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The tube feet of sea urchins and sea stars contain functionally different mutable collagenous tissues

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Summary

Echinoderms possess mutable collagenous tissues (MCTs), which are capable of undergoing rapid changes in their passive mechanical properties mediated by secretions from a specific cell type, the juxtaligamental cell. In this study, the possible presence of MCTs in the tube feet of the echinoid *Paracentrotus lividus* and the asteroid *Marthasterias glacialis* was investigated by measuring their extensibility, tensile strength, stiffness and toughness after different treatments known to influence the physiological state of MCTs. Calcium removal reversibly induced a significant plasticization of the tube feet of both species. When exposed to cell-disrupting solutions, the tube foot stem of sea urchins and sea stars showed a significant increase in strength, stiffness and toughness in the absence of calcium. This response, combined with the ultrastructural observation of juxtaligamental-like cells in the connective tissue, confirms that an MCT is present in both echinoid and asteroid tube feet. It was observed, however, that the tube foot stems of *P. lividus* and *M. glacialis* are affected differently by exposure to cell-disrupting solutions in the presence of calcium, indicating that their MCTs could be functionally different. In their soft state, MCTs could assist the muscles in tube foot protraction, bending and retraction; in their stiff state, they could play a role in the energy-sparing maintenance of position; for example, during strong attachment to the substratum to resist hydrodynamically generated loads.

Key words: connective tissue, mechanical property, ultrastructure, Asteroidea, Echinoidea.

Introduction

The connective tissue of echinoderms has the unique ability to change its passive mechanical properties in response to environmental and mechanical stimuli (Motokawa, 1984, 1988; Wilkie, 1984, 1988). These collagenous tissues are said to be mutable and have a widespread occurrence in all echinoderm classes. Being present in numerous distinct organs, they play a role in various functions such as autotomy mechanisms, locomotion, energy-efficient maintenance of posture, and defence (see Wilkie, 1996 for a review). All mutable collagenous tissues (MCTs) have in common the capacity to undergo rapid, nervously mediated changes in their mechanical properties in a physiological time scale (Wilkie, 1996; Wilkie et al., 2004).

MCTs are composed of collagen fibres with varying sizes, conformation and spatial arrangement, each fibre consisting of numerous spindle-shaped collagen fibrils (Trotter et al., 2000; Wilkie et al., 2004). An elastomeric network of fibrillin microfibrils surrounds and separates the collagen fibres (Trotter et al., 2000; Wilkie et al., 2004). This microfibrillar network maintains the organization of collagen fibrils as they slide with respect to one another during lengthening and shortening of the tissue; it may also provide long-range restoring force (Thurmond and Trotter, 1996; Trotter et al., 2000). Both collagen fibrils and microfibrils are interconnected by a matrix of proteoglycans and glycoproteins (Trotter et al., 2000; Wilkie et al., 2004). Furthermore, MCTs always contain a special type of cell, the so-called juxtaligamental cells, which are characterized by the presence in their cytoplasm of numerous electron-dense, membrane-bound granules. Nerve terminals and axon-like profiles have often been observed in contact with, or in the vicinity of, these secretory cells (Wilkie, 1996). Juxtaligamental cells are believed to contain molecules that regulate interactions between the collagen fibrils (Trotter and Koob, 1995; Koob et al., 1999; Trotter et al., 2000). The temporary nature of these interactions accounts for the capacity of MCTs to switch reversibly from a stiff to a compliant state (Tipper et al., 2003; Wilkie et al., 2004).

Tube feet are the external appendages of the echinoderm water-vascular system and present a remarkable diversity of forms and functions (Flammang, 1996). Although it has often

been suggested that an MCT is present in echinoderm tube feet (Florey and Cahill, 1977; Motokawa, 1984; Byrne, 1994; Flammang, 1996), this has never been demonstrated experimentally. In sea urchins and sea stars, tube feet are disc-ending (i.e. they consist of a proximal extensible stem and a distal disc-shaped extremity) and are involved in attachment to the substratum and locomotion. In the present study, the material properties of the tube foot stem of the echinoid *Paracentrotus lividus* and the asteroid *Marthasterias glacialis* were investigated in order to search for evidence of the presence of an MCT.

Materials and methods

Adult individuals of *Paracentrotus lividus* (Lamarck 1816) and *Marthasterias glacialis* (Linné 1758) were collected intertidally in Brest and Morgat (Brittany, France), respectively. They were maintained in re-circulating aquariums at 14–15°C and 33‰.

Preparation of specimens and bathing solutions

Two experiments were conducted on both species to establish the tensile properties of their tube feet under various conditions. In the first, each individual was dissected under water to prevent tissue desiccation, and the five ambulacra were separated into five fragments, each representing a fifth of the test in sea urchins or one arm in sea stars. Each ambulacrum was then incubated for 1 h at room temperature and under slight agitation in one of the five following solutions: (1) ASW (artificial seawater made up of 445 mmol l^{-1} NaCl, 60 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl, 2.4 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} Hepes and 10 mmol l^{-1} $CaCl_2$; pH 7.8); (2) ASW-EGTA (in which $CaCl_2$ was replaced by 2.5 mmol l^{-1} EGTA); (3) ASW-EGTA-TX (in which 1% Triton-X100 was added to the ASW-EGTA solution); (4) ASW-TX (ASW solution with 1% Triton-X100); and (5) DW (deionised water). The mechanical tests were performed in the solutions and, for each ambulacrum, 10 adoral tube feet from the sea urchins or 10 mid-arm tube feet from the sea stars were tested. The whole protocol was repeated with three different individuals of each species. For two individuals of *P. lividus*, the ambulacra that had been bathed in ASW-EGTA were returned to ASW and incubated for an additional 1 h (ASW-EGTA → ASW). Mechanical tests were repeated on 10 adoral tube feet per ambulacrum.

In the second experiment, individuals from both species were anaesthetized by incubation in ASW containing 0.1% propylene phenoxetol (ASW-PP) for 1 h at room temperature. This anaesthetic was used to prevent non-specific, manipulation-induced stiffening of the connective tissue (Motokawa and Tsuchi, 2003). It was preferred to the commonly used $MgCl_2$ because the latter modifies the ionic environment of the tissues and thus affects connective tissue mechanical properties (Wilkie, 1996; Santos and Flammang, 2005). Subsequently, individuals were dissected in the ASW-PP solution, and the ambulacra were incubated for an additional 1 h either in the ASW-PP solution or in the same solution containing 1% Triton-X100 (ASW-PP-TX). As in the first experiment, three individuals per species and 10 tube feet per ambulacrum were tested.

