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ORIGINAL ARTICLE

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The attachment complex of brachiolaria larvae of the sea star *Asterias rubens* (Echinodermata): an ultrastructural and immunocytochemical study

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Abstract The attachment complex of brachiolaria larvae of the asteroid *Asterias rubens* comprises three brachiolar arms and an adhesive disc located on the preoral lobe. The former are used in temporary attachment and sensory testing of the substratum, whereas the latter is used for permanent fixation to the substratum at the onset of metamorphosis. Brachiolar arms are hollow structures consisting of an extensible stem tipped by a crown of dome-like ciliated papillae. The papilla epidermis is composed of secretory cells (type A, B and C cells), non-secretory ciliated cells, neurosecretory-like cells and support cells. Type A and B secretory cells fill a large part of the papilla epidermis and are always closely associated. They presumably form a duo-gland adhesive system in which type A and B cells are respectively adhesive and de-adhesive in function. The adhesive disc is an epidermal structure mainly composed of secretory cells and support cells. Secretory cells produce the cement, which anchor the metamorphic larva to the substratum until the podia are developed. The relatedness between the composition of the adhesive material in the brachiolaria attachment complex and in the podia of adults was investigated by immunocytochemistry using antibodies raised against podial adhesive secretions of *A. rubens*. Type A secretory cells were the only immunolabelled cells indicating that their temporary adhesive shares common epitopes with the one of podia. The attachment pattern displayed by the individuals of *A. rubens* during the perimetamorphic period—temporary, permanent, temporary—is unique among marine non-vertebrate Metazoa.

Keywords Adhesion · Sensory testing · Perimetamorphic period · Cross-immunoreactivity · Duo-gland system · Asteroidea

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Introduction

One of the most widespread developmental patterns in Asteroidea is the indirect pelagic planktotrophic development through bipinnaria and brachiolaria larval stages (see McEdward and Miner 2001 for review). Brachiolaria larvae differ from bipinnaria larvae by the presence of specialized attachment structures on the preoral lobe comprising three brachiolar arms and an adhesive disc (Gemmill 1914; Dawidoff 1948; Barker 1978; Gondolf 2000). These structures form together the brachiolaria attachment complex, which plays a crucial role during the perimetamorphic period (Haesaerts et al. 2003). The behaviour of brachiolaria larvae during this period was investigated by Gemmill (1914) and Barker (1977) for the forcipulatid Asteroidea *Asterias rubens* Linné, 1758, and *Stichaster australis* (Verrill, 1870) and *Coscinasterias calamaria* (Gray, 1840), respectively. Brachiolar arms provide temporary adhesion, allowing several successive cycles of attachment and detachment, and sensory testing of the substratum while the adhesive disc is involved in permanent adhesion which marks the onset of metamorphosis.

Though the morphology and functioning of the brachiolaria attachment complex remain poorly known, similarities were pointed out between brachiolar arms in larvae and podia in postmetamorphic individuals (Barker 1978; Hermans 1983; Flammang 1996). The present study deals with the asteroid *Asterias rubens*, a species in which the ultrastructure of the podia and the composition of their adhesive have already been investigated (Flammang et al. 1994, 1998a). It aims to describe the

fine morphology of the brachiolaria attachment complex with a particular interest for the secretory structures, and to investigate the relatedness between the adhesive material composition in both larval attachment complex and podia of adults.

Materials and methods

Larval rearing

Ripe individuals of *A. rubens* were collected intertidally in Audresselles (Pas-de Calais, France) in March 1999. They were transported to the Marine Biology Laboratory of the University of Mons, kept in marine aquariums with running seawater and fed mussels. Spawning was induced by injecting 0.2 ml of a 100 μM 1-methyladenine solution in each arm. Released gametes were collected with Pasteur pipettes, and eggs were fertilized in Petri dishes at room temperature. Embryos were then transferred (ca. 250 embryos/l) in tanks filled with 50 l of aerated and previously filtered seawater (FSW, 0.22 μm) at 14°C, and larvae were fed daily diatoms, *Phaedactylum tricornutum* Bohlin, 1897. Seawater was left unchanged during the rearing, and late brachiolaria larvae were collected about 9 weeks after fertilization (Haesaerts et al. 2003).

Microscopy

For scanning electron microscopy (SEM), larvae were fixed in Bouin's fluid for 12 h. Some additional larvae were fixed in glutaraldehyde and post-fixed in osmium tetroxide (see below), a method that does not preserve the cuticle and thus reveals the underlying structures (Ameye et al. 2000). Specimens were dehydrated in graded ethanol series, dried by the critical point method (with CO_2 as transition fluid), mounted on aluminium stubs, coated with gold in a sputter coater, and observed with a JEOL JSM-6100 scanning electron microscope.

For light microscopy (LM) and transmission electron microscopy (TEM), larvae were fixed in 3% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.8, adjusted to 1,030 mOsm/l with NaCl) for 30 min at 4°C, rinsed in cacodylate buffer, and post-fixed for 1 h in 1% osmium tetroxide in the same buffer. After a final buffer wash, they were dehydrated in graded ethanol series and embedded in Spurr. For LM analysis, thin sections (1 μm) were cut with a Reichert OmU2 ultramicrotome equipped with a glass knife, stained with an equivolumic mixture of 1% Azur II and 1% methylene blue solutions, observed and photographed with a Leitz Orthoplan light microscope equipped with a Leica DC 300F digital camera. For TEM analysis, ultrathin sections (70–80 nm) were cut with a Leica UCT ultramicrotome equipped with a diamond knife. The sections were contrasted with uranyl acetate and lead citrate and observed with a Zeiss LEO 906 E transmission electron microscope.

Immunochemistry

Immunohistochemical analyses were done on larvae fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 2 h at room temperature and rinsed in the same buffer for 30 min. An indirect immunofluorescence method was used on both larvae in toto and paraffin sections (7 μm in thickness) prepared by routine procedures (see Gabe 1968). Whole larvae and sections were permeabilised in PBS containing 0.25% Triton-X-100 for 1 h, and then pre-incubated for 30 min in 10% normal swine serum (Dako, Denmark) in PBS, in order to block non-specific antigenic sites. Antisera were obtained by immunising rabbits with the adhesive material of the podia of *A. rubens* (Flammang et al. 1998a). They were diluted 1:100 in PBS containing 1% Tween 20 and 3% bovine serum albumine (BSA; PBS-Tween-BSA), and applied overnight at 4°C. After three washes in PBS, samples were incubated for 1 h at room temperature in FITC (fluorescein isothiocyanate)-conjugated swine anti-rabbit immunoglobulins (Dako, Denmark) diluted in 1:50 in PBS-Tween-BSA. After a final wash in PBS, both whole larvae and sections were mounted in Vectashield (Vector, CA, USA) and observed with a Leica TCS 4D confocal laser scanning microscope. Propidium iodide was added as DNA stain to the mounting medium to facilitate tissue identification.

