Form and function of tentacles in pteriomorphian bivalves

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Abstract

Tentacles are remarkable anatomical structures in invertebrates for their diversity of form and function. In bivalves, tentacular organs are commonly associated with protective, secretory, and sensory roles. However, anatomical details are available for only a few species, rendering the diversity and evolution of bivalve tentacles still obscure. In Pteriomorphia, a clade including oysters, scallops, pearl oysters, and relatives, tentacles are abundant and diverse. We investigated tentacle anatomy in the group to understand variation, infer functions, and investigate patterns in tentacle diversity. Six species from four pteriomorphian families (Ostreidae, Pinnidae, Pteriidae, Spondylidae) were collected and thoroughly investigated with integrative microscopy techniques, including histology, scanning electron microscopy, and confocal microscopy. Tentacles can be classified as middle fold tentacles (MFT) and inner fold tentacles (IFT) according to their position with respect to the folds of the mantle margin. While MFT morphology indicates intense secretion of mucosubstances, no evidence for secretory activity was found for IFT. However, both tentacle types have appropriate ciliary distribution and length to promote mucus transportation for cleaning and lubrication. Protective and sensory functions are discussed based on different lines of evidence, including secretion, cilia distribution, musculature, and innervation. Our results support the homology of MFT and IFT only for Pterioidea and Ostreoidea, considering their morphology, presence of ciliated receptors at the tips, and branched innervation pattern. This is in accordance with recent phylogenetic hypotheses that support the close relationship between these superfamilies. In contrast, major structural differences indicate that MFT and IFT are probably not homologous across all pteriomorphians. By applying integrative microscopy, we were able to reveal

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anatomical elements that are essential for the understanding of homology and function when dealing with such superficially similar structures.

Keywords: anatomy, invertebrates, mantle, microscopy, molluscs, secretory, sensory.

Research Highlights

Detailed microscopical analysis of tentacles in pteriomorphian bivalves not only provided anatomical evidence to describe the functional morphology of such diverse organs, but also revealed possible shared traits for the clades Pterioidea and Ostreoidea.

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1. Introduction

Tentacular organs, including palps, tentacles, and papillae, are among key features in the taxonomy and biology of most invertebrate groups. Tentacles may be generically defined as slender, flexible appendages in animals, usually comprehending contractible, soft-tissue extensions of the body (Brusca, Moore, & Shuster, 2016). Despite clearly not being homologous between phyla, tentacles have evolved multiple times in numerous taxa, performing different roles deeply associated with the lifestyle and ecology of the organism (Roberts & Moore, 1997; Zhadan & Tzetlin, 2002; Audino, Marian, Wanninger, & Lopes, 2015; Tamberg & Shunatova, 2017). In this context, three main functions can be assigned to tentacular organs, i.e., feeding, protective, and sensory.

In several invertebrate species, tentacles are related to feeding behavior through the collection of food particles by ciliary mechanisms. In sea cucumbers and sipunculans, for example, the oral tentacles are used in nonselective deposit feeding, or water filtering, by means of a combination of hydrostatic pressure, complex musculature, and mucus coating (Cameron & Fankboner, 1984; Pilger, 1982). In most suspension feeders, such as bryozoans, phoronids, entoprocts, and many polychaetes, tentacles are organized in crowns responsible for both water flow and food particle collection by mucociliary transportation (Dubois, Barillé, Cognie, & Beninger, 2005; Nielsen & Riisgård, 1998; Riisgård, 2002; Schwaha & Wanninger, 2015; Tamberg & Shunatova, 2016, 2017).

In cnidarians, tentacle crowns are commonly found around the mouth of a polyp or at the edge of the umbrella in medusae (Shimizu & Namikawa, 2009). In anemones, corals, and tube anemones, tentacles bear a great diversity of nematocysts and

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spirocysts, the former used for stinging and capturing prey, while the latter are used for binding the prey to the feeding tentacles (Rifkin, 1991; Thorington & Hessinger, 1998). Among cephalopod mollusks, nautilids use a complex adhesive gland system in the digital tentacles to catch and bind the prey (von Byern, Wani, Schwaha, Grunwald, & Cyran, 2012).

Tentacles can also be used in aggressive interactions or protective strategies. In many anemones and antiphatarian corals, specialized tentacles are developed in response to other cnidarian species, frequently resulting in extensive nematocyst discharge (Goldberg, Grange, Taylor, & Zuniga, 1990; Watson & Mariscal, 1983). An alternative form of protection can involve tentacle autotomy, as observed in some medusae, preventing damage caused by tentacle entanglement with prey, predators, or conspecifics (Bickell-Page & Mackie, 1991). In file clams (Limidae), specialized tentacles are thought to be involved in protective responses through secretion of distasteful substances (Gilmour, 1967).

In most mollusks, with the evident exception of cephalopods, tentacles are not directly involved in feeding processes; instead, they participate in sensory roles. In gastropods, for example, cephalic and mantle tentacles are sensory organs associated with the acquisition of olfactory cues involved in a variety of social, feeding, and reproductive behaviors (Croll, 1983; Künz & Haszprunar, 2001).

In many bivalve taxa, multiple tentacles are distributed along the mantle margin, comprising an impressive diversity of form and function (Yonge, 1983). The bivalve mantle is formed by left and right lobes, delimiting the mantle cavity, and their free margins bear tissue extensions named mantle folds (Carter et al., 2012). Tentacles may be present either on the middle mantle fold, on the inner mantle fold, or on both (Yonge,

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1983). Within Bivalvia, the largest diversity of tentacles is found within the Pteriomorphia. This large clade, which includes oysters, scallops, mussels, and relatives, are characteristic for their numerous tentacles along the mantle margin. In scallops (Pectinidae), long tentacles have been thoroughly examined for two species, *Nodipecten nodosus* (Linnaeus, 1758) and *Placopecten magellanicus* (Gmelin, 1791), and revealed to be specialized sensory organs (Audino et al., 2015; Moir, 1977). Enormous tentacles are also present in Limidae, possibly performing sensory roles (Gilmour, 1963, 1967). Tentacle morphology and distribution have been examined in pearl oysters and relatives (Pterioidea), revealing shared attributes, such as tentacle presence on two mantle folds and distribution along the entire mantle margin (Tëmkin, 2006).

Tentacular organs are an interesting system to be explored in the light of multidisciplinary approaches investigating evolutionary novelties and the evolution of complex phenotypes. As illustrated above, tentacles comprise key anatomical features to understand the diversification of form and function. However, externally they are all superficially similar, which requires a detailed anatomical investigation to detect variation. In this study, we have applied integrative microscopy techniques to investigate the functional anatomy of tentacles in representatives of major pteriomorphian lineages, aiming at understanding variation, inferring functions, and investigating patterns in tentacle diversity.

2. Material and methods

Taxa sampling

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Samples were obtained for six pteriomorphians species collected during low tide in São Sebastião's coast (State of São Paulo, Brazil): *Isognomon bicolor* (C. B. Adams, 1845), *Pinctada imbricata* Röding, 1798 and *Pteria colymbus* (Röding, 1798) (Pteriidae), *Ostrea equestris* Say, 1834 (Ostreidae), *Pinna carnea* (Pinnidae), and *Spondylus ictericus* Reeve, 1856 (Spondylidae). Sampled localities included rocky shores (23°48'54.9"S 45°24'25.3"W, 23°49'44.7"S 45°26'29.6"W, 23°46'18.8"S 45°21'21.3"W) for *I. bicolor*, *Pi. imbricata*, *O. equestris*, and *S. ictericus*; gorgonian clusters (23°49'40.5"S, 45°24'46.8"W) for *Pt. colymbus*; and sand flats (23°45'57.2"S 45°21'00.2"W) for *P. carnea*. Eight to ten individuals were collected per species, except for *P. carnea* and *S. ictericus*, which included one specimen each. Mantle margin samples were obtained after dissecting animals previously anesthetized in a 7.5% solution of $MgCl_2$ for 3 h. Representatives of the Limidae and Pectinidae have been previously examined (Audino et al., 2015; Gilmour, 1967) and were not included in our study.

For histology and scanning electron microscopy (SEM), fragments of the mantle margin bearing tentacles were fixed for 3 h at 4°C in a modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer with osmolarity adjusted to 1 Osm with sucrose), and stored in cacodylate buffer (Audino & Marian, 2018). For confocal laser scanning microscopy (CLSM), samples were fixed in 4% paraformaldehyde for 2 h and stored in 0.1 M phosphate buffer (PB) (Audino & Marian, 2018).

Microscopy techniques

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For scanning electron microscopy (SEM), post-fixation procedures were conducted as described in Audino et al. (2015), including 30 min in 1% OsO_4 in cacodylate buffer, followed by 15 min in 1% tannic acid in buffer solution, and 15 min in a fresh solution of 1% OsO_4 at 4ºC. Analysis and image acquisition were performed on a Zeiss DSM 940. For confocal laser scanning microscopy (CLSM), samples were permeabilized in PB containing 2% Triton-X 100 (PBT) overnight. To evidence musculature, samples were incubated in a 1:40 dilution of Alexa Fluor 488 phalloidin (Molecular Probes, USA) in PBT for 24 h at room temperature in the dark. For cilia and nerve investigation, samples were incubated in a 1:400 dilution of alpha-tubulin antibody (monoclonal antibody, B-5-1-2) with Alexa Flour 488 conjugate (Molecular Probes, USA) in the same conditions as described for phalloidin. After three washes of 15 min in PBT, samples were mounted on microscope slides in ProLong Diamond Antifade Mountant with DAPI (Molecular Probes, USA). Analysis was performed on a Zeiss LSM 880 (Zeiss, Germany) and image stacks were digitally merged in the software ZEN lite 2.3 (Zeiss, Germany).

