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The ultrastructure and histology of the perinotal epidermis and defensive glands of two species of *Onchidella* (Gastropoda: Pulmonata)

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ABSTRACT

Histology and electron microscopy were used to describe and compare the structure of the perinotal epidermis and defensive glands of two species of shell-less marine Systellommatophora, *Onchidella capensis* and *Onchidella hildae* (Onchidiidae). The notum of both species is composed of a layer of epithelial and goblet cells covered by a multi-layered cuticle. Large perinotal multi-cellular glands, that produce thick white sticky mucus when irritated, are located within the sub-epidermal tissue. The glands are composed of several types of large secretory cell filled with products that stain for acidic, sulphated and neutral mucins, and some irregularly shaped support cells that surround a central lumen. The products of the secretory cells are produced by organelles that are basal in position. The entire gland is surrounded by a well-developed capsule of smooth muscle and collagen, and in addition smooth muscle surrounds the cells within the glands. Based on the size of the gland cells, their staining properties, and the appearance of their stored secretions at the transmission electron microscope level, five different types of secretory cells were identified in *O. capensis* and four in *O. hildae*. The products of these cells, which are released by holocrine secretion, presumably mix in the lumen of the duct as they are forced out by contraction of the smooth muscle. The structural similarity of these glands to those of siphonariids, suggest that they have a common ancestry.

1. Introduction

The Onchidioidea, of the sub-order Systellommatophora, are a superfamily of mainly marine intertidal, shell-less pulmonates (Britton, 1984), although there has been debate as to their higher taxonomic placement (Marcus and Marcus, 1956; Jensen, 1992; Dayrat, 2009). Current phylogenies place the Onchidioidea within the Pulmonata (Mordan and Wade, 2008; Dayrat, 2009). One family, the Onchidiidae, with 19 genera, one of which is *Onchidella*, is currently recognised within the Onchidioidea (Dayrat, 2009).

Members of the Onchidiidae are usually 10–70 mm long, oval in shape with a broad, large foot (flanked by the hyponotum) and a papillate or tuberculate notum. The notum is covered by a cuticle that may also contain siliceous spicules (Marcus, 1979; Smith and Stanisic, 1998). Although the Onchidiidae are common in some habitats, little is known about their biology and ecology. Most onchidiids live amphibiously in the eulittoral zone of sandy, muddy and rocky shores as well as estuaries, where they probably feed on the organic film of algae, diatoms and bacteria on the surface of the substratum (e.g. Arey and Crozier, 1921; Watson, 1925; Fretter, 1943; Wägele et al., 2006; Dayrat, 2009). *Onchidella* spp. avoid direct sunlight and prefer to forage at low tide when the sky is overcast (Arey and Crozier, 1921; Pepe and Pepe, 1985; Weiss and Wägele, 1998). The Onchidiidae are chiefly found throughout the tropics of the Pacific and Indian oceans (Stringer, 1969; Kenny and Smith, 1987; Barker, 2001; Dayrat, 2009). The genus *Onchidella*, however, whilst having an extensive global distribution including temperate habitats, is absent from the tropical Indo-West Pacific (Dayrat, 2009).

Like other shell-less molluscs, the Onchidiidae are potentially vulnerable to attack by predators. *Onchidella* spp. appear to avoid predation, however, by producing secretions from glands sited along the edge of the notum. Because of their position these glands have been referred to as perinotal glands (Watson, 1925; Marcus, 1979). These glands may also be situated in erectile papillae which are not obvious in the undisturbed animal, but are readily distinguished when it is disturbed (Arey, 1937; Arey and Barrick, 1942; Young et al., 1986). In response to stimulus (physical, chemical and electrical) the glandular secretions are discharged simultaneously or singly, the secretion being released as a stream of milky fluid (Fretter, 1943) which is acidic and burning to the taste (Arey and Barrick, 1942). Young et al. (1986) showed that the secretion from the repugnatorial perinotal glands of *Onchidella borealis* repelled intertidal predatory asteroids, and whilst intertidal crabs ate dead *O. borealis*, they did not consume live ones that are capable of

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releasing their defensive secretion. Chemical analysis of secretions from *Onchidella binneyi* and six species of the genus *Onchidium* has revealed that the glands contain isomeric polypropionates (Ireland and Faulkner, 1978 and see Darias et al., 2006 for review), and it is likely that these are the compounds that deter predators. Relatively little is known about the structure and histology of the glands of onchidellids. Most studies to date on gland structure are brief light microscope descriptions or illustrations mainly using routine histological stains. Joyeux-Laffuie (1882) was the first to describe epidermal glands in his study of *Onchidella celtica* (as *Oncidium celticum*). Von Wissel (1898) described similar glands in *Onchidella marginata* and *Onchidella juan-fernandeziana* (as *Oncidiella* spp.). Other light microscope studies of glands of *Onchidella* include those of Watson (1925) on *Onchidella pulchella* and *Onchidella capensis*, Arey and Barrick (1942) on *Onchidella floridana* (as *Onchidium floridanum*), Fretter (1943), Gabe and Prenant (1950) as well as Binot (1965) and Weiss and Wägele (1998) on *Onchidiella celtica*, Marcus (1979) on six species of *Onchidella* (*O. celtica*, *O. incisa*, *O. indolens*, *O. accrensis*, *O. capensis* and *O. philippei*) and Young et al. (1986), Weiss and Wägele (1998) and Wägele et al. (2006) on *Onchidella borealis*. Whilst Arey and Barrick (1942) observed that the glands of *Onchidella floridana* were composed of seven different types of cells, Binot (1965) only recognised five types in *Onchidella celtica* (as *Onchidiella*).

