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Histochemical characterization of the adhesive organ of three *Idiosepius* spp. species

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Abstract

An adhesive organ is a prominent characteristic of the genus *Idiosepius*. Histological, histochemical and ultrastructural methods were applied to elucidate the nature of secretion of the epithelial cells of three *Idiosepius* species. The adhesive organs of *Idiosepius biserialis* and *Idiosepius pygmaeus* consist of five distinct cell types that can be distinguished morphologically and by the composition of their secretions. Histochemical analysis revealed that three cell types contain different sugar units and basic proteins, whereas the interstitial cells lacked secretory material. Acidic and sulfated substances were absent in *Idiosepius* secretions. The adhesive organ, but not the secretory material of the glandular cells, contained O-linked oligosaccharides. The histochemical analysis of the secretory products suggested that adhesion and release are not effected by a "duo-gland" adhesive system as in *Euprymna scolopes*. *Idiosepius* presumably uses a transitory adhesion, perhaps induced by secretion of a highly viscous gel. Release might be caused by contraction of the mantle musculature and/or chemical release mechanisms such as dilutors or enzymes.

Key words: Adhesion, adhesive gland system, adhesive mechanisms, cephalopoda, glue compounds, histochemistry, *Idiosepius*, Mollusca

Adhesive substances with a variety of functions are widely known among marine animals. The best investigated groups are mussels, polychaetes and barnacles, which adhere permanently to a substrate (Lacombe and Liguori 1969, Walker 1972, Waite 1986, 1987, Stewart et al. 2004, Kamino 2006). Current experimental approaches have concentrated on identifying and characterizing the mussel adhesive proteins (MAP) and adhesive substances of barnacles (Waite et al. 1989, Waite 1992, 2002, Waite and Qin 2001, Wiegemann and Watermann 2003, Dalsin and Messersmith 2005, Wiegemann 2005, Sagert et al. 2006).

Many marine organisms use a chemical mechanism for temporary attachment, e.g., Turbellaria

(Rieger and Tyler 1974, Tyler 1976), Gastrotricha (Tyler and Rieger 1980), Nematoda (Adams and Tyler 1980) and Polychaeta (Gelder and Tyler 1986). This type of adhesion is effected by the so-called duo-gland system, including two types of secretory cells; one type secretes an adhesive secretion and the other secretes a releasing secretion (Hermans 1983). By contrast, transitory adhesion allows movement and effects attachment via a secreted viscous gel. This type of adhesion is characteristic of gastropods and echinoderms (Grenon and Walker 1980, 1981, Smith 1991b, 2002, Smith et al. 1993, Flammang 1996, Pawlicki et al. 2004).

Two genera of cephalopods (*Euprymna*, Sepioliidae and *Idiosepius*, Idiosepiidae) produce glue from adhesive glands that form the adhesive organ (Nesis 1982, Norman 2003, von Byern and Klepal 2006). *Euprymna scolopes* live in benthic habitats near the shore, hiding during the day in the sediment (Moynihan 2002). The animals secrete glue to coat themselves completely with sand. When threatened, they release sand instantly to

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confuse predators (Shears 1988, Norman 2003). Ultrastructural and histochemical studies indicate that *Euprymna scolopes* has a duo-gland adhesive system consisting of goblet and ovate cells that cover the body (Singley 1982). Nonsecretory interstitial cells are positioned between these cells. The goblet cells contain large, electron-dense granules (neutral polysaccharides) that are responsible for adhesion. The fine granular secretory material of the ovate cells is enclosed in large vesicles and appears to contain basic proteins. During secretion, these proteins transform to highly sulfated acidic proteins. Singley (1982) assumed that the acidic mucoproteins cause release from substrate.

Idiosepius species live in shallow waters near shore between sea grass and mangrove beds. They are camouflaged during the day by sticking to the underside of sea grass leaves or algae (Moynihan 1983, Hylleberg and Natewathana 1991a,b, Jackson 1992). Hiding there, the animals wait to capture prey swimming by. Females, moreover, adhere for spawning (Natsukari 1970, Jackson 1992, Lewis and Choat 1993, Kasugai 2000).

A conspicuous morphological characteristic of this family is that the adhesive organ, also known as the adhesive gland, is on the posterior part of the dorsal mantle. By contrast to *Euprymna*, the adhesive organ of *Idiosepius* is restricted to the posterior portion of the fin region of the dorsal mantle side (Steenstrup 1881, Sasaki 1921). Structurally, the adhesive organ can be distinguished easily from the epithelium. Sasaki (1921) described the adhesive organ as follows: "It is represented by a longitudinal corrugated area extending along the posterior three-fourths of the back. The folds run quite irregularly without any definite mode of arrangement, and show also fine furrows and pits."

Earlier reports by Sasaki (1921) indicated that five cell types can be distinguished histologically in the adhesive organ of *Idiosepius paradoxus*, viz., columnar, granular, goblet, fusiform, and basal cells. The columnar cells extend from the basal membrane to the surface of the organ and are filled with fine granules. The cells are ovoid proximally, tapering distally and forming a bundle at the surface. The interstitial cells, also known as fusiform cells, occur around the columnar cells. According to Sasaki (1921), "the cells have a short process distally and a long fiber proximally." The columnar cells are responsible for adhesion, whereas the fusiform cells respond to touch and stimulate the secretion of adhesive substances from the columnar cells (Sasaki 1921). Additional large cell types in the adhesive organ include the granular and the goblet cells. The granular cells

are oblong, tube-shaped and contain larger granules than the columnar cells, whereas goblet cells are ovoid and contain readily visible fine granular material. The basal cells are small and do not extend to the surface. No information is available concerning either the function of the granular, goblet and basal cells or release mechanisms (Sasaki 1921).

For the study reported here, histological, histochemical and ultrastructural methods were applied to elucidate the nature of the secretions from the epithelial cells of three *Idiosepius* species: *I. biserialis*, *I. paradoxus* and *I. pygmaeus*. Our investigations explored the kinds of compounds involved in the adhesion/release mechanisms of this genus. Moreover, our study permits comparison of *Idiosepius* glue composition with *Euprymna* and other glue-producing molluscs.

Material and methods

Adult specimens of *Idiosepius pygmaeus* with a mantle length (ML) of 8.5–14.5 mm for males and 14–30 mm for females were captured in April 2004 in a mangrove forest at Klong Mudong, Phuket Island, Thailand (7°48.107' N, 98°24.472' E). *Idiosepius biserialis* (males ML 4.5–7.5 mm and females ML 6–10 mm) were collected in shallow intertidal sea grass areas at Inhaca Island, Moçambique (26°00.215' S, 32°54.721' E and 26°02.300' S, 32°54.166' E) in October 2004. *Idiosepius paradoxus* (males ML 8–16 mm and females ML 9–23 mm) were caught in intertidal sea grass areas at Nagoya (34°43.021' N, 136°58.208' E) and Ushimado, Japan (34°35.567' N, 134°07.340' E) in April 2005. All animals were kept in seawater tanks for 1 week and fed mysids and shrimp.

Preparation and fixation

All three species of *Idiosepius* were anesthetized with 3% (v/v) ethanol-seawater solution, measured and decapitated. The mantle was fixed by one of the following methods: 10% formalin in sea water or in 70% ethanol at room temperature (approximately 30°C); two acetic-alcohol-formalin (AAF) mixtures (AAF I, Lillie (1949) and AAF II (Böck 1989) for 24 h at room temperature; Carnoy solution (Kiernan 1999) for 1 h at room temperature; 2.5% glutaraldehyde or Karnovsky's solution (Karnovsky 1965) with phosphate buffer (pH 7.4, plus 10% sucrose) for 6 h at 4°C or 6 h at room temperature. The mantle of *I. pygmaeus* was prefixed *in toto* for 5 min in these fixatives at room temperature. To accelerate the infiltration of the

fixatives, the adhesive organ was excised from the mantle and cut in to 2 mm² pieces. Fixation was continued for 8 h at 4°C or 5 h at room temperature. The ventral mantle was fixed *in toto* without dissection.