For histology, samples were completely dehydrated in ethanol and embedded in resin (Leica Historesin Kit, Germany). Serial sections of 4 µm were obtained with a Leica RM2255 microtome (Leica, Germany). To evidence secretory cells, periodic acid-Schiff stain (PAS) and alcian blue (AB) were applied for mucosubstances (Bancroft & Stevens, 1982), and mercury-bromophenol blue (BB) was applied for protein aggregates (Pearse, 1985). Other staining methods included hematoxylin and eosin (HE; Behmer et al., 1976), Gomori trichrome stain (Humanson, 1962), and toluidine blue and basic fuchsin (TF; Junqueira, 1995). The histological material and stubs (SEM) are deposited

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at the Museum of Zoology 'Prof. Adão José Cardoso' of the State University of Campinas (ZUEC, UNICAMP).

3. Results

In the studied animals, tentacles can be classified in two types according to their location in the mantle margin, i.e., in which mantle fold they are located. Inner fold tentacles (IFT) are marginal projections of the long inner fold, varying from filiform to flattened structures. Middle fold tentacles (MFT) can be marginal or submarginal projections, usually organized in commarginal rows. Anatomical features of IFT and MFT are described below for each species studied and summarized in Table 1. In some samples, including those of *I. bicolor*, *P. carnea* and *S. ictericus*, intense pigmentation or ciliary abundance prevented observation of tentacular nerves by CLSM.

Pteria colymbus (Pteriidae)

MFT are marginal structures, filiform and more pigmented (brownish) at the base than the tip (Fig. 1a). MFT are distributed on the entire fold extension, being more abundant ventrally, where they are proportionally larger than those at the dorsal region. They display two main sizes, short (ca. 1.25 mm) and long (ca. 2.5 mm), short tentacles being about half of the size of the adjacent long tentacles (Fig. 1a). Two types of subepithelial secretory cells are abundant in the MFT; one type has granular content with affinity for eosin and BB (Fig. 1b, c) and the other for PAS and AB (Fig. 1d). The tentacle epithelium is covered by tufts of short cilia (Fig. 1e) that are more densely distributed at the tentacle's base. A cluster of long cilia is located at the tip of each tentacle (Fig. 1f). MFT are very muscular structures with numerous longitudinal fibers

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(Fig. 1g) continuous with the mantle radial muscles. Thin fibers also form a delicate transverse musculature (Fig. 1g). MFT are provided with central nerves that branch towards the tip (Fig. S1a).

IFT are marginal, slightly pigmented, and present along the entire margin. In the anterior and posterior regions, they are filiform, short, appearing as small papillae (Fig. 1a). In contrast, IFT are longer (ca. 2.5 mm) and slightly flat in the ventral region, mainly along the incurrent aperture (Fig. 1h). In this region, some IFT are branched (Fig. 1h), reaching similar lengths as the MFT. Pigmentation is also more intense ventrally, with brown, yellow, and white spots (Fig. 1h). Despite intense secretory activity in the inner mantle fold (data not shown), gland cells were not identified in the IFT. Each tentacle is densely covered by cilia, both in short non-branched (Fig.1i) and large branched tentacles (Fig. 1j, k). Isolated clusters of cilia were not observed at the tip. Musculature in IFT is similar to MFT, including longitudinal and transverse fibers, although muscle bundles ramify in branched tentacles (Fig. 1l). IFT innervation includes multiple, branching tentacular nerves (Fig. S1b).

Pinctada imbricata (Pteriidae)

MFT are marginal, filiform, and pigmented at the base (brownish and greyish). They are very similar to those observed in *Pt. colymbus*, including the presence of short and long tentacles. In the ventral region, MFT are proportionally longer and more abundant than those in the dorsal region. Intense secretory activity is suggested by subepithelial secretory cells with affinity for PAS and AB (Fig. 2a). Affinity for eosin – but not for BB – was also observed in other subepithelial cells. Each MFT has a cluster of long cilia at the tip (Fig. 2b), while shorter cilia are sparsely distributed along the

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tentacle. Longitudinal musculature runs along the lengths of the tentacles (Fig. 2c), but transverse fibers were not observed. Innervation includes multiple, branching tentacular nerves (Fig. S1c).

IFT are marginal and slightly pigmented along the entire margin, including white spots. They are longer and wider ventrally (ca. 3 mm long), especially along the incurrent aperture, where most of them are branched and slightly flat (Fig. 2d). In general, tissue organization of IFT is similar to the that of the inner fold, except for the absence of secretory cells in the former (Fig. 2e). Tufts of cilia are sparsely distributed on their surfaces (Fig. 2f, g), and a ciliary cluster is present at the tip of each branch (Fig. 2h). The musculature is comprised of a few transverse fibers and numerous longitudinal muscles, which ramify in branched tentacles. The nerves are highly branched (Fig. 2h), even in non-branched tentacles, from the base to the tips. Small nerves directly reach the ciliary tufts, especially those at the tips where numerous ciliary roots and nerves appear to be connected (Fig. 2h).

Isognomon bicolor (Pteriidae)

MFT are marginal and filiform; pigmentation can vary from light to dark grey. Along the entire margin, adjacent tentacles have similar sizes (from 0.5 to 1 mm), although they are proportionally longer and more abundant in the ventral region. Intense secretory activity should be present, evidenced by subepithelial secretory cells with large granules with affinity for both eosin (Fig. 3a) and toluidine blue (stained in light blue; Fig. 3b), but not for PAS or alcian blue (Fig. 3c). Epithelial secretory cells, in turn, are evidenced by TF (stained in pink; Fig. 3b) and AB (Fig. 3c). Tentacles are densely covered by cilia at their bases, but tufts are largely scarce on the inner surface (i.e., the

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surface opposed to the valve) (Fig. 3d). A cluster of cilia is located at the tip (Fig. 3e). Musculature includes transverse and longitudinal fibers, the latter being more developed than the former (Fig. 3f). Innervation was not observed.

IFT are marginal, slightly flat, and darkly pigmented, although pigmentation is less intense than the rest of the inner fold. Adjacent tentacles are similar in size (from 0.5 to 1 mm) and IFT are present along the entire margin, being longer ventrally and non-branched. Secretory cells are lacking, and cilia cover the surfaces of the tentacles, being more concentrated distally (Fig. 3g), where a cluster is present (Fig. 3h). Musculature includes longitudinal and transverse fibers. Branched innervation seems present, with nerves reaching the long ciliary roots at the tip (Fig. 3h).

Ostrea equestris (Ostreidae)

MFT are submarginal, filiform, and pigmented with brownish to grayish spots. The position of the tentacles on the inner surface of the fold (i.e., the surface of the fold opposed to the valve) includes distal and proximal commarginal rows. Shorter tentacles (ca. 1 mm) are located on the distal row, near to the edge, while longer tentacles (ca. 2 mm) are proximal. In the ventral region, MFT are proportionally longer and more abundant, particularly along the incurrent aperture. Short and long tentacles likely display intense secretory activity, as indicated by subepithelial and epithelial secretory cells. Two types of subepithelial secretory cells are present. Cells with more sparsely distributed granules were stained in dark blue with toluidine blue (Fig. 4a) and had affinity for BB (Fig. 4c); those with more densely distributed granules stained in light blue with toluidine blue (Fig. 4a) and had a strong affinity for eosin (Fig. 4b). Weak affinity for PAS and AB was observed for both cell types. Each MFT has abundant tufts

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of cilia on its epithelium and a dense cluster of cilia at the tip (Fig. 4d, f). MFT are very muscular; this system is dominated by longitudinal fibers (Fig. 4e). Tentacle innervation is provided by branching nerves from the base reaching the ciliary rootlets, which are more evident at the apical tufts (Fig. 4f).

IFT are marginal (Fig. 4g), filiform, usually lightly pigmented, and adjacent tentacles are similar in size (ca. 1.5 mm). Along the margin, IFT are longer ventrally, but never branched. Secretory cells were not detected. Cilia on the tentacle surface are distributed in numerous longitudinal rows (Fig. 4h, i), forming a dense cluster at the tip (Fig. 4j). Tentacle musculature is formed by longitudinal fibers, with no evidence of transverse fibers (Fig. 4k). Innervation includes multiple branching nerves from the base to the tip.

Pinna carnea (Pinnidae)

Only IFT are present. They are marginal, slightly flattened, and pigmentation includes tiny yellow, brown, and whitish spots (Fig. 5a). IFT occur only in the posterior region, being larger along the incurrent and excurrent apertures (Fig. 5a). Secretory cells were not detected in histological sections, although droplets of possible mucosubstances were observed interspersed with the short cilia that cover the entire tentacle surface (Fig. 5b, c). No clusters of cilia were observed at the tentacle tips. Musculature is formed by longitudinal and transverse fibers (Fig. 5d). Innervation was not observed.