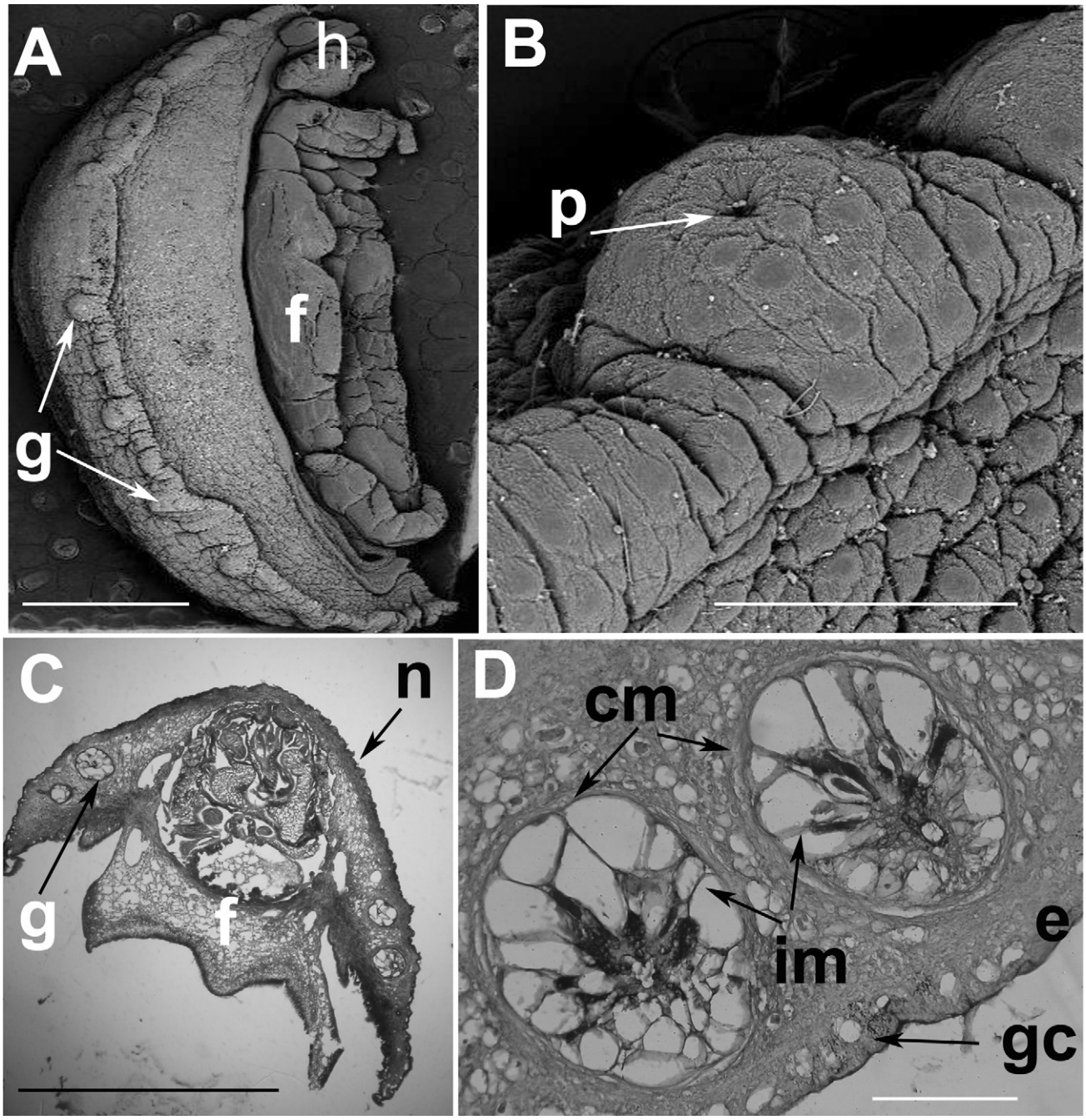


Fig. 1. Scanning electron microscopy and light microscopy of *Onchidella capensis*. (A) Lateral view; SEM image of the perinotal glands (g, gland; h, head; f, foot). (B) A higher magnification image of an individual papilla and gland pore (p). (C) Light micrograph of a mid-transverse section showing the position of the glands embedded in the perinotal region (g, gland; n, notum; f, foot), stained with toluidine blue. (D) 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.

Table 1
Summary of the histochemical staining results of the perinotal glands of two species of *Onchidella*.

	Mucopolysaccharides	Acid mucins	Neutral mucins	Sulphated mucins	Carboxylated mucins	Protein	Collagen	Muscle fibre
Onchidella capensis	+	+	+	+	–	+	+	+
Onchidella hildae	+	+	+	+	–	+	+	+