For cryostat investigations, tissue samples from *I. paradoxus* were pre-fixed in 4% (v/v) formalin-seawater or embedded directly in Tissue Tec© (Sakura) and frozen in liquid nitrogen.

Unfortunately, it was impossible to anesthetize or stabilize *Idiosepius* during the attachment stage. The animals are easily frightened and escape immediately after disturbance. The mucus on the sea grass leaves always was lost during fixation and sample processing.

Embedding

Ethanol, AAF and Carnoy fixed material was dehydrated in a graded ethanol series, cleared three times for 20 min each in methylbenzoate as well as in benzene and infiltrated overnight with paraffin. Formalin fixed samples were washed in running water for 24 h, dehydrated in ethanol, washed in methylbenzoate, benzene and infiltrated in paraffin. To ensure the absence of water soluble compounds, Carnoy fixed samples were washed in absolute ethanol and treated further as above. Glutaraldehyde and Karnovsky fixed samples were washed three times for 20 min in buffer solution at room temperature, dehydrated in ethanol, cleared with methylbenzoate and benzene, and embedded in paraffin.

Sectioning

Sections 5–7 µm thick were mounted on glass slides with Ruyter solution (Ruyter 1931). Sections of Carnoy fixed material washed in absolute alcohol were mounted on glass slides in 1:1 acetone:methylbenzoate. Tissue Tec© stabilized samples were cut at 7 µm, mounted on glass slides and dried at room temperature before use.

Histology and histochemistry

Applying Singley's (1982) methods for *Euprymna scolopes*, several histological and histochemical tests were employed to elucidate the nature of the epithelial secretions of *Idiosepius*. A summary providing an overview of all tested dyes follows. All standard methods were used on paraffin embedded material. In some cases the time exposure was modified from that specified by Singley (1982).

The trichrome method AZAN (Heidenhain 1905) was used to provide an overview of the glandular system and structural details. The Cajal method modified according to Stephens and Young (1969) and Young (1971), and the Bodian technique (Bodian 1936) were used to demonstrate the presence of nerve fibers in the adhesive organ of *Idiosepius*.

The following chemical techniques showed that neutral hexose sugar units are present: the periodic acid-Schiff (PAS) method (McManus and Mowry 1960) alone or with prior treatment in dimedone for 3 h (Bulmer 1959), borohydride reduction and phenylhydrazine for 3 h, acetylation for 2 and 9 h, and acetylation-deacetylation for 24 h; alcoholic PAS (Pearse 1968); and the colloidal iron method (Hale 1946) for 1 h with prior methylation and in combination with hematoxylin or PAS (McManus and Mowry 1960, Mowry 1963).

The following staining methods were used to differentiate neutral and acidic mucosubstances in the adhesive organ of *Idiosepius*: the periodic acid-diamine method according to Spicer and Jarrels (1961) and Spicer (1965) for 7, 24 and 48 h at pH 4.0; the diamine and mixed diamine technique (Spicer and Jarrels 1961, Spicer 1965) for 24 and 48 h at pH 4.0; alcian blue 8GX (McManus and Mowry 1960) at pH 1.0 and 2.5 for 2 h at 20°C alone or with prior methylation, saponification, deamination with nitrous acid for 4, 12, 24 and 48 h, benzil blockade of arginine and in combination with PAS; azure A (Spicer 1960) in either HCl-phosphate or phosphate-citrate buffer at graded pH levels (30 min in pH 0.5, 1.0 and 3.2; and 0.1% toluidine blue in 30% ethanol for 20 min (Kramer and Windrum 1954).

Carbohydrates were characterized enzymatically using neuraminidase in combination with PAS and prior saponification and acid hydrolysis (Kiernan 1999). Furthermore, the following lectins (100 µg/ml; incubated for 30 min at room temperature) were used according to the method of Kiernan (1999): FITC-labeled concanavalin agglutinin (ConA) (Sigma, St. Louis, MO), specific for α-D-mannose/α-D-glucose and FITC-labeled peanut agglutinin (Sigma), specific for β-D-galactose, both diluted in 0.05 M TRIS/HCl (pH 7.2, containing Trace metal solution [0.01 M each calcium, magnesium, manganese chlorides; Kiernan 1999] and sodium azide); FITC-labeled soybean agglutinin (EY Laboratories, San Mateo, CA), specific for N-acetyl-D-galactosamine; TRITC-labeled wheat germ agglutinin (EY Laboratories), specific for N-acetyl-D-glucosamine, and FITC-labeled *Sambucus nigra* lectin (EY Laboratories), specific for

N-acetylneuraminic acid- α -2; FITC-labeled *Anguilla anguilla* lectin (EY Laboratories), specific for α -L-fucose; FITC-labeled *Galanthus nivalis* lectin (EY Laboratories), specific for mannose; all diluted in 0.01 M phosphate buffered saline (PBS; pH 7.2, containing 0.15 M NaCl and sodium azide). Inhibition was carried out by incubation of diluted fluorescent-labeled lectin with 0.2 M inhibitory carbohydrate for 60 min at room temperature before applying to the sections. Control for autofluorescence was tested by incubating sections in buffer solution without fluorescent-labeled lectin.

Proteins were detected using the ninhydrin-Schiff method (Pearse 1968): 16–20 h at 37°C in 0.5% ninhydrin and 30 min in Schiff's reagent (Merck, No. 109033); mercuric bromphenol blue with deamination as control (Durrum 1950, Mazia et al. 1953), acid solochrome cyanine (Pearse 1968), 1% acid solochrome cyanine for 10 min at 20°C in 0.1 M citric acid solution, pH 2.1; Biebrich scarlet for 1 h at 20°C in 0.04% Biebrich scarlet in phosphate buffer at pH 6.0 (Spicer and Lillie 1961) and at pH 8.0, 9.5 and 10.5 in Laskey's glycine buffer (McManus and Mowry 1960) and in addition, with prior deamination with nitrous acid for 4 and 12 h; fast green FCF (FG): 0.1% fast green for 30 min at pH 8.1 (Böck 1989); and fast red and ferric ferricyanide reaction (Kiernan 1999).

Sudan black B (McManus 1946) was used for frozen samples to identify lipids within the adhesive organ of *Idiosepius paradoxus*.

Scanning electron microscopy

Adult *I. paradoxus* were anesthetized with 3% (v/v) ethanol-seawater solution, measured, fixed in 70% ethanol, dehydrated in a graded series of ethanols, washed several times in acetone, dried with hexamethyldisilazane, mounted on stubs and coated with gold in a Polaron 5800 sputter coater. The samples were examined using a Philips XL 20 scanning electron microscope.

Results

Fixation effects

Fixation of the adhesive organ in formalin-seawater and ethanol caused loss of adhesive substances and gave a weak staining reaction. Treatment with both AAF variations produced intense staining, but also structural disruptions. Fixation with Carnoy resulted in good staining and was preferable to AAF owing to better tissue preservation. Glutaraldehyde and Karnovsky fixatives gave

the most vivid staining and showed the most cytological details.

Nonaqueous treatment did not enable us to detect any water soluble carbohydrates in the adhesive organ. Dehydration of Carnoy fixed samples with 2,2-dimethoxypropane caused strong irreversible contraction and tissue hardening.

Histological observation

The mantle epidermis of *I. biserialis* was 30 ± 5 μ m thick and that of *I. pygmaeus* 40 ± 6 μ m. The epidermis consisted of a single layer of columnar epithelial cells and some mucous cells. Beneath the basal membrane were collagen, muscles, nerve fibers and chromatic elements. The adhesive organ was distinguished easily from the remaining body epithelium in both species by its greater thickness (60–80 μ m in *I. biserialis* and 80–100 μ m in *I. pygmaeus*) and its five cell types (columnar, granular, goblet, interstitial and basal cells). These cells can be distinguished morphologically and by their secretory components (Fig. 1).

