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Characterization of the adhesive dermal secretion of *Euprymna scolopes* Berry, 1913 (Cephalopoda)

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ABSTRACT

Bio-adhesion is a common and crucial process in nature and is used by several different species for camouflage, prey capture, hatching or to avoid drifting. Four genera of cephalopods belonging to four different families (*Euprymna*, Sepiolidae; *Idiosepius*, Idiosepiidae; *Nautilus*, Nautilidae; and *Sepia*, Sepiidae) produce glue for temporary attachment. *Euprymna* species live in near-shore benthic habitats of the Indo-Pacific Ocean, are nocturnal and bury into the seafloor during the day. The animals secrete adhesives through their epithelial glands to completely coat themselves with sand. In cases of danger, they instantaneously release the sandy coat as a sinking decoy to deflect predators. Earlier morphological investigations have shown that the adhesive gland cells of *Euprymna scolopes* are scattered on the dorsal epidermis. It has been proposed that neutral mucopolysaccharides, secreted by one gland type (goblet cells), are responsible for adhesion, whereas the release of the glue could be caused by acidic mucoproteins produced by ovate cells in the ventral epidermis. The ultrastructural re-investigation of the *Euprymna* epithelium in this study has indicated the presence of a new gland type (named flask cell), exclusively located in the dorsal epithelium and always neighboured to the known goblet cells. Based on our histochemical observations, the secretory material of the ovate cells does not display a strong reaction to tests for acidic groups, as had been previously assumed. Within the dermis, a large muscle network was found that was clearly distinctive from the normal mantle musculature. Based on our data, an antagonistic gland system, as previously proposed, seems to be unlikely for *Euprymna scolopes*. We hypothesize that the adhesive secretion is formed by two gland types (goblet and flask cells). The release of the sand coat may occur mechanically, i.e. by contraction of the dermal mantle muscle, and not chemically through the ovate cells.
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1. Introduction

Many organisms are remarkable in their ability to secrete specialised permanent or reversible adhesives that operate in hugely diverse environments, for different purposes and under different conditions (see respective chapters in Smith and Callow, 2006; von Byern and Grunwald, 2010). These biological adhesives show good performances underwater, on rough, dry or dirty substrates, and over a wide range of temperatures. Within a few seconds, some organisms can adhere permanently for the rest of their lives, while others use temporary adhesives to enable locomotion, prey capture or protection. 500 million years of evolution have optimised these glues (von Byern et al., 2010a) for the needs and requirements of the organisms producing them. There is a high diversity of bioadhesives in the marine and terrestrial kingdoms (e.g., algae – Dimartino et al., 2016; platyhelminths – Lengerer et al., 2016; echinoderms – Flammang, 2006; insects – Betz, 2010; Gorb and Koch, 2014; molluscs – Sagert et al., 2006; Smith, 2006, 2010; Silverman and Roberto, 2010; and vertebrate species such as hagfish – Fudge et al., 2010; or salamanders – von Byern et al., 2015; to mention just a few of them) and within the last years many have been characterized in detail on a morphological, chemical and/or molecular level. But despite this progress, we still know very little about the composition, production, secretion and mechanical properties of the vast majority of these bioadhesives.

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In cephalopods, four genera belonging to four different families (*Nautilus* sp., Nautilidae; *Sepia* sp., Sepiidae; *Euprymna* sp., Sepiolidae; and *Idiosepius* sp., Idiosepiidae) are known to produce glue in an adhesive area on the mantle or on the tentacles (von Byern and Klepal, 2006; Cyran et al., 2010). The adhesive substances of these animals are used in different ways and studies of their mechanisms allow comparisons among them.

Idiosepius possesses only a small area on the dorsal mantle that is involved in adhesion. The animals attach themselves to sea grass or algae for camouflage and/or prey capture (Sasaki, 1921; Moynihan, 1983; Suwanmala et al., 2006; Cyran et al., 2008, 2011). Adhesion in four *Sepia* species (*S. tuberculata*, *S. pulchra*, *S. typica*, *S. papillata*) is mainly induced mechanically by a defined dermal structure on the ventral mantle (Scott, 2005). Additionally, chemical substances secreted from this adhesive area might be used to increase the strength of attachment (Boletzky and Roeleveld, 2000; von Byern et al., 2011). In *Nautilus* the adhesive structures only occur on the digital tentacles. They are used to hold prey and to attach to the substratum or to other individuals during mating (von Byern et al., 2012). *Euprymna scolopes* spends much of its life buried in sand; it has developed a special technique to attach sand grains to its dorsal mantle and head using adhesives. It rakes sand over itself to form a "sand coat" (Singley, 1982; Shears, 1988). This sand coat acts as camouflage on the matching substratum during the day when the animals remain buried in the sandy seafloor. When threatened, the animals instantaneously release the sand to deflect predators (Singley 1982, 1983; Shears, 1988). The morphological study by Singley (1982) showed that three types of gland cells (interstitial, ovate and goblet cells) occur in this dorsal adhesive region, whereas two of them (interstitial and ovate cells) are also present ventrally. Singley (1982) presumed a so-called duo-gland adhesive system (Hermans, 1983) to be responsible for adhesion and release in *E. scolopes*, in which one cell type (goblet cells) produces neutral mucopolysaccharides (as demonstrated by PAS staining) for adhesion. The ovate cells, on the other hand, secrete basic proteins which become acidic in contact with sea water, causing a release of the glue from the epithelium. In an earlier publication (Klinger et al., 2010), we provided basic information on the glandular structure of *Euprymna* and indicated some morphological and histochemical differences to the results of Singley (1982). In addition to the three cell types described by Singley (1982), we found an additional glandular cell type (previously only called cell type 4 by Klinger et al. (2010), renamed in the present study as flask cells) that was prominent in the dorsal mantle epithelium. Based on this finding we suggest a mechanism of adhesion and release that differs from the proposed duo-gland hypothesis.

With the present research article, we provide a detailed ultrastructural and histochemical description of the epithelial gland system of *Euprymna* as a basis for other researchers working in this field. Characterization of the *Euprymna* adhesive secretion system will improve our knowledge of the adhesive mechanisms in cephalopods and allow a more detailed comparison of their function and usage within cephalopods and other mollusk groups.

2. Materials and methods

Adult specimens of *E. scolopes* were collected in the waters off the coast of Manoa, Hawaii with the permission of Dr. Heinz Gert de Couet from the University of Hawaii, Manoa, USA.