For immunocytochemistry, larvae were fixed, embedded and cut as for TEM analyses. Ultrathin sections were collected on gold grids, pre-incubated for 30 min in 10% normal swine serum in PBS and incubated overnight at 4°C with the antisera diluted 1:100 in PBS-Tween-BSA. After PBS rinsing, sections were immunogold-stained for 1 h at room temperature in goat anti-rabbit immunoglobulins conjugated to 15 nm gold particles (Sigma-Aldrich Chemie, Germany) diluted 1:10 in PBS-Tween-BSA. After three washes in PBS and distilled water, they were then contrasted with uranyl acetate and lead citrate and observed with a Zeiss LEO 906 E transmission electron microscope.

For each experiment, a section of a podium of *A. rubens* was included as a positive control. Negative controls were carried out by (1) substituting the primary antiserum with PBS-Tween-BSA, and (2) substituting the primary antiserum with the corresponding preimmune serum diluted 1:100 in PBS-Tween-BSA.

Results

External morphology

The brachiolaria attachment complex of *A. rubens* is located on the most anterior part of the preoral lobe, on

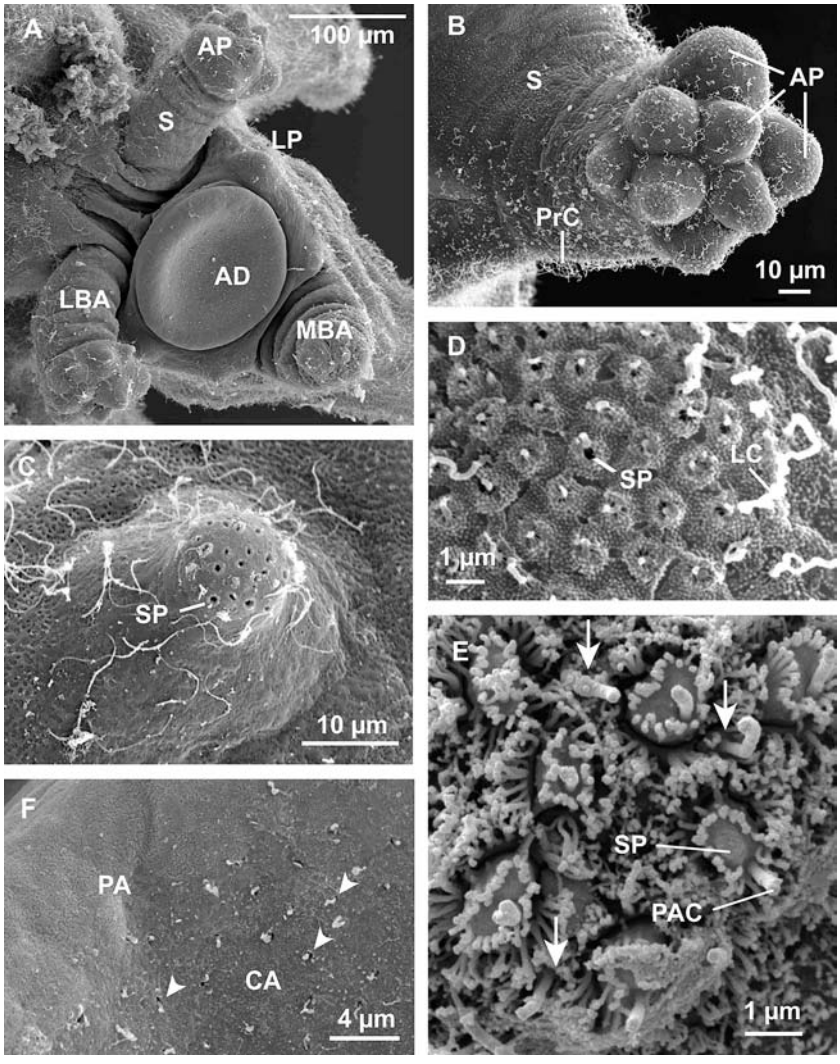

Fig. 1 *Asterias rubens* (SEM). External morphology of the attachment complex of the brachiolaria larva. **a** General view of the attachment complex. **b** Extended brachiolar arm. **c** Lateral papilla. **d**, **e** Details of the tip of a papilla [larvae fixed in Bouin's fluid (**d**) and glutaraldehyde (**e**)] *Arrows* in **e** indicate short straight transcuticular cilia. **f** Details of the adhesive disc surface (*arrowheads* indicate the presence of short transcuticular cilia). *AD* adhesive disc, *AP* apical papillae, *CA* central area, *LBA* lateral brachiolar arm, *LC* long cilium, *LP* lateral papillae, *MBA* median brachiolar arm, *PA* peripheral area, *PAC* pore-associated cilium, *PrC* preoral ciliary tract, *S* stem, *SP* secretory pore

the ventral side of the larval body. It consists of an adhesive disc surrounded by three brachiolar arms, one being median and two others lateral (Fig. 1a). The attachment complex is surrounded by the preoral ciliary tract which extends to the tip of each arm (Fig. 1b). Those arms share the same external morphology though the median one may be slightly larger and longer than the other two. The length of the arms varies from 100 µm to 200 µm. Each arm is divided into a proximal stem and a distal crown made of eight to twelve apical papillae (Fig. 1b). The tip of each papilla is dome-shaped, measures about 20 µm in diameter, and presents from 50 to 100 secretory pores. Each pore is surrounded by a ring of microvilli from which protrudes a short cilium (Fig. 1d, e). Two other types of transcuticular cilia occur that are not pore-associated, viz. long twisted cilia and short straight cilia. The latter are restricted to the papilla tip and are scattered between secretory pores while the twisted ones occur all over the papillae (arrows, Fig. 1e, and Fig. 1b–d, respectively). A mat of microvilli covers each brachiolar arm (Fig. 1e).

The adhesive disc is a round concave structure of about 100–150 µm in diameter (Fig. 1a). Its thickened edge is smooth while its concave central part has short cilia and a few cuticular protrusions (Fig. 1f). The adhesive disc is surrounded by three to five lateral papillae whose morphology is similar to those of arm tip papillae (Fig. 1a, c).

Internal morphology

The brachiolar arms are hollow cylindrical structures occupied by an extension of the preoral coelom. Both their stem and tip are made up of four tissue layers that are, from inside outwards, a mesothelium surrounding the lumen, a connective tissue layer, a subepidermal nerve plexus and an epidermis covered by a cuticle (Fig. 2a). Constituting tissues are thicker at the papilla level, the thickening affecting mostly the epidermal layer (Fig. 2b).