Measurement of mechanical properties

Mechanical traction tests were performed with a Mecmesin-Versa Test motorized stand fitted with an electronic force gauge that measures forces up to 2.5 N (Mecmesin AFG 2.5 N, Horsham, UK), connected to a computer collecting the data. The precision of the tensile force measurements was 0.0005 N. Ambulacra were placed upside down, and a small surgical clip was attached to one tube foot in the portion of the stem just under the disc and then pulled perpendicular to the specimen (in the direction of the natural extension) at a constant rate of 25 mm min^{-1} (Santos and Flammang, 2005), until failure. Failure never occurred at the clip. Before pulling the tube foot, the initial length of the stem (distance between the base of the tube foot and the clip) was measured at the point at which the force started to increase and reached 0.0015 N. The initial length of the tube foot, together with the time required to break the tube foot at a constant extension rate, was subsequently used to calculate the stem final length at failure. From these data, several material properties were calculated: the true strain, the true stress, the tangent modulus of elasticity and the breaking energy density (Fig. 1). True values of strain and stress were used instead of nominal values because of the high extensions observed for both species' tube feet (Shadwick, 1992).

The true strain (ε), a unitless parameter, was calculated as the Napierian logarithm of the ratio of each incremental value of length (L) to the initial length (L_0) of the tube foot (extension ratio). Strain expresses the deformation of the tube foot in response to a certain force and, at the point at which the stem fails (at final length), is a measure of the material's extensibility:

$$\varepsilon = \ln (L/L_0) . \quad (1)$$

The true stress (σ) was calculated as the product of the extension ratio and tensile stress [force (F) divided by cross-sectional area (S)]. It is expressed in N m^{-2} or Pa (Pascal). The maximum value of stress (at breaking force) is an indicator of the strength of the tube foot:

$$\sigma = (F/S)(L/L_0) . \quad (2)$$

The connective tissue cross-sectional area was used for the calculations of the true stress instead of the stem wall cross-sectional area because this tissue appears clearly as the layer bearing most of the load exerted on a tube foot. Indeed, Florey (cited in Florey and Cahill, 1977) reported that the connective tissue resists extensions with forces bigger by one or two orders of magnitude than those developed by the muscle when stimulated to maximal contraction. The ultrastructure of the epidermis and nerve plexus suggests that they are even weaker.

The modulus of elasticity (E) was calculated as the tangent

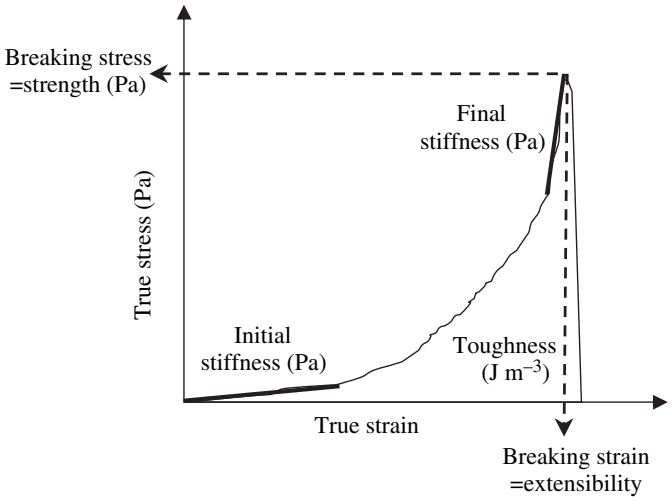

Fig. 1. Typical J-shaped stress–strain curve for the stem of echinoid and asteroid tube feet, showing the different material properties measured.

to the slope of the stress–strain curve. It is a measure of the tube foot stiffness and is also expressed in Pa:

$$E = (\sigma_2 - \sigma_1)/(\varepsilon_2 - \varepsilon_1) \, . \quad (3)$$

The stress–strain curve obtained for tube feet of both species was typically J-shaped (Fig. 1). Characteristically, there was an initial region of low resistance of the tube foot to the applied force followed by a region presenting a sudden increase in stress until rupture. Thus, an initial and final stiffness were calculated, corresponding, respectively, to the stiffness of the tube foot at the beginning and at the end of extension.

Finally, we calculated the breaking energy density as the integral of force multiplied by extension (i.e. the area under a force–extension curve) divided by the volume of stem connective tissue (calculated as the product of tube foot initial length multiplied by mean connective tissue cross-sectional area). This parameter is an indicator of the energy needed to extend and break the tube foot. It is also referred to as the toughness and is expressed in J m^{-3}.

For both species, the results were analysed in order to look for significant differences in the mechanical properties of the tube feet between different individuals and between the different bathing solutions. Data were analysed by two-way analysis of variance (ANOVA) followed by the multiple comparison test of Tukey. When necessary, logarithmic transformation was used to achieve homoscedasticity. The variability explained by each factor is derived from the sum of squares.

Morphological analyses

The mean values of the cross-sectional areas of each tissue layer of the tube foot stem from each species were obtained using tube feet dissected from the animals used for the mechanical tests. For both species, 10 tube feet were cut off the ambulacra that remained in ASW and were fixed in Bouin's fluid for 24 h. They were subsequently dehydrated in a sequence of graded ethanols, embedded in paraffin wax using a routine method, and cut transversely into 7 μm-thick sections with a Microm HM 340 E microtome. The sections were mounted on clean glass slides and stained with Masson's trichrome. Measurements were made with a Leica Laborlux light microscope equipped with a graduated eyepiece on sections taken halfway between the base and the disc of the tube foot. A mean value of the connective tissue cross-sectional area was calculated from measurements of connective tissue diameter and thickness made on 10 tube feet. This mean value was used in the calculation of stem mechanical properties for the particular animal from which the tube feet were dissected.

For transmission electron microscopy (TEM), tube feet from both species were fixed for 3 h at 4°C in 3% glutaraldehyde in cacodylate buffer (0.1 mol l^{-1}, pH 7.8; adjusted to 1030 mOsm l^{-1} with NaCl). Then they were rinsed in cacodylate buffer, post-fixed for 1 h in 1% OsO_4 in the same buffer, dehydrated in graded ethanols, and embedded in Spurr resin. Transverse ultrathin sections (70–80 nm in thickness) were cut with a Leica UCT ultramicrotome equipped with a diamond knife, collected on copper grids and stained with uranyl acetate and lead citrate. Ultrathin sections were observed with a Zeiss Leo 906E transmission electron microscope, and morphometric measurements were obtained using analySIS® software (Soft Imaging System GmbH, Münster, Germany).

Results

Morphometry and ultrastructure of tube foot stem

The tube feet of the two species have a similar histological structure, the stem wall consisting of an outer epidermis, a basiepidermal nerve plexus, a connective tissue layer and an inner myomesothelium that surrounds the water-vascular lumen. Table 1 summarizes the morphometric measurements made on individuals of the two species and their tube feet. In order to compare the two species, the cross-sectional area of each tissue layer was calculated as a percentage of the stem cross-sectional area. The stem wall cross-sectional area was 63.4% and 77.4% of the stem total cross-sectional area (i.e. including the water-vascular lumen) for sea urchin and sea star tube feet, respectively. In sea urchin tube feet, the epidermis (including the basiepithelial nerve plexus) and the connective tissue were the dominant layers of the stem wall, while in sea star tube feet the stem wall was occupied mainly by the epidermis and the myomesothelium. It is important to note that the biggest differences were found in the connective tissue cross-sectional area, which was 39% of the stem wall cross-sectional area in sea urchin tube feet and only 8.4% in sea stars.

The TEM study revealed several ultrastructural differences in the connective tissue layer of sea urchin and sea star tube feet. In both species, this layer was organized into an outer sheath of longitudinally orientated collagen fibres and an inner sheath of helicoidally orientated fibres (see also Flammang, 1996). The outer sheath was almost 20 times thicker than the

Table 1. *Mean morphometric values of echinoid and asteroid individuals and their tube feet*

	Species	
	Paracentrotus lividus	*Marthasterias glacialis*
Size of individuals (mm) (*N*=3)	Test diameter: 43.27±4.28 Test height: 22.74±1.86	Arm length*=67.7±11.5
Tube foot measurements (in ASW) (mm)		
Stem diameter	0.309±0.054	1.016±0.150
Stem initial length	5.80±2.12	8.23±1.52
Tissue cross-sectional areas (mm^2)		
Stem wall	0.047±0.018	0.647±0.208
Epidermis and nerve plexus	0.018±0.007	0.270±0.110
Connective tissue	0.018±0.008	0.054±0.021
Myomesothelium	0.010±0.004	0.324±0.112
Stem wall cross-sectional area relative to stem cross-sectional area (%)	63.38±16.16	77.4±6.9
Tissue cross-sectional area relative to stem wall cross-sectional area (%)		
Epidermis and nerve plexus	38.5±4.3	41.3±6.7
Connective tissue	39.1±4.7	8.4±2.4
Myomesothelium	22.4±3.8	50.2±7.3

Values are means ± S.D. (*N*=30 unless specified).
*Distance between the centre of the disc and the tip of the longest arm.

inner one in the tube feet of *P. lividus*, but the two sheaths were similar in thickness in those of *M. glacialis* (compare Fig. 2A,B). Within the outer sheath of sea urchin tube feet, the collagen fibres were densely packed and embedded in an electron-lucent matrix (Fig. 2C,E). Each fibre consisted of fibrils with a maximum diameter of 113±32 nm (*N*=20) and separated from each other by 20–40 nm. Within the outer sheath of sea star tube feet, on the other hand, the fibres were more dispersed, being enclosed and separated by an electron-dense network of microfibrils (Fig. 2D,F,G). Each fibre contained fibrils with a maximum diameter of 232±28 nm (*N*=20) and separated from each other by 10–30 nm.

In both species, only the outer sheath of the connective tissue layer contained cells possessing numerous electron-dense granules. These cells were numerous and similar in appearance to the juxtaligamental cells present in echinoderm MCTs. In sea urchin tube feet, the juxtaligamental-like cells were interspersed between the collagen fibres of the outer sheath of the connective tissue layer (Fig. 2A). They had small cell bodies and were filled with electron-dense secretory granules with diameters of ~130 nm (Fig. 2E). In sea star tube feet, these cells appeared as bundles of cell processes with occasional cell bodies, also located in the middle of the outer sheath of the connective tissue layer (Fig. 2B). Two populations of cell processes may be distinguished on the basis of the size of their electron-dense secretory granules: one with granules of ~150 nm in diameter, and one with granules of ~250 nm in diameter (Fig. 2F).

Mechanical properties of the stem

In the first experiment, the effect of five solutions on the mechanical properties of tube feet was tested. Artificial seawater (ASW) was used as a standard solution. ASW-EGTA was used as a calcium-free medium (EGTA is a calcium chelator that removes the endogenous calcium from the tissues). The other three solutions were treatments that disrupted cellular membranes: two made use of the non-ionic detergent Triton-X100, either in the presence (ASW-TX) or absence (ASW-EGTA-TX) of calcium, and the third, deionised water (DW), worked by osmotic shock.

Fig. 3 represents examples of typical J-shaped stress–strain curves obtained for sea urchin and sea star tube feet in three of the five solutions. The tube foot stems of these two echinoderm species seem to have different mechanical properties, echinoid tube feet being apparently stronger, stiffer and tougher than asteroid tube feet.

The whole data set for material properties of the tube feet was analysed by a two-way ANOVA using both individuals and bathing solutions as the independent factors (Table 2). This analysis showed that, in sea urchin tube feet, solutions do not influence extensibility but explain about 12.4% of the variability in initial stiffness and 33–41% of the variability in strength, final stiffness and toughness. Individuals, on the other hand, accounted for about 50% of the variability in tube foot extensibility and between 20 and 30% of the variability in the other mechanical properties. Interactions between the two factors were found for extensibility and both initial and final stiffness. In sea star tube feet, bathing solutions explained only 10% of the variability in tube foot extensibility, but 30–50% of the variability in the other mechanical properties. Conversely, in asteroids, individuals never explained more than 4–17% of the variability in tube foot mechanical properties. Interactions between solutions and individuals were found, however, for all the parameters considered.

In both species, there was no significant difference in tube foot extensibility between samples incubated in ASW-EGTA

and ASW (Fig. 4A,F). However, the treatment with ASW-EGTA significantly decreased the tensile strength, stiffness and toughness of the tube feet in comparison with ASW (Fig. 4B–E,G–J). The tube foot plasticization due to the absence of calcium was fully reversed when sea urchin ambulacra were put back and incubated in ASW after their bath in ASW-EGTA (ASW-EGTA >> ASW) (Fig. 5). Sea urchin tube feet showed similar extensibility and initial stiffness in the three cell-disrupting solutions, with values comparable to those obtained in ASW (Fig. 4A,C). By contrast, the cell-disrupting

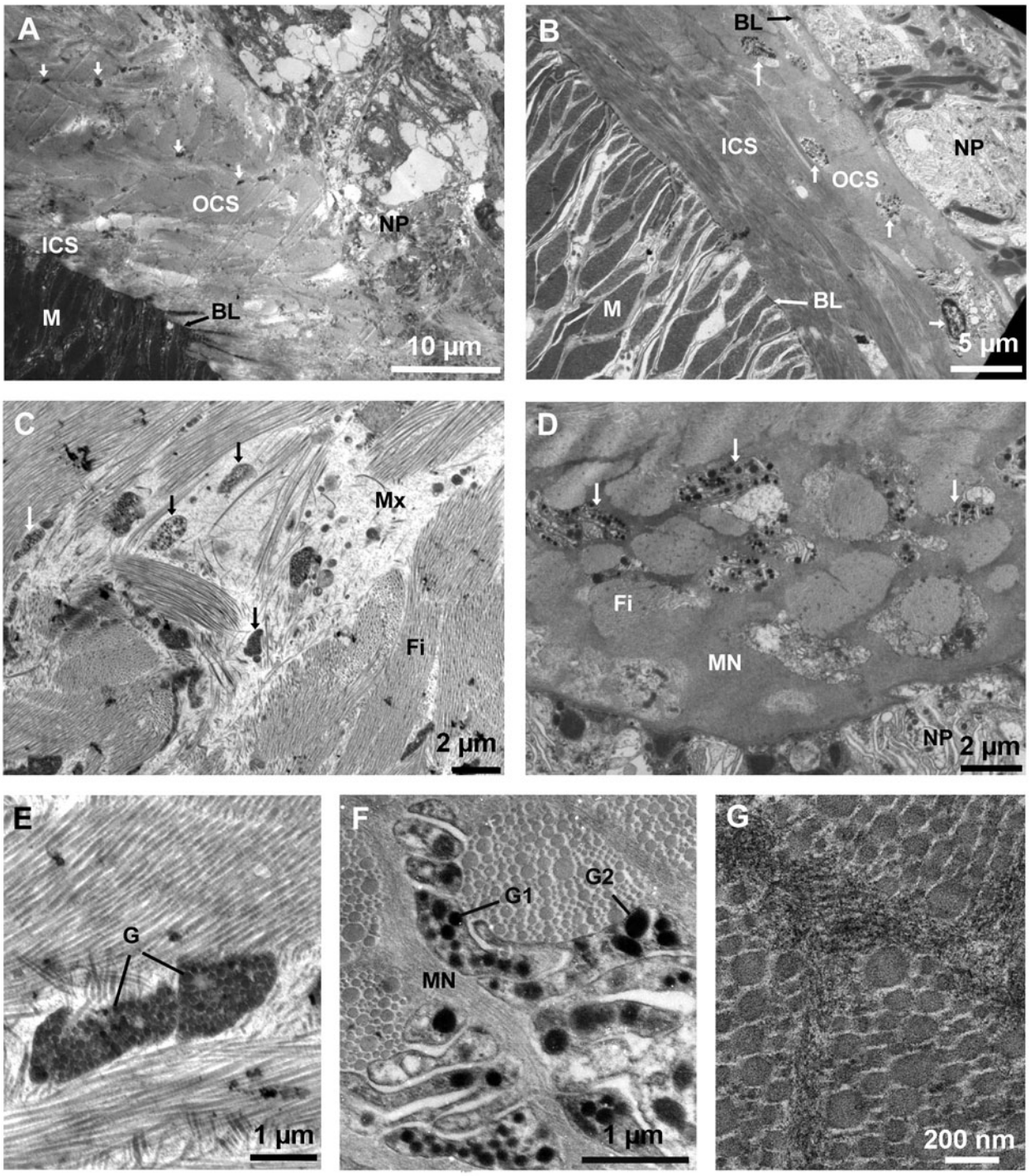

Fig. 2. Ultrastructure (TEM) of the tube foot connective tissue layer in *Paracentrotus lividus* (A,C,E) and in *Marthasterias glacialis* (B,D,F,G). Arrows indicate juxtaligamental-like cells intercalated between collagen fibres. (A,B) General views of a transverse section through the tube foot stem. (C,D) Details of the outer connective tissue sheath. (E,F) Juxtaligamental-like cells. (G) Detail of the microfibrillar network. Abbreviations: BL, basal lamina; Fi, fibres; G, granules; G1 and G2, type 1 and 2 granules; ICS, inner connective tissue sheath; OCS, outer connective tissue sheath; NP, nerve plexus; M, myomesothelium; MN, microfibrillar network; Mx, matrix.

solutions significantly increased tube foot strength and final stiffness in comparison with tissues incubated in ASW, and *a fortiori* in comparison with tissues incubated in ASW-EGTA (Fig. 4B,D). Tube foot toughness was significantly higher in ASW-EGTA-TX, but not in the other cell-disrupting solutions, than in ASW (Fig. 4E). However, stem initial stiffness and toughness were significantly higher in the cell-disrupting solutions than in ASW-EGTA (Fig. 4C,E). In sea stars, tube foot extensibility, strength, final stiffness and toughness were not significantly affected by the three cell-disrupting solutions in comparison with ASW but were significantly affected in comparison with ASW-EGTA (Fig. 4F,G,I,J). Tube foot initial stiffness was significantly higher in the three cell-disrupting solutions than in ASW and ASW-EGTA (Fig. 4H).