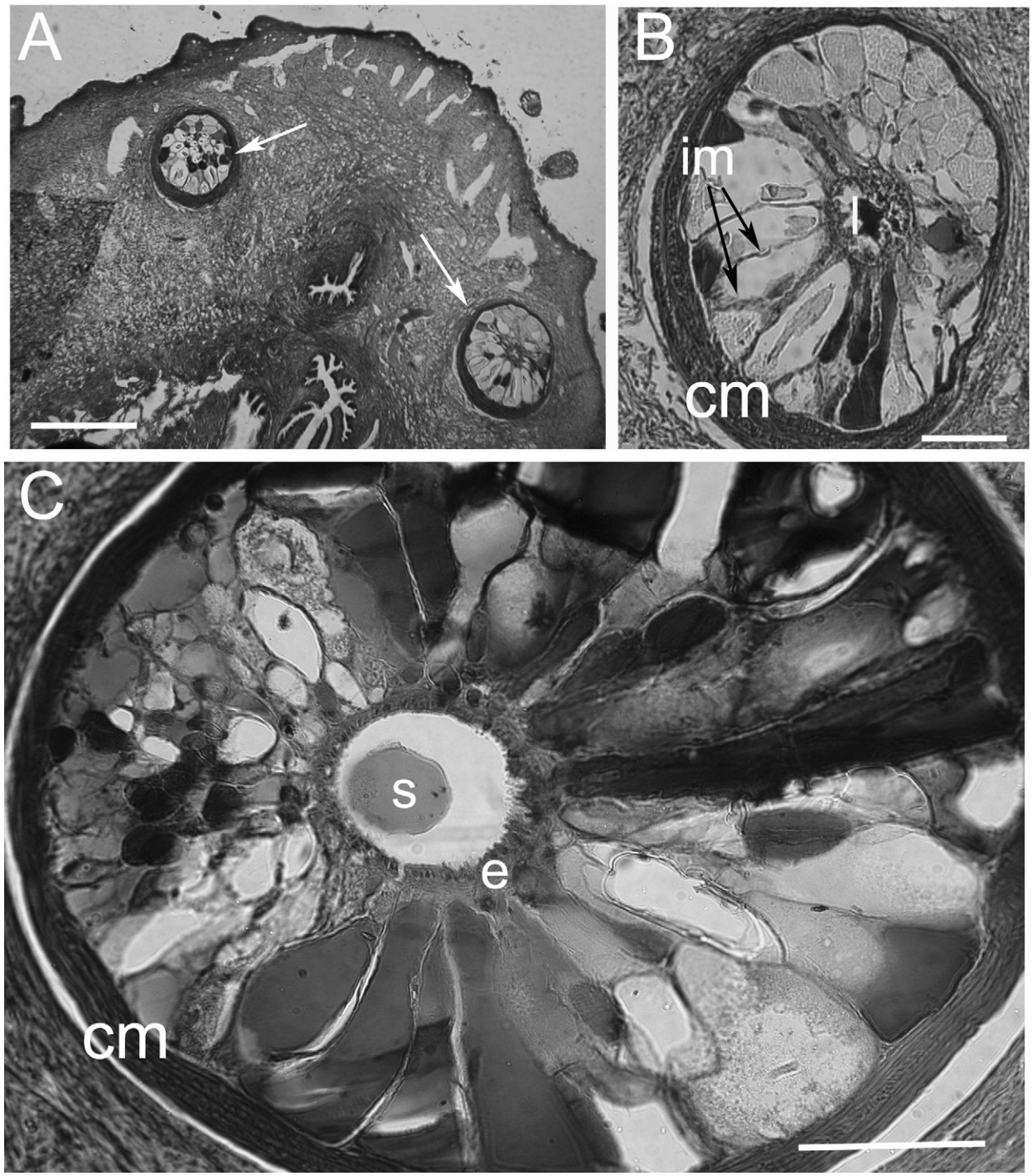
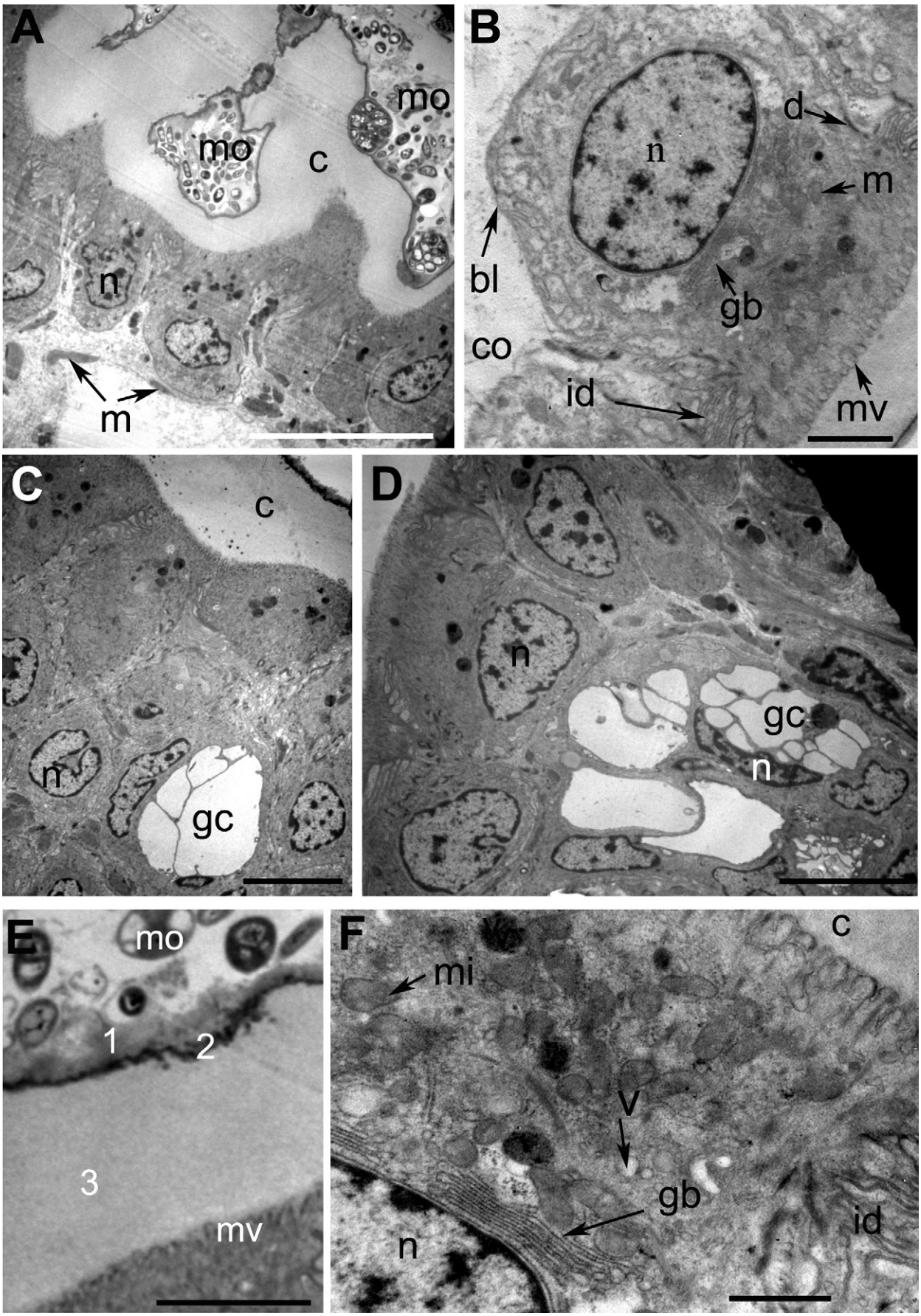


