Morphological, ultrastructural, and histochemical investigation of epipodial sensory structures of *Haliotis tuberculata* (Gastropoda: Haliotidae)

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""Epipodial sensory structures of the abalone"

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Abstract

Molist, P., Álvarez Nogal, R., Collado, G. Morphological, ultrastructural, and histochemical investigation of epipodial sensory structures of *Haliotis tuberculata* (Gastropoda: Haliotidae)

In this paper we described the microstructure and ultrastructure of the epipodial papillae and epipodial tentacles of *Haliotis tuberculata* using light and electron microscopy. The epipodial papillae vary morphologically; they are subdivided into several unequal sub-papillae whose surface is covered by small micropapillae. The epipodial tentacles are large extendable conic-elongated structures whose surface is differentiated in two regions: a dorsal with long corrugated folds, and another ventral composed by three parts, a basal with the same structure as the dorsal, a middle with shorter corrugated folds and another apical with large micropapillae. Although the thin sections and ultrastructure examination show that the epithelium of both organs is morphologically similar, and composed by supporting cells, sensory cells, and different type of secretory cells, there is a certain specialization in their secretory product. Although, the epithelium of both structures was positive for acidic glycoconjugates, the tentacle epithelium was also positive for neutral sugars. Further specific differences were revealed by lectin histochemistry. Because papillae and tentacles can be extend or retract depending on environmental conditions, they probably have tactile and olfactory functions.

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Introduction

The abalones of the genus *Haliotis* Linnaeus, 1758 inhabit the coastal areas of most continents. They are an important food source around the world, with more than 56 species (Geiger and Owen 2012), many of which are one of the most commercially important shells consumed in Asian, Europe, North America, South Africa and Australia, with China being the largest producer of *Haliotis diversicolor* (Reeve 1846). In Europe, *Haliotis tuberculata* Linnaeus, 1758 is the only species in the family Haliotidae which is harvested commercially (Mgaya 1995). Nevertheless, in Galician, Spain, the fishery of this species was prohibited in 1993 due to the presence of PST toxin at levels which are persistently over the legal limit. By unknown reasons toxicity has been decreased since 2000 until 2009 and consequently the Galician government allowed its culture and marketing after the announcement in the Diario Oficial de Galicia (May 6, 2009).

Apart from the shell, the body surface of the *Haliotis* is divided in four portions: head, foot (side and sole), mantle and epipodium. The epipodium is a complex of sensory structures found in the vestigastropods, which extends along the edge of the body, between the mantle and the superior margin of the foot (Cox 1962; Crisp 1981). The epipodium is more elaborate in *Haliotis* than in any other vetigastropod taxa; it is a development of the foot and is elaborately supplied with nerves from the cerebral and pleuro-pedal ganglia (Cox 1962). The color and the composition of the epipodium present interspecific differences and has been used for systematic and taxonomic studies in the group (Cox 1962; Salvini-Plawen and Haszprunar 1987; Simone 1998; Geiger 1999; Collado 2008; Collado *et al.* 2012*a,b*). Some authors referring to the epipodium as part of the mantle edge (Voltzow 1994) or periostracal groove (Sud *et al.* 2002; Mcdougall *et al.* 2011); others however called it skirt (Na *et al.* 2006*a*), in this case being part of the foot, and finally there are authors that study the epipodium as a structure apart from the foot and mantle (Crofts 1929; Macdonald and Maino 1964; Hickman and McLean 1990; Simone 1998; Wanichanon *et al.* 2004; Collado *et al.* 2012*a,b*). Among the most conspicuous structures of the organ are the epipodial sensory tentacles and epipodial papillae that we study here. Although the epipodium nomenclature and composition vary among authors or among the species, we follow Na *et al.* (2006*a*)

that ~~consider~~ *H. diversicolor* epipodium compose of epipodial papillae and epipodial tentacles (they called these structures as skirt hillocks and skirt tentacles).

In order to understand the anatomy and functional morphology of the epipodium of the vetigastropods, the aim of this study is to investigate the structure and glycoconjugate composition of the papillae and epipodial tentacles of *H. tuberculata*. Crofts (1929) studied the gross anatomy of the epipodium in this species. Here we use lectin histochemistry and conventional histochemical and electron microscopy techniques to study in more details both morphological structures. Lectins are specific carbohydrate-binding proteins that promote interactions between molecules and cells. They are widely used in biochemistry, for the study of glycoproteins as well as in cell biology and medical research. The presence of glycosaminoglycans and glycoprotein has been demonstrated using lectins in tissues of mollusks (Robledo *et al.* 1997; Calabro *et al.* 2005), including *H. tuberculata* (Bravo-Portela *et al.* 2012). Cephalic tentacles were excluded of this study because they have been previously studied using techniques similar to those employed here (Künz and Haszprunar 2001; Na *et al.* 2006*b*).

Material and methods

Four adult specimens of *H. tuberculata* were collected in 2009 by diving from the coastal region of Ria of Vigo, (NW Spain). Small pieces of the epipodium were cut from the medium edge part of the body after the animals were anesthetized by immersion in 5% MgCl2 in seawater and then fixed according to the technique implemented. For lectin histochemistry and conventional histochemical techniques tissue samples were fixed in formol Baker for 24–48 h, embedded in paraffin and sectioned to 8 µm thick. For histochemical analysis, sections were subjected to periodic acid-Schiff to reveal glycoconjugates containing neutral sugars, and stained with alcian blue (AB) and this colorant in combination with high iron diamine (HID) to observe acidic glycoconjugates and separate sulphated and carboxylated glycoconjugates (Molist *et al.* 2011, Bravo *et al.* 2012). For lectin histochemistry we follow the methods described by Bravo et al. (2012). For transmission electron microscopy (TEM), small pieces of the epipodium were dissected and fixed for two hours in 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer in a solution isosmolar to seawater. The samples were rinsed with the same buffer, postfixed for two hours in 2% osmium tetroxide at 4ºC, and embedded in Spurr's resin. Ultrathin sections were obtained in an ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a Jeol JEM1010 TEM. For scanning electron microscopy (SEM), small pieces of the epipodium were fixed for 2

h in 2.5% glutaraldehyde in filtered sea water, washed in cacodylate buffer, critical point dried in CO2, covered with gold, and observed with a Philips XL30 SEM.

Results

Anatomical and SEM observations

The external surface of epipodium, pale yellow mottled to dark brown in colour, is a highly folded and lobed complex structure rich in papillae and conspicuous green colour tentacles of different length. Epipodial papillae and tentacles are shown in Figure 1A-K. By SEM both epipodial structures, papillae and tentacles, can be observed together (Fig. 1A). The epipodial papillae vary in morphology and length along the edge of the epipodium. They are subdivided into several unequal sub-papillae whose surface is covered by small micropapillae which are located at irregular intervals (Fig. 1B,C). The epipodial tentacles are large extendable conic-elongated structures that show two regions in its external longitudinal surface, a dorsal and another ventral (Fig. 1D,E). The dorsal surface is corrugated; it has long folds separated by long grooves. Scattered on the folds are found scarce small micropapillae at irregular intervals (Fig. 1E,H), some of which become larger towards the apical portion of the tentacle (Fig. 1E). The ventral surface is composed by three parts, a basal corrugated with long folds and grooves, a middle less corrugated with shorter folds that are transformed into papillae toward the tip of the tentacle, and another apical with large micropapillae (Fig. 1D-G,I), some of them with ciliary tufts (Fig 1J,K).

TEM observations

The epithelium of the epipodial papillae is simple, columnar, and composed by supporting cells, sensory cells and secretory cells. The epithelial supporting cells (Fig. 2A-D) in the papillae contain pigmented melanin-granules and melanosomes in the apical cytoplasm, which is recovered by a dense microvillus margin (Fig. 2A,B). The melanosomes produce the brown color of the papillae observed even macroscopically. The nucleus of the supporting cells, located at its base, is oval with chromatin rather pale and with different patterns of organization and appearance. Microfilaments are deeply developed forming patent bundles which criss-cross the entire cytoplasm and seem to be connected with the basal lamina (inset in Fig. 2A); they are joined together in a thicker bundle that cross all the cytoplasm until the apical zone, making up the core of the well developed microvillus (Fig. 2B). A zonula adherens as the

most apical intercellular junctions was found (Fig 2C). The epithelium has a variety of epithelial secretory cells interspersed between the pigmented melanin supporting cells; types A, B, E and F [after Bravo-Portela et al. 2012) are well represented (Fig. 2A,D,E). In general, in their apical zone all of them are full with secretory vesicles and/or granules while the nucleus occupies the basal region. The type A secretory cells have the cytoplasm packed with white vesicles (Fig. 2A). The type B secretory cells have near all its cytoplasm packed with pale-gray vesicles containing finely granular material (Fig 2A). The type E secretory cells have near all its middle and apical cytoplasm packed with similar and denser vesicles than type B (as seen under TEM) (Fig 2A) . The type F secretory cells have great part of the cytoplasm packed with vesicles of different size and higher electron density (Fig 2D). Additionally, we found a particular secretory cells, call here type "H", which have the basal, middle and apical zone of the cytoplasm full with electron-dense granules of different size (Fig. 2E); they are in some cases larger than vesicles found in the other cells, and are in general larger than melanosomes found in the supporting cells. The sensory cells have oval nuclei with dense spots of chromatin irregularly spaced within the nucleus and associated to the nuclear envelope; this cells present cilia in the apex (Fig. 2F).

The epithelium of the epipodial tentacles of *H. tuberculata* is morphologically similar to that of the papillae. However, supporting cells present a varied of vesicles with a granular and filamentous content (Fig 2G), from electron lucent to electron dense. Some of these pigmented vesicles are very close to a well developed Golgi complex (Fig. 2H). By their ultraestructural morphology, these vesicles correspond to those containing a phycobilin-like pigment, as previously described in the side foot of *H. tuberculata* (Bravo-Portela et al. 2012).This pigment causes the green color of the tentacle. In the epipodial tentacle, secretory cells are abundant and varied; they show types A, B, E and F in common with epipodial papillae. In addition to a new cell type in the epipodium, the type C has been often observed mainly in the apical part of the tentacle. The secretory vesicles contain different distribution of electron dense and electron lucent material with a dotted appearance (Fig 2I).

Some histochemical differences were found with respect to the presence of different types of epithelial secretory cells as revealed by histochemical methods (see below). The ultrastructure of epipodial papillae and tentacles of *H. tuberculata* is schematized in the Figure 3. This scheme is an idealization of observations obtained with TEM (Fig. 2 A-I).

Histochemical observations

Conventional hematoxylin and eosin staining show the simple columnar epithelium covering the epipodial papillae and epipodial tentacles of the epipodium (Fig. 4A); however the distribution of secretory cells is revealed using histochemical methods. The AB positive secretory cells are distributed moderately in the epithelium of both papillae (Fig. 4B) and tentacles (Fig. 4C) demonstrating the presence of acidic glycoconjugates. The AB positivity of the epithelium and conjunctive tissue disappeared after methilation; it was recovered only in the connective tissue following saponification. This meant that desulphation was properly made and that most of the AB-positive secretory cells contained acidic glicoconjugates with sulphated groups. The HID/AB supported the desulphatation results. The majority of the HID/AB- positive cells in papillae and tentacles stain black or dark brown (Fig. 4D) demonstrating the presence of acidic sulphated glycoconjugates and the lack of carboxylated ones. With the periodic-acid-Schiff (PAS) technique, no staining was observed in the papillae, however a moderate number of secretory cells intensely stained were found in the tentacles (Fig. 4E). PAS-positive cells were distributed mainly in the apical portion of the tentacle. The enzymatic control with amylase technique excluded the presence of glycogen in these cells so PAS positivity is due to neutral sugars and/or sialic acid.

Lectin histochemistry. Lectins have been organized by the specific binding affinity for the sugar residues which are fucose, mannose, galactose, N-acetyl-galactosamine, N-acetyl-glucosamine and sialic acid. L-fucose residues were detected using three lectins (AAA, LTA, UEA I). AAA lectin binded to secretory cells of the epithelial papillae only after desulphation (Fig. 4F); in contrast LTA (Fig. 4G) and UEA I (Fig. 4H) stained secretory cells in the tentacle. UEAI-positive secretory cells are distributed by the most apical large micropapillae of the tentacle whereas LTA positive cells are found dispersed along the whole epithelium of the tentacle. The mannose/glucose-residues were detected with both lectins used in this study; ConA and GNA display a strong reaction for the apical portion of the papillae and tentacle epithelial cells (figs. 4I,J,K). Moreover, GNA lectin binds to scarce tentacle secretory cells (Fig. 4K). In the case of PNA lectin, which recognize the sequence galactose-N-acetyl-galactosamine, a moderate amount of positive secretory cells were found only after desulphation in the papillae (Fig 4L), in contrast to the scarce number in the tentacles (Fig. 4M). The terminal N-acetyl-galactosamine specific lectin (DBA) binds to very scarce secretory cells of the papillae (Fig. 4N) and to the external surface of the tentacle epithelium (Fig. 4O). No binding to WGA was found before the desulphation treatment but a moderate amount of papillae secretory cells showed a strong reaction to N-acetyl-glucosamine lectin (Fig.

4P) after the treatment. Moreover, a weakly staining is visible at the luminal surface of the tentacle epithelial cells (Fig. 4Q). Use of the SNA and MAA lectins showed that sialic acid was not found in the epithelium of the *Haliotis* epipodium.

Discussion

The external surface of epipodium of *H. tuberculata* is rich in papillae and tentacles. They may be extended or retracted depending on the animal behavior and environmental conditions, as we observed in animals maintained in aquaria conditions. Cox (1962) performed experiments with a series of different materials that suggested that abalones can discriminate between food items and nonfood items: *Touching the epipodium with any substance other than macroalgae causes an abalone to withdraw exposed body portions. When touched with a piece of kelp, however, an abalone will extend its epipodium, grasp the kelp and pull it towards its mouth.* Among several California abalones the epipodium was signed as one of the most reliable characters for determining specific identification (Cox, 1962). More recently, Simone (1998) also found conspicuous differences among abalone species from Brazil and Caribbean Sea and Geiger (1999) around the world.

Based on SEM observations, in the present study we found that the external ventral surface of the tentacles of *H. tuberculata* was composed by three parts, a basal with long corrugated folds, a middle with shorter highly corrugated folds and another apical with large micropapillae. Although this was not reported by Croft (1929), our findings are similar to those described by Wanichanon *et al.* (2004) in the entire surface of the tentacle of *H. asinina*. Thus, the characteristic long folds found on the dorsal surface of the tentacles of *H. tuberculata* may be use as taxonomic character to distinguish it from *H. asinina*. Wanichanon *et al.* (2004) also described on the surface of the basal folds "many short bulbous papillae", each with ciliary tufts on the top. These structures correspond to our small micropapillae found in the surface of the epipodial papillae and in the dorsal, basal and middle part of epipodial tentacles. We found large micropapillae with ciliary tufts on the top in the ventro-apical part of the tentacles of *H. tuberculata*, as revealed by SEM observations. Na *et al.* (2006) found in *H. diversicolor* small and large micropapillae (they called these structures papillae). They were also observed in *H. asinina*; they may function mostly as chemoreceptors (Wanichanon *et al.* 2004). Wanichanon *et al.* (2004) also reported that the ciliated sensory cells of the large micropapillae of epipodial tentacles (and cephalic tentacles) are "structurally

very similar to the olfactory epithelium or taste buds of vertebrates", suggesting convergence of external sensory characters among these taxa.

Although the external morphology of the epipodial papillae and epipodial tentacles of *H. tuberculata* vary regarding the presence in its surface of small micropapillae and large micropapillae, respectively, the epithelium of both organs show a similar cellular ultrastructure. They are composed by at least three cell kinds: supporting cells, sensory cells, and different types of secretory cells. A similar finding was reported by Crofts (1929) in the cephalic tentacles of this species and Wanichanon *et al.* (2004) in *H. asinina*. Comparatively, the supporting cells have denser microvilli than other epithelial cells located in the foot epithelium (Bravo-Portela *et al.* 2012) and mantle (personal observations). The presence of a "brush border" in the epithelial cells of the foot of *H. tuberculata* has been suggested as indicative of absorptive functions (Bravo-Portela *et al.* 2012). The supporting cells have oval nuclei with different chromatin organization and a clearer appearance than other epithelial cell kinds suggesting a different state of the cellular cycle or a different cell function. The supporting epipodial cells are pigmented with melanin and phycobilin granules which cause the brown and green color to the papillae and tentacle respectively. These types of pigmented epithelial cells have been described located on the grooves and the crests of the side foot of *H. tuberculata* (Bravo *et al.* 2001). Sensory cells were scarce, difficult to observe. They were reported in *H. asinina* by having a tuft of cilia projecting from the apical zone of the cells of the epipodial tentacles (Wanichanon *et al.* 2004). TEM observation revealed the presence of at least six types of secretory cells (type A, B, C, E, F and G) in the epithelium of the papillae and tentacles of *H. tuberculata*. With the exception of the cell that we identified as type G, the other secretory cells are very similar to those described by Bravo-Portela *et al.* (2012) in the foot epithelium of this species. Both parts of the epipodium share the majority of the secretory cells but type G is only found in the epipodial papillae and type C in the epipodial tentacles. The mucus of mollusks has a leading role in a number of physiological processes, even having role at the community level (Davies and Hawkins 1998). The mucus may act to facilitate locomotion, adhesion to substrate, feeding, respiration and digestion, as well as protection, lubrication and defense (see Davies and Hawkins 1998). Mucus also has a significant capacity for absorbing water in terrestrial mollusks (Verdugo 1990). Beside sensory function, ciliated cells of the foot can help spread the mucus to distribute the mucus for gliding. A similar function of spreading may occur at the epithelial epipodium.

Our results identified neutral and acidic glycoconjugates among sugars residues of the papillae and tentacles of *H. tuberculata*, which is typical of the molluscan mucus (Davies and Hawkins 1998). Acidic sulphated glycoconjugates were identified in both structures. It has been suggested that acidic GAGs increase the viscosity of invertebrate mucus (Davies and Hawkins 1998). As the tentacles and papillae are used to explore the environment, finding food and receiving chemical stimuli (Croft 1929, Wanichanon *et al.* 2004), they maybe need to have high viscosity. However, noticeable difference found between both structures was the identification of neutral glycoproteins only in the tentacles. As the tentacles are larger, *Haliotis* probably uses these glycoproteins to increase the protection and adhesion to the food. Lectin histochemistry shows further specific differences among the compounds present in the epithelium of papillae and tentacles. L-fucose-residues were detected in both structures (as revealed by lectins AAA, LTA, and UEA-I). However, AAA lectin binds to secretory cells of the epithelial papillae and LTA and UEAI to those of the tentacle suggesting that different sugars residues are located in these epipodial structures. The UEA-I and LTA lectins bind to fucose residues whereas AAA lectin labels fucose resifues alpha (1-6) linked to N-acetyl-glucosamine. Furthermore, others specific L-fucose-residues were detected between the two regions of the tentacles, with UEAI secretory cells mostly distributed in the large micropapillae and LTA cells found along its epithelium. Different patterns of fucosylation has been described in the foot epithelium of *H. tuberculata* and others species of mollusks (Gutternigg *et al.* 2007; Bravo-Portela *et al.* 2012). The staining pattern of the mannose/glucose binding lectins of the papillae and tentacle epithelium of *H. tuberculata* was similar with both lectins used in this study (ConA and GNA) suggesting a similar distribution in both epipodial structures. Contrary, the galactose-N-acetyl-galactosamine-sequence, as revealed by the PNA lectin, was found mostly distributed in secretory cells of the papillae epithelium, which could be indicated that they are implicated in protective and lubrication functions (Bravo-Portela *et al.* 2012). The presence of N-acetyl-galactosamine and N-acetyl-glucosamine in secretory cells of the papillae and in the tentacle epithelium of *H. tuberculata*, as revealed with the lectins DBA and WGA, respectively, is indicative of the presence of sulphated glycosaminoglycans (Bravo-Portela *et al.* 2012), compounds that increase viscosity of the epithelial secretions (Davies and Hawkins 1998). SNA and MAA-negative reaction lectins revealed that no sialic acid is present in the epipodium epithelium of *H. tuberculata*. This agrees with the results reported by Bravo-Portela *et al.* (2012) for the foot epithelium of this species.

In conclusion, our data suggest that the secretory cells of the epipodium contain GAGs which could be constituents of proteoglycans. Moreover the tentacle secretory cells are characterized by the presence of glycoproteins with fucose and mannose residues.

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Figure legends

Figure 1. A. SEM micrograph showing an epipodial papilla (p) and an epipodial tentacle (t) of the epipodium of *Haliotis tuberculata*. The epipodial papillae are divided in subpapillae (sp). Bar: 100 µm. B. SEM micrograph showing small micropapillae (sm) on the surface of the epipodial papillae. Bar: 20 µm. C. Small micropapillae from B seen to higher magnification. Bar: 2 µm. D. SEM micrograph showing the dorsal (d) and ventral (v) regions of the epipodial tentacles. Bar: 500 µm. E. Figure D view at higher magnification to show small micropapillae on the dorsal corrugated surface of the epipodial tentacle and large micropapillae (lp) on the ventral surface. Bar: 100 µm. F. SEM micrograph showing the ventral region of the epipodial tentacle with its basal (b), middle (m) and apical (a) portion. Bar: 300 µm. G. Figure F view at higher magnification to show the middle and apical portion of the epipodial tentacles. Bar: 100 µm. H. SEM micrograph showing small micropapillae on the surface of the dorsal portion of the epipodial tentacles. Bar: 20 µm. I. Large micropapillae of the epipodial tentacles seen to higher magnification. Bar: 10 µm. J, K. Cilia in the apical surface of the large micropapillae of the epipodial tentacles. Bar in J, 5 µm; bar in K, 2 µm.

Figure 2. TEM micrograph showing the simple, columnar epithelium of epipodial papillae of *Haliotis tuberculata*. A. Supporting cells (Sc) with melanosomas (m), microfilaments (mf), and dense microvillous (mv). Conspicuous secretory cells can also be seen (A, B, E). A thin basal lamina (bm) supports the epithelium. Bar: 2.5 µm. Inset. Microfilaments connect the basal lamina (bm). Bar: 1 µm. B, C. Apical zone of the supporting cells showing microfilaments (mf), microvillous (mv) and a zonula adherens. Bar in B 0,5 µm; in C 200nm. D. Higher magnification of the epithelium showing secretory cells (type F) with basal nuclei (n). Bar: 2 µm. E. TEM micrograph showing supporting cells (Sc) and secretory cell of type "H" with basal nuclei (n) surrounded by vesicles. Bar: 2 µm. F. Ciliated cell (Cc) with oval nuclei (n) and cilia (Ci) in the apex. Bar: 5 µm G. TEM micrograph showing phycobilin-like pigment vesicles (fv) in the supporting cells of the tentacles. Bar: 0,5 µm. H. A well developped Golgi

complex (Gc) in close association with the phycobilin-like pigment vesicles (fv). Bar: 0,5 µm. I. Type C secretory cells among supporting cells of the tentacles. Bar: 2 µm.

Figure 3. Schematic representation of the papillae (A) and tentacle (B) epithelium showing supporting cells, sensory cells, and secretory cells (A, B, C, E, F and H) of Haliotis tuberculata. Basal membrane (bm), cilia (ci), melanosomas (m), phycobilin vesicles (fv), microfilaments (mf), microvillus (mv), nuclei (n). Bar: A, B 5 µm.

Figure 4. Histochemistry and histological sections describing epithelium of the epipodial papillae and epipodial tentacles of *Haliotis tuberculata*. A. Simple columnar epithelium stained with hematoxylin and eosin. B, C. The AB-positive secretory cells in the epithelium of both papillae (B) and tentacles (C) with acidic glycoconjugates. D. HID/AB-positive cells (stain black or dark brown) with acidic sulphated glycoconjugates. E. PAS positive mucous cells in the tentacles (no staining was observed in the papillae). F. After desulphation, AAA lectin binds to secretory cells of the epithelial papillae (tentacles were not reactive). G, H. LTA (G) and UEAI (H)-positive secretory cells in the tentacle (papillae were not reactive). I, J, K. With ConA and GNA-positive epithelial cells of the papillae and tentacles; GNA lectin (K) binds to scarce secretory cells of the tentacles. L, M. PNA-positive secretory cells (L) after desulphation in the papillae and scarce positive reaction in the tentacles (M). N, O. DBA-scarse positive reaction secretory cells in the papillae (N) and luminal surface of the tentacle epithelium (O). P. A moderate amount of secretory cells of the papillae showed a strong reaction to Nacetylglucosamine lectin after desulphation; WGA-negative reaction was found before treatment. Q. Weakly WGA positive reaction in the luminal surface of the tentacle epithelial. Bars: A-F 500µm; G. 250µm; H-K. 500µm; L-P 100µm.