermal cells and the mucous glands.

A most important role of the epidermis is that of protection. Chemical defence is an important determinant of survival for many sessile invertebrates (Lindquist, 2002). Many molluscs obtain protection from predators by producing secretions from gland cells which occur in large numbers in, and beneath, the epidermis (Zylstra, 1972; Bubel, 1984). The most characteristic secretion produced by these cells is mucus. Mucus covers the body making it slimey and therefore difficult for some predators to grasp the mollusc. Mucus also enables harmful substances to be driven away from the body and it has been suggested that the mucus may have anti-biotic properties (Ireland and Faulkner, 1978). Further protection from predators is due to defensive or repugnatorial secretions from glands present in the mantle edge and throughout the epidermis of gastropods – particularly those that have become naked through loss or reduction of the shell (Thompson, 1969; Bubel, 1984). In many cases the mucus secreted in response to stress contains noxious secondary metabolites (Lindquist, 2002).

The molluscan epidermis is a relatively simple structure consisting of a single layer of columnar or cuboidal epithelial cells on a basement membrane. The external surface is covered with microvilli that hold the “slime” or mucous layer in place (Bubel, 1984). The basal lamina overlies a layer of connective tissue permeated by muscle fibres, often referred to as tonofibrils or tonofilaments (Zylstra, 1972; Bubel, 1984). The function of these muscle fibres is possibly cytoskeletal and they have a contractile function, serving as a sort of muscular system for the cell (Zylstra, 1972). These contractile properties may play a role in the dynamic alterations in the structure of the microvilli and also play a role in the process of endocytosis. Ciliated, non-ciliated epithelial cells and goblet or gland cells are the main types of cell found in the epidermis (Zylstra, 1972). The epidermis is well innervated and contains primary sensory cells and a number of sense organs, thus enabling molluscs to detect and avoid detrimental conditions.

There are several different types of gland cells associated with the molluscan epidermis. There are intra-epithelial gland cells found between the columnar or cuboidal epithelial cells. These are single goblet cells and are thought to produce the mucous secretion that covers the epidermis and this mucus is thought to play a role in the cleaning the microvilli and facilitating ionic exchange. Beneath the epidermis of many species are larger, multi-cellular glands and these are the glands that are thought to produce the repugnatorial secretions used in defence against predators, being toxic to fish at levels as low as 10 μml^{-1} (Hochlowski *et al*., 1983). In addition, the secretions have been shown to be bioactive by effectively suppressing bacterial growth (Biskupiak and Ireland, 1983; Bancroft and Gamble, 2002; Ponder and Lindberg, 2008)) and delaying the fertilization of sea urchin eggs at 1 μl^{-1}. It is therefore not surprising that gastropods have been used medicinally as early as 450BC (Ponder and Lindberg, 2008). Their mucus was used to treat burns and other skin disorders and to relieve pain. In the 18th century various gastropod preparations were also used for relieving the symptoms of tuberculosis and kidney problems. More recently the pharmacologically active ingredients have been used to treat drug resistant bacterial infections and used in cancer treatments (Davies-Coleman and Beukes, 2004; Ponder and Lindberg, 2008).

Many molluscs may be distasteful to predators for a variety of reasons. Mucous secretions from the repugnatorial glands frequently appears to contain a variety of pharmacologically active components (Ireland and Faulkner, 1978; Davies-Coleman and Beukes, 2004; Davies-Coleman, 2006; Darias *et al*., 2006). These may be synthesised by the animal itself or may be derived from the toxicants in the food that the animal eats (Trueman and Clarke, 1988). Molluscan defensive secretions are essentially inorganic and contain predominantly SO_4^{2-} anions (sulphates) and are often strongly acidic (Thompson, 1983, 1988).

All species of southern African *Siphonaria* exude a toxic sticky white mucus when irritated that repels most predators. They are not consumed by whelks, oyster catchers or the giant cling fish (Branch, 1981, 1988; Branch and Cherry 1985; McQuaid *et al*., 1999; Davies-Coleman and Garson, 1998). The mucous secretion has been described as bitter to the taste (Hodgson 1999).

Mucus is largely made up of mucins. Mucins and glycogen are the two main entities to be considered in tissue carbohydrate demonstration, mucins being the largest group, comprising ‘mucopolysaccharides’, ‘mucosubstances” and ‘glyco-conjugates’ (Bancroft and Gamble, 2002).

The role of glycogen in metabolism as a glucose precursor is well established, but the precise function of mucin in all its forms is not yet fully understood. Most surfaces are covered with mucin which has a lubricatory function, but it has been suggested that this mucin coating also provides a suitable environment for ionic and molecular diffusion. In addition, Bancroft and Gamble (2002) suggested that the epithelial mucous layer may also exert an “anti-adhesive” effect on bacteria.

Macrolides (antibiotic drugs whose activity stems from a macrolide ring) from bacteria and fungi are amongst the most important commercial antibiotics used as therapies against respiratory infections, against Legionnaires disease and for minor infections in patients sensitive to penicillin antibiotics (Ponder and Lindberg, 2008). Propionates are important structural and biosynthetic building blocks of antibiotics. The Mollusca are the most important source of marine polypropionates (Davies-Coleman and Garson, 1998; Davies-Colman and Beukes, 2004; Darias *et al*., 2006). Studies on the chemistry of 13 species of *Siphonaria* have revealed that they produce polypropionate metabolites (Davies-Coleman and Garson, 1998). It is thought that these metabolites, which are secreted as part of the mucus from the epidermal glands, are noxious. Some of the polypropionates are biologically active, being toxic to fish at levels as low as 10µg ml^{-1} (Hochlowski *et al*.1983). This chemical defence is very effective in some species, *S. capensis, S. serrata, S. concinna* and *Kerguelenella lateralis* suffer very little predation and many predators that eat patellogastropods, refuse to eat these siphonariids when presented with them (Branch & Cherry, 1985; Branch, 1988; Hodgson, 1999; McQuaid *et al.*, 1999, Davies – Coleman, 2006; Darias *et al.*, 2006). Branch (1981) speculated that the unpalatability of most siphonariids results in low predation and may be responsible for the success of this genus in tropical waters.

The Onchidellidae, a group of shell-less marine molluscs, are another group of marine pulmonate gastropod that produce defensive or repugnatorial secretions. These secretions have also been found to contain polypropionate metabolites (Darius *et al.*, 2006). A final group of marine pulmonate limpets that produces sticky milky-white mucus when irritated or threatened is the Trimusculidae (Order: Eupulmonata). This mucus will also deter predators, but the chemicals produced in the mucus of the trimusculids have been identified as labdane diterpenes (Gray *et al*., 1998; Darias *et al.*, 2002; Davies-Coleman and Beukes, 2004; Davies-Coleman, 2006). The difference between the active ingredients of siphonariids and trimusculids is perhaps further evidence that although these two taxa have much in common they are not closely related (Hodgson, 1999).

The primary function of the shell is to provide protection from predators. However, many successful gastropods [nudibranchs and tectibranchs (Opisthobranchia)] lack an external shell but produce a strong acid (pH 1-2) when threatened. The acid is a mixture of sulphuric acid and hydrochloric acid. This acid secretion is believed to have a defensive function, making the mollusc unpalatable to its predators. It has been suggested that this could be an evolutionary driving force (Cimino and Ghiselin, 2001). In the epidermis these acid secreting glands appear either as empty columnar cells, or empty sub-epidermal sacs. Sub-epidermal glands may be unicellular or multi-cellular, but usually both are surrounded by a network of muscle fibres which contract to discharge the gland secretion. The distribution varies according to species, but these glands are normally found in the foot, mantle, gills and generally exposed epidermis associated with mucous gland cells and ciliated cells (Edmunds, 1968). Edmunds (1968) also proposed that the mucous glands produce a layer of mucus that either protects the epidermal cells from acid damage or that the mucus provides a viscous medium that prevents the acid from being too rapidly dispersed and diluted in sea water.

The structure of the defensive glands of the opisthobranchs has been fairly extensively studied (Thompson, 1983, 1988; Wägele *et al*., 2006). Although the review by Wägele *et al*. (2006) on Opisthobranch defensive glandular structures included some information on the defensive glands of pulmonates (siphonariids and onchidellids), little is known about the detailed structure and functioning of pulmonate defensive glands. The purpose of this study therefore is to examine and compare the ultrastructure and histology of the defensive glands of six different species of marine pulmonates from the three different orders. The results from the order Basommatophora (Siphonariidae) are presented in Chapter 3, from the Systellommatophora (Onchidellidae) in Chapter 4 and from the Eupulmonata (Trimusculidae) in Chapter 5. Whilst this study focuses on the structure of the glands and their secretory products, as well as the organelles involved in their formation and secretion, the following hypotheses are tested:

1. As the taxa studied are from different pulmonate orders there will be differences in the gland structure that could provide phylogenetic characters.

2. It is already known that the mucus of siphonariids and onchidellids contains polypropionate metabolites, but that of the trimusculids contains labdane diterpenes, so a difference in the structure of the glands is predicted.

Because the methods used were similar for all species, a single description of these (Chapter 2) is presented. The thesis ends with a general discussion (Chapter 6) of the results and comparison of the structure of the glands of the three groups.

Chapter 2. Materials and Methods

2.1. Collection of specimens

Specimens of marine pulmonates (at least five per species) from three different orders were collected from different locations on the southern and south eastern coasts of South Africa as well as the Pacific coast of Panama (details of collecting sites are presented in Chapters 3, 4 and 5). The live animals were transported back to the laboratory dissected, fixed and processed for histology and electron microscopy.
As the preparative techniques and instrumentation used was similar for all animals, a generic description follows. Any details specific to a taxon are presented in the materials and methods section of the relevant chapter.

2.2. Scanning Electron Microscopy (SEM)

For SEM animals were collected and placed directly into a fixative solution of 2.5% glutaraldehyde in filtered sea water (4º C). After 12 hours *Siphonaria* spp. and *Trimusculus costatus* were removed from their shells and the lateral regions dissected and re-immersed in the fixative solution (volume: tissue ratio approximately 10:1) for a further 4 hours. The tissue samples were then rinsed in 0.1M sodium cacodylate buffer (pH 7.4) and dehydrated through an ethanol series (30% - 100%) and then critical point dried using a Polaron critical point dryer. The dried samples were mounted on SEM stubs using double sided graphite tape, sputter coated with gold using a Balzers Union sputtering device and viewed under a Tescan Vega scanning electron microscope (at 20kV). Digital images were captured using the Vega imaging system.

2.3. Light Microscopy/ Histology

After collection the animals were dissected and fixed in aqueous Bouin's fluid for at least 48 hours. Tissues were then dehydrated in a graded ethanol series followed by two changes of xylene (1 hour and 1.5 hours) before being embedded in Paraplast.

Sectioning (7 µm) of wax embedded material was carried out on a Leica RM 2035 microtome and the sections were stained using a number of histological stains (Table 2.1). In addition, semi-thin sections (about 1 µm thick) of transmission electron microscopy prepared material (see below) were stained with Toluidine blue for neutral and acid mucopolysaccharides. Details of precise staining protocols are given in Appendix I.

Images of sections were captured with an Olympus Camedia digital camera attached to an Olympus BX 50 microscope.

Table 2.1. List of histochemical stains used on light microscope sections. All from Bancroft and Gamble (2002) except Bromophenol Blue technique that was taken from Humason (1979).

Stain	Result
Toluidine blue	Neutral mucopolysaccharides = blue Acid mucopolysaccharides = red to violet
Alcian blue/ PAS	Acid mucins = blue Neutral mucins = magenta Nuclei = pale blue
Aldehyde fuchsin/alcian blue	Sulphated mucins = purple Carboxylated mucins = blue
Southgate's mucicarmine	Mucins = red Nuclei = blue Background = unstained
Haemotoxylin and eosin	Nuclei = blue/black Cytoplasm = varying shades of pink Muscle fibres = Deep pink/red Red blood cells = orange/red Fibrin = deep pink
Masson's trichrome	Nuclei – blue/ black Cytoplasm, muscle and erythrocytes = red Collagen = blue
Bromophenol blue	Proteins and peptides not removed by washing = blue

2.4. Transmission Electron Microscopy (TEM)

The animals were collected and some samples placed directly into a fixative solution of 2.5% glutaraldehyde in filtered sea water (4º C, volume: tissue ratio 10:1) for 12 hours. Other specimens were placed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4 º C, volume: tissue ratio 10:1) for 12 hours. After fixation small (about 1 mm^3) pieces of the lateral regions of each animal were dissected and then re-immersed in the fixative for a further 4 hours (4º C). The fixed pieces of tissue were then rinsed in 0.2 M sodium cacodylate buffer followed by secondary fixation in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer for 90 minutes.

After the secondary fixation the tissue pieces were rinsed in 0.2 M sodium cacodylate buffer and then dehydrated through a graded ethanol series (30% - 100%). The absolute ethanol was replaced with two changes of propylene oxide for 15 minutes each, followed by 25% resin: 75% propylene oxide mixture, 50% resin: 50% propylene oxide mixture and 75% resin: 25% propylene oxide mixture for 90 minutes each. The tissue was then immersed in 100% resin and left to infiltrate at 4º C overnight.

Finally the tissue was placed in 100% resin (Araldite/Taab 812 mixture; Cross, 2001) in the embedding moulds and polymerised at 60º C for 36 hours. A second batch of samples were prepared in the same way, but infiltrated and embedded with Agar Low Viscosity resin. These samples were polymerised for 24 hours at 60º C.

Semi-thin sections of approximately 1 µm in thickness were cut from the polymerised blocks using an RMC MT-7 ultramicrotome and stained for light microscopy using a

1% Toluidine blue solution. After determining the correct region of tissue using semi-thin sections stained with Toluidine blue and viewed under a light microscope, ultra thin sections (approximately 100 nm) were cut using a diamond knife. The ultra thin sections were stained for the TEM using a 5% aqueous solution of uranyl acetate for 30 minutes followed by lead citrate (Reynolds) for 5 minutes. The stained sections were then viewed in a JEOL 1210 TEM at 100kV. Digital images were captured using AnalySIS software.

Chapter 3. Structure and histology of the lateral pedal epidermis and glands of three species of *Siphonaria*

3.1. Introduction

The chemical ecology of organisms continues to attract a great deal of research. One of the main study areas in molluscan chemical ecology is that of chemical defence. Many opisthobranch and pulmonate gastropods produce mucous secretions that deter predators (Wägele *et al*., 2006). These defensive secretions can contain a variety of secondary metabolites that may be synthesised by the animal itself or derived from its diet (Simkiss, 1988; Davies and Hawkins, 1998; Davies-Coleman and Garson, 1998; Davies-Coleman, 2006; Wägele *et al*., 2006; Moore, 2006). Gastropod mucous secretions are produced from a variety of epidermal and sub-epidermal glands. Whilst the general structure of these glands is well described (for reviews see Bubel, 1984; Simkiss, 1988; Voltzow, 1994; Gosliner, 1994; Luchtel *et al*., 1997) there are relatively few studies that have correlated gland structure to defensive secretions (but see Bickell-Page, 1991; Wägele *et al*., 2006).

The Siphonariidae are a primitive family of marine basommatophoran pulmonate limpets that are particularly abundant in the intertidal regions of warm temperate to tropical rocky shores (Hodgson, 1999). The success of these limpets is probably not only due to their physiological and behavioural adaptations, but also to the ability of most species to avoid predation (Branch, 1981; Hodgson, 1999). This is believed to be achieved by secreting sticky white mucus from glands located in the dorso-lateral regions of the head/foot when irritated (de Villiers and Hodgson, 1984; Hodgson, 1999). This mucus contains polypropionate metabolites (Davies-Coleman and Garson, 1998; Cimino and Ghiselin, 2001; Darias *et al*., 2006; Davies-Coleman, 2006), some of which are biologically active and toxic to fish at levels as low as 10 µg ml^{-1} (Hochlowski *et al*., 1983). Chemical defence is very effective in some species of *Siphonaria*, and many predators that readily consume patellogastropods refuse to eat siphonariids (Branch, 1981; Branch & Cherry, 1985; McQuaid *et al*., 1999). The role of mucus in defence, however, has been questioned because some siphonariids do suffer predation (Cook, 1980; Cimino and Ghiselin, 2001; Yamamoto, 2004). A notable example of this is *Siphonaria gigas* that can be consumed by predators (Garrity and Levings, 1983; Levings and Garrity, 1984) and is exploited for food by humans, possibly because this species does not produce polypropionates (Faulkner pers. comm. cited in Ortega, 1987). *Siphonaria gigas* is found in the mid-intertidal of rocky shores from the Gulf of California to Peru (Hubendick, 1947). It is the largest species of *Siphonaria,* attaining a shell length of up to 80 mm (Levings and Garrity, 1983; Levings and Garrity, 1984). Whether it possesses epidermal glands is not known.

However, whilst the chemical nature of siphonariid secondary metabolites and their effects, as well as the effects of the mucous secretions of some species, are well established, the morphology of the structures that produce these secretions is poorly understood. Fretter and Graham (1954) noted marine pulmonates such as *Siphonaria* spp. possessed large glands on the sides of the foot and they provided an illustration (figure 8) of a longitudinal section through one such gland. A single illustration of a lateral pedal gland from *S. javanica* was also presented in the recent paper by Wägele *et al*. (2006, figure 9E). In a study of *S. hispida*, Marcus and Marcus (1960) also noted the presence of such lateral pedal (or marginal) glands and suggested that they had a repugnatorial function. The aim of this study, therefore, was to provide a more detailed description of the putative defensive glands and compare their structures in different species of *Siphonaria,* focussing on two species that are known to be avoided by predators namely *S. capensis* (Quoy and Gaimard, 1833), and *S. serrata* (Fischer, 1807) and one that can be consumed *S. gigas* (Sowerby, 1825).

3.2. Materials and methods

Three species of marine Siphonariidae were collected for this study. Two species from the east coast of South Africa and one species from the Pacific coast of Panama. *Siphonaria capensis* and *S. serrata* were collected from rocks in the intertidal zone at Kenton-on-sea (33º 37’ 12” S; 26º 52’ 32” E), Eastern Cape Province, South Africa. *Siphonaria gigas* was collected from the intertidal rocks of Naos island (adjacent to the Smithsonian Tropical Research Institute), Panama City, Panama (9º 04’ 48” N; 79º 40’ 48” W). A minimum of five individuals per species were collected. The live animals were transported back to a laboratory, dissected and small portions of the outer lateral pedal tissue fixed for light and electron microscopy. The methodology of preparation is presented in Chapter 2.

3.3. Results

3.3.1. General observations and scanning electron microscopy

Numerous white glands, visible to the naked eye, are embedded in the translucent lateral body wall in *Siphonaria capensis* and *S. serrata* (Fig. 3.1A). These glands are distributed around the entire animal. By contrast the lateral pedal area of *S. gigas* is black in colour, and glands are not visible. Scanning electron microscopy of the lateral regions of the foot of all species, however, reveals the presence of pores that are the openings of glands. There was a significant difference (t-test, P = <0.001) in the density of pores between *S. capensis* and *S. serrata*. In *S. capensis* the mean pore density was about 74/ mm^2 whereas in *S. serrata* it was only 57/ mm^2 (Table 3.1). The mean pore diameter was similar in both species, about 29 and 23 µm in *S. capensis* and *S. serrata* respectively (Fig. 3.1B; Table 3.1). In *S. gigas* two sizes of pores are visible, the first having a diameter of 10 µm and the second about 1 µm (Fig. 3.1C). The density of the larger pores was about 34 / mm^2 (Table 3.1).

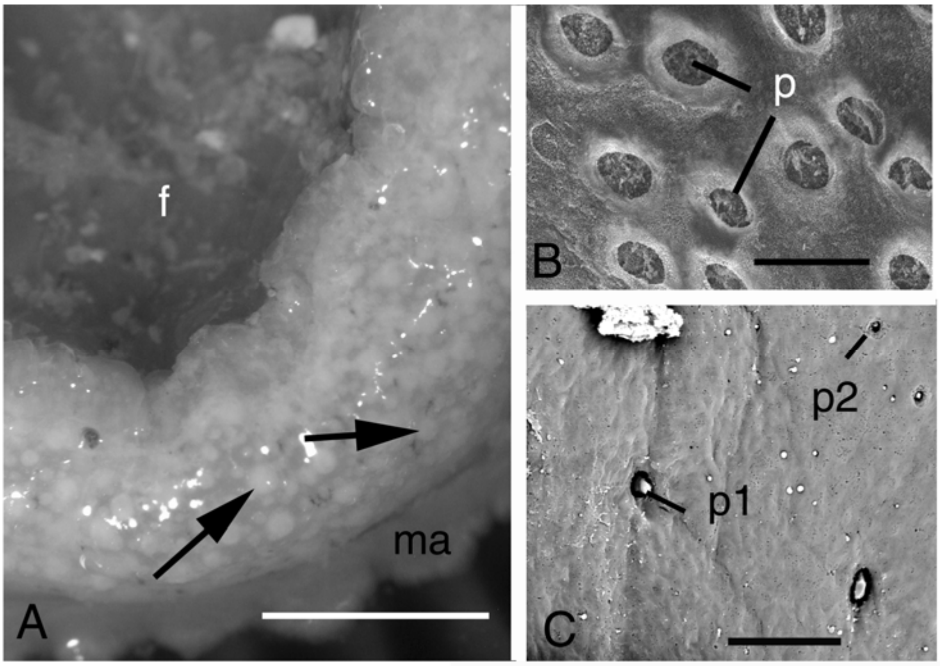

Figure 3.1.A. Ventral view of part of the foot of *Siphonaria capensis* showing position of lateral pedal glands. **B**. Scanning electron micrograph of the lateral pedal region of *S. serrata* showing pore openings to glands. **C**. Scanning electron micrograph of the lateral pedal region of *S. gigas* showing opening to two sizes of pore (p1 and p2). f = foot; ma = mantle; p1 and p2 = pore.

Scale bars: A = 0.5 mm; B and C = 50 µm.

Table 3.1. Dimensions of the multi-cellular sub-epithelial glands, gland openings (pores) and pore density. Length and width dimensions are maximum values observed from longitudinal sections.

Species	Length (μm)	Width (μm)	Area (μm^2)	Pore diameter (μm)	Pore density (number/mm^2)
S. serrata	210	122	14134	23.1 ± 3.6	59.5 ± 10.8
S. capensis	280	157	31173	29.3 ± 4.2	78.4 ± 10.8
S. gigas	312	82	15257	10.1 ± 2.9	33.4 ± 10.5

Table 3.2. Histochemical results from the dorso-lateral pedal glands of three species of *Siphonaria*.

Species	Cell type	Mucopolysaccharides	Neutral mucins	Acid mucins	Sulphated mucins	Carboxylated mucins	Protein
S. capensis	I	+	+	-	+	-	-
	II	++	-	++	++	-	-
S. serrata	I	+	+	-	+	-	-
	II	++	-	++	++	-	-
S. gigas		++	++	-	++	-	-

3.3.2. Light microscopy

***Siphonaria capensis* and *S. serrata*.**

Examination under the light microscope, using Toluidine blue, showed *Siphonaria capensis* and *S. serrata* have an epidermis and glands that are very similar in structure. The glands also have a similar distribution in that they are found all around the head and foot. The epidermis consists of a single layer of cuboidal epithelial cells that sit on a basal lamina (Figure 3.2A, B). These epithelial cells contain a large nucleus situated towards the base of the cell and have microvilli on their external surface. Numerous small goblet cells are present between the epithelial cells (Figure 3.2E). The goblet cells have a flattened nucleus at the base of the cell and most of the cell is made up of mucus that stains violet with toluidine blue, indicating the production of acid mucopolysaccharides.

In both *S. capensis* and *S. serrata* large multi-cellular sub-epithelial glands are present in the lateral pedal regions. The glands (about 280 µm long x 160 µm diameter in *S. capensis* and 210 µm long x 121 µm diameter in *S. serrata*, Table 3.1) are surrounded by a muscle layer and open to the epidermal surface via a small duct and pore between the epithelial cells (Figure 3.2A, B). Each gland consists of numerous product containing pear-shaped cells that surround a central lumen. The contents of the gland cells stain differentially indicating the presence of more than one secretary product and two types of secretory cell. Cell type I stains weakly for neutral and sulphated mucins whereas type II stains strongly for acidic and sulphated mucins (Table 3.2; Figure 3.2C and D).

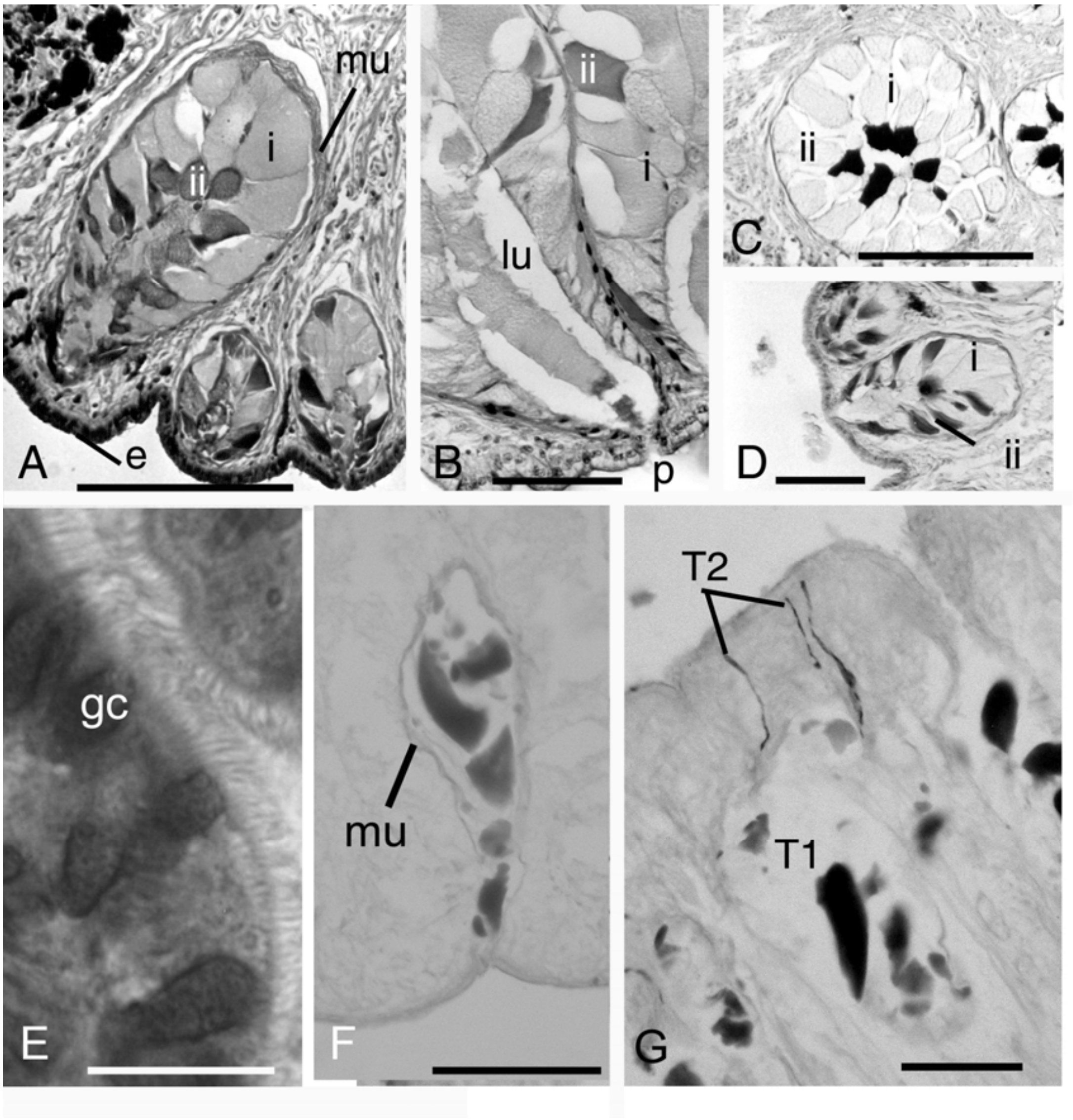

Figure 3.2. A. Longitudinal section through a *Siphonaria capensis* lateral pedal gland stained in Mallory's trichrome, showing two types of secretory cell (i and ii) and gland surrounded by smooth muscle. **B**. Higher magnification of longitudinal section through a *S. capensis* gland (stained in Mallory's trichrome) showing central lumen and pore, as well as two types of secretory cell (i and ii). **C** and **D**. Transverse and longitudinal sections of glands of *S. capensis* stained in alcian blue (C, pH 1 and D, pH 2.5). **E**. Light microscope section of the epidermis and goblet cells of *S. capensis* stained with toluidine blue. **F**. Longitudinal section through a Type 1 gland (multi-cellular) of *S. gigas*; section stained in aldehyde fuschin/alcian blue. **G**. Section through the lateral pedal region of *S. gigas* showing both type 1 (T1) and type 2 (T2) glands. Section stained in aldehyde fuschin/alcian blue. e = epithelium; gc = goblet cell; lu = lumen; mu; muscle layer; p = pore; i = type I gland cell; ii = type II gland cell.

Scale bars: A = 50 mm, B and C = 50 µm, D = 20 µm, E = 20 µm, F and G = 100 µm.

Siphonaria gigas.

The epithelium consists of a single layer of columnar epithelial cells (approximately 8 µm long x 3 µm in diameter) with a large, well developed nucleus seated on a basal lamina (Figure 3.2G). The external surface is fringed with microvilli. In between the epithelial cells are goblet cells (not illustrated as similar to those of S. capensis, Figure 3.2F) filled with an amorphous substance, thought to be mucous, that is secreted directly onto the epithelium.

Two types of sub-epithelial gland are present in the lateral pedal regions of *S. gigas*. The first type are large multi-cellular sub-epithelial glands (about 310 µm long x 82 µm diameter) with a central lumen, opening to the outside via a small duct (Figure 3.2F). Unlike those of *S. capensis* and *S. serrata* the multi-cellular glands are not surrounded by a well-developed muscle layer. Futhermore each gland possesses one type of secretory cell only that stains for sulphated and neutral mucins only (Table 3.2). The second type of gland is unicellular, long and narrow, containing a secretory product that also stains for sulphated and neutral mucins (Figure 3.2G; Table 3.2).

3.3.3. Transmission electron microscopy

***Siphonaria capensis* and *S. serrata*.**

As the ultrastructure of the epidermal layer and the lateral pedal glands of *Siphonaria capensis* and *S. serrata* are very similar, a single description only follows.

The epithelial layer is made up of a single layer of cuboidal epithelial cells situated on a basal lamina and attaches to it by hemidesmosomes (Figure 3.3A and E). The epithelial cells contain a basal nucleus (about 4 µm in diameter) and microvilli (about

3.5 µm long) on the external surface. Their cytoplasm contains numerous small mitochondria, Golgi bodies and small lipid droplets (Figure 3.3F). The intra-epithelial goblet cells have a flattened nucleus and a few other organelles (mitochondria, Golgi bodies and lipid droplets) near the base of the cell, the rest of the volume of the cell is filled with an amorphous substance thought to be mucus (Figure 3.3A). Adjacent to some goblet cells are ciliated epithelial cells, although the goblet cells themselves do not appear ciliated (Figure 3.3C and D). These ciliated cells are elongated and have an elongated nucleus. Cells containing pigment granules are found between the cuboidal epithelial cells (Figure 3.3C).

Transmission electron microscopy confirmed that the multi-cellular glands are surrounded by a band of smooth muscle and collagen about 4.8 µm thick (Figures 3.4A and 3.5C). The muscle fibres are orientated in longitudinal and circular directions around each gland. The two secretory cell types (type I and II) are clearly distinguished by the appearance of their content (Figures 3.4A and 3.5A). The majority of the volume of type I cells (which can be up to 36 µm long by 12 µm diameter) is occupied by a relatively electron-lucent substance with a reticulated appearance (Figure 3.4A and B) which is presumed to be mucus. The cytoplasm and the irregularly shaped nucleus are confined mainly to the base of the cell (Figure 3.4A and C). In some type I cells the basal cytoplasm contains numerous mitochondria, rough endoplasmic reticulum, well-developed Golgi bodies, glycogen and accumulating secretory product (Figure 3.4C). Numerous vesicles (50 – 55 nm in diameter) can be seen associated with the Golgi cisternae (Figure 3.4C). Presumably these vesicles contain the mucus produced by these cells. In type I cells that are swollen with product the base contains large amounts of glycogen and very few other organelles (Figure 3.4D). The apical regions of the cell membranes of type I cells do not bear microvilli.

The secretory product of mature type II cells is more electron-dense, vesicular in appearance with a granular-like content (Figure 3.5A, B and C). The irregularly shaped nucleus and cytoplasm, which contains a few well developed Golgi bodies, is restricted to the base of these cells (Figure 3.5B, C and D). Numerous vesicles are associated with the end of the Golgi cisternae and some appear to be fusing with the accumulating cell product (Figure 3.5D). Apically the cell membrane does not have any microvilli.

Each gland opens to the outside via a pore lined by non-ciliated cuboidal epithelial cells (Figure 3.6A). The lumen of nearly all glands sectioned contained products from both types of secretory cell (Figure 3.6B, C), the product from type II cells being the more electron-dense. These secretory products appear to be released into the lumen of the gland by holocrine secretion, and in the case of type II cells, initially as vesicles (Figure 3.6D). In addition to the two secretory cell types, non-secretory support cells are present in the glands. These cells are joined to adjacent support cells and gland cells by desmosomes (Figure 3.6F). The support cells are irregular in shape, have long (up to 2.5 µm) apical microvilli, and partially line the lumen of the gland (Figure 3.6A, B and F). Thin strands of cytoplasm from these cells penetrate between the gland cells (Figure 3. 4A and 3.6E). The cytoplasm of these cells often contains large amounts of glycogen and lipid droplets (Figure 3.6F).

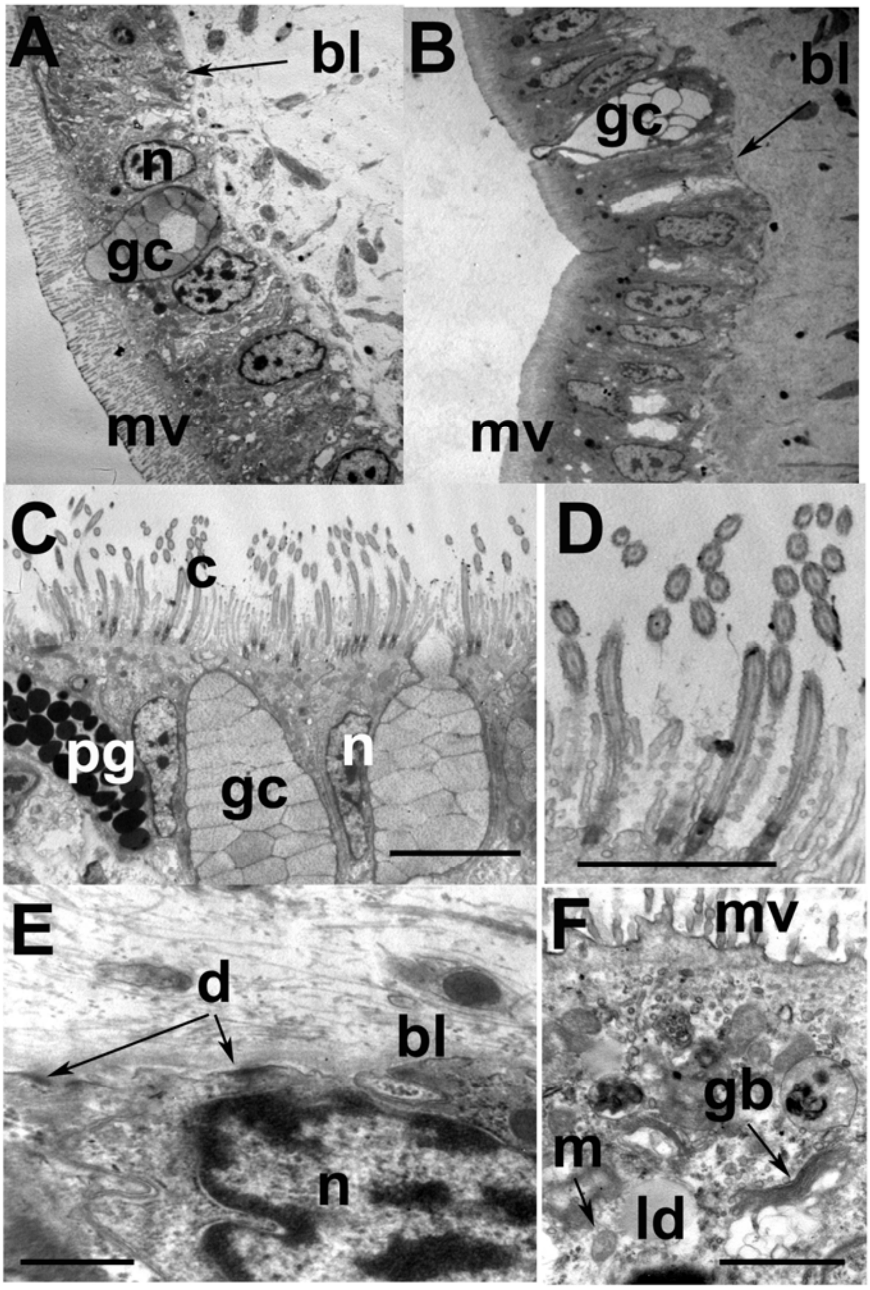

Figure 3.3. A. Transmission electron microscope image of the epithelial layer of *Siphonaria capensis* showing the epithelial cells and their nuclei (n), the goblet cells (gc) and the microvilli (mv). **B**. A TEM image of the epithelial layer of *Siphonaria gigas* showing the columnar epithelial cells with their nuclei (n), goblet cells (gc) and the microvilli border (mv). **C**. A higher magnification of the goblet cells (gc), the associated ciliated cells, with their nuclei (n) and cilia (c) and a cell containing pigment granules (pg). **D**. A high magnification TEM image of the cilia . **E**. A high magnification TEM of the base of an epidermal cell showing the basal lamina (bl) and hemi-desmosomes (d). **F**. A high magnification TEM image of the apical region of an epidermal cell showing the microvilli (mv), mitochondria (m), lipid droplets (ld) and Golgi body (gb). (**C** – **F** *S.capensis*).

Scale bars: A = 5 µm, B = 10 µm, C = 5 µm, D = 2 µm, E = 0.5 µm and F = 10 µm.

Siphonaria gigas.

The epidermal layer is comprised of columnar epithethial cells (about 14.2 µm long x 2.6 µm wide) on a basal lamina. These epithelial cells contain a well-developed nucleus (about 7.1 µm in length and 2.3 µm in width) mitochondria, Golgi bodies and numerous vesicles, the apical surface is covered with microvilli (approximately 1.7 µm in length). The intra-epithelial goblet cells have a flattened nucleus at the base and are filled with mucus. The adjacent epithelial cells do not possess cilia (Figure 3.3B).

The large multi-cellular glands (type 1) of *S. gigas* are surrounded by a few smooth muscle myofibrils and collagen fibres only (Figure 3.7A and B). In addition the surrounding connective tissue contains elongate cells that contain large numbers of electron-dense vesicles (Figure 3.7C) that are presumed to be pigment granules. Only one type of secretory cell could be identified in the multi-cellular glands. Basally this cell type has an irregularly shaped nucleus (Figure 3.7B) and some cytoplasm containing small mitochondria, a few well-developed Golgi bodies and smooth endoplasmic reticulum (not illustrated). Most of the volume of these cells is occupied by large electron-dense vesicles embedded in an electron-lucent matrix (Figure 3.7A, B and D). The content of the cells is released into the lumen of the duct by holocrine secretion (Figure 3.7D). In addition to the secretory cells, irregularly shaped support cells lie between the gland cells (Figure 3.7D). The support cells have well developed, elongate, apical microvilli and a cytoplasm containing and irregularly shaped nucleus, multi-vesicular-bodies, small vesicles, and rough endoplasmic reticulum (not illustrated).

Type 2 glands are comprised of a basal region containing an irregularly shaped nucleus, a few organelles and granular secretory vesicles (Figure 3.7C) and a long, tubular ‘neck’ about 3 μm diameter that extends to the outside (Figure 3.7E). The tubular neck also contains an electron-dense granular product (Figure 3.7F). The neck of the gland penetrates between the columnar epithelial cells (Figure 3.7E) and opens to the outside via a small pore.

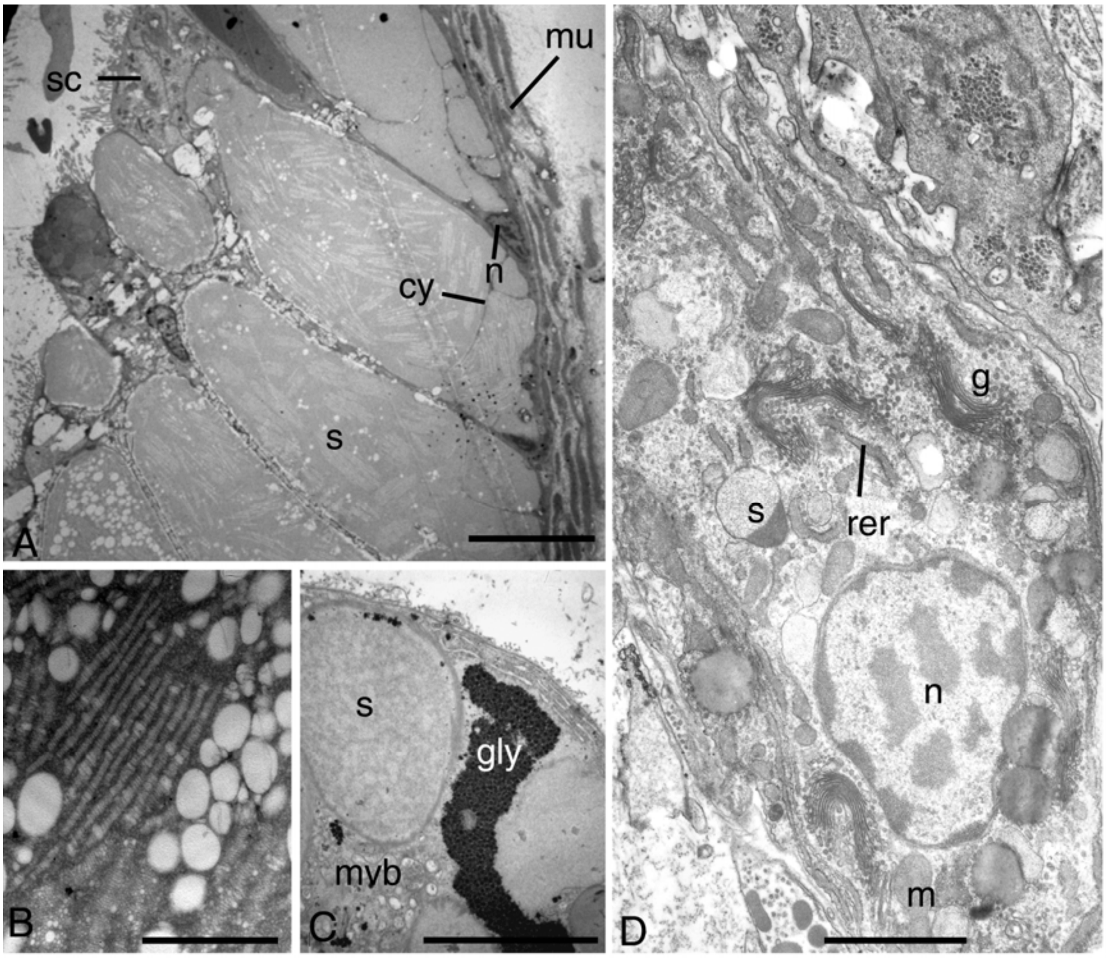

Figure 3.4. Transmission electron microscope images of *S. capensis* type I secretory cells.

A. Section through gland showing several type I secretory cells (s) and support cells (sc) surrounded by a muscle/collagen layer (mu). **B**. Higher magnification of secretory product of a type I cell. **C**. Section through the base of a type I secretory cell showing secretory product (s), glycogen (gly) and a multi-vesicular body (mvb). **D**. Section through a developing type I cell showing cytoplasm containing numerous Golgi bodies (g) surrounded by vesicles, rough endoplasmic reticulum (rer), mitochondria (m) and developing secretory product (s). cy = cytoplasm; n, nucleus.

Scale bars: A = 10 µm, B = 0.1 µm, C = 2 µm and D = 3 µm.