Spondylus ictericus (Spondylidae)

MFT are submarginal, filiform, and slightly pigmented with yellow and whitish spots (Fig. 6a). Brown and white pigmentation are more intense on the outer surface,

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i.e., the side that faces the valve. MFT are located on the inner surface of the fold, occupying proximal to distal positions (Fig. 6b). Some tentacles are modified as eyestalks, bearing an eye at their tip (Fig. 6a). Extremely long tentacles (more than 5 mm) are also present in a proximal position on the fold, being much longer than adjacent tentacles (ca. 2 mm) (Fig. 6a). MFT are uniformly distributed along the entire margin, with no evident difference in density or size with body regions. The epithelium contains numerous secretory cells stained by PAS and alcian blue (Fig. 6c), with low affinity for TF and none for BB or eosin. Long tentacles show sparse tufts of cilia on the epithelium (Fig. 6d), while the remaining tentacles have cilia organized in longitudinal rows (Fig. 6e). No clusters of cilia were observed at the tentacle tips. Droplets of mucosubstances were observed on the ciliary rows, possibly being secreted by the adjacent glandular epithelium (Fig. 6f). Musculature and innervation could not be investigated by confocal microscopy, but histological sections indicate abundance of longitudinal and transverse fibers, as well as a central tentacular nerve (Fig. S1d).

IFT are very small marginal projections, similar to papillae (Fig. 6a). They are uniformly distributed along the entire margin, being unpigmented and translucent. No evidence for secretory activity was detected. Short cilia cover all tentacle surfaces while long cilia are present in sparsely distributed tufts (Fig. 6g, h) but are not concentrated in a cluster at the tip. Despite their small size, IFT are provided with longitudinal muscles and tiny transverse muscle fibers (Fig. 6i). Innervation was not observed.

4. Discussion

Gross-morphology and distribution of tentacles

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All species studied herein have tentacles on both mantle folds, except for *Pinna carnea*, which has tentacles only on the inner fold. In addition, tentacles may be distributed along the entire mantle margin or be restricted to some regions. The former condition is observed in most studied species, with tentacle distribution along the mantle extension. Nevertheless, tentacles are more abundant and larger ventrally in pteriid and ostreid species (Table 1). The latter condition is illustrated by *Pinna carnea*, with tentacles restricted to the posterior region, bordering the incurrent and excurrent apertures (Yonge, 1953).

In infaunal and semi-infaunal bivalves, IFT are common on the siphons as sensory and protective structures distributed close to their apertures (Yonge, 1983). A great diversity in shape and number of siphonal tentacles was described for infaunal bivalves from the families Veneridae, Donacidae, and Tellinidae, which have multiple branched tentacles on the incurrent siphon (Fishelson, 2000; Narchi, 1972; Piffer, Arruda, & Passos, 2011; Sartori & Domaneschi, 2005; Sartori, Printrakoon, Mikkelsen, & Bieler, 2008; Vitonis, Zaniratto, Machado, & Passos, 2012). Despite the absence of a siphon, a similar condition is present in *Pteria colymbus* and *Pinctada imbricata* – both species have well-developed, branched tentacles restricted to the ventral, incurrent region. Carnivorous bivalves from the order Anomalodesmata also bear siphonal tentacles (e.g., *Cardiomya cleryana*; Machado, Morton, & Passos, 2017). More rarely, tentacles may also occur in the anterior portion of the pedal opening, as observed in Cyammiidae (Passos & Machado, 2014).

Among the Pteriomorphia, tentacles have been studied in detail only for a few species, although general observations were described for many groups (Waller, 1975). In file clams (Limidae), MFT pigmentation ranges from translucent white to intense red.

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In these bivalves, they vary from short to extremely long structures, sometimes annulated, and always restricted to the middle fold, where they are usually densely distributed (Allen, 2004; Gilmour, 1963, 1967; Mikkelsen & Bieler, 2003). In the Pectinoidea, scallops (Pectinidae) and thorny oysters (Spondylidae) have MFT formed on the inner surface of the middle fold (i.e., the surface opposed to the valve), also including very long tentacles (Audino et al., 2015; Dakin, 1928b; Moir, 1977). Tentacles on the inner mantle fold are also common in scallops; given that they are located along the margin, they are named "guard tentacles" of the mantle cavity aperture (Audino et al., 2015; Dakin, 1909; Drew, 1906). Although previous studies have not recorded IFT in Spondylidae (Dakin, 1928a), our data for *Spondylus ictericus* show robust evidence for their presence. Possibly due to their extremely small size or preservation artifacts, they have passed unnoticed in previous investigations.

Pearl oysters and relatives (Pterioidea) are another pteriomorphian lineage provided with numerous tentacles on both middle and inner mantle folds, also with variation in size and pigmentation (Tëmkin, 2006). Our data for the pteriids *Isognomon bicolor*, *Pinctada imbricata*, and *Pteria colymbus* are in accordance with the pattern of tentacle distribution previously described for both folds, and also with the presence of branched IFT in the latter two genera (Tëmkin, 2006). Interestingly, our results for *Ostrea equestris* revealed great similarity to Pteriidae in respect to the increase of IFT size and abundance towards the ventral region (incurrent aperture), as also observed in *Crassostrea* spp. (Amaral & Simone, 2014). In contrast to pteriids, however, MFT are submarginal in *Crassostrea* and *Ostrea*, with proximal (long) and distal (short) tentacles (Amaral & Simone, 2014; present study).

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Tentacle functions and anatomical patterns

The increase of tentacular size towards the incurrent and excurrent regions is a common trend for many bivalve groups, especially for those with siphons (Sartori et al., 2008). Even in bivalves with free mantle margins (i.e., devoid of siphons), the position of the inner mantle fold may be muscularly adjusted to delimit and control the aperture of incurrent and excurrent regions (Owen & McCrae, 1979). Tentacles at the incurrent region are supposed to prevent the entrance of large particles into the mantle cavity, especially in turbulent environments, where large particles would obstruct water flow through the gills and mantle cavity. Our results show abundant musculature in the IFT, regardless of their size or of the presence of lateral branches. The musculature of IFT should allow for fine adjustments in tentacle position, possibly protecting the mantle cavity apertures. In contrast to previous hypotheses on tentacles as non-specialized extensions of the mantle margin (Waller, 1975), our data are in accordance with studies on Pterioidea arguing for the structural and functional complexity of those structures (Tëmkin, 2006). As described herein, elongated IFT at the incurrent region are observed in Ostreidae, Pinnidae, and Pteriidae (all of them currently placed within Ostreida, Fig. 7), as well as in most siphoned bivalves.

In particular cases, tentacles can also play defensive roles against potential predators. For example, *Limaria hians* (former *Lima hians*, Limidae) can autotomize parts of its tentacles, or even the entire organ, with subsequent release of distasteful mucus to avoid predation (Gilmour, 1963, 1967). In the phylogenetically distant Galeommatidae, anatomical data on *Galeomma takii* also suggest autotomy and secretory activity as similar defensive mechanisms. When stimulated, papillae on the

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middle fold, which almost completely cover the shell, are capable of autotomy, secreting a noxious substance (Morton, 1973).

Secretory activity appeared to be largely present in the species studied (Table 1). Our results show that secretion of mucosubstances is an important role performed by MFT, including neutral (PAS-positive) and acidic polysaccharides (AB-positive) and basic glycoproteins (BB-positive), secreted by subepithelial and epithelial secretory cells. Similar results were obtained for other Pteriidae, with acidophilic secretory cells and neutral glycoproteins secretion in the MFT of *Pinctada margaritifera* (Jabbour-Zahab, Chagot, Blanc, & Grizel, 1992). The production of mucus by the tentacles is expected to keep these organs clean through the agglutination of undesirable particles or small organisms, propelling them to the mantle surface for rejection (Beninger & St-Jean, 1997; Prezant, 1990). Another piece of evidence reinforcing this hypothesis is the short, densely distributed cilia covering the surface of MFT in the studied species, which display characteristics likely associated with mucociliary transportation (Sleigh, 1989; Sleigh, Blake, & Liron, 1988).

Secretory activity in MFT was also shown for *Nodipecten nodosus* (Audino et al., 2015), including acidic and neutral mucosubstances very similar to those of *Spondylus ictericus*. In the case of *N. nodosus*, cilia are densely distributed over the tentacle surface (Audino et al., 2015). In contrast, *S. ictericus* exhibits longitudinal rows of cilia along the MFT that are supposed to promote mucus propulsion, which is supported by the presence of secretory droplets in adjacent areas.

Despite intense secretory activity in the inner mantle fold of many bivalve species (Audino & Marian, 2018; Jabbour-Zahab et al., 1992; Richardson, Runham, & Crisp, 1981), IFT show little or no evidence of secretory cells in the species investigated

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herein. Nevertheless, our results indicate these organs should be able to propel the mucus secreted proximally by the inner fold, possibly helping to lubricate the mantle margin. Droplets of possible mucosubstance observed in the tentacles of *P. carnea* and cilia distribution in the pteriids and in *O. equestris* support this hypothesis. Moreover, ciliary rows observed in *O. equestris* and ciliary patches in the pteriids match the conditions to provide mucociliary transportation of mucus rafts required for cleansing and lubrication (Sleigh, 1989). Both roles are important for animals living in benthic environments, especially where there is a high concentration of suspended particles and debris that can cover, obstruct or damage the mantle margin.

Bivalve tentacles are also regarded as sensory organs due to putative mechano- and chemoreceptors organized in ciliary receptor cells. Nevertheless, both functional types can hardly be distinguished based exclusively on morphological criteria (Owen & McCrae, 1979). Scallops and limids are, by far, the most investigated examples of bivalves having specialized sensory MFT. In *Placopecten magellanicus* and *Nodipecten nodosus*, ciliary tufts, supposedly acting as mechanoreceptors, are distributed on papillae at the distal portion of long, exploratory tentacles (Audino et al., 2015; Moir, 1977). Ciliary papillae were not observed on the MFT of *Spondylus ictericus*, a representative of a family phylogenetically close to Pectinidae (Pectinoidea, Fig. 7). However, ciliary tufts are sparsely distributed on the surfaces of long tentacles, and they are most likely related to sensory perception.

