COMPARATIVE STRUCTURE OF THE LATERAL PEDAL DEFENSIVE GLANDS OF THREE SPECIES OF *SIPHONARIA* (GASTROPODA: BASOMMATOPHORA)

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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 multicellular glands, but these were largest 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 observed with transmission electron microscope (TEM) the product had a reticulate appearance. By contrast type II gland cells stained positively for acidic mucins and the secretory product was formed as large granules. The products from both types of cell, which appeared to be secreted by holocrine secretion, mixed in the lumen of the duct. Individuals of *S. gigas* had two types of lateral pedal glands, a large multicellular gland and a tubular unicellular gland. The multicellular 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 positively for neutral and sulphated mucins.

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, Ballesteros & Avila, 2006). These defensive secretions can contain a variety of secondary metabolites that may be synthesized by the animal itself or derived from its diet (Simkiss, 1988; Davies-Coleman & Garson, 1998; Davies-Coleman, 2006; Moore, 2006; Wägele et al., 2006). Gastropod mucous secretions are produced from a variety of epidermal and subepidermal glands. Whilst the general structure of these glands is well described (for reviews see Bubel, 1984; Simkiss, 1988; Godiner, 1994; Voltzow, 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 basal 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 upper lateral regions of the headfoot when irritated (Hodgson, 1999). This mucus contains polypropionates (Davies-Coleman & Garson, 1998; Cimino & Ghiselin, 2001; Darias, Cueto & Díaz-Marrero, 2006; Davies-Coleman, 2006), some of which are biologically active and toxic to fish at levels as low as 10 μg/ml (Hochlowski et al., 1983). Chemical defence is very effective in some species of *Siphonaria*, and many predators that readily consume patello-gastropods refuse to eat siphonariids (Branch, 1981; Branch & Cherry, 1985; McQuaid, Cretchley & Rayner, 1999). The role of mucus in defence, however, has been questioned because some siphonariids do suffer predation (Cook, 1980; Cimino & Ghiselin, 2001; Yamamoto, 2004). A notable example of this is *Siphonaria gigas* that can be consumed by predators (Garrity & Levings, 1983) and is exploited for food by humans, possibly because this species does not produce polypropionates (J. Faulkner, personal communication 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 & Garrity, 1984). Whether it possesses epidermal glands is not known.

Thus, while 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. Freter & Graham (1954) noted that marine pulmonates such as *Siphonaria* species possessed large glands on the sides of the foot, and they provided an illustration (fig. 8) of a longitudinal section through one such gland. A single illustration of a lateral pedal gland from *Siphonaria javanica* was also presented in the recent paper by Wägele et al. (2006; fig. 9E). In a study of *S. hispida*, Marcus & 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*,

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focusing on two species that are known to be avoided by predators [S. capensis Quoy & Gaimard, 1833, and S. serrata (Fischer, 1807)] and one that can be consumed (S. gigas Sowerby, 1825).

MATERIAL AND METHODS

Siphonaria capensis and S. serrata were collected from rocks in the intertidal zone at Kenton-on-Sea (34°S, 27°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 (9°N, 79°W). A minimum of five individuals per species were collected. The five live animals were transported back to a laboratory, dissected and small portions of the outer lateral pedal tissue fixed for light and electron microscopy. Specimens were not relaxed prior to fixation.

Light microscopy

Tissues were fixed for at least 24 h in aqueous Bouin’s solution, dehydrated and embedded in Paraplast via xylene. Sections (5–7 μm thick) were cut on a Leitz rotary microtome. In addition, semi-thin sections (c. 1 μm thick) of tissues that had been prepared for transmission electron microscopy (see below) were also cut. 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. 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 fuchsine–alcian blue (AF–AB) for separating sulphated and carboxylated mucins. For proteins sections were stained with fuschin–alcian blue (AF–AB) for separating sulphated and neutral mucins (pH 1.5 and 2.5), periodic acid Schiff (AB–PAS) for differentiating between acid and neutral mucins, and aldehyde fuchsin–alcian blue (AF–AB) for separating sulphated and carboxylated mucins. For proteins sections were stained with fuschin–alcian blue (AF–AB) for separating sulphated and neutral mucins.

In the correct region of tissue using the semi-thin sections, ultrathin 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 3 min. Sections were then viewed in a Jeol 1210 TEM at 90 kV.

RESULTS

General observations and SEM

Numerous white glands, visible to the naked eye, are embedded in the translucent lateral body wall (head-foot) in Siphonaria capensis and Siphonaria serrata (Fig. 1A). These glands are distributed around the entire animal and exude a thick, white sticky mucus in response to mechanical stimulation. By contrast the lateral pedal area of Siphonaria gigas is black in colour, and glands are not visible. However, when stimulated mechanically a sticky transparent mucus, similar in appearance to pedal mucus, is released. SEM of the lateral regions of the feet 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 c. 74/mm² whereas in S. serrata it was only 57/mm² (Table 1). The mean pore diameter was similar in both species, c. 29 and 23 μm in S. capensis and S. serrata, respectively (Fig. 1B; Table 1). In S. gigas two sizes of pores are visible, the first having a diameter of 10 μm and the second c. 1 μm (Fig. 1C). The density of the larger pores was c. 34/mm² (Table 1).

Light microscopy

In S. capensis and S. serrata large multicellular sub-epithelial glands are present in the lateral pedal regions. The glands (c. 280 μm long × 160 μm diameter in S. capensis and 210 μm long × 122 μm diameter in S. serrata, Table 1) are surrounded by a muscle layer and open to the epidermal surface via a small duct and pore between the epithelial cells (Fig. 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 secretory product and two types of secretory cell. Cell type I stains positively for neutral and sulphated mucins only, whereas type II stains very positively for acidic and sulphated mucins only (Table 2; Fig. 2C, D).

Two types of subepidermal gland are present in the lateral pedal regions of S. gigas. The first type is a large multicellular sub-epithelial gland (c. 312 μm long × 82 μm diameter) with a central lumen, opening to the outside via a small duct (Fig. 2E). Unlike those of S. capensis and S. serrata, the multicellular glands are not surrounded by a well-developed muscle layer. Furthermore, each gland possesses one type of secretory cell only that stains for sulphated and neutral mucins only (Table 2). The second type of gland is unicellular, long and narrow, containing a secretory product that also stains for sulphated and neutral mucins (Fig. 2F; Table 2).
Transmission electron microscopy

As the ultrastructure of the lateral pedal glands of *S. capensis* and *S. serrata* are very similar, only a single description follows. TEM confirmed that the glands are surrounded by a band of smooth muscle and collagen c. 4.8 μm thick (Figs 3A, 4C). 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 (Figs 3A, 4A). 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 (Fig. 3A, B) which we presume to be mucus. The cytoplasm and the irregularly shaped nucleus are confined mainly to the base of the cell (Fig. 3A, C). In some type I cells the basal cytoplasm contains numerous mitochondria, rough endoplasmic reticulum, well-developed Golgi bodies, glycogen and accumulating secretory product (Fig. 3D). Numerous vesicles (50–55 nm in diameter) can be seen associated with the Golgi cisternae (Fig. 3D). 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 (Fig. 3C). The apical regions of the cell membranes of type I cells do not bear microvilli.

The secretory product of type II cells is more electron-dense and vesicular in appearance with a granular-like content (Fig. 4A–C). The irregularly shaped nucleus and cytoplasm that contains a few well-developed Golgi bodies are restricted to the bases of these cells (Fig. 4B–D). Numerous vesicles are associated with the end of the Golgi cisternae and some appear

Table 1. Dimensions of the multicellular glands, gland openings (pores) and pore density in *Siphonaria* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Area (μm²)</th>
<th>Pore diameter (μm)</th>
<th>Pore density (number/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Siphonaria serrata</em></td>
<td>210</td>
<td>122</td>
<td>14,134</td>
<td>23.1 ± 3.6</td>
<td>56.7 ± 12.8</td>
</tr>
<tr>
<td><em>Siphonaria capensis</em></td>
<td>280</td>
<td>157</td>
<td>31,173</td>
<td>29.3 ± 4.2</td>
<td>74.0 ± 15.8</td>
</tr>
<tr>
<td><em>Siphonaria gigas</em></td>
<td>312</td>
<td>82</td>
<td>15,257</td>
<td>10.1 ± 2.9</td>
<td>~34</td>
</tr>
</tbody>
</table>

Length, width and area are maximum dimensions observed from light microscope longitudinal sections (20 glands measured from 5 individuals per species). Pore diameters and densities (mean ± SD; n = 5 for *S. capensis* and *S. serrata*, and n = 3 for *S. gigas*) were determined by SEM. The densities and diameters of large pores only are presented for *S. gigas*.
to be fusing with the accumulating cell product (Fig. 4D). Apically the cell membrane does not have any microvilli.

Each gland opens to the outside via a pore lined by nonciliated cuboidal epithelial cells (Fig. 5A). The lumens of nearly all glands sectioned contained products from both types of secretory cell (Fig. 5B, 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 (Fig. 5D). In addition to the two secretory cell types, nonsecretory support cells are present in the glands. These cells are joined to adjacent support cells and gland cells by desmosomes (Fig. 5F). The support cells are irregular in shape, have long (up to 2.5 μm) apical microvilli and partially line the lumen of the gland (Fig. 5A, B, F). Thin strands of cytoplasm from these cells penetrate between the gland cells (Figs 3A, 5E).
The cytoplasm of these cells often contains large amounts of glycogen and lipid droplets (Fig. 5F).

The large multicellular glands (type 1) of *S. gigas* are surrounded by a few smooth muscle myofibrils and collagen fibres only (Fig. 6A, B). In addition the surrounding connective tissue contains elongate cells that contain large numbers of electron-dense vesicles (Fig. 6D, F) that we presume to be pigment granules. Only one type of secretory cell could be identified in the multicellular glands. Basally this cell type has an irregularly shaped nucleus (Fig. 6B) and some cytoplasm containing small mitochondria, a few well-developed Golgi bodies and smooth endoplasmic reticulum (not illustrated).

### Table 2. Histochemical results from the multicellular lateral pedal glands of three species of *Siphonaria* (+, positive; ++, very positive; −, negative)

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell type</th>
<th>Mucopolysaccharides</th>
<th>Neutral mucins</th>
<th>Acid mucins</th>
<th>Sulphated mucins</th>
<th>Carboxylated mucins</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Siphonaria capensis</em></td>
<td>I</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Siphonaria serrata</em></td>
<td>I</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Siphonaria gigas</em></td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>++</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
Most of the volume of these cells is occupied by large electron-dense vesicles embedded in an electron-lucent matrix (Fig. 6A–C). The content of the cells is released into the lumen of the duct by holocrine secretion (Fig. 6C). In addition to the secretory cells, irregularly shaped support cells lie between the gland cells (Fig. 6C). The support cells have well-developed, elongate, apical microvilli and a cytoplasm containing an irregularly shaped nucleus, multivesicular 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 (Fig. 6F), and a long tubular ‘neck’ c. 3 μm diameter that extends to the outside (Fig. 6D). The tubular neck also contains an electron-dense granular product (Fig. 6E). The neck of the gland penetrates between the

Figure 4. Siphonaria serrata, TEM images of type II secretory cells. A. Longitudinal section of apical region of type II 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). Abbreviations: co, collagen; mu, muscle layer; s, secretory product; sc, support cell. Scale bars: A = 10 μm; B = 5 μm; C = 2 μm; D = 0.5 μm.
DEFENSIVE GLANDS OF *SIPHONARIA*

Figure 5. **A.** TEM section through the pore region (arrow) of a multicellular gland of *Siphonaria capensis* showing the epithelium (e), a support cell (sc) 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 (lu) 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 (lu) 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 regions of support cells joined by desmosomes (one arrowed). Note the lipid droplets (lp) in the cytoplasm. 

Scale bars: **A, C, E** = 10 μm; **B, D** = 5 μm; **F** = 2 μm.
Figure 6. TEM 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), pigment granules (pyg).

**E.** Higher magnification of the secretory vesicles of type 2 gland.

**F.** Transverse section through the basal regions of several type 1 (T1) glands.

Abbreviations: co, collagen; lu, lumen; mu, muscle layer; n, nucleus; pyg, pigment granules; s, secretory product. Scale bars: A = 5 µm; B, C = 2 µm; D, F = 10 µm; E = 1 µm.
columnar epithelial cells (Fig. 6D) and opens to the outside via a small pore.

**DISCUSSION**

The dorso-lateral pedal regions of all three species of *Siphonaria* studied possess large multicellular 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 & Graham (1954: fig. 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 & Graham’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 positively for neutral and sulphated mucins, and the second type a product that stains very 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.

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; Li & Graham, 2007; Werneke et al., 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.

While *S. gigas* also has multicellular 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 subepithelial gland cells of the opisthobranch *Cadilla luteomarginata* and *C. lutes* described by Wägele et al. (2006) and it is interesting to note that the phylogeny of Grande et al. (2004) placed *Siphonaria* within the opisthobranchs. Whether the structurally simpler multicellular glands of *S. gigas* are ancestral to those of *S. capensis* and *S. serrata*, or vestigial, remains to be determined. A phylogeny of the siphonariids onto which gland structure is mapped will be a useful tool for answering these questions.

The abundance of the lateral pedal glands in the species of *Siphonaria* studied suggests 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.N. Hodgson, personal observation) and repels predators (Hodgson, 1999; McQuaid et al., 1999). Whether the capacious exudate contains polypropionate metabolites, however, is not known. Furthermore the location of such chemicals at a cellular level is equivocal. This is because all chemical extractions to date have been from entire animals, and not from specific glands or their secretions. Ireland & Faulkner (1978), however, did extract polypropionates from the mucous secretions of *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 do not have a defensive role, as this species is consumed by fish and humans (Garrity & Levings, 1983; Ortega, 1987). Defence against predators in *S. gigas* probably takes a combination of its size, extreme tenacity and habitat. This species 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 & Levings, 1983).

Finally, the gland secretions of siphonariids may also play a role in protecting them from desiccation, microbial attack or the attachment of ectocommensals. Molluscan mucus is known for its anti-bacterial properties (Yamaguchi et al., 2000) and Hochlowski & Faulkner (1983) 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, Hawkins & Cross, 1983), no such unicellular organisms have been found attached to the epidermis of South African siphonariids (J. van As, personal communication).

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