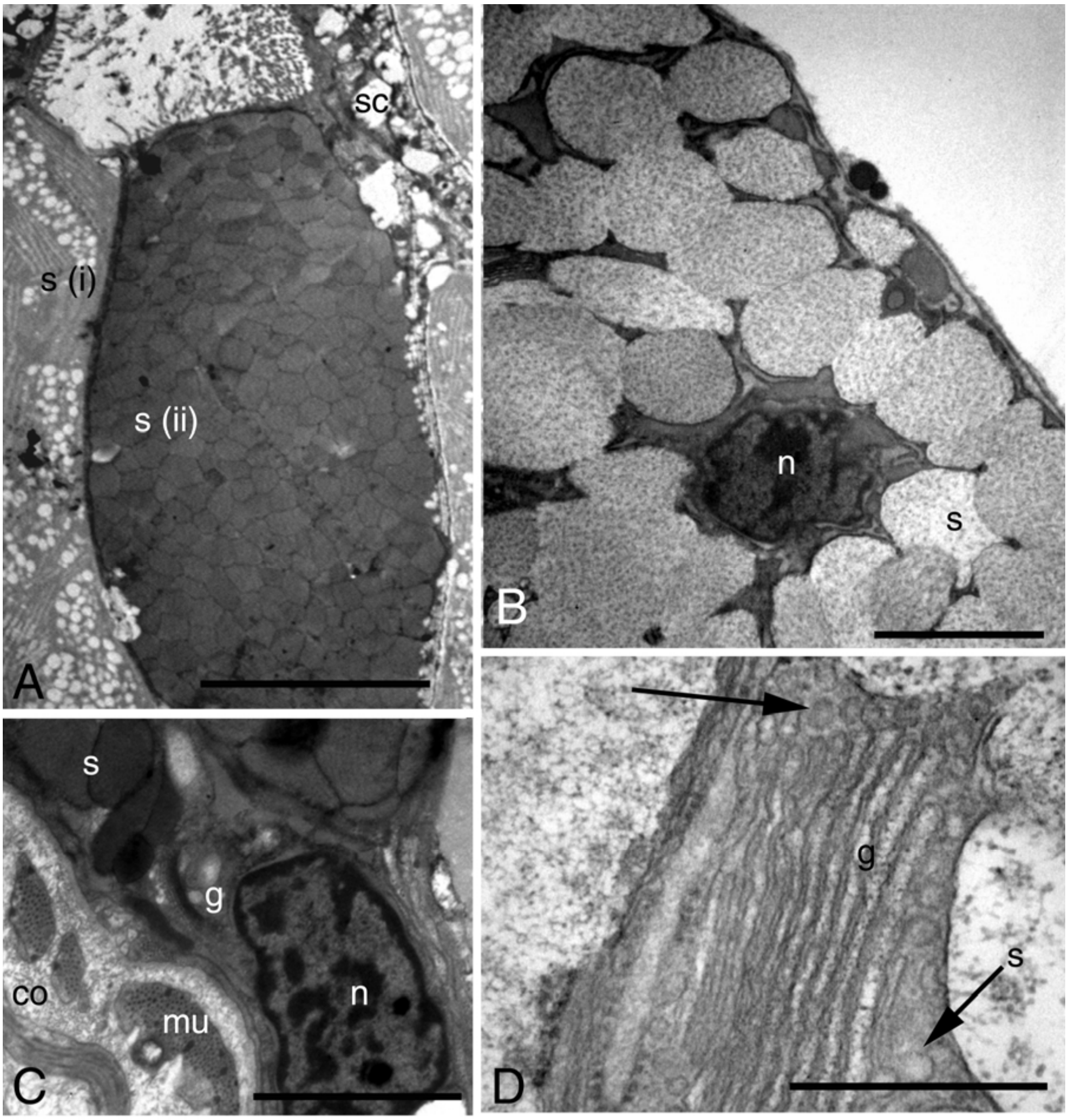

Figure 3.5. Transmission electron microscope images of type I secretory cells from *Siphonaria serrata*.
A. Longitudinal section of apical region of type I cell (s (ii)) with a type I cell (s (i)) adjacent to it. **B**. Section through the basal region of a type II cell showing nucleus (n) surrounded by secretory product (s). **C**. Section through basal region of type II cell showing nucleus (n), cytoplasm with a small Golgi body (g) and secretory product (s). **D**. Higher magnification of a Golgi body (g) with peripheral vesicles (arrow). Co, collagen; mu = muscle layer; s, secretory product; sc, support cell.

Scale bars: A = 10 μm, B = 5 μm, C = 1 μm and D = 0.5 μm.

3.4. Discussion

The lateral pedal epidermis of all three species of *Siphonaria* mainly consists of a single layer of either cuboidal (*S. capensis* and *S. serrata*) or columnar (*S. gigas*) epithelial cells that sit on a basal lamina. This is typical of the epidermis of most molluscs (Bubel, 1984). In addition all three species of *Siphonaria* possesses inter-epithelial goblet cells between the epithelial cells. These single goblet cells undoubtedly produce the characteristic mucous secretion that covers the epidermis and this is thought to play a role in the cleaning of the microvilli and facilitating ionic exchange (Bubel, 1984). This mucus which contains acidic mucopolysaccharides may also play a role in driving harmful microbes away from the body.

The lateral pedal region of all three species of *Siphonaria* studied possesses large multi-cellular glands. Although the glands of *S. capensis* and *S. serrata* differ in size and abundance, they are structurally and histochemically similar. Their structure is also similar to that presented diagrammatically by Fretter (1954, figure 8), being composed of numerous secretory cells and support cells that surround a central lumen, with a capsule of muscle surrounding the entire gland. This type of gland was categorized by Wägele *et al.* (2006) as a mantle dermal formation. Fretter's diagram clearly indicates the possibility of two types of secretory cell within the gland (although both types were simply labelled as 'gland cells'), and this has been confirmed by this study. One type produces a secretion that stains weakly for neutral and sulphated mucins, and the second type a product that stains positively for acidic mucins. The latter type of mucin is a common feature of molluscan mucoid secretions (Wägele *et al.*, 2006). These two products are presumably mixed in the lumen of the gland during exudation.

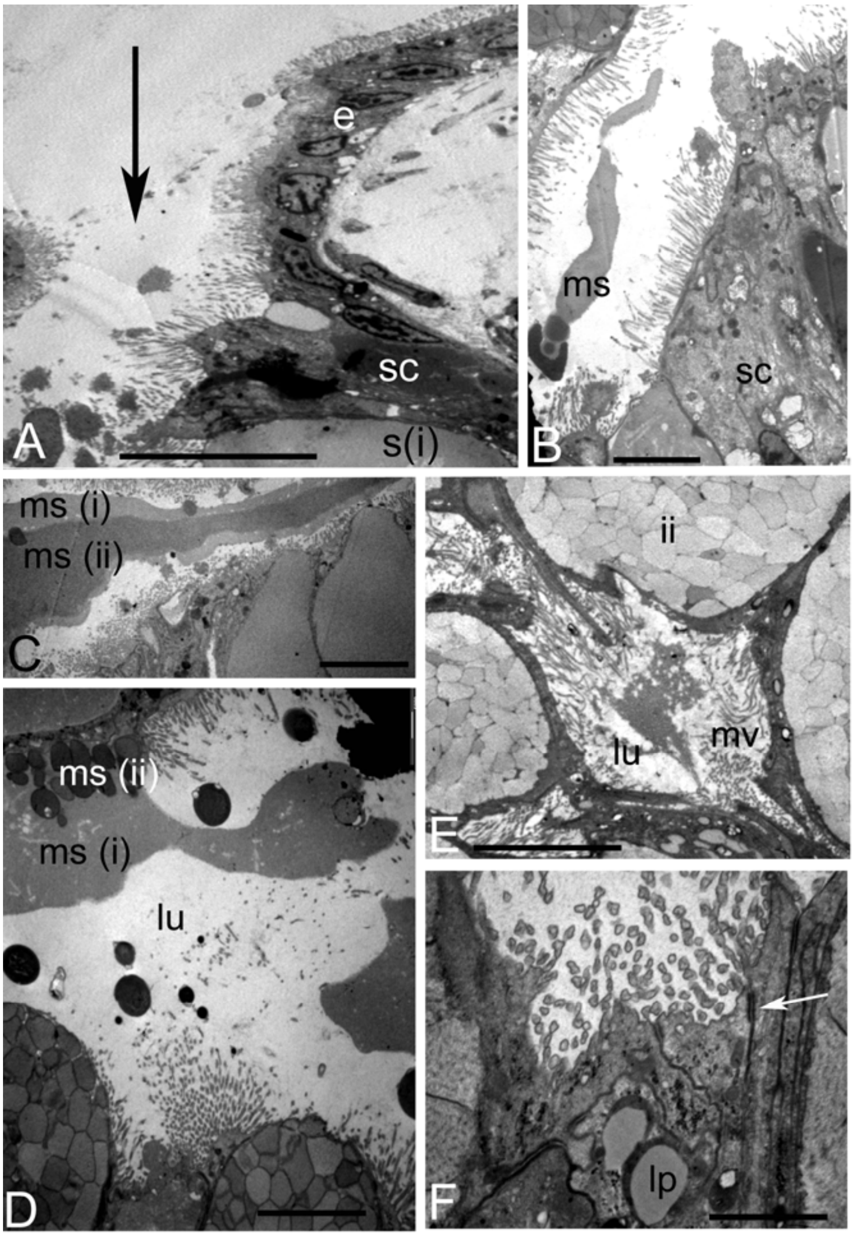

Figure 3.6. **A**. TEM section through the pore region (arrow) of a multi-cellular gland of *Siphonaria capensis* showing the epithelium (e), a support cell (cs) and Type I secretory cell (s(i)). **B**. Longitudinal section though the lumen showing mucoid secretion (ms) in lumen and apical region of a support cell (sc). **C**. Lumen with secretory product from type I (ms (i)) and type II (ms (ii)) secretory cells. **D**. Transverse section through the lumen of a gland showing secretory product from type I cells (ms (i)) and secretory vesicles from type II glands (ms (ii)). **E**. Transverse section through the lumen of a gland showing several type II secretory cells (ii) separated by support cells with well developed microvilli (mv). **F**. Higher magnification of the apical region of support cells joined by desmosomes (one arrowed). Note the lipid droplets (lp) in the cytoplasm.

Scale bars: A = 10 μm, B = 5 μm, C = 10 μm, D = 5 μm, E = 10 μm and F = 2 μm.

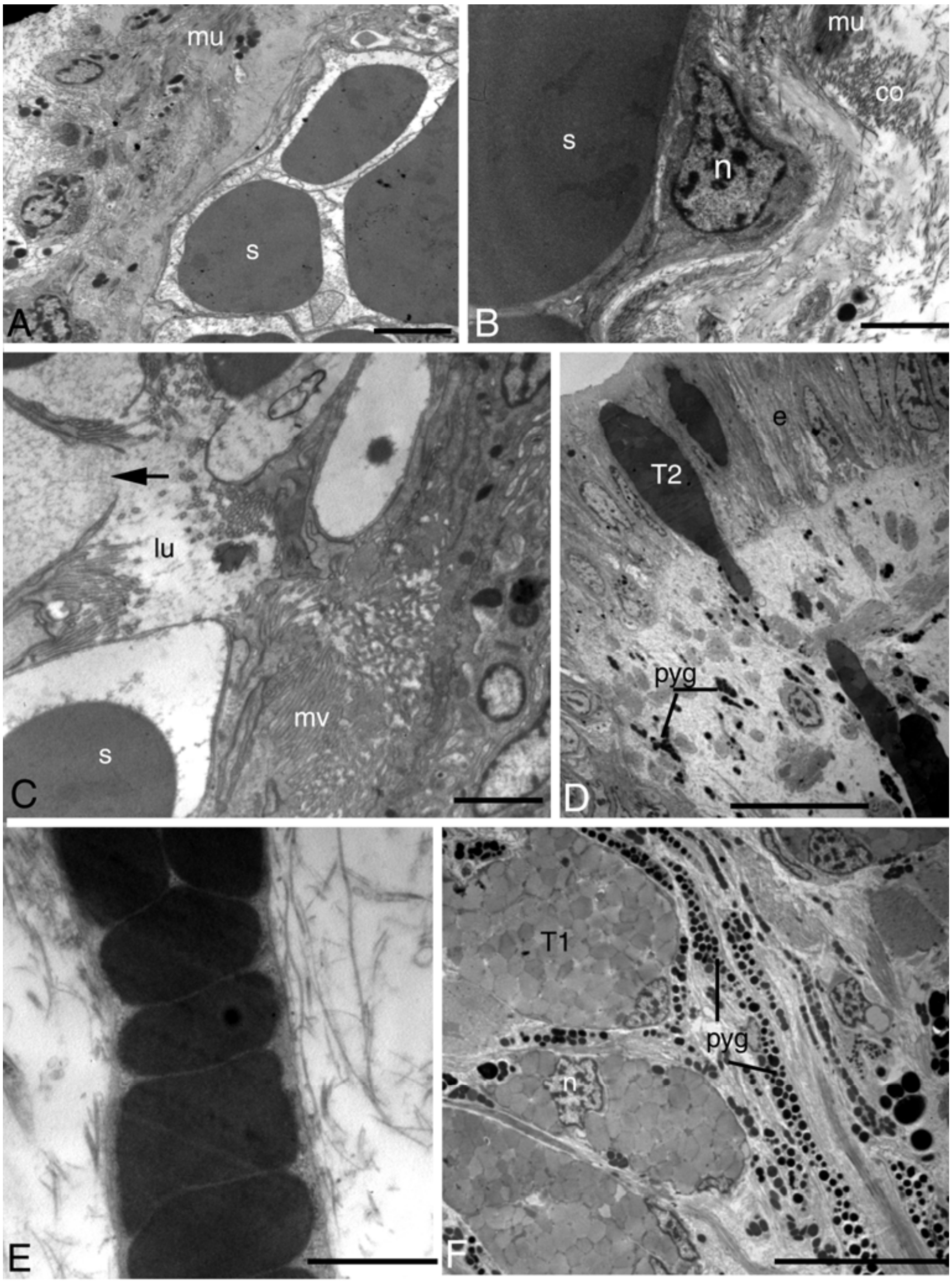

Figure 3.7. Transmission electron microscope images of the glands of *Siphonaria gigas*. **A**. Section through part of a multicellular gland (type 1) showing secretory vesicles (s). **B**. Section through the basal region of a type 1 gland showing nucleus (n) in peripheral cytoplasm. **C**. Transverse section though part of the lumen of a type 1 gland. Note the release of secretory product into the gland (arrow) and elongate microvilli (mv) of the support cells. **D**. Longitudinal section through parts of a type 2 (tubular, T2) gland part of which runs between the columnar epithelium (e). **E**. Higher magnification of the secretory vesicles of type 2 gland. **F**. Transverse sections through the basal regions of several type 1 (T1) glands. co = collagen; lu, lumen; mu = muscle layer; n = nucleus; pyg = pigment granules; secretory product.

Scale bars: A = 5 μm, B = 2 μm, C = 2 μm, D = 10 μm, E = 1 μm and F = 10 μm.

The secretory product of the gland cells in all species did not stain for proteins. This suggests that they are either not produced in the defensive gland secretions, or are in amounts too low to be detected using a simple histochemical stain. The paucity of rough endoplasmic reticulum in all the secretory cells suggests that they produce little protein. This is unlike the adhesive mucous secretions of gastropods that can have a significant protein content (*e.g*. Pawlicki *et al*., 2004; Werneke *et al*., 2007; Li and Graham, 2007; Smith *et al*., 2009). These proteins play a central role in adhesion and cause stiffening of the mucus. Detailed biochemical analysis of *Siphonaria* defensive mucus is now required to determine whether proteins are present.

Whilst *S. gigas* also has multi-cellular lateral pedal glands, they are smaller, less abundant and differ in structure to those of *S. capensis* and *S. serrata*. *Siphonaria gigas* glands possess one type of secretory cell only whose product does not contain acidic mucins. Morphologically these glands have a greater resemblance to the sub-epithelial gland cells of the opisthobranchs *Cadlina luteomarginata* and *C. laevis* described by Wägele *et al*. (2006) and it is interesting to note that the molecular phylogeny of Grande *et al.* (2004) placed *Siphonaria* within the opisthobranchs. Whether the structurally simpler multicellular glands of *S. gigas* are more primitive than those of *S. capensis* and *S. serrata*, or vestigial, remains to be determined. A phylogeny of the siphonariids onto which gland structure is mapped may help in this regard. In addition to multi-cellular glands, *S. gigas* also possessed unicellular sub-epidermal glands, similar in structure to the tubular glands of other opisthobranchs and pulmonates (Storch and Welsch, 1972; Yamaguchi *et al*., 2000).

The multi-cellular glands in all species studied were encapsulated by smooth muscle, although this was not well developed in *S. gigas*. A similar arrangement has been described for the dermal glands of several other gastropods (Bubel, 1984; Wägele *et al.*, 2006). The multi-cellular glands of the opisthobranch *Melibe leonia* are also surrounded by muscle, but in this species the glands are encased by striated muscle (Bickell-Page, 1991). Whatever the type of muscle, synchronised contraction of the muscle capsule must be responsible for squeezing secretions out of the glands. The lack of a well organised muscle layer around *S. gigas* glands may mean that they are not able to project their glandular secretions in the same way as *S. capensis* and *S. serrata*. In *Siphonaria* spp. the trigger for the discharge of the secretions is probably mechanical, although ciliated sensory cells such as those described in association with the repugnatorial glands of *Melibe leonia* (Bickell-Page, 1991) were not found in the siphonariids studied.

The abundance of the lateral pedal glands in *Siphonaria* suggest that they play an important role in their biology. In *S. capensis* and *S. serrata* they undoubtedly contribute towards defence. The secretory product is thick and sticky, contains both acidic and sulphated mucopolysaccharides, is bitter to the taste (A.Hodgson pers. obs.) and repels predators (Hodgson, 1999; McQuaid *et al.*, 1999). Whether the secretory exudate contains polypropionate metabolites, however, is not known. Furthermore their location at a cellular level is also equivocal. This is because all chemical extractions to date have been from entire animals, and not from specific glands or their secretions. Ireland and Faulkner (1978) however did extract polypropionates from the mucous secretions of the pulmonate *Onchidella binneyi,* and it is likely that the exudate from the glands of siphonariids contains such chemicals. By contrast the lateral pedal glands of *S. gigas* clearly less effective as some predators are not deterred and this species is consumed by fish and humans (Garrity and Levings, 1983; Ortega, 1987). Defence against predators in *S. gigas* is probably a combination of its size, extreme tenacity and habitat. *S. gigas* is more common on irregular substrata in wave-exposed areas where predators find it more difficult to feed (Hodgson, 1999). In addition *S. gigas* creates well developed home scars that have been shown to be a defence against fish predators (Garrity and Levings, 1983).

Finally, the gland secretions of siphonariids may also play some role in protecting them from desiccation, microbial attack, or the attachment of ectocommensals. Molluscan mucus is known for its anti-bacterial properties. Hochlowski and Faulkner, (1983, 1984) and Yamaguchi *et al*, (2000) have shown that the metabolites from siphonariids act as antibiotics. Whilst the epidermis of many patellogastropods is the site of attachment of ectocommensals such as peritrich ciliates (Hodgson *et al.*, 1985), no such unicellular organsisms have been found attached to the epidermis of South African siphonariids (J. van As, pers. comm.).

Chapter 4. Structure and histology of the lateral pedal epidermis and glands of two species of *Onchidella*

4.1. Introduction

The Onchidioidea, of the suborder Systellomatophora, is a superfamily of mainly marine intertidal, slug-like pulmonates (Britton, 1983). There has been debate as to whether they should be classified as pulmonates or opisthobranchs (Marcus and Marcus, 1954; Jensen, 1992; Dayrat, 2009), although it is generally accepted that both pulmonates and opisthobranchs arose from closely related prosobranch groups (Stringer, 1968; Barker, 2001). Current phylogenies place the Onchidioidea within the Pulmonata (Mordan and Wade, 2008). One family the Onchidiidae, with nineteen genera, one of which is *Onchidella*, is currently recognised within the Onchidioidea (Dayrat, 2009). The systematics of this taxon is rather confused (Dayrat, 2009).

Members of the Onchidiidae are usually 10-70 mm long, oval in shape with a broad, large foot and a papillate or tuberculate notum. Anteriorly they have a retractable pair of tentacles, each bearing a terminal eye (Smith and Stanisic, 1998). The notum is covered by a cuticle that may also contain siliceous spicules or branchial plumes (Marcus, 1979; Smith and Stanisic, 1998). The large foot is flanked by the hyponotum. Onchidiids live amphibiously in sheltered areas in the eulittoral zone and in estuaries (Arey and Crozier, 1921; Watson, 1925; Fretter, 1943; Marcus and Marcus, 1956; Weiss and Wägele, 1998; Dayrat, 2009). They are able to breathe air during low tide when the water recedes and feed on the organic film of algae, diatoms and bacteria on the surface of the substratum. They avoid direct sunlight and prefer to forage at low tide when the sky is overcast (Weiss and Wägele, 1998). The Onchidiidae are chiefly found in the tropics and subtropics of the Pacific and Indian oceans (Kenny and Smith, 1987), although a few species extend into the temperate regions of the Northern and Southern hemispheres (Stringer, 1968; Marcus, 1979). Although the Onchidiidae are common in some habitats, little is known about their biology and ecology.

The genus *Onchidella* is one of the most speciose, with about 18 species (Weiss and Wägele, 1998). Species of *Onchidella* are medium to large slug-like, shell-less marine molluscs (Kay *et al*., 1998) that are usually oval in shape. The mantle cavity is reduced or absent, and the dorsal mantle forms a leathery, dorsally arched notum which covers the head and sides of the body, and extends into the hyponotum on the ventral surface. The notum is either smooth or covered with papillae or tubercles that may contain accessory eyes or branchial plumes (Watson, 1925; Fretter, 1954; Kay *et al*., 1998).

Like other shell-less molluscs, the onchidellids are potentially vulnerable to attack by predators. They appear to avoid predation, however, by producing secretions from glands in the mantle edge when irritated. These glands were referred to as perinotal glands in earlier work (Watson, 1925; Marcus, 1979). Young *et al.* (1986) have shown that the secretion from the perinotal or repugnatorial glands of *Onchidella borealis* repels intertidal predatory asteroids, and whilst intertidal crabs will eat dead *O. borealis*, they do not consume live ones that are capable of releasing their defensive secretion. Chemical analysis of secretions from six species of the genus *Onchidium* have revealed that they contain largely isomeric polypropionates (see Darias, *et al*. 2006 for review), and it is likely that these are the compounds that deter predators. Furthermore, *Onchidella* probably also has these chemicals. Relatively little is known about the structure and histology of the glands of *Onchidella.*

Work to date on gland structure has mainly been at the light microscope level using routine histological stains. Joyeux-Laffuie (1882) was the first to describe epidermal glands in his study of *O. celtica*. Von Wissel (1898) described similar glands in *O. marginata*. Watson (1925) used light microscopy to describe the structure and histology of the epidermis and the marginal glands *Onchidella pulchella* and *O. capensis,* and Arey (1937) and Arey and Barrick (1942) described the structure of the repugnatorial glands of *Onchidium floridanum. Onchidium floridanum* has ten to fourteen of these glands along the edge of the mantle. They are situated in erectile papillae which are not obvious in the undisturbed animal but are readily distinguished when the animal is disturbed. In response to stimulus (physical, chemical and electrical) the glands were found to discharge simultaneously or singly. The secretion from these glands is forcibly expelled in a stream of milky fluid (0.2 mm in diameter and reaching a distance of 15 cm) which is acidic and burning to the taste (Arey and Barrick, 1942). Arey and Barrick (1942) also described the marginal pedal glands as having seven different types of secretory cells. Binot (1965) used light microscopy and histological staining to study the glands of *O. celtica* and described its glands as having five different types of secretory cells.