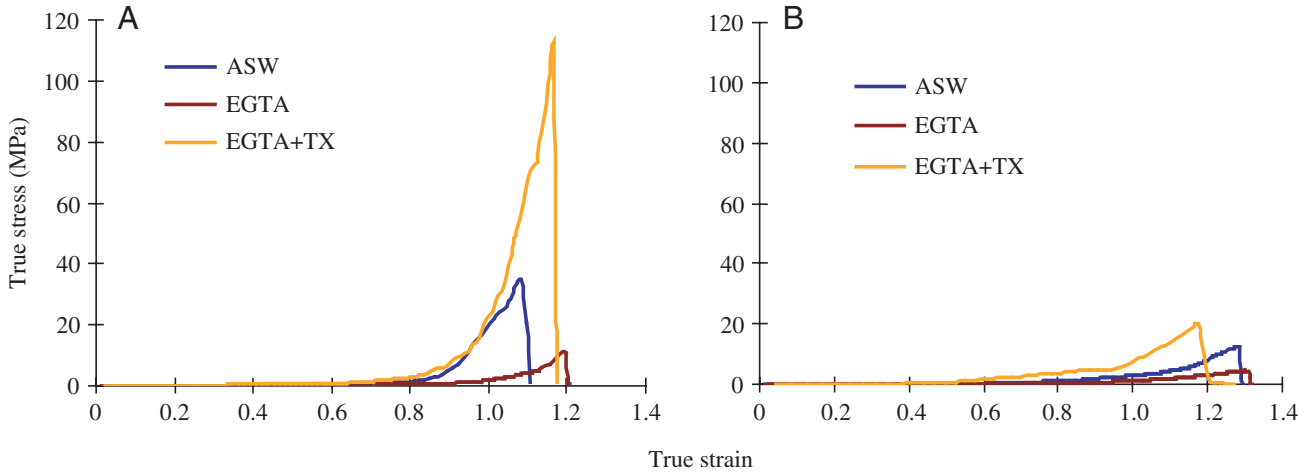

Fig. 3. Selected stress–strain curves for the tube feet of *Paracentrotus lividus* (A) and *Marthasterias glacialis* (B), illustrating stem connective tissue material properties in three different bathing solutions. See text for abbreviations.

Table 2. *Variability (%) in the material properties of echinoid and asteroid tube foot stem explained by the two factors considered (bathing solution and individual)*

	Material properties				
	Extensibility	Strength	Initial stiffness	Final stiffness	Toughness
First experiment					
Paracentrotus lividus					
Solution	0.87*	41.44	12.35	33.05	35.39
Individual	48.68	25.00	19.88	31.46	23.14
Solution × individual	10.46	2.48*	13.83	4.67	2.54*
Residual	39.99	31.08	53.94	30.82	38.94
Marthasterias glacialis					
Solution	10.11	39.27	28.40	50.27	28.64
Individual	16.76	9.91	3.92	8.72	8.57
Solution × individual	21.80	10.13	19.52	8.65	11.62
Residual	51.32	40.68	48.16	32.36	51.17
Second experiment					
Paracentrotus lividus					
Solution	20.75	59.10	14.78	42.45	45.83
Individual	10.04	3.36*	0.91*	3.12*	3.50*
Solution × individual	0.49*	1.16*	3.04*	0.30*	2.84*
Residual	68.73	36.38	81.26	54.13	47.82
Marthasterias glacialis					
Solution	41.31	2.02*	2.55*	13.65	2.84*
Individual	0.12*	15.77	25.55	5.87*	10.58
Solution × individual	3.90*	0.41*	0.74*	7.76*	3.00*
Residual	54.66	81.80	71.16	72.72	83.58

Asterisk indicates non-significant percentages ($P_{2\text{-way ANOVA}} \geqslant 0.05$).

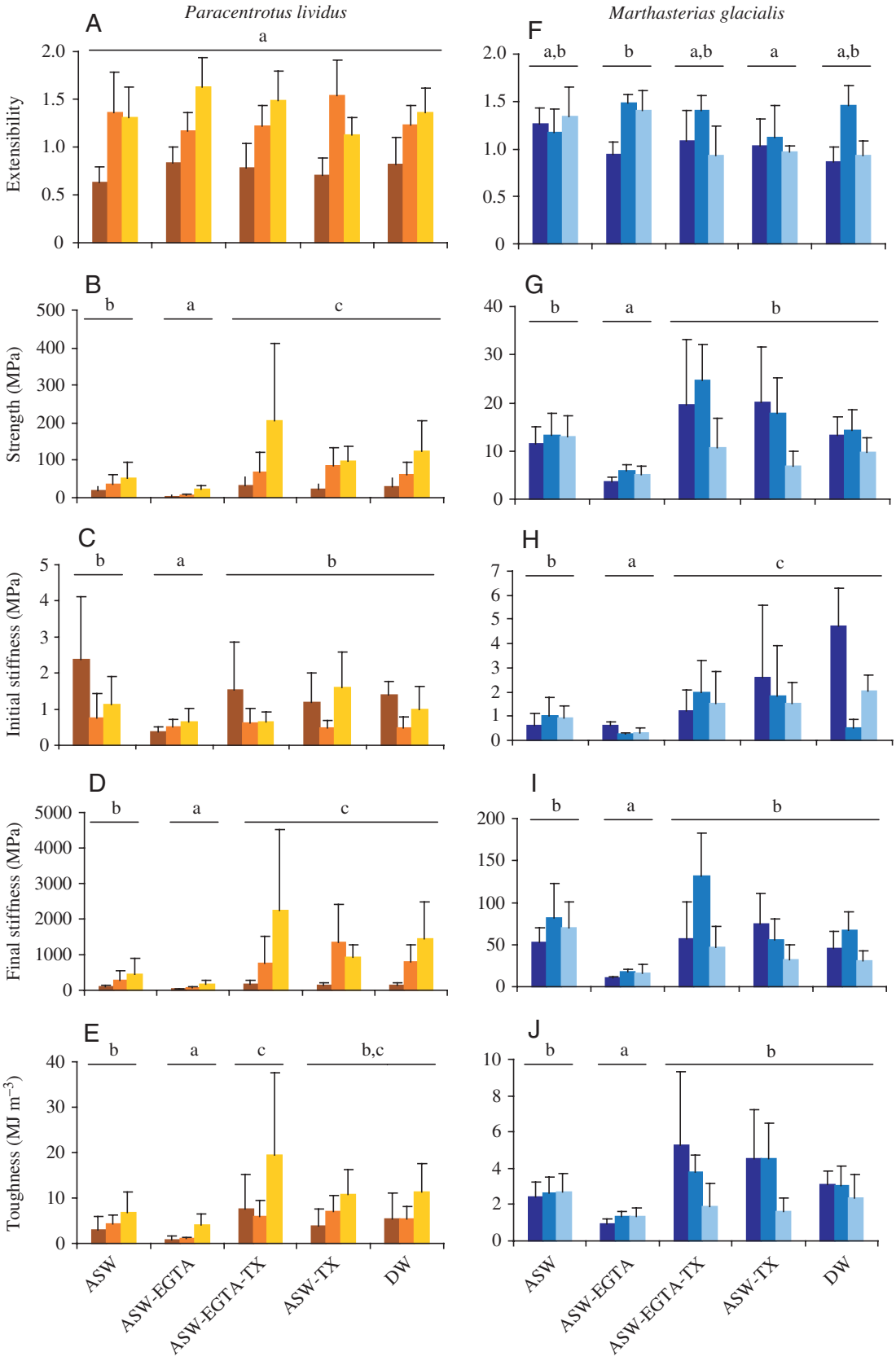

Fig. 4. Variation of the mechanical properties of the tube foot stem of *Paracentrotus lividus* (A–E) and *Marthasterias glacialis* (F–J) with different bathing solutions. Values are means + S.D. for 10 tube feet from one of three animals, each animal being indicated by a different colour. Data analysed using a two-way ANOVA; means sharing at least one letter are not significantly different ($P_{Tukey} \geqslant 0.05$). See text for abbreviations.