The mesothelium (coelomic epithelium) is pseudostratified and has two cell types, adluminal and myoepithelial cells (Fig. 2a, c). Both contact the underlying basal lamina. Adluminal cells line the coelomic cavity and bear a long vibratile cilium. Myoepithelial cells oc-

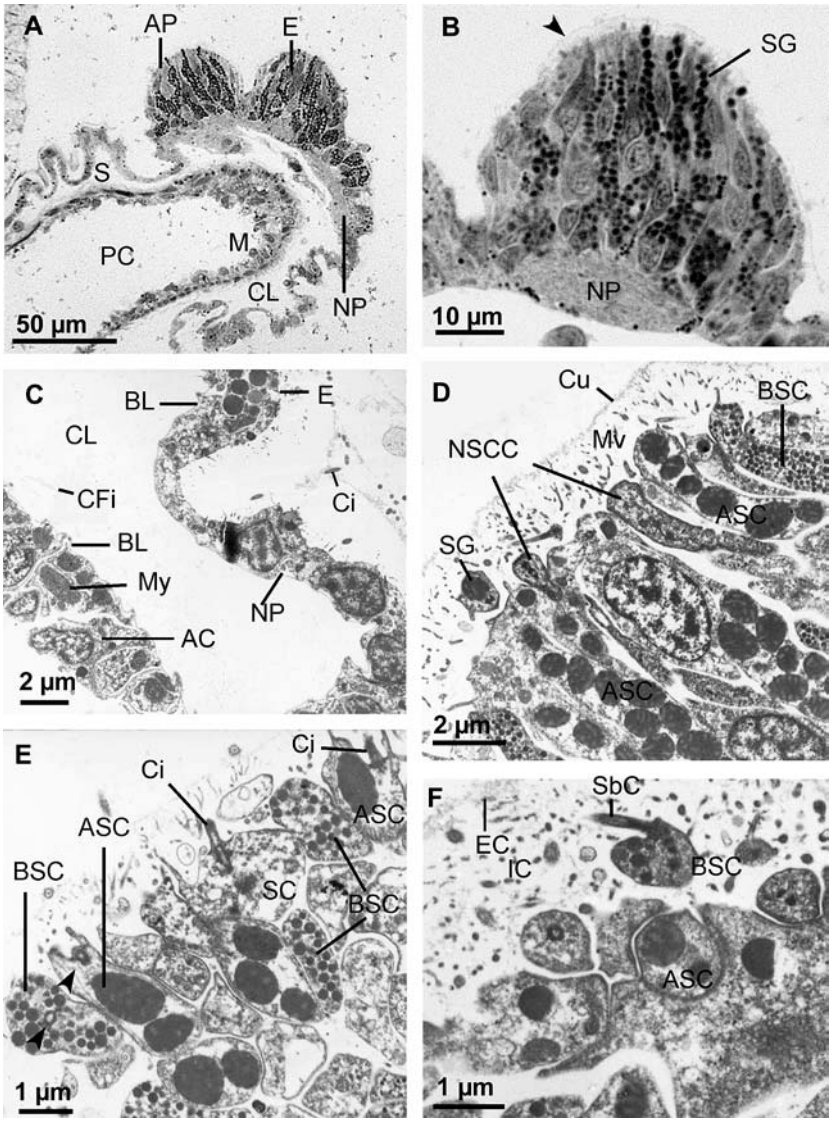

Fig. 2 *Asterias rubens*. Fine structure of the brachiolar arms. (**a**, **b** LM; **c**–**f** TEM). **a** Longitudinal section through a brachiolar arm. **b** Detail of an apical papilla (*AP*) [*arrowhead* indicates the presence of the cuticle(*Cu*)]. **c** Section through the stem of a brachiolar arm.**d**–**f** Details of the epidermis (*E*) of the apical part of a papilla, showing (**d**) non-secretory ciliated cells (*NSCC*), (**e**) support cells (*SC*), type A and type B (*BSC*) secretory cells and (**f**) and their subcuticular cilia (*SbC*), (*arrowheads* in **e** indicate basal bodies). *AC* adluminal cell, *BL* basal lamina, *CFi* collagen fibre, *Ci* cilium, *CL* connective tissue layer, *E* epidermis, *EC* external granular cuticle, *IC* internal filamentous cuticle, *M* mesothelium, *Mv* microvilli, *My* myoepithelial cell, *NP* nerve plexus, *PC* preoral coelom, *S* stem, *SG* secretory granule

cur between adluminal cell bodies and the basal lamina, and contain a bundle of myofilaments. Myofibrils in myoepithelial cells present two orientations, one being perpendicular to the other. The connective tissue layer is made up of an amorphous material that encloses a few collagen fibres and some mesenchymal cells (Fig. 2a, c). The nerve plexus consists of tangled neurites underlying the epidermal cells and containing clear or dense-core small vesicles (Fig. 2b). The plexus is more developed in the papillae than in the stem.

The epidermis of the brachiolar arms is monostratified. All epidermal cells are connected apically by junctional complexes made up of a distal zonula adherens and a proximal septate junction (Fig. 4). The whole epidermis is coated with a bi-layered cuticle consisting of an internal filamentous layer and an external granular layer (Fig. 2d, f). The stem epidermis contains only covering cells that may be ciliated or not, and whose cytoplasm encloses numerous vacuoles of variable electron density, size and shape (Fig. 2c). As for the papilla epidermis, it is made up of six different cell types: three types of secretory cells (the A, B and C cell types; see Barker 1978 for terminology), non-secretory ciliated cells (NSCC), neurosecretory-like cells (NSC) and support cells (SC; Fig. 4). Among secretory cells, types A and B are abundant and uniformly distributed in the papillae, whereas type C cells are less numerous and mostly located at its periphery (Figs. 2b, 3a). Type A and B cells are narrow and elongated while type C cells are of various shapes (Figs. 3a, 4). The cytoplasm of type A cells is filled with large ellipsoidal membrane-bound heterogeneous granules of high electron density (about 1 µm × 0.7 µm) (Figs. 2d,e, 3c, d). Developing granules are larger, more loosely packed (Fig. 3a, d), and always associated with Golgi stacks and RER cisternae (Fig. 3c, d). Granules are expelled by a pore, delimited by a ring of apical microvilli. Type A cells also bear a short cilium that protrudes on the margin of the secretory pore (Figs. 1e, 2e). The pore associated cilia of type A cells correspond to those seen on SEM pictures

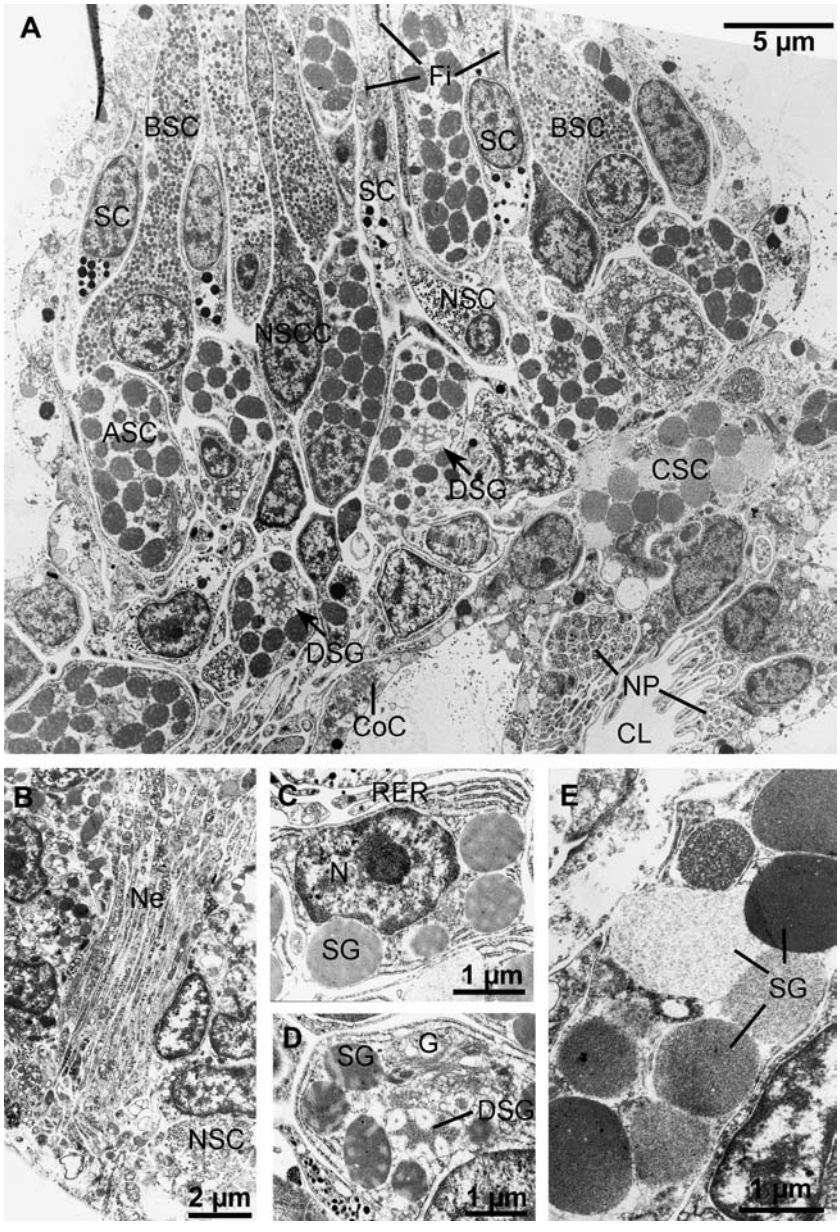

Fig. 3 *Asterias rubens*. Ultrastructure of the papillae (TEM).**a** Longitudinal section through a papilla showing various types of cells and basiepidermal nerve plexus (*NP*). **b** Detail of the nerve plexus with neurites (*Ne*) and neurosecretory-like cells (*NSC*) located in the basal part of the epidermis. **c**, **d** Details of type A secretory cells (*ASC*). **e** Detail of type C secretory cell (*CSC*). *BSC* type B secretory cell, *CL* connective tissue layer, *CoC* covering cell, *DSG* developing secretory granule, *Fi* bundle of filaments, *G* Golgi stack, *N* nucleus, *RER* rough endoplasmic reticulum, *SC* support cell, *SG* secretory granule

(Fig. 1d, e). Type B secretory cells bear a short subcuticular cilium, and are filled with densely packed small spherical granules (about 0.2 µm in diameter) (Fig. 2f). These are membrane-bound and contain a homogeneous electron-dense material. Type C secretory cells are not ciliated and have spherical, membrane-bound granules (up to 1.2 µm in diameter) whose content is of variable electron density (Fig. 3a, e). Non-secretory ciliated cells are regularly interspersed between type A and B secretory cells (Figs. 2d, 3a, 4). They bear a short transcuticular cilium and are located in the central area of the papillae. Neurosecretory-like cells may be observed in the epidermal layer and appear to stretch out to the papilla tip (Figs. 3a, d, 4). They are not ciliated and contain dense-core vesicles of about 70 nm in diameter similar to those observed in the neurites of the subepidermal plexus. Both non-secretory ciliated cells and neurosecretory-like cells are closely associated with the nerve plexus, which may send fascicules of neurites towards the epidermis (Fig. 3b). Support cells are uniformly distributed among the other cell types (Figs. 2e, 3a, 4). They are crossed by a bundle of transcytoplasmic filaments joining their apical and basal membranes (Fig. 3a). They may enclose some dense core vesicles that are mostly localised beneath the nucleus. Support cells bear a cilium which corresponds to the long twisted cilia observed on SEM pictures (Fig. 2e). The three to five lateral papillae located around the adhesive disc (Fig. 5a) share the same ultrastructure with the apical papillae of the brachiolar arms.

The adhesive disc is a local thickening of the epidermis covering the preoral lobe (Fig. 5a). Like the

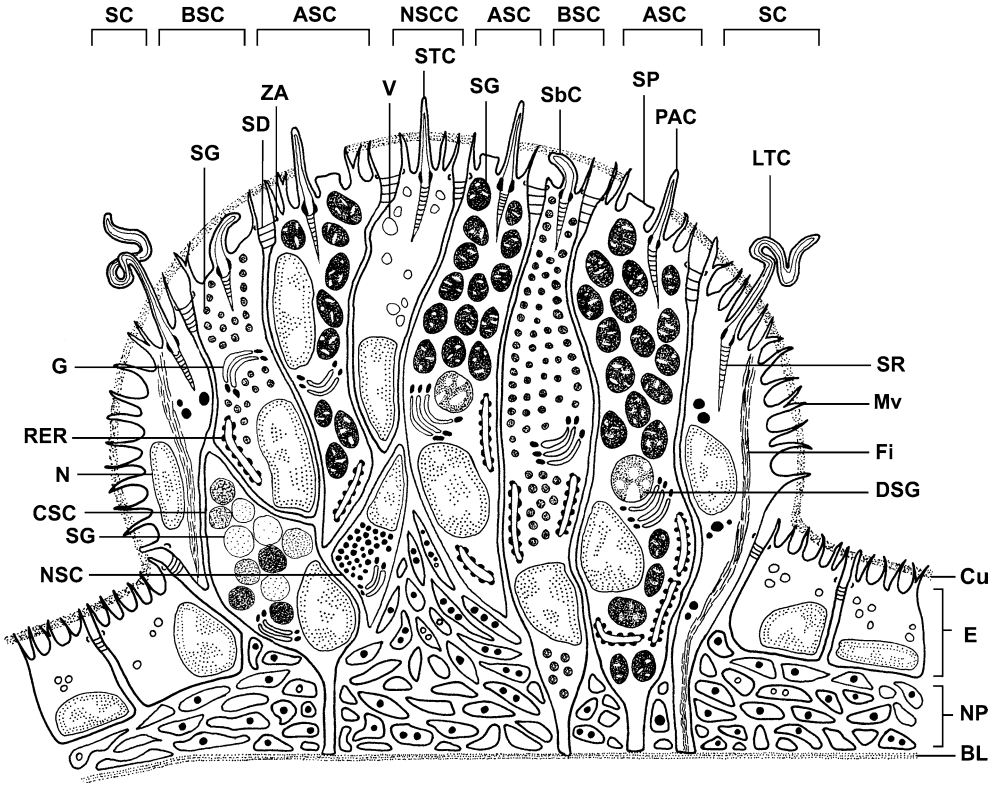

Fig. 4 *Asterias rubens.* Schematic drawing of a longitudinal section through the papilla epidermis of a brachiolar arm. *ASC* type A secretory cell, *BL* basal lamina, *BSC* type B secretory cell, *CSC* type C secretory cell, *Cu* cuticle, *DSG* developing secretory granule, *E* epidermis, *Fi* bundle of filaments, *G* Golgi stack, *LTC* long transcuticular cilium, *Mv* microvilli, *N* nucleus, *NP* nerve plexus, *NSC* neurosecretory-like cell, *NSCC* non-secretory ciliated cell, *PAC* pore-associated cilium, *RER* rough endoplasmic reticulum, *SbC* subcuticular cilium, *SC* support cell, *SD* septate junction, *SG* secretory granule, *SP* secretory pore, *SR* striated rootlet, *STC* short transcuticular cilium, *V* vesicle, *ZA* zonula adherens

brachiolar arms, the preoral lobe wall is made up of an epidermis, a subepidermal nerve plexus, a connective tissue layer and a mesothelium (coelomic epithelium). The mesothelium underlining the disc is similar to the one of brachiolar arms, except the occurrence of scattered granular cells with small spherical dense core granules of about 100 nm in diameter (Fig. 5b). The fine structure and thickness of the connective tissue correspond to those of its equivalent in the brachiolar arms (Fig. 5b). The nerve plexus, however, is much less developed and only a few neurites occur between and under the epidermal cells.

The monostratified epidermis is made of three types of cells, secretory cells (type D cells), neurosecretory-like cells and support cells (Figs. 5b, c, 6). Type D cells bear a short transcuticular cilium and are uniformly distributed in the disc central part. They are full of large spherical to ellipsoidal membrane-bound granules of medium electron density (about 1.7 μm in diameter). These granules have a woven-like fibrous content and are often associated with Golgi stacks and RER cisternae in the cell most basal part (Fig. 5c, d). Equally important in number, support cells occur between secretory cells. They are connected to each others by junctional complexes (identical to those observed in the brachiolar arm epidermis) and crossed by a well-developed bundle of filaments joining the basal part and the tip of each cell. This bundle forms a basal root-like structure protruding in the connective tissue layer which acts probably as anchoring structure (Figs. 5b, 6). At the apex, filaments extend into microvilli at the tip of which cuticular protuberances may be observed (Fig. 5e). Neurosecretory-like cells contain numerous small dense-core, spherical to ellipsoidal granules (about 100 nm in diameter). Their basal process mixes with the neurites, yet their upper part does not reach the epidermal apex (Fig. 5b). A few additional vacuolated cells may be observed in the disc of late brachiolaria larvae only. These are mostly localised at the disc periphery (Fig. 5a).

Immunochemistry

Observations in confocal microscopy showed that on both whole larvae and sections, there was a strong and reproducible immunolabelling, mainly restricted to the brachiolaria attachment complex, whereas no labelling was seen with the pre-immune sera. There was only a weak response in both the adhesive disc and the ciliary bands, the immunoreactivity being obvious in the papillae of the brachiolar arms as well as in the lateral papillae (Fig. 7b—d). A higher magnification allowed to see that immunoreactivity was concentrated in intracellular granules (Fig. 7e) whose size and abundance, together with the shape of the cells housing them, strongly suggest that the immunoreactive cells correspond to type A secretory cells. A weak response was also noted in the nerve plexus of the adhesive disc, and in the disc and arm stem cuticle (Fig. 7d).

Confirmation of type A secretory cells immunoreactivity was given by immunocytochemistry using gold particles as they were indeed the only labelled cells (i.e.

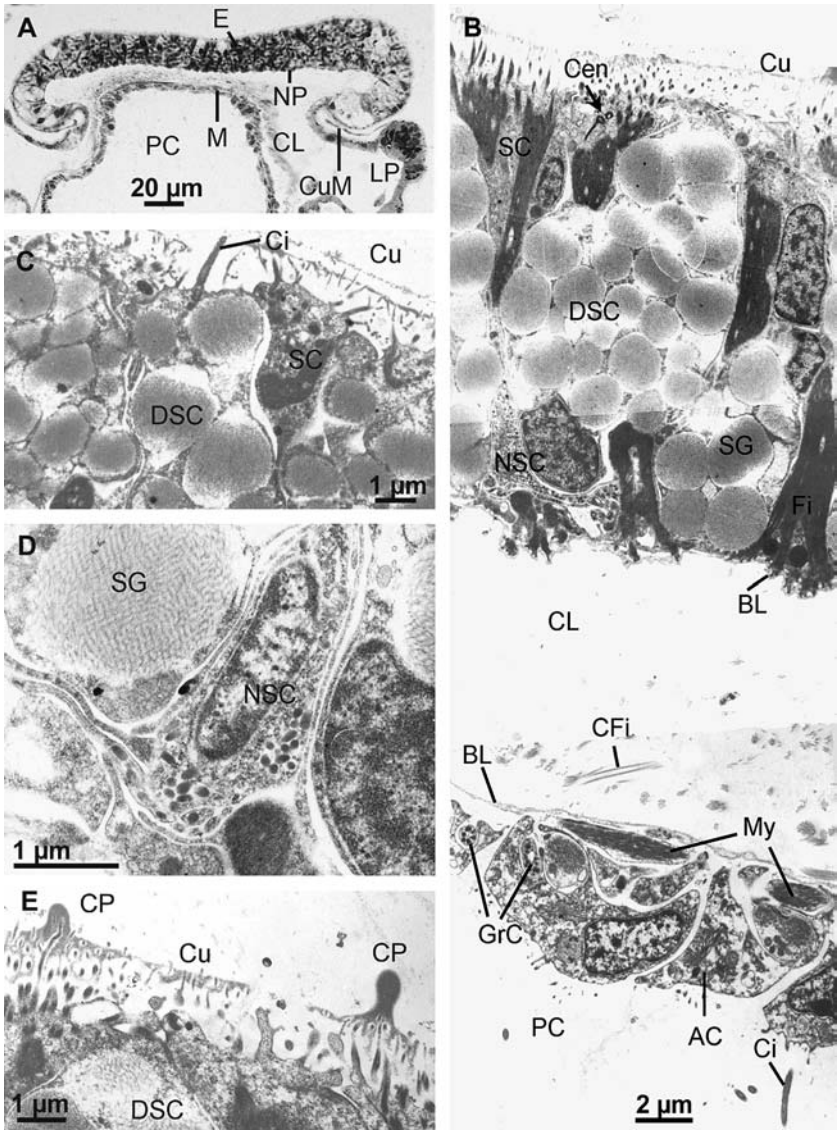

Fig. 5 *Asterias rubens*. Fine structure of the adhesive disc. (**a** LM; **b**–**e** TEM). **a** Section through the adhesive disc. **b** Section through the different tissue layers making up the adhesive disc. **c** Apex of the adhesive disc epidermis showing type D secretory cell (*DSC*) apex with cilium (*Ci*). **d** Details of neurosecretory-like cell (*NSC*) and type D secretory granule (*SG*). **e** Details of the cuticle (*Cu*) with cuticular protrusion (*CP*) covering the disc. *AC* adluminal cell, *BL* basal lamina, *Cen* centriole, *CFi* collagenous fibre, *CL* connective tissue layer, *CuM* cuticular material, *E* epidermis, *Fi*, bundle of filaments, *GrC* granular cell, *LP* lateral papilla, *M* mesothelium, *My* myocyte, *NP* nerve plexus, *PC* preoral coelom, *SC* support cell, *SG* secretory granule

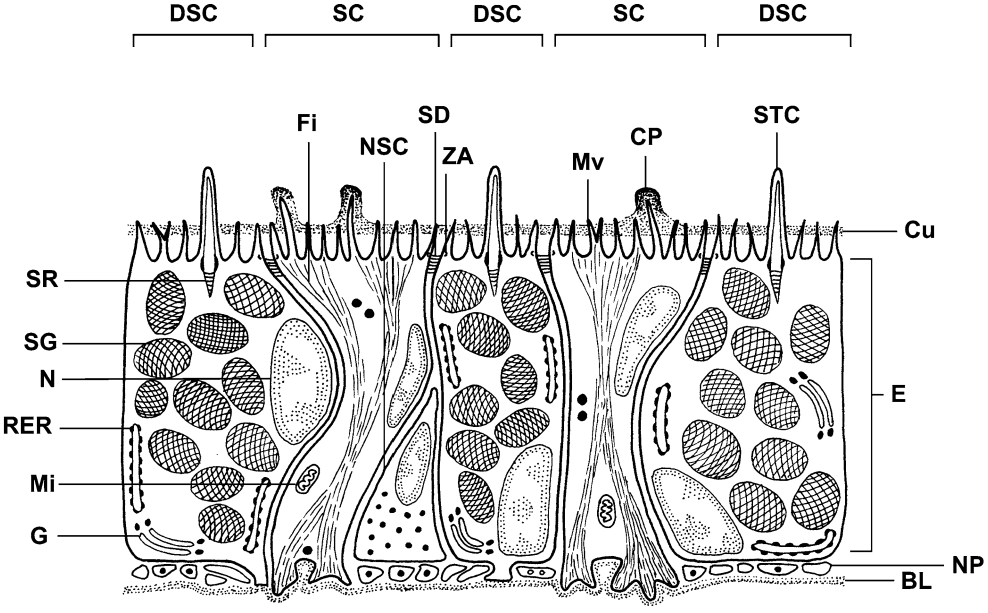

Fig. 6 *Asterias rubens*. Schematic drawing of a section through the disc epidermis. *BL* basal lamina, *CP* cuticular protrusion, *Cu* cuticle, *DSC* type D secretory cell, *E* epidermis, *Fi* bundle of filaments, *G* Golgi stack, *Mi* mitochondria, *Mv* microvilli, *N* nucleus, *NP* nerve plexus, *NSC* neurosecretory-like cell, *RER* rough endoplasmic reticulum, *SD* septate junction, *SG* secretory granule, *SR* striated rootlet, *STC* short transcuticular cilium, *ZA* zonula adherens

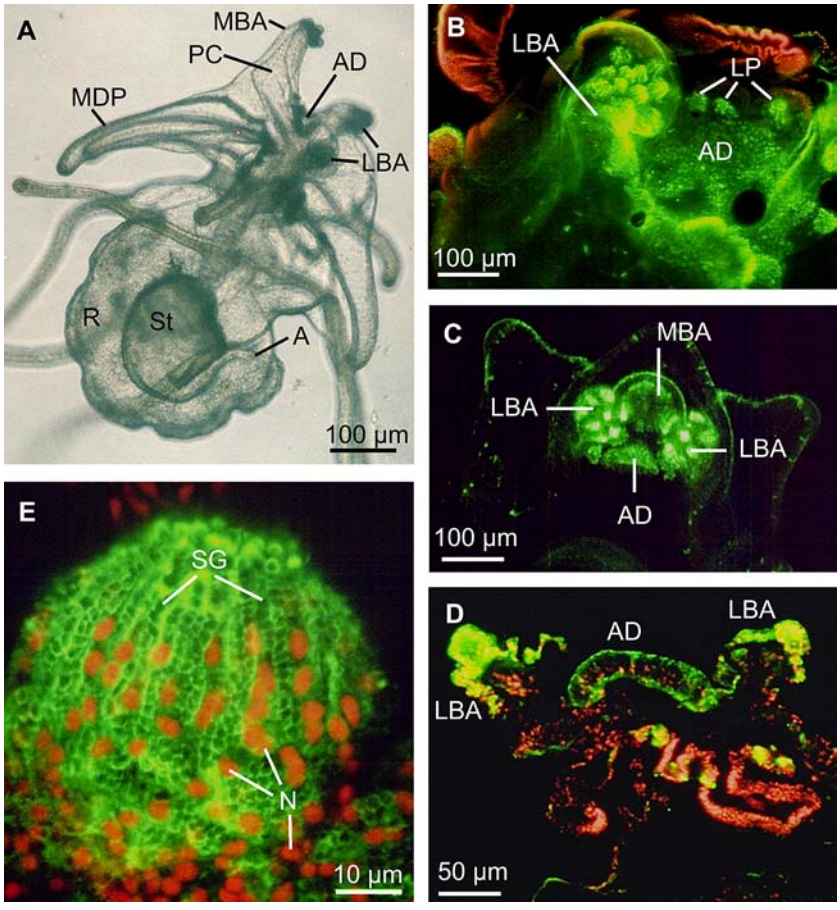

Fig. 7 *Asterias rubens*. Immunofluorescent labelling of brachiolaria larvae with antibodies raised against the adhesive material of the podia (immunoreactive structures are labelled in *green* while nuclei appear in *red*). **a** Whole mount view a competent brachiolaria in transmitted light. **b**–**d**. General views of the attachment complex [whole mount (**b**,**c**) and section (**d**)]. **e** Details of a papilla (whole mount) *A* anus, *AD* adhesive disc, *LBA* lateral brachiolar arm, *LP* lateral papillae, *MBA* median brachiolar arm, *MDP* mediodorsal process, *N* nucleus, *PC* preoral coelom, *R* rudiment, *SG* secretory granule, *St* stomach

they contained the only labelled granules; compare Fig. 8a to Fig. 8b and 8c). The cells and tissues of the disc and arm stem did not show any labelling.

Discussion

Functional morphology of the attachment complex

It is well known that the brachiolaria attachment complex plays a major role during settlement and metamorphosis in forcipulatid Asteroidea (Gemmill 1914; Barker 1977; Haesaerts et al. 2003). These authors reported that competent brachiolaria larvae contact the substratum, orient their ventral side down and temporarily attach by one or two of the three brachiolar arms through their apical papillae. The larvae then move slowly along the substratum by alternatively attaching and detaching each brachiolar arm ('walking larvae'). This exploration phase can last from a few seconds to several minutes. In presence of adverse stimuli or in absence of positive stimuli, larvae resume swimming. Positive stimuli induce metamorphosis and brachiolar arms gradually splay out, bringing the adhesive disc in close contact to the substratum. A permanent attachment then occurs ('larval fixation') which marks the beginning of the metamorphosis (Strathmann 1978; Burke 1983).

Function of the papillae is twofold, i.e. temporary adhesion and sensory testing of the substratum. Their epidermis is made up of six cell types among which three types of secretory cells (type A, B and C) were already observed by Barker (1978) in brachiolaria larvae of two species of forcipulatid Asteroidea, *Coscinasterias calamaria* and *Stichaster australis*. According to him, granules of type A cells contain neutral mucosubstances and would be adhesive in function. Each type A cell bears a short cilium associated to a pore surrounded by a ring of microvilli by which the secretion is expelled. One may hypothesize that type A cell cilium might be involved in substratum mechanoreception and could possibly trigger release of secretion upon contact. Barker (1978) suggested that type B secretory cells may act in providing additional adhesive material. On the contrary, Hermans (1983) and Flammang (1996) hypothesized that both types of secretory cells are involved in a duo-glandular system in which type A cells secrete an adhesive material and type B cells a de-adhesive material, enabling the brachiolar arms to make several cycles of attachment and detachment. The function of type C secretory cells remains enigmatic. Yet their localisation at the outer margin of the papilla and their limited number suggest they do not take part in temporary attachment. As for non-secretory ciliated cells, they are morphologically similar to sensory cells of primary podia of echinoid larvae which are known to be involved

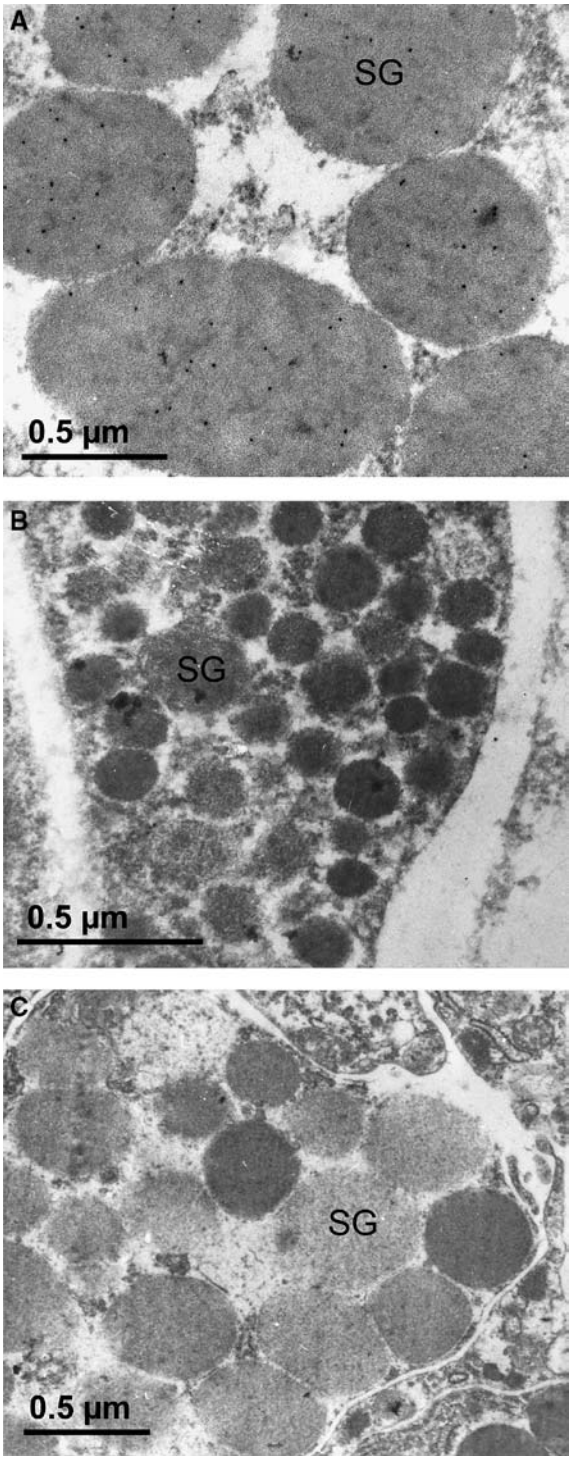

Fig. 8 *Asterias rubens* (TEM). Immunogold labelling of type A (**a**), type B (**b**) and type C (**c**) secretory cells in the brachiolar arms with antibodies raised against the adhesive material of the podia. *SG* secretory granules

in substratum recognition for metamorphosis (Burke 1980; Flammang et al. 1998b). In brachiolaria larvae of *A. rubens*, we suggest non-secretory ciliated cells trigger the behavioural sequence leading to larval fixation, their transcuticular cilia being ideally located to detect cues from the substratum. A similar role was already proposed by Barker (1978).

The fixation of the brachiolaria to the substratum is achieved by the adhesive disc. This adhesion can be described as permanent. Although the newly metamorphosed postlarva detaches from the substratum and becomes motile, detachment occurs at the level of the stalk connecting the disc to the rest of the body, the disc itself remaining cemented to the substratum (Gemmill 1914; Strathmann 1978; Haesaerts et al. 2003). The single type of secretory cells (type D) occurring in the adhesive disc epidermis, thus, contains the cement which will permanently attach the larva during metamorphosis. Their secretory granules are strikingly similar to those described by Barker (1978), meaning therefore that the cement in *A. rubens* is likely to be a proteinaceous material as it is the case for *C. calamaria* and *S. australis*. Once again, mechanical stimulation of the short transcuticular cilium born by type D cells would trigger cement release. According to Barker (1978), cement release is an all or nothing response that completely modifies the structure of the disc. The same is likely to be true in *A. rubens*, in which no secretory pores were observed on the disc surface, suggesting the occurrence of an abrupt release of the granule contents. This material forms a mass of cement in which the bundle of filaments from support cells anchor, resulting in a strong attachment of the larva to the substratum. This clearly reduces its chances to be removed by wave action.

Comparison with the podia

The attachment complex of brachiolaria larvae has no equivalent in post-metamorphic asteroids. However, the brachiolar arms share many functional, morphological and molecular similarities with the podia (Table 1). Functionally, both organs are involved in temporary adhesion, allowing asteroid larvae and adults to repeatedly attach to and detach from the substratum (Hermans 1983). Morphologically, they are both divided into a proximal highly flexible stem and a distal sensory-secretory area. The latter consists of eight to twelve dome-like papillae in the brachiolar arms and of a flattened disc in the podia. Their histological organisation comprises four tissue layers surrounding a fluid-filled cavity of coelomic origin. In *A. rubens*, and more generally, in every echinoderm species investigated so far, the podium epidermis encloses a duo-gland adhesive system that always comprises two types of cells: adhesive cells containing large heterogeneous secretory granules and de-adhesive cells full of small homogeneous granules (Flammang et al. 1994; Flammang 1996). Clearly, the epidermal organisation of brachiolar arm papillae also correspond to a duo-glandular system as described in the podia, type A and B secretory cells being adhesive and de-adhesive in function, respectively (Table 1). Indeed, type A secretory cells are similar to

adhesive cells of podia of adults, except that they bear a pore-associated cilium which does not occur in the latter. Furthermore, the adhesive function of these cells is supported by the immunochemical results as their secretory granules cross-react strongly with antibodies raised against the adhesive secretions of the podia. This indicates that adhesives from both brachiolar arms and podia are related, probably sharing identical molecules, or, at least, identical epitopes on their constituents. As for type B secretory cells, they are remarkably similar to the de-adhesive cells of the podia of *A. rubens* (Flammang et al. 1994, 1998a). Both are filled with small electron-dense granules and bear a subcuticular cilium. Like the de-adhesive cells in the podia (Flammang et al. 1998a), type B secretory cells were never immunolabelled. Finally, the analogy between brachiolar arms and podia is further consolidated by the observation that the same homeobox genes are expressed during their respective developments (Lowe and Wray 1997).