Young *et al*. (1986) established that in *O. borealis* the number of repugnatorial glands increases with the size of the animal, large individuals having more than twenty. The glands were described as many flask shaped secretory cells surrounding a lumen and covered by a thick muscular sheath, embedded in connective tissue. The lumen of each multi-cellular gland opens to the outside on a distinct, individual marginal papilla (Young *et al.*, 1986). More recently, Weiss and Wägele (1998) used light and scanning electron microscopy to describe the morphology, anatomy and histology of three species of onchidellid (*O. celtica, O. indolens* and *O. borealis*), with emphasis on intraspecific and interspecific variability and to look for new characters to assess the systematic position of the Onchidiidae.

Therefore, morphological details at the ultrastructural level of these important defensive structures are lacking. The aim of this study was to examine and describe the structure of the repugnatorial glands of onchidellids using both light and electron microscopy. Two species were available for study: *Onchidella capensis* and *O. hildae*.

4.2. Materials and methods

Onchidella capensis (Watson, 1925) was collected from underneath rocks in the intertidal zone at Kommetjie on the Cape Peninsula (34° 8’ 25” S, 18° 19’ 45” E) South Africa, and a second species, *Onchidella hildae* (Hoffman, 1928), was collected by A.N.Hodgson (Rhodes University) from Naos Island on the Pacific coast of Panama (9° 04’ 48” N, 79° 40’ 48” W).

The live animals (a minimum of 5 individuals of each species) were transported back to the laboratory and tissue from the perinotal regions of the epidermis of both species was prepared for light microscopy and histology, scanning electron microscopy and transmission electron microscopy using the same techniques described in Chapter 2.

4.3. Results

4.3.1. General observations and scanning electron microscopy

Onchidella capensis was described by Watson (1925) as having about 12 marginal glands on either side of the body. These glands are visible to the naked eye as slightly raised papillae that are lighter in colour and they appear to be smaller towards the head region and larger along the sides of the animal (Figure 4.1). The glands are not particularly obvious in an unthreatened animal, but when irritated the edge of the notum is raised and the papillae become more pronounced and conical in shape (Watson, 1925). There are several other types of glandular structure in *O. capensis*, but for the purpose of this study only the large multi-cellular glands are described.

Under the scanning electron microscope the glands are visible along the edge of the notum (Figure 4.2A). The gland pores (approximately 15 µm in diameter) are situated on raised papillae along the lateral edge of the notum (Figure 4.2B). No pores were visible on the notum due to the presence of a thick cuticle which extends into the region of the hyponotum.

As specimens of *Onchidella hildae*, a species from Panama, were provided as fixed tissues, no observational data on live animals is available. Scanning electron microscopy was also not possible as very few individuals were available for study.

4.3.2. Light microscopy

Examination of sections stained with Toluidine blue show *O. capensis* to have large, pear-shaped, sub-epidermal multi-cellular glands. These glands contain some cells that stained positively for mucopolysaccharides and others that do not stain at all. These pear-shaped glands are situated deep in the thick marginal tissue of the notum (Figure 4.2C) and have a duct leading to a pore at the mantle edge (not illustrated). The largest glands can be up to 0.35 mm in diameter and 0.51 mm in length. They are surrounded by a well-organised capsule of layers of smooth muscle (about 35 µm in thickness), orientated in both a longitudinal and circular direction (Figure 4.2D). Staining with Toluidine blue showed the cells of each gland varied in their staining properties, some being strongly stained (dark blue) to only slightly stained (very light blue).

The Alcian blue – PAS technique showed that the base of the some cells stained magenta indicating neutral mucins, but the cells closest to the lumen of the gland and the secretion in the lumen stained blue, indicating acidic mucins (Table 4.1). The gland cells stained variably positive for sulphated mucins with aldehyde fucshin, indicating different cell types, but did not stain for carboxylated mucins (Table 4.1). Staining with Masson’s trichrome showed that the individual gland cells within the multi-cellular gland are surrounded by a thin layer of muscle (Figure 4.2D). Staining with Bromophenol blue showed the presence of protein in both the cuticle and in the largest cells of the defensive gland (Table 4.1).

The large multi-cellular glands of *O. hildae* have a diameter of about 0.78 mm and are enclosed in an even thicker, multi-layered capsule of muscle fibres (up to 110 µm in thickness), surrounded by tissue in which there is a dense matrix of inter-lacing fibres of smooth muscle and collagen (Figure 4.3). The muscle in the capsule surrounding the multi-cellular gland is organised in both a circular and longitudinal direction. Like *O. capensis*, most of the gland cells within the multi-cellular gland stained variably positively, using Toluidine blue, for mucopolysaccarides, but a few of the cells did not stain at all (Table 4.1). The Alcian blue – PAS technique showed that some cells stained magenta indicating neutral mucins, but the cells closest to the lumen and the secretion in the lumen stained blue, indicating acidic mucins (Table 4.1).

The gland cells stained differentially positive for sulphated mucins, but negatively for carboxylated mucins (Table 4.1). The Masson’s trichrome stain showed that there is a multi-layer muscle capsule surrounding the glands and the internal partitions of muscle between the different gland cells within the gland. (Figure 4.3B).

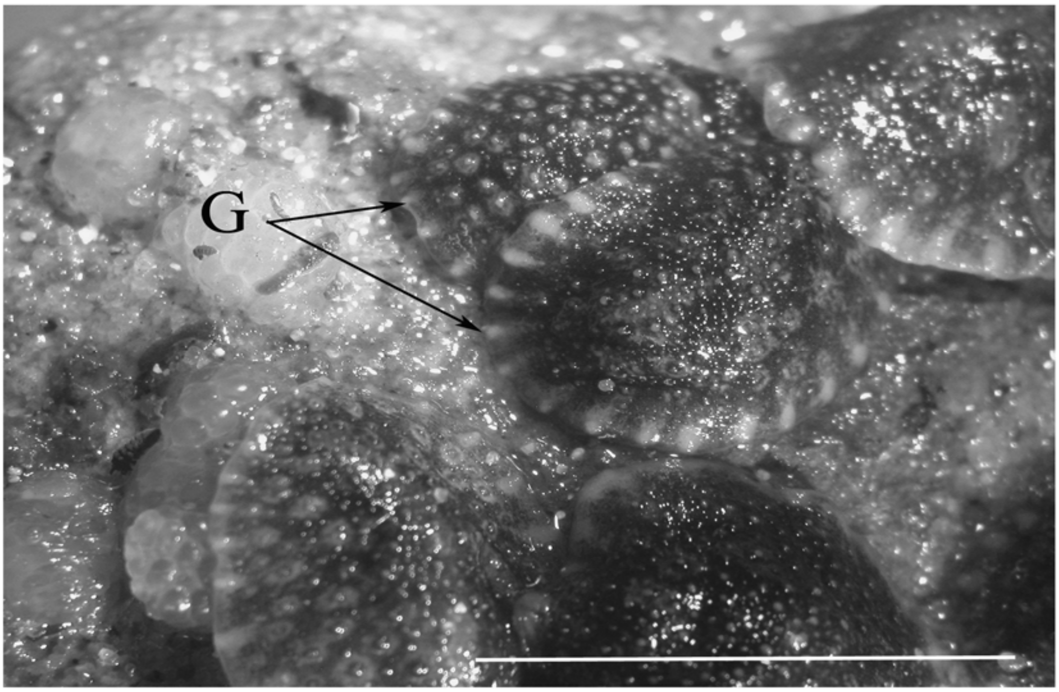

Figure 4.1. *Onchidella capensis*, live animals, showing the position of the perinotal glands (G).

Scale bar = 1cm.

Table 4.1. Summary of the histochemical results for the multi-cellular, lateral marginal glands of two species of *Onchidella.*

	Mucopolysaccharides	Acid mucopoly-saccharides	Neutral Mucins	Sulphated mucins	Carboxylated mucins	Protein	Collagen	Muscle fibre
Onchidella capensis	+	+	+	+	-	+	+	+
Onchidella hildae	+	+	+	+	-	+	+	+

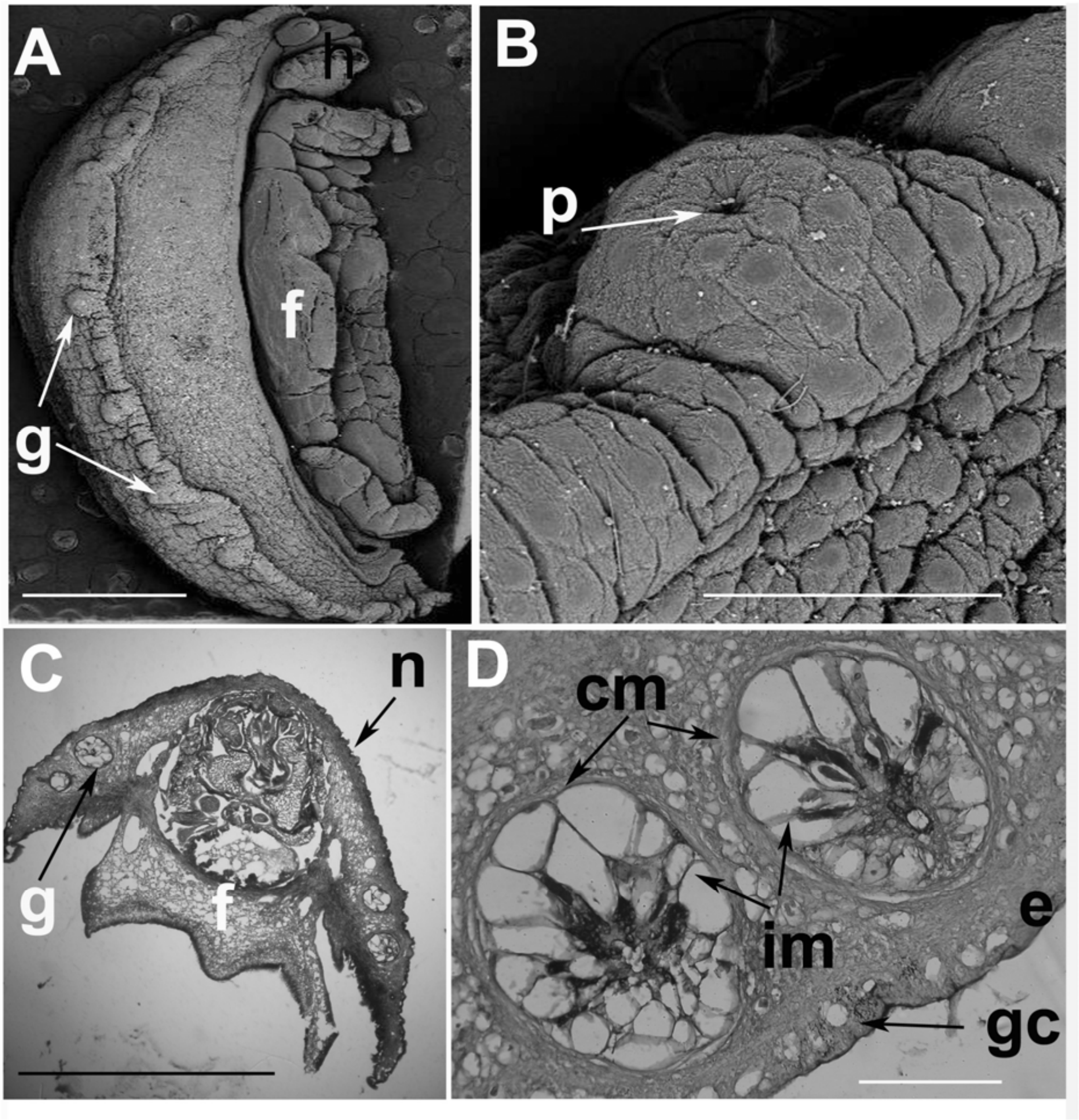

Figure 4.2. Scanning electron microscopy and light microscopy of *Onchidella capensis*. **A**. Lateral view; low power SEM image of the lateral marginal glands (g, gland; h, head; f, foot). **B**. A higher magnification image of an individual papilla and gland pore (p, pore). **C**. A low power light micrograph of a mid-transverse section showing the position of the glands embedded in the hyponotum (g, gland; n, notum; f, foot), stained with Toluidine blue. **D**. A higher power light micrograph of the glands stained with aldehyde fuchsin (cm, capsule muscle; im, intra-gland muscle; gc, goblet cell; e, epidermis).

Scale bars: A = 2 mm; B = 200 µm; C = 5 mm and D = 100 µm.

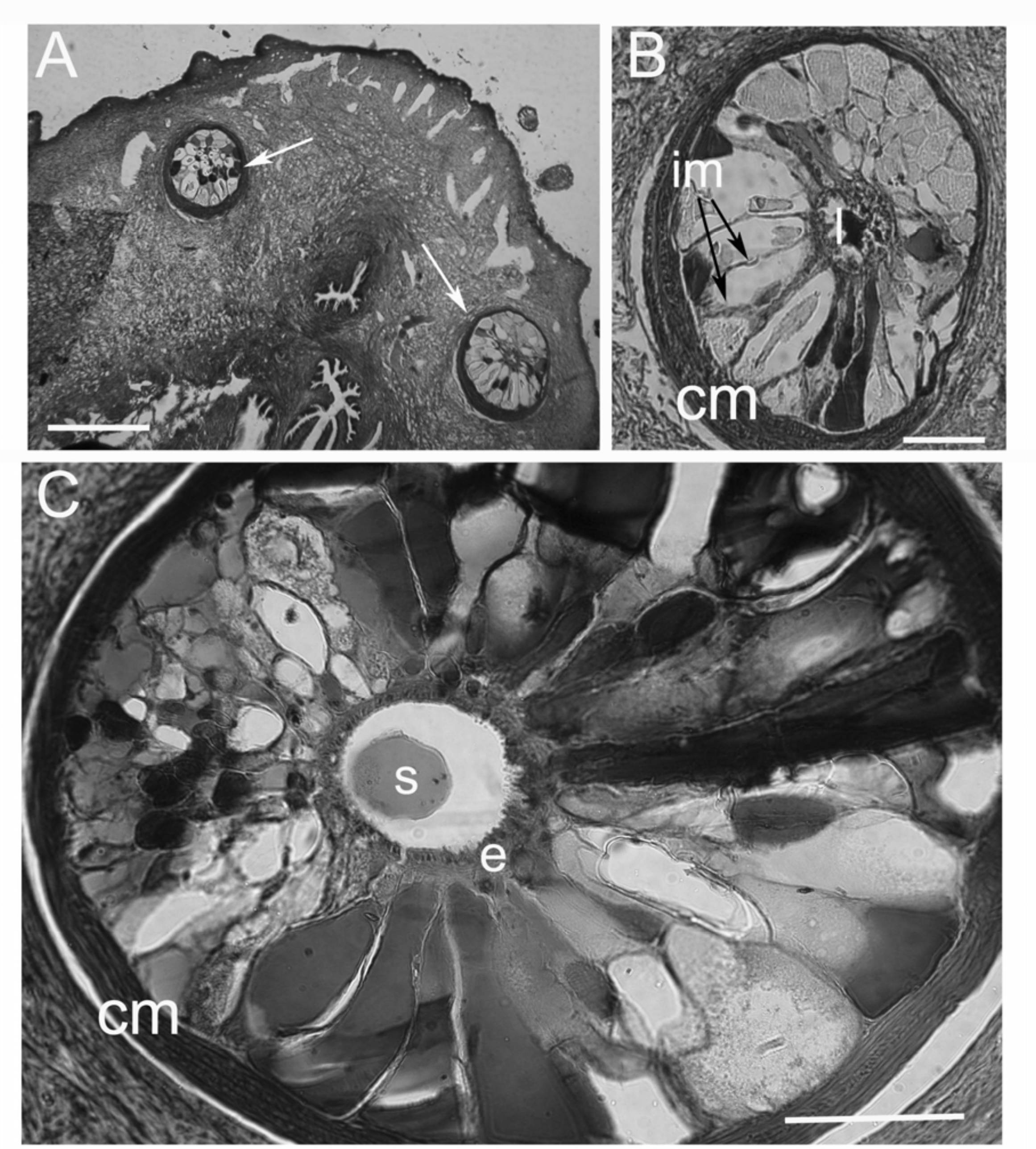

Figure 4.3. Light microscopy of the lateral pedal glands of *O. hildae.*
A. Low power light microscope image of a section through the body of *O. hildae* (stained with Masson's trichrome) showing the position of the multi-cellular glands (arrows). **B**. Light microscope image of a transverse section through an individual gland (stained with Masson's trichrome) showing the capsule muscle layer (cm), the intra gland muscle (im) and the lumen (l). **C**. Higher power image of a transverse section through a multi-cellular gland stained with Toluidine blue showing the capsule muscle layer (cm), the lumen with secretion (s) and the epithelial layer surrounding the lumen (e).

Scale bars: A = 1 mm; B = 200 μm; C = 100 μm.

4.3.3. Transmission electron microscopy

Onchidella capensis

The epidermal layer of *O. capensis* comprises a single layer of pear-shaped epithelial cells (about 11.7 µm in length and 7.6 µm in width) seated on a basal lamina, beneath which are collagen and muscle fibres (Figure 4.4A and B). The epithelial cells contain a single large nucleus (about 3.5 µm in diameter), situated towards the base of the cell, as well as vesicles, mitochondria, Golgi bodies (with up to 5 cisternae) and other organelles (Figure 4.4F). There is interdigitation of the lateral cell membranes of the epithelial cells (Figure 4.B and F). The external, or apical, surface of the epithelial cells bears numerous short microvilli (less than 1 µm in length) that are overlain by a cuticle that varies in thickness from 5 – 10 µm (Figure 4.4A, B, C, D and E). The cuticle has three distinct layers, a thin electron-lucent layer on the outside (possibly mucus), on top of a thin electron dense-layer with a thick layer of electron-lucent, homogenous material closest to the epithelial layer (Figure 4.4E). The external surface of the cuticle is covered in a dense mass of micro-organisms (algae, diatoms and bacteria) (Figure 4.4E).

Secretory or goblet type cells occur in or just below the epithelial layer (Figure 4.4C and D). These cells are largely filled with a homogenous electron-lucent secretory product, presumably mucus, and have a flattened, irregularly shaped nucleus and organelles at the base of the cell (Figure 4.4C and D).

The large sub-epithelial multi-cellular glands are pear-shaped and have a duct leading to the pore at the centre of the raised papilla on the marginal region of the notum. The glands are surrounded by a well organised capsule of smooth muscle (Figure 4.5). These bands of muscle surround the entire gland and are arranged in layers circling both the length and circumference of the gland. They continue along the length of the secretory duct.

The gland is composed of five different types of secretory cells (based on the appearance of their product), all of which are largely filled with secretion and have a flattened nucleus and other organelles at their base. The most prevalent cell type herein after referred to as Type 1, has a secretory product that is granular in appearance and occupies the region of the gland closest to the outer muscle capsule (Figure 4.6A and C). These cells are also the largest cell type (up to 200 µm in length and 103 µm in width). The second and third type of gland cell are smaller than the Type 1 cells and contain a substance with a homogenous appearance that stains darkly in Type 2 and lightly in Type 3 (Figure 4.6C and E). The fourth cell type has a secretory product that has a vesicular appearance (Figure 4.6E), with the vesicles having a maximum diameter of 1.5 µm. The secretory product of the fifth cell type (Type 5) also has a vesicular appearance (Figure 4.6B), but unlike Type 4 cells the vesicles of Type 5 cells have a heterogeneous appearance and are surrounded by a granular matrix.

The individual gland cells are separated by smooth muscle fibres and narrow support cells containing large numbers of mitochondria, rough endoplasmic reticulum, vesicles and glycogen (Figure 4.6C, D and F). The lumen of the gland and the duct are lined with irregularly shaped epithelial cells (Figure 4.6E). These epithelial cells (about 4.8 µm in width and 5.4 µm in length) have an elongate nucleus (about 5 µm in length), mitochondria, Golgi bodies and microvilli on the apical surface. The gland cells discharge their contents into the central lumen of the multi-cellular gland between the epithelial cells via holocrine secretion (Figure 4.6E).

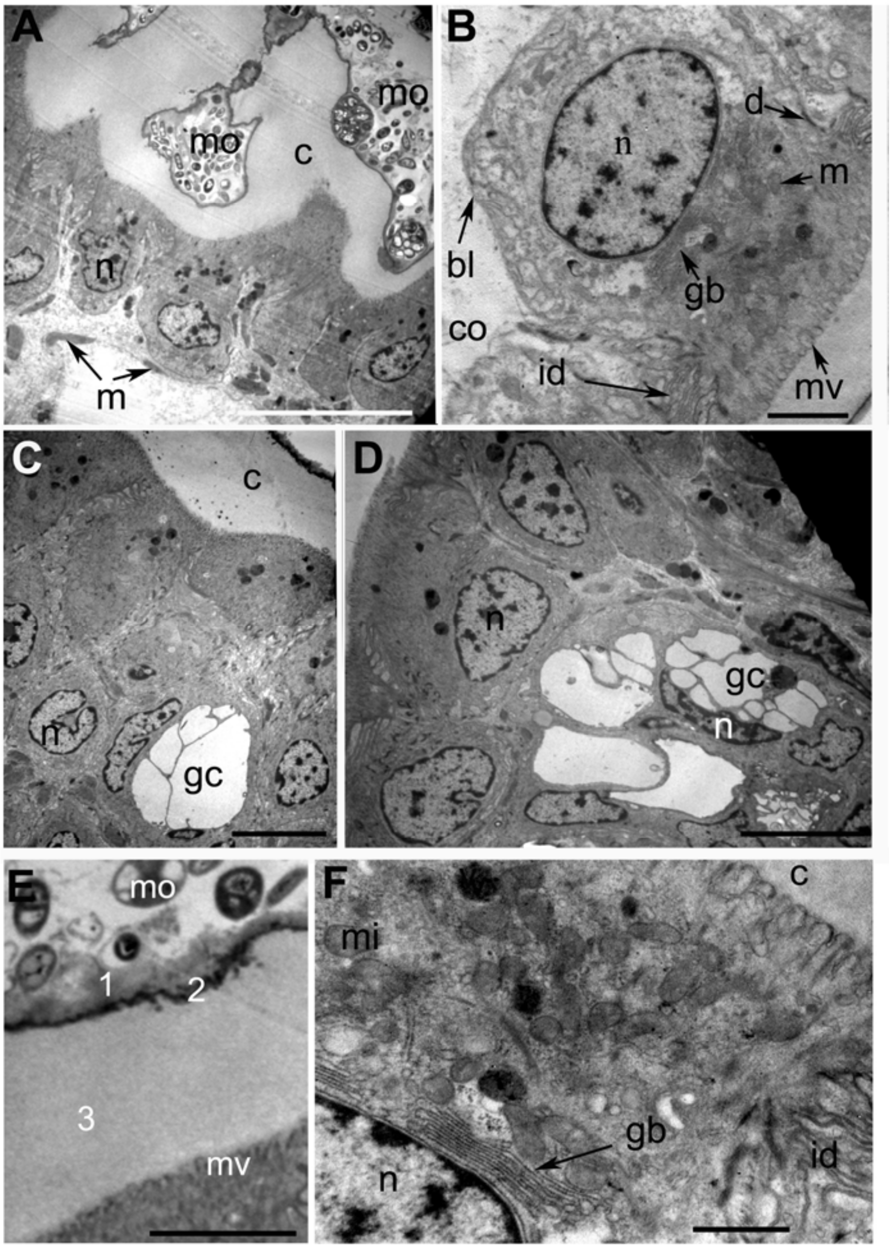

Figure 4.4. Transmission electron microscope images of the epidermal layer of *O. capensis*.

