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Authors

Penney, BK
Sigwart, JD
Parkinson, DY

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Spicules and skeletons: mantle musculature of two species of dorid nudibranchs (Gastropoda: Nudibranchia: Doridina)¹

B.K. Penney,

Department of Biology, Saint Anselm College, Box 1742, Manchester, NH 03102, USA.

J.D. Sigwart,

Queen's University Belfast, Marine Laboratory, Portaferry BT22 1PF, County Down, Northern Ireland; Senckenberg Research Institute, 25 Senckenberganlage, Frankfurt 60325, Germany.

D.Y. Parkinson

Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Mail Stop 15-317, Berkeley, CA 94720, USA.

Abstract

Molluscs often possess complex calcified elements in addition to the shell, but how these elements function and relate to other tissues is often poorly understood. Dorid nudibranchs typically possess innumerable calcareous spicules arranged in complex networks. To describe how these spicules interact with muscles and connective tissue, we reconstructed tomographic digital models using serial sections and synchrotron micro-computed tomography. In two species with dramatically different spicule network morphologies, musculature was divided into a dorsal layer of crossed fibres, a ventral layer of branching radial fibres, and scattered dorsoventral fibres in between. These two species differed in the size of their dorsal tubercles, which was reflected in the organization of dorsal musculature, and in the amount and organization of connective tissue. In *Platydoris sanguinea* Bergh, 1905, dense mats of spicules sandwiched a layer of connective tissue with fewer spicules and muscle insertions only onto the ventral spicules. In *Cadlina luteomarginata* MacFarland, 1966, thick tracts of spicules are surrounded by a sheath of connective tissue. Muscles surround and insert into the dorsal tubercle spicule layer. Thus, both species appear to use the spicule network for muscle antagonism and transfer of motion, but the different arrangement of elements suggests that they use this skeleton in quite different ways.

Résumé:

Les mollusques présentent fréquemment des éléments calcifiés complexes en plus de leur coquille, mais le fonctionnement de ces éléments et leur relation avec d'autres tissus ne sont souvent pas bien compris. Les nudibranches doriens possèdent typiquement d'innombrables spicules calcaires disposés en réseaux complexes. Pour décrire l'interaction de ces spicules avec les muscles et les tissus conjonctifs, nous avons reconstitué des modèles numériques tomographiques en utilisant des séries de coupes et la microtomodensitométrie par synchrotron. Chez deux espèces

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Corresponding author: Brian K. Penney (bpenney@anselm.edu).

dont les réseaux de spicules présentent des morphologies très différentes, la musculature est divisée en une couche dorsale de fibres entrecroisées, une couche ventrale de fibres radiales ramifiées et, entre les deux, des fibres dorsoventrales dispersées. Ces deux espèces diffèrent sur le plan de la taille de leurs tubercules dorsaux, ce qui se reflète dans l'organisation de la musculature dorsale, et de la quantité et de l'organisation du tissu conjonctif. Chez *Platydorid sanguinea* Bergh, 1905, de denses tapis de spicules bordent de part et d'autre une couche de tissu conjonctif comptant moins de spicules et des insertions de muscles seulement sur les spicules ventraux. Chez *Cadlina luteomarginata* MacFarland, 1966, d'épais faisceaux de spicules sont entourés d'une gaine de tissu conjonctif. Des muscles entourent la couche de spicules du tubercule dorsal et la pénètrent. Ainsi, les deux espèces semblent utiliser le réseau de spicules pour l'antagonisme de muscles et le transfert de mouvement, mais les différentes configurations d'éléments indiqueraient qu'elles utilisent ce squelette de manières très différentes.

Keywords

Cadlina luteomarginata ; *Platydorid sanguinea* ; nudibranch; skeleton; spicule; connective tissue; musculature; computed tomography; histology

Mots-clés:

Cadlina luteomarginata ; *Platydorid sanguinea* ; nudibranche; squelette; spicule; tissu conjonctif; musculature; tomodensitométrie; histologie

Introduction

Molluscs produce a variety of hard parts, from shells to plates to spicules, and understanding the form and function of this diversity is important to revealing how molluscs evolved (Chen et al. 2015; Sigwart 2017). In particular, one group of shell-less molluscs — dorid nudibranchs (Heterobranchia: Nudibranchia: Doridina) — have extensive networks of internal calcareous spicules (Penney et al. 2020). These elements are unusual among mollusc sclerites in that they are formed by intra-cellular rather than extra-cellular secretions, and they are buried deep in mesodermal tissue rather than sitting above the epidermis (Hyman 1967). Spicule networks differ among dorid species, and the morphology of network arrangement is broadly aligned to phylogeny within the suborder Doridina (Penney et al. 2020). The spicule networks are large — up to 50% of an individual's dry mass (Carefoot 1967; Penney 2006) — and apparently reflect a substantial investment by the animal, yet we have little idea what function they serve. Prior work suggests that the spicules have only a minor role in defense, so their primary role is inferred to be structural (Penney 2002, 2006). Dorid spicule networks could be characterized as extensive unbraced frameworks, similar to those seen in the demosponges (Vogel 1988). However, sponges lack muscles; how might this type of network interact with the musculature in a mobile animal?

In molluscs, muscles interface with shell through specialized epithelium called “tendon cells”; connective tissue fibers may extend into larger shell elements (Tompa and Watabe 1976; Bonar 1978). Muscle termini that do not insert onto the shell instead generally fan out into connective tissue (Hyman 1967). Dorids may interface with their hard elements

differently, as collagen fibers appear to insert into the connective tissue sheath surrounding spicules in caryophyllidia — specialized dorsal sensory tubercles — of *Rostanga arbutus* (Angas, 1864) and *Jorunna* sp. (Foale and Willan 1987). However, most species lack specialized caryophyllidia (Valdés 2002), and connections to outlying structures may not be representative of the broader whole-body network. Connections between muscles and spicules were also found in another dorid, *Cadlina luteomarginata* MacFarland, 1966 (Penney 2006). In both observations of potential connection between the dorid musculature and the spicule network, it is unclear whether muscle insertions are widespread throughout the general network, and whether muscle fibres attach to spicules at both ends.

To investigate these connections, we used light microscopy and synchrotron micro-computed tomography (micro-CT) to reconstruct three-dimensional digital tomographic models of the spicule network and the musculature of dorid notum tissue. The notum is a thickened but flexible mantle tissue that extends outward from the body wall, much like the cap of a mushroom, and has some of the highest investment of spicules in the animal (Penney 2002). We compared two target species in separate families representing different network morphologies: thick, consolidated tracts of spicules in *C. luteomarginata*, and a dispersed, cobweb-like network with caryophyllidia in *Platydorid sanguinea* Bergh, 1905 (Penney 2008; Penney et al. 2020). If dorid spicule networks act as a hard element skeleton, we would expect to see regular and potentially patterned insertion of muscles to the spicules throughout the spicule network and not restricted to specialized structures like tubercles. Alternatively, if dorid spicules primarily contribute a stiffening agent in a muscular hydrostat, then we might expect to see more irregular distribution of spicules relative to musculature and connective tissue.

Materials and methods

Cadlina luteomarginata (B.K. Penney, personal collection) and *Platydorid sanguinea* (California Academy of Sciences, CASIZ 110458) were initially fixed in formalin for a short time and then transferred to 70% ethanol for preservation and storage. Tissue samples approximately 4 mm long and 1 mm wide were taken through the notum edge from one specimen each, near the midpoint of the animal, and were selected to ensure the sample included at least one complete dorsal tubercle. Tissues were processed for serial sectioning following established protocols (Ruthensteiner 2008; Sigwart et al. 2014), including post-fixation in 1% osmium tetroxide, decalcification in 2% EDTA, serial dehydration in acetone and embedding in epoxy resin (Sigma 45359 Epoxy Embedding Kit), serial sectioning at 1 μm using a Leica Ultracut E ultramicrotome, and high contrast monochromatic staining using Richardson's stain. Resulting sections represent sagittal cuts through the notum. Sections were cover-slipped in Araldite (Sigma), then every section for a range of 200+ sections photographed on a compound microscope (Olympus BX41 or BX60) with images captured via digital camera (Olympus E-600 or DP71) mounted on the microscope trinocular. The resulting digital image stacks were imported to AMIRA version 5.3.3 and were aligned using a combination of automatic and manual alignment adjustments. Muscle, nerve, and connective tissues were identified based on histological structures, and spicules were identified by spaces formed by decalcification; these materials were highlighted

throughout the stack before surface rendering and smoothing to produce three-dimensional tomographic models.

Prior work could only identify the general extent of the total network rather than individual spicules (e.g., Penney et al. 2020: fig. 6F). For high-resolution models, mantle tissue samples were taken as above and scanned at the Hard X-ray Tomography Beamline (8.3.2) of the Advanced Light Source, Lawrence Berkeley National Laboratory (MacDowell et al. 2012). Synchrotron CT provides lower energy X-rays, higher X-ray flux, monochromatic, and higher coherence light compared with benchtop machines. Monochromatic 20 keV X-rays were used to illuminate the sample, and detection was accomplished with a 50 μm thick LuAG:Ce scintillator, a 10 \times Olympus objective in an Optique-Peter lens system, and a PCO.edge sCMOS detector. Initial tomographic reconstructions were performed with Xi-CAM and TomoPy (Gursoy et al. 2014; Pandolfi et al. 2018). Stacks were converted to 8 bit format, cropped in Fiji (Schindelin et al. 2015), and spicule networks segmented in Dragonfly version 4.0 (Object Research Systems).

Results

Platydoris sanguinea

The notum had a one-cell thick epidermis on both the dorsal surface and the ventral surface; the dorsal epidermis is partially missing on this specimen (Fig. 1A). Loose connective tissue ran from the dorsal to the ventral basal lamina, and all other components described were embedded in this layer. No hemocoel channels were found. On the dorsal surface, each tubercle (caryophyllidia) was supported by a ring of 4–7 vertical spicules (Figs. 2A and 3B). These were surrounded by a ring of 5–11 spicules that radiated outward and ventrally; the dorsal ends of this outer ring are only slightly ventral to, and in contact with, the dorsal ends of the inner vertical spicules. The ventral ends of the radial spicules were positioned just above a mat of irregularly arranged, overlapping, horizontal spicules (Fig. 2B). This horizontal sheet was several spicules thick (Fig. 3A). On the ventral side of this sheet, large spicules were oriented downwards at approximately 30°–45° to the surface (Fig. 3A, not shown in Figs. 2A–2C). These met similar large spicules oriented upward from a basal horizontal mat of spicules, sometimes via vertically oriented spicules, to form pillarlike structures. Horizontal locations of these pillars were not coordinated with the location of caryophyllidia. The basal horizontal mat was similar in structure to the dorsal horizontal mat (Fig. 3A, not shown in Figs. 2A–2C). A thick mat of vertically oriented spicules was embedded in the ventral horizontal mat and formed the last spicule layer above the ventral epidermis (Fig. 3C).

Surrounding the spicules associated with the caryophyllidia, the dorsal muscle sheet was composed of longitudinal and transverse fibres approximately 20 μm thick, forming a nearly orthogonal grid (Fig. 2A). Spicules from the caryophyllidia passed through the openings of this grid, with no connections between spicules and muscles (Fig. 2A). The central area of the notum was filled with thin, dorsoventral muscle fibres (Figs. 2A and 2C); these fibres were evenly distributed except where interrupted by the large pillars of spicules connecting the two horizontal sheets. These dorsoventral fibres appeared to bypass the pillar spicules and inserted into the horizontal spicule layers. A thick nerve (Fig. 2A)

branched and anastomosed to form a plexus, and connected to both the dorsal and the ventral muscle layers (Figs. 1B, 1C, and 2A). The ventral muscle layer consisted of two parts: a transverse proximal layer 10 μm thick and a more ventral, thinner ($<5 \mu\text{m}$) layer of obliquely longitudinal fibres just above the basal lamina (Fig. 1D). The longitudinal fibres of the ventral muscle sheet inserted onto vertical spicules that formed the basal mat (Fig. 1D). Neither set of muscle fibres branched within the portion of tissue that was reconstructed.

Cadlina luteomarginata

The notum had a one-cell thick epidermis on both the dorsal surface and the ventral surface (Fig. 4A). Connective tissue was only found surrounding the spicule tracts, which created a trabecular arrangement with many interconnected sinuses (Fig. 5A); other tissues such as muscle fibres and nerves were mostly in these hemoceolic sinuses rather than in the connective tissue (Figs. 4B and 5A). On the dorsal surface, each tubercle was supported by a column of connective tissue filled with dozens of spicules (Figs. 5A and 6). This column extended ventrally to the trabecular network. The column passed through the grid of dorsal musculature, where it was surrounded by a thicker band of muscle fibres (Fig. 5A). Underneath this dorsal muscle grid, running through the interstices of the trabecular network, was the nerve plexus (Figs. 4A and 5B). Below this, large radial muscle fibres extended and branched distally. Longitudinal muscle fibres ran parallel to the anterior–posterior axis (Figs. 4B and 5A), but they were so thin that they were difficult to detect at this magnification and they did not appear to form a coherent pattern.

There were few dorsoventral muscle fibres that ran from one basal lamina to the other. Instead, short fibres in the central mantle were largely associated with the spicule network, running horizontally as well as dorsoventrally just outside the connective tissue sheath (Figs. 5C). These sometimes showed insertion points similar to those seen in *P. sanguinea* (Figs. 4C and 4F), sometimes appeared to fan out into the connective tissue (Fig. 4D), and sometimes appeared to bypass areas of the connective tissue to insert elsewhere (Figs. 4E and 4G).

Discussion

In both *P. sanguinea* and *C. luteomarginata*, muscles insert into connective tissue near spicules. The connective tissue did not appear to penetrate the spicules, as in some molluscan shells (Tompa and Watabe 1976) or the Sharpey's fibers of vertebrates; further studies using transmission electron microscopy could detect if such connections exist. In both a consolidated and more diffuse spicule network, there are a dorsal muscular grid, dorsoventral muscle fibres, a ventral muscle sheet, and central nerve plexus, within the notum tissue. These are common elements in molluscan tissues (Hyman 1967; Satterlie 2015), apparently even when the mantle is expanded and partially skeletonized as in dorids. Muscle fibres sometimes insert into the spicule network and sometimes fan out into the connective tissue. Most gastropods have a mix of soft tissue and hard insertions, e.g., the pedal retractor muscle that connects the columella to the felt of foot musculature (Hyman 1967), but such a high number of these connections is unusual among molluscs.