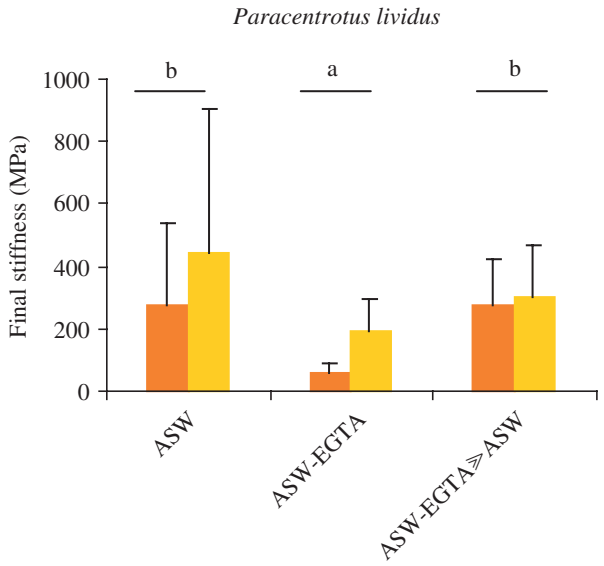

Fig. 5. Variation of the final stiffness of the tube foot stem of *Paracentrotus lividus* with different bathing solutions. Values are means + S.D. for 10 tube feet from one of two animals, each animal being indicated by a different colour. Data analysed using a two-way ANOVA; means sharing at least one letter are not significantly different ($P_{Tukey} \geqslant 0.05$). See text for abbreviations.

In the second experiment, tube feet were anaesthetized in ASW with 1% propylene phenoxetol and then tested in the absence (ASW-PP) and the presence (ASW-PP-TX) of a cell-disrupting agent. In this experiment, the anaesthetising agent was used to make sure all the tube feet were in the same relaxed state at the beginning of the mechanical tests.

The two-way analysis of variance of the whole data set for tube foot material properties (Table 2) showed that, in sea urchins, individuals influence only extensibility, explaining 10% of its variability, whereas solutions explain the largest proportion of the overall variability in tube foot mechanical properties (15–60%). No interactions between the two factors were observed. In sea stars, solutions accounted for 41.3 and 13.7% of the variability in tube foot extensibility and final stiffness, respectively, but did not influence the other properties. The variability in the latter was partly explained (10–25%) by the individuals. Once again, no significant interactions were found between the two factors.

As in the first experiment, sea urchin tube feet showed again higher tensile strength, final stiffness and toughness in the cell-disrupting solution (Fig. 6B,D,E). However, in this experiment, the presence of the cell-disrupting agent also increased the extensibility and the initial stiffness of anaesthetized tube feet (Fig. 6A,C). In sea stars, there was no significant difference between tube feet incubated in the two solutions except for stem extensibility and final stiffness, which decreased in the cell-disrupting solution (Fig. 6F–J).

Discussion

Sea urchin and sea star tube foot stems present the stress–strain behaviour of a typical pliant composite made up of a fibrous phase of collagen and a matrix phase of other proteins and proteoglycans (Vincent, 1990; Vogel, 2003). Such biomaterials show a J-shaped stress–strain curve in which the first, low-modulus, region is usually interpreted as the stretching of the elastomeric matrix and/or the progressive unfolding, reorientation and recruitment of the collagen fibres in the direction of pull; the second, high-modulus, region is attributed to the transfer of load to the aligned collagen fibres (Vincent, 1990). These curves, in which high extensibility is combined with progressive stiffening to produce high toughness, are characteristic of shock-absorbing materials such as mammalian skin and artery (Vincent, 1990; Vogel, 2003). Echinoid and asteroid tube feet are therefore mechanically well-designed to resist hydrodynamic forces generated by waves and currents (see also Santos and Flammang, 2005).

There are, however, several differences between the tube feet of sea urchins and sea stars as far as morphology and mechanics are concerned. In sea stars, the connective tissue cross-sectional area represents only ~10% of the stem wall cross-sectional area as opposed to ~40% in sea urchins. Another major morphological difference lies in the proportion between the outer connective tissue sheath, in which the collagen fibres are longitudinally orientated (Nichols, 1966; Florey and Cahill, 1977), and the inner sheath in which the fibres are arranged as a crossed-fibre helical array (Skyler McCurley and Kier, 1995). In *Paracentrotus lividus*, the former is ~20 times thicker than the latter, while in *Marthasterias glacialis* they are of equal importance. Mechanically, on the other hand, echinoid tube feet are stronger, stiffer and tougher than asteroid tube feet. All these differences probably reflect variations in the functioning of the tube feet. In addition to their role as a holdfast, tube feet in both sea urchins and sea stars are involved in locomotion. Echinoid tube feet function as traction systems, attaching to the substratum and contracting, hence pulling the sea urchin, whereas asteroid tube feet act as struts that are used as levers (Lawrence, 1987). A much more important protraction force is presumably needed in the latter case because the tube feet not only extend to reach the substratum but also lift and support the weight of the animal during locomotion. This protraction force is achieved by an increase in hydrostatic pressure within the water-vascular fluid, and it is the function of the inner connective tissue sheath to resist the hoop stress (which tends to increase diameter) induced by this pressure (Skyler McCurley and Kier, 1995; Vogel, 2003). It is not surprising, therefore, that this sheath is more developed in the tube foot stem of *M. glacialis* than in *P. lividus*. As the collagen fibres in this sheath are likely to be disposed at a high angle to the longitudinal axis of the stem (in the asteroids *Luidia clathrata* and *Astropecten articulatus* this angle is 67° in fully extended tube feet; Skyler McCurley and Kier, 1995), it is unlikely that they resist longitudinal stress, this being the function of the outer sheath of collagen fibres. If this is the case, our values of stem tensile strength and stiffness are underestimated as they were calculated using the total cross-sectional area of the

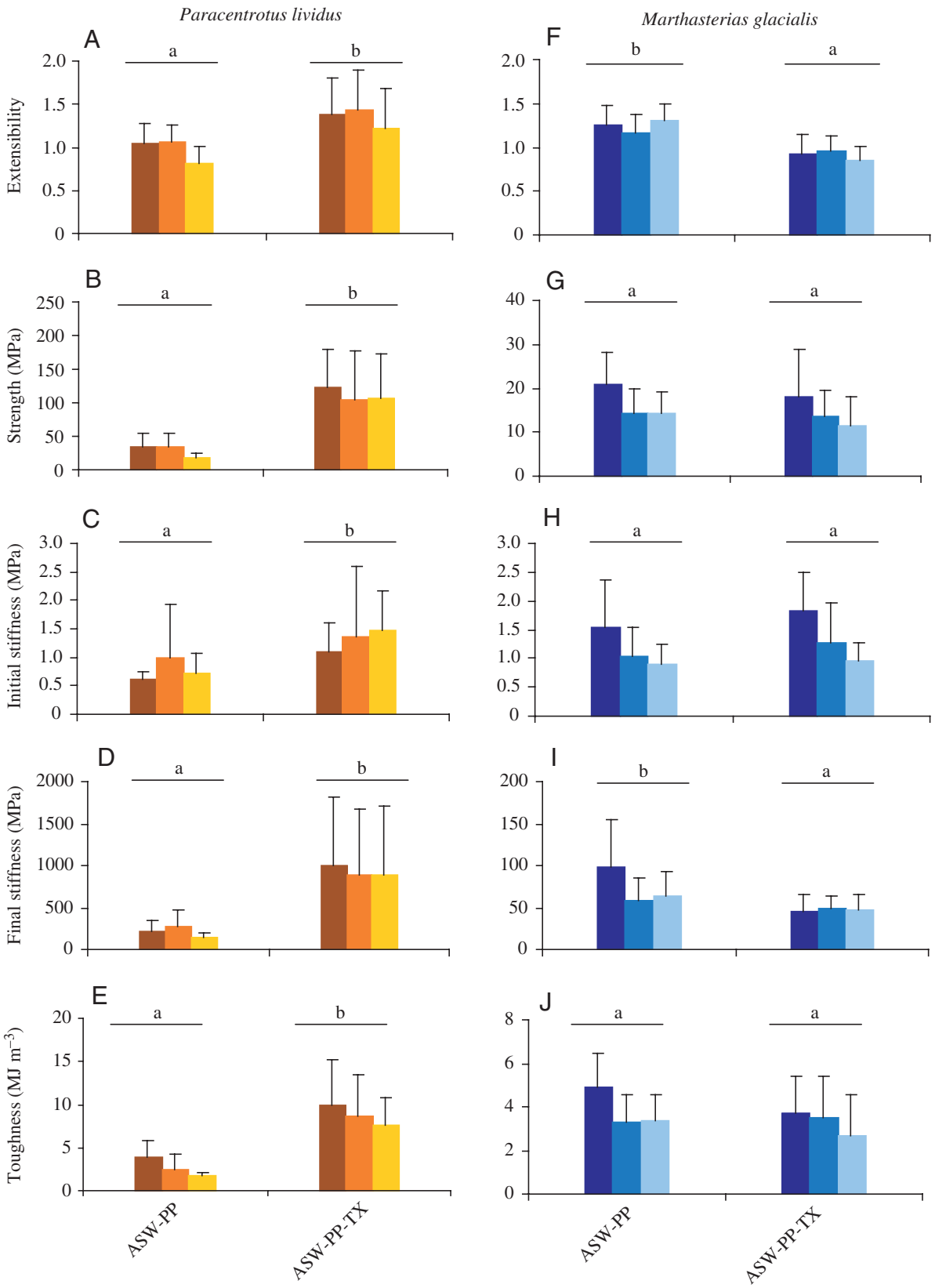

Fig. 6. Variation of the mechanical properties of the tube foot stem of *Paracentrotus lividus* (A–E) and *Marthasterias glacialis* (F–J) with different bathing solutions. Values are means + S.D. for 10 tube feet from one of three animals, each animal being indicated by a different colour. Data analysed using a two-way ANOVA; means sharing at least one letter are not significantly different ($P_{Tukey} \geqslant 0.05$). See text for abbreviations.

whole connective tissue layer and not the cross-sectional area of the outer sheath (see Eqns 2, 3). According to the proportion of both sheaths in the two species, this underestimation is more important for sea star tube feet, meaning that their actual values of strength and stiffness would therefore be much closer to those calculated for the sea urchin tube foot stem. However, estimating the relative proportions of both connective tissue sheaths is difficult in light microscopy, and processing every tube foot for TEM is impracticable. As only intraspecific comparisons have been made in this study, the cross-sectional area of the whole connective tissue layer has been used for calculations of mechanical properties, and the comparisons between the different individuals and treatments used remain valid.

In the tube feet of both species, the outer connective tissue sheath contained a large amount of cell processes with electron-dense granules, which are remarkably similar in appearance to the juxtaligamental cells always present in echinoderm MCTs (Wilkie, 1996). This is initial evidence for the presence of an MCT in sea urchin and sea star tube feet because there is no known MCT that lacks these cells, whereas they are absent from the few definitely non-mutable collagenous structures examined (Wilkie, 2002; Wilkie et al., 2003). There are, nevertheless, differences between sea star and sea urchin tube feet. In the former, two populations of juxtaligamental-like cell processes may be distinguished on the basis of the size of their secretory granules, while in the latter, only one population occurs. The co-occurrence of two or more populations of granule-containing cells has been described in many echinoderm MCTs (Wilkie, 1996).

Although morphology strongly suggests the presence of an MCT in sea urchin and sea star tube foot stems, only mechanical testing can demonstrate this presence unequivocally. In our first experiment, the tube foot stems of *P. lividus* and *M. glacialis* were tested in five treatments known to influence the physiological state and thus the mechanical properties of different MCTs (see Wilkie, 2002 for a review). Mechanical measurements were performed on tube feet whose connective tissue was in its natural, standard state (ASW), depleted of calcium (ASW-EGTA) or exposed to the contents of disrupted cells in the absence (ASW-EGTA-TX; DW) and presence (ASW-TX) of calcium. As previously observed in other MCTs, tube feet from both species became compliant when in a state of calcium depletion, indicating that their mechanical properties are influenced by calcium concentration (Wilkie, 1996; Wilkie et al., 2004). Indeed, previous studies suggested that calcium depletion can inhibit calcium-dependent macromolecular associations that regulate the viscosity of the matrix (Motokawa, 1988; Wilkie, 1988). Although this effect is non-specific (it was also observed in non-mutable collagenous structures; Wilkie et al., 2003), it mimics the soft state of MCTs (Motokawa and Tsuchi, 2003). Cell-disrupting treatments, on the other hand, induce a stiffening response in the two most studied MCTs, i.e. the sea urchin spine ligament (Szulgit and Shadwick, 1994) and the holothuroid dermis (Trotter and Koob, 1995). An identical response was observed in sea urchin tube feet treated with the three cell-disrupting treatments, their stems being consistently stronger and stiffer (final stiffness) in these solutions than in ASW. These solutions, however, had no effect on stem extensibility, initial stiffness and toughness. On the other hand, all mechanical properties (except extensibility) were significantly increased when the tube feet were treated with cell-disrupting solutions in the absence of calcium in comparison with the treatment in ASW-EGTA. This is different from what occurs in the echinoid compass-rotular ligament, a non-mutable collagenous tissue in which mean breaking load was not significantly different in ASW-EGTA and in DW (Wilkie et al., 2003). Cell-disrupting agents had a different action on sea star tube feet. Indeed, tube foot stems of *M. glacialis* bathed in these solutions showed similar extensibility, strength, final stiffness and toughness as those incubated in ASW. Only the initial stiffness increased significantly after cell perforation. However, similar to what occurred in *P. lividus*, all stem mechanical properties were significantly affected by incubation in the calcium-free cell-disrupting solutions in comparison with incubation in ASW-EGTA.

In both species, important animal–animal variability was observed, accounting for as much as 50% of the variation in the mechanical properties measured. Such an interindividual variability has been reported in other mechanical studies of MCTs (e.g. Trotter and Koob, 1995), but this is the first time it has been quantified. To avoid this phenomenon, which masks the effect of the different bathing solutions, some authors have used specimens rested for up to 24 h (Motokawa and Tsuchi, 2003) or anaesthetized specimens (Wilkie et al., 1999). In our second experiment, individuals were anaesthetized to avoid any non-specific stiffening of the connective tissue under nervous control. The mechanical properties of anaesthetized tube feet were similar to those obtained in ASW but with a lower interindividual variability. In *P. lividus*, the addition of the cell-disrupting solution induced once again a significant strengthening and stiffening of the tube feet. Moreover, this time, extensibility, initial stiffness and toughness also increased in this solution. In *M. glacialis*, the lysing agent did not cause strengthening but decreased final stiffness and extensibility.

In both *P. lividus* and *M. glacialis*, the results of the mechanical tests together with the presence of juxtaligamental-like cells in the connective tissue clearly show that an MCT is present in their tube feet. However, the tube foot stems of echinoids and asteroids are affected differently by the cell-disrupting solutions, indicating that their MCTs could be functionally different. This hypothesis is supported by the observation that there is a single type of juxtaligamental-like cell in the connective tissue of echinoid tube feet but two types of cells in asteroid tube feet. The influence of our bathing solutions on the mechanical properties of the tube feet of *P. lividus* matches exactly the influence of similar treatments on sea urchin spine ligament and holothuroid dermis (see, for example, Szulgit and Shadwick, 1994; Trotter and Koob,

1995). These observations suggest that, following cell lysis, a stiffening factor is released from the juxtaligamental-like cells in the extracellular matrix. Recently, such a factor, named tensilin, was isolated from sea cucumber dermis and characterized (Tipper et al., 2003). This is a collagen-fibril binding protein that, when released from the juxtaligamental-like cells, has the ability to induce dermis stiffening (Tipper et al., 2003; Wilkie et al., 2004).

As far as the tube foot MCT of *M. glacialis* is concerned, our observations in the calcium-free solutions indicate that, following cell perforation, a stiffening factor is released into the extracellular matrix. This factor seems to act differently from that of *P. lividus*, having a very limited effect in the presence of calcium. Hence, in sea stars, the standard state of tube foot MCT appears to be mechanically similar to its stiffened state, as evidenced by the lack of effect of cell-disrupting solutions on tensile strength, final stiffness and toughness. Yet, solutions inducing cell lysis have an effect on the tube foot stem extensibility and initial stiffness, two mechanical properties that are presumably directly linked to the elastic network of microfibrils (Vincent, 1990; Thurmond and Trotter, 1996; Vogel, 2003). Moreover, this elastic component of the connective tissue seems to be much more developed in the tube feet of *M. glacialis* than in those of *P. lividus*. Therefore, the asteroid stiffening factor could in some way interact with the microfibrillar network. Interestingly, the possibility that MCT stiffness may be altered by changing the interactions between the collagen fibrils and the microfibrils has already been proposed (Szulgit and Shadwick, 2000). Clearly, more experiments are needed to clarify the functioning of the MCT in asteroid tube feet.

It is certainly an adaptive advantage for both echinoids and asteroids to have tube foot stem connective tissue with mutable mechanical properties. In order to protract the tube foot, ampulla muscles contract to force the ambulacral fluid into the lumen, inducing a gradual stretching of the stem. To retract, the retractor muscles of the tube foot contract, expelling the ambulacral fluid back into the ampulla. When there is a differential contraction of these muscles, the tube foot bends to the more contracted side (Flammang, 1996). Therefore, in the compliant or destiffened state, MCT would deform with low energetic costs, assisting the ampulla muscles during tube foot elongation as well as retractor muscles during retraction and bending. In the stiffened state, MCT would also have a prominent role in the energy-sparing maintenance of position, for example during strong attachment to the substratum to resist hydrodynamically generated loads.

List of symbols and abbreviations

ASW	artificial seawater
DW	deionised water
E	modulus of elasticity
EGTA	ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid (calcium chelator)
F	force
L	length
L_0	initial length
MCT	mutable collagenous tissue
PP	propylene phenoxetol
S	cross-sectional area
TEM	transmission electron microscopy
TX	Triton-X100 (cell-disrupting agent)
ε	true strain
σ	true stress

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