A. Low power TEM of the epidermal layer showing the epithelial cells with nuclei (n), cuticle (c), micro-organisms (mo) and muscle fibres (m). **B**. A higher power image of an individual epithelial cell showing the nucleus (n), the Golgi bodies (gb), mitochondria (m), interdigitation (id), desmosome (d), collagen (co) and basal lamina (bl). **C**. TEM of the epithelial layer and the underlying gland cell (gc) with its nucleus. **D**. Higher power TEM of the sub-epithelial region with gland cells (gc) and nuclei (n). **E**. Higher power image of the three layers of the cuticle (1, 2 and 3), micro-organisms (mo) on the surface and the microvilli (mv) on the surface of the epithelial cells. **F**. High magnification of an epithelial cell showing Golgi body (gb), nucleus (n), mitochondria (mi), vesicles (v), interdigitation (id) and the cuticle (c).

Scale bars: A = 10 µm; B = 2 µm; C = 5 µm; D = 5 µm; E = 5 µm; F = 1 µm.

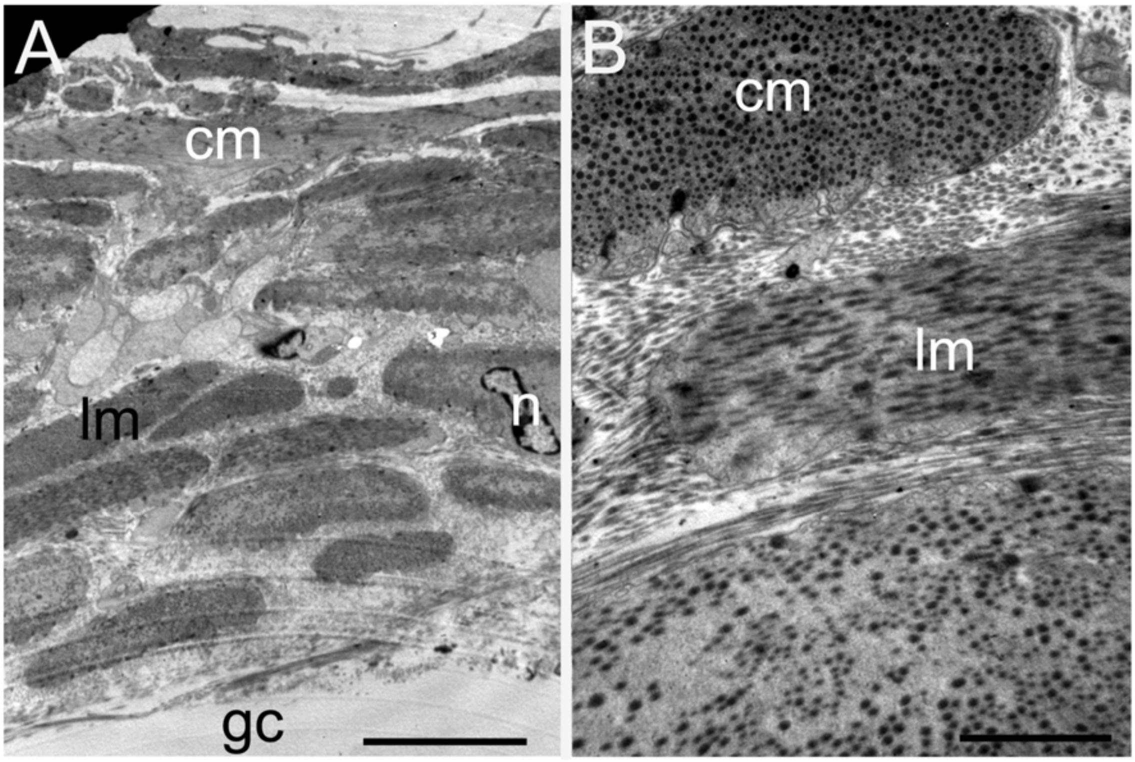

Figure 4.5. TEM of *O. capensis* muscle capsule.
A. Cross section of the muscle capsule surrounding the multi-cellular gland showing the longitudinal muscle (lm), the circular muscle (cm) and nucleus (n). **B**. A higher magnification of the longitudinal muscle (lm) and the circular muscle (cm).

Scale bars: A = 5 µm and B = 1 µm.

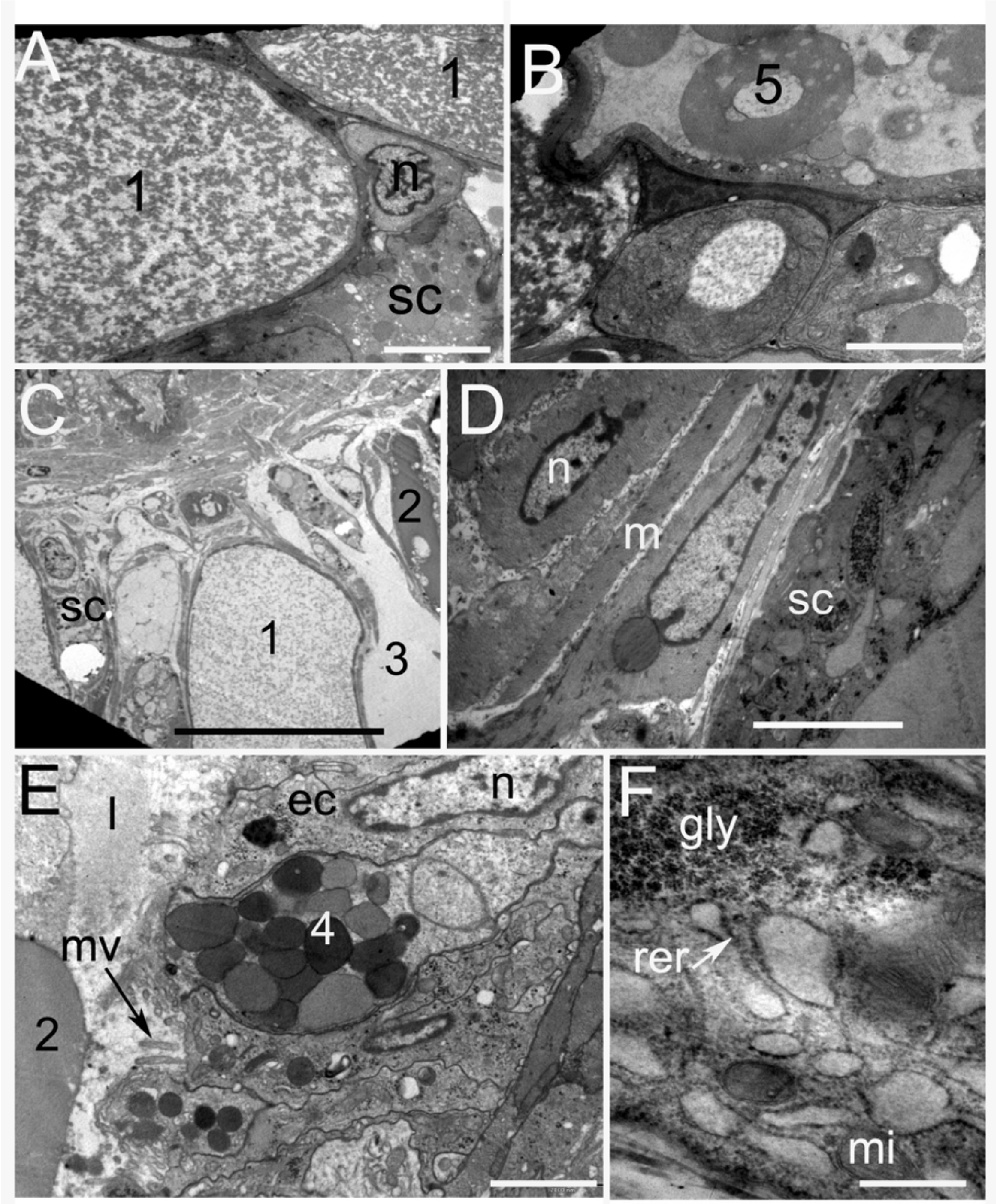

Figure 4.6. Transmission electron microscopy of the marginal multi-cellular glands of *O. capensis.* **A**. Type 1 gland cells (1) and support cell (sc) with nucleus (n). **B**. Type 5 gland cell (5). **C**. Type1 (1), 2 (2) and 3 (3) gland cells and a support cell (sc). **D**. Muscle tissue (m) between the cells of the gland and a support cell (sc). **E**. The lumen (l) of the gland, a Type 2 and Type 4 gland cell, and epithelial cell (ec) with microvilli (mv) and nucleus (n). **F**. High magnification of a support cell showing mitochondria (mi), rough endoplasmic reticulum (rer) and glycogen (gly).

Scale bars: A = 5μm; B = 2μm; C = 20μm; D = 2 μm; E = 2μm and F = 0.5 μm.

Onchidella hildae

The epidermis of *O. hildae* consists of a layer of flask-shaped epithelial cells (about 8.1 µm in length and 3.9 µm in width) that have a large irregularly shaped basal nucleus, lysosomes and have short microvilli (about 1 µm in length) on their apical surface (Figure 4.7A). The epithelial cells are seated on a basal lamina and are connected to it via desmosomes (Figure 4.7C). The epithelial cells are connected to each other via interdigitation of the lateral cell membranes. Beneath the epidermal layer there is a matrix of muscle fibres, collagen and pigment granules (Figure 4.7A and B). The epidermal layer is covered by a thick bi-layered cuticle (varying from about 7 µm to 12 µm in thickness) consisting of a thin electron-dense layer overlying a thicker electron-lucent layer that in places is extended as papillae (Figure 4.7A). The cuticle is densely covered in micro-organisms (algae, diatoms and bacteria) and at times these micro-organisms appear to be embedded in the cuticle (Figure 4.7A).

Large multi-cellular glands are deeply embedded within the hyponotum and connect to the external pore via a duct. These glands are encapsulated in a thick layer of smooth muscle, encircling the gland both longitudinally and circularly (Figure 4.7D). The muscle layer extends along the length of the duct, although it is slightly thinner than the layer surrounding the gland body.

Based on the appearance of the product, four different types of gland cells could be identified in the glands. The predominant cells are large cells that occur mostly in the lower half of the gland. They have a flattened nucleus and organelles at the base of the cell and contain large quantities of granular product (Type 1) (Figure 4.8A and C). Cell types 2 and 3 are smaller and filled with a homogenous product, staining rather darkly in Type 2 and lightly in Type 3 (Figure 4.8C and E). The fourth cell type has a vesicular appearance and occurs closer to the lumen of the gland (Figure 4.8B and F). The different cell types are separated by strands of muscle and support cells that contain a nucleus, large numbers of mitochondria, Golgi bodies and vesicles (Figure 4.8D).

Both the gland lumen and the lumen of the collecting duct are lined with a layer of cuboidal cells (Figure 4.8E). These cells have microvilli on the apical surface and their cytoplasm forms a finger like projection in some places. As with *O. capensis* the gland secretions appear to discharge into the lumen by holocrine secretion (Figure 4.8F).

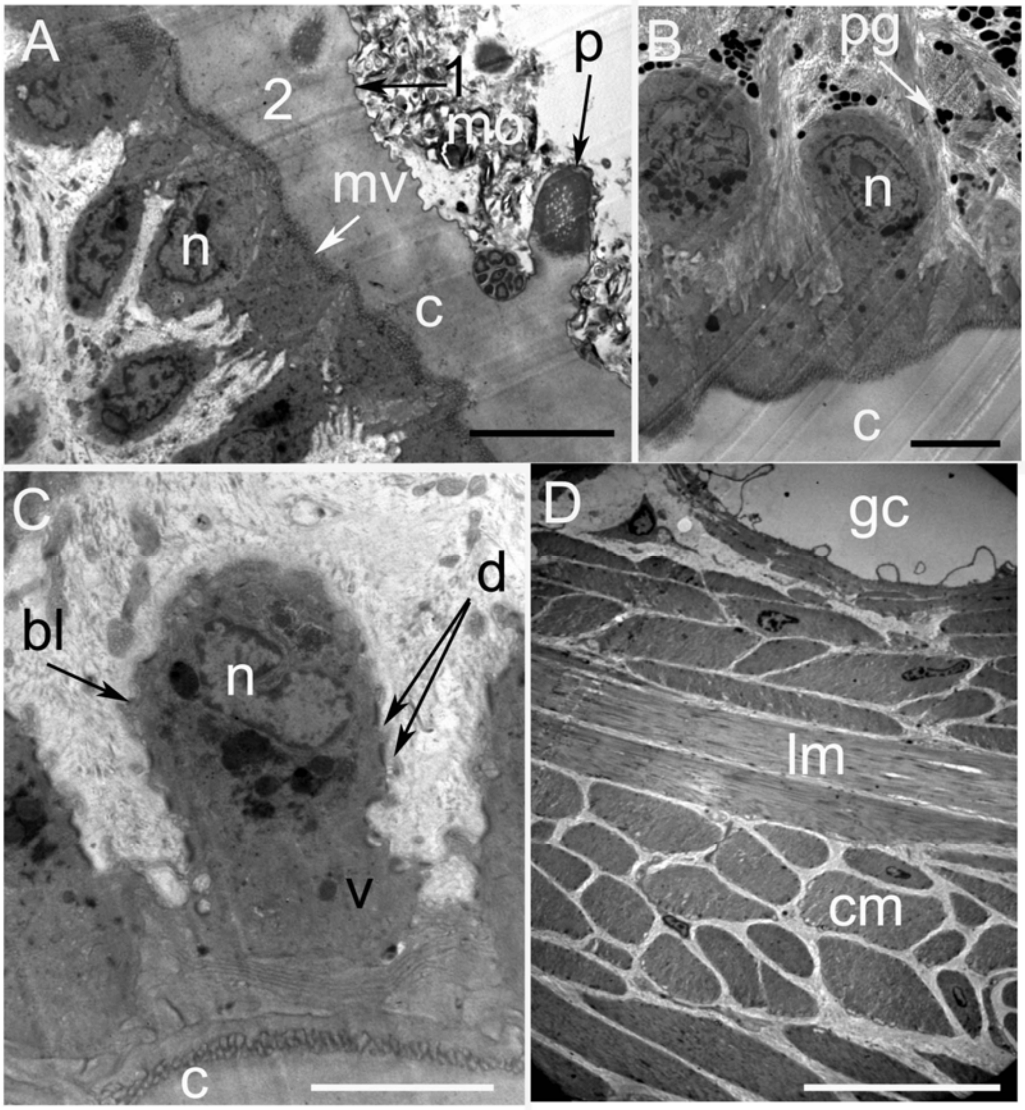

Figure 4.7. Transmission electron microscopy of *O. hildae* epidermis.
A. Low magnification of the epithelial cells with nuclei (n), cuticle (c), papilla (p), micro-organisms on the surface of the cuticle (mo), microvilli (mv) and the layers of the cuticle (1 and 2). **B**. A slightly higher magnification of the epithelial cells, nuclei (n) and pigment granules (pg). **C**. Higher magnification of an individual epithelial cell showing the nucleus (n), vesicles (v), basal lamina (bl) and desmosomes (d). **D**. the muscle layer (m) surrounding the multi-cellular gland and the base of a gland cell (gc).

Scale bars: A = 5 μm; B. = 5 μm; C = 10 μm; D = 10 μm.

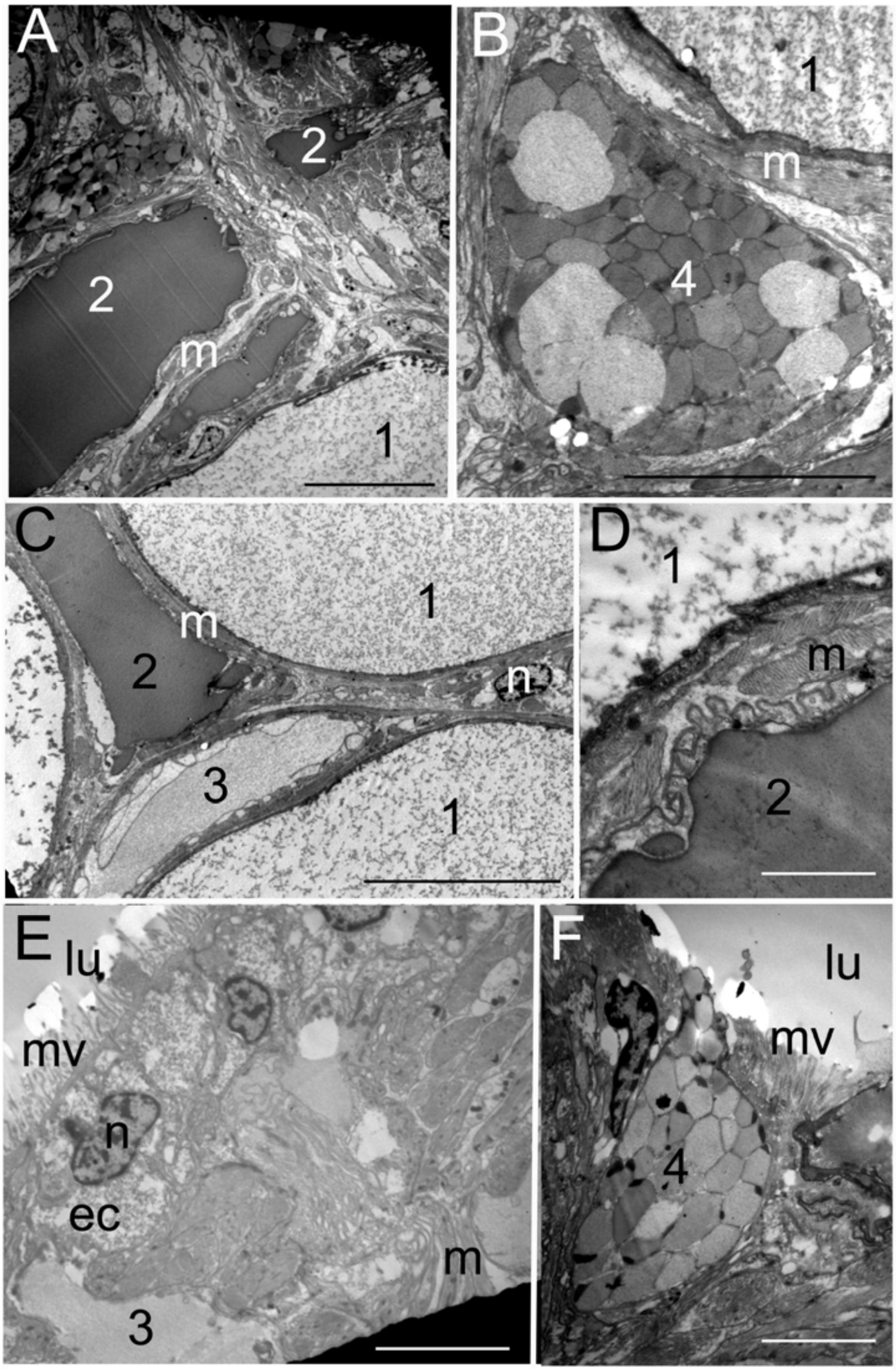

Figure 4.8. Transmission electron microscopy of the multi-cellular glands of *O. hildae.*
A. Type 1 (1) and 2 (2) gland cells and the layers of muscle (m) in between. **B**. Type 1 (1) and 4 (4) gland cells and muscle (m). **C**. Junction between type 1, 2 and 3 gland cells with muscle (m) and the nucleus of a support cell (n). **D**. The muscle (m) between type 1 and 2 gland cells. **E**. The epithelial cells (ec), with microvilli (mv) lining the lumen (lu) of the gland, muscle (m) and type 3 cell. **F**. A type 4 cell secreting its product into the lumen (lu) which is lined with microvilli (mv).

Scale bars: A = 10 µm; B = 5 µm; C = 10 µm; D = 1 µm; E = 5 µm; F = 2 µm.

4.4. Discussion

As shell-less, soft-bodied animals, onchidellids are potentially vulnerable to desiccation and predation. Whilst onchidellids tend to forage at low tide on overcast days (Weiss and Wägele, 1998; pers. obs.), the cuticle, epidermal layer and its mucous secretions must provide protection from dehydration, particularly during foraging. The epidermal layer of *Onchidella capensis* and *O. hildae* is similar in structure, being composed of an uneven layer of pear-shaped epithelial cells beneath a relatively thick cuticle. The cuticle of *O. capensis* and *O. hildae* stained light blue with Toluidine blue, indicating the presence of mucopolysaccharides. The cuticle also stained blue with bromophenol blue, indicating the presence of protein. The cuticle thickness ranged between 5 – 10 µm in *O. capensis* and 7 – 12 µm in *O. hildae.* These results are similar to the findings of Weiss and Wägele (1998) for *O. celtica*, which has a cuticle about 8 µm in thickness and also stains light blue with Toluidine blue. Under the transmission electron microscope the cuticle of *O. capensis* is composed of three layers a thin, electron-lucent outer layer, beneath which is a thin electron-dense layer, and a thick third layer. The cuticle of *O. hildae* appears to have only two layers, a thin electron-dense layer on the outside and a thick, electron-lucent inner layer. It is possible that the outermost layer in *O. capensis* is a layer of mucus and this may have been removed from the surface of *O. hildae* during the processing of tissue.

Scanning electron microscopy of the hyponotum of *O. capensis* and *O. hildae* did not reveal any pores correlating to the openings of goblet cells, as seen in siphonariids (see Chapter 3). However, it is possible that this was an artefact of fixation and specimen preparation. This is because light and transmission electron microscopy revealed numerous small goblet cells in the epithelium of *O. capensis* and *O. hildae*.

Goblet cells presumably produce a thin layer of mucus over the epidermis, as in other gastropods (Bubel, 1984). The openings to the goblet cells may have also been obscured by the dense layer of diatoms, algae and other micro-organisms that either covered, or were encapsulated in, the cuticle. The micro-organisms, particularly the diatoms with their silicaceous skeleton, posed problems with cutting sections for the TEM, resulting in scratch marks on the sections even when using a diamond knife.

Like all shell-less gastropods onchidellids rely on chemical defence to deter predators (Watson, 1925; Young *et al*., 1986; Weiss and Wägele, 1998; Darias *et al*., 2006). All studies to date have revealed that onchidellids posses large marginal, multi-cellular, epidermal glands (Joyeux-Laffuie, 1882; Von Wissel, 1898; Watson, 1925; Binot, 1965; Marcus, 1979; Weiss and Wägele, 1998; Wägele et al., 2006; present study) that produce a repugnatorial secretion. Like the multi-cellular epidermal glands of siphonariids (see Chapter 3), those of onchidellids are surrounded by layers of smooth muscle. The muscular capsule was particularly well developed in *O. hildae*. In addition, this study has shown that unlike siphonariids, muscle fibres run between the gland cells of *O. capensis* and *O. hildae*. The well developed muscle layer and the strands of muscle running between the different gland cells indicates that the glands can be constricted to forcibly propel their secretions along the length of the duct and away from the body of the animal. Arey (1937) records that, in response to disturbance (mechanical, electrical or chemical), *O. floridanum* projected a thin stream of secretion up to 15 cm towards the source of the disturbance. In a further study, Arey (1942) found that it was easier to elicit a glandular discharge when the animal was submerged in water and that only a thin thread of coagulated mucous was secreted which did not break up into a spray as it did in air. The distance that the secretion travelled in water was only a centimetre or so. The erectile papillae of some species may enable the animal to direct a stream of secretion directly towards the predator. Young *et al*. (1986) established that *O. borealis* will direct its papillae towards the source of irritation and will project the defensive secretion several millimetres towards the source. Each gland seems to discharge independently and they do not necessarily discharge the entire contents, but can fire repeatedly. In the presence of certain predators e.g. starfish, the gland can release the entire contents of the lumen but then requires a period of regeneration before it is capable of firing again. This was not established for *O. capensis* and *O. hildae*, but sections through the marginal epidermis showed gland in different stages "fullness" possibly indicating the glands containing less secretory product had recently fired their contents.

The number of cell types that comprise the marginal multi-cellular glands of onchidellids varies between species. Based on their appearance at the light microscope level, Arey (1937) and Arey and Barrick (1942) found seven different cell types within the glands of *Onchidella floridanum*, whereas Gabe and Prenant (1950) only found five cell types in *Onchidella celtica*. This was later confirmed by the histological study of Binot (1965) who named them Types 1 – 5. Gland cell Type 1 was the largest cell type (220 – 320 µm long and 85 – 120 µm wide) and therefore these cells occupied the largest proportion of the gland. In this electron microscope study, *O. capensis* was found (based on morphological appearance of the gland product) to have five different types of gland cell, whereas *O. hildae* only had four. As in *O. celtica* Type 1 cells in both *O. capensis* and *O. hildae* were the largest (200 µm in length and 103 µm in width). Light microscopy and transmission electron microscopy, however, revealed that there was a marked similarity in the structure of the gland and the cell types of *O. capensis* and *O. hildae.* Both have a layer of epithelial cells lining the lumen of the gland and the secretory duct (this layer of epithelial cells was evident in the drawings of *O. capensis* by Marcus, 1979). These epithelial cells have microvilli on the apical surface lining the lumen of the gland, presumably to aid the movement of the gland secretions along the length of the duct, towards the external pore. Furthermore, the results of the histological and histochemical staining of the glands of showed that the secretory product is largely made up of acidic mucopolysaccharides and neutral and sulphated mucins. Arey (1937) found the pH of the gland secretion of *O. floridanum* to be pH 2.7. Binot (1965) described the secretory product of the gland cells of *O. celtica* as acid and lipid complexes. The functional significance of possessing different cell types within a gland is unknown. Presumably each cell type produces a unique product that mixes in the lumen to produce the final defensive mucous secretion. This mucus contains polypropionate metabolites (Darias *et al.*, 2006) but which cells within the gland produces these chemicals is not known.

Marcus (1979) in study of the morphology and taxonomy of onchidellids concluded that whilst the number of marginal repugnatorial glands varied between species, e.g. eight on either side for *O. brattstroemi* and nine for *O. miusha* (two Atlantic species described by Marcus, 1979), twelve on either side for *O. capensis* (Watson, 1925) and sixteen to eighteen in *O. borealis* (Weiss and Wägele, 1998) there was also variation within a species. This was largely due to age and size of the individual (Marcus, 1979). Young *et al.* (1986) also concluded that age and size affected the size and number of glands. Nevertheless, Marcus (1979) did suggest that the structure of these glands might provide taxonomic characters. The results of this study, however, would suggest that this is unlikely. Gland size within a species will vary with state of discharge, as will the appearance of the cell types within a gland. In addition, there appears to be a great deal of similarity in the structure of the glands between species. Nevertheless, studies on a greater number of species may reveal taxonomic patterns.

Gray (1840, cited by Wagele, 2008) recognised the close relationship between the Opisthobranchia and the Pulmonata, which he combined in the Heterobranchia (or Euthyneura by later authors). Fretter and Graham (1943) and Zilch (1959) decided that the Onchidioidea should be placed in the Opisthobranchs, but Van Mol (1967) demonstrated the homogenous nature of the procerebrum in pulmonates and onchidoideans and the absence in the opisthobranchs. Since then, authorities have agreed that the Onchidioidea are allied to the pulmonates, but the closeness of that relationship is the focus of debate (Britton, 1983; Ponder and Lindberg, 2008). In this study the defensive glands of *O. capensis* and *O. hildae* were found to resemble those of the siphonariids (Chapter 3) adding support to onchidellids being placed within the Pulmonata. Fretter and Graham (1954) produced a drawing of a siphonariid defensive gland and noted that *O. celtica* possessed similar glands, but with a longer duct. She speculated that due to the loss of the protective shell and operculum that prevent the animal from shutting up entirely when disturbed the animal has similar or even more elaborate glands. The structure of the glands of *O. capensis* and *O. hildae* would appear to substantiate this theory.

Chapter 5. Structure and histology of the lateral pedal glands of *Trimusculus costatus* (Trimusculidae)

5.1. Introduction

The family Trimusculidae (Order: Eupulmonata) are a group marine pulmonates with small, cap-shaped, thick, round to ovate, shells (commonly called “button shells” due to their small, uniformly round shells). Shell length is usually less than 20 mm (Kay *et al*., 1998). Trimusculids are amphibious (Purchon, 1977) and can breathe in both air and water. They are found in the lower regions of the intertidal zone of rocky shores. *Trimusculus costatus* (Krauss, 1848) is the only known member of this genus found along the coast of southern Africa (Kilburn and Rippey, 1982; Davies-Coleman and Beukes, 2004), and is a relatively small pulmonate molluscs with a round, conical shell, less than 20 mm in length and 10 mm in height. This species congregates in large groups under overhanging rocks, caves and in crevices between the rocks along exposed shores, where it will remain moist and cool (Kilburn and Rippey, 1982). Trimusculids lead a mostly sedentary life style and feed by means of a mucous net that they secrete in order to filter out food particles in the water column at high tide (van Wyk *et al*., 2008). Like other intertidal shelled pulmonates, trimusculids could be subject to predation by a variety of sub-tidal and inter-tidal predators (Rice, 1985).

Intertidal gastropods have been shown to employ a wide variety of defensive strategies to avoid predation including tough shells, cryptic coloration, adherence to a homing scar and the production of defensive or repugnatorial chemical secretions (Wagele *et al.*, 2006). Like siphonariid limpets (see Chapter 3) trimusculids employ chemical defence.

Rice (1985) studied the anti-predator chemical defence of *Trimusculus reticulatus* in relation to the predatory sea star *Pisaster ochraceus.* Starved individuals of *P. ochraceus* were offered *T. reticulatus* and a species of prosobranch limpet upon which to feed and repeatedly chose to consume the patellogastropod limpet and avoided eating *T. reticulatus*. *Trimusculus reticulatus* secretes a milky-white mucus when attacked. When this mucus was painted onto the shells of the prosobranch limpet they were consumed less readily. Rice (1985) proposed that the mucus from *T. reticulatus* contained a compound that temporarily stuns the tube feet of the attacking sea star, although the sea star appeared to suffer no long term effects from repeated exposure to the compound. *T. costatus* also produces copious amounts of thick white mucus when it is disturbed or removed from the rock face (personal observation).

Subsequent to the work of Rice (1985) it has been established that *T. reticulatus* from California and *T. conica* from New Zealand both produce defensive secretions that contain labdane diterpenes (Darias *et al*, 2006). *Trimusculus reticulatus* was the first species studied and diterpenes were isolated from both the whole animal and from the mucus secreted by it to repel starfish (Darias *et al.*, 2006). It has also been established that these diterpenes are concentrated in the mantle and foot of both, while the viscera contain no diterpene compounds (Manker and Faulkner, 1996; Darius *et al.*, 2006). Four new diterpenes have been also isolated from *T. peruvianus* (collected in the intertidal regions of Los Cruces, Chile) and two from *T. costatus* which is endemic to South Africa. Both the compounds isolated from *T. costatus* exhibited anti-feeding activity against the predatory fish *Pomadasys commersonnii* (spotted grunter) and proved toxic to *Artemia salina* (brine shrimp) (Gray *et al.*,1998).

Thus whilst some work has been done on the chemistry of the secretions of the lateral pedal glands of trimusculids, nothing is known about the anatomy of these glands that may produce them. The aim of this study therefore was to examine the dorso-lateral epidermis of a representative trimusculid, *T. costatus*, to determine what mucous secretion glands may be present.

5.2. Materials and methods

Specimens of *Trimusculus costatus* (Krauss, 1848) were collected at low tide from underneath rocks in the lower regions of the intertidal zone at Cintsa (32^{o} 49’ 50” S, 28^{o} 6’ 34” E) on the south-east coast of South Africa. The live animals were transported back to the laboratory and the tissues from the dorso-lateral margins of the head and foot of five individuals were dissected, fixed and processed for light microscopy (histology and histochemistry), scanning electron microscopy and transmssion electron microscopy using the same methods and techniques as for *Siphonaria* and *Onchidella* (Chapter 2).

5.3. Results

5.3.1. General observations and scanning electron microscopy

Trimusculus costatus produces copious amounts of thick white mucus when threatened or dislodged from its home scar. This mucus is secreted from numerous glands that are visible to the naked eye (Figure 5.1A and B), situated in the lateral marginal regions of the foot.

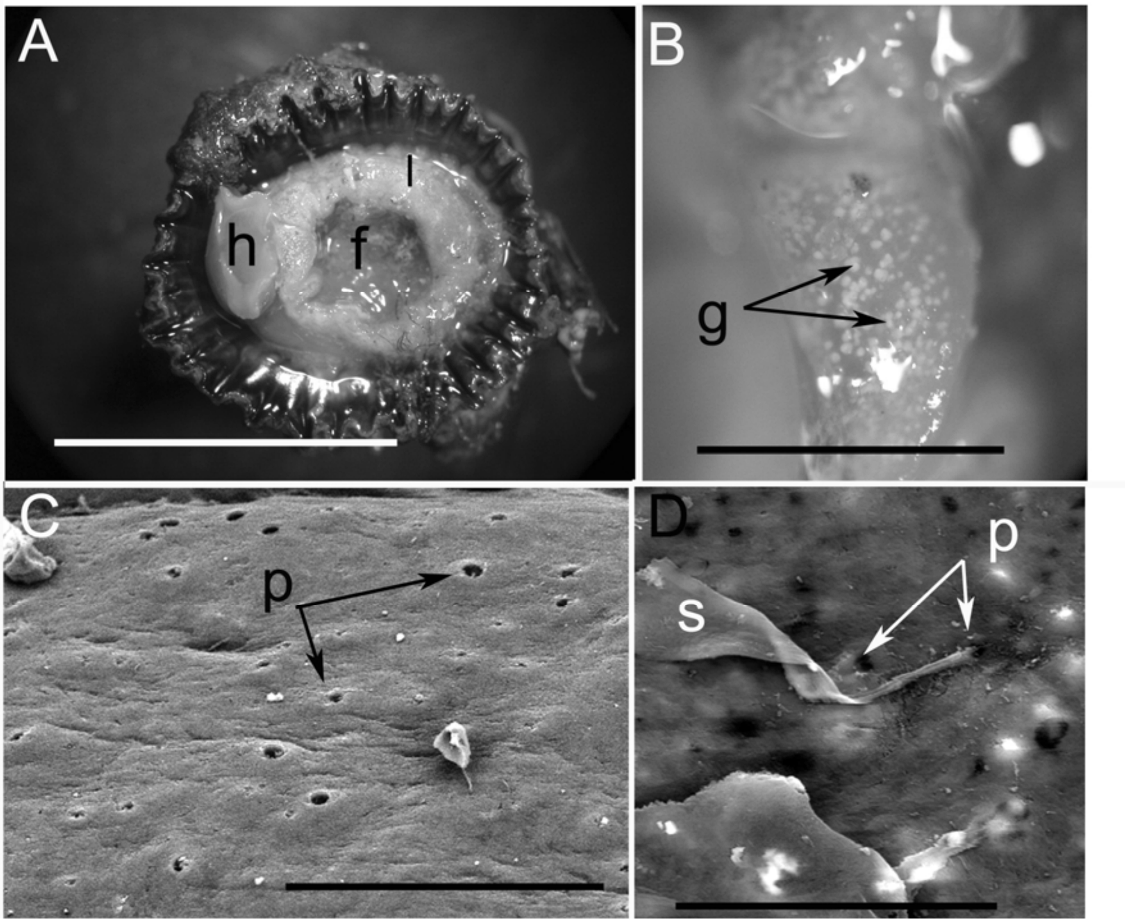

Figure 5.1. *Trimusculus costatus* epidermal glands.
A. Ventral view of *T. costatus* showing the head (h) and foot (f) and lateral regions (l) sampled for histology and TEM. **B**. Higher magnification of the marginal regions of the foot showing the glands (g). **C**. Scanning electron micrograph of the marginal epidermis of the foot showing the pores (p). **D**. SEM of pores (p) and the ribbons of secretion (s).

Scale bars: A = 1 cm; B = 200 µm; C = 100 µm; D = 100 µm.

Examination of the lateral epidermal region of the head and foot, the region in which the visible glands are situated, under the scanning electron microscope shows the presence of pores in the epidermis (Figure 5.1C). These pores vary in size (Figure 5.1 C and D) with diameters ranging from about 1 µm to 8 µm. Ribbons of secretion can be seen extruding from some of the pores (Figure 5.1D).

5.3.2. Light microscopy/ histology

Staining with toluidine blue shows that the marginal regions of the foot of *T. costatus* are densely packed with glandular tissue (Figure 5.2A). There are small glandular structures near the epidermal layer and larger glandular structures deeper within the tissue (Figure 5.2B). Several different types of gland structure were observed and they stained variably for mucopolysaccharides when stained with Toluidine blue. Some of the glands did not stain at all, appearing as unstained spaces (Figure 5.2A, B, C and D), but others stained blue indicating neutral mucopolysaccharides. Other gland structures stained magenta indicating the presence of acidic mucopolysaccharides (Table 5.1; Figure 5.2A).

The alcian blue- PAS stain showed positive results for neutral mucins only and the combined aldehyde fuchsin / alcian blue gave positive results for sulphated mucins and negative results for carboxylated mucins (Table 5.1; Figure 5.2). Staining with Masson’s trichrome showed no collagen present in the glandular tissue, but haemotoxylin/ eosin confirmed the presence of muscle and fibrin in the surrounding tissue (Table 5.1). Sections stained with Bromophenol blue showed the presence of protein in both the epithelial layer and in the gland cells.

Table 5.1. Histchemical results from the dorso-lateral pedal glands of *Trimusculus costatus*

	Mucopolysaccharides	Acidic mucpolysaccharides	Neutral mucins	Acid mucins	Sulphated mucins	Carboxylated mucins	Protein	Collagen	Muscle fibre
Trimusculus costatus	+	+	+	-	+	-	+	-	+

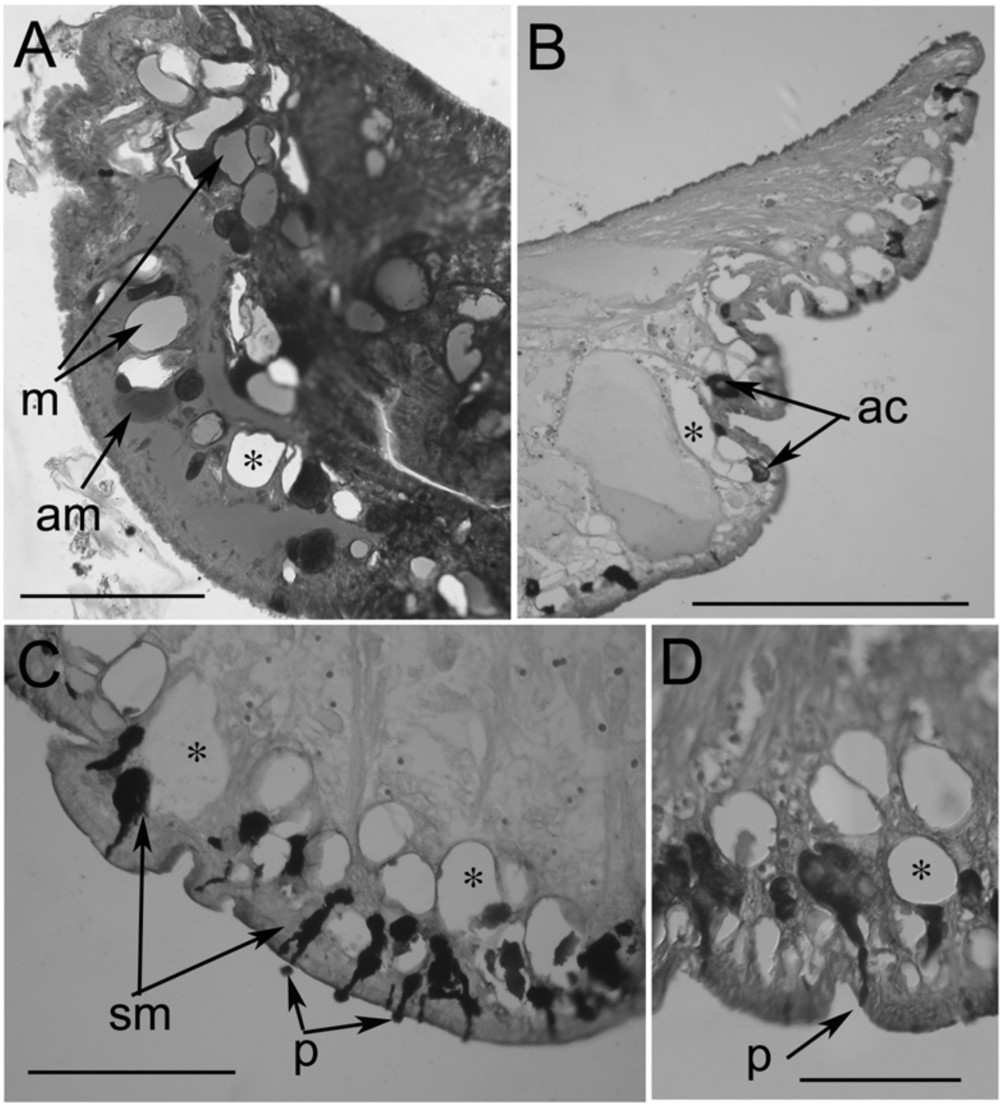

Figure 5.2. Light microscopy of *Trimusculus costatus.*
Sections through the lateral pedal glands, stained with **A**. Toluidine blue (am, acid mucopolysaccarides; m, mucopolysaccharides; *, unstained glandular structures). **B**. Alcian blue-PAS (ac, acid mucins; *, unstained glandular structures). **C**. Aldehyde fuchsin (sm, sulphated mucins; p, pore; *, unstained glandular structures). **D**. A higher magnification of glands, stained with aldehyde fuchsin (p, pore; * unstained glandular structures).

A = semi-thin resin section, 1 µm thick, **B – D** = Paraplast sections, 5 µm thick.

Scale bars. A = 100 µm, B = 1 mm, C = 100 µm and D = 100 µm

5.3.3. Transmission electron microscopy

The epidermis of the lateral region of the foot of *T. costatus* (Figure 5.3) consists of columnar epithelial cells (about 14.0 µm in length and 7.4 µm in width) seated on a basal lamina (Figure 5.3C). This is largely a single layer of epithelial cells, but in certain areas where the epithelial layer has become invaginated there appears to be more than one layer of epithelial cells. These cells generally contain a large, variably shaped nucleus (about 6.1 µm long and 4.1 µm wide), and the usual complement of organelles, numerous vesicles and microvilli (approximately 2.2 µm in length) on the apical surface (Figure 5.3A, B and C). The cells are connected to each other and the basal lamina via desmosomes and hemi-desmosomes respectively (Figures 5.3B and C). Goblet cells are found between the columnar epithelial cells (Figure 5.3C). There are two different types of goblet cell, one having cytoplasm with a granular appearance and well developed apical microvilli (Type 1; Figure 5.3C) and the second a more globular content with no microvilli (Type 2; Figure 5.3C). Both types of goblet cells appear to secrete their contents to the exterior via holocrine secretion. Micro-organisms, bacteria, algae and diatoms are often caught in the microvilli on the epithelial surface (Figure 5.C inset).

In some places the epidermal layer invaginates towards larger unicellular glandular cells and the epidermal cells in the invaginated epithelium have cilia rather than microvilli on the apical surface (Figure 5.4A and B). Secretions from the gland cells are extruded from pores between the epithelial cells (Figure 5.2C and D; Figure 5.4C and D).