Fig. 2. Light microscopy of the perinotal glands of *O. hildae*. (A) Light microscope image of a section through the body of *O. hildae* (stained with Masson's trichrome) showing the position of the multi-cellular glands in transverse section (arrows). (B) Light microscope image of a transverse section 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) 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 epidermal layer surrounding the lumen (e). Scale bars: A = 1 mm; B = 200 μm; C = 100 μm.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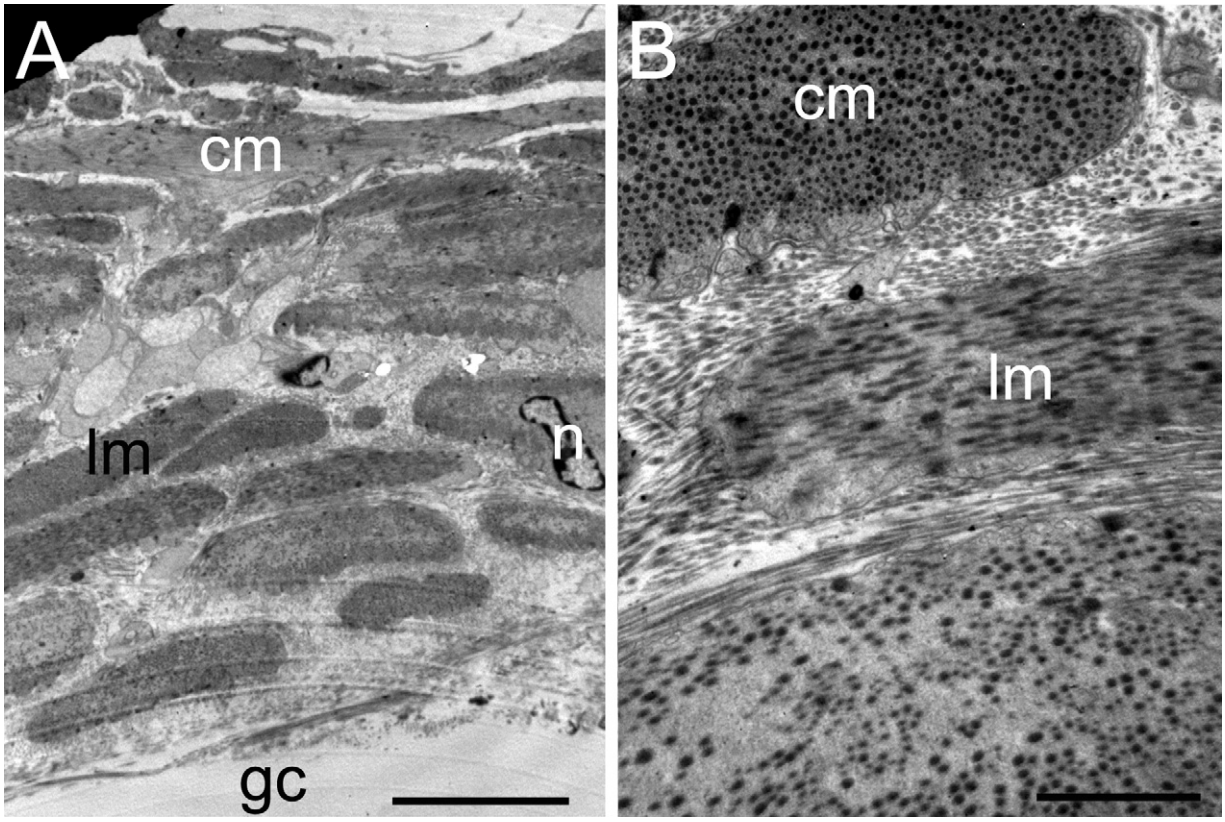


Fig. 4. Transmission electron microscopy images of *O. capensis* muscle capsule. (A) Cross-section of the muscle capsule surrounding the multi-cellular gland showing the longitudinal (lm), the circular (cm) smooth muscle fibres and nucleus (n). (B) A higher magnification of the longitudinal muscle (lm) and the circular muscle (cm). gc, gland cell. Scale bars: A = 5 μm and B = 1 μm.

As morphological details at the ultrastructural level of these important defensive structures are lacking, this study aimed to provide a more detailed description of the structure of the perinotal glands of onchidellids using both light and electron microscopy. Two species were available for study, *Onchidella capensis* and *Onchidella hildae*.

2. Materials and methods

2.1. Materials

Onchidella capensis Watson, 1925 was collected from underneath rocks in the intertidal zone at Kommetjie on the Cape Peninsula (33°S, 18°E) South Africa. *Onchidella hildae* (Hoffman, 1928) was collected from Naos Island on the Pacific coast of Panama (9°N, 80°W). The live animals (15 *O. capensis*, 5 *O. hildae*) were transported back to the laboratory and tissue from the lateral, marginal regions of the notum of both species was prepared for histology, scanning, and transmission electron microscopy.

2.2. Light microscopy

Tissues from five *O. capensis* and three *O. hildae* were fixed for at least 24 h in aqueous Bouin's solution, dehydrated and embedded in Paraplast® Plus via xylene. Sections (5–7 μm thick) were cut on a Leica RM2035 microtome. Some sections were stained in haematoxylin and eosin to show the general structure and distribution of the glands. To examine whether connective and muscle tissues were associated with the glands, other sections were stained in Masson's trichrome. In addition, semi-thin sections (approximately 1 μm thick) of tissues that had been prepared for transmission electron microscopy (see below) were also cut. Mucosubstances were stained in toluidine blue (semi-thin resin sections), alcian blue (pH 1.5 and 2.5), periodic acid Schiff (AB–PAS) for differentiating between acid and neutral mucins, and aldehyde fuschin–alcian blue (AF–AB) for separating sulphated and carboxylated mucins. For the detection of proteins, sections were stained with mercuric bromophenol blue. All staining protocols were taken from Humason (1979) and Bancroft and Gamble (2002). Gland dimensions (maximum length and width) were obtained by taking digital images of sections, using an Olympus Camedia digital camera mounted on an Olympus BX50 microscope. Images were then analysed using the image analysis software, AnalySIS Version 3 (Soft Imaging System GmbH).

2.3. Scanning electron microscopy

Since only five specimens of *Onchidella hildae* were collected, scanning electron microscopy of this species was not undertaken.

Fig. 3. Transmission electron microscope images of the epidermal layer in the perinotal region of *O. capensis*. (A) The epidermal layer showing the epidermal cells with nuclei (n), cuticle (c), micro-organisms (mo) and muscle fibres (m). (B) An individual epidermal cell showing the nucleus (n), the Golgi bodies (gb), mitochondria (m), interdigitation (id), desmosome (d), collagen (co) and basal lamina (bl). (C) Epidermal layer and the underlying goblet cell (gc) with its basal nucleus. (D) The sub-epidermal region with a unicellular gland cells (gc) and nucleus (n). (E) Image of the three layers of the cuticle (1, electron-lucent outer layer; 2, electron-dense middle layer; and 3, electron-lucent inner layer), micro-organisms (mo) on the surface, and the microvilli (mv) 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.