Interactions between hard elements and muscles generally take one of two forms: either as (1) stiffening gelatinous tissue, as in cnidarian spicules and holothurian ossicles, or (2) points for muscle insertion that allow muscle antagonism and transfer of motion, as in sea star ossicles, mollusc shells, and vertebrate bones (Koehl 1982; Brusca and Brusca 2003). The first arrangement remains functionally a hydrostat, whereas the second functions as a hard element skeleton. The dorid arrangement appears intermediate, with muscle fibers pulling on discrete points of a network. A gradient presumably also exists in echinoderms, where most asteroids have muscles and connective tissue inserting into ossicles (Blowes et al. 2017), unlike holothurians that have dispersed ossicles and depend on a more classic hydrostat. This intermediate of muscles attaching to a stiffened network may be worth further exploration as a transitional form.

Spicule networks may provide a cheap way to allow circular and longitudinal muscles to oppose each other without undue deformation of the notum. As circular muscles antagonize longitudinal muscles in typical hydrostatic skeletons, dorsoventral muscles must contract to prevent changes in thickness (Kier 1988, 2012). In many muscular hydrostats, connective tissue fibres prevent thickness changes, reducing the physiological cost of unneeded contraction (Kier 2012). Here, the spicule network may instead prevent the notum from changing thickness, also reducing the structural cost. Spicules may provide a cheaper alternative than investing protein into connective tissue fibres, as the calcium carbonate portions of gastropod shells have been found to be much cheaper than the organic portion (Palmer 1983, 1992). Indeed, the high growth rate of the dorid *Doris pseudoargus* (Rapp, 1827) has been attributed to supplementation of organic growth by spicules; this may be an important adaptation to the sponge-consuming lifestyle of this group (Carefoot 1967). Change in notum width in vivo is also restricted due to the transverse parts of the network, so most movement will be flexions of the notum edge. This is similar to the movement seen in squid fins (Johnsen and Kier 1993), albeit with a different tissue composition.

A somewhat separate potential function of the spicule network may be to provide a “pop up” recovery. Connective tissue can provide elasticity to a form, as seen in squid mantle (Kier 1988). Dorid nudibranchs, like many other benthic molluscs, often deform themselves to crawl into protective crevices, but re-inflate hydrostats or alter deformed parts to regain normal shape on emergence. It may be useful for the notum to return to its shape without expending energy or time. This is additionally important for dorids, given that they may avoid predator attack in part by resembling stationary colonies of non-preferred invertebrates (Todd 1981). An unbraced framework of hard elements in gelatinous connective tissue could provide a cheap way to construct such concealment.

Despite the gross similarities between these species, the arrangements of, and connections between, elements differ greatly. *Cadlina luteomarginata* has its spicules bound into cable-like structures embedded in connective tissue, with the dorsal mantle musculature wrapping around and inserting at the base of the tubercle and large hemocoelic spaces around the network (Fig. 7B). *Platydoris sanguinea* has a less defined spicule network in the middle of the mantle, and the connective tissue occupies almost the whole central space without hemocoelic channels (Fig. 7A). Muscle bundles extending from the caryophyllidia base have been reported in *R. arbutus* and *Jorunna* sp. (Foale and Willan 1987). We did not

find these in *P. sanguinea*, even though it possesses caryophyllidia and the resolution of our visualizations should have been sufficient to detect them. The size of openings in the dorsal grid roughly correlated with the size of the tubercles (Figs. 2A and 5A). The ventral networks in *P. sanguinea* showed clear longitudinal muscle fibres that inserted into the vertical spicule layer; longitudinal muscle fibres were weak or lacking in *C. luteomarginata*. *Platydorid sanguinea* also had clear dorsoventral muscle fibres that ran from the dorsal muscle grid to the ventral spicule and muscle layers. In *C. luteomarginata*, muscle fibres in the middle of the mantle followed the spicule network just outside of the connective tissue and inserted into it; these fibres were oriented in multiple directions, not just dorsoventrally. We know of at least two other spicule network forms: overlapping sheets of large spicules and dispersed spicules (Penney 2008; Penney et al. 2020). Future work should explore how muscles and connective tissues differ in these groups and how the forms may have evolved.

Although it is clear that these two species have very different spicule–muscle constructions, we know too little about their ecology to generate adaptive hypotheses for the different organizations. In sea stars, body wall thickness correlates with the need for defence versus the need for flexibility (Blake 1990). The relative proportion of hemocele space to musculature varies among hydrostatic skeletons (Kier 1988) and less fluid-filled space generally means finer, but slower, control of movement (Kier 2012). Other trabecular fibre networks are found in the mammalian penis, eye, and subarachnoid space, and may play a role in mechanical resilience and fluid flow (Tamm 2009; Mortazavi et al. 2018; Warburton et al. 2019). Another possible explanation is that tissue from some caryophyllid-bearing dorids tears more easily than the *C. luteomarginata* mantle (B.K. Penney, personal observation) and some use autotomy as a defense (Todd 1981), so differences may relate to how the two species deal with predators.

The spicular networks of dorids fit into the broad diversity of calcified elements among molluscs, and these networks also demonstrate a diversity of forms (Penney et al. 2020). Although most skeletons fall into one of two classes — hard elements with muscular bundles or hydrostatic with muscles in sheets or complex three-dimensional arrays (Kier 1988) — the dorid spicule skeleton may fall somewhere in between. Molluscs frequently have additional calcified elements beyond the canonical shell or shells. Examples of these secondary structures include the calcareous spicular cuticle in chitons and aplousobranch molluscs (Sigwart 2017), evolutionary oddities such as the scaly-foot gastropod *Chrysomallon squamiferum* Chen, Linse, Copley and Rogers, 2015 (Chen et al. 2015), but also additional ordinary calcareous elements embedded within the tissue such as the ligamental lithodesma in some bivalves and the gastropod opercula. Studying the function of spicule networks and other “atypical” calcareous inclusions could also help us better understand the diversity of body forms seen in the Mollusca.

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References

- Blake DB 1990. Adaptive zones of the class Asteroidea (Echinodermata). *Bull. Mar. Sci* 46(3): 701–718.
- Blowes LM, Egertová M, Liu Y, Davis GR, Terrill NJ, Gupta HS, and Elphick MR 2017. Body wall structure in the starfish *Asterias rubens*. *J. Anat* 231(3): 325–341. doi:10.1111/joa.12646. [PubMed: 28714118]
- Bonar DB 1978. Fine structure of muscle insertions on the larval shell and operculum of the nudibranch *Phestilla sibogae* (Mollusca: Gastropoda) before and during metamorphosis. *Tissue Cell*, 10(1): 143–152. doi:10.1016/0040-8166(78)90013-7. [PubMed: 644573]
- Brusca RC, and Brusca GJ 2003. *Invertebrates*. 2nd ed. Sinauer Associates, Sunderland, Mass.
- Carefoot TH 1967. Growth and nutrition of three species of opisthobranch molluscs. *Comp. Biochem. Physiol* 21(3): 627–652. doi:10.1016/0010-406X(67)90459-8. [PubMed: 6058882]
- Chen C, Copley JT, Linse K, Rogers AD, and Sigwart J 2015. How the mollusc got its scales: convergent evolution of the molluscan scleritome. *Biol. J. Linn. Soc* 114(4): 949–954. doi:10.1111/bij.12462.
- Foale SJ, and Willan RC 1987. Scanning and transmission electron microscope study of specialized mantle structures in dorid nudibranchs (Gastropoda: Opisthobranchia: Anthobranchia). *Mar. Biol* 95(4): 547–557. doi:10.1007/BF00393098.
- Gursoy D, De Carlo F, Xiao X, and Jacobsen C 2014. TomoPy: a framework for the analysis of synchrotron tomographic data. *J. Synchrotron Radiat* 21(5): 1188–1193. doi:10.1107/S1600577514013939. [PubMed: 25178011]
- Hyman LH 1967. *The Mollusca I. The invertebrates* McGraw-Hill Book Company, San Francisco, Calif.
- Johnsen S, and Kier WM 1993. Intramuscular crossed connective tissue fibres: skeletal support in the lateral fins of squid and cuttlefish (Mollusca: Cephalopoda). *J. Zool. (Lond.)*, 231(2): 311–338. doi:10.1111/j.1469-7998.1993.tb01921.x.
- Kier WM 1988. The arrangement and function of molluscan muscle. In *The Mollusca: form and function*. Edited by Trueman ER, Clarke MR, and Wilbur KM. Vol. 11. Academic Press, Inc., New York. pp. 211–252.
- Kier WM 2012. The diversity of hydrostatic skeletons. *J. Exp. Biol* 215(Pt. 8): 1247–1257. doi:10.1242/jeb.056549. [PubMed: 22442361]
- Koehl MAR 1982. Mechanical design of spicule reinforced connective tissue: stiffness. *J. Exp. Biol* 98: 239–267.
- MacDowell AA, Parkinson DY, Haboub A, Schaible E, Nasiatka JR, Yee CA, et al. 2012. X-ray micro-tomography at the Advanced Light Source. *SPIE Optical Engineering + Applications In Proceedings SPIE 8506, Developments in X-Ray Tomography VIII*, 850618, San Diego, California, 17 October 2012. Available from doi:10.1117/12.930243.
- Mortazavi MM, Quadri SA, Khan MA, Gustin A, Suriya SS, Hassanzadeh T, et al. 2018. Subarachnoid trabeculae: a comprehensive review of their embryology, histology, morphology, and surgical significance. *World Neurosurg*. 111: 279–290. doi:10.1016/j.wneu.2017.12.041. [PubMed: 29269062]
- Palmer AR 1983. Relative cost of producing skeletal organic matrix versus calcification: evidence from marine gastropods. *Mar. Biol* 75(2–3): 287–292. doi:10.1007/BF00406014.
- Palmer AR 1992. Calcification in marine molluscs: How costly is it? *Proc. Natl. Acad. Sci. U.S.A* 89(4): 1379–1382. doi:10.1073/pnas.89.4.1379. [PubMed: 11607278]
- Pandolfi RJ, Allan DB, Arenholz E, Barroso-Luque L, Campbell SI, Caswell TA, et al. 2018. Xi-cam: a versatile interface for data visualization and analysis. *J. Synchrotron Radiat* 25(Pt. 4): 1261–1270. doi:10.1107/S1600577518005787. [PubMed: 29979189]

- Penney BK 2002. Lowered nutritional quality supplements nudibranch chemical defense. *Oecologia*, 132(3): 411–418. doi:10.1007/s00442-002-0993-6. [PubMed: 28547419]
- Penney BK 2006. Morphology and biological roles of spicule networks in *Cadlina luteomarginata* (Nudibranchia, Doridina). *Invertebr. Biol* 125(3): 222–232. doi:10.1111/j.1744-7410.2006.00055.x.
- Penney BK 2008. Phylogenetic comparison of spicule networks in cryptobranchiate dorid nudibranchs (Gastropoda, Euthyneura, Nudibranchia, Doridina). *Acta Zool.* 89(4): 311–329. doi:10.1111/j.1463-6395.2008.00320.x.
- Penney BK, Ehresmann KR, Jordan KJ, and Rufo G 2020. Micro-computed tomography of spicule networks in three genera of dorid seaslugs (Gastropoda: Nudipleura: Doridina) shows patterns of phylogenetic significance. *Acta Zool.* 101(1): 5–23. doi:10.1111/azo.12266.
- Ruthensteiner B 2008. Soft part 3D visualization by serial sectioning and computer reconstruction. *Zoosymposia*, 1: 63–100. doi:10.11646/zoosymposia.1.1.8.
- Satterlie RA 2015. Organization of the dorsoventral musculature in the wings of the pteropod mollusc *Clione limacina*. *Invertebr. Biol* 134(4): 282–290. doi:10.1111/ivb.12103.
- Schindelin J, Rueden CT, Hiner MC, and Eliceiri KW 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol. Reprod. Dev* 82(7–8): 518–529. doi:10.1002/mrd.22489. [PubMed: 26153368]
- Sigwart J 2017. Zoology: Molluscs all beneath the sun, one shell, two shells, more, or none. *Curr. Biol* 27(14): R708–R710. doi:10.1016/j.cub.2017.05.075. [PubMed: 28743018]
- Sigwart J, Sumner-Rooney L, Schwabe E, Heß M, Brennan G, and Schrödl M 2014. A new sensory organ in “primitive” molluscs (Polyplacophora: Lepidopleurida), and its context in the nervous system of chitons. *Front. Zool* 11(1): 7. doi:10.1186/1742-9994-11-7. [PubMed: 24447393]
- Tamm ER 2009. The trabecular meshwork outflow pathways: Structural and functional aspects. *Exp. Eye Res* 88(4): 648–655. doi:10.1016/j.exer.2009.02.007. [PubMed: 19239914]
- Todd CD 1981. The ecology of nudibranch molluscs. *Oceanogr. Mar. Biol. Annu. Rev* 19: 141–234.
- Tompa AS, and Watabe N 1976. Ultrastructural investigation of the mechanism of muscle attachment to the gastropod shell. *J. Morphol* 149(3): 339–352. doi:10.1002/jmor.1051490304. [PubMed: 957445]
- Valdés A 2002. A phylogenetic analysis and systematic revision of the cryptobranch dorids (Mollusca, Nudibranchia, Anthobranchia). *Zool. J. Linn. Soc* 136(4): 535–636. doi:10.1046/j.1096-3642.2002.00039.x.
- Vogel S 1988. *Life’s devices*. Princeton University Press, Princeton, N.J.
- Warburton NM, Bateman PW, and Fleming PA 2019. Anatomy of the cavernous muscles of the kangaroo penis highlights marsupial–placental dichotomy. *J. Anat* 234(3): 306–315. doi:10.1111/joa.12930. [PubMed: 30613968]

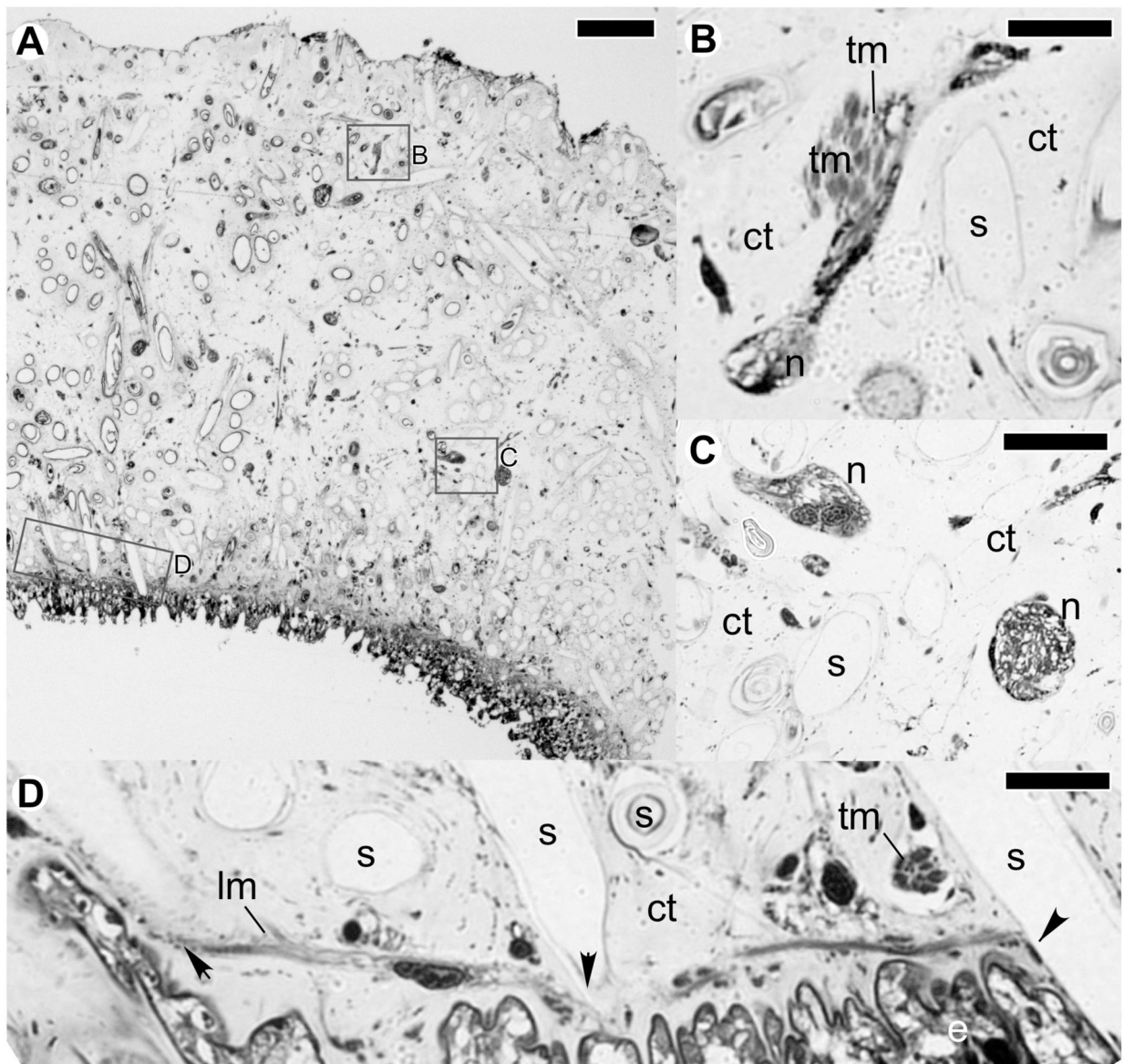


Fig. 1. Photomicrographs of *Platydorís sanguinea* mantle in sagittal section. (A) Overview. Letters indicate the location of detail panels. Dorsal is to the top of the image. Scale bar = 100 µm. (B) Detail of nerve–muscle interaction within the dorsal network. Scale bar = 10 µm. (C) Detail of nerve plexus branches. Scale bar = 20 µm. (D) Detail of muscle insertions. Arrowheads indicate insertions of longitudinal muscle into the basal spicule mat. Scale bar = 10 µm. Abbreviations: ct, connective tissue; e, epidermis; lm, longitudinal muscle; n, nerve; s, spicule; tm, transverse muscle.

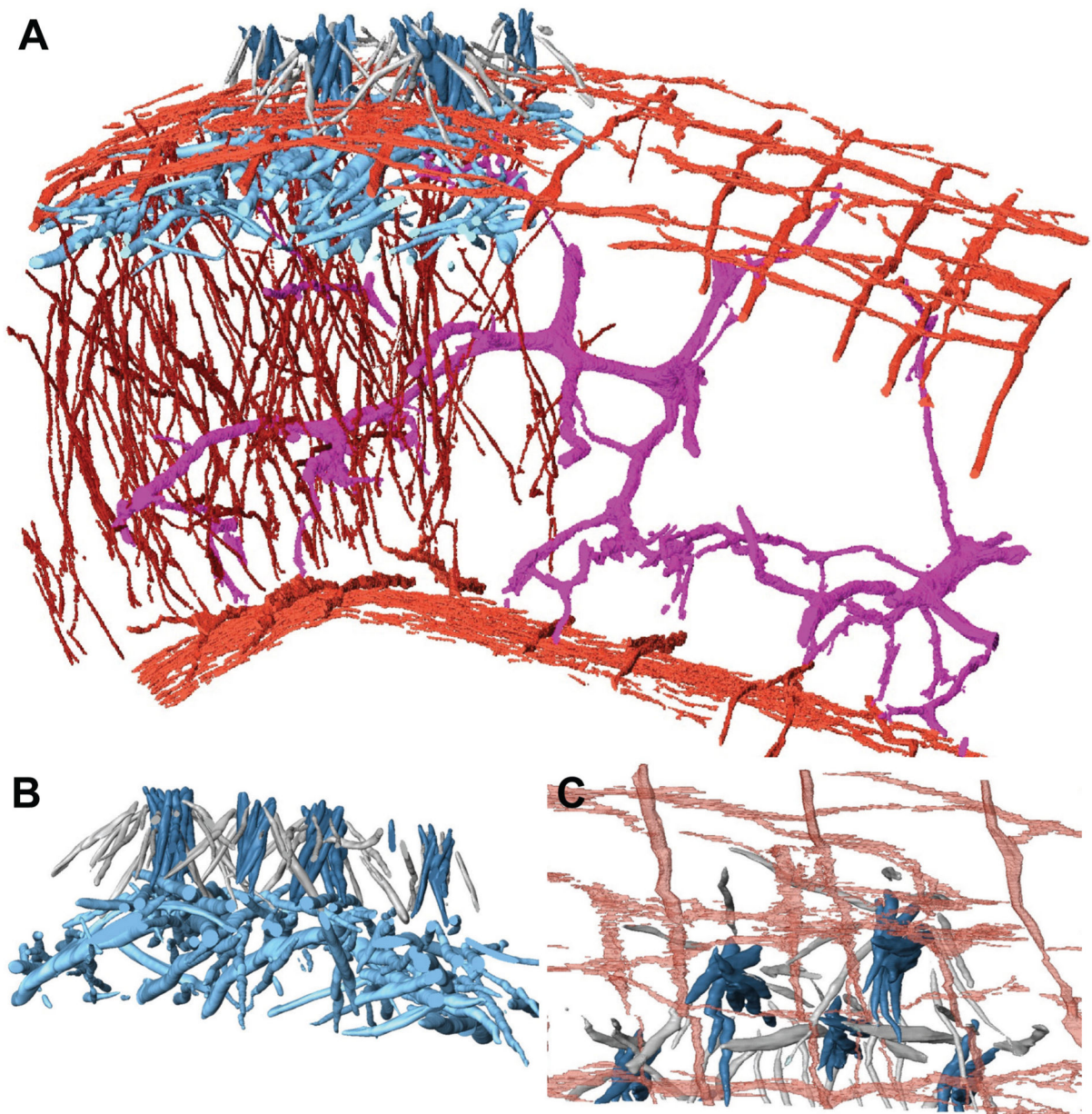


Fig. 2. Reconstruction of *Platydoris sanguinea* mantle edge tissue based on semithin slices. (A) Three-quarter view of tissue organization. Dorsal is to the top and anterior is to the right. Only a few caryophyllidia are shown and the dorsoventral muscle fibres are excluded on the right side of the panel to better show the ventral structures. (B) Side view of dorsal surface without musculature showing the relationship of the caryophyllidia to the dorsal sheet of underlying spicules. (C) Three-quarter view of dorsal surface from below showing the relationship between the caryophyllidia and the dorsal muscle grid, with spicules hidden and the muscles partly transparent. In all panels, the connective tissue–spicule network is translucent, dorsal and ventral musculature is light red, dorsoventral muscles are dark red,

nerve is magenta, caryophyllidia spicules are dark blue, radial spicules are light grey, and horizontal spicules are light blue.

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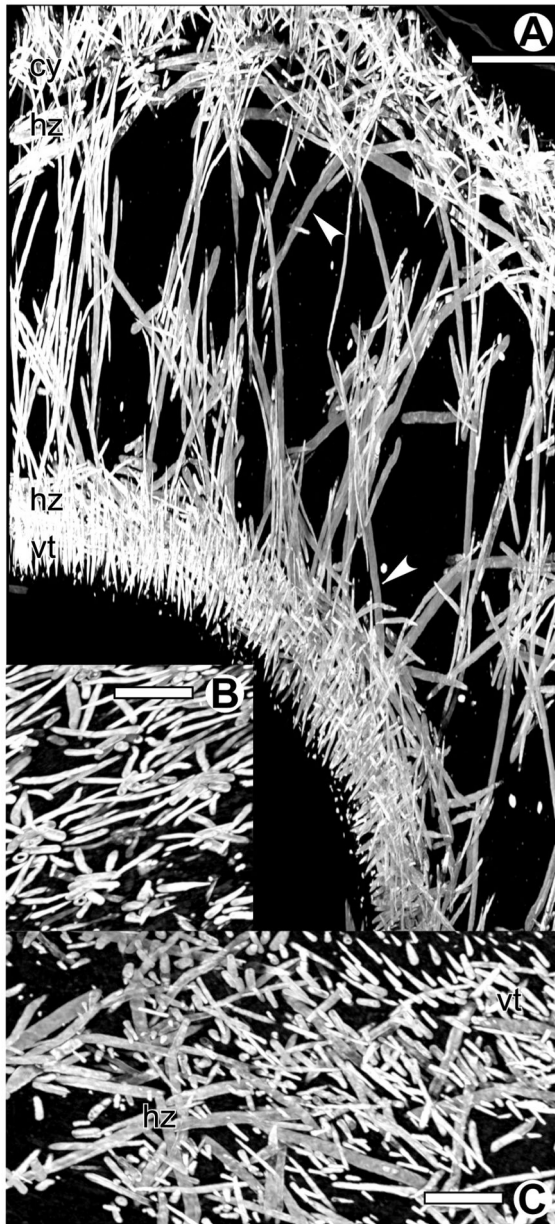


Fig. 3. Micro-CT of *Platydoris sanguinea* mantle spicule network: (A) virtual cross section 0.3 mm slab through network showing multiple layers, with vertical pillars of spicules and the angled support spicules (arrowheads), scale bar = 1 mm; (B) virtual frontal section 0.1 mm through mantle surface showing arrangement of caryophyllidia, scale bar = 0.5 mm; (C) virtual frontal section 0.1 mm through ventral mantle, scale bar = 0.5 mm. Abbreviations: cy, caryophyllidia; hz, horizontal sheet spicules; vt, vertical spicules.

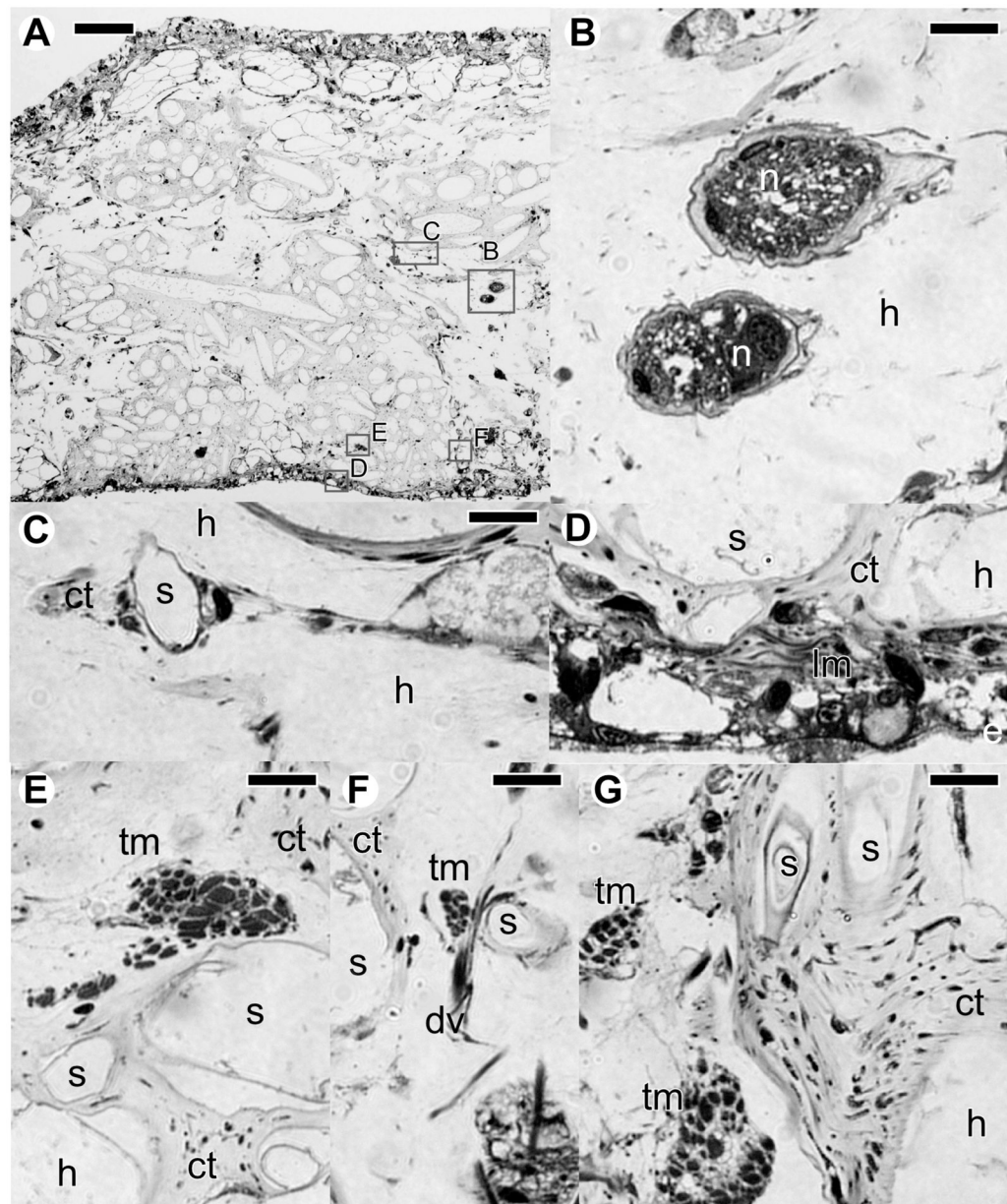


Fig. 4. Photomicrographs of *Cadlina luteomarginata* mantle in sagittal section. Dorsal is to the top of all images. (A) Overview. Letters indicate the location of detail panels, except G. Scale bar = 100 µm. (B) Detail of nerve plexus branches. Scale bar = 10 µm. (C–F) Details of muscle interaction with the spicule network and the overlying connective tissue sheath. Scale bar = 10 µm. (G) Detail of muscle interaction with connective tissue sheath surrounding a tubercle base. Note this image is from a different section than the other panels, scale bar = 10 µm. Abbreviations: ct, connective tissue; dv, dorsoventral muscle; e, epidermis; h, hemocoel; lm, longitudinal muscle; n, nerve; s, spicule; tm, transverse muscle.

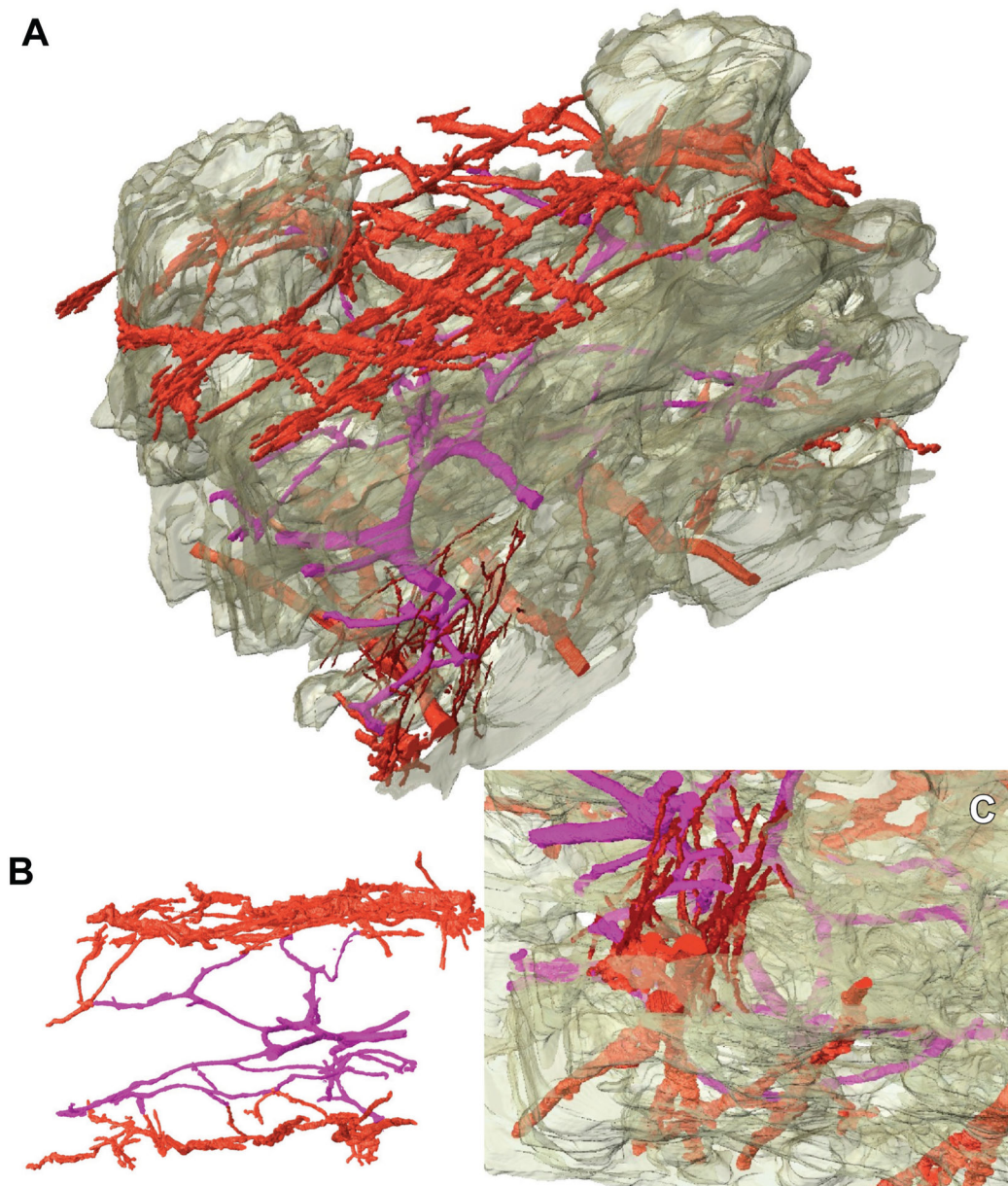


Fig. 5. Reconstruction of *Cadlina luteomarginata* mantle edge tissue based on semithin slices. (A) Three-quarter view of dorsal surface showing the dorsal musculature and interaction with connective tissue–spicule bundles. Dorsal is to the top and anterior is to the right. (B) Three-quarter dorsal view showing the relative position of dorsal muscle grid, nerve plexus, and ventral radial fibres, with the connective tissue omitted for clarity. (C) View inside a section of the trabecular network showing the relationship of the nerve plexus, ventral musculature, and dorsoventral muscle fibres to the connective tissue–spicule bundles. Only a small area of dorsoventral muscle fibres have been reconstructed for clarity. In all panels, the connective tissue–spicule network is translucent, dorsal and ventral musculature is light red, dorsoventral muscles are dark red, and nerve is magenta.

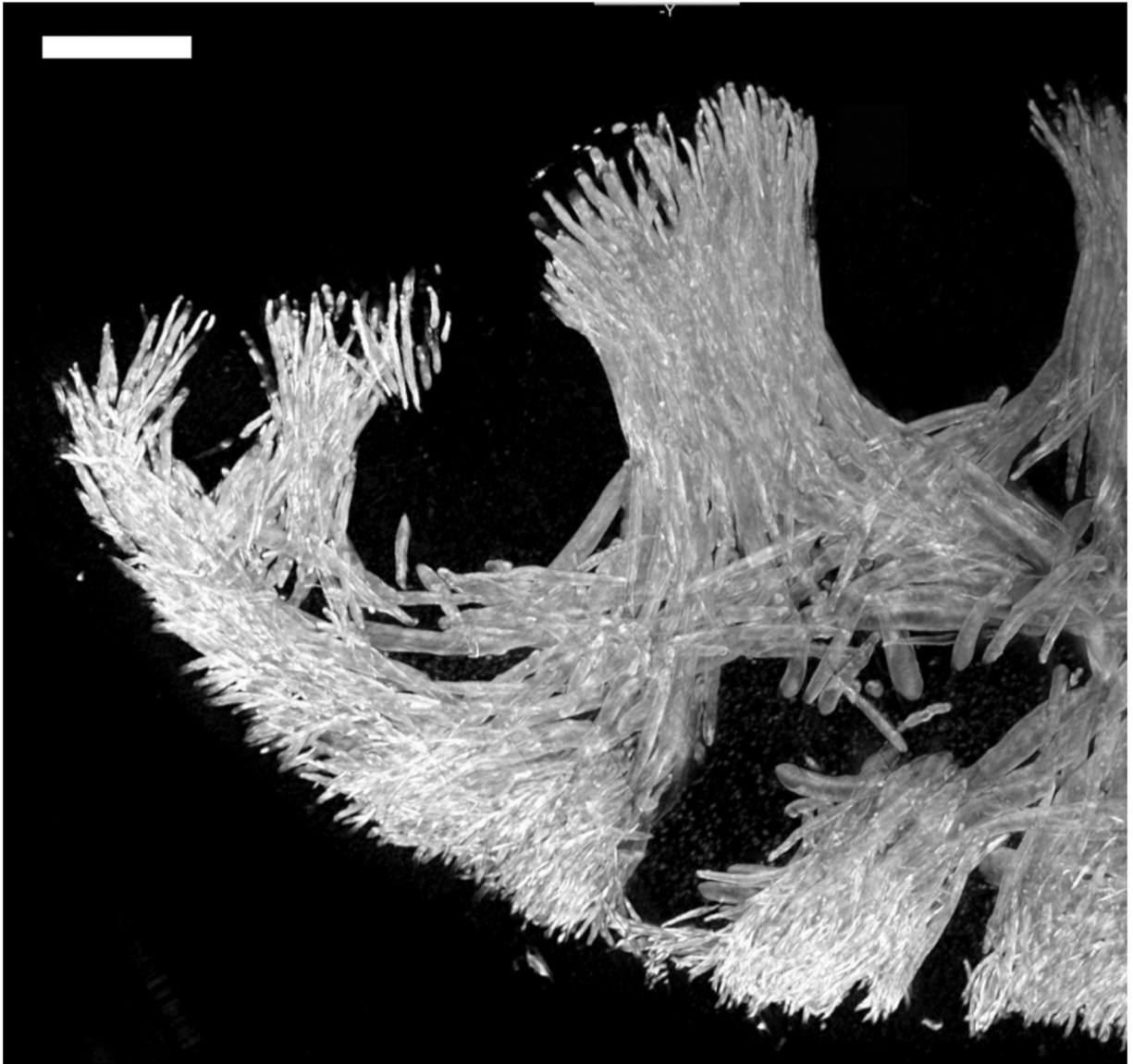


Fig. 6. Micro-CT of *Cadlina luteomarginata* mantle spicule network, virtual cross section 0.3 mm slab. Scale bar = 0.5 mm.

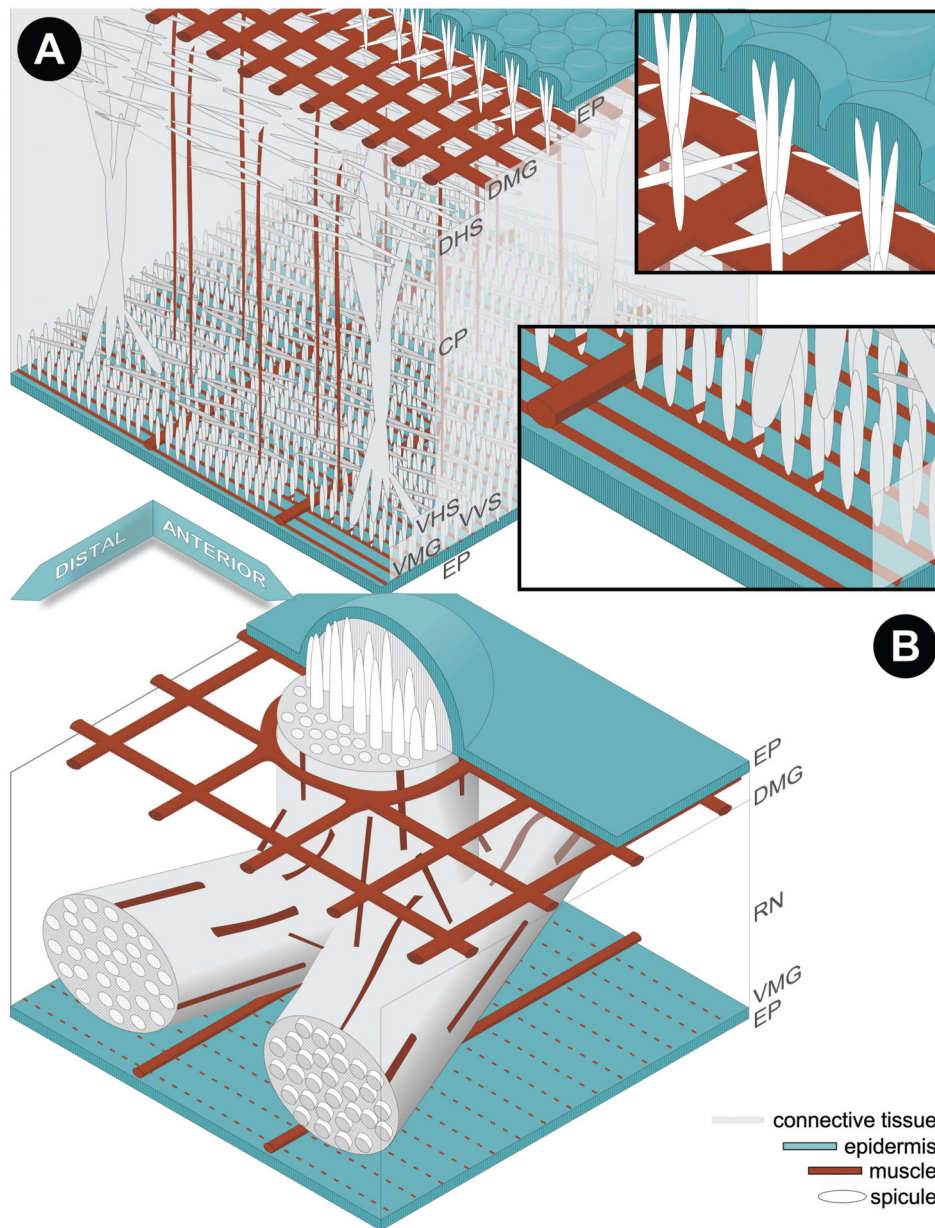


Fig. 7. Summary drawings of mantle architecture in isometric views. (A) *Platydoris sanguinea*, cut away to eight layers. Top inset: dorsal surface at 3× magnification showing caryophyllidia bases. Bottom inset: ventral surface at 3× magnification showing longitudinal muscles inserting into vertical spicule array. (B) *Cadlina luteomarginata*, cut away to five layers. Abbreviations: EP, epidermis; DMG, dorsal muscle grid; DHS, dorsal horizontal sheet; CP, central pillars; VHS, ventral horizontal sheet; VVS, ventral vertical spicules; VMG, ventral muscle grid; RN, reticulated network. Not drawn to scale.