Sensory ciliated receptors are also common at the tip of siphons and on siphonal tentacles, as observed in Donacidae and Tellinidae (Hodgson & Fielden, 1984; Vitonis et al., 2012), and on papillae along the middle fold, as in *Mysella charcoti* (Lasaeidae) (Passos, Domaneschi, & Sartori, 2005). In the present study, a dense ciliary cluster

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associated with abundant innervation was consistently observed at the tip of MFT in *Isognomon bicolor*, *Ostrea equestris*, *Pinctada imbricata*, and *Pteria colymbus*. These results suggest the presence of putative sensory receptors at the tip of MFT as a shared pattern for Ostreoidea and Pterioidea (Fig. 7). The widespread distribution of ciliary clusters and ciliated cells in the studied MFT indicate tentacles are important sensory organs for interacting with the surrounding environment, possibly detecting types of substrate, predators, suspended particles, and other ecological cues.

Ciliated receptors were also observed at the tip of IFT in some pteriid and ostreid species studied herein (Table 1), which suggests that that these putative homologous structures play important sensory roles in addition to the protection of the mantle cavity aperture. The extensive innervation associated with ciliated receptors supports this hypothesis. However, lack of data for *Pinna carnea* and *Spondylus ictericus* prevents further conclusions on innervation patterns. In the pteriid and ostreid species studied herein, MFT and IFT innervation is characterized by a branching pattern throughout the tentacle, resulting in numerous small nerves running along the organ and reaching the ciliated cells (possibly sensory receptor cells). This suggests another common pattern shared by Pterioidea and Ostreoidea (Fig. 7), which is distinct, for example, from tentacle innervation in the pectinid *N. nodosus*, in which a single central nerve emits very tiny projections to the epithelium (Audino et al., 2015).

5. Conclusions

Although the evolutionary origin and diversification of tentacles during Pteriomorphia radiation are still speculative, our results support the homology of MFT and IFT in Pterioidea and Ostreoidea, considering their larger size and abundance

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ventrally, presence of ciliary clusters at the tip, and branched innervation pattern (Table 1). Even though information for additional taxa would be necessary to support such a conclusion, our results are in accordance with phylogenetic hypotheses that recover the sister relationship between these superfamilies (Tëmkin, 2010; Lemer et al., 2016; Fig. 7). In contrast, profound anatomical differences, such as tentacle innervation, cilia organization, and size distribution along dorsal to ventral regions (Table 1), indicate IFT and MFT are not homologous across Pteriomorphia. In addition, given that tentacle diversification may be associated with similar selective pressures, as suggested by the recurrent evolution of protective IFT in numerous bivalve lineages (e.g., Sartori et al., 2008; Vitonis et al., 2012), evolutionary convergence should be expected when exploring tentacle evolution in Pteriomorphia.

Just like most invertebrates, bivalves have evolved numerous types of tentacles associated with diverse functions. The detailed anatomical analysis of pallial tentacles in selected representatives of pteriomorphian bivalves revealed possible shared characters for the clades Pterioidea and Ostreoidea, as well as the first anatomical description of tentacles in Spondylidae and Pinnidae. Sensory and protective roles are likely performed by both MFT and IFT, as well as cleansing and lubrication by MFT. In conclusion, by applying integrative microscopy to study the structure and infer the functional morphology of such diverse organs, we were able to reveal anatomical elements that are essential for the understanding of homology when dealing with such superficially similar structures.

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Figure Legends

Figure 1. Mantle tentacles of *Pteria colymbus* (Pteriidae). Middle fold tentacles (MFT) in (b-g) and inner fold tentacles (IFT) in (h-l). (a). MFT (black arrows) and IFT (white arrows) in the posterodorsal region. (b). Longitudinal section of a tentacle showing subepithelial secretory cells with granular content (arrowheads); HE. (c). Same as (b), but stained with BB, evidencing protein aggregates (arrowheads). (d). Same as (b), but stained with AB, evidencing acidic mucosubstances (arrowheads). (e). Ciliary tufts covering the tentacle surface at its base; scanning electron micrograph (SEM). (f). Cilia cluster at the tip of the tentacle; SEM. (g). Tentacle musculature with abundant longitudinal fibers; confocal micrograph (CLSM). (h). Tentacles on the inner fold; some of them are branched (white arrows), ventral region. (i). Dense cilia distribution on short, not-branched tentacles; SEM. (j). Branched tentacle with typical flattened shape; SEM. (k). Detail from the previous image, showing cilia distribution; SEM. (l). Musculature in a branched tentacle, including branching fibers; CLSM.

Figure 2. Mantle tentacles of *Pinctada imbricata* (Pteriidae). Middle fold tentacles in (a-c) and inner fold tentacles in (d-h). (a). Longitudinal section showing subepithelial secretory cells with acidic mucosubstances (arrowheads); AB. (b). Ciliary cluster at the tip of the tentacle; scanning electron micrograph (SEM). (c). Muscle fibers (yellow) along the tentacle, nuclei in blue; confocal micrograph (CLSM). (d). Branched tentacles in the ventral region. (e). Longitudinal section of the tentacle, showing the absence of secretory content; PAS. (f). Branched tentacle with sparse tufts of cilia; SEM. (g). Detail of the epithelium of a branched tentacle; SEM. (h). Branched tentacle innervation

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(in green) with branching nerves reaching the ciliary rootlets at the tips of the branches; nuclei in blue; CLSM.

Figure 3. Mantle tentacles of *Isognomon bicolor* (Pteriidae). Middle fold tentacles in (a-f) and inner fold tentacles in (g-h). (a). Longitudinal section showing subepithelial secretory cells with granular content (arrowheads); HE. (b). Same as (a), but showing subepithelial secretory cells with granular content stained in light blue (arrowhead), and epithelial secretory cells stained in pink (arrows); TF. (c). Longitudinal section showing epithelial secretory cells with acidic mucosubstances (arrowheads); AB. (d). Cilia distribution on the tentacle; scanning electron micrograph (SEM). Ciliary tufts are largely scarce on the inner surface. (e). Detail of the ciliary cluster at the tip of the tentacle; SEM. (f). Tentacle musculature with prominent longitudinal fibers; confocal micrograph (CLSM). (g). Cilia distribution on tentacle surface; SEM. (h). Cilia distribution (in green), including long rootlets possibly related to innervation; nuclei in blue; CLSM.

Figure 4. Mantle tentacles of *Ostrea equestris* (Ostreidae). Middle fold tentacles in (a-f) and inner fold tentacles in (g-k). (a). Longitudinal section showing two types of subepithelial secretory cells, which either have more densely (white arrowheads) or more sparsely distributed granules (black arrowheads); TF. (b). Same as (a), but stained with HE, showing the eosinophilic nature of the secretory cells with more densely distributed granules (white arrowheads). (c). Same as (a), but stained with BB, evidencing the proteinaceous content of the secretory cell type with sparsely distributed granules (black arrowheads). (d). Cilia distribution on the distal portion of the tentacle;

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scanning electron micrograph (SEM). (e). Mantle and tentacle musculature; confocal micrograph (CLSM). (f). Branching nerves (in green) reaching the ciliary rootlets from the ciliary cluster at the tip of the tentacle; nuclei in blue; CLSM. (g). Longitudinal section of the mantle margin, showing an enlarged, marginal inner fold tentacle (te) with no evidence of secretory cells; HE. (h). Cilia distribution on the distal portion of the tentacle; SEM. (i). Detail of (h), showing ciliary tufts; SEM. (j). Detail of the cilia cluster at the tip; SEM. (k). Tentacle musculature; CLSM.

Figure 5. Inner fold tentacles of *Pinna carnea* (Pinnidae). (a). Posterior view of the tentacles located on the margin of the inner mantle fold at the incurrent region. (b). Uniform ciliary distribution on the tentacle; scanning electron micrograph (SEM). (c). Detail of cilia and droplets of possible mucosubstances (arrowheads); SEM. (d). Tentacle musculature with longitudinal fibers greatly contracted; confocal micrograph.

Figure 6. Mantle tentacles of *Spondylus ictericus* (Spondylidae). Middle fold tentacles (MFT) in (b-f) and inner fold tentacles (IFT) in (g-i). Scanning electron micrographs in (d-h). (a). Tentacle types on the mantle margin: MFT (black arrows), including very long tentacles (arrowhead), and very small IFT (white arrows; inset). (b). Longitudinal section of the mantle margin, showing tentacles formed on the inner surface of the middle fold; HE. (c). Different types of epithelial secretory cells distinguished by neutral (magenta) and acidic (blue) mucosubstances; AB and PAS combined method. (d). Sparse tufts of cilia on the distal portion of a long tentacle. (e). Cilia organized in longitudinal rows on the surface of tentacles. (f). Detail from (e), showing a droplet (arrowhead) released close to the ciliary row, indicating possible secretion of

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mucosubstances. (g). Small tentacles on the margin of the inner fold. (h). Ciliary tufts sparsely distributed on the tentacle surface. (i). Muscle fibers of a small tentacle; confocal micrograph. Abbreviations: if, inner mantle fold; pe, pallial eye; te, tentacles.

Figure 7. Schematic representation of the Pteriomorphia phylogenetics, including current orders (in bold) and superfamilies. Redrawn after Lemer et al. (2016).

