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Body wall structure in the starfish *Asterias rubens*

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SUMMARY

The body wall of starfish is composed of magnesium calcite ossicles connected by collagenous tissue and muscles and it exhibits remarkable variability in stiffness, which is attributed to the mechanical mutability of the collagenous component. Using the common European starfish *Asterias rubens* as an experimental animal, here we have employed a variety of techniques to gain new insights into the structure of the starfish body wall. The structure and organization of muscular and collagenous components of the body wall were analysed using trichrome staining. The muscle system comprises interossicular muscles as well as muscle strands that connect ossicles with the circular muscle layer of the coelomic lining. The collagenous tissue surrounding the ossicle network contains collagen fibres that form loop-shaped straps that wrap around calcite struts near to the surface of ossicles. The 3D architecture of the calcareous endoskeleton was visualized for the first time using X-ray microtomography, revealing the shapes and interactions of different ossicle types. Furthermore, analysis of the anatomical organisation of the ossicles indicates how changes in body shape may be achieved by local contraction/relaxation of interossicular muscles. Scanning synchrotron small-angle X-ray diffraction (SAXD) scans of the starfish aboral body wall and ambulacrum were used to study the collagenous tissue component at the fibrillar level. Collagen fibrils in aboral body wall were found to exhibit variable degrees of alignment, with high levels of alignment probably corresponding to regions where collagenous tissue is under tension. Collagen fibrils in the ambulacrum had a uniformly low degree of orientation, attributed to macrocrimp of the fibrils and the presence of slanted as well as horizontal fibrils connecting antimeric ambulacral ossicles. Body wall collagen fibril D-period lengths were similar to previously reported mammalian D-periods, but were significantly different between the aboral and ambulacral samples. The overlap/D-period length ratio within fibrils was higher than reported for mammalian tissues. Collectively, the data reported here provides new insights into the anatomy of the body wall in *A. rubens* and a foundation for further studies investigating the structural basis of the mechanical properties of echinoderm body wall tissue composites.

INTRODUCTION

The phylum Echinodermata comprises five extant classes: the Asteroidea (starfish), Ophiuroidea (brittle stars), Echinoidea (sea urchins and sand dollars), Holothuroidea (sea cucumbers) and Crinoidea (sea lilies and featherstars) (Brusca et al., 2016). A characteristic feature of echinoderms is their endoskeleton, which comprises magnesium calcite ossicles or plates together with varying proportions of associated collagenous and muscular tissue. At one end of the spectrum are sea urchins where the body wall is comprised of fused calcite plates that form a rigid and inflexible endoskeleton (Birenheide et al., 1996, Takemae and Motokawa, 2005, Ribeiro et al., 2011, Ribeiro et al., 2012b, Motokawa and Fuchigami, 2015). At the other end of the spectrum are sea cucumbers, where the body wall is largely comprised of flexible collagenous tissue that contains only a scattering of tiny calcite spicules (Motokawa, 1984, Byrne, 2001). Intermediate compositions are found in asteroids, ophiuroids and crinoids, where the body wall is comprised of calcite ossicles or plates that are interlinked by muscles and collagenous ligaments, which confer skeletal flexibility (Motokawa, 1984, Birenheide and Motokawa, 1996, O'Neill, 1989, Eylers, 1976, Motokawa, 2011). Furthermore, there is variability between starfish species in the proportions of the calcite ossicles, muscle and collagenous tissue in the body wall (Heddle, 1967, Motokawa, 2011).

The first detailed investigation of the structure and mechanical properties of the starfish body wall was a study by Eylers (1976) on *Asterias forbesi* Desor, 1848 (Forcipulatida). The structure of the ray skeleton was investigated by analysis of preparations in which the majority of the soft tissue had been dissolved to reveal the architecture of the network of calcite ossicles. Different ossicle types were identified in different regions of the ray, including the ambulacral and adambulacral ossicles on the oral side and the marginal, reticular and carinal ossicles in the aboral body wall. Subsequently, O'Neill (1989) analysed the spatial organisation of ossicles in the aboral body wall of the starfish *Echinaster spinulosus* Verrill, 1869 (Spinulosida) and identified two ossicle types - oblate disks and ellipsoid bars, which form a reticular lattice of hexagonal rings.

91   The ultrastructure of ossicles in the starfish *Pisaster giganteus* Stimpson, 1857 (Forcipulatida)

92   has been investigated using powder X-ray diffraction, infrared spectroscopy, elemental analysis and

93   scanning electron microscopy (SEM) (Gayathri et al., 2007, Martina et al., 2005). This revealed that

94   starfish ossicles are composed of single crystals of macroporous magnesium-rich calcite (stereom),

95   similar to the mineralized skeletal elements of echinoids and some ophiuroids (Addadi et al., 2003,

96   Aizenberg et al., 2001, Long et al., 2014, Raz et al., 2000). X-ray microtomography (Micro-CT) has

97   also been employed to analyse the 3D structure of echinoderm endoskeletons, but the use of this

98   technique has largely focused on fossil specimens (Rahman et al., 2015, Dominguez et al., 2002,

99   Zamora et al., 2012) and only more recently have extant species been examined (Ziegler et al.,

100   2010, Ziegler et al., 2014).

101   The presence of muscles and connective (collagenous) tissue connecting, in parallel, adjacent

102   ossicles of the starfish body wall has been revealed by histochemical staining of thin sections

103   (O'Neill, 1989, Eylers, 1976, Smith, 1936, Hamann, 1885). O'Neill (1989) described the

104   collagenous component of the body wall as a "loosely woven, three-dimensional fabric in which

105   some of the fibres were 'felted' (frayed together)" and where interconnected fibres distribute load

106   across the tissue and prevent delamination under strain. Besides the load bearing collagenous tissue

107   component, O'Neill (1989) also described the interossicular (or "reticular") muscles and muscle

108   layers associated with the coelomic lining of the body wall.

109   Changes in posture of the starfish ray are mediated by the interossicular muscles (Heddle,

110   1967), whereas changes in the stiffness of the body wall are thought to be mediated by

111   interossicular "catch"-type collagenous tissue or mutable collagenous tissue (MCT) (Motokawa,

112   1984, Wilkie, 2005). MCT is a characteristic of all echinoderms, with a unique ability to change

113   stiffness rapidly under the control of the nervous system (Wilkie, 2002, Wilkie, 1996, Takemae and

114   Motokawa, 2005, Wilkie, 2005). The mechanisms of echinoderm MCT are not fully understood.

115   However, it has been proposed that the mutability is conferred not by changes in the mechanical

116   properties of the collagen fibrils but by changes in the stiffness of the interfibrillar matrix (Cluzel et

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117   al., 2001, Ribeiro et al., 2012a, Wilkie, 2005). Recently, this mechanism was demonstrated at the

118   nanoscale using sea cucumber dermis as an experimental system (Mo et al., 2016). Furthermore,

119   proteins that may mediate changes in interfibrillar stiffness have also been identified (Motokawa,

120   1984, Trotter et al., 1996, Trotter et al., 1999, Koob et al., 1999, Tipper et al., 2003, Tamori et al.,

121   2006, Yamada et al., 2010, Birenheide et al., 1998).

122   Hitherto, the techniques used to study the soft tissue phase (collagenous and muscle

123   components) of the starfish body wall have been predominantly histological (e.g. trichrome

124   staining) (Heddle, 1967, Eylers, 1976, O'Neill, 1989, Smith, 1936). However, scanning and

125   transmission electron microscopy (SEM and TEM) have also been used to analyse the ultrastructure

126   of fixed echinoderm collagen tissue or extracted fibrils (Trotter et al., 1994, Thurmond and Trotter,

127   1994, Barbaglio et al., 2015, Ribeiro et al., 2011). But fixation and extraction can induce changes in

128   the native structure of the collagen fibrils and therefore alternative methods without use of fixatives

129   are also needed. Microfocus scanning synchrotron small-angle X-ray diffraction (SAXD) is one

130   such technique that has proven to be a powerful and informative approach for analysis of

131   collagenous tissue structure at the nanoscale (Fratzl, 2008, Sasaki and Odajima, 1996a, Puxkandl et

132   al., 2002, Yang et al., 2015, Krauss et al., 2009). Collagenous tissues are amenable for SAXD

133   because collagen molecules are arranged in a highly ordered manner within fibrils, resulting in

134   alternating electron-dense and electron-light regions along the long axis of the fibrils. The

135   combined width of adjacent electron-dense and electron-light regions is known as the D-period,

136   which is ~65-67 nm in mammals, depending on factors such as tissue type and level of hydration

137   (Fratzl, 2008). When collagenous tissue is irradiated with X-rays, the two phase periodic

138   arrangement of electron densities leads to diffraction peaks in the meridional direction, enabling the

139   D-period and fibril orientation to be determined. As long as exposure times to X-rays are not too

140   long (from seconds down to milliseconds depending on the brilliance of the X-ray source), the

141   tissue can be examined in its native state (Jeffries et al., 2015). In this way, SAXD has been used

142   successfully to analyse the structural and mechanical properties of, for example, mammalian

143   tendons, bone and antlers (Karunaratne et al., 2016, Gupta et al., 2006, Zimmermann et al., 2011,

144   Yang et al., 2015). Furthermore, it was use of SAXD that recently enabled interfibrillar stiffening of

145   echinoderm MCT to be demonstrated at the nanoscale, using sea cucumber dermis as an

146   experimental system (Mo et al., 2016).

147   To obtain new insights into relationships between body wall structure and function in

148   starfish, here we have used histology, micro-CT and SAXD to perform a detailed analysis of the

149   structural properties of the body wall in the common European starfish *Asterias rubens* Linnaeus,

150   1758 (Forcipulatida).

MATERIALS AND METHODS

Animals

Starfish (*A. rubens*) used in this study were collected at low tide from Foreness Point near Margate in Kent. Animals were transported in seawater to Queen Mary University of London where they were then maintained in an aquarium with circulating artificial seawater at ~11°C, corresponding to the sea water temperature for this species naturally. Specimens ranging in diameter from 3 to 7 cm were used for studies reported in this paper. Animals were fed periodically with the mussel *Mytilus edulis* Linnaeus, 1758 (Mytiloida).

Masson's trichrome staining

To enable analysis of the histological composition of the body wall in *A. rubens*, Masson's trichrome staining was used, which differentiates collagenous (blue) from non-collagenous (red) tissues. Intact animals or severed rays were fixed for 24 h in Bouin's fixative, which comprised 75 ml of saturated picric acid in seawater, 25 ml 40% formaldehyde solution and 5 ml glacial acetic acid. Following fixation, specimens were decalcified at 4°C for 2-5 days in a freshly prepared solution of 1% ascorbic acid in 0.15 M sodium chloride. Prior to fixation, some specimens were narcotized in seawater containing 3.5% added $MgCl_2$, which causes muscle relaxation (Mayer, 1909). This was done to reduce the tissue damage caused during fixation and tissue processing that was observed in untreated specimens. Following fixation and decalcification, starfish rays were dehydrated through an ethanol series, immersed in xylene and then embedded in paraffin wax. Serial transverse sections of rays were prepared using a Leica RM2145 microtome and mounted on chrome alum gelatin coated slides. Following dewaxing in xylene and rehydration, slides were stained using the Masson's trichrome method. Sections were rehydrated and then placed in Weigert's heamatoxylin solution for 10 min and then washed in running tap water for 10 min at room temperature. After rinsing with distilled water, slides were placed in Biebrich scarlet-acid

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177 fuchsin solution for 15 min and then washed in distilled water. Differentiation of staining was

178 achieved by placing the slides in phosphomolybdic-phosphotungstic acid solution until collagenous

179 tissue no longer appeared red (normally 10-15 min). Slides were then transferred into aniline blue

180 solution for 5-10 min, followed by washes (3 x 1 min) in distilled water and then a 1-5 min

181 differentiation step in 1% acetic acid. After washing in distilled water, slides were quickly

182 dehydrated in 95% (1 x 10 sec) and 100% ethanol (3 x 10 sec), cleared in xylene and then mounted

183 using DPX, a resin-based mounting medium (Thermo Fisher Scientific Inc.).

184

185 **X-ray microtomography**

186 Micro-CT was used to enable analysis of the structure and organization of the complex

187 network of calcareous ossicles that form the endoskeleton of the body wall in *A. rubens*. Starfish

188 were fixed (overnight at 4°C) with a 4% solution of paraformaldehyde in artificial seawater and

189 then washed in phosphate buffered saline (PBS; pH 7.4). The rays were cut off and embedded in

190 4% low melting point agarose dissolved in PBS, contained within 5 ml polystyrene tubes, which

191 were then stored at 4°C. The complete tubes were scanned on the custom designed MuCAT 2

192 micro-CT scanner at Queen Mary University of London. This uses a high dynamic range CCD

193 camera in time-delay integration mode to achieve accurate, high contrast tomographic images

194 (Davis et al., 2013). The accelerating voltage was set to 40 kV with a tube current of 405 μA. The

195 voxel size was set to 15 μm; 1201 projections were recorded in 19 hours (the long scan time is

196 required to obtain a high contrast ratio and two complete scans were required to cover the ~20 mm

197 length of the rays). Immediately following the scan, a multi-element calibration carousel (Evershed

198 et al., 2012) was scanned in order to characterise the X-ray spectrum. Since direct measurement of

199 the X-ray spectrum is almost impossible (with the degree of accuracy required) a modeled estimate

200 of the spectrum is made. Attenuation measurements from the carousel are used to optimize the

201 model to create a good working representation of the spectrum. With *a priori* knowledge of at least

202 the most absorbing components of the specimen, a correction curve can be created that maps the

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203 projections derived from polychromatic radiation to an estimate of those that would be derived from

204 monochromatic radiation. This both corrects for beam-hardening errors and allows measurements

205 of mineral concentration to be made from the reconstructed image. While high magnesium calcite is

206 likely the appropriate mineral in the case of starfish ossicles (Gayathri et al., 2007), as we did not

207 know the exact chemical composition and were not interested in absolute mineral concentration

208 measurements, hydroxyapatite mineral was used to create the correction curve. Reconstructed

209 volume images were viewed from different orientations using in-house "Tomview" software and

210 rendered using the Drishti volume rendering package to produce 3D views (Limaye, 2012).

211

212 ***In situ* synchrotron small-angle X-ray diffraction**

213 ***SAXD experiments on starfish aboral body wall and ambulacrum strips***

214 Experiments were performed using the beamline I22 synchrotron light source at Diamond

215 Light Source (DLS; Oxfordshire, UK). Live specimens of *A. rubens* were transported in a portable

216 tank to DLS, where they were maintained in cool seawater until immediately prior to dissection of

217 body wall samples from two individuals. Strips of the midline aboral body wall of rays were

218 prepared using a pair of parallel-blades that were separated by a 6 mm gap. Strips of the

219 ambulacrum on the oral side of rays were prepared by cutting between the rows of ambulacral and

220 adambulacral ossicles on both sides of the ray (see Figs. 1 and 2). The SAXS scans of the

221 ambulacrum and aboral body wall were carried out at different times separated by wide-angle X-ray

222 diffraction measurements on other samples at the same beamline.

223 For static scanning SAXD tests, the strips were placed in a 50 mm × 50 mm plastic clamp,

224 with a hole of 35 mm × 23mm in the centre. The hole was covered with Ultralene film (Spex

225 SamplePrep, Metuchen, NJ, USA), which has minimal X-ray scattering in the small-angle regime.

226 Artificial seawater (ASW, prepared according to the manufacturer's instructions using Tropic

227 Marin® Sea Salt) was added in between the two layers of film where the sample was held in order

228 to provide a physiological environment for the samples. The edges of the body wall samples were

229 marked with X-ray absorbent lead tape to aid detection of the sample centre when scanning with the

230 X-ray beam.

231   The sample holder was clamped on a 2-axis motorized stage and a synchrotron X-ray beam

232 with a wavelength of 0.8857Å and beam diameter 15 μm was used to acquire the SAXS spectra.

233 Spectra were collected using a silicon hybrid pixel PILATUS 2M detector system (Kraft, 2010).

234 The sample-detector distance was 1008.2 mm for aboral body wall samples and 847.2 mm for

235 ambulacral samples, calculated based on a measurement of a calibration standard consisting of

236 silver behenate. The detector had a 2D resolution of 1475 × 1678 pixels with pixel dimensions of

237 172×172 μm^2.

238   The centre of the sample on the plastic frame was first located by carrying out a 2D X-ray

239 absorption (radiography) scan of the sample across the X-ray beam with a photodiode placed

240 downstream of the sample. The strong X-ray absorption by the lead markers enabled location of

241 regions of interest on the specimen. 2D SAXD area-scans, with 40 and 100 μm x- and y-step

242 distances used for ambulacral and aboral body wall, respectively, were acquired in the regions of

243 interest using software for customized control of motorized stage movement and detector SAXD

244 pattern acquisition, available at the beamline via the GDA open source framework

245 (http://www.opengda.org/). Different spatial resolutions were used for the two body regions

246 because of their intrinsically different size. To avoid radiation damage by repeated exposure of the

247 same tissue location to the beam, the 2-axis motorized stage was translated by an offset of 30 μm

248 (~2 × beam diameter) in the x-direction between carrying out the 2D diode and SAXS scan grids.

249

250 ***SAXD data analysis***

251 *Identification of diffraction peaks arising from collagen D-period*

252   2D SAXD spectra were first corrected for air and background scattering by the empty cell,

253 using photodiode measurements of the transmission of the sample and the empty cell, as is standard

254 practice in SAXD data acquisition (Pabisch et al., 2013). The corrected 2D spectra were initially

255 averaged azimuthally across a 0-360° sector (with a wavevector range from 0.2 to 0.9 nm^{-1}) to

256 identify points in the 2D scan where SAXD peaks arising from collagen are present (subsequent

257 more detailed analysis of fibril orientation, D-period and peak intensity is described below). The

258 Bragg peaks arising from the 65-68 nm axial D-period of the collagen fibrils were identified

259 qualitatively from expected positions in the spectrum, with the n = 5th order (at q ~ 0.46 nm^{-1})

260 being the clearest peak.

261

262 *Determination of collagen fibril orientation*

263   To determine collagen fibril orientation in body wall samples, the angular distribution of the

264 SAXD intensity of the 5th order reflection $I_5(\chi)$ was used. $I_5(\chi)$ was calculated by averaging the

265 intensity radially in a narrow band around the peak position of the 5th order reflection, i.e. over the

266 wavevector range 0.45 – 0.55 nm^{-1}. The intensity profile was calculated for the full angular range in

267 χ from 0 to 360°. To correct for diffuse background scattering, the average of the intensity in two

268 narrow rings (just outside the ring of 0.45-0.55 nm^{-1} containing the n = 5 reflection) was subtracted

269 from the ring centred on the peak position, following the same procedure as described for bone

270 collagen in Karunaratne et al. (2016). The corrected intensity $I_5(\chi)$ exhibited two peaks separated by

271 180° and hence was fitted to the sum of two Gaussians separated by 180° using the Gnuplot

272 graphics and fitting program (www.gnuplot.info). Peak amplitude, peak width ($\Delta\chi_0$) and peak

273 position (χ_0) were determined from the fit. χ_0 determines mean fibril orientation and the reciprocal

274 of peak width $\left(\frac{1}{\Delta\chi_0}\right)$ was used as a measure of the degree of fibrillar orientation around the mean

275 fibril orientation. Lower values of $\left(\frac{1}{\Delta\chi_0}\right)$ imply a more random dispersion of fibril directions

276 whereas higher values imply a more aligned arrangement.

277   Following identification of fibril direction, the D-period for each 2D pattern was calculated

278 from azimuthally averaged intensity profiles (denoted I_q, with q the radial wavevector; the total

279 intensity of a particular peak order (the integrated intensity under the Bragg peak) will be denoted

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280 with a subscript, for example the 5th order peak intensity will be denoted as I_{q5}). Specifically, the 2D

281 spectra were azimuthally averaged again in a pie-shaped sector centred at the mean fibril

282 orientation, and covering 20° on either sides of χ_0. This procedure ensures that the signal is not

283 attenuated by including angular sectors, which are far from the principal fibril directions and have

284 no scattering intensity from the collagen meridional peaks. The most clearly visible orders were the

285 fifth (n = 5, I_{q5}) and eighth (n = 8, I_{q8}), with the fifth order having the strongest intensity.

286

287 ***Determination of fibril D-period and peak intensity***

288 As the fifth order Bragg peak from the collagen D-period was the most prominent, it was

289 selected for fitting a Gaussian peak function with a linear baseline (background) term for each

290 SAXD spectrum. The peak position q_5, peak amplitude A_5, and peak width-at-half-maximum w_{mq5}

291 were determined from the fits, as reported previously in (Mo et al., 2016). The meridional peak

292 width is influenced by instrumental broadening of the peak due to the finite resolution of the

293 detector. The instrumental broadening of the peak can be calculated from diffraction patterns of

294 collagen fibrils with highly crystalline organisation, such as in dried rat tail tendon or chicken

295 collagen (Krauss et al., 2009). The corrected full-width-at-half-maximum (FWHM) was calculated,

296 as described in Krauss et al. (2009), according to the formula $w_{q5} = \sqrt{w_{mq5}^2 - w_{crys}^2}$ where w_{q5} is

297 the corrected FWHM, w_{mq5} is the measured FWHM of the sample of interest and w_{crys} is the FWHM

298 of the sample with near crystalline organisation (here chicken collagen was used). The peak width

299 w_{q5} represents the variability of q_5 at each scan point. As the D-period length is calculated using the

300 formula $D = 5 \times \frac{2\pi}{q5}$ (as calculated similarly for the third order meridional reflection in bone

301 collagen: e.g. (Karunaratne et al., 2012)), w_{q5} is also a measure of the variability of D-period length

302 at any scan point. The range of D-values at each scan point can therefore be derived from the above

303 after determining the maximum and minimum q values $q_5 \pm \frac{1}{2}w_{q5}$, respectively (the use of the half-

304 width as representing maximum and minimum is a convention choice). The azimuthal average of

305   the intensity at the peak position (I_{q5} and I_{q8} referring to the intensities of the 5th and 8th order peaks

306   respectively) contains contributions from both the collagen phase as well as from all the other

307   components of the tissue. The diffraction peak intensity is from collagen (I_{q5col}) and the background

308   scattering is contributed to by the mineral (I_{q5min}) components of the tissue. Therefore to calculate

309   I_{q5col}, the background scattering (I_{q5min}) was approximated as a linear fall-off over the small q-range

310   around the peak and subtracted from I_{q5} to get I_{q5col}. The linear background scattering was

311   determined by calculating a linear regression over a combination of several points to the left and

312   right of the peak.

313   As not all scan points were on collagen-containing regions, a filtering process was used to

314   identify SAXS patterns with sufficient collagen for analysis, based on the multi-step procedure

315   outlined above. Points with little scattering intensity are expected to give artifactual results for

316   fitting, such as very small or negative peak width, low peak amplitude or very large errors in the fit.

317   After visual inspection of the Bragg peaks of some selected patterns, integrated profiles and fit

318   results, only scan points satisfying the following empirical criteria were determined to have

319   substantial amounts of collagen and were included in the data analysis: i) positive $\Delta\chi_0$, ii) $w_{q5} >$

320   0.0085 nm^{-1} (very noisy and low intensity $I(q)$ tended to have very small $w_{q5} < 0.0001$ nm^{-1}) and iii)

321   sum of errors (the sum of % error of the peak fit parameters q_{05}, A_5 and w_{q5}) < 17 %. This

322   corresponded to ~22% of all the scan points.

323

324   ***Determination of the overlap : D-period ratio in collagen fibrils***

325   Structural information on the intrafibrillar arrangement of tropocollagen molecules can be

326   obtained from the differences in peak intensities between the different orders of the SAXD

327   spectrum. Specifically, assuming the gap zone inside the fibril to have a length G and the overlap

328   zone to have a length O and a uniform electron density in each zone, with $O + G = D$, then it can be

329   shown (Sasaki and Odajima, 1996a) that the ratio of the peak intensities of the n_1^{th} peak to that of

330   the n_2^{th} peak is:

331 $$\frac{I_{n_1}}{I_{n_2}} = \left(\frac{n_2}{n_1}\right)^2 \left(\frac{\sin\left(\pi n_1 \frac{O}{D}\right)}{\sin\left(\pi n_2 \frac{O}{D}\right)}\right)$$

332 The two most prominent peaks in the radial profile I_q were the 8th and 5th order. Hence, from the

333 ratio of the intensities of the $n_1 = 8^{th}$ order peak to the $n_2 = 5^{th}$ order peak (I_{q8}/I_{q5}) the ratio O/D was

334 determined by numerically solving the above equation for O/D, given an experimentally measured

335 intensity ratio. It is noted that the assumption of a constant electron density is likely not strictly

336 accurate, as has been found in vertebrate collagen types (Antipova and Orgel, 2010), but in the

337 absence of a detailed information on the amino acid sequences and structure of collagen in starfish

338 body wall, we use it as an approximation.

RESULTS

To facilitate interpretation of data obtained using histological staining, micro-CT and SAXD (see below), figure 1 shows the anatomy of a dissected specimen of *A. rubens*.

Histology of the body wall of *Asterias rubens*

Figure 2 shows a trichrome stained section of a decalcified ray from *A. rubens*, with the calcite ossicles that are present *in vivo* appearing as voids because of the decalcification process. Here we focus our attention on the structure and organization of collagenous (blue) and non-collagenous (red) tissues that surround and interlink the ossicles, which are shown at higher magnification in Fig. 3.

Adjacent ossicles in the body wall are interlinked by muscles and the sizes and shapes of these muscles vary between ossicle types. Examples of the small muscles that link ossicles in the aboral body wall are shown in Fig. 3, which shows interossicular muscles linking adjacent reticular ossicles (Fig. 3A, B). What these images also show is that muscle fibres of the interossicular muscles appear to be wrapped around voids that would be filled by calcite struts near the ossicle surface *in vivo*. In close association with the interossicular muscle is the collagenous tissue that surrounds and also interconnects adjacent ossicles. The collagenous tissue occupies the bulk of the space between ossicles and surrounding ossicles, with fibres forming a dense latticework (Fig. 3A, B). It can clearly be seen that the collagen fibres form loop-shaped straps that wrap around the voids that would be filled by calcite struts near the ossicle surface *in vivo* (Fig. 3C). Indeed the entire surface of each ossicle is penetrated by collagen fibres that project into the ossicle.

The coelomic epithelium of the aboral body wall is underlain by two muscle layers. Firstly, a layer of longitudinally orientated muscle, which is thickened along the midline to form what is known as the apical muscle. Secondly, a layer of circularly oriented muscle, from which are derived muscle strands that insert onto the inner surface of body wall ossicles (Fig. 3C).

365   The largest interossicular body wall muscles are the antagonistic pairs of muscles that link

366   adjacent antimeric ambulacral ossicles (abductor supra- and adductor infra-ambulacral muscles) and

367   that link ambulacral and adambulacral ossicles (inner and outer lateral transverse muscles) (Figs. 2,

368   3D), which participate in enabling changes in ray posture (Heddle, 1967). The collagen fibres

369   connecting the antimeric and longitudinally adjacent ambulacral ossicles are oriented

370   predominantly horizontally (Fig. 3D), but slanted fibres connecting longitudinally overlapping

371   ossicles between and within each side of the ambulacrum can also be observed in Fig. 3E. Crimping

372   of collagen fibres in the interambulacral ligament located above the adductor infra-ambulacral

373   muscle can be seen in Fig 3F.

374

375   **X-ray microtomography reveals the 3D architecture of the starfish ray skeleton**

376   Based on data obtained using micro-CT to analyze the ray skeleton of *A*. *rubens*, here we

377   present images that show for the first time the 3D architecture of the starfish skeleton in

378   unprecedented detail (Fig. 4). A detailed description of the images shown in Fig. 4 is presented in

379   the associated figure legend and therefore here we will focus on a few key observations. What the

380   images show most strikingly is the variety of shapes of the different ossicle types and the geometry

381   of their interactions. Thus, the ambulacral ossicles are elongated, flattened and tightly packed with

382   their aboral "head" region orientated over the adjacent proximal ambulacral ossicle (Fig. 4D). These

383   contrast with the more cuboid-shaped adambulacral ossicles that provide basal support for the

384   ambulacral ossicles (Fig. 4B). The marginal ossicles that form the lateral body wall region are more

385   varied in shape and size but are quite tightly packed. Larger irregular shaped marginal ossicles form

386   longitudinal overlapping rows of ossicles, which are connected radially by overlapping smaller,

387   lozenge-shaped ossicles (Figs. 4B and D). The reticular ossicles that form the aboral skeleton have a

388   similar variety of ossicle shapes and sizes but these form a more open skeletal meshwork than the

389   marginal ossicles (Fig. 4C). Lastly, the carinal ossicles form a single row of overlapping ossicles

390   along the midline of the aboral ray skeleton, arranged like a fallen line of dominos toppled from the

391   tip-end of the ray (Figs. 4 A and B).

392   Another feature that can be observed from the images shown in Fig. 4 are the shapes and

393   sizes of what appear as unfilled gaps where the X-ray attenuation is below the threshold set for the

394   "hard tissue" image rendering, and which is indistinguishable from the surrounding medium but

395   would be filled with tissue *in vivo*. For example, linking the "head" regions of the ambulacral

396   ossicles are several muscles (Fig. 2) and the size and shapes of the spaces occupied by these

397   muscles can be observed in Fig. 4D. Voids that would be filled by a narrow fluid-filled tube linking

398   the tube foot podium and ampulla can also be seen in Fig 4D, where they form two rows on each

399   side of the ambulacrum in an alternating manner with a void proximal or distal to the head region of

400   each pair of adjacent ambulacral ossicles. At the other end of the ambulacral ossicles, it can be seen

401   that their bases insert in a groove formed by adjacent adambulacral ossicles (Figs. 4B-D), i.e. the

402   ambulacral ossicles are not arranged directly above the adambulacral ossicles but are staggered so

403   that these two interacting ossicle types alternate with respect to each other along the length of the

404   ray.

405

406   **X-ray transmission maps of aboral body wall and the ambulacrum reveal the positions of**

407   **ossicles and spines**

408   In order to interpret SAXD data with respect to anatomical organisation of the starfish body

409   wall, X-ray transmission maps were first obtained. Scanning X-ray transmission images of the

410   aboral body wall and ambulacrum revealed their inhomogeneous structure, with regional variation

411   in X-ray transmission and hence conversely X-ray absorption (Figs. 5A1, 5A2; the longitudinal axis

412   of the starfish ray is indicated by an arrow in these figures). Regions with high X-ray absorption

413   correspond with the location of the mineralized ossicles and associated appendages (e.g. spines, * in

414   Fig. 5A1). Thus, the lattice-like structure of the aboral endoskeleton that can be seen with micro-CT

415   (Figs. 4A and 4B) is also revealed in X-ray transmission maps (Fig. 5A1). In Fig. 5A1 the outline of

17

416   the row of carinal ossicles is indicated with dashed lines and it can be seen that the regions of

417   highest X-ray absorption (blue in Fig. 5A1) in the aboral body wall correspond with the positions of

418   spines located on the outer surface of the body wall. In the ambulacrum, the overall level of X-ray

419   absorption is much higher than in the aboral body wall, consistent with the presence of the regular

420   and densely packed rows of pairs of long ladle-shaped ambulacral ossicles (Fig. 5A2, ambulacral

421   ossicle head, AMOh). However, regions of lower X-ray absorption are evident and these assist in

422   identifying boundaries between adjacent ossicles. The regions of lowest X-ray absorption are along

423   the mid-line of the ambulacrum, which is where the heads of the ambulacral ossicles align and are

424   interlinked by muscles and collagenous tissue (Fig. 5A2).

425

426   **SAXD reveals the inhomogeneous distribution of collagen in the starfish body wall**

427   The amount of collagen present in tissue is proportional to the intensity of the 5th order

428   Bragg peak with the diffuse background scattering (I_{q5min}) subtracted from it (I_{q5col}, see methods). By

429   mapping the I_{q5col} across the body wall samples (Fig 5B1 and 5B2), the areas with the highest

430   concentrations of collagen (red) can be seen. Note, however, that the intensities shown in Fig 5B1

431   and 5B2 are in arbitrary units and intensities cannot be directly compared between the aboral body

432   wall (Fig. 5B1) and the ambulacral (Fig. 5B2) samples because signal intensity is affected by

433   sample thickness. The large ovoid regions without distinguishable collagen diffraction spectra

434   correspond to voids in aboral body wall skeleton and collagenous tissue that are overlain on the

435   body wall surface by papulae (thin-walled finger-like protrusions that mediate gas exchange in

436   starfish) and pedicellariae (pincer-like organs that remove debris and encrusting organisms from the

437   body wall surface), as seen Fig. 1D. In the ambulacrum, collagen spectra are detectable at scan

438   points along the midline between the antimeric ambulacral ossicles (arrow heads in Fig. 4B1),

439   which corresponds with the position of interossicular ligaments that link the ambulacral ossicles

440   (Figs. 3D, 3E and 3F).

441   A map of the ratio of diffraction intensity from collagen to the mineral scattering intensity

442   (I_{q5col}/ I_{q5min} Fig. 5C1 and 5C2) shows that the background scattering from the ossicles is

443   proportionally higher in the ambulacrum compared to the aboral body wall (note the different

444   scales). This can be attributed to thicker ossicles in the ambulacrum and/or proportionally higher

445   amounts of collagen in the aboral body wall.

446

447   **SAXD reveals collagen fibril orientation and differences in the degree of fibril orientation in**

448   **starfish aboral body wall and ambulacrum**

449   Measurement of the azimuthal intensity distribution of the 5th order reflection $I_5(\chi)$ enabled

450   determination of collagen fibril orientation in body wall samples. Thus, Figs. 5D1 and 5D2 show

451   vector plots overlaid on grayscale X-ray transmission maps, with the orientation of vectors

452   representing mean collagen fibril orientation. In the aboral body wall the carinal ossicles are

453   surrounded by fibrils aligned with the longitudinal axis of the starfish ray (black arrow). Fibrils

454   adjacent to the reticular ossicles are orientated towards the voids in the ossicular lattice (white

455   arrows), perpendicular to the ossicle surface.

456   In addition to fibril orientation, the extent to which fibrils are parallel (called degree of

457   orientation here) can be determined from the inverse of the width of the azimuthal integrated

458   intensity profile $\left(\frac{1}{\Delta\chi_0}\right)$. Azimuthally wide diffraction peaks (large $\Delta\chi_0 > 10°$) indicate high variation

459   in the angular orientation of fibres. The scaled (from 0-1, normalised using a standard minimum and

460   maximum for all the samples) mean degree of orientation for aboral body wall samples (0.13, SD

461   0.20) was twice that of ambulacrum samples (0.06, SD 0.03). These correspond to azimuthal

462   diffraction peaks with mean FWHM of ~17° and ~35°, respectively. The degree of variation at

463   different scan points is illustrated by the lengths of the vectors in Figs. 5D and 5D'. The longer the

464   vector, the more parallel are the fibres at each scan point. The vector length varies more in the

465   aboral body wall (the standard deviation of the vector length in the aboral samples is nearly 7 times

466   that of ambulacral samples), i.e. the variation in degree of orientation was higher in the aboral body

467   wall samples than in the ambulacrum.

468

469   **D-period length and Overlap/D ratio of collagen fibrils differ between starfish aboral body**

470   **wall and ambulacrum**

471   Collagen fibril D-period lengths (see Fig. 6A, which shows a diagram of collagen fibril

472   organization) calculated using the fifth order meridional Bragg peak position q_5, for each scanning

473   point with collagen intensity above the threshold revealed a significant difference in D-period

474   length between the aboral body wall and ambulacrum (P<0.001; two tailed t-test assuming equal

475   variance) (Fig. 6B, D-period distribution). The mean D-period for scanning points in aboral body

476   wall was 65.27 nm (SD 0.60, the combined number of scan points from the two aboral body wall

477   samples included in the analysis; n=504) and in the ambulacrum was 66.95nm (SD 0.62, n=269).

478   The D-period length was also more variable within each scan point in the ambulacral groove than in

479   the aboral body wall. The range of D-values for each scan point was calculated as described in the

480   methods ($w_{m(aboral)}$ = 0.012 nm^{-1}, $w_{m(ambulacrum)}$ = 0.015 nm^{-1}, $w_{crys(chicken)}$ = 0.011 nm^{-1}) and the mean

481   ranges were D ± 0.72 nm for ambulacrum and D ± 0.36 nm for the aboral body wall, again a

482   possible indication of X-rays penetrating through multiple collagenous layers in the ambulacrum

483   with differential D-period lengths.

484   The O/D overlap ratios calculated using the ratio of I_8/I_5 (by numerically solving the

485   following equation for O/D $\frac{I_8}{I_5} = \left(\frac{5}{8}\right)^2 \left(\frac{sin\left(\pi 8\frac{O}{D}\right)}{sin\left(\pi 5\frac{O}{D}\right)}\right)$) were also significantly different between the two

486   regions of the body wall (P<0.001; two tailed t-test assuming equal variance; Fig. 6C, O/D

487   distribution). One limitation with utilizing the step function and experimentally measured intensity

488   ratios to estimate O/D is the lack of supporting structural data on echinoderm collagens. Therefore,

489   when using the above mentioned equation to solve O/D it was assumed that electron densities

490   within overlap and gap regions were constant and that the starfish collagen O/D ratio would be

491 between 0.4-0.5, i.e. similar to reported O/D values for mammalian collagen fibrils (Sasaki and

492 Odajima, 1996a), as to the best of our knowledge there have been no reported studies of

493 echinoderm collagen fibril organisation being radically different from mammalian fibrils (see

494 discussion for more details). The O/D ratio was lower in the aboral body wall (0.468, SD 0.015)

495 than in the ambulacrum samples (0.479, SD 0.009). If the differences in D-period length and O/D

496 ratio are both taken into account, the average lengths of overlap and gap regions in the aboral body

497 wall are 30.55 and 34.72 nm, respectively ($O + G = D$, therefore $O = 0.468{\times}D$ and $G =$

498 $(1 - O){\times}D$). Average O and G lengths for the ambulacrum were 32.07 and 34.88 nm, respectively,

499 showing that with a longer D-period the length of the overlap region increases whilst the gap length

500 remains more or less constant.

501

DISCUSSION

We report here a detailed analysis of body wall structure in the starfish *A. rubens*, employing histological, micro-CT and SAXD techniques. Use of these complementary approaches has provided new insights into the functional anatomy of the starfish body wall, as discussed below.

Trichrome staining reveals the anatomy of body wall interossicular muscles and ligaments in *A. rubens*

Trichrome staining of sectioned starfish has been employed previously for analysis of the structure and composition of the body wall, revealing that it is a composite of calcite ossicles linked by interossicular muscles and ligaments (O'Neill, 1989, Motokawa, 2011, Wilkie, 2001, Wilkie et al., 1995, Ben Khadra et al., 2015). The application of this histological staining technique to the common European starfish *A. rubens* adds to these body of data. In particular, high magnification photographs show how interossicular ligaments and muscles insert onto adjacent ossicles, with collagen fibrils and muscle fibres, respectively, wrapping around struts near to the ossicle surface (Fig. 4D'), findings that are consistent with a previous study on the longitudinal interambulacral muscles of the starfish *Pycnopodia helianthoides* Brandt, 1835 (Forcipulatida) (Wilkie et al., 1995). No intermediate tendons linking muscles with ossicles were observed. Thus, cavities in the calcite stereom near to the surface of the ossicle contain the loop-shaped straps of both muscle fibres and collagen fibrils (Figs. 3A,B). This anatomical arrangement provides multiple sites of interaction between the soft (muscle and collagen) and hard (ossicle) tissue of the starfish body wall and may enable stiffened collagenous ligaments to bear load when starfish are static and in between changes in posture initiated by muscle contraction.

Analysis of the structure of the aboral body wall in the starfish *E. spinulosus* (O'Neill, 1989) revealed that it comprises an ossicular layer consisting of ossicles and collagenous tissue located between inner and outer collagenous dermal layers, muscle and epithelium, with the collagenous

22

528 tissue thickest in the inner dermal layer. The organisation of collagen fibrils in the ossicular layer

529 resembles an orthogonal web where the bulk of the collagen fibrils surround the ossicles but do not

530 insert directly into them. This contrasts with the body wall of *A. rubens*, where collagenous tissue is

531 closely associated with the ossicles and many fibrils penetrate into the ossicles to form loop-shaped

532 straps around calcite struts, as discussed above. More recently, Motokawa (2011) analysed the

533 structure of the body wall in the starfish *Linckia laevigata* Linnaeus, 1758 (Valvatida), revealing

534 that in this species the aboral body wall has an exceptionally thick inner dermal collagenous layer.

535 By way of comparison, in *A. rubens* the aboral body wall ossicles are evenly surrounded by

536 collagenous tissue without thickening of the inner dermis (Figs. 2, 3; (Wilkie, 2001)). The presence

537 of a thickened inner dermal collagenous layer in *E. spinulosus* and *L. laevigata* accounts for the

538 increased stiffness and rigidity of the body wall in these species by comparison with *Asterias*

539 (Motokawa, 2011, Eylers, 1976, Marrs et al., 2000). Differences in body wall structure in the

540 Asteroidea have been discussed in detail previously with respect to phylogeny, evolutionary

541 success, ecology and habitat, defence strategies, mode of feeding and reproductive behaviour

542 (Feuda and Smith, 2015, Mah and Blake, 2012, Blake, 1990, Gale, 2011). For example, the

543 mechanical properties of the body wall influence the flexibility of the rays limiting and/or enabling

544 specific feeding behavior (Gale, 2013) and the thickened dermal tissue in the sturdy rays of tropical

545 shallow water valvatids and spinulosids such as *L. laevigata* and *E. spinuslosis* has also been

546 attributed as an adaptation against extensive predation (Blake, 1983).

547

548 **X-ray microtomography reveals the 3D structure of body wall ossicles in *A. rubens***

549 To the best of our knowledge this is the first study to employ micro-CT to analyse the 3D

550 anatomy of the body wall ossicular skeleton of an extant starfish species. Use of this technique has

551 generated high resolution images that are informative from a functional perspective. The shapes and

552 interactions of the different ossicle types that form the ray body wall in *A. rubens* are revealed in

553 unprecedented detail.

554   The lateral, reticular and carinal ossicles, which form the skeleton of the aboral body wall,

555   form a mesh-like structure with overlapping lozenge-shaped ossicles organized in rings that

556   surround voids in the body wall skeleton. This "loose" arrangement of ossicles is of functional

557   significance for gas exchange because the voids are overlaid by clusters of papulae (Figs. 1, 2),

558   finger-like extendable and retractable exvaginations of the coelomic lining of body wall. The

559   external epithelium and coelomic epithelium of the papulae are only separated by thin layers of

560   collagenous tissue and muscle and thus they provide a large surface area for gas exchange between

561   the coelomic fluid and external seawater (Brusca et al., 2016). The mesh-like organization of

562   ossicles that form the aboral body wall skeleton is also of functional relevance for changes in body

563   posture in *A. rubens*. Thus, the contractile state of small interossicular muscles that link the aboral

564   body wall ossicles (Figs. 3A, B) will determine the shape and size of a ring of ossicles. This can be

565   seen in Fig. 4C, where the shape and size of the voids in aboral skeleton are highly variable,

566   reflecting the particular posture of the ray when the animal was fixed. Changes in the shape and

567   posture of the rays are required for several types of starfish behaviour. For example, when starfish

568   are upturned by strong water currents they exhibit a righting response, during which the rays twist

569   and/or bend to enable the tube feet on the oral side of the ray to gain contact with the substratum

570   (Pollis and Gonor, 1975). Likewise, when starfish feed on prey such as mussels they adopt a

571   humped posture, with the rays bent so that tube feet proximal to the mouth are able to attach to the

572   valves of the mussel and tube feet in the distal region of the rays maintain attachment to the

573   substratum (Norberg and Tedengren, 1995). A similar posture is adopted during spawning (gamete

574   release), increasing the dissemination of gametes in the water column (Himmelman et al., 2008).

575   Furthermore, flexion of rays is aided by contraction of the apical muscle, a midline thickening of

576   the longitudinally orientated muscle beneath the coelomic epithelium (Figs. 2, 3C).

577   In comparison to the aboral ray skeleton, the ossicles that form the oral ambulacral region of

578   the rays in *A. rubens* are very different in their shape, size and packing. Thus, the ambulacral

579   ossicles are long, thin and tightly packed, with their bases inserting in a groove formed by

580   longitudinally adjacent cuboid-shaped adambulacral ossicles. This anatomy can be inferred from

581   detailed analysis of serial sections of starfish rays (Fig. 3) and scanning electron micrographs of

582   individual ossicles (Wilkie et al., 1995), but analysis of images generated from micro-CT is much

583   easier and quicker. Furthermore, the micro-CT data complement histological data in providing 3D

584   representations of ossicle shapes and the "gaps" between adjacent ossicles. For example, it can be

585   seen how the aboral "head" region of each ambulacral ossicle is shaped (Fig. 4D) to accommodate

586   the muscles and ligaments that link antimeric and longitudinally adjacent ossicles (Fig. 3D).

587   Similarly, indentations in the shaft of the ambulacral ossicles (Fig. 4D) accommodate the fluid-

588   filled tubes that connect each tube foot podium with its associated bulb-shaped ampulla (Fig. 2).

589   Both carinal and ambulacral ossicles are organized in an overlapping manner, with each

590   distal ossicle overlapping the more proximal one inclining towards the central disk. This overlap

591   enables the bending, extension and retraction of the whole ray when the apical muscle within the

592   aboral body wall and the interossicular longitudinal muscles in the ambulacrum contract or relax.

593   Heddle (1967) described body wall structure in the starfish *Luidia ciliaris* Philippi, 1837

594   (Paxillosida) and *Astropecten irregularis* Pennant, 1777 (Paxillosida), paying attention to the

595   organisation of antagonistic pairs of muscles involved in postural changes during locomotion and

596   burrowing of the animals. It was noted that these burrowing starfish have three pairs of antagonistic

597   muscles involved in abduction and adduction of the normally A-shaped ambulacral groove. Only

598   two of these pairs can be observed in *A. rubens* – the transverse supra- and infra-ambulacral

599   muscles and inner and outer lateral muscles (Fig. 2). The lack of a third antagonistic muscle pair is

600   explained by the lack of superambulacral ossicles, structures found only in burrowing starfish that

601   are evolutionarily distantly related to the Forcipulata (Heddle, 1967, Feuda and Smith, 2015). The

602   superambulacral ossicles and associated muscles are involved in changing the ray shape from a

603   walking posture to a posture suitable for digging substrate from underneath the animal using tube

604   feet. *A. rubens* is a non-burrowing starfish and only changes its ray shape to crawl into crevices if

605   stranded on the beach at low tide to avoid exposure to air and predators. Contraction of supra-

606   ambulacral and outer lateral abductor muscles flatten the shape of the starfish ray, increasing the

607   angle between antimeric ambulacral ossicles forming the ambulacral arch. Contraction of infra-

608   ambulacral and inner lateral adductor muscles induces a more rounded and tubular ray shape

609   bringing the rows of tube feet directly below the animal enabling locomotion with only vertical

610   stress on tube feet.

611

612   **SAXD reveals properties of collagen in the body wall of *A. rubens*.**

613   Use of trichrome staining revealed the location of collagen in sections of the body wall of *A.*

614   *rubens*, as discussed above. However, there are limitations in this approach to analysis of

615   collagenous tissue. Firstly, only thin (~10 μm) slices of tissue are sampled in each section, so a 3D

616   perspective requires complex and time-consuming reconstructions. Secondly, tissue fixation and

617   processing for histological analysis causes changes in tissue structure (e.g. shrinkage) (Howat and

618   Wilson, 2014) that may affect interpretation of the structural organization of collagen. Both of these

619   limitations are circumvented with the application of the SAXD method to unfixed starfish body

620   wall strips. Furthermore, SAXD can reveal ultrastructural properties of the fibrils (degree of

621   orientation, O/D ratio) that cannot be determined with light/electron microscopy-based histological

622   methods.

623   X-ray absorption maps revealed the positions of spines above reticular ossicles and the

624   shape of the "gaps" between the ambulacral ossicles, aiding the interpretation of the collagen X-ray

625   diffraction data. The SAXD scans themselves revealed an inhomogeneous distribution of collagen

626   within the aboral body wall. Areas lacking detectable amounts of collagen correspond to voids in

627   the body wall skeleton that underlie clusters of papulae on the body wall surface (Figs. 1, 2). The

628   amount of collagenous components in comparison to mineral (ossicular) components was found to

629   be higher in the aboral body wall in comparison to ambulacrum, consistent with the differences in

630   the thickness of the ossicles in the two locations. The 2D orientation of individual collagen fibrils

631   can be observed in high magnification images of trichrome sections (Figs. 3E, F), but SAXD

632   enables quantification of mean fibril orientation *in vivo* and the degree of orientation of collagen

633   fibrils, enabling comparisons between different locations in the body wall. The degree of fibril

634   orientation within the aboral body wall was highly variable between scan points (the standard

635   deviation of the vector length in the aboral samples was nearly 7 times that of ambulacral samples).

636   This probably reflects the complex lattice-like organization of the ossicles in the aboral body wall

637   where some parts of the tissue are under tension (high degree of orientation) and some completely

638   relaxed (dispersed fibril orientation). In contrast, the ambulacral groove fibrils had a uniformly low

639   degree of orientation (the mean vector length; degree of orientation for ambulacral samples was half

640   of the mean length in the aboral body wall; note the different scales in Figs. 5D1 and 5D2). It might

641   be expected that collagen linking the highly regularly organized ambulacral ossicles would have a

642   high degree of orientation. However, the thickness of the sample and the multidirectional fibres and

643   fibrils (Fig. 3E) with macrocrimp (Fig. 3F) connecting antimeric ambulacral ossicles probably

644   explains the uniform mean orientation with low degree of orientation in the ambulacrum.

645   The reported D-value ranges (65.3±0.6nm for the aboral body wall and 67.0±0.6nm for

646   ambulacral) are similar to the range of D-values reported for mammalian collagen fibrils (65-67nm,

647   (Krauss et al., 2009, Yang et al., 2015, Sasaki and Odajima, 1996a) and sea cucumber and sea

648   urchin fibrils reported by Trotter et al. (1994) (65.7±0.5 nm), but they are higher and less variable

649   than those reported by Ferrario et al. (2017) (62.7±2.8 nm for sea urchin, 63±4.7 nm for starfish and

650   66±1.6 nm for sea cucumber) and Ribeiro et al. (2012b) (59.2±6.2 nm for sea urchin). The variation

651   in D-period length within echinoderms could reflect the differences in techniques (TEM can only be

652   performed on fixed tissue/extracted fibrils, SAXS on the other hand requires no pre-treatment of

653   tissue) and/or differences between species (all the previous echinoderm studies referred to in this

654   paper used TEM images to measure the D-period). The O/D ratios reported here (0.468, SD 0.015

655   for aboral body wall and 0.479, SD 0.009 for ambulacrum) are the first to be determined in an

656   echinoderm. Interestingly, these are higher than previously described O/D ratios of 0.42-0.46

657 (depending on collagen type) for mammalian fibrils (Antipova and Orgel, 2010, Sasaki and

658 Odajima, 1996b); however, the functional significance of this difference remains to be determined.

659   Comparison of the internal structure of collagen fibrils in the ambulacrum and aboral body

660 wall of *A. rubens* revealed a significantly higher D-period length, variability of D at each scan point

661 and O/D ratio in the ambulacrum. The trend is consistent across and between the samples from each

662 body location and possibly reflects differential fibril composition at sites experiencing varying

663 mechanical demands. At least three mechanisms explaining forms of intrafibrillar deformation in

664 response to increasing tension have been described and recognised in the past (Sasaki and Odajima

665 (1996a), see Fig. 6A for reference of fibril structure). Initially, extension of tissue parallel to the

666 main fibril direction straightens crimped fibrils. Thereafter fibrils can deform by three mechanisms

667 (i) homogeneous elongation of individual tropocollagen molecules (the length of D increases, O/D

668 remains the same, refer to Fig. 6A), (ii) molecular slippage where only the gap between

669 longitudinally adjacent molecules increases (no change in D, but decrease in O/D) and/or (iii)

670 inhomogeneous elongation of the molecules (elongation of only gap or overlap regions).

671 Additionally the length of D has been shown to vary between and within tissues even when they are

672 not under tension (Fang et al., 2012).

673   Until now, the correlation between D and O/D has only been studied in the context of

674 changes at a single tissue location during/before and after extension. This study focuses on two

675 different locations with no external strain applied. Therefore, the observed differences in D-values

676 and O/D ratios may not solely or primarily represent differences in the state of tension in the tissue.

677 The structure of fibrils is determined by constituent tropocollagen molecules (e.g. hetero vs

678 homotrimeric and type of collagen alpha chains) (Orgel et al., 2006, Antipova and Orgel, 2010) and

679 the length of D varies between mammalian collagenous tissues (Fang et al., 2012). The differences

680 in the observed D and O/D values between aboral body wall and ambulacrum could therefore arise

681 either from differential strain conditions and/or the molecular structure of the fibrils. Fibrillar

682 collagen isolated from *Asterias amurensis* Lütken, 1871 (Forcipulatida) body wall consists of

683 (α1)$_2$$\alpha$2 heterotrimers, similar to mammalian type I collagen (Kimura et al., 1993). This study,

684 however, does not specify whether the collagen was isolated from whole starfish or aboral body

685 wall only. Nor have any amino acid sequences of *A. amurensis* or *A. rubens* α1 and α2 procollagen

686 chains been determined, to the best of our knowledge. Hence we cannot, at this stage, make any

687 statements about the difference (or not) between aboral and ambulacral collagen molecular

688 structure. We also acknowledge that without structural knowledge of the starfish collagen

689 molecules at the amino acid level, the method of calculating O/D ratios using experimentally

690 measured intensity ratios has some limitations and the interpretation of the data may be reviewed in

691 the future. Nevertheless, the O/D ratios determined here in *A. rubens* give an indication of innate

692 differences between collagenous tissue in different body locations.

693

694 **Future directions**

695   The present study provides detailed insights into ossicle shape and organisation, the structural

696 characteristics of collagenous tissue and the positions of muscles in the body wall of *A. rubens*.

697 Determination of the anatomical organisation of ossicles may provide a basis for experimental

698 investigation of structure-function relationships. This study also highlights the lack of knowledge of

699 *in vitro/vivo* ultrastructural properties of collagen fibrils from non-mammalian animals.

700 Comparative studies between tissues within species and between animals from different clades are

701 required to understand mechanisms such as collagenous tissue mutability. Our study is a first step in

702 this direction.

703   Further studies using *in situ* micromechanical testing during SAXD scanning could bring

704 insights into mechanisms of intrafibrillar deformation and how mechanisms of collagenous tissue

705 mutability work in concert with spatial gradients in collagen fibril composition/re-orientation and

706 the interconnecting ossicular network leading to large scale mechanical and postural changes in

707 starfish. Using the combination of micromechanical testing and SAXD, we have recently shown

708 that the mechanism of collagen mutability in sea cucumbers involves changes in interfibrillar

709   stiffening rather than in any intrinsic changes in the properties of the collagen fibrils themselves

710   (Mo et al., 2016). In inhomogeneous ossicle-tissue networks like the starfish body wall, changes in

711   MCT properties could mechanically affect the links to and between the ossicle network, leading to

712   flexion, torsion etc. Changes in fibril strain and orientation during *in vitro* micromechanical testing

713   can be tracked via changes in the SAXD patterns reflective of four different mechanisms for

714   collagenous tissue elongation: (i) straightening of the fibrillar crimps, (ii) fibril reorientation, (iii)

715   interfibrillar displacement (changes in the interfibrillar cohesion) and (iv) stretching of the fibrils

716   themselves (Gupta, 2008, Sasaki and Odajima, 1996a).

717   Combined information on the material properties, shapes and organization of the ossicles and

718   known material properties of the collagenous and muscle components could also be utilized to build

719   a model of starfish arm movement. The model would provide information about the unique mode

720   of locomotion and postural behavior of starfish. In addition, the model could be used to simulate

721   how the mechanics of composite materials can be modulated through changing the architecture, to

722   make them softer/stiffer and more/less extensible by changing the fibril to mineral component ratio

723   and/or internal structure and spatial organization of one or more of the elements (e.g. the amount of

724   collagen linking the ossicles or the orientation of the fibrils).

725

726   **Conclusion**

727   In conclusion this study provides a detailed description of body wall structure in the starfish

728   *A. rubens*, illustrating the value of a multi-technique approach when characterizing tissue

729   composites. The data obtained provides a structural basis for investigation of the mechanical

730   properties of the starfish body wall both *in vitro* and *in silico,* using *A. rubens* as a model organism.

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AUTHOR CONTRIBUTIONS

Concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript: LMB, HSG, MRE; acquisition of data, data analysis: ME, YL, GRD, NJT; all authors contributed to revising of the manuscript and approved the manuscript.

FIGURE LEGENDS

748

749 **Figure 1. General anatomy of starfish *A. rubens***

750 The main image shows a specimen of the starfish *A. rubens* with one of the rays transversely

751 dissected and with the aboral body wall of two other rays removed to reveal the digestive glands

752 (pyloric caeca, PC) and the ridge of ambulacral ossicle heads (AMOh). Inset A shows a close up of

753 the transverse cross section of the ray with ambulacral ossicles (AMO) and tube feet (TF) on the

754 oral side of the cross section and pyloric caeca attached to the inner side of the aboral body wall.

755 The positions of the apical carinal ossicle (CO) and lateral reticular ossicles (ROs) are labelled.

756 Inset B shows the outer surface of the aboral body wall centered on the mid-line of the arm showing

757 the positions of spines (SPs) that are located over the row of carinal ossicles and clusters of papulae

758 (CPa). Inset C shows a close up of a pedicellaria, a pincer shape defensive organ. Inset D shows a

759 close up of the inner surface of the aboral body wall with voids in the body wall that are overlain by

760 clusters of papullae (CPa) on the outer body wall surface (see inset C). The position of the

761 longitudinally orientated apical muscle (AM) is outlined. The scale bar on the main figure is 1 cm.

762

763 **Figure 2. Trichrome stained transverse section of a decalcified ray from *A. rubens*.**

764 The voids formed by decalcification of the body wall ossicles show the positions and shapes of the

765 different ossicle types: the ambulacral ossicles (AMO), the adambulacral ossicles (ADO), the

766 marginal ossicles (MO), the reticular ossicles (RO) and the carinal ossicle (CO). The red speckling

767 within the ossicle voids are stained cells, which are located in pores between the calcite struts of the

768 ossicle stereom *in vivo*. Surrounding the ossicle network can be seen a dense meshwork of

769 collagenous tissue (blue), which forms the bulk of the soft tissue in the body wall. The

770 interossicular muscles that link adjacent ossicles can also be seen. These are most prominent

771 adorally, where there are large muscles that link adjacent ambulacral ossicles (longitudinal supra-

772 ambulacral muscle, LSM; transverse infra-ambulacral muscle, TIM; transverse supra-ambulacral

muscle, TSM) or that link ambulacral ossicles with adambulacral ossicles (inner and outer transverse lateral muscles; TLM; longitudinal lateral muscles, LLM). The smaller interossicular muscles (IOM) linking ossicles of the aboral skeleton are also evident but these are seen more clearly at higher magnification (see Fig. 3). Occupying the coelomic space internal to the body wall can be seen the prominent pair of digestive glands (pyloric caeca, PC), which are connected via mesenteries (*) to the aboral coelomic lining (ACL) of the body wall. The coelomic lining is detached from the body wall in this stained section, which is an artifact probably caused by shrinkage of the body wall dermis during tissue processing. Note that the aboral lining of the coelom is thicker in the midline position due to the presence of the longitudinally oriented apical muscle (AM), which causes aboral flexion of the ray when it contracts *in vivo*. The prominent V-shaped radial nerve cord (RNC) can be seen between the two rows of tube feet podia (TF), which are connected to the intracoelomic bulb-shaped ampullae (AMP) by tubular connections that run between adjacent ambulacral ossicles (as seen here on the right side). Note also other appendages that are associated with the external body wall surface, including spines (SP), pedicellariae (Pe) and papulae (Pa) that overlay voids (V) between the ossicles forming the aboral body wall skeleton. Scale bar is 150 μm.

Figure 3. Trichrome stained sections of starfish body wall showing ossicles and associated muscles and collagenous tissue.