In contrast, the adhesive disc is an epidermal structure that encloses a single type of secretory cells. These cells are used only once, at the onset of metamorphosis and are responsible for permanent adhesion. It was not surprising therefore that the adhesive disc epidermis did not show any immunolabelling with antibodies raised against the temporary adhesive from the podia. It should be noted, however, that one of the adhesive cell types in the podia of *A. rubens* contains granules filled with a fibrillar structure resembling those of adhesive disc secretory cells (Table 1). Flammang et al. (1998a) showed that the immunolabelling of these particular granules was fixation-dependent and that the fibrils they contain were not labelled when fixed by a protocol similar to the one used in this study. Thus, molecular similarities between the cement of the adhesive disc and some constituents of the podial adhesive cannot be ruled out completely.

The cuticle and the nerve plexus of the adhesive disc were also weakly immunolabelled. Such positive response in the cuticle is easy to explain since the antisera comprise antibodies raised against the cuticular material which is partly enclosed in the podial footprint material (Flammang et al. 1998a). The immunolabelling observed in the nerve plexus of the adhesive disc is more difficult to explain but a similar slight cross-reactivity has also been reported in the nerve plexus of podia of other asteroid species (Santos et al. 2005).

Perimetamorphic attachments in benthic marine metazoans

All benthic invertebrates going through a larval stage and attaching to the substratum as adults use attachment mechanisms during their perimetamorphic period.

Table 1 Comparison of the fine structure of larval and adult adhesive systems in *Asterias rubens*

	Brachiolar arms(present work)	Adhesive disc (present work)	Podia (Flammang et al. 1994, 1998a)
Structure and function	*Cylincrical processes ending by 8 to 12 dome-like papillae* Sensory testing Locomotion Temporary attachment	*Round concave structure thickened in the edge* Permanent attachment	*Cylindrical processes ending by a disc* Sensory testing Locomotion Temporary attachment
Internal morphology	Coelomic cavity surrounded by four tissue layers	Epidermal structure laying above thin tissue layers	Coelomic cavity surrounded by four tissue layers
Cell types present in the epidermis	–secretory cells (A, B, C) –sensory cells –neurosecretory-like cells –support cells	–secretory cells (D) –neurosecretory-like cells –support cells –vacuolated cells	–secretory cells (NCS1, NCS2, CS) –sensory cells –support cells
–Secretory cells **name**, *function* –description –*immunoreactivity*	**Type A** *adhesive* –heterogeneous, ellipsoidal, electron-dense granules (1×0.7 μm) –transcuticular cilium –*immunoreactivity (+)* **Type B** –*de-adhesive* –homogeneous, spherical, electron-dense granules (0.2 μm) –subcuticular cilium –*no immunoreactivity (-)* **Type C** *unknown* –homogeneous, spherical granules of varible electron density (1.2 μm) –no cilium observed –*no immunoreactivity (-)*	**Type D** *adhesive* –homogenous, ellipsoidal to spherical granules (about 1.7 μm) of medium electron density and fibrous texture –transcuticular cilium –*no immunoreactivity (-)*	**Non-ciliated secretory 1 (NCS1)** *adhesive* –heterogeneous, ellipsoidal granules with a central fibrillar bundle (1×0.6 μm) –no cilium –*high immunoreactivity (+ +)* **Non-ciliated secretory 2 (NCS2)** *adhesive* –heterogeneous, spherical dense-cored granules (0.5 μm) –no cilium –*high immunoreactivity (+ +)* **Ciliated secretory (CS)** *de-adhesive* –homogeneous, spherical, electron-dense granules (0.25-0.45 μm) –subcuticular cilium –*no immunoreactivity (-)*

Three successive attachment phases may usually be distinguished during this period: (1) premetamorphic attachment allowing competent larvae to search for a favourable site for metamorphosis, (2) metamorphic attachment (sometimes referred to as fixation) securing the organism during this crucial step of its development, and (3) postmetamorphic attachment, the future adult attachment mechanism (Crisp 1984). In general, invertebrates which remain mobile as adults use a single type of adhesion throughout their perimetamorphic period. For example, during settlement, pediveligers of Gastropoda (Mollusca) adhere to the substratum through a viscous film of mucus produced by their foot and on which they creep (transitory adhesion; Flammang 1996). This type of adhesion is then conserved up to the adult form (Bonar 1978; Hadfield 1978; Walker 1987). Similarly, competent larvae of Echinoidea and Holothuroidea (Echinodermata) attach to and detach from the substratum with their primary podia (temporary adhesion; Flammang 1996). Once again, this type of adhesion is maintained throughout their life (Strathmann 1978; Burke 1983, Flammang 1996). In both cases, the adhesive organs are basically identical, both morphologically and functionally, in larval and adult forms (Bonar 1978; Cameron and Fankboner 1984; Burke 1980). Sessile invertebrates, on the other hand, cannot rely on a single type of adhesion during their perimetamorphic period. Indeed, these organisms, which as adults use permanent adhesion and live cemented to the substratum, need a non permanent type of adhesion during settlement to enable them to move around while exploring the substratum (Crisp 1984). For premetamorphic attachment, they can therefore use either transitory adhesion like larvae of tubicolous Polychaeta (Postwald 1978; Eckelbarger 1978), larvae of Bryozoa (Loeb and Walker 1977; Reed and Woollacott 1982), or pediveligers of Bivalvia (Mollusca; Cranfield 1973; Lane and Nott 1975); or temporary adhesion like cyprids of Cirripedia (Crustacea; Yule and Walker 1987), or larvae of Ascidiacea (Urochordata; Cloney and Torrence 1984). From metamorphic attachment onwards, all these organisms then rely on permanent adhesion to remain cemented to the substratum (Crisp 1984). In each group of sessile invertebrates, both metamorphic and post-metamorphic adhesives are closely related in composition and, in general, consist almost exclusively of proteins (see Crisp 1984 and Walker 1987 for review). Among marine invertebrates, Asteroidea are therefore unique in using temporary adhesion during settlement, permanent adhesion for fixation, and then reversing to temporary adhesion for their whole postmetamorphic life. Moreover, each type of attachment is associated with a special type of adhesive organ (brachiolar arms, adhesive disc, and podia, respectively), what is also uncommon. However, the existence of similarities between the different attachments as far as the composition of the adhesives is concerned is more like what occurs in other invertebrates and presumably reflects an economy at the genetic level.

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