2.1. Preparation and fixation

Following the guidelines published by Fiorito et al. (2015) the animals were anesthetized with 3% (v/v) ethanol–seawater solution until they showed no sign of ventilation and reaction to an external stimulus and then immediately decapitated. The dorsal and ventral mantle as well as the arms were fixed in an acetic–alcohol–formalin (AAF) mixture (Böck, 1989) for 3 h at 25 °C or in Carnoy solution (Kiernan, 1999) for 3 h at 25 °C for histological and histochemical analyses. For ultrastructural studies, tissue samples were preserved in 2.5% glutaraldehyde with a sodium-cacodylate buffer (0.1 M, pH 7.4, plus 10% sucrose) for 6 h at 25 °C.

2.2. Histology, histochemistry, immunocytochemistry

Both the Carnoy- and AAF-fixed materials were cleared three times at 20 min each, in methylbenzoate as well as in benzene, and infiltrated overnight with paraffin. Sections (5–7 μm thick) were cut, mounted on glass slides with Ruyter solution (Ruyter, 1931) and dried at room temperature before use.

The histochemical analyses were carried out according to von Byern et al. (2012) using Azan trichrome staining to provide an overview of the glandular system and structural details. Periodic acid–Schiff (PAS) staining (McManus and Mowry, 1960) was used to detect the presence of neutral hexose sugar units. Control and blocking of PAS was tested by prior treatment in dimedone for 3 h (Bulmer, 1959), by borohydride reduction and phenylhydrazine for 3 h, acetylation for 2 and 9 h, and acetylation–deacetylation for 24 h (Kiernan, 1999).

Proteins were detected using three methods: Alcian Blue 8GX (McManus and Mowry, 1960) at pH 1.0 and 2.5 for 2 h at 20 °C; Biebrich Scarlet (0.04%) for 1 h at 20 °C in phosphate buffer at pH 6.0 (Spicer and Lillie, 1961); and Laskey's glycine buffer at pH 8.0, 9.5 and 10.5 (McManus and Mowry, 1960) as well as Toluidine Blue O (in 0.2 M acetate buffer at pH 5) according to Mulisch and Welsch (2010).

The immunocytochemical analysis of the muscles and nerve fibers was carried out on 100 μm thick vibratome sections prepared with a microtome (Leica VT 1200S; Leica Microsystems, Wetzlar, Germany) and incubated with 2.5% Alexa Fluor TRITC-conjugated phalloidin (R415; Invitrogen, Carlsbad, CA, USA) and 1:100 diluted acetylated α tubulin (T-6793; Sigma–Aldrich, St. Louis, MO, USA) with FITC-labeled secondary antibody M308012 (Invitrogen) (see protocol in Wollesen et al., 2008, 2009) and observed with a confocal laser scanning microscope (TCS SP5X; Leica Microsystems).

Carbohydrates were characterized enzymatically on paraffin sections (5–7 μm thick) using the following lectins (50 mg/ml; incubated for 30 min at room temperature): FITC-labeled concanavalin agglutinin (ConA), specific for a-D-mannose/a-D-glucose; Texas Red labeled peanut agglutinin (PNA), specific for lactose/β-galactose; TRITC-labeled soybean agglutinin (SBA), specific for N-acetyl-D-galactosamine; FITC-labeled wheat germ agglutinin (WGA), specific for N-acetyl-D-glucosamine; TRITC-labeled *Galanthus nivalis* lectin (GNA), specific for mannose; and FITC-labeled *Ulex europaeus* agglutinin (UEA), specific for α-L-fucose. All lectins were diluted with the respective buffers as specified by the manufacturer (EY Laboratories, San Mateo, CA, USA). Inhibition was carried out by incubating diluted fluorescent-labeled lectin with 0.2 M inhibitory carbohydrate for 60 min at room temperature before application to the sections. Autofluorescence was controlled by incubating sections in buffer solution without fluorescent-labeled lectin.

2.3. Ultrastructure

Glutaraldehyde-fixed samples were washed three times for 30 min in buffer solution at room temperature and stored for further processing. For post-fixation, the samples were immersed for 1.5 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (same pH as above) and dehydrated in a graded series of ethanol. For transmission electron microscopy (TEM) examination, the sam-

ples were embedded in epoxy resin; ultrathin sections (50–70 nm) were mounted on copper slot grids coated with formvar in dioxane, stained with uranyl acetate and lead citrate, and examined in two TEMs (Zeiss Libra 120 and Zeiss EM 902; Carl Zeiss AG, Oberkochen, Germany).

3. Results

The epidermis consists of a columnar epithelium with a microvilli layer on the surface and an undulated basal region. The dorsal mantle epidermis is about 34–60 μm thick, while the mantle epithelium on the ventral side measures up to 43 μm and the epithelium on the fins is around 13–25 μm thick. Beneath the 0.3 μm thick basal lamina there are several differently orientated muscle layers. The dermis has a thickness of around 78–90 μm, and no size differences could be measured between the dorsal and ventral sides. Incubation with wheat germ agglutinin (WGA), and peanut (PNA) and soybean agglutinin (SBA) revealed strong reactivity to N-acetyl-D-glucosamine in the epidermis but not the dermis. Additionally, granular aggregations of labelled lectin could be observed around the ovate cells as well as at the basal lamina (Fig. 1A). The control without lectin and incubation with the appropriate sugar was negative. All other applied lectins showed no reactivity with the epithelium layer.

Ultrastructural observations showed that four different types of gland cells (interstitial, ovate, goblet and flask) are present in the dorsal mantle epithelium (Fig. 2) and the interstitial and ovate cells are also present ventrally. All types span the whole width of the epithelium and release secretory material to the tissue surface.

The **interstitial cells** are polymorphic and occur throughout the entire mantle, but are less frequent ventrally. These cells represent the dominant cell type and separate ovate from goblet cells in the adhesive region. The apical surface of the cells is covered by a dense layer of 1.3–1.5 μm long microvilli (Singley, 1982: 0.5–0.6 μm) (Fig. 3A). Their nuclei are located either at the basal or apical end of the cell and are spherical to ovate-shaped. Numerous organelles, especially mitochondria but also rough endoplasmic reticulum (RER) and Golgi bodies, are evenly distributed. Mitochondria are often present between the longitudinally oriented filaments along the cell membranes. The cells possess small vesicles (300–500 nm in diameter) (Fig. 3B) containing material of varying electron density. Singley (1982) did not demonstrate any secretion by these cells, but our observations show evidence of (rare) exocytosis (Fig. 3B). The small size of these vesicles, however, masks a clear positive histochemical reaction as was observed in the other cell types. The chemical nature and function of the secretory content has not yet been characterized.

The **ovate cells** appear the largest of all the gland cells and are ovate to sac-like. They occur in the adhesive dorsal as well as in the ventral mantle epithelium and on the bases of the fins. The surface of the cells is covered with microvilli with a length of 0.8–2 μm. Surrounding the nucleus, various amounts of RER and a few Golgi bodies could be observed within the cytoplasm. A reticulum of 5–8 nm long filaments occurs within the peripheral cytoplasm. This reticulum becomes rearranged and tightly packed during secretory activity. However, these filaments produce deep folds in the plasma membrane from the apical end toward the basal end of the cell. Ovate cells contain a fine granular secretory material of uniform appearance, which flattens the nucleus against the basal surface. Cells appear in different stages of activity and three phases can be distinguished: (i) with very dense secretory material accumulated in the centre of the cell (Fig. 3C), (ii) with loose secretory material evenly distributed over the entire cell area (Fig. 3D), or (iii) completely empty after releasing the entire secretory material. It remains uncertain whether the ovate cells "refill" themselves with

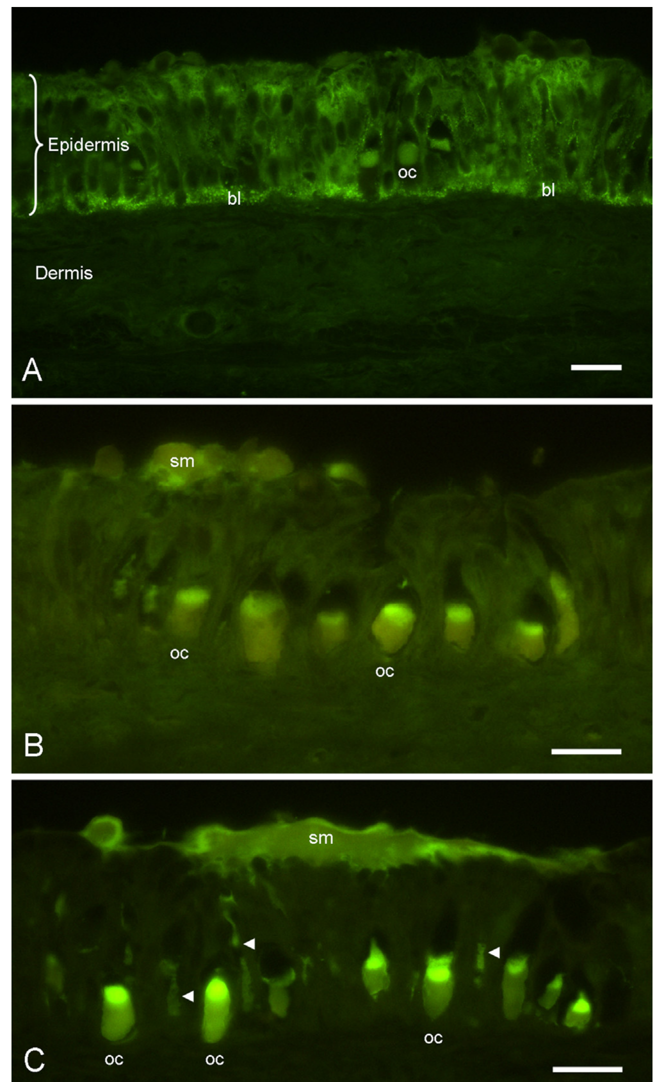

Fig. 1. (A) The epidermis of *Euprymna scolopes* (here, on the ventral side of the mantle) shows strong reactivity for N-acetyl-D-glucosamine (wheat germ agglutinin – WGA) while the dermis is unreactive. Around the ovate cells (oc) and in the region of the basal lamina (bl), lectin-labelled granular aggregations could be observed. (B) The secretory content of the ovate cells (oc) as well as the secreted material (sm) show a strong affinity to concanavalin agglutinin (ConA) which is specific for a-D-mannose/a-D-glucose. (C) A strong reactivity for α-L-fucose (*Ulex europaeus* agglutinin – UEA) could be observed dorsally in the secretory material (sm) of the goblet cells (white arrowheads) as well as the ovate cells (oc). Scale bars = 25 μm.

secretory material or induce apoptosis. They show a direct release of (secretory) material through an opening in the cells' apical surface.

The secretory content of the ovate cells appears to consist of highly sulfated proteins; the granules are unreactive for sugars (PAS negative) but remain strongly reactive for basic proteins (Biebrich Scarlet staining at pH 6.5 and 8.5 but only weakly reactive at pH 9.5 and 10.5) (Fig. 3E). The secreted material at the outer surface and the cell bases also showed a weak reaction for acidic proteins (Alcian Blue at pH 1.0 and stronger at pH 2.5) (Fig. 3F). Although the ovate cells do not stain for sugars histochemically (PAS negative), the secretory material nevertheless reacts positively to lectin labeling: incubation with *Ulex europaeus* agglutinin (UEA) and concanavalin agglutinin (ConA) (Fig. 1B and C) revealed a strong presence of α-L-fucose and a weaker presence of a-D-mannose/a-D-glucose in the glandular content and secreted material ventrally and dorsally. The bright spot at the tip of the secretory content (present with UEA