Supplementary Figure S1. Innervation of MFT (a, c, d) and IFT (b) in pteriomorphian species. Confocal micrographs in (a-c) and histological longitudinal section in (d). (a). Central tentacular nerves that branch towards the tip (*Pteria colymbus*). (b). Branching tentacular nerves (*Pteria colymbus*). (c). Branching tentacular nerves (*Pinctada imbricata*). (d) Central tentacular nerve (*Spondylus ictericus*).

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Table 1. Comparative morphology of inner (IFT) and middle fold tentacles (MFT) in Pteriomorphia. "Morphology" refers to size variation among adjacent tentacles (1 – similar size or 2 – varying size) and presence of branched tentacles (3). In the ventral region of the body, tentacles can be either (1) larger and more abundant in comparison to the anterior and posterior regions, or (2) similar to the other regions. Absence of a trait is indicated by "–" and unavailable information by "?". For additional anatomical information, please see the results described in the main text. All data generated by the present study, except those for *Limaria hians* (sources: Gilmour 1967, Owen & McCrae 1979) and *Nodipecten nodosus* (source: Audino et al. 2015).

Species	Position on the fold	Morphology	Ventral region	Secretory cells	Cluster of cilia at the tip	Musculature	Innervation
			MFT				
Pteria colymbus	marginal	2	1	eosin & BB; PAS & AB	present	LM, TM	BN
Pinctada imbricata	marginal	2	1	eosin; PAS & AB	present	LM	BN
Isognomon bicolor	marginal	1	1	eosin & TF (light blue); AB & TF (pink)	present	LM, TM	?
Ostrea equestris	submarginal	2	1	eosin & TF (light blue); BB & TF (dark blue)	present	LM, TM	BN
Pinna carnea	–	–	–	–	–	–	–
Limaria hians	submarginal	2	2	?	–	LM, TM	CN
Nodipecten nodosus	submarginal	2	2	PAS & AB	–	LM, TM	CN
Spondylus ictericus	submarginal	2	2	PAS, AB & TF (light blue)	–	LM, TM	CN
			IFT				
Pteria colymbus	marginal	2, 3	1	–	–	LM, TM	BN
Pinctada imbricata	marginal	2, 3	1	–	present	LM, TM	BN
Isognomon bicolor	marginal	1	1	–	present	LM, TM	BN
Ostrea equestris	marginal	1	1	–	present	LM	BN
Pinna carnea	marginal	1	–	–	–	LM, TM	?
Limaria hians	–	–	–	–	–	–	–
Nodipecten nodosus	submarginal	1	2	–	–	LM, TM	CN
Spondylus ictericus	marginal	1	2	–	–	LM, TM	?

Abbreviations: AB, alcian blue; BB, mercury-bromophenol blue; BN, branching nerves; CN, central nerve; LM, longitudinal muscles; PAS, periodic acid-Schiff stain; TF, toluidine blue and basic fuchsin; TM, transverse muscles.

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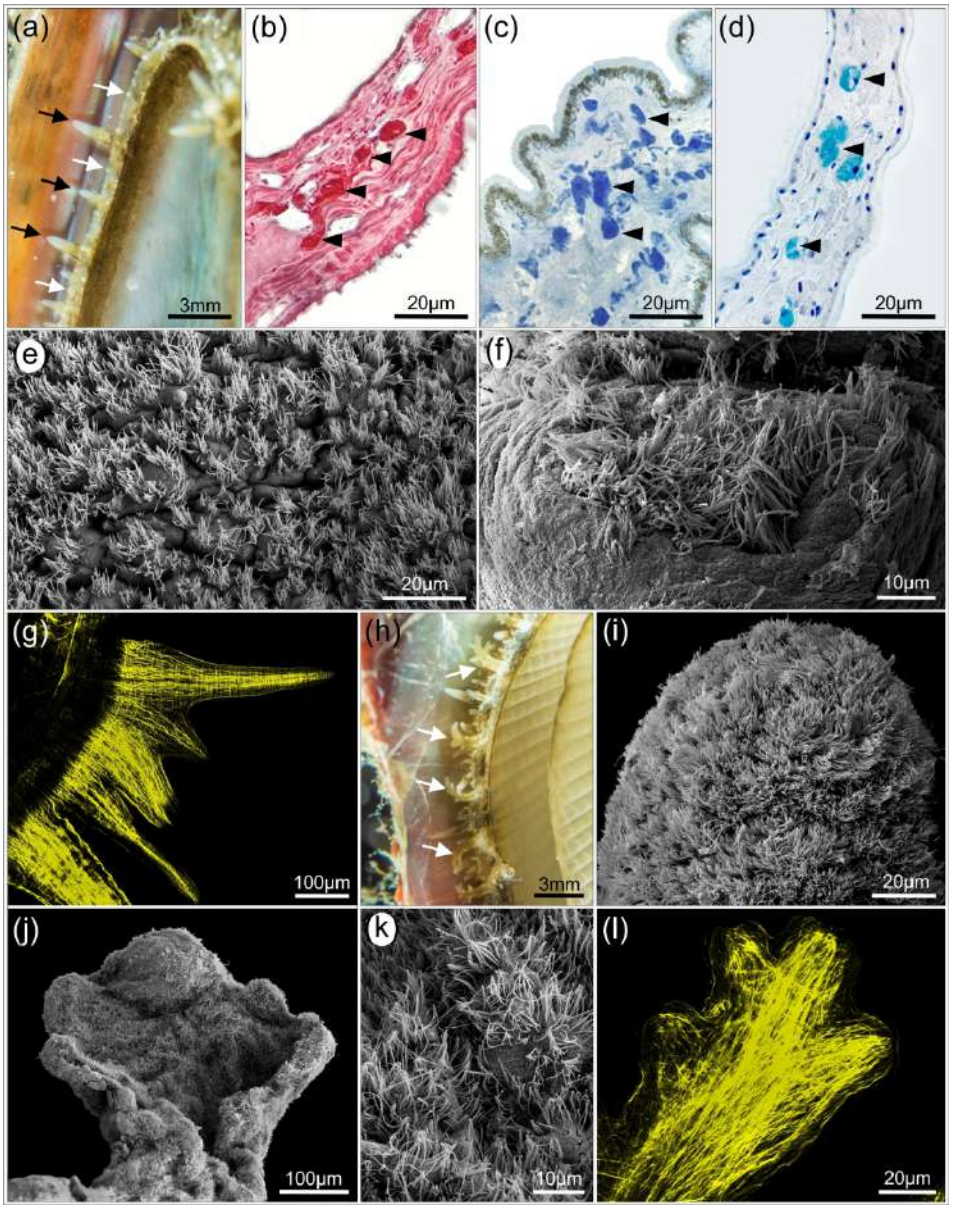
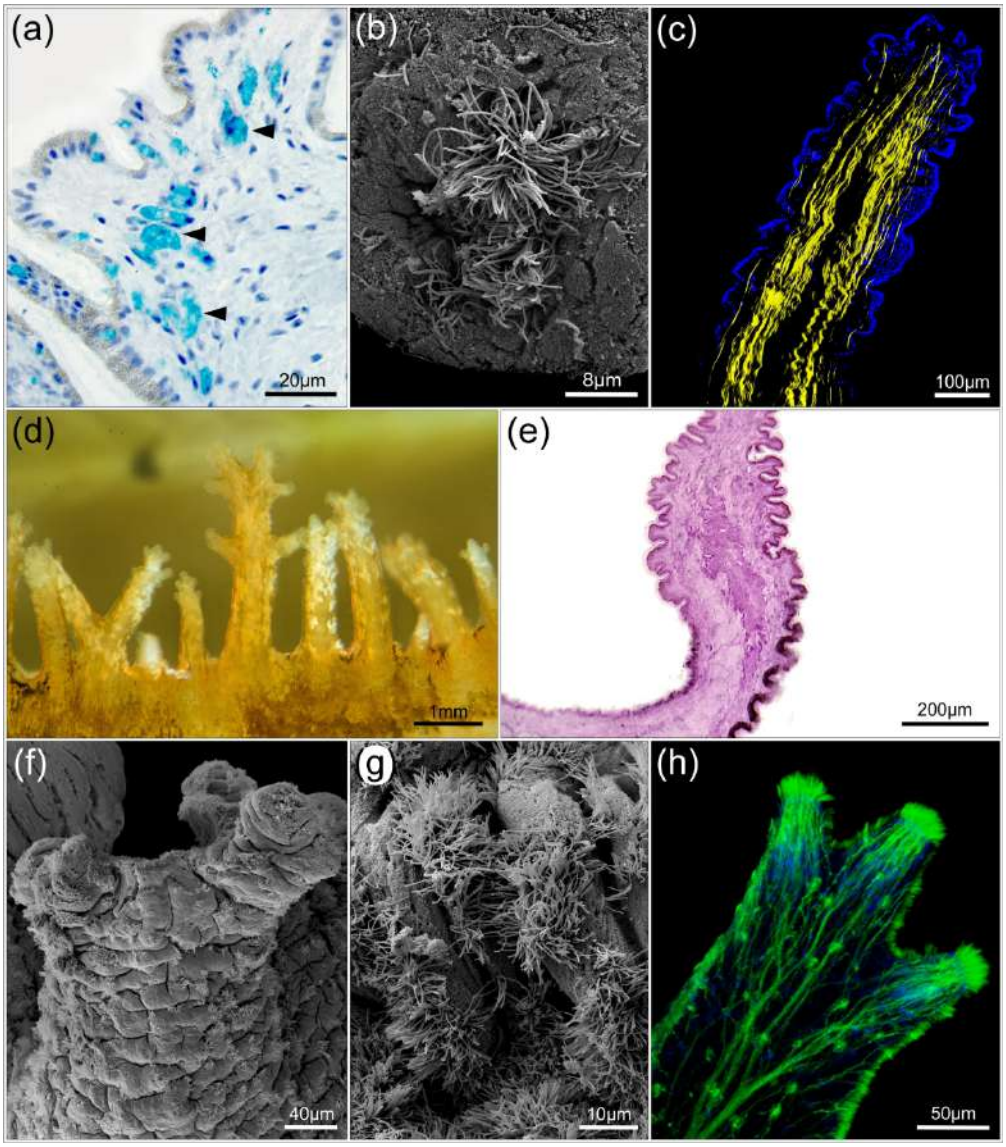
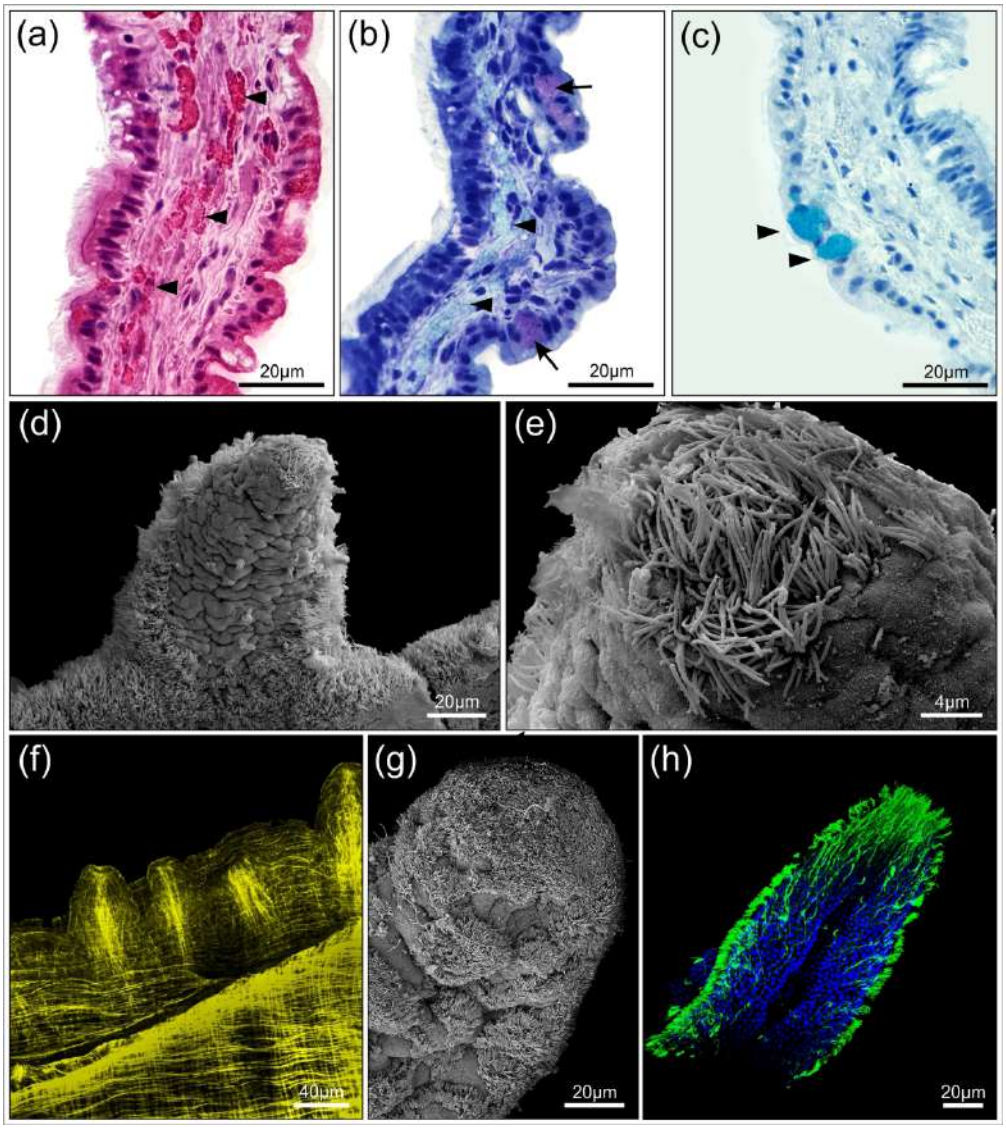


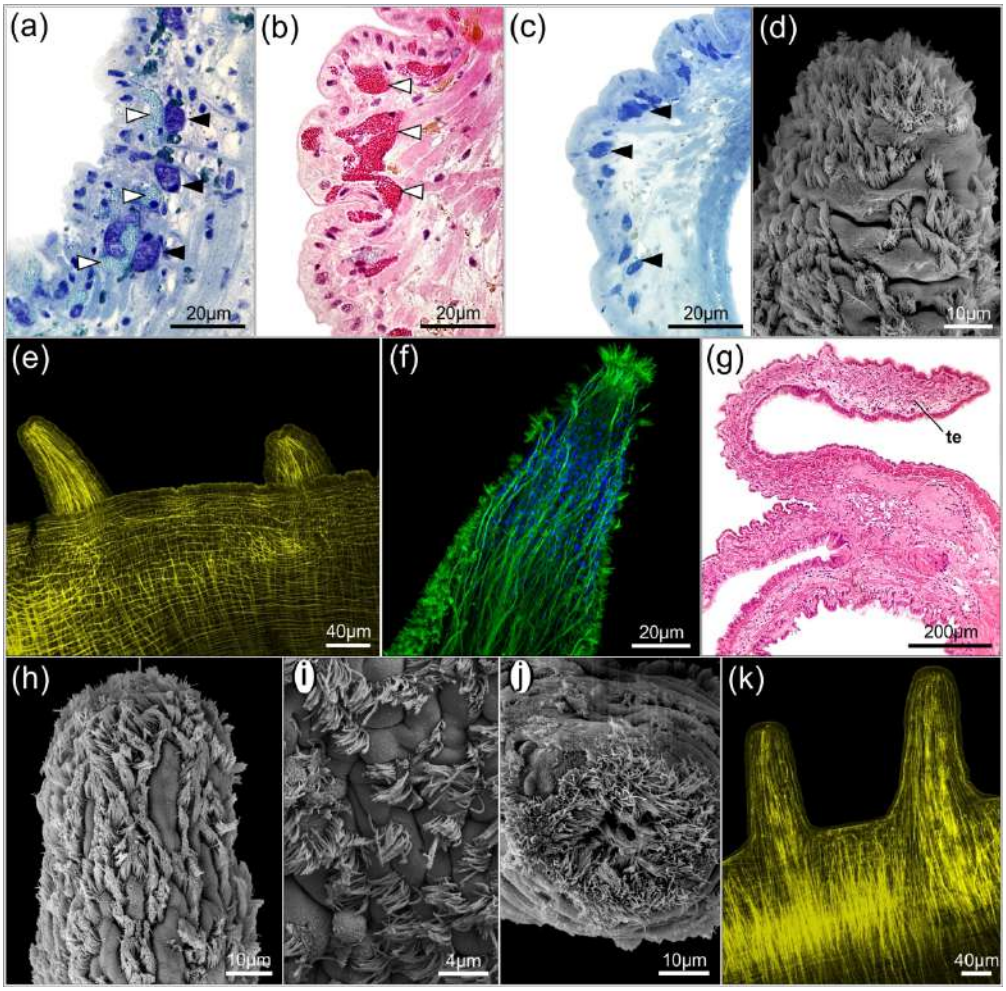
Figure 1. Mantle tentacles of *Pteria colymbus* (Pteriidae). Middle fold tentacles (MFT) in (b-g) and inner fold tentacles (IFT) in (h-l). (a). MFT (black arrows) and IFT (white arrows) in the posterodorsal region. (b). Longitudinal section of a tentacle showing subepithelial secretory cells with granular content (arrowheads); HE. (c). Same as (b), but stained with BB, evidencing protein aggregates (arrowheads). (d). Same as (b), but stained with AB, evidencing acidic mucosubstances (arrowheads). (e). Ciliary tufts covering the tentacle surface at its base; scanning electron micrograph (SEM). (f). Cilia cluster at the tip of the tentacle; SEM. (g). Tentacle musculature with abundant longitudinal fibers; confocal micrograph (CLSM). (h). Tentacles on the inner fold; some of them are branched (white arrows), ventral region. (i). Dense cilia distribution on short, not-branched tentacles; SEM. (j). Branched tentacle with typical flattened shape; SEM. (k). Detail from the previous image, showing cilia distribution; SEM. (l). Musculature in a branched tentacle, including branching fibers; CLSM.



