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Abstract: The viscoelastic properties of vertebrate connective tissues rarely undergo significant changes within physiological timescales, the only major exception being the reversible destiffening of the mammalian uterine cervix at the end of pregnancy. In contrast to this, the connective tissues of echinoderms (sea urchins, starfish, sea cucumbers etc.) can switch reversibly between stiff and compliant conditions in timescales of around a second to minutes. Elucidation of the molecular mechanism underlying such mutability has implications for the zoological, ecological and evolutionary field. Important information could raise also for veterinary and biomedical science, particularly regarding the pathological plasticization or stiffening of connective tissue structures. In this investigation we analyzed aspects of the ultrastructure and biochemistry in two representative models, the compass depressor ligament and the peristomial membrane of the edible sea urchin Paracentrotus lividus, compared in three different mechanical states. The results provided further evidence that the mechanical adaptability of echinoderm connective tissues does not imply necessarily changes in the collagen fibrils themselves. The higher GAG content registered in the peristomial membrane with respect to the compass depressor ligament suggested a diverse role of these molecules in the two mutable collagenous tissues. The possible involvement of glycosaminoglycans in the mutability phenomenon will need further clarification. Significant changes in glycosaminoglycan content were detected during the shift from a compliant to a standard condition only in the compass depressor ligament. Similarities in terms of ultrastructure (collagen fibrillar assembling) and biochemistry (two alpha chains) were found between the two models and mammalian collagen. Nevertheless differences in collagen immunoreactivity, alpha chain migration on SDS-PAGE and BLAST alignment highlighted the uniqueness of sea urchin collagen with respect to mammalian collagen.

Milan, 26/09/2014

To
**Dr. S. N. Gorb,**
Editor
ZOOLOGY

Dear Prof. Gorb,

please find enclosