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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-

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