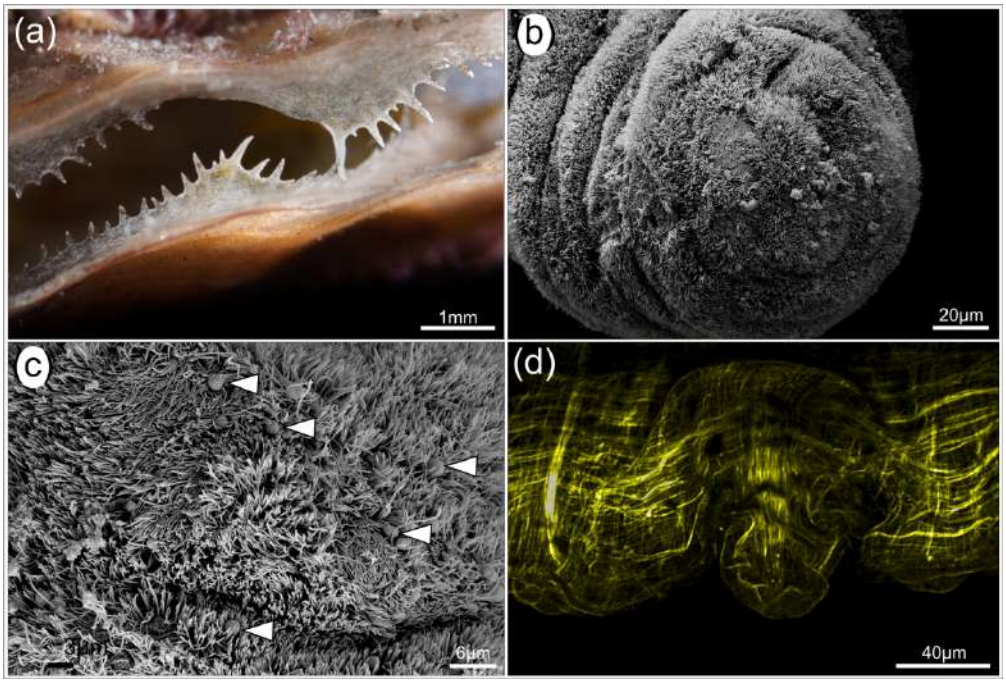
Mantle tentacles of *Pinctada imbricata* (Pteriidae). Middle fold tentacles in (a-c) and inner fold tentacles in (d-h). (a). Longitudinal section showing subepithelial secretory cells with acidic mucosubstances (arrowheads); AB. (b). Ciliary cluster at the tip of the tentacle; scanning electron micrograph (SEM). (c). Muscle fibers (yellow) along the tentacle, nuclei in blue; confocal micrograph (CLSM). (d). Branched tentacles in the ventral region. (e). Longitudinal section of the tentacle, showing the absence of secretory content; PAS. (f). Branched tentacle with sparse tufts of cilia; SEM. (g). Detail of the epithelium of a branched tentacle; SEM. (h). Branched tentacle innervation (in green) with branching nerves reaching the ciliary rootlets at the tips of the branches; nuclei in blue; CLSM.



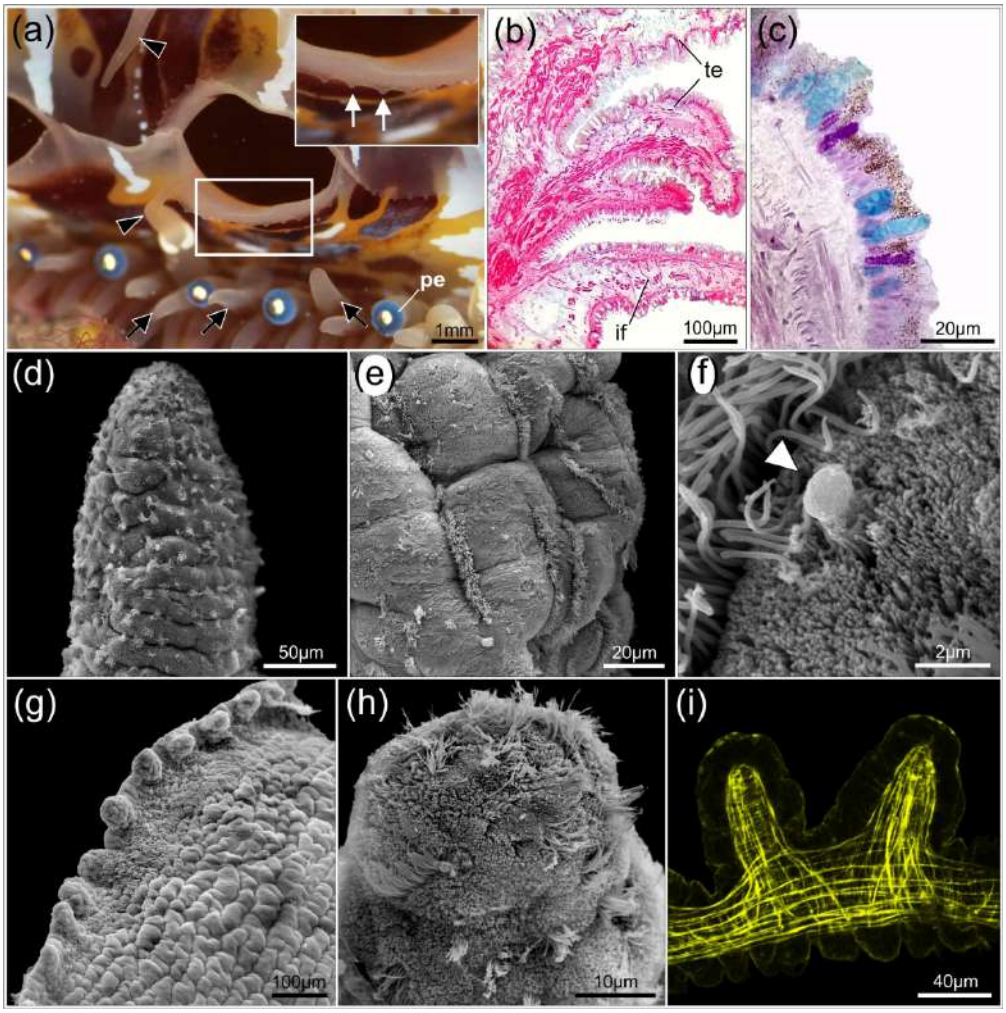
Mantle tentacles of *Isognomon bicolor* (Pteriidae). Middle fold tentacles in (a-f) and inner fold tentacles in (g-h). (a). Longitudinal section showing subepithelial secretory cells with granular content (arrowheads); HE. (b). Same as (a), but showing subepithelial secretory cells with granular content stained in light blue (arrowhead), and epithelial secretory cells stained in pink (arrows); TF. (c). Longitudinal section showing epithelial secretory cells with acidic mucosubstances (arrowheads); AB. (d). Cilia distribution on the tentacle; scanning electron micrograph (SEM). Ciliary tufts are largely scarce on the inner surface. (e). Detail of the ciliary cluster at the tip of the tentacle; SEM. (f). Tentacle musculature with prominent longitudinal fibers; confocal micrograph (CLSM). (g). Cilia distribution on tentacle surface; SEM. (h). Cilia distribution (in green), including long rootlets possibly related to innervation; nuclei in blue; CLSM.



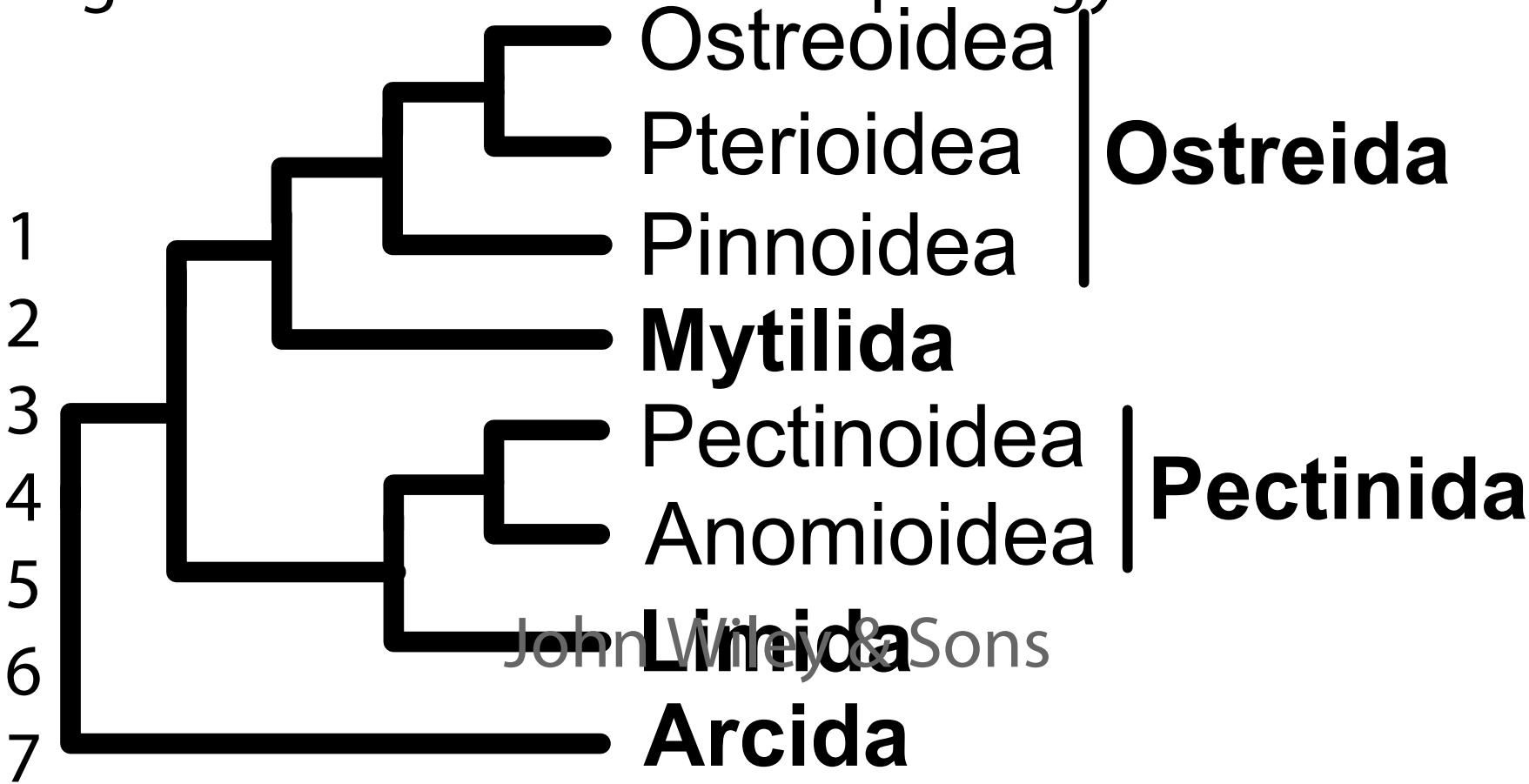
Mantle tentacles of *Ostrea equestris* (Ostreidae). Middle fold tentacles in (a-f) and inner fold tentacles in (g-k). (a). Longitudinal section showing two types of subepithelial secretory cells, which either have more densely (white arrowheads) or more sparsely distributed granules (black arrowheads); TF. (b). Same as (a), but stained with HE, showing the eosinophilic nature of the secretory cells with more densely distributed granules (white arrowheads). (c). Same as (a), but stained with BB, evidencing the proteinaceous content of the secretory cell type with sparsely distributed granules (black arrowheads). (d). Cilia distribution on the distal portion of the tentacle; scanning electron micrograph (SEM). (e). Mantle and tentacle musculature; confocal micrograph (CLSM). (f). Branching nerves (in green) reaching the ciliary rootlets from the ciliary cluster at the tip of the tentacle; nuclei in blue; CLSM. (g). Longitudinal section of the mantle margin, showing an enlarged, marginal inner fold tentacle (te) with no evidence of secretory cells; HE. (h). Cilia distribution on the distal portion of the tentacle; SEM. (i). Detail of (h), showing ciliary tufts; SEM. (j). Detail of the cilia cluster at the tip; SEM. (k). Tentacle musculature; CLSM.