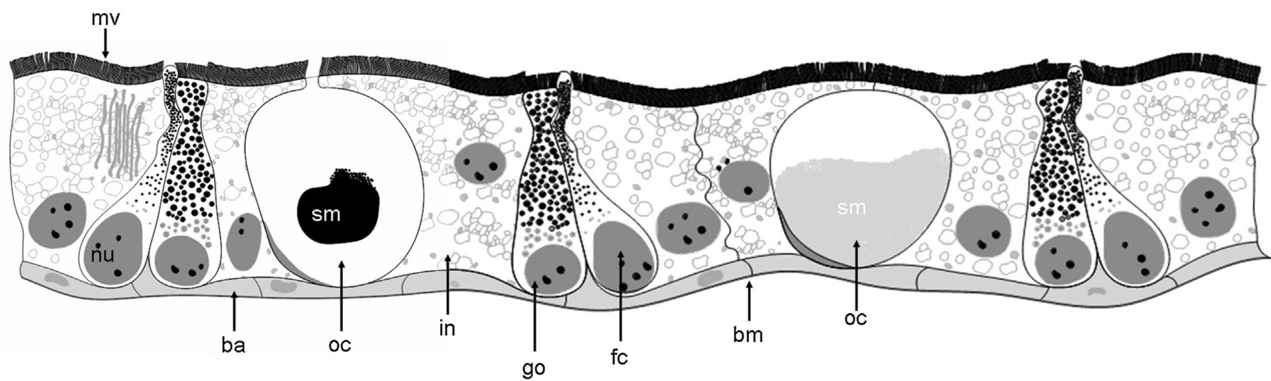

Fig. 2. Schematic drawing of the dorsal region of the mantle epithelium of *Euprymna scolopes* showing the various glandular cell types: interstitial cells (in), ovate cells (oc), goblet cells (go) and the newly found flask cells (fc). While the nucleus (nu) of the ovate cells is mostly flattened against the basal cell pole, in the other three types it usually appears spherical to ovate. The epithelium is bordered by a dense microvilli layer (mv) to the outer surroundings and a basal cell layer (ba) and basal membrane (bm) to the dermal muscle region. In the interstitial cells, longitudinal filaments (fi) could be observed. The secretory material (sm) in the ovate cells appears either uniformly distributed or as a central compact core as shown here. Image re-produced with permission by Klinger et al., 2010.

and ConA only) within the cell cavity is caused by an aggregation of the material, presumably during fixation and embedding; this could also be observed at the ultrastructural level. All other applied lectins showed no reaction with the secretory material.

The **goblet cells** represent the third cell type and are restricted to the dorsal mantle and the fin base. Often, this cell type occurs in groups, but always interspersed among interstitial cells. The cells have a round shape in cross section and taper towards the apical pole. Towards the apex, the cell membranes have many folds, displaying a branching profile in longitudinal section. The microvilli of goblet cells are shorter than those of ovate cells, with a length of approximately 0.7 μm. Spherical to ovate, the nuclei occur at the basal end and are girded by organelles such as a RER and multiple Golgi bodies and mitochondria. The Golgi apparatus release electron-lucent vesicles from the basal region toward the apical surface of the cell. These vesicles become more electron-dense as they approach the apical cell pole. They are membrane-bound, have a diameter of 0.8–1 μm and always have a uniform appearance with a homogenous density and spherical shape (Fig. 4A). Especially in actively secreting cells, various microtubules are oriented along the longitudinal axes of the goblet cells and often appear in close proximity to the secretory granules. The secretory process of the goblet cells is distinguished from the way interstitial and ovate cells secrete. Instead of a direct release of secretory material through an opening in the surface, goblet cells pinch off the most apical part of the cell content, including cytoplasm and secretory vesicles (Fig. 3B). During active secretion, granules break up into smaller units.

Histochemical observations confirm the data of Singley (1982) that the secretory material of the goblet cells contains neutral sugars (positive PAS reaction) (Fig. 4D) and some basic proteins (Biebrich Scarlet at pH 6.0–10.5). As shown for the ovate cells, the secretory content of the goblet cells also reacted positively for α-L-fucose (lectin *Ulex europaeus* agglutinin UEA) (Fig. 1C) only; all other tested lectins showed no positive affinity. The secreted material in the dorsal region also showed a strong affinity for α-L-fucose; however, it remains unclear whether one or both gland types (goblet and/or ovate) produced this secretion (Fig. 1C).

The newly described **flask cells** occur only in the dorsal epithelium and are always tightly adjacent to a goblet cell. As shown in the goblet cells, the vesicles of this new cell type are also spherical, membrane-bound and contain electron-dense secretory material (Fig. 4A and C). These vesicles are evenly distributed within the cell and have a diameter of 0.2–0.4 μm. Tubuli inside the cell transport them to the surface for subsequent secretion. This cell type is elongated yet more slender than the goblet cell and has a rounded base; therefore we named this cell type flask cells. Typically, microvilli are absent at the surface of the cell, but the surface area is covered with glycocalix. The elongated nuclei as well as the cell organelles (mitochondria, RER and Golgi apparatus) are located at the basal end. In contrast to goblet cells, but similar to ovate cells, this cell type releases secretory material through a small opening on the surface (Fig. 4C).

Similar to the secretory material of the interstitial cells, the granules of the flask cells are also too small for a clear histochemical and immunocytochemical characterization. Further investigations are necessary to gain more information about the reactivity of the secretory material to the specific tests.

Within the dermis, a prominent muscle network could be observed above the chromatic elements (Fig. 5A and B). The muscles pass longitudinally and transversely through the mantle and come close to the basal membrane. This dermal muscle is distinct from the normal mantle muscle; nevertheless, connections between both muscle systems could be observed.

4. Discussion

Singley (1982) was the first and, until now, the only author to investigate ultrastructurally and histochemically the epithelium of *E. scolopes*. According to this author, three different cell types (interstitial, ovate and goblet cells) are present in the *E. scolopes* epithelium (Table 1). The goblet cells are restricted to the dorsal adhesive region while the interstitial and ovate cells also occur in the ventral area. Apart from their location, the two distinct gland types (ovate and goblet cells) also clearly differ in their appearance and chemical composition (Table 1). According to Singley (1982), the adhesion of the sand particles is caused by the neutral mucopolysaccharides secreted by the goblet cells, while the bonding release is caused by the secretory material of the ovate cells. This glandular material consists of basic proteins, which, through the influence of the surrounding sea water, are transformed into highly acidic mucoproteins. Singley (1982) thus suggested that acidic groups are present yet masked in the unsecreted material of the ovate cells. He assumed that both adhesion and the bonding break in *Euprymna* are based on a duo-gland adhesive system, as shown for other interstitial organisms such as Turbellaria (Tyler, 1976), Gastrotricha (Boaden, 1968; Teuchert, 1977) and Archiannelida (Martin, 1978).

Based on the re-examination of the epithelium of *Euprymna* in the present study, several deviations and new details of the adhesive region can be added to Singley's findings from 1982.

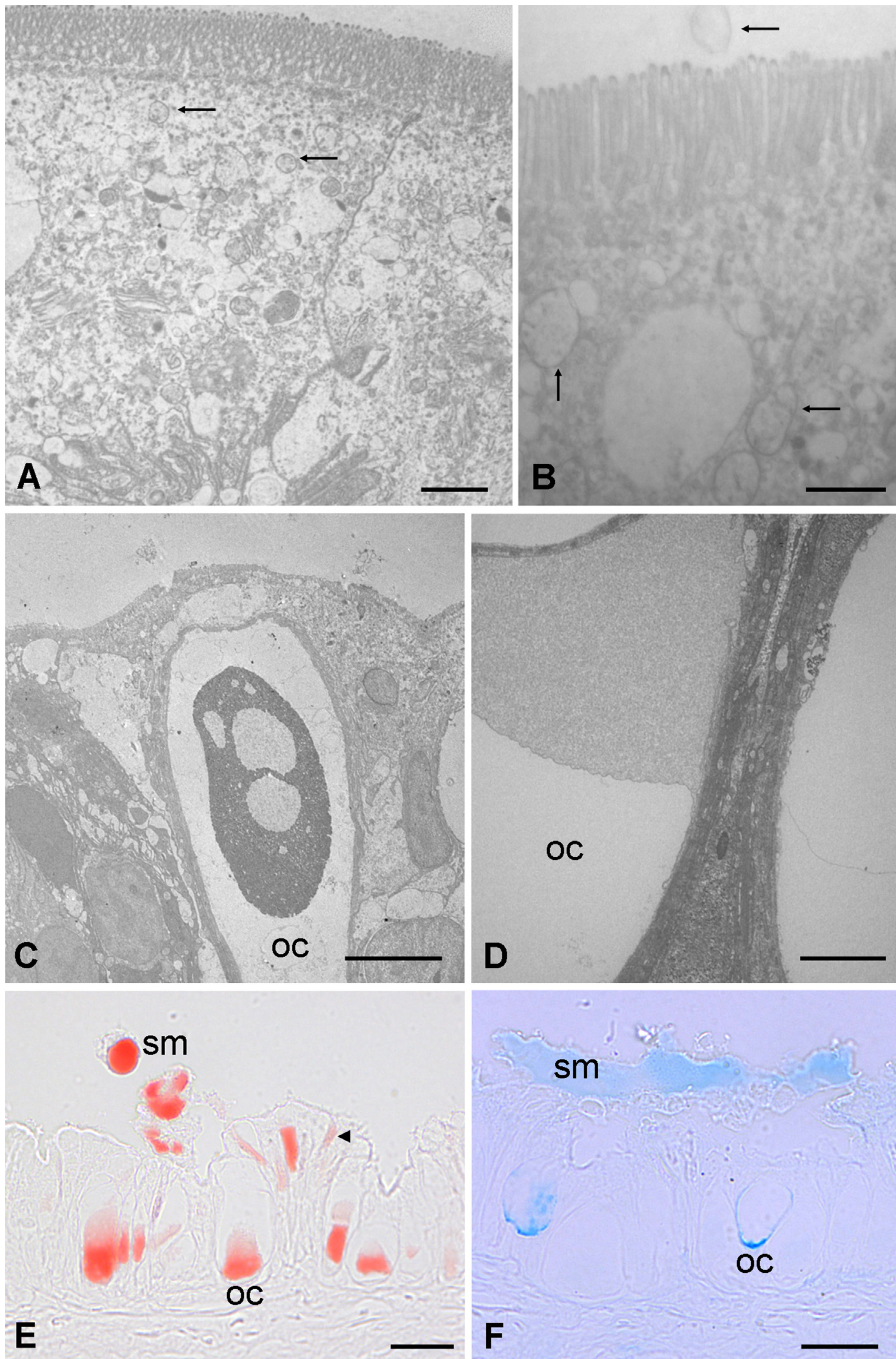

Fig. 3. (A) and (B) Electron microscope image of the interstitial cells with vesicles (black arrows) within the cells and after exocytosis. The secretory material of the ovate cells (oc) appears either (C) as a compact, electron-dense core or (D) fine-grained, electron-lucent and tightly filling the cell lumen. The glandular content of the ovate cells as well as the secreted material (sm) react positive for (E) basic proteins at pH 8.5 (Biebrich Scarlet staining) and slightly positive for (F) acidic proteins (Alcian Blue reaction at pH 1.0). Besides, the goblet cells (arrowhead in E) also stain slightly for basic proteins. Scale bars in A = 2 μm, B = 1 μm, C = 20 μm, D = 5 μm, E and F = 25 μm. (E) and (F) reproduced with permission by Klinger et al. (2010).

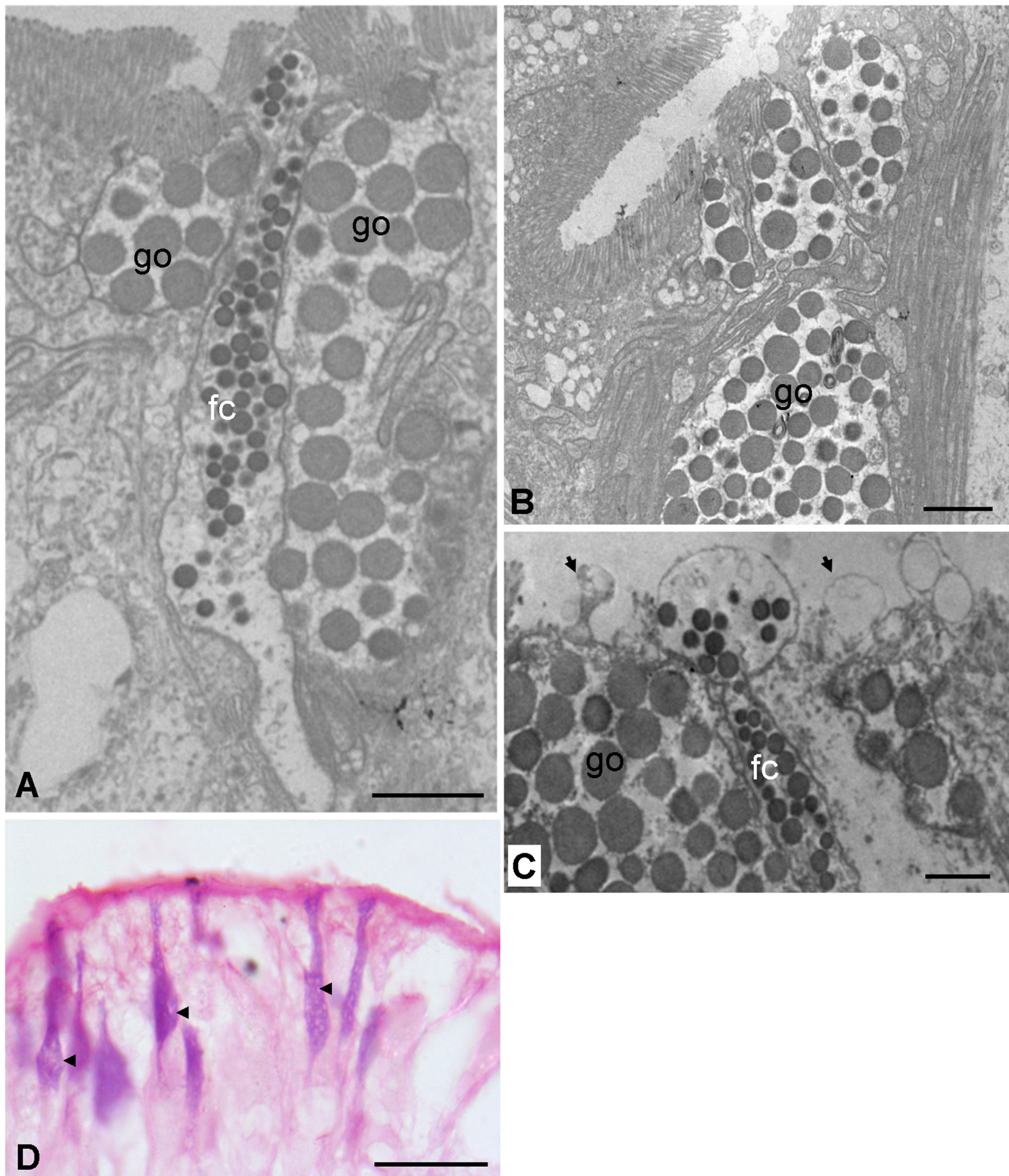

Fig. 4. (A) Goblet cells (go) and the newly found flask cells (fc) always appear next to each other. (B) In the goblet cells the most apical part of the cell content including cytoplasm and secretory vesicles is pinched off during secretion. (C) Release of the secretory material from the goblet cells (black arrowheads) and the flask cells. (D) PAS staining confirms the presence of carbohydrates in the goblet cells (black arrowheads). Scale bars in A and B = 2 μm, C = 5 μm and D = 20 μm.

While Singley (1982) did not demonstrate any secretion by the interstitial cells, our observations demonstrated mild exocytosis. This secretory process might be essential in forming the glycocalix, which covers the microvilli and their interspaces at the apical surface of the cells. The present study also detected microvilli on the apical surface of the ovate cells, whereas Singley (1982) reported a lack of a microvilli layer.

The present re-characterization, however, shows some slight histochemical differences to the descriptions in Singley (1982) concerning the evacuating ovate cells. According to this author, the glandular material of the ovate cells performs a shift from basophilia (Biebrich Scarlet staining) to acidophilia (Alcian Blue staining) induced by the surrounding sea water. This secreting ovate cell type was referred to as “evacuating ovate cells”, solely

Table 1
Comparison of the results from Singley (1982) and the present study (highlighted in grey) showing morphological similarities and differences between all gland types present in *Euprymna* skin. N.D. = not detected.

	Interstitial cell		Ovate cell		Evacuating ovate cells		Goblet cell		Cell Type 4	
	Singley (1982)	Present Study	Singley (1982)	Present Study	Singley (1982)	Present Study	Singley (1982)	Present Study	Singley (1982)	Present Study
Location	dorsal and ventral epithelium	dorsal and ventral epithelium	dorsal and ventral epithelium	dorsal and ventral epithelium	Same characteristics as for the ovate cells	Cell type not detected in this study	dorsal epithelium	dorsal epithelium	Not present in Singley (1982)	dorsal epithelium
Shape	N.D.	polymorphic	ovate	ovate to sac-like			rounded at the base, taper toward apical end	elongate pear-shaped, infolded laterally		elongate pear-shaped
Gimmick	thick and thin filaments	thick and thin filaments	5-8 nm thick filaments	peripheral filaments			microtubules	microtubules along cell axes		
Arrangement	N.D.	singular	N.D.	singular			N.D.	singular		always in close vicinity to goblet cell
Secretory Content	small membrane bound vesicles but no exocytosis	vesicles and exocytosis	Fine granular appearance	fine granules, partial release of granules			granules ø 0.5-0.7 µm, partial secretion	granules ø 0.5-1.3 µm, partial release of granules		granules ø 0.2-0.4 µm, partial release of granules
Histochemistry	N.D.	epithelium = ++ lectin WGA	++ proteins (pH 6.0); ++ proteins (pH 9.5)	++ proteins (pH 6.0); + proteins (pH 8.5); + proteins (pH 1.0); ++ lectin UEA	++ proteins (pH 9.5); ++ proteins (pH 1-3)		++ neutral sugars	++ neutral sugars		undetermined

based on the chemical modification of the secretory material but not because of morphological modifications (Singley, 1982). In the present study the pH shift (determined by Biebrich Scarlet, Alcian Blue and Toluidine Blue O staining) in the secreted material of the ovate cells was not observed, the secretory material at the cell surface only showed a very weak staining reaction for acidic proteins. We cannot exclude that our samples did not reach the same stage as those of Singley (1982), i.e. the secretions were not exposed to the sea water long enough to cause the pH shift. In the present study, the slight positive staining of the secreted mucus of the ovate cells with Alcian Blue is also evident; nevertheless, we assume that the ovate cell secretory content is a mixture of basic and acidic proteins.

The present morphological and histochemical studies show similar results for the secretory material of the goblet cells compared with those of Singley (1982). Although goblet cells showed no reaction in tests for basic and acidic proteins, there was a strong reaction for sugars in both studies.

In addition to Singley's (1982) characterization of three known gland types, we found a new cell type in the *Euprymna* epithelium that we called flask cell. The fact that this gland type could only be observed tightly adjacent to the goblet cells in the adhesive dorsal epithelium indicates a strong involvement in adhesive production and formation. It can be refuted that this cell type only represents an early developmental stage of the goblet cells as this gland type does not only appear in different animals but also in different regions within the specimens.

4.1. Comparison with other adhering cephalopods

A detailed overview of all gland types in both the adhesive and normal epithelia for all four cephalopod genera (*Nautilus* sp., *Sepia* sp., *Euprymna* sp. and *Idiosepius* sp.) can also be found elsewhere (Cyran et al., 2010). Summarizing the studies of all four genera (Table 2), the adhesive system of *Euprymna*, with its proposed doublet gland type system (goblet and flask cells), shows the highest congruence with that of *Sepia*. Although in *Sepia* the adhesive area is located ventrally on the mantle, it likewise possesses a gland type with large granules (type 1, ∅ 1–1.5 mm, positive reaction to PAS and Biebrich Scarlet, negative lectin staining) and another gland type with smaller granules (type 2, ∅ 50–250 nm, positive reaction to PAS and Biebrich Scarlet, negative lectin staining) (von Byern et al., 2010b, 2011). Regarding their appearance, the *Euprymna* gland types are elongate and pear-shaped, similar to those of *Sepia* (Cyran et al., 2010).

Although the adhesive organ of *Idiosepius* is located on the dorsal mantle side, as in *Euprymna*, and involves two related gland types, in *Idiosepius* they are differently arranged but uniformly distributed within the adhesive epithelium: around 8–15 columnar cells (granules: ∅ 1 µm, positive reaction to PAS, Biebrich Scarlet, and the lectins SBA (N-acetyl-D-galactosamine), PNA (lactose/β-galactose), UEA (α-L fucose)) are aggregated around a central sensory cell. The second gland type, the granular gland (granules: ∅ 2–5 mm, positive reaction to PAS and Biebrich Scarlet, specific for Con A (mannose), SNA (N-acetylneuraminic acid) and SBA (N-acetyl-D-galactosamine) lectins) is, in contrast, solitary (Cyran et al., 2008; von Byern et al., 2008; Cyran and von Byern, 2010). Regarding gland appearance, the *Euprymna* goblet cells resemble the *Idiosepius* columnar gland more closely than the granular gland, but there is no counterpart to the *Euprymna* flask cells in the *Idiosepius* adhesive system.

In *Nautilus* there are likewise two PAS-positive gland types: (1) columnar cells (granules: ∅ 1 mm, lectins UEA (α-L-fucose) and WGA (N-acetyl-D-glucosamine)); (2) gland type 1 (granules: ∅ 1.5–1.8 mm, lectin UEA) are present in the oral side of the digital tentacles, however, the gland types are neither aggregated nor arranged but appear singularly (von Byern et al., 2010a, 2012). On the aboral side, only one gland type (goblet cells) is present, which has granules of the same size and content (PAS reaction, lectin UEA) as the other two gland types (von Byern et al., 2010a, 2012). As in *Idiosepius*, no counterpart to the *Euprymna* flask cells could be observed in *Nautilus* either.

Table 2
Overview of the secretory gland types present in the four glue-producing cephalopod species *Euprymna* spec., *Idiosepius* spec., *Sepia tuberculata* and *Nautilus* spec. Cells which occur exclusively in the adhesive epithelium are highlighted in grey.

	Euprymna scolopes Present Study				*Idiosepius spec.* Cyran et al. (2010)				
	Interstitial	Ovate	Goblet cells	Cell Type 4	Interstitial	Goblet	Saccular	Columnar	Granular
Location	Dorsal and ventral mantle epithelium	Dorsal and ventral mantle epithelium	Adhesive dorsal mantle epithelium	Adhesive dorsal mantle epithelium	Dorsal and ventral mantle epithelium	Dorsal and ventral mantle epithelium	Dorsal and ventral mantle epithelium	Adhesive dorsal mantle epithelium	Adhesive dorsal mantle epithelium
Shape	polymorphic	ovate to sac-like	elongate pear-shaped, infolded laterally	elongate pear-shaped	polymorphic	cylindrical	sac-to balloon-shaped	elongate pear-shaped	cylindrical
Organelles	mitochondria, rER, Golgi	rER, Golgi	Golgi, rER, mitochondria	mitochondria, rER, Golgi	Golgi network, cytoskeleton	rER	rER, filament	rER, Golgi	rER, Golgi
Gimmick	Thick and thin filaments	peripheral filaments	microtubules along cell axes	-	longitudinal bundles	-	lateral filaments	-	-
Arrangement	singular	singular	singular	always in close vicinity to goblet	singular	singular	singular	aggregates of 8-15 cells	singular
Secretory Content	Small membrane bound vesicles	Fine granular appearance	granules ø 0.5-1.3 µm	granules ø 0.2-0.4 µm	glycocalix	fine-grained material	fine-grained material	granules ø 1 µm	granules ø 2-5 µm
Histochemistry	Epithelium = ++ lectin WGA	++ proteins (pH 6.0) + proteins (pH 1.0) + proteins (pH 1.0) UEA	++ neutral sugars	undetermined	-	+ neutral sugar ++ protein (pH 6.0-10.5)	-	++ neutral sugar + protein (pH 6.0-10.5)	++ neutral sugar ++ protein (pH 6.0-10.5)
Secretory release	exocytosis	partial release of granules	Partial release of granules	partial release of granules	exocytosis	release of entire secretory content	?	partial release of granules	partial release of granules

	Sepia tuberculata Cyran et al. (2010)					*Nautilus pompilius* Cyran et al. (2010)				
	Interstitial	Type 3	Goblet	Type 1	Type 2	Type 2	Type 3	Columnar	Type 1	Goblet
Location	Dorsal and ventral mantle epithelium	Dorsal and ventral mantle epithelium	Dorsal and ventral epithelium, prominent around suckers	Adhesive ventral mantle epithelium	Adhesive ventral mantle epithelium	No-adhesive aboral tentacle area	No-adhesive aboral tentacle area	Adhesive tentacle area (thick epithelium)	Adhesive tentacle area (thick epithelium)	Adhesive tentacle area (thin epithelium)
Shape	high prismatic	elongate pear-shaped	oval to pear-shaped	elongate pear-shaped	elongate pear-shaped	elongate pear-shaped	cylindrical	long slender	cylindrical	round to oval
Organelles	Golgi network, cytoskeleton	rER, Golgi	rER, Golgi	rER, Golgi	?	?	?	rER, Golgi	rER, Golgi	?
Gimmick	longitudinal bundles	-	-	tubuli between granules	tubuli between granules	-	-	translucent granular core	translucent granular core	-
Arrangement	singular	singular	singular	appear as doublet/triplet aggregation		singular	singular	singular	singular	singular
Secretory Content	glycocalix	granules ø 1µm	partly filled with fine-grained content	granules ø 1-1.5 µm different types	granules ø 50-250 nm	granules ø 2 µm different density	granules ø 1.3-1.5 µm	granules ø 1 µm	granules ø 1.5-1.8 µm	granules ø 1.2-1.7 µm
Histochemistry	-	++ proteins (pH 6.0 + 8.5)	++ proteins (pH 2.5) cell membrane ++ neutral sugars	++ neutral sugar ++ proteins (pH 6.0-10.5)	+- proteins (pH 2.5)	++ neutral sugars +- proteins (pH 1.0&2.5)	++ neutral sugars	+- neutral sugars	++ neutral sugars	++ neutral sugars
Secretory release	exocytosis	?	?	simultaneously, partial release of granules		partial release of granules	partial release of granules	partial release of granules	partial release of granules	partial release of granules

Interstitial and ovate cell counterparts are also present in the other cephalopods (Table 2). In *Euprymna*, *Idiosepius* and *Sepia*, interstitial cells are present in the adhesive and non-adhesive epidermis, presumably involved in glycocalix formation. Interstitial cells are lacking in the epithelium of *Nautilus*.

The *Euprymna* ovate cells are similar in location (adhesive and non-adhesive epithelium), appearance and fine grained material to the goblet cells of *Sepia* and the saccular-shaped glands in *Idiosepius*. However, there are histochemical differences between the three gland types. The *Sepia* ovate cells react positively to PAS and Alcian Blue and the lectins SBA (N-acetyl-D-galactosamine) and GNA (mannose) (von Byern et al., 2010b, 2011). The saccular-shaped glands of *Idiosepius* remain unreactive to sugars, proteins and lipids (von Byern et al., 2008; Cyran and von Byern, 2010). In *Nautilus* two gland cells (gland types 2 and 3) appear in the non-adhesive region; however, both gland types consist of granules (Ø

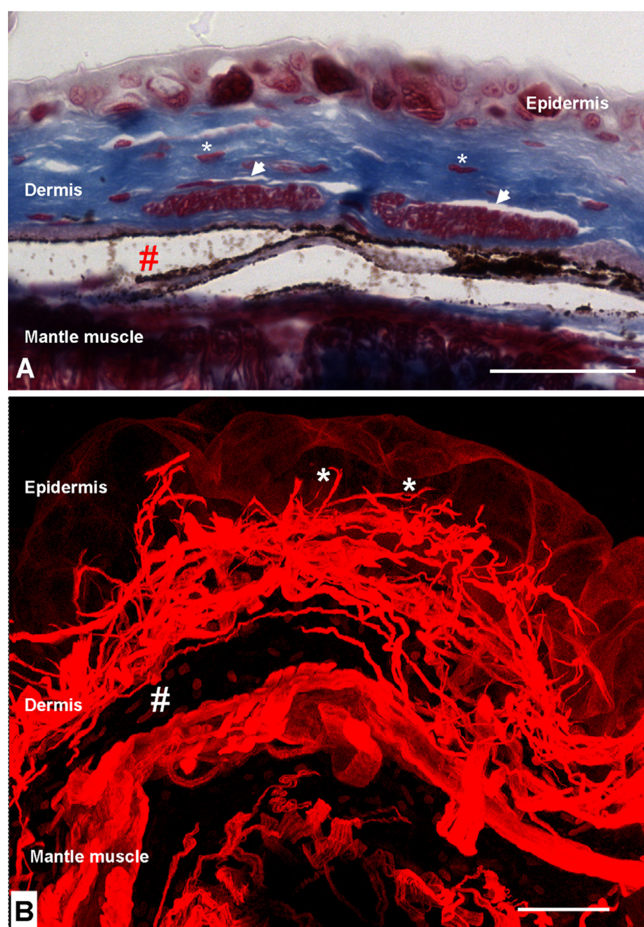

Fig. 5. (A) Azan staining of the dorsal *Euprymna* skin showing the thick muscle bundles (white arrowheads) located in the dermis layer above the chromatophores (red hashtag). Small fibers (white asterisks) from the muscle bundles pass through the dermis towards the epidermis. (B) Immunocytochemical labelling with phalloidin indicates the presence of a dense musculature network in the dermis above the chromatophores (white hashtag). Small fibers (white asterisks) pass through the dermis towards the epidermis. Scale bars = 50 μm.

1.3–2 μm), are positive to PAS and Alcian Blue staining and are reactive to the lectins UEA and WGA (von Byern et al., 2010a, 2012).

4.2. Attachment mechanism

The present re-characterization of the epithelium of *Euprymna* speaks against an antagonistic system (i.e. duo-gland system; Hermans, 1983), as suggested by Singley (1982). The ovate and interstitial cells presumably do not play a major role in bond formation, and bond breaking caused by acidic mucoproteins from the ovate cells could not be confirmed. Both cell types are also present in the non-adhesive mantle tissue and are likely to produce mucus to cover and protect the animal's surface.

The fourth cell type seems to be too small to be the sole producer of a sufficient amount of secretory material for adhesion or to discard the thick sand coat. A synergistic two-component system, as proposed for other cephalopods, involving both cell types (goblet and flask cells) in adhesive production seems to be more likely for *Euprymna*. In *Idiosepius*, two gland types stain positively for neutral sugars and basic proteins and contain granules of different sizes (Cyran et al., 2008; von Byern et al., 2008; Cyran and von Byern, 2010). In *Sepia* the granules of one gland cell (gland type 1) likewise react for sugar and basic proteins (PAS and Biebrich Scarlet staining), while the secretory material of gland type 2 consists of smaller granules and presumably of acidic proteins (Alcian Blue staining) (von Byern et al., 2010b, 2011). In *Nautilus* both gland types react positively for neutral sugars, although one gland type contains granules of around 1 μm in diameter and the other gland type contains granules of 1.5–1.8 μm (von Byern et al., 2010a, 2012).

The dense dermal muscle network described for *Euprymna* in the present study may be involved in breaking the bond of the sand coat through simple muscle contraction and release. The animal would be able to react faster than with a chemically induced release. Furthermore, a release process using the dermal musculature system would allow better control. Yet, this option was never mentioned by Singley (1982). Such a mechanically effected glue release also seems to be the primary mechanism in *Nautilus* and *Sepia* (von Byern et al., 2011, 2012). In *Idiosepius* the absence of an explicit mantle musculature beneath the adhesive area (Cyran et al., 2008; von Byern et al., 2008) speaks against such a release mechanism; instead, a detachment by animal movement is more likely (Suwanmala et al., 2006).

In summary, the present study on the adhesive epithelium in *E. scolopes* suggests that the bonding of the sand coat occurs chemically through a combination of the secretory content of the goblet and flask cells. Instead of a chemical release by the ovate cells, the release of the glue is effected mechanically, induced by the dermal musculature.

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