A. Adjacent reticular ossicles (RO) are linked by an interossicular muscle (IOM) and are embedded within a collagenous tissue meshwork (blue). The calcareous struts of the ossicles appear as voids, due to decalcification of the tissue, and the cellular stroma appears red. **B**. High magnification image of adjacent reticular ossicles showing how muscle fibres of an interossicular muscle (IOM) insert between and around strut voids near the surface of each ossicle (white arrows). The wrapping of collagen fibres (blue) around ossicle strut voids (black arrows) can be clearly seen in this image. **C**. Muscle strands (arrows) derived from the circular muscle layer (CML) above the apical muscle

799 (AM; longitudinal muscle) extend through the collagenous inner dermis (blue) and insert on the

800 carinal ossicle (CO). **D.** Ambulacral ossicle heads (AMOhs) are inter-connected by transverse

801 supra-ambulacral muscle (TSM) and longitudinal supra-ambulacral muscles (LSMs). The

802 ambulacral ossicles (AMOs) are furthermore connected by transverse infra-ambulacral muscle

803 (TIM). Other abbreviations as in Fig 2. **E.** Collagen fibres (blue) below the transverse supra-

804 ambulacral muscle (TSM) connect antimeric ambulacral ossicle heads strapping around ossicle

805 struts. **F.** The collagen fibres (blue) above the transverse infra-ambulacral muscle (TIM) have a

806 predominantly transverse horizontal orientation with a macro-crimp (wavy appearance). The scale

807 bar in 3F corresponds to: 50 μm in A, 25 μm in B, 50μm in C, 100 μm in D, 12.5 μm in E and

808 7.8μm in F.

809

810 **Figure 4. The ray skeleton of *Asterias rubens*, as revealed by X-ray microtomography.**

811 **A.** Low-magnification overview of the ray skeleton from a top (aboral) view. Along the midline of

812 the aboral skeleton can be seen the row of overlapping carinal ossicles (COs). Either side of the

813 carinal ossicles are a loose meshwork of reticular ossicles (ROs) and through the gaps bounded by

814 rings of reticular ossicles can be seen the two rows of ambulacral ossicles (AMOs) on the oral side

815 of the ray. Note also the numerous spines located external to the ossicle network; three spines

816 located above the carinal ossicles are labeled with arrow heads. **B.** Transverse segment of a starfish

817 ray showing the ambulacral skeleton formed by two rows of ambulacral ossicles (AMO), which are

818 supported orally by the cuboid-shaped adambulacral ossicles (ADO). Lateral to the adambulacral

819 ossicles are the densely packed marginal ossicles (MO). The aboral region of the ray skeleton is

820 formed by a loose meshwork of reticular ossicles (RO) and the single row of carinal ossicles (CO).

821 Spines (SPs) can be seen on the body wall surface **C.** The aboral ray skeleton viewed from its

822 underside, showing the overlapping row of carinal ossicles (COs) along the midline and the loose

823 meshwork of reticular ossicles (ROs) on either side of the carinal ossicles. This image also

824 illustrates how changes in orientation of the carinal and reticular ossicles, mediated *in vivo* by

contraction/relaxation of interossicular muscles, affects skeletal structure. Thus, on the left side of the image ossicles form ring-shaped structures, whereas as on the right hand side of the image the ossicles form oblong-shaped structures. **D.** The ambulacral skeleton viewed at high magnification, looking towards the tip of the ray. The image shows how the slender and tightly packed ambulacral ossicles (AMO) are orientated at an angle, leaning away from the tip-end of the ray. Furthermore, it can be seen that the aboral "head" (AMOh) of each ambulacral ossicle overlaps an adjacent ossicle more proximal to the central disk. The large gaps between the adjacent "heads" of ambulacral ossicles (arrow heads and asterisks) are occupied *in vivo* by longitudinally and transversely orientated interossicular muscles, respectively (which can also be seen in Figs. 2 and 3). Dashed lines show where tubular connections of the tube feet and ampullae are located **D'.** Scanning electron micrograph showing the calcite struts and pores of ambulacral ossicle stereom at high magnification. **E.** External view of the marginal ossicles of the body wall. At this high magnification it can be seen that overlapping ossicles with appendages (spines, SP and pedicellariae, Pe) are arranged in longitudinally orientated rows and these ossicles are interlinked radially by smaller ossicles without appendages (arrow heads). Scale bars for A and C, 2 mm; B and D 1 mm; D' 40μm; E 500μm.

Figure 5. Transmission, I_{q5col}; I_{q5col} / I_{q5min} ratio and vector maps of *A. rubens* aboral body wall and ambulacrum

A1 and A2. X-ray transmission maps of an aboral body wall (A1) and an ambulacrum sample (A2). The contour scale corresponds to darker regions as regions with higher density of the tissue (higher absorption). Areas of high density in blue (*) indicate areas of ossicular overlap. The insets are close ups of micro-CT images of corresponding body locations. **B1 and B2.** The Intensity of fifth order Bragg peaks mapped across the aboral (B1) and ambulacrum (B2) samples. Red corresponds to the highest amount of collagen. **C1 and C2.** The ratio of fifth order Bragg peak intensity from collagen fibrils (I_{q5col}), to the intensity of diffuse SAX scattering (which arises mainly from mineral

851   components of the tissue) (I_{q5min}) mapped across the aboral (C1) and ambulacral samples (C2). **D1**

852   **and D2.** Vector and circle plots of collagen fibril structure overlapped with a transmission map of

853   the aboral (D1) and ambulacral samples (D2). The orientations of the vectors are parallel to the

854   orientation of the collagen fibrils. The length of the vector is inversely proportional to $\Delta\chi_0$. The

855   degree of fibril orientation $\left(\frac{1}{\Delta\chi_p}\right)$ increases with the vector length. The scale vector lengths in the

856   bottom left corner correspond to $\left(\frac{1}{\Delta\chi_p}\right)$ of 0.23, 0.46, 0.70 and 0.93 [Au] or $\Delta\chi_0$ of 5, 2.5, 1.67 and

857   1.25 degrees. It is notable that all the vectors in D2 are shorter than the scale vector corresponding

858   to $\Delta\chi_0$ of 5°, in comparison to 8% of vectors in D1 being longer than the scale vector for $\Delta\chi_0$ of 5°.

859

860   **Figure 6.** *A. rubens* **body wall collagen fibril D-period length and Overlap/D ratio.**

861   A. A simplified diagram of collagen triple helical molecule arrangement within a collagen fibril.

862   The striated collagen fibrils are formed of triple helical collagen molecules that are arranged in a

863   staggered manner leading to electron dense and light regions (overlap, O; gap, G). The combined

864   length of one overlap and gap region equals a D-period (D). The length of the D-period and overlap

865   to D ratio (O/D) can be used as a measure to characterise collagen fibrils. B. The histogram shows

866   the distribution of D-period length in aboral body wall (red solid line) and ambulacral groove (black

867   dotted line) divided in bins of D < 64.5 nm; 64.5 nm ≤ D < 65.5 nm, 65.5 nm ≤ D < 66.5 nm, 66.5

868   nm ≤ D < 67.5 nm, 67.5 nm ≤ D < 68.5 nm and 68.5 nm ≤ D < 69.5 nm. Aboral body wall collagen

869   fibrils have a lower mean D-period than ambulacral ones. C. The histogram shows the distribution

870   of O/D ratios in aboral body wall (red solid line) and ambulacral groove (black dotted line) divided

871   in bins with 0.01 ratio intervals. A lower O/D ratio is observed for the aboral body wall relative to

872   the ambulacrum.