Inner fold tentacles of *Pinna carnea* (Pinnidae). (a). Posterior view of the tentacles located on the margin of the inner mantle fold at the incurrent region. (b). Uniform ciliary distribution on the tentacle; scanning electron micrograph (SEM). (c). Detail of cilia and droplets of possible mucosubstances (arrowheads); SEM. (d). Tentacle musculature with longitudinal fibers greatly contracted; confocal micrograph.



Mantle tentacles of *Spondylus ictericus* (Spondylidae). Middle fold tentacles (MFT) in (b-f) and inner fold tentacles (IFT) in (g-i). Scanning electron micrographs in (d-h). (a). Tentacle types on the mantle margin: MFT (black arrows), including very long tentacles (arrowhead), and very small IFT (white arrows; inset). (b). Longitudinal section of the mantle margin, showing tentacles formed on the inner surface of the middle fold; HE. (c). Different types of epithelial secretory cells distinguished by neutral (magenta) and acidic (blue) mucosubstances; AB and PAS combined method. (d). Sparse tufts of cilia on the distal portion of a long tentacle. (e). Cilia organized in longitudinal rows on the surface of tentacles. (f). Detail from (e), showing a droplet (arrowhead) released close to the ciliary row, indicating possible secretion of mucosubstances. (g). Small tentacles on the margin of the inner fold. (h). Ciliary tufts sparsely distributed on the tentacle surface. (i). Muscle fibers of a small tentacle; confocal micrograph. Abbreviations: if, inner mantle fold; pe, pallial eye; te, tentacles.



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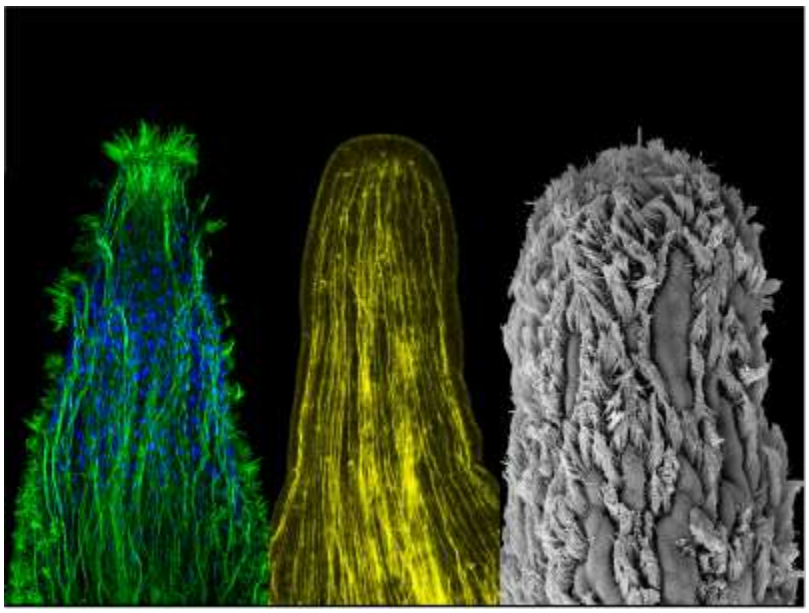

Graphical Abstract Image
Detailed microscopy analysis of mantle tentacles in pteriomorphian bivalves not only provided anatomical evidence to infer the functional morphology of such diverse organs, including sensory roles, mucociliary transportation, and protective functions, but also revealed possible shared traits for the clades Pterioidea and Ostreoidea (oysters and relatives) and the first anatomical description of tentacles in Spondylidae (thorny oysters) and Pinnidae (pen shells). Left, tentacle innervation and ciliary tufts; center, tentacle musculature; right, tentacle cilia.

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Detailed microscopy analysis of mantle tentacles in pteriomorphian bivalves not only provided anatomical evidence to infer the functional morphology of such diverse organs, including sensory roles, mucociliary transportation, and protective functions, but also revealed possible shared traits for the clades Pteriidae and Ostreidae (oysters and relatives) and the first anatomical description of tentacles in Spondylidae (thorny oysters) and Pinnidae (pen shells). Left, tentacle innervation and ciliary tufts; center, tentacle musculature; right, tentacle cilia.

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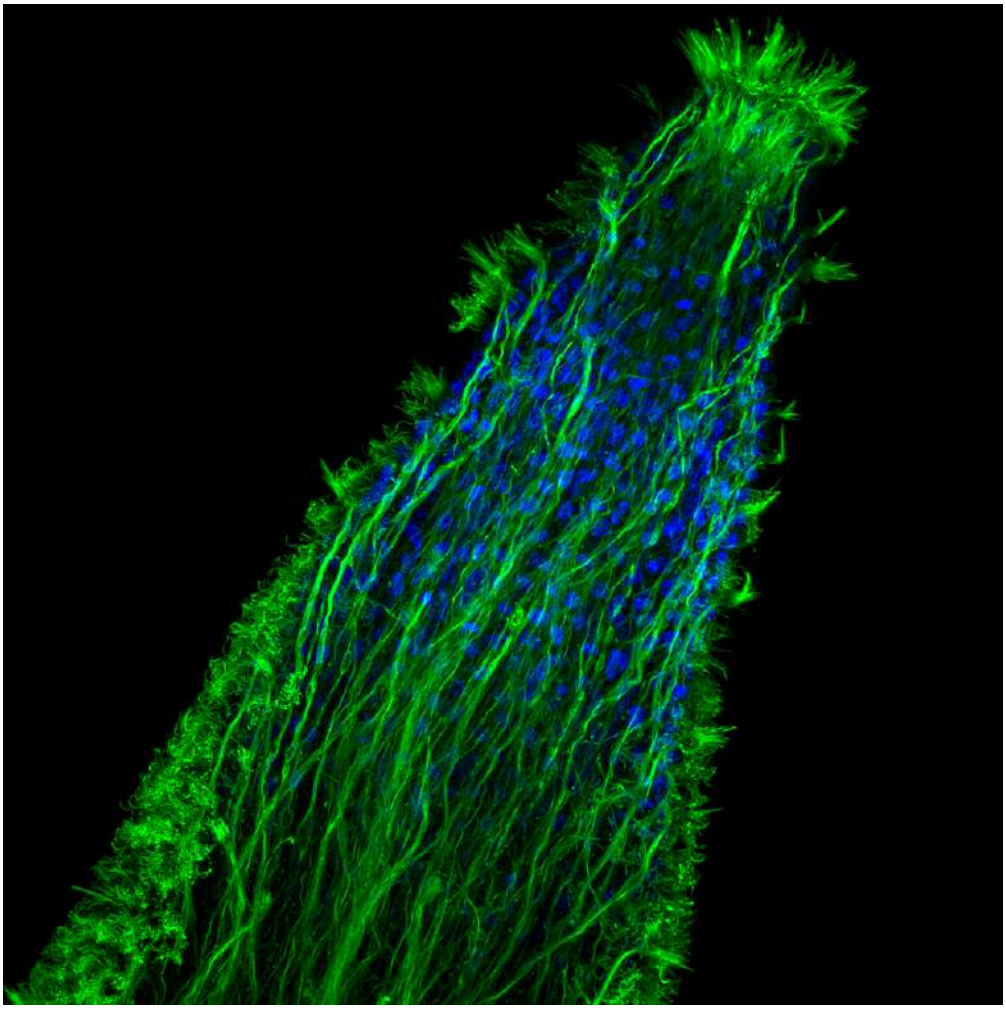

Candidate image for journal cover. Middle fold tentacle of *Ostrea equestris* showing ciliary tufts and innervation in green (anti-α-tubulin), and nuclei in blue (DAPI).