For scanning electron microscopy of *O. capensis*, five individuals were placed directly into cold (4 °C) 2.5% glutaraldehyde in filtered seawater. After 12 h fixation the animals were rinsed in cold (4 °C) 0.2 M sodium cacodylate buffer (pH 7.2), dehydrated through an ethanol series (30–100%) and then critical point dried using a Polaron critical point dryer. Specimens 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 20 kV). All quantitative analyses were done using the Scandium SEM Imaging Platform (based on AnalySIS FIVE).

2.4. Transmission electron microscopy

Small pieces of tissue from several regions of the perinotum (lateral epidermal tissue) from five individuals of *O. capensis* and two *O. hildae* were fixed in 2.5% glutaraldehyde in filtered seawater (4 °C) for 12 h. Tissues were then rinsed in 0.2 M sodium cacodylate buffer (pH 7.2) followed by secondary fixation in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer for 90 min. After rinsing in 0.2 M sodium cacodylate buffer, tissues were dehydrated through a graded ethanol series (30–100%) and embedded in an Araldite-Taab 812 resin mixture (Cross, 1989) via propylene

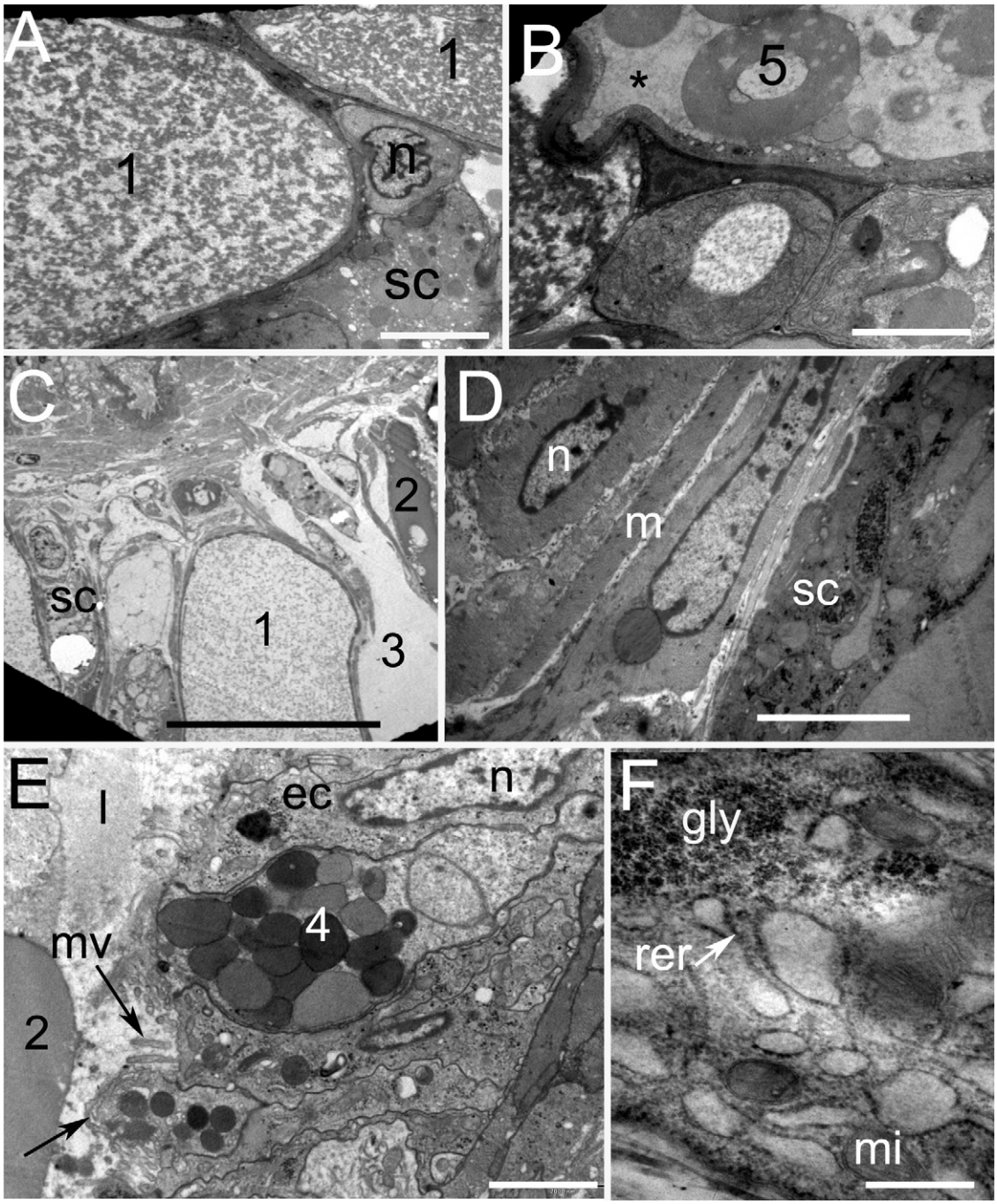


Fig. 5. Transmission electron microscopy images of the perinotal multi-cellular glands of *O. capensis*. (A) Type 1 gland cells (1) and support cell (sc) with nucleus (n). (B) Type 5 gland cell (5) showing vesicles in granular matrix (*). (C) Type 1 (1), 2 (2) and 3 (3) gland cells and a support cell (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). Arrow shows product being secreted. (F) High magnification of a support cell showing mitochondria (mi), rough endoplasmic reticulum (rer) and glycogen (gly). Scale bars: A = 5 μm; B = 2 μm; C = 20 μm; D = 2 μm; E = 2 μm and F = 0.5 μm.

oxide. 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 1% toluidine blue. After determining the correct region of tissue using the semi-thin sections, ultra-thin sections (silver/gold interface) were cut using a Microstar diamond knife. The thin sections were stained in a 5% aqueous solution of uranyl acetate for 30 min followed by lead citrate for 5 min. Sections were then viewed in a Jeol 1210 TEM at 90 kV.

3. Results

3.1. General observations and scanning electron microscopy

Onchidella capensis has about 12 marginal glands on either side of the body (Watson, 1925). These glands are visible to the naked eye as slightly raised papillae that are lighter in colour. The pores of the glands (each approximately 15 μm in diameter) are situated on raised papillae along the lateral edge of the notum (Fig. 1A and B). No pores were visible on the notum due to the presence of the thick cuticle (see Section 3.2) which extends into the region of the hyponotum.