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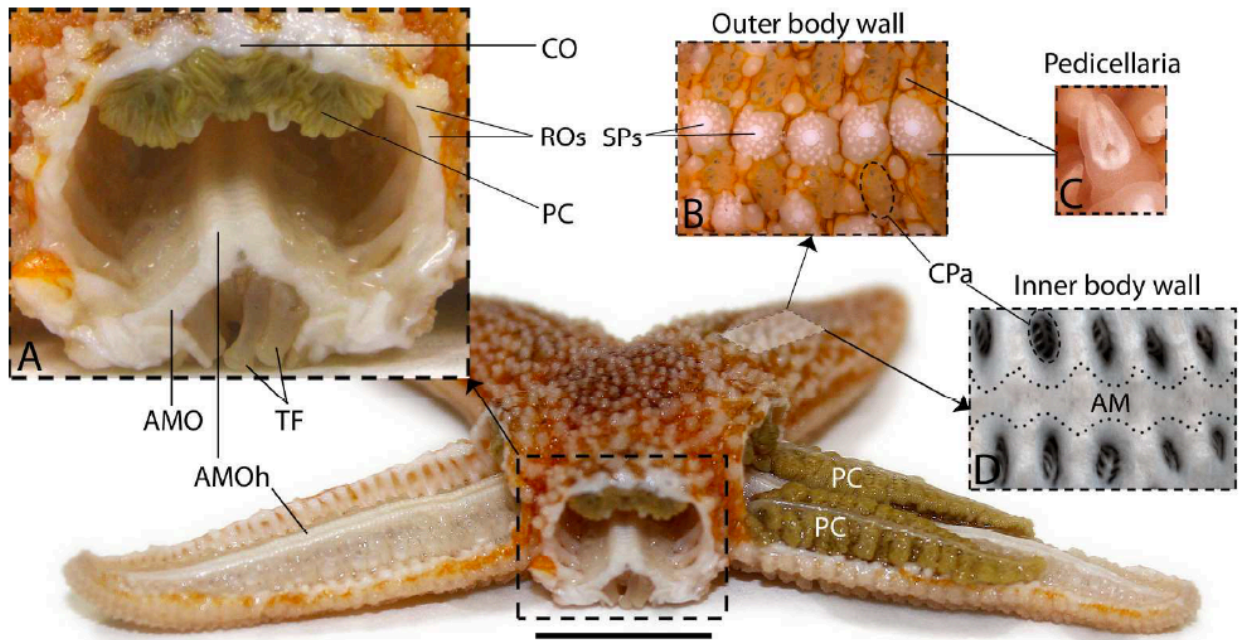
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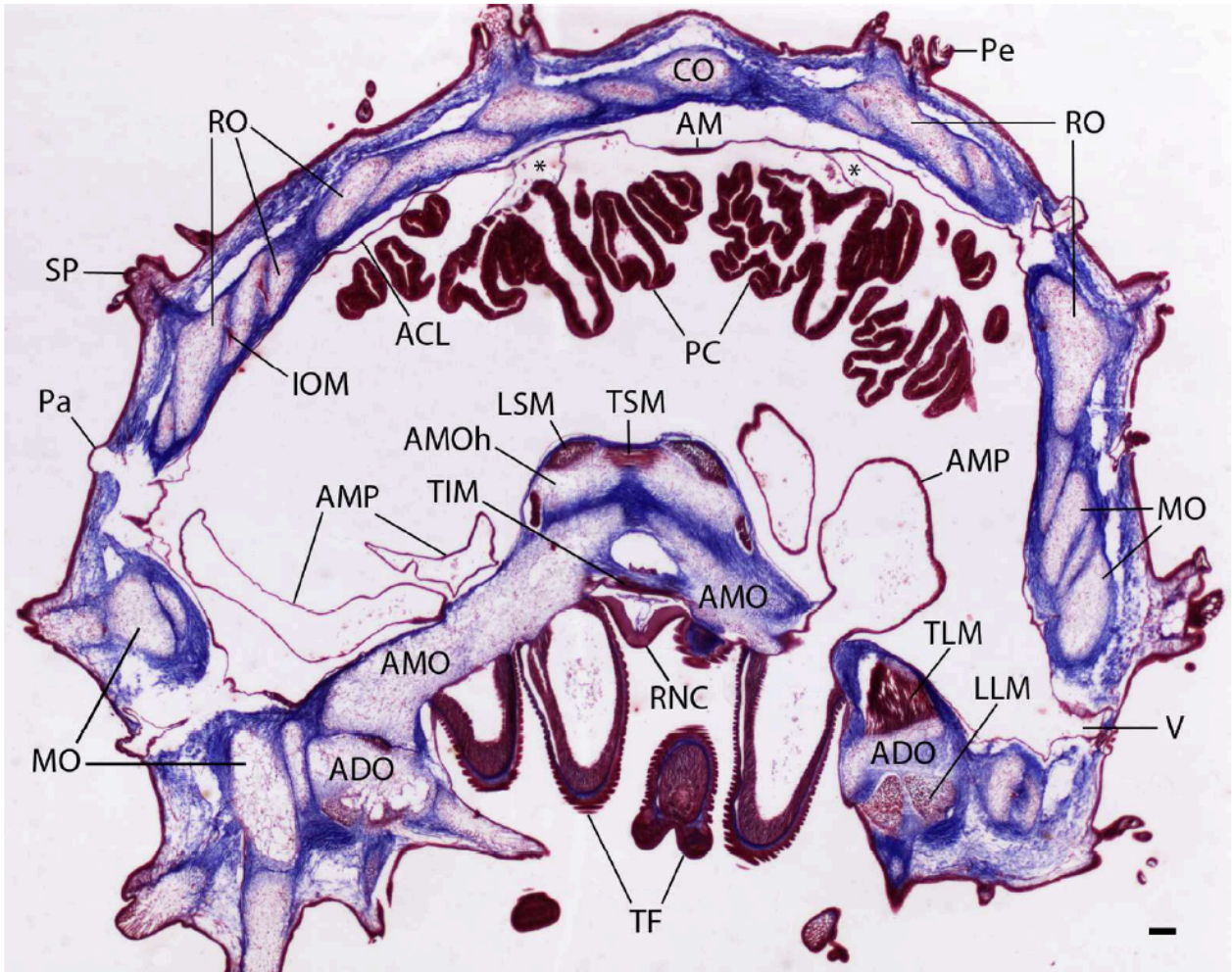1056   14416-21.
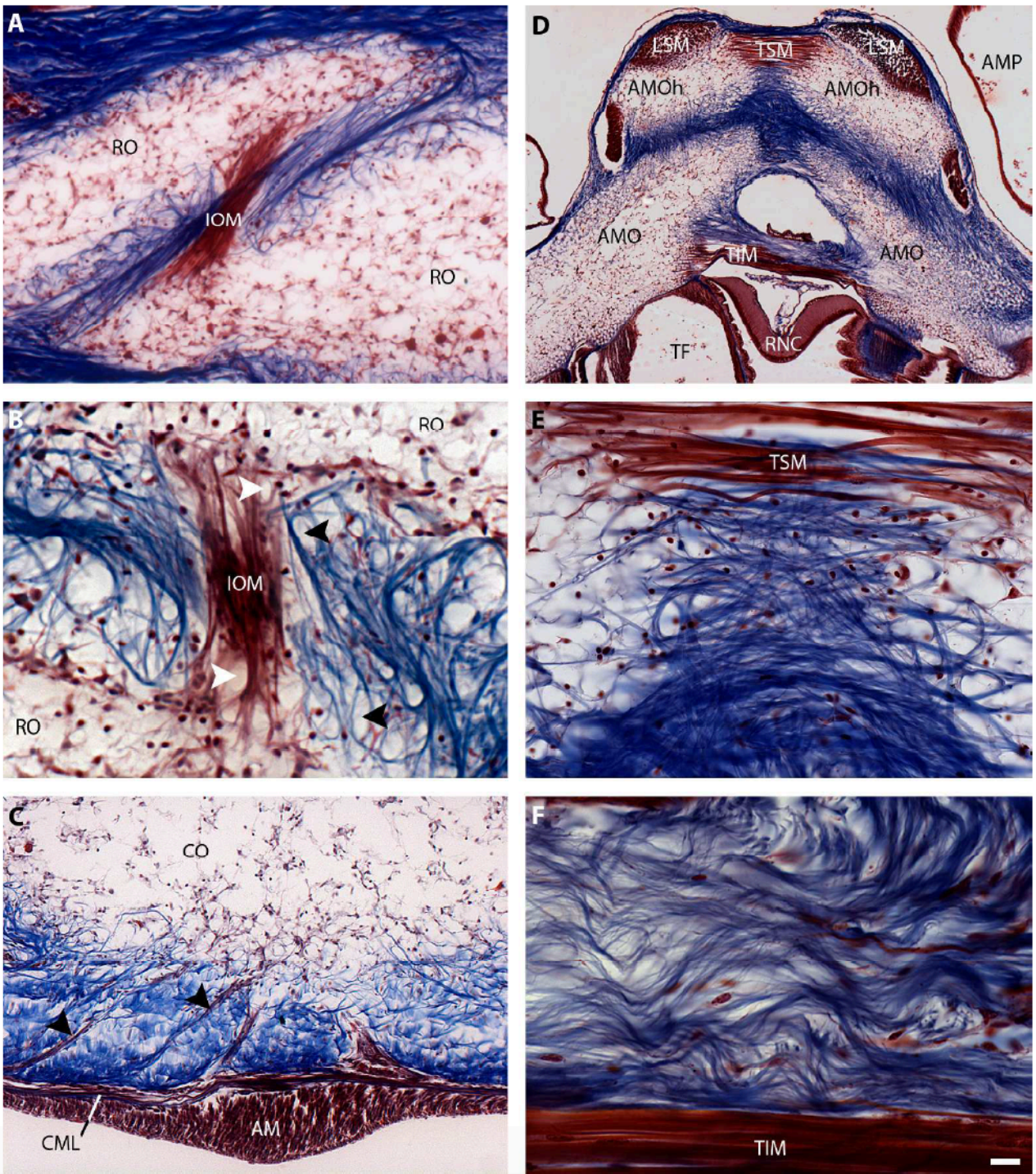1057

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Figure 1
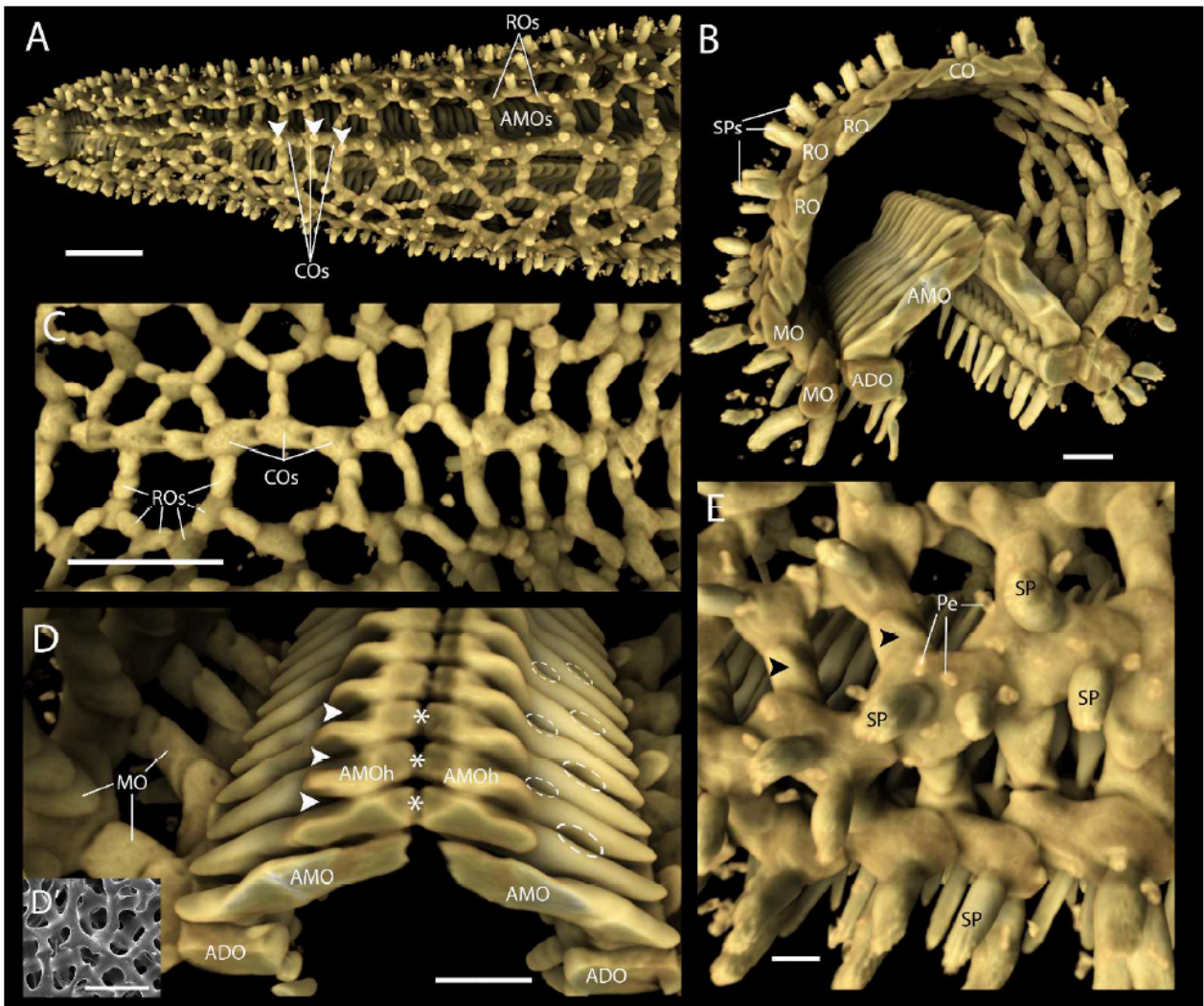
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