Columnar cells formed a battery and were pear shaped and tapered toward the surface, where they ended in a hump. They contained vacuoles that were densely filled with fine granules, 1 μ m in diameter. Their nuclei were located at their base. Granular cells were oblong and tube shaped. The cells were packed tightly with spherical to polygonal granules of uniform density, 3–5 μ m in diameter. As in the columnar cells, the nuclei of the granular cells were located basally. Goblet cells were round to sac shaped, tapering toward their apical ends. The secretory material was finely granular. Their nuclei also were located basally. Interstitial cells between the secretory cells were long and slender. Their nuclei were in a central position. This cell type was free of secretory material. Basal cells lined the basal membrane, with no contact with the surface of the epithelium. They contained vacuoles of uniform density and their nuclei were located basally.

In both *Idiosepius* species, the columnar cells were present predominantly in the middle area of the adhesive organ and less frequently near the edges of the mantle and fin region. This is in contrast to the granular and goblet cells, which were less frequent in the middle, but dominant near the boundary of the mantle and fin area.

Histochemical observations

The histochemical reactivity of the cell types is summarized in Table 1. In the surface layer, neutral

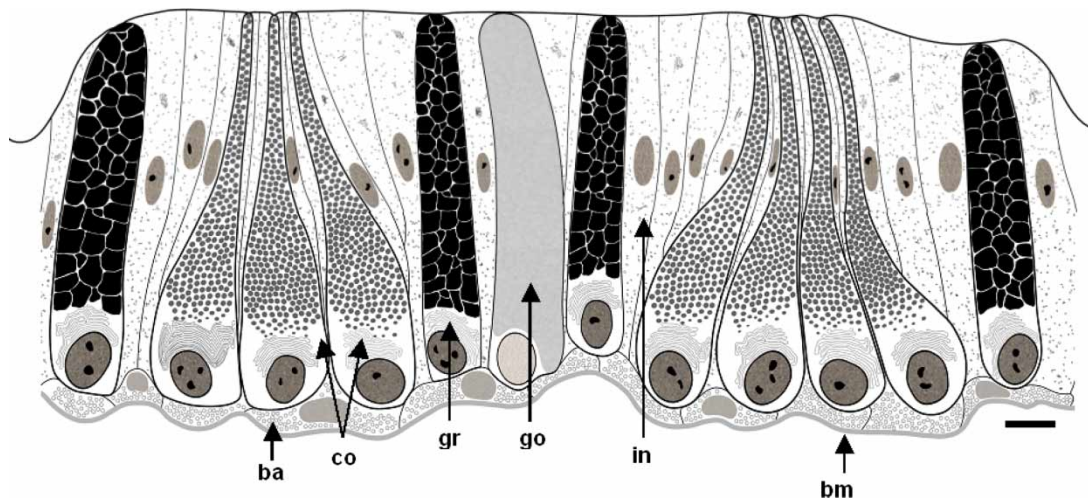


Fig. 1. Schematic drawing of the adhesive organ of *Idiosepius* columnar cells (co), granular cells (gr), goblet cells (go), interstitial cells (in), basal cells (ba) and basal membrane (bm). Scale bar = 20 μ m. Drawing by Cyran et al. (2005).

sugar units, identified by PAS, periodic acid-p-diamine and colloidal iron, covered the apical surface of the adhesive organs of both *Idiosepius* species. Proteins or acid mucosubstances could not be detected. Incubation with peanut and soybean agglutinin revealed strong reactivity to galactose and N-acetyl-D-galactosamine (Fig. 2I). The control without lectin and incubated with the appropriate sugar was negative. There was moderate N-acetylneuraminic acid labeling and a weak reaction for mannose/glucose.

In Columnar cells the secretory product was strongly periodate-reactive (Fig. 7B, D). Staining without periodate oxidation or conversion of the hydroxyl groups by acetylation produced no reactions. Dimedone treatment before PAS resulted in lack of staining. A weak reaction was observed for periodic acid-p-diamine. Moreover, no metachromatic effect was induced by diamine and mixed diamine. The secretory material was light brown like the cytoplasm of the cells. The reactivity with colloidal iron was moderate, whereas a strong reaction occurred with hematoxylin or PAS; prior methylation had no effect on the reaction. No γ -metachromasia was effected by toluidine blue and alcian blue demonstrating acid groups. Bromphenol blue, Biebrich scarlet at all pH levels, and fast green showed weakly positive reactions for basic proteins that could be neutralized by deamination. Ninhydrin-Schiff, acid solochrome cyanine, Sudan black B, fast red and ferric ferricyanide produced no staining.

Staining of the secretory material of the columnar cells with *Anguilla anguilla* lectin showed strong reactivity for fucose, moderate reactivity with ConA and *Galanthus nivalis* lectin for mannose/

glucose, and a weak affinity of *Sambucus nigra* lectin for N-acetylneuraminic acid. Columnar cells were strongly reactive to galactose and N-acetyl-D-galactosamine (Fig. 2I), but the secretory material showed no reactivity.

The secretory material in granular cells was likewise strongly periodate-reactive and unreactive to any blocking or control tests (Fig. 7B, D). With longer staining periods, the secreted substances reacted strongly with periodic acid-p-diamine (Fig. 2B). Treatment with colloidal Iron resulted in moderate staining, but no metachromatic effect was caused by either diamine or mixed diamine. Blocking of basic side chains of the protein by deamination indicated the presence of acid mucosubstances (pH 2.5 after 12 h) in this cell type of the adhesive organ. Alkaline solutions of Biebrich scarlet and fast green yielded moderate reactivity that was blocked by deamination. Bromphenol blue reacted strongly, while ninhydrin-Schiff, acid solochrome cyanine, Sudan black B, fast red and ferric ferricyanide showed no staining. The secretory material of the granular cells was strongly labeled by ConA, and *Galanthus nivalis* lectin demonstrated affinity for mannose and glucose (Fig. 2E, F). Controls with appropriate sugars provided partial inhibition (Fig. 2G). Galactose, N-acetylneuraminic acid and N-acetyl-D-galactosamine (peanut agglutinin *Sambucus nigra* lectin and soybean agglutinin) were detected in the granular cells, while the secretory material showed no reactivity (Fig. 2I).

The secretory vesicles of goblet cells were moderately reactive to PAS, periodic acid-p-diamine, diamine and mixed diamine. Dimedone failed to block staining and yielded a moderate reaction. Carnoy, AAF and ethanol fixed samples caused

	<i>Idiosepius pygmaeus</i>									
	surface		columnar		granular		goblet		interstitial	basal
Heidenhain's AZAN Fixation I	++	(B)	++	(B)	++	(B)	+	(B)	++ (R)	++ (R)
Heidenhain's AZAN Fixation II	+	(R)	++	(T-R)	++	(T)	+	(B)	+	(R)
Fixation I PAS	+++	(P)	+++	(P)	+++	(P)	++	(P)	-	-
Fixation II PAS	+++	(P)	+++	(P)	+++	(P)	++	(Y-N)	-	-
PAS-control	-		-		-		-		-	-
PAS-alc.	+++	(P)	+++	(P)	+++	(P)	++	(P)	-	-
Dimedone (3 h) - PAS (Fixation I/Fixation II)	+	(P/P)	-		-		++	(P/Y)	-	-
Acetylation (2 h)-PAS	-		-		-		-		-	-
Acetylation (9 h)-PAS	-		-		-		-		-	-
Acetylation (2 h)-Deacetylation (24 h)-PAS Fixation I	+++	(P)	+++	(P)	+++	(P)	++	(P)	-	-
Acetylation (9 h)-Deacetylation (24 h)-PAS Fixation I	+++	(P)	+++	(P)	+++	(P)	++	(P)	-	-
Acetylation (9 h)-Deacetylation (24 h)-PAS Fixation II	+++	(P)	+++	(P)	+++	(P)	++	(Y-N)	-	-
Neuraminidase-PAS	+++	(P)	+++	(P)	+++	(P)	+++	(P)	-	-
Acid hydrolysis-Neuraminidase-PAS	+++	(P)	+++	(P)	-		-		-	-
Control Neuraminidase (without enzyme)-PAS	+++	(P)	+++	(P)	+++	(P)	+++	(P)	-	-
Fixation I Borohydrid-PAS	+++	(P)	+++	(P)	+++	(P)	+++	(P)	-	-
Fixation II Borohydrid-PAS	+++	(P)	+++	(P)	+++	(R-N)	+++	(Li)	-	-
Fixation I Phenylhydrazine-PAS	+++	(P)	+++	(P)	+++	(P)	+++	(P)	-	-
Fixation II Phenylhydrazine-PAS	+++	(P)	+++	(P)	+++	(N)	+++	(Y-N)	-	-
PAD (7 h)	-		-		++	(N)	+	(N)	-	-
PAD (24 h)	-		+	(N)	+++	(N)	++	(N)	-	-
PAD (48 h)	-		+	(N)	+++	(N)	++	(N)	-	-
D (24 h)	-		+	(N)	++	(N)	++	(N)	-	-
D (48 h)	-		+	(N)	++	(N)	++	(N)	-	-
MD (24 h)	-		-		++	(N)	++	(N)	-	-
MD (48 h)	-		+	(N)	++	(N)	++	(N)	-	-
Cl (1 h)	+	(B)	++	(B)	++	(B)	+++	(T-Y)	+	(B)
Cl (2 h)-Haematoxylin	+++	(B)	++	(B)	++	(B)	+++	(T-Y)	+	(B)
Cl (2 h)-PAS	+++	(Lr)	+++	(Lr-B)	+++	(B)	+++	(T-Y)	+	(B)
Methylation-Cl (2 h)-PAS	+++	(Lr)	+++	(Lr-B)	+++	(B)	+++	(T-Y)	+	(B)
AB (pH 1.0)	-		-		-		-		-	-
AB (pH 2.5)	-		-		-		-		-	-
Methylation-AB (pH 2.5)	-		-		-		-		-	-
Methylation-Saponification-AB (pH 2.5)	-		-		-		-		-	-
Deamination-AB (pH 1.0) 4 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 1.0) 12 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 1.0) 24 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 1.0) 48 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 2.5) 4 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 2.5) 12 h	-		-		++	(B)	++	(B)	-	-
Deamination-AB (pH 2.5) 24 h	-		-		++	(B)	++	(B)	-	-
Deamination-AB (pH 2.5) 48 h	-		-		++	(B)	++	(B)	-	-
Benzil blockade-AB (pH 1.0)	-		-		-		-		-	-
Benzil blockade-AB (pH 2.5)	-		-		-		-		-	-

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AA (pH 0.5)	-	-	-	-	-	-
AA (pH 1.0)	-	-	-	-	-	-
AA (pH 3.2)	-	-	-	-	-	-
TB	-	-	-	-	-	-
Methylation-TB	-	-	-	-	-	-
NS	-	-	-		+ (N)	-
ASC	-	-	-	-	-	-
BPB	-	+ (B)	+++ (B)	+++ (B)	-	-
BPB control	-	-	-	-	-	-
BS (pH 6.0)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
BS (pH 8.0)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
BS (pH 9.5)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
BS (pH 10.5)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
Deamination-BS (pH 6.0) 2 h	-	-	-	-	-	-
Deamination-BS (pH 6.0) 4 h	-	-	-	-	-	-
Deamination-BS (pH 10.5) 4 h	-	-	-	-	-	-
Deamination-BS (pH 10.5) 12 h	-	-	-	-	-	-
Fast Green	-	+ (G)	++ (G)	+++ (G-C)	-	-
Fast Red	-	-	-	-	-	-
Ferric ferricyanide reaktion	-	-	-	-	-	-
Sudan Black B	-	-	-	-	-	-
ConA	+	++	+++	+++	-	-
Control without ConA	-	-	-	-	-	-
ConA with a-D-mannose	-	++	++	+	-	-
ConA with a-glucose	-	+	++	+	-	-
GNA	-	+	++	++	-	-
Control without GNA	-	-	-	-	-	-
GNA with a-D-mannose	-	-	-	-	-	-
PNA	+++	++	++	++	++	++
Control without PNA	-	-	-	-	-	-
PNA with Gal	-	-	-	-	-	-
WGA	-	-	-	+	-	-
Control without WGA	-	-	-	-	-	-
WGA with GlcNAc	-	-	-	-	-	-
SNA	++	+	++	+	-	-
Control without SNA	-	-	-	-	-	-
SNA with NeuAc	-	-	-	-	-	-
SBA	+++	+++	+++	+++	++	++
Control without SBA	-	-	-	-	-	-
SBA with GalNAc	-	-	-	-	-	-
AAA	-	++	-	-	-	-
Control without AAA	-	-	-	-	-	-
AAA with L-fucose	-	-	-	-	-	-

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Table 1 (continued)

	<i>Idiosepius biserialis</i>									
	surface		columnar		granular		goblet		interstitial	basal
Heidenhain's AZAN Fixation I	++	(B)	++	(B)	++	(B)	+	(B)	++ (R)	++ (R)
Heidenhain's AZAN Fixation II	+	(R)	++	(T-R)	++	(T)	+	(B)	+	(R)
Fixation I PAS	+++	(P)	+++	(P)	+++	(P)	++	(P)	-	-
Fixation II PAS	+++	(P)	++	(P)	++	(P)	++	(Y-N)	-	-
PAS-control	-		-		-		-		-	-
PAS-alc.	+++	(P)	++	(P)	++	(P)	++	(P)	-	-
Dimedone (3 h)-PAS (Fixation I/Fixation II)	+	(P/P)	-		-		++	(P/Y)	-	-
Acetylation (2 h)-PAS	-		-		-		-		-	-
Acetylation (9 h)-PAS	-		-		-		-		-	-
Acetylation (2 h)-Deacetyl (24 h)-PAS Fixation I	+++	(P)	++	(P)	++	(P)	++	(P)	-	-
Acetylation (9 h)-Deacetyl (24 h)-PAS Fixation I	+++	(P)	++	(P)	++	(P)	++	(P)	-	-
Acetylation (9 h)-Deacetyl (24 h)-PAS Fixation II	+++	(P)	++	(P)	++	(P)	++	(Y-N)	-	-
Neuraminidase-PAS	+++	(P)	+++	(P)	+++	(P)	+++	(P)	-	-
Acid hydrolysis-Neuraminidase-PAS	+++	(P)	+++	(P)	-		-		-	-
Control Neuraminidase (without enzyme)-PAS	+++	(P)	+++	(P)	++	(P)	++	(P)	-	-
Fixation I Borohydrid-PAS	+++	(P)	+++	(P)	++	(P)	++	(P)	-	-
Fixation II Borohydrid-PAS	+++	(P)	+++	(P)	++	(R-N)	+++	(Y-N)	-	-
Fixation I Phenylhydrazine-PAS	+++	(P)	+++	(P)	++	(P)	+++	(P)	-	-
Fixation II Phenylhydrazine-PAS	+++	(P)	+++	(P)	++	(N)	+++	(Y-N)	-	-
PAD (7 h)	-		-		++	(N)	+	(N)	-	-
PAD (24 h)	-		+	(N)	++	(N)	++	(N)	-	-
PAD (48 h)	-		+	(N)	++	(N)	++	(N)	-	-
D (24 h)	-		+	(N)	++	(N)	++	(N)	-	-
D (48 h)	-		+	(N)	++	(N)	++	(N)	-	-
MD (24 h)	-		-		++	(N)	++	(N)	-	-
MD (48 h)	-		+	(N)	++	(N)	++	(N)	-	-
Cl (1 h)	-		+	(N)	++	(N)	+++	(T-Y)	-	-
Cl (2 h)-Haematoxylin	+++	(B)	+	(B)	++	(B)	+++	(T-Y)	-	-
Cl (2 h)-PAS	+++	(Lr)	+++	(P)	++	(P)	+++	(N-C)	-	-
Methylation-Cl (2 h)-PAS	+++	(Lr)	+++	(P)	++	(P)	+++	(N-C)	-	-
AB (pH 1.0)	-		-		-		-		-	-
AB (pH 2.5)	-		-		-		-		-	-
Methylation-AB (pH 2.5)	-		-		-		-		-	-
Methylation-Saponification-AB (pH 2.5)	-		-		-		-		-	-
Deamination-AB (pH 1.0) 4 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 1.0) 12 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 1.0) 24 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 1.0) 48 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 2.5) 4 h	-		-		-		++	(B)	-	-
Deamination-AB (pH 2.5) 12 h	-		-		++	(B)	++	(B)	-	-
Deamination-AB (pH 2.5) 24 h	-		-		++	(B)	++	(B)	-	-
Deamination-AB (pH 2.5) 48 h	-		-		++	(B)	++	(B)	-	-

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Benzil blockade-AB (pH 0.0)	-	-	-	-	-	-
Benzil blockade-AB (pH 2.5)	-	-	-	-	-	-
AA (pH 0.5)	-	-	-	-	-	-
AA (pH 1.0)	-	-	-	-	-	-
AA (pH 3.2)	-	-	-	-	-	-
TB	-	-	-	-	-	-
Methylation-TB	-	-	-	-	-	-
NS	-	-	-	+ (N)	-	-
ASC	-	-	-	-	-	-
BPB	-	+ (B)	+++ (B)	+++ (B)	-	-
BPB control	-	-	-	-	-	-
BS (pH 6.0)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
BS (pH 8.0)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
BS (pH 9.5)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
BS (pH 10.5)	-	+ (Lr)	++ (R)	+++ (Dr)	-	-
Deamination-BS (pH 6.0) 1 h	-	-	-	-	-	-
Deamination-BS (pH 6.0) 2 h	-	-	-	-	-	-
Deamination-BS (pH 10.5) 4 h	-	-	-	-	-	-
Deamination-BS (pH 10.5) 12 h	-	-	-	-	-	-
Fast Green	-	+ (G)	++ (G)	+++ (G-C)	-	-
Fast Red	-	-	-	-	-	-
Ferric ferricyanide reaktion	-	-	-	-	-	-
Sudan Black B	-	-	-	-	-	-
ConA	+	++	+++	+++	-	-
Control without ConA	-	-	-	-	-	-
ConA with a-D-mannose	-	++	++	+	-	-
ConA with a-glucose	-	+	++	+	-	-
GNA	-	+	++	++	-	-
Control without GNA	-	-	-	-	-	-
GNA with a-D-mannose	-	-	-	-	-	-
PNA	+++	++	++	++	++	++
Control without PNA	-	-	-	-	-	-
PNA with Gal	-	-	-	-	-	-
WGA	-	-	-	+	-	-
Control without WGA	-	-	-	-	-	-
WGA with GlcNAc	-	-	-	-	-	-
SNA	++	+	++	+	-	-
Control without SNA	-	-	-	-	-	-
SNA with NeuAc	-	-	-	-	-	-
SBA	+++	+++	+++	+++	++	++
Control without SBA	-	-	-	-	-	-
SBA with GalNAc	-	-	-	-	-	-
AAA	-	++	-	-	-	-
Control without AAA	-	-	-	-	-	-
AAA with L-fucose	-	-	-	-	-	-

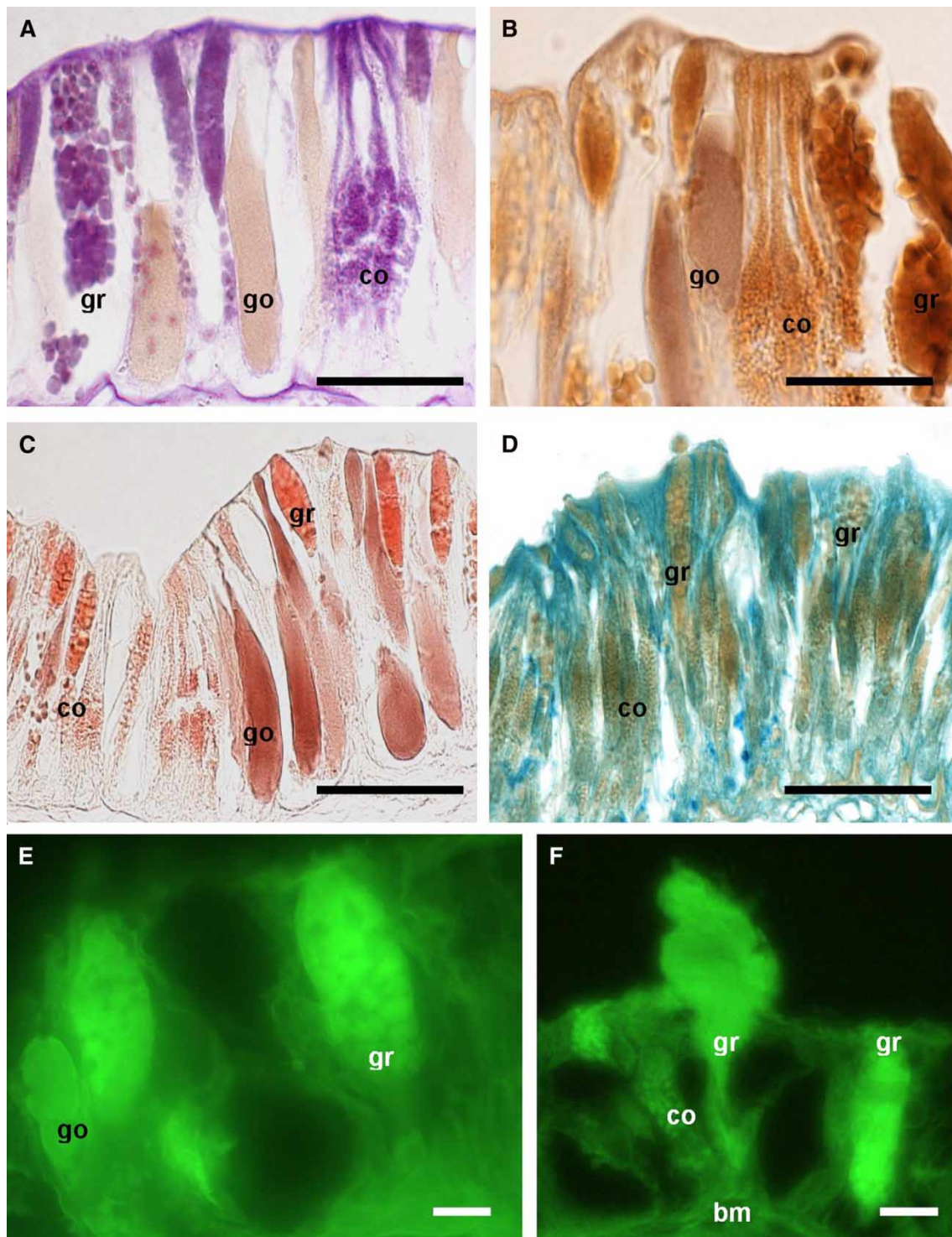


Fig. 2 (Continued)

purple coloration by PAS, while glutaraldehyde and Karnovsky fixatives produced a bicolor effect on the coloration (Fig. 2A); the cells were yellow at their base and brown toward the apex. Aldehyde

blocking by the borohydride reduction and phenylhydrazine had no effect. Colloidal iron induced a bicolor effect also, but independent of the fixative (Fig. 2D). The secretory product of these cells

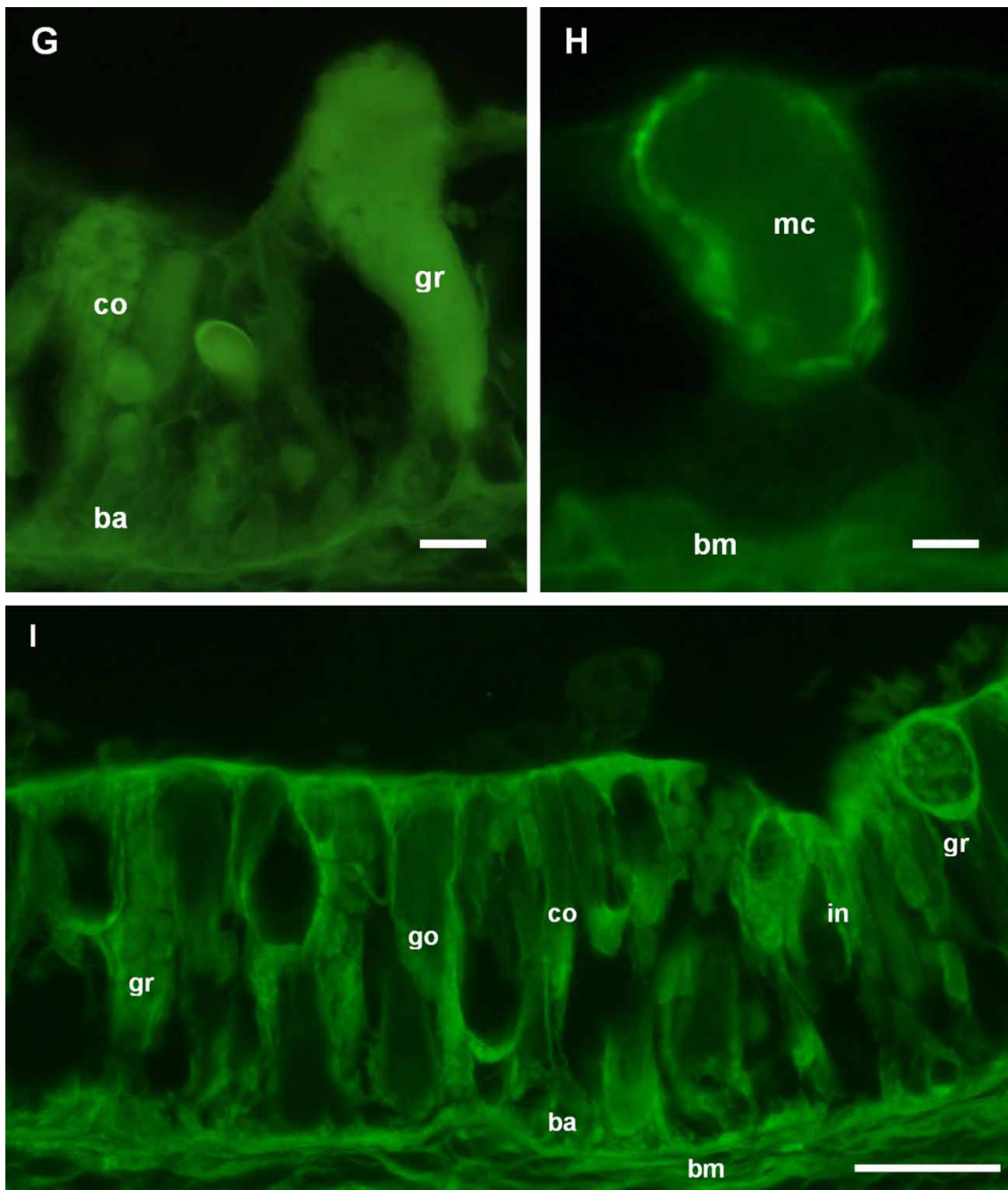


Fig. 2. Histochemical reactions of the cell types in the adhesive organ of *Idiosepius*. A) PAS with glutaraldehyde fixation produced a biphasic effect on the coloration of the secretory material of the goblet cells. B) Periodic acid-p-diamine. C) Biebrich scarlet. D) Colloidal iron with hematoxylin. E and F) FITC-ConA. G) Inhibition of ConA binding by D-glucose. H) FITC-ConA of mucus cells (mc) in the mantle epithelium. I) FITC-soybean agglutinin. Abbreviations of characteristic cell types as in Fig. 1. Scale bar = 20 μ m.

stained strongly turquoise near the basal membrane, but yellow in the parts of the cells tapering toward the apical end. The secretory vesicles stained strongly with Biebrich scarlet at all pH levels (Fig. 2C) as well as with fast green and bromphenol blue. Ninhydrin-Schiff showed a weak

reaction, while acid solochrome cyanine, fast red, ferric ferricyanide and Sudan black B showed no staining. In this cell type, acid mucosubstances (pH 1.0) could be demonstrated within the cell vacuoles after deamination with alcian blue, but these were absent in secreted material.

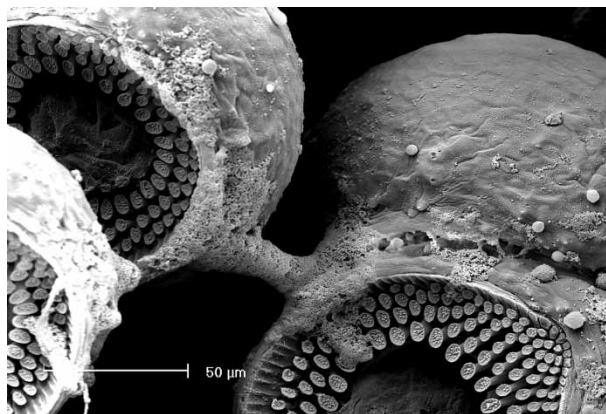


Fig. 3. SEM of mucin on the tentacle suckers in *I. paradoxus*. Even suckers contained glandular cells; their composition and function is unknown. It is possible that they provide skin protection or egg attachment during spawning. Scale bar = 50 μm .

The secretory material of the goblet cells was strongly reactive for mannose, as shown by ConA and *Galanthus nivalis* lectin (Fig. 2E, F), and fucose, as shown by *Anguilla anguilla* lectin. Like the columnar and granular cells, the goblet cells showed a strong affinity for galactose and N-acetyl-D-galactosamine (Fig. 2I), whereas no reaction was detected in the secretory material. Incubation with *Sambucus nigra* lectin resulted in weak staining.

The contents of interstitial cells did not react with most of the histochemical tests applied. Colloidal iron induced weak staining only slightly stronger than background, while the cell membrane was moderately reactive for galactose and N-acetyl-D-galactosamine (Fig. 2I).

The secretory vesicles of basal cells did not react with any of the histochemical tests applied, while the cell membrane was moderately reactive to peanut and soybean agglutinin.

The mucous cells of the mantle epidermis reacted strongly positive with Biebrich scarlet (no difference in reactivity at different pH levels) and fast green. The secretory material of the cells was strongly reactive for D-mannose as shown by *Galanthus nivalis* lectin, while the cells were reactive for glucose as shown by ConA (Fig. 2H).

Scanning electron microscopy observations

The mantle epidermis of *I. paradoxus* was smooth, with cilia either singly or in tufts, on the ventral and dorsal sides, except on the adhesive organ. Droplets of secretory material were present on the mantle epidermis, the arms and the suckers (Fig. 3).

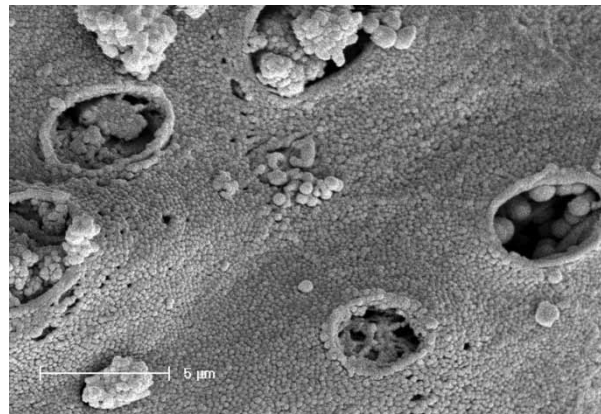


Fig. 4. The adhesive organ was completely covered by microvilli except for the pore openings. Scale bar = 5 μm .

The electron microscopic analysis of the adhesive organ showed furrows, pits and secretion pores on the surface, which was covered completely by microvilli (Fig. 4). The organ extended from the dorsal mantle to the fins and was clearly delineated from the surrounding epidermis (Fig. 5).

Two kinds of secretory pores could be distinguished on the surface of the adhesive organ (Fig. 6). The first type was round or ovoid and grouped on an elevation, while the second type was single and situated between the elevations. The material inside the pores consisted of round granules, which aggregated to form humps (Fig. 7A). Granule size and the structure of this pore field correlated with the columnar cells seen in histological sections

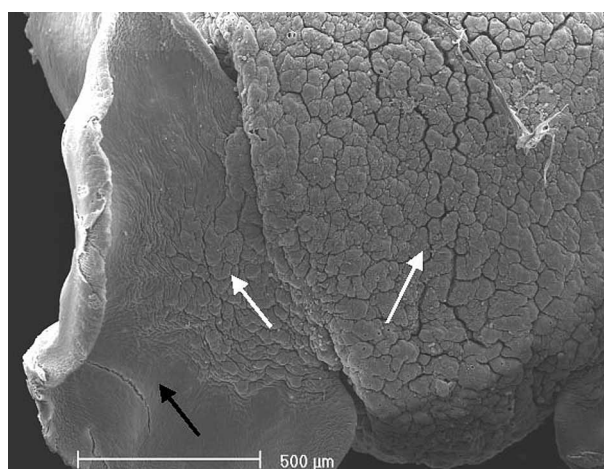


Fig. 5. SEM of the adhesive organ of *I. paradoxus*. The adhesive organ (white arrow) was restricted to the dorsal mantle side and fins, and was distinguished easily from the mantle epithelium (black arrow) by its different structure. Scale bar = 500 μm .

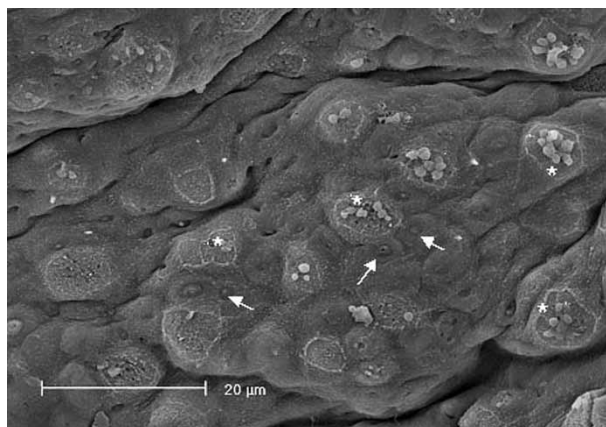


Fig. 6. Surface of the adhesive organ with elevated pore fields (*) and single pores (arrow) between the elevations. Scale bar = 20 μm .

(Fig. 7b). The single pore type secreted uniform, drop-shaped granules, indicating that these are the pores of the granular cells (Fig. 7 C,D, and Fig. 8).

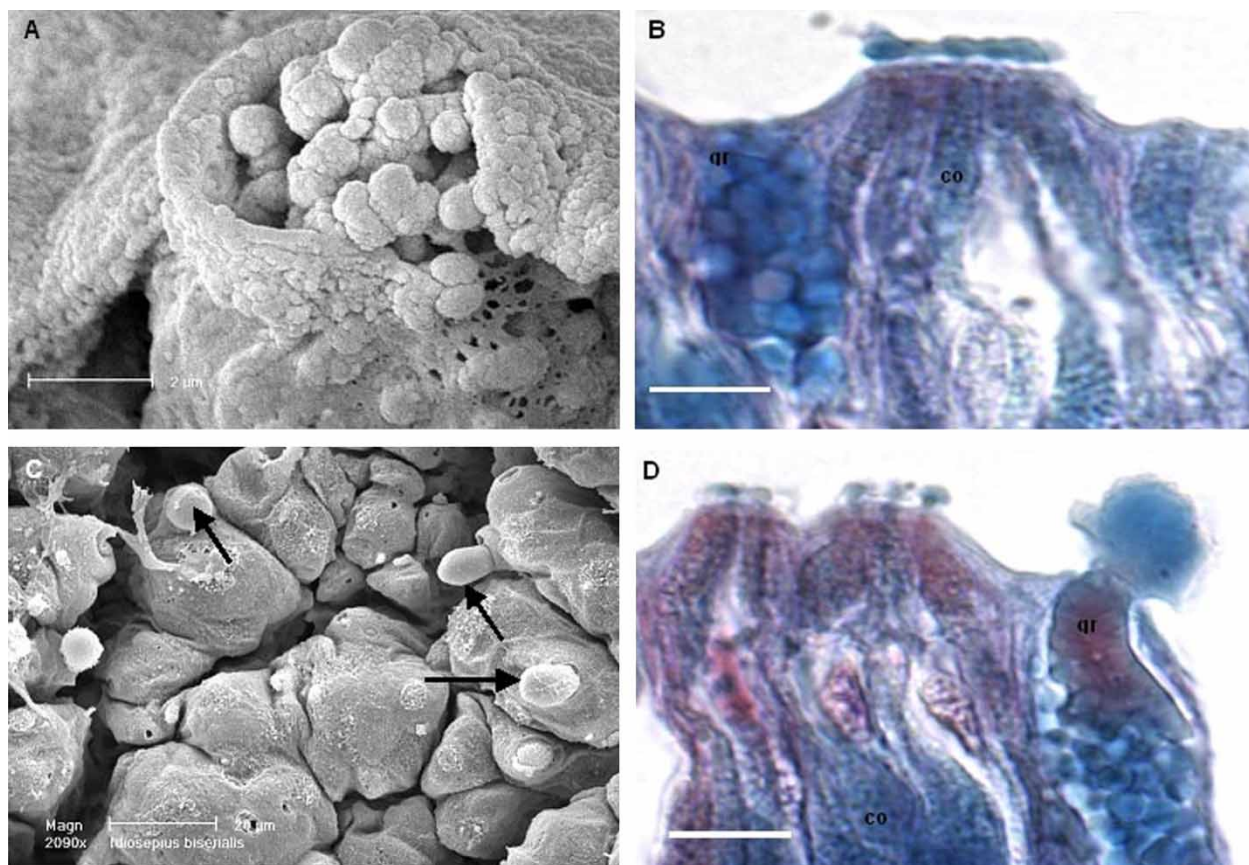


Fig. 7. A) Material inside the pore was granular and rounded and produced lumps during secretion. B) The size of the granules and the humps of these pores corresponded with the histological characteristics of the columnar cells (co) (alcian blue-PAS). C) Single pores secreted uniform drop-shaped granules (arrow). D) The position of the single pores and histological results indicated that the secretion from this kind of pore corresponds to granular cells (gr) (alcian blue-PAS). Scale bar = 20 μm .