3.2. Light microscopy

3.2.1. Onchidella capensis

Onchidella capensis has large, pear-shaped, sub-epidermal multi-cellular glands. These glands contain some cells that stain positively for mucins and others that do not stain at all. The glands are situated within the thick marginal tissue of the notum (Fig. 1C) and have a duct leading to a pore at the mantle edge (not illustrated). Of 30 glands measured, the largest were 0.35 mm in

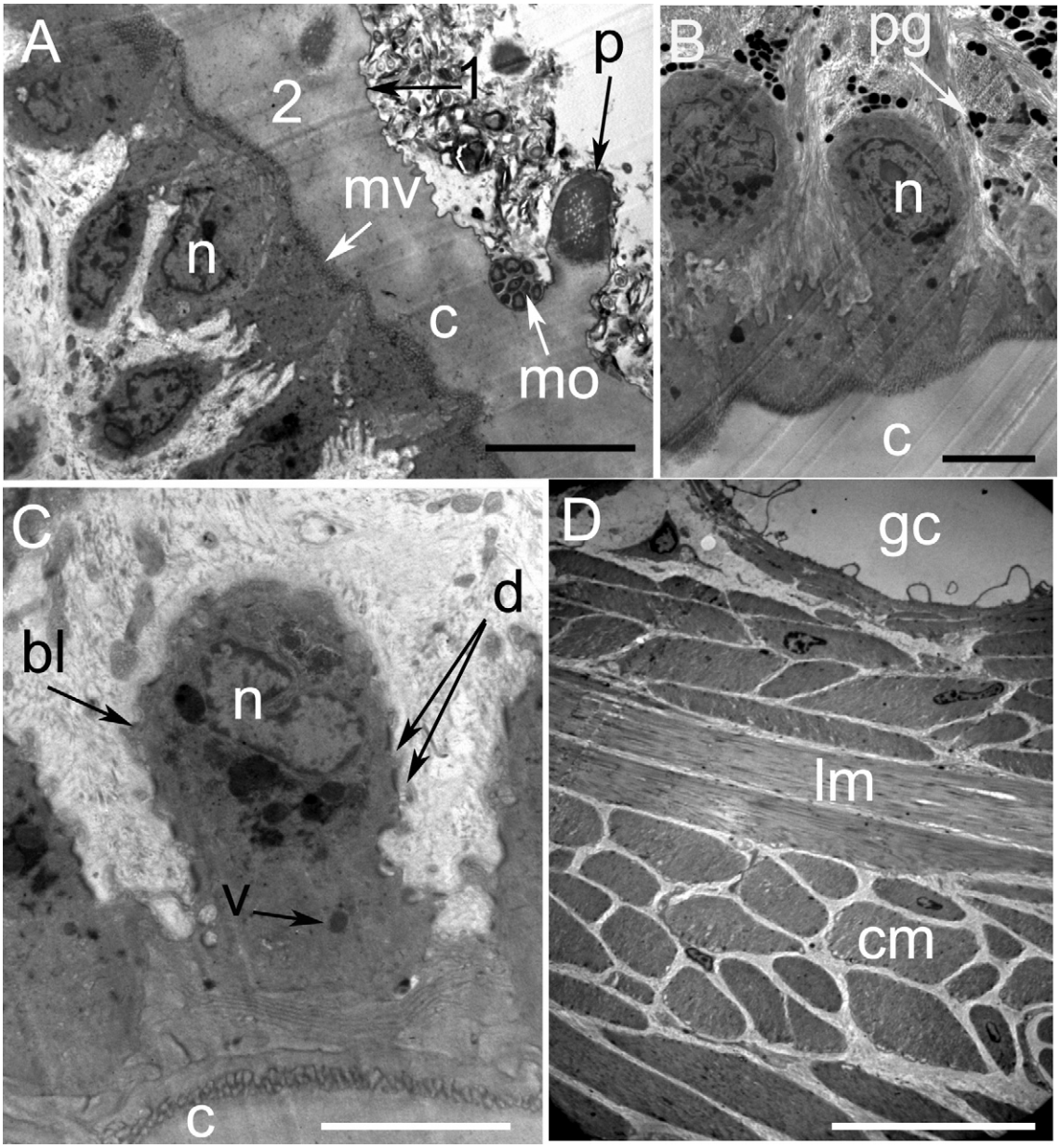
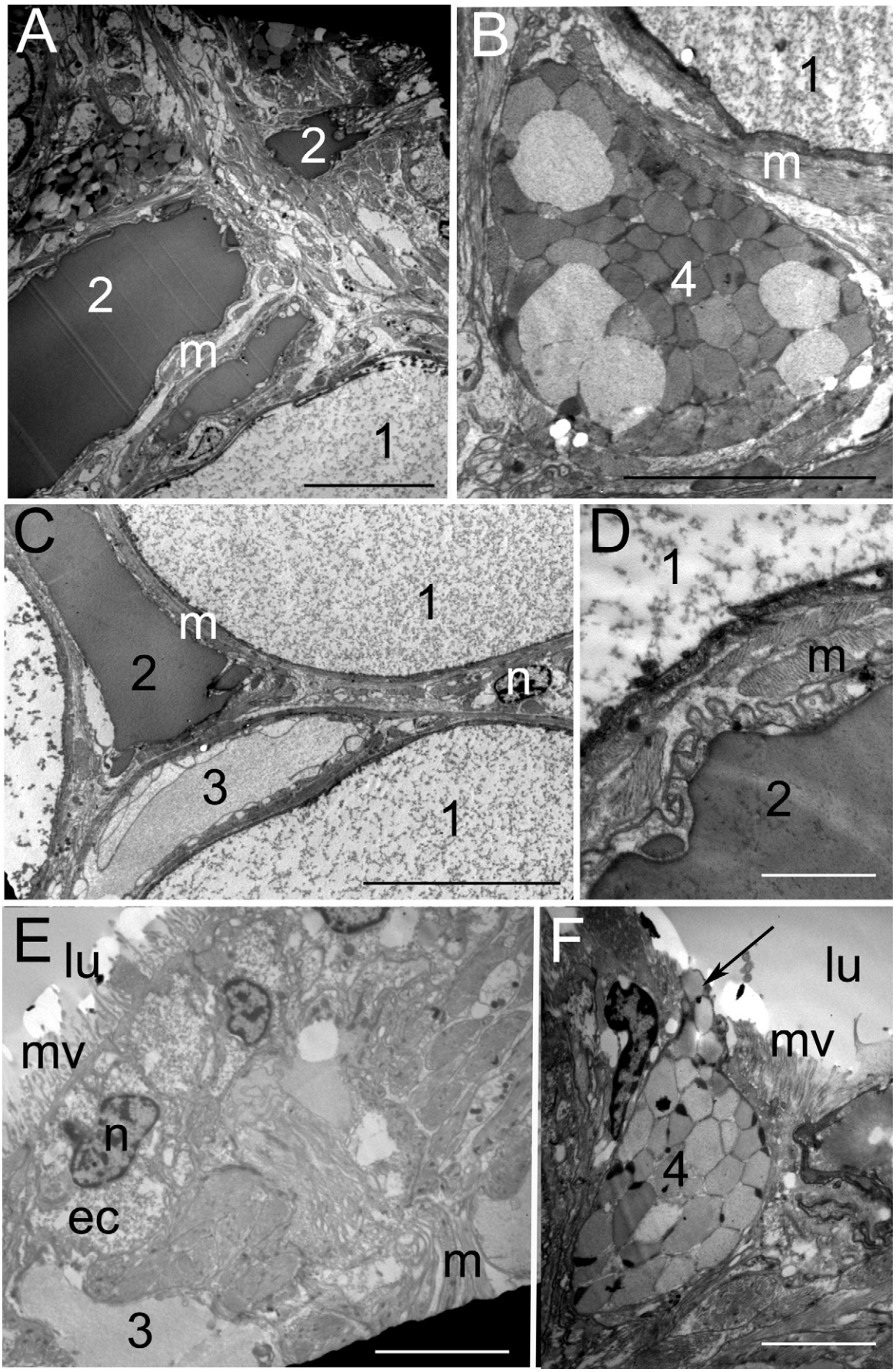