Beneath the epidermal layer, the tissue is largely made up of muscle fibres and gland cells. Based on their cell contents three different types of large unicellular gland cell can be recognised. All three types are not surrounded by a capsule of muscle although there are muscle fibres in the tissues surrounding the gland cells (Figure 5.5C) and the matrix of the foot is densely packed with muscle fibres. Each gland secretes its contents via a pore between the epithelial cells (Figure 5.2C and D; Figure 5.4C)

The contents of the largest and most common cell type (Type 1) appear unstained (Figure 5.5A and B). These cells can be more than 30 µm in length and have a flattened basal nucleus (Figure 5.5A). The second type of gland cell (Type 2) has a uniform more electron-dense content (Figure 5.5B, C) with an irregularly shaped basal nucleus (Figure 5.5C). The content of the third gland cell type (Type 3) consists of vesicles with a granular appearance (Figure 5.5B and D).

In all three gland cell types most of the gland cell is filled with secretory product but there is a basal nucleus, Golgi bodies, vesicles, glycogen and rough endoplasmic reticulum at the base of the cell (Figure 5.5A and C; Figure 5.6A, B and C).

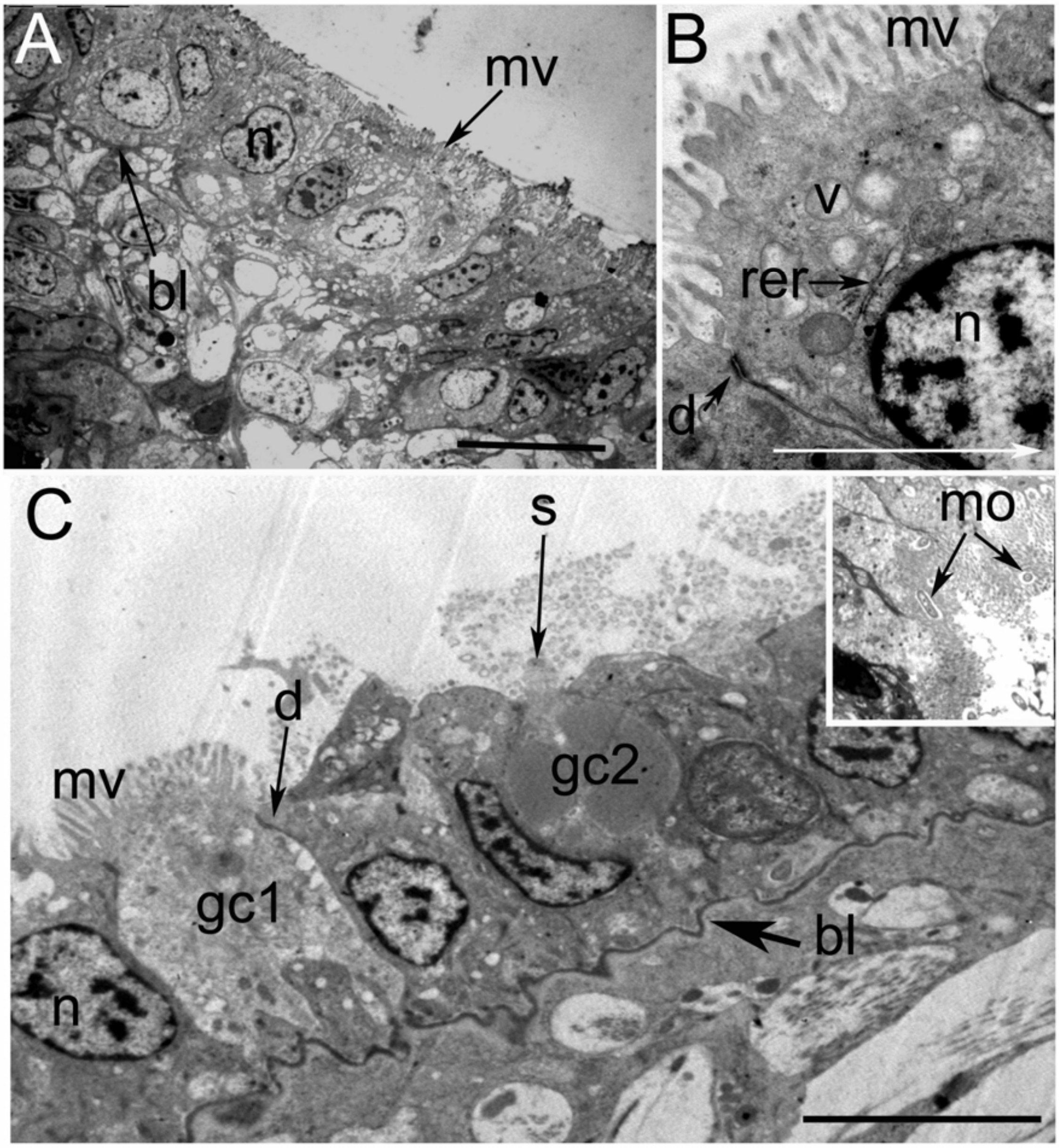

Figure 5.3. Transmission electron microscopy of *Trimusculus costatus* epidermis.

A. Low power micrograph showing the columnar epithelial cells containing the nucleus (n), seated on a basal lamina (bl) with microvilli (mv) on the apical surface. **B**. A higher power micrograph of an epithelial cell. (n, nucleus; d, desmosome; v, vesicle; mv, microvilli; rer, rough endoplasmic reticulum). **C**. The epithelial layer with goblet cells (gc1 and gc2), nucleus (n), microvilli (mv), desmosome (d) and the basal lamina (bl) and the Goblet cell contents being secreted (s).Inset; Micro-organisms (mo) in the microvilli.

Scale bars: A = 10 µm; B = 5 µm; C = 5 µm.

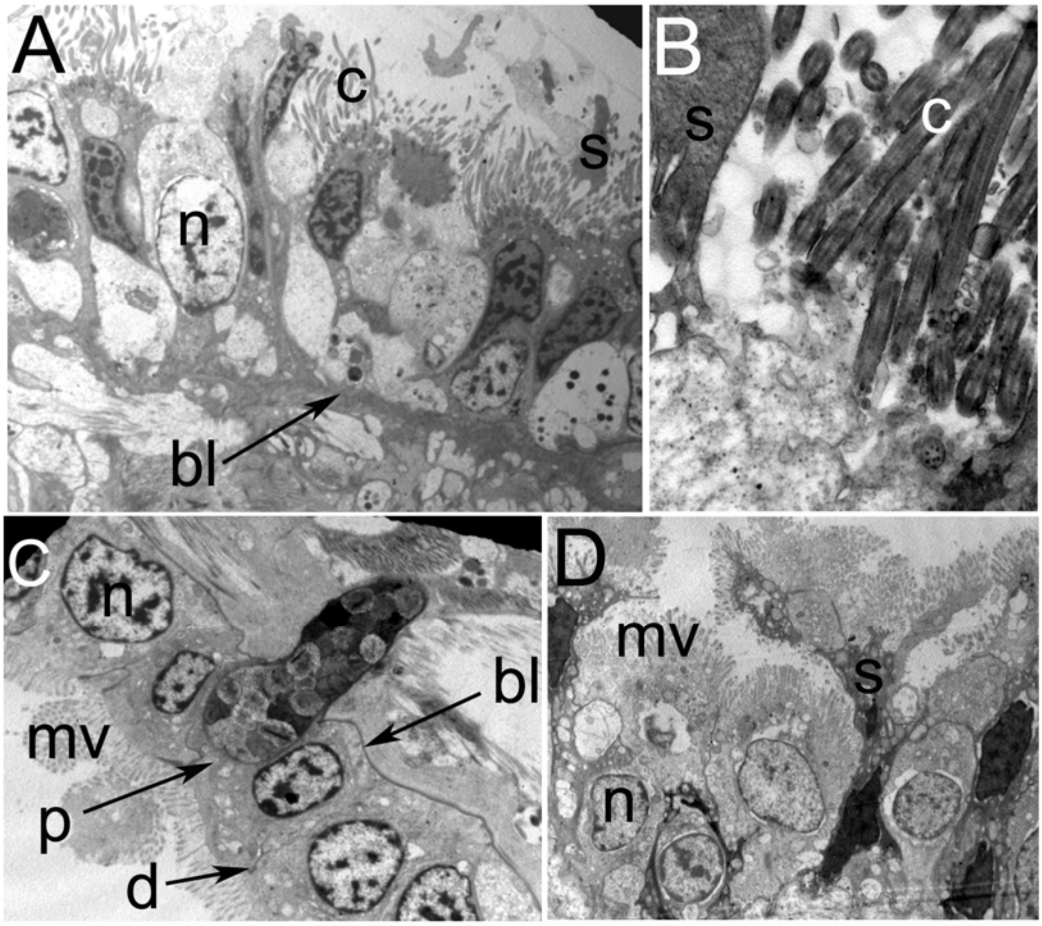

Figure 5.4. Epithelial layer of the epidermis of *T. costatus*.
A. Ciliated epithelial cells (n, nucleus; s, secretion; c, cilia; bl, basal lamina). **B**. Higher magnification of the apical surface of an epithelial cell (c, cilia; s, secretion). **C**. Glandular secretion (Type 3) exiting between epithelial cells (bl, basal lamina; mv, microvilli; d, desmosome; p, pore). **D**. Type 2 secretion exiting between epithelial cells (n, nucleus; mv, microvilli; s, secretion).

Scale bars: A = 10 μm; B = 1 μm; C = 5 μm; D = 5 μm

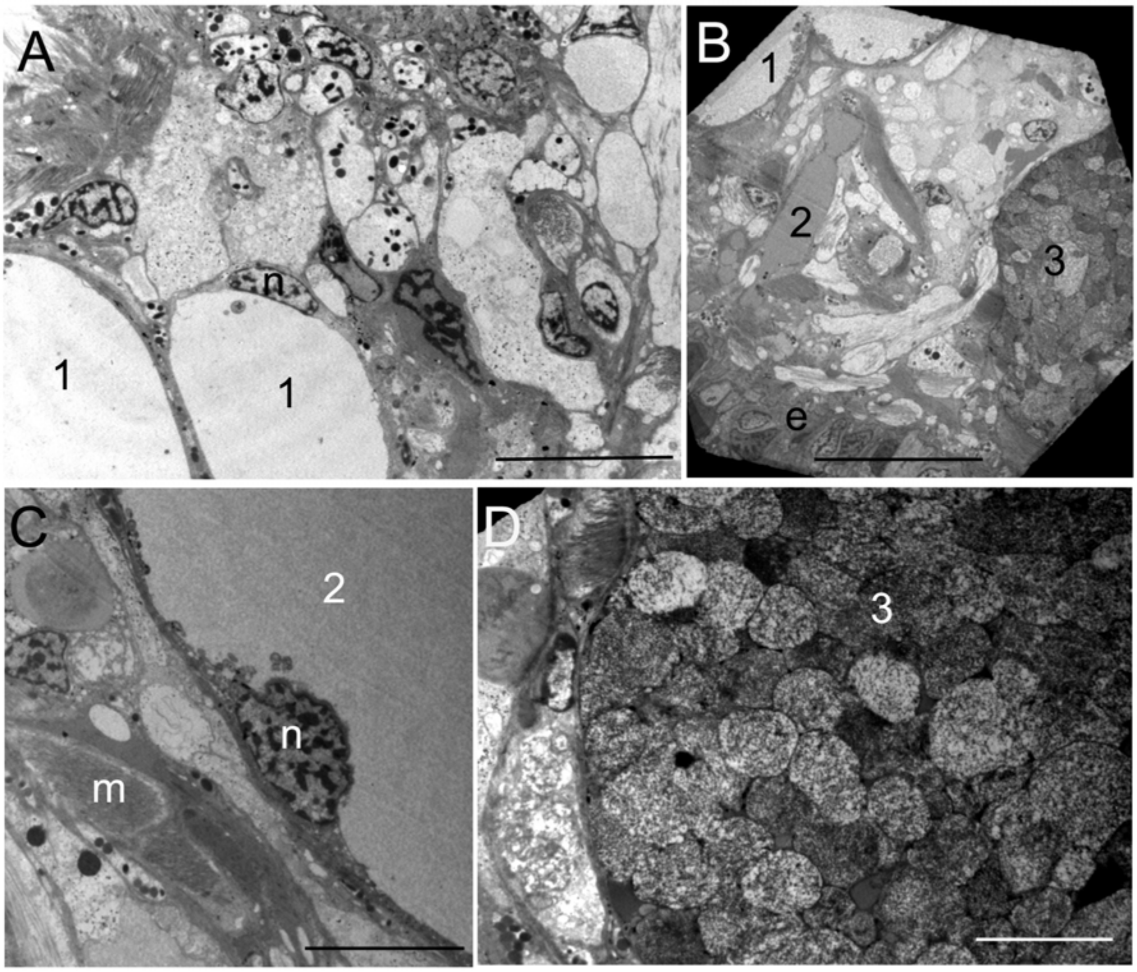

Figure 5.5. TEM of *T. costatus* gland cells.
A.Type 1 gland cells (1), nucleus (n). **B**. Type 1, 2 and 3 gland cells beneath the epithelial layer (e). **C**. The base of a Type 2 cell (2) with its basal nucleus and surrounding muscle fibres (m). **D**. The cell content of a Type 3 gland cell (3).

Scale bars: A = 10 µm; B = 20 µm; C = 10 µm; D = 10 µm

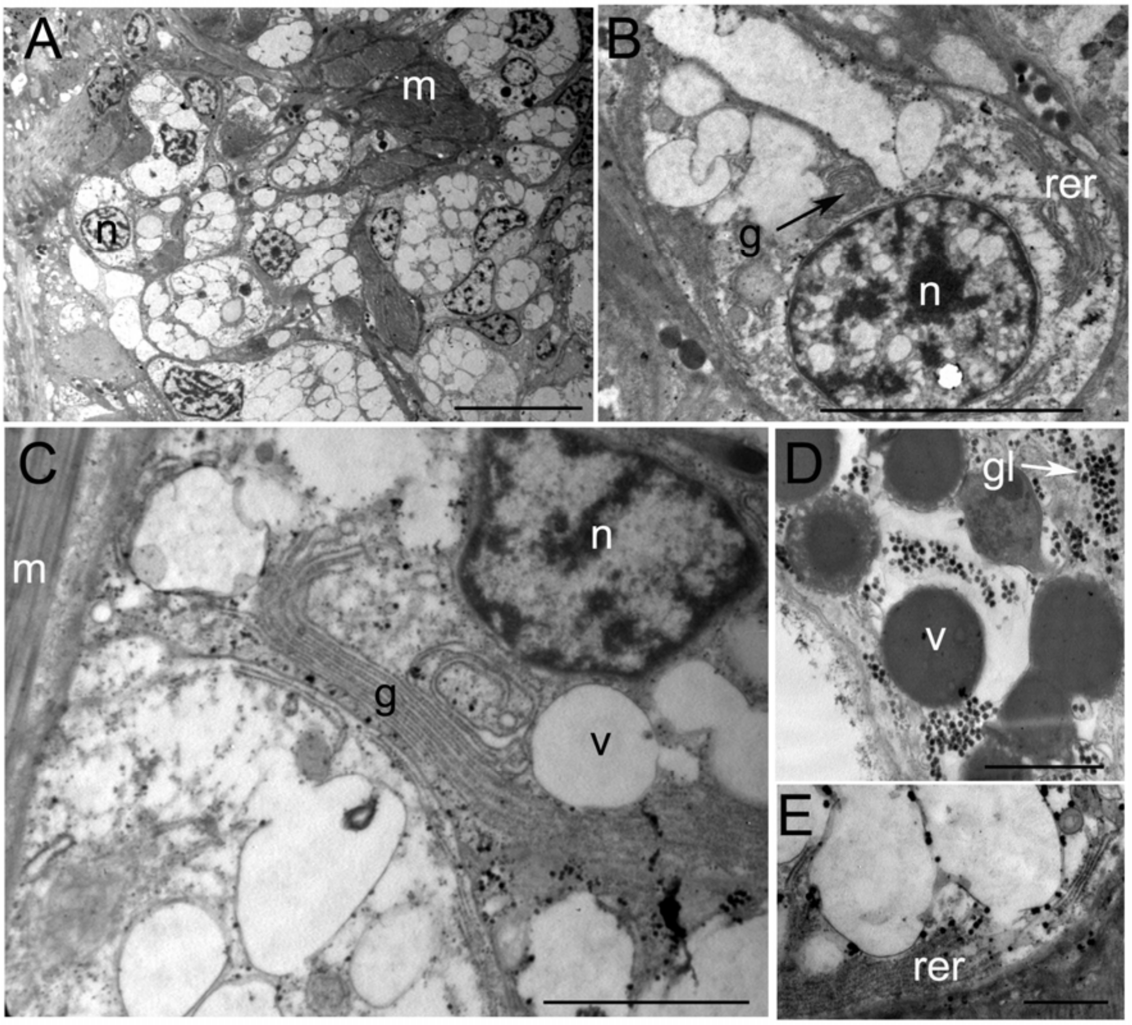

Figure 5.6. TEM of *T. costatus* gland cells.
A. The base of Type 3 gland cells (n, nucleus; m, muscle fibres). **B**. Higher magnification of an individual gland cell (type 3) showing the basal nucleus (n), rough endoplasmic reticulum (rer) and a Golgi body (g). **C**. High magnification of a gland cell (Type 3) showing the nucleus (n), Golgi body (g) and vesicles (v) and muscle fibres (m). **D**. Glycogen (gl) and vesicles (v) at the base of a Type 1 gland. E. Rough endoplasmic reticulum (rer) at the base of a Type 3 gland cell.

Scale bars: A = 10 µm; B = 5 µm; C = 2 µm; D = 1 µm; E = 1 µm.

5.4. Discussion

Trimusculus costatus are sedentary animals that live under rocky overhangs and caves in the lower regions of the littoral zone and could be subject to bacterial attack. Large numbers of bacteria were found in amongst the micovilli. Ireland and Faulkner (1978) found that some species of the genus *Onchidella* produce a defensive compound which is distasteful to predators and acts as an anti-bacterial agent. It is possible that the mucus secreted by *T. costatus* is also an anti-bacterial agent and whilst not totally eliminating bacteria may prevent the accumulation of epibionts on these sedentary limpets. Wägele *et al.* (2006) suggests that opisthobranch mucous secretions play a role in preventing the adhesion of bacteria to the animal and it is possible that the secretions of trimusculids play a similar role. The acidic or sulphated nature of the secretions may help in this role.

The glands of *T. costatus* gave positive results for acid, neutral and sulphated mucins, but negative results for carboxylated mucin. The gland cells and the epithelium stained positively for proteins and peptides. The proteins may play a role in the rhelogical propertites of the mucus (Pawlicki *et al.*, 2003; Smith *et al.*, 2009).

The defensive glands found in the mantle of *T. costatus* are very different to those found in the species of *Siphonaria* and *Onchidella* examined in this study. *Trimusculus costatus* does not have the large multi-cellular glands encapsulated in a well developed muscle layer, but has three different types of individual sub-epidermal gland cells that appear to secrete their contents individually via pores. It is not clear how this is achieved in the absence of a well-developed capsule of muscle as in siphonariids and onchidellids (see Chapters 3 and 4). Nevertheless the foot tissue is highly muscular and general contractions of the body wall may be responsible for mucus extrusion. The mucus, secreted when an animal is stressed, has a repugnatorial or defensive function against certain predators (Rice, 1985; Gray *et al*., 1998). Rice (1985) suggests that the mucus, produced by *T. reticulatus* when the animal is stressed, interferes with the predator's (the seastar *Pisaster giganteus*) ability to capture it by rendering the attacker's tube feet temporarily functionless, rather like a local anaesthetic. This has the effect of making *Trimusculus* hard to detect and makes it difficult for the predator to remove the prey from the substratum to which it is attached. Thus *T. reticulatus* is much more difficult to eat and the predator eventually gives up and searches for easier prey (Rice, 1985).

Trimusculus spp. live in dense populations and this increases the efficiency of the effect of the mucous secretion on the prey (Rice, 1985). Another important consequence of this type of defensive strategy is that it enables the trimusculid to deter predators without moving (Rice, 1985). Trimusculids live upside down on the roofs of caves and on the undersurfaces of rocky overhangs, so they are largely inaccessible to many predators *e.g.* birds, so it is likely that they have developed predator specific defensive secretions and Rice (1985) found that *T. reticulatus* has developed predator-specific defense against sea stars.

The defensive mucous secretions of *Siphonaria* and *Onchidella* contain polypropionates, whilst the active ingredients of *Trimusculus* mucus have been identified as labdane diterpenes (Gray *et al.*, 1998; Darius *et al.*, 2006; Davies-Coleman, 2006).

The gland cells within the foot of *T. costatus* resemble the defensive gland cells found in many species of opisthobranch (Fretter and Graham, 1954; Storch and Welsch, 1972; Thompson, 1983; Thompson and Garhercole, 1986; Thompson, 1988 and Wagele *et al.*, 2006). In addition, the labdane diterpenes from trimusculids resemble those isolated from opisthobranchs (Darius *et al.*, 2006), supporting a close opisthobranch – eupulmonate relationship. Gray (1840, according to Haszprunar, cited by Wägele *et al*., 2008) recognized the close relationship between opisthobranchs and pulmonates, which he combined in the Heterobranchia (or Euthyneura by later authors). More recently Haszprunar (1985, 1988, cited in Ponder and Lindberg, 2008) revived the name Heterobranchia (as cited by Wagele *et al*., 2008). Ultrastructural studies on the sperm structure of *T. costatus* and *T. reticulatus* show characteristics of heterobranch sperm (Hodgson and Healy, 1998). Furthermore the taxonomically useful differences in the dimensions and shape of the acrosome, nucleus and midpiece of the sperm occur between the species and these results support the decision to transfer the Trimusculidae from the Siphonarioidea to a separate superfamily: Trimusculoidea (Hodgson and Healy, 1998). The different chemistry of the defensive secretions of *Trimusculus* and *Siphonaria* (Davies-Coleman and Garson, 1998; Davies-Coleman and Beukes, 2004; Davies-Coleman, 2004; Darias, 2006) and the different ultrastucture of the glands (this study – see Chapter 3) producing these secretions appear to support this decision.

Chapter 6. General discussion

Marine molluscs adopt many different defensive strategies. These include cryptic appearance, the formation of spicules, the uptake of cnidarian nematocysts (opisthobranchs), the incorporation of toxic metabolites from prey and the *de novo* synthesis of defensive chemicals (Wägele *et al*., 2006). It is well documented that many species of marine pulmonate limpet are avoided by predators due to defensive or repugnatorial secretions produced by glands on the lateral pedal regions of the animal (Fretter, 1954; Marcus and Marcus, 1960; deVilliers and Hodgson, 1984; Hodgson, 1999; Wägele *et al*., 2006).

Nearly all opisthobranch and pulmonate species that have been investigated to date have more epidermal glands than just the repugnatorial or defensive glands and the ventral region of the foot in particular is highly glandular because it is used for crawling and adhering to the substratum (Wägele *et al*., 2006). During this study only the lateral epidermal glands, known to produce secretions in response to stress, were examined. All six marine species, from the three different pulmonate orders (Basommatophora, Systellommatophora and Eupulmonata), examined in this study had gland cells between the epithelial cells (intra-epithelial glands) of the epidermis as well as larger sub-epithelial glands (termed mantle dermal formations by Wägele *et al*., 2006).

6.1. A comparison of the intra-epithelial glands

The intra-epithelial glands are single goblet cells that secrete their mucus directly out of the cell, usually via holocrine secretion. These intra-epithelial gland cells are thought to produce the mucus that covers the epidermis and probably plays a role in cleaning the microvilli, facilitating ionic exchange and reducing desiccation (Davies and Hawkins, 1998; Wägele *et al.*, 2006). In addition the mucus makes the animal "slimey" and therefore difficult for some predators to grasp. The epithelial cells of all three groups of pulmonates stained positively for mucopolysaccharides. The intra-epithelial goblet cells stained positively for acidic mucopolysaccharides. It has been suggested that these mucous secretions might have an anti-bacterial function, but this has not been tested (Ireland and Faulkner, 1978; Wägele *et al*., 2006). Bancroft and Gamble (2002) suggested that the mucus prevents bacteria from adhering to the epidermis of animals.

The goblet cells of *Siphonaria* spp. and *Onchidella* spp. were similar in structure and also similar to those described for other molluscs (Watson, 1925; Storch and Welsch, 1972; Wägele *et al*., 2006). The intra-epithelial goblet cells of both the siphonariids and onchidellids examined in this study have a flattened nucleus and a few other organelles (mitochondria, Golgi bodies and lipid droplets) near the base of the cell, the rest of the volume of the cell is filled with an amorphous substance thought to be mucus. Adjacent to the goblet cells are ciliated epithelial cells, although the goblet cells themselves do not appear ciliated.

Whilst the epithelial cells of the epidermis of *Trimusculus costatus* are similar to those of the siphonariids and onchidellids, they posses two types of intra-epithelial gland cells (goblet cells). The first type has a cytoplasm with a granular appearance and well-developed apical microvilli and the second a more globular content with no microvilli. *Trimusculus costatus* are sedentary animals that live under rocky overhangs and caves in the lower regions of the littoral zone and could be more vulnerable to bacterial attack. Large numbers of bacteria were found in amongst the micovilli. Ireland and Faulkner (1978) found that some species of the genus *Onchidella* produce a defensive compound which is distasteful to predators and acts as an anti-bacterial agent. It is possible that the mucus secreted by *T. costatus* is also an anti-bacterial agent and whilst not totally eliminating bacteria may reduce the adhesion of bacteria on these sedentary limpets. Further work is required to determine whether other species of *Trimusculus* have more than one type of goblet cell.

6.2. A comparison of the sub-epithelial glands

Both the siphonariids and the onchidelliids have large multi-cellular, sub-epidermal, marginal glands that must play a role in defence against predators (Watson, 1925; Fretter, 1954; Binot, 1965; Storch and Welsch, 1972; Marcus, 1979; Young et al., 1986, Weiss and Wägele, 1998; Wägele *et al*., 2006; and this study). There are many similarities in the structure of these glands. By contrast trimusculids have defensive glands that are very different to those of the siphonariids and onchidellids (see Table 6.1).

All three species of *Siphonaria* (Basommatophora) examined in this study had multi-cellular, sub-epithelial glands but, in addition, only *S. gigas* had unicellular, sub-epithelial glands (Chapter 3). *Siphonaria gigas* is also the only known species of siphonariid that is palatable to humans. Neither of the two species of Systellommatophora (*Onchidella capensis* and *O. hildae*) had unicellular, sub-epithelial glands, but the only species of Eupulmonata examined in this study (*T. costatus*), had only unicellular sub-epithelial glands (Table 6.1) and no multi-cellular defensive glands.

The multi-cellular, defensive glands of the siphonariids and onchidellids have a number of structural and chemical similarities (Table 6.1). Species from both orders have flask shaped multi-cellular glands with more than one type of secretary cell that release their contents into a central lumen where they mix. The mixed product of the gland cells is then secreted via a duct exiting through a pore in the epidermal layer. The entire gland and its duct are encapsulated in smooth muscle. Both the siphonariids and the onchidellids produce pharmacologically active polypropionate metabolites (Davies-Coleman and Garson, 1998; Table 6.1). The exception to the above features is *S. gigas*. Its glands, whilst multi-cellular, are comprised of one type of gland cell only, the muscle layer is poorly developed and they do not produce polypropionates (Table 6.1).

There are some notable morphological differences in the gland structure between *Siphonaria* spp. and *Onchidella* spp. The glands of the onchidellids are generally larger and have a much thicker, multi-layered muscle capsule surrounding the gland. In addition, they have muscle fibres arranged between the individual gland cells within the capsule. There is also a layer of epithelial cells lining the lumen of the gland (Table 6.1). Siphonariids do not have this inter-cellular muscle nor the epithelial layer lining the lumen of the gland. Another difference between siphonariid and onchidellid glands is that the glands of the latter have a more elongated duct and

O. capensis and *O. hildae* have raised papillae surrounding the pores or gland openings. It is possible that these morphological traits of onchidellids may be derived features associated with shell loss.

The sub-epidermal glands of *Trimusculus costatus* are not only very different in structure to those of the siphonariids and onchidellids, but the chemistry of trimusculid gland secretions is also different (Table 6.1). *Trimusculus costatus* does not have large multi-cellular glands encapsulated in a well developed muscle layer, but has a variety of individual unicellular gland cells, of varying size, that appear to secrete their contents via pores between the epithelial layer. Unlike siphonariids and onchidellids, trimusculids produce secretions containing labdane diterpenes (Davies-Coleman and Beukes, 2004; Darias *et al.*, 2006).

The gland cells within the foot of *T. costatus* resemble the defensive gland cells found in many species of opisthobranch (Fretter, 1954; Storch and Welsch, 1972; Thompson, 1983; Thompson and Gathercole, 1986; Thompson, 1988; Wägele *et al.*, 2006). Furthermore, trimusculid labdane diterpenes also resemble those isolated from opisthobranchs (Darius *et al.*, 2006). Whilst ultrastructural studies on the sperm structure of *T. costatus* and *T. reticulatus* show that they have characteristic heterobranch sperm features (Hodgson and Healy, 1998), differences in sperm structure, gland structure and chemistry of the defensive secretions of *Trimusculus*, when compared to *Siphonaria*, all support the removal of the Trimusculidae from the Siphonarioidea to a separate superfamily: Trimusculoidae (see Hodgson and Healy, 1998). Furthermore it suggests that trimusculids are more closely related to opisthobranchs than the siphonariids and onchidellids.

Table 6.1. The characteristics of the multi-cellular, sub-epidermal glands of some marine pulmonates.

Species	Unicellular sub-epithelial glands	Multi-cellular sub-epithelial glands	External muscle capsule surrounding multi-cellular glands	Muscle between gland cells within multi-cellular glands	Pores with raised papillae	More than one gland cell type within multi-cellular glands	Pharmacologically active poly-propionate derivatives	Pharmacologically active labdane diterpenes	Reference
S. capensis	No	Yes	Yes	No	No	Yes	Yes	No	Marcus, 1979; Davies-Coleman and Beukes, 1999; this study
S. serrata	No	Yes	Yes	No	No	Yes	Yes	No	Davies-Coleman and Beukes,1999; this study
S. gigas	Yes	Yes	No	No	No	No	No	No	Garrity and Levings, 1983; Ortega, 1987; this study
Siphonaria sp.	-	Yes	Yes	No	No	Yes	-	-	Fretter, 1954
O. binneyi	-	-	-	-	Yes	-	Yes	No	Ireland and Faulkner, 1978
O. borealis	Yes	Yes	Yes	-	Yes	-	Yes	No	Young *et al*., 1986; Weiss and Wägele, 1998

O. capensis	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Watson, 1925; this study
O. celtica	-	Yes	Yes	-	Yes	Yes	-	-	Gabe and Prenant, 1950; Binot, 1967
O. floridanum	-	Yes	Yes	-	Yes	Yes	-	-	Arey, 1937; Arey and Barrick, 1942
O. hildae	No	Yes	Yes	Yes	Yes	Yes	Yes	No	This study
O. indolens	-	Yes	-	-	Yes	-	-	-	Weiss and Wägele, 1998
O.marginata	-	Yes	Yes	-	Yes	Yes	-	-	Von Wissel, 1898
O. pulchella	-	Yes	Yes	-	Yes	Yes	-	-	Watson, 1925
T. costatus	Yes	No	No	No	No	-	No	Yes	Gray *et al.*, 1998

T. reticulates	-	-	-	-	-	-	-	Yes	Darias *et al*., 2006
T. conica	-	-	-	-	-	-	-	Yes	Darias *et al*., 2006

6.3. The evolution of defensive glands in marine pulmonates

The Gastropoda is the most speciose molluscan class. The group has radiated enormously in comparison to other molluscan classes and shows great variety in external form, anatomy, behaviour and physiology resulting in the vast diversity of this clade (Ponder and Lindberg, 2008). The Gastropoda also surpass all other molluscan classes in the habitats that they occupy. The first fossils of molluscs date back to the Pre-Cambrian – Cambrian boundary (approximately 550 million years ago). The oldest known heterobranchs have been found in the middle Paleozoic (550 – 350 million years ago) and the group is fairly diverse and abundant in the late Paleozoic. Heterobranchs had diverged from other gastropods by the Devonian period (420 – 350 million years ago) but the relationships of the Paleozoic heterobranchs to the extant ones is unclear (Ponder and Lindberg, 2008).

Pulmonate fossil history is patchy. Pulmonate shells are made of aragonite, which does not preserve well and many of the species are shell-less (*e.g.* onchidellids) or have a reduced shell. In addition, systematic interpretation of known fossils is often extremely difficult as there is much convergence in the shell form of unrelated taxa (Ponder and Lindberg, 2008). Whilst pulmonate phylogenies vary and are still unresolved, most agree that the siphonariids are a basal family within the Pulmonata (Hodgson, 1999; Barker, 2001; Mordan and Wade, 2008), the trimusculids and onchidellids being more derived. Fossil evidence of siphonariids has been found in the middle and upper Jurassic (approximately 200 million years ago), whilst trimusculids appear to be more recent with fossil evidence found only in the Oligocene (approximately 34 million years ago) (Mordan and Wade, 2008). Due to the lack of a shell there is no known fossil evidence for the onchidellids, but as they lack a shell, they probably evolved later than the siphonariids. Studies by Pickford (1995) on East African gastropod fossils however suggest that there has been little evolution, above species level, in the last 20 million years (Ponder and Lindberg, 2008). The fact that the siphonariids are considered closer to the ancestoral position of pulmonates indicates that their multi-cellular glands are less complex when compared to those of onchidellids. Thus the development of a thicker muscle capsule and muscle fibres between the gland cells in the onchidellids is a more derived development. However, because trimusculids have very different glands to those of siphonariids and onchidellids, defensive glands have probably evolved more than once within marine pulmonates.

6.4. Conclusions

1. The lateral epidermis of siphonariids, onchidellids and trimusculids possesses numerous well-developed repugnatorial glands that must contribute to the success of these intertidal animals in warm temperate to tropical habitats where the risk of predation is particularly great.

2. Siphonaria gigas is the only siphonariid species identified to date that does not appear to have pharmacologically active chemicals in the secretions from the lateral epidermal glands. It is also the largest species in this study and it is possible that their large size alone is adequate to deter most predators. Whether *S. gigas* has simpler glands as a result of them loosing their importance as they increased in size, or never produced defensive secretions, remains unknown.

3. The similarity in the structure of the multi-cellular glands and the chemical secretions of the Onchidellidae and Siphonariidae suggests that these two taxa are more closely related to each other than to the Trimusculidae although this hypothesis is not supported by current phylogenies.

4. Differences in the structure of the glands between the Onchidellidae and Siphonariidae are possibly related to the fact that onchidellids are shell-less and are therefore more reliant on gland secretions for defence.

4. The differences in the gland structure and chemistry of trimusculids, when compared to siphonariids and onchidellids, suggest that defensive glands have evolved more than once within marine pulmonates. This may be in response to different defensive needs.

5. In Chapter 1 the following hypotheses were proposed:

A. As the taxa studied are from different pulmonate orders there will be differences in the gland structure that could provide phylogenetic characters.
B. It is already known that the mucus of siphonariids and onchidellids contains polypropionate metabolites, but that of the trimusculids contains labdane diterpenes, so a difference in the structure of the glands is predicted.

The results of this study support the first hypothesis to a certain extent, but at this stage it is unlikely that the features of the gland structure of the different species are sufficient to provide phylogenetic characters at the species level.

The structure of the multi-cellular glands of the polypropionate producing siphonariids and onchidellids were very different to the labdane diterpene producing trimusculids, supporting the second hypothesis. Further work on a wider variety of species from each genus and different genera within the Siphonarioidea, Onchidioidea and Trimusculoidae is now needed to test these hypotheses more rigorously.

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Appendix I

Bouin's fluid:

Saturated aqueous picric acid solution	75 ml
40% formaldehyde	25 ml
Glacial acetic acid	5 ml

Fixation may vary from a few hours to 18 hours.

Embedding:

Day 1:

1. Dehydrate in 70% ethanol	1 hour
2. Dehydrate in 95% ethanol	1 hour (2x)
3. Dehydrate in 100% ethanol	1.5 hours (3x)
4. Leave in 100% ethanol overnight.	

Day 2:

1. Xylene	1 hour
2. Xylene	1.5 hours
3. Immerse in Paraplast wax (under vacuum)	1 hour
4. Change to fresh Paraplast wax (under vacuum)	1.5 hours
5. Change to fresh Paraplast wax (under vacuum)	2 hours
6. Embed in Paraplast wax and leave to set.	

Sectioning of wax embedded material was carried out on a Leica RM 2035 microtome and the sections were then stained for histology.

1. Toluidine blue

Semi-thin (or "survey") sections were stained with Touidine blue to determine the general tissue structure. The differential staining intensities produced are quite effective at demonstrating tissue and cellular components, providing a means of screening samples to determine the presence of features within the block and select relevant areas for thin sectioning.

Method (Cross 2001):

1. Semi-thin sections were covered with a 1% Toluidine blue dissolved in 2.5% aqueous sodium carbonate solution on a hot plate (70º -80º Centigrade) for approximately 30 seconds.
2. Wash in running water and allow to dry.
3. The sections can be viewed as is, or dehydrated through an ethanol series, dried and mounted with Gurr's DPX mounting medium.

Results

Neutral mucopolysaccharides = blue
Acid mucopolysaccharides = red to violet

2. Combined alcian blue-PAS technique for acid and neutral mucins

Acid mucins and neutral mucins are clearly separated by this technique, which is also a useful technique to demonstrate the presence of any mucins. The rational is that by first staining all acid mucins with the alcian blue, those acid mucins which are also PAS-positive will not react in the subsequent PAS reaction; only the neutral mucins will, resulting in a good colour distinction between acid and neutral mucins.

1.Wax embedded tissue was sectioned using a Leica RM2035 microtome.
2. The sections were then dewaxed and brought to water,
3. Stained for 5 minutes in a solution of alcian blue (1% alcian blue in 3% aqueous acetic acid),
4. Washed in water
5. Followed by washing in distilled water.
6. Stained for 5 minutes in 1% aqueous periodic acid.
7. Rinse well in distilled water.

8. Stained for 15 minutes in Schiff's reagent
9. Washed in running tap for 10 minutes
10. Stained lightly in haematoxylin (Mayer's): differentiated and blued
11. Washed in water
12. Rinsed in absolute ethanol
13. Cleared in xylene and mounted.

Results:

Acid mucins = blue
Neutral mucins = magenta
Mixture of the above = the colour will depend on the dominant entity and will range from blue-purple through purple to a violet or mauve colour
Nuclei = pale blue

3. Combined aldehyde fuchsin – alcian blue method

This technique is a reliable means of separating sulphated from carboxylated mucins. The rationale depends on the greater affinity of aldehyde fucshin for sulphated mucins, so by first staining with this solution they are stained purple and by subsequently staining with alcian blue, the carboxylated forms only will be stained blue.

Preparation of the stain:

Aldehyde fuchsin solution

Basic fuchsin	1 g
Paraldehyde	2 ml
Concentrated hydrochloric acid	1 ml
Ethanol	60 ml
Distilled water	40 ml

Dissolve the basic fuchsin in the alcohol-distilled water. Add the hydrochloric acid and the paraldehyde. Allow to 'ripen' for 2-7 days at room temperature, then filter. Store at 4 º C.

Alcian blue:

Alcian blue	1 g
3% acetic acid	100 ml

Resulting pH = 2.5

Method

1. Dewax sections and a positive control and bring to water.
2. Aldehyde fuchsin solution, 20 minutes
3. Rinse well in 70% ethanol, then in water
4. Alcian blue solution, 5 minutes
5. Rinse in water
6. Dehydrate in absolute ethanol
7. Clear in xylene and mount in DPX mountant

Results

Sulphated mucins = purple
Carboxylated mucins = blue

4. Southgate’s mucicarmine

Mucins are specifically stained, although failure of the tissue to stain does not rule out these substances. Neutral and some of the strongly acidic sulphated mucins fail to show appreciable staining. “The rationale underlying the remarkable specificity of mucicarmine for mucins is not fully understood. The probable mechanism is that the aluminium salts in the solution form a chelate compound with the carmine, thus conferring a net positive charge on the molecule and consequent binding to the tissue polyanions.” (Bancroft and Gamble, 2002). It has been suggested that this specific affinity of mucicarmine is at least partly due to its large molecular size. This would allow the dye complex to penetrate and bind to acidic substrates of low density *i.e.* mucins: other acidic substances such as nucleic acids are of a high density and so exclude the mucicarmine.

Preparation of the stain:

Grind 1g carmine and place in a large (500 ml volume) conical flask. Add 100 ml of 50% alcohol and mix. Add 1g aluminium hydroxide, mix, and add 0.5 g anhydrous aluminium chloride. Mix and boil gently for 2.5 minutes. Cool, filter and store at 4 degrees C.

Method

1. Dewax sections and bring to water.
2. Stain the nuclei with one of the conventional haematoxylin stains (not Ehrlich’s haematoxylin). Differentiate well, and blue in the usual way.
3. Mucicarmine, 20 minutes
4. Wash in water
5. Rinse in absolute ethanol
6. Clear in xylene and mount

Results

Mucins = red
Nuclei = blue
Background = unstained

5. Haematoxylin and eosin stain for paraffin sections

Due to its comparative simplicity and ability to demonstrate an enormous number of different tissue structure, haemotoxylin and eosin is probably the most widely used histological stain. Essentially the haemotoxylin component stains the cell nuclei blue/black, with good intranuclear detail, while the eosin stains cell cytoplasm and most connective tissue fibres in varying shades and intensities of pink, orange and red.

Method

1. Dewax sections, hydrate through graded alcohol series to water.
2. Remove fixation pigments if necessary.
3. Stain in alum haemotoxylin of choice for a suitable time
4. Wash well in running water until sections “blue” for 5 minutes.
5. Differentiate in acid alcohol (15% HCl in 70% ethanol) for 5 –10 seconds.
6. Wash well in tap water until sections are again “blue” (10 – 15 minutes).
7. Stain in 15 eosinY for10 minutes.
8. Wash in running water for 1-5 minutes.
9. Dehydrate though alcohols, clear and mount.

Results

Nuclei = blue black
Cytoplasm = varying shades of pink
Muscle fibres = deep pink/red
Red blood cells = orange/red
Fibrin = deep pink

6. Masson’s trichrome technique

The term “trichrome stain” is a general name for a number of techniques for the selective demonstration of muscle, collagen fibres, fibrin and erythrocytes. When the protein component of a tissue is exposed to a fixative an interaction occurs between the protein chains and the fixative. The nature of the reaction and the end result will vary according to the exact composition of the protein and the fixative used. A three-dimensional insoluble protein network is formed, different proteins will form networks with different physical characteristics, including different pore sizes. Trichrome staining uses the different pore sizes of different proteins, and the subsequent different reactions, to differentiate between the different tissue types. Three stains are used, one of which may be a nuclear stain.
In order to achieve adequate and even staining of the connective tissue fibres, dyes utilised in the trichrome method are prepared as low pH solutions (pH 1.5 to pH 3.0).

Fixation

Bouin's fixative

Solution A

Acid fucshin	0.5 g
Glacial acetic acid	0.5 ml
Distilled water	100 ml

Solution B

Phosphomolybdic acid	1.0 g
Distilled water	100 ml

Solution C

Methyl blue	2.0 g
Glacial acetic acid	2.5 ml
Distilled water	100 ml

Method

1. Deparaffinise sections and bring to water.
2. Remove mercury pigment by iodine, sodium thiosulfate sequence.
3. Wash in tap water.
4. Stain nuclei by Celestin blue-haematoxylin method.
5. Differentiate with 1% acid alcohol.
6. Wash well in tap water.
7. Stain in acid fuchsin solution A, 5 minutes.
8. Rinse in distilled water.
9. Treat with phoshomolybdic acid solution B, 5 minutes.
10. Drain.
11. Stain with methyl blue solution C, for 2 – 5 minutes.
12. Rinse in distilled water.
13. Treat with 1% acetic acid 2 minutes.
14. Dehydrate through alcohols.
15. Clear in xylene, mount in permanent mounting medium.

Results

Nuclei	Blue-black
Cytoplasm, muscle and erythrocytes	Red
Collagen	Blue

7. Bromophenol blue (Humason, 1979)

Fixation

Bouin’s fixative

Solution:

Mercuric chloride	10.0 g
Bromophenol blue	100.0 g
Distilled water	100.0 ml

Method:

1. Deparraffinise sections.
2. Transfer to absolute alcohol: 1 – 2 minutes.
3. Hydrate slides to water.
4. Stain in Bromophenol solution: 15 minutes.
5. Wash in aqueous acetic acid: 20 minutes.
6. Immerse in tap water: 3 minutes.
7. Dehydrate rapidly, clear and mount.

Results

Blue = proteins and peptides not removed by washing.