Discussion

Most earlier studies of the genus *Idiosepius* have provided information about life cycles, behavior and physiology (Yamamoto 1988, Pecl and Moltchanivskyj 1999, Yamamoto et al. 2003, Eyster and van Camp 2003), but no investigation of the mechanism of adhesion has been made. The results of our work show that adhesive substances are exclusively responsible for adhesion in *Idiosepius*. No muscles or nerve fibers are related to the gland cells of *I. biserialis* and *I. pygmaeus*, so adhesion induced by reduced pressure, as in cephalopod suckers (Kier and Smith 1990, 2002, Smith 1991a, 1996), can be excluded. Rather, adhesive substances secreted from the columnar, goblet and granular cells are responsible for adhesion in *Idiosepius*.

Our histological and SEM observations of the adhesive organ of *I. pygmaeus* and *I. biserialis* agreed

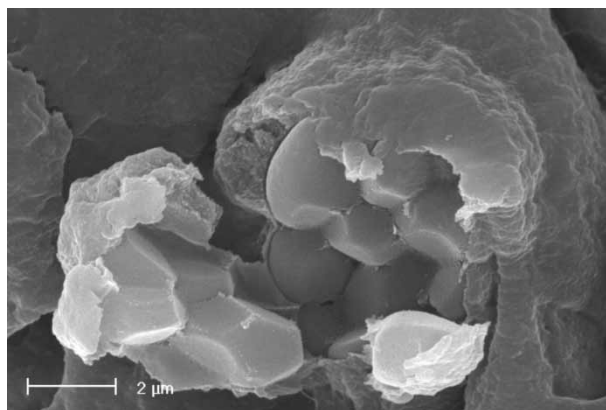


Fig. 8. The granular cell type with larger polygonal granules, packed tightly together. Scale bar = 2 μm .

with the description of Sasaki (1921) for *I. paradoxus*. Compared with *Euprymna scolopes* (Singley 1982), the adhesive organ of *Idiosepius* clearly differed in the number of cell types, but showed similarities in the morphology and secretory components of these cell types. The goblet cells of *Euprymna* resembled the granular cells of *Idiosepius* and the ovate cells of *Euprymna* were similar to the goblet cells of *Idiosepius*. The interstitial cells were similar in both species. The columnar and basal cells were restricted to the adhesive organ of *Idiosepius*.

Despite the morphological similarities of the cell types of the two genera, we follow the terminology used by Sasaki (1921; see also Packard 1988, Budelmann et al. 1997). The histochemical results of our study showed that all three granular cell types in the adhesive organs of *I. biserialis* and *I. pygmaeus* contained neutral carbohydrates and proteins (von Byern et al. 2005). Acidic mucosubstances, lipids and sulfated proteins were not detected in the adhesive organs.

Secretory products of the columnar cells displayed weak reactivity with basic protein stains, whereas the secretory product of the granular cells showed moderate staining with Biebrich scarlet and fast green but strong reactivity to mercuric bromphenol blue. By contrast, the goblet cells showed a strongly positive reaction with Biebrich scarlet, fast green FCF and mercuric bromphenol blue, suggesting a large amount of basic proteins in the secretory material. The weak reaction of the secretory material in the goblet cells to ninhydrin-Schiff might be a staining effect rather than a positive reaction.

Lectin analysis indicated that the sugar composition differs among the glandular cells. Mannose sugars are present predominantly in the granular

and goblet cells, but were moderately in the columnar cells, which showed a high affinity for fucose. Based on these results, we assume that the secretory material of the glandular cells consists of a protein-carbohydrate complex. The different staining reactions showed that the ratio of proteins to carbohydrates varies considerably among the different cell types. Columnar cells contain primarily sugars, such as fucose, and relatively little protein. Granular cells contain a balanced ratio of sugar and proteins, whereas the sugar primarily is mannose with a small proportion of glucose. Goblet cells have a larger proportion of protein and a smaller proportion of sugar, primarily mannose and some glucose. Acidic substances were not detected in any *Idiosepius* species.

The tissue of the adhesive organ, but not the secretory material, of the three glandular cells showed strong affinity for galactose, N-acetyl-D-glucosamine and N-Acetylneuraminic acid indicating the presence of O-linked oligosaccharides.

Several studies have shown that acid mucopolysaccharides (proteoglycans) occur in the skin of cephalopods, and most studies have provided information about the chondroitin sulfate side chains of these compounds (Kinoshita et al. 2001, Kinoshita-Toyoda et al. 2004). In *Loligo opalescens*, 70% of the chondroitin is highly sulfated (Srinivasan et al. 1969, Radhakrishnamurthy et al. 1970), whereas the proteoglycans in the skin of *Todarodes pacificus* contain nonsulfated chondroitin (Anno et al. 1964, Kawai et al. 1966, Isobe and Seno 1971). In *Illex coidentii*, three major groups of polysaccharides are present in the skin, two containing chains of chondroitin and the other over-sulfated chondroitin sulfate (Karamanos et al. 1988, 1990, Vynios and Tsiganos 1990, Vynios et al. 2000). Several studies have characterized the different chemical forms of chondroitin sulfate based on repeating disulfated disaccharide units, such as the over-sulfated chondroitin isoform E in squids (Habuchi et al. 1971, Inoue et al. 1986, Ito and Habuchi 2000). Chondroitin sulfate is a ubiquitous component of the extracellular matrix of connective tissues and plays different roles in the physiology of animals (Kinoshita et al. 1997; Habuchi et al. 2002). It has no apparent influence on secretion.

The study reported here reveals similarities and differences in the secretory material of the adhesive organs of *Euprymna scolopes* and *Idiosepius*. The three glandular cells of *Idiosepius* and the goblet cells of *Euprymna* contain neutral hexose sugars. The interstitial cells of *E. scolopes*, *I. biserialis* and *I. pygmaeus* showed no histochemical reaction and

presumably are not involved in the secretion of mucous substances for adhesion or release. To the contrary, the ovate cells of *Euprymna* contain basic proteins, which become highly acidic during secretion (Singley 1982). In all three secretory cell types of the *Idiosepius* adhesive organ, basic proteins associated with a protein-carbohydrate complex also were present. A change of these proteins to an acidic complex in secreting cells could not be observed in *I. biserialis* and *I. pygmaeus*. The appropriate tests (alcian blue, azure A, toluidine blue) were negative.

The histochemistry of the secretory products suggests that adhesion and release of *Idiosepius* is not based on a duo-gland adhesive system as in *E. scolopes* (Singley 1982). *Idiosepius* probably attaches to surfaces by transitory mechanisms like many gastropods (Grenon and Walker 1980, 1981, Smith 1991b, 2002, 2006, Smith et al. 1993, Flammang 1996, Pawlicki et al. 2004). Adhesion produced only by proteins, as is the case for sessile organisms such as mussels and barnacles (Sagert et al. 2006), can be excluded for *Idiosepius*.

The differential distribution of the three types of glandular cells in the adhesive organ of *Idiosepius* may lead to contradictory conclusions about the mechanism of adhesion and release. The viscous mucus secreted from one, two or all three glandular cells jointly generates the adhesive mucus. The columnar cells in the center produce a strong adhesion effected by a carbohydrate-rich gel, whereas granular and goblet cells at the boundary secrete a more proteinaceous mucus. The centrally located columnar cells achieve attachment while the secretion of the boundary cell types effect dilution of the secretion and break the connection. Other release mechanisms such as contraction of the mantle musculature and/or chemical release mechanisms by other dilators also are conceivable. So far, observations of the animals' behavior give no clue concerning the mechanisms involved in release. The different adhesive substances and mechanisms of release in *Idiosepius* and *Euprymna* serve different ecological and behavioral adaptations.

Euprymna scolopes uses its glue to cover itself with a coat of soft sediment and can release it quickly from its body. *Idiosepius* attaches with a small adhesive area to different substrates such as seaweed, seagrass leaves and roots. An easy, but slow, disconnection is effected without a special releasing substance. Investigations of the adhesive force and material properties of the glue as well as of structural deformation of the adhesive organ in response to the roughness of the substratum will

help to elucidate the mechanisms of release in *Idiosepius*.

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