Fig. 6. Transmission electron microscopy images of *O. hildae* epidermis. (A) Epithelial cells with nuclei (n), cuticle (c), papilla (p), micro-organisms on the surface of the cuticle (mo), microvilli (mv) and the layers of the cuticle (1, electron-dense outer layer; and 2, electron-lucent inner layer). (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) longitudinal (lm) and circular (cm) muscle layer 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.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diameter and 0.51 mm in length. They are surrounded by a capsule consisting of layers of smooth muscle, orientated in both a longitudinal and circular direction (Fig. 1D) and some collagen. This muscle capsule is thickest (about 35 μm thick) around the base of the gland. The secretory cells of each gland vary in size, with cells decreasing in size from the basal to the apical region of the gland. Staining with toluidine blue showed that the secretory cells varied in their staining properties, from being strongly stained (dark blue) to only slightly stained (very light blue). Many showed metachromasia.

The alcian blue–PAS technique showed that the base of 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 1). The gland cells stained with different intensities for sulphated mucins with aldehyde fucshin, indicating different cell types, but did not stain for carboxylated mucins (Table 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 (Fig. 1D). Staining with bromophenol blue showed the presence of protein in both the cuticle and in the largest cells of the defensive gland (Table 1).

3.2.2. *Onchidella hildae*

The large multi-cellular glands of *O. hildae* have a maximum diameter of about 0.8 mm ($n = 30$) and are enclosed in an even thicker (when compared to *O. capensis*), multi-layered capsule (up to 110 μm thick at the base of the gland) of muscle, surrounded by tissue composed of a dense matrix of inter-lacing fibres of smooth muscle and collagen (Fig. 2). 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 decrease in size apically and many stained positively for mucopolysaccharides with toluidine blue (Table 1), but a few of the cells did not stain at all. 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 1). The gland cells stained positively for sulphated mucins, but negatively for carboxylated mucins (Table 1). Masson's trichrome stain showed that in addition to the multi-layer muscle capsule surrounding the glands, there are internal partitions of muscle between the different gland cells within the gland (Fig. 2B).

3.3. Transmission electron microscopy

3.3.1. *Onchidella capensis*

The epidermal layer of the perinotal region of *O. capensis* comprises a single layer of pear-shaped epithelial cells (about 12 μm in length and 8 μm in width) seated on a basal lamina, beneath which collagen and muscle fibres are located (Fig. 3A and B). The epithelial cells contain a single large nucleus (about 3–4 μm in diameter), situated towards the base of the cell, as well as vesicles, mitochondria, Golgi bodies (with 4 or 5 cisternae) and other organelles (Fig. 3F). There is interdigitation of the lateral cell membranes of the epithelial cells (Fig. 3B 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 μm to 10 μm (Fig. 3A–C, 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 (Fig. 3E). The external surface of the cuticle is covered in a dense mass of micro-organisms (algae, diatoms and bacteria) (Fig. 3E).

Goblet cells occur in or just below the epithelial layer (Fig. 3C 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 (Fig. 3C 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 (see Arey and Barrick, 1942 and Marcus, 1979 for diagrams of this arrangement in *Onchidella* spp.). The glands are surrounded by a well organised capsule of smooth muscle (Fig. 4). 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 several 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, which we have called Type 1, has a secretory product that is granular in appearance and occupies the region of the gland closest to the outer muscle capsule (Fig. 5A 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 cells are smaller than the Type 1 cells and contain a substance with a homogenous appearance that is more electron-dense in Type 2 when compared to Type 3 (Fig. 5C and E). The fourth cell type has a secretory product that has a vesicular appearance (Fig. 5E), 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 (Fig. 5B), but unlike Type 4 cells the vesicles of Type 5 cells have a heterogeneous appearance and are surrounded by a granular matrix.

The cells within a gland are separated by smooth muscle fibres and narrow support cells containing large numbers of mitochondria, rough endoplasmic reticulum, vesicles and glycogen (Fig. 5F). The lumen of the gland and the duct are lined with irregularly shaped epithelial cells (Fig. 5E). These epithelial cells (about 4–5 μm in width and 5–6 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 (Fig. 5E).

3.3.2. *Onchidella hildae*

The epidermis of *O. hildae* consists of a layer of flask-shaped epithelial cells (about 8 μm in length and 4 μm in width) that have a large irregularly shaped basal nucleus, lysosomes and short microvilli (about 1 μm in length) on their apical surface (Fig. 6A). The epithelial cells are seated on a basal lamina and are connected to it via desmosomes (Fig. 6C). The epithelial cells are connected to each other via interdigitation of their cell epidermal membranes. Beneath the epidermal layer there is a matrix of muscle fibres, collagen and pigment granules (Fig. 6A 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 (Fig. 6A). The cuticle is densely covered with micro-organisms (algae, diatoms and bacteria) and at times these micro-organisms appear to be embedded in the cuticle (Fig. 6A).

Fig. 7. Transmission electron microscopy images 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 Types 1–3 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 (arrow) 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.

Large multi-cellular glands are deeply embedded within the hyponotum and connect to the external pore via a duct in a similar way to that illustrated for other *Onchidella* spp. (see Arey and Barrick, 1942 and Marcus, 1979 for diagrams). These glands are encapsulated in a thick layer of smooth muscle, encircling the gland both longitudinally and circularly (Fig. 6D). 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 products, four different types of gland cells could be identified in the glands. The predominant cells are large cells that occur mostly in the basal region 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) (Fig. 7A 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 (Fig. 7C and E). The fourth cell type has a vesicular appearance and occurs closer to the lumen of the gland (Fig. 7B 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 (Fig. 7D).

Both the gland lumen and the lumen of the collecting duct are lined with a layer of cuboidal epithelial cells (Fig. 7E). 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 discharge into the lumen by holocrine secretion (Fig. 7F).

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 (e.g. Pepe and Pepe, 1985; Weiss and Wägele, 1998; observations made during this study), the cuticle, epidermal layer and its mucous secretions provide protection from dehydration, particularly during foraging activity. The epidermal layer of *Onchidella capensis* and *Onchidella hildae* is similar in structure, being composed of an uneven layer of pear-shaped epithelial cells beneath a relatively thick cuticle. The cuticle of both species stained light blue/magenta with toluidene blue, indicating the presence of mucopolysaccharides. It also stained blue with bromophenol blue, indicating the presence of protein. The cuticle thickness, which ranged between 5–10 μm in *O. capensis* and 7–12 μm in *O. hildae,* is similar to that of *O. celtica* (about 8 μm in thickness) which also stains light blue with toluidine blue (Weiss and Wägele, 1998). Transmission electron microscopy revealed that 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 thicker more electron-lucent third layer. The cuticle of *O. hildae*, however, 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 perinotum of *O. capensis* did not reveal any pores correlating to the openings of goblet cells, as seen in the epidermis of siphonariids (Pinchuck and Hodgson, 2009). It is possible that this was an artefact of fixation and specimen preparation. This is because light and transmission electron microscopy revealed numerous small epithelial and sub-epithelial goblet cells in *O. capensis* and *O. hildae*. Such secretory cells have been observed in the epidermis of other Onchidiidae (e.g. Watson, 1925; Awati and Karandikar, 1940; Arey and Barrick, 1942). These cells presumably produce a thin layer of mucus over the epidermis, as in other gastropods (Bubel, 1984), but may also be involved in the secretion of the cuticle. 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 silicated skeleton, posed problems with cutting sections for the TEM, resulting in scratch marks on the sections even when using a diamond knife.

Like many shell-less gastropods onchidellids rely on chemical defence to deter predators (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; Arey and Barrick, 1942; Gabe and Prenant, 1950; Binot, 1965; Marcus, 1979; Weiss and Wägele, 1998; Wägele et al., 2006; present study) that produce a repugnatorial secretion. Arey and Barrick (1942) and Marcus (1979) identified two types of multi-cellular gland in onchidellids. In this study, however, all multi-cellular glands had the same morphology. Like the multi-cellular epidermal glands of siphonariids (Pinchuck and Hodgson, 2009), 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 could be constricted more forcibly to 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), *Onchidella floridana* projected a thin stream of secretion up to 15 cm towards the source of the disturbance. In a further study, Arey and Barrick (1942) found that it was easier to elicit a glandular discharge when *O. floridana* was submerged in water and that a thread of coagulated mucus was secreted which did not break up into a spray as it did in air. 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 *Onchidella borealis* would direct its papillae towards the source of irritation and could project the defensive secretion several millimetres towards the source. Each gland seemed to discharge independently and did not necessarily discharge the entire contents, but could fire repeatedly. In the presence of certain predators, e.g. starfish, the gland could release the entire contents of the lumen but then required a period of regeneration before it was capable of firing again. This was not established for *O. capensis* and *O. hildae*, but sections through the marginal epidermis showed glands in different stages of "fullness" possibly indicating that those containing less secretory product had recently released 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 floridana*, 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 (220–320 μm long and 85–120 μm wide) and therefore these cells occupied the greatest proportion of the gland. In this study, *O. capensis* was found (based on morphological appearance of the gland products) to have five different types of gland cell in all glands, 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 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. Furthermore, the results of the histological and histochemical staining of the glands showed

that the secretory product is largely made up of acidic mucins, neutral and sulphated mucins. Arey (1937) found the pH of the gland secretion of *Onchidella floridana* to be pH 2.7. Binot (1965) described the secretary product of the gland cells of *Onchidella celtica* as acid and lipid complexes. The functional significance of possessing different cell types within a gland in relation to function is unknown. Presumably each cell type produces a unique product that mixes in the lumen to produce the final defensive mucous secretion. This mucus, like that produced by siphonariids, contains polypropionate metabolites and has antibacterial properties (Ireland and Faulkner, 1978) but which cells within the gland produce these chemicals is not known.

The number of marginal repugnatorial glands varies between onchidellid species, ranging from as few as eight to as many as 32 (Watson, 1925; Arey, 1937; Arey and Barrick, 1942; Stringer, 1969; Marcus, 1978, 1979; Weiss and Wägele, 1998). However the number of glands can also vary within a species, which has been attributed to age and size of the individual (Marcus, 1979; Young et al., 1986). Nevertheless, Marcus (1979) did suggest that the structure of these glands might provide taxonomic characters. The results of this study, however, suggest that this is unlikely. Gland size within a species will also 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.

Current phylogenies place the onchidellids within the Pulmonata (Mordan and Wade, 2008; Dayrat, 2009). The structural similarities of the multi-cellular defensive glands of *O. capensis* and *O. hildae* (this study) and those of siphonariids (Pinchuck and Hodgson, 2009) add support to this placement, and suggest that such structures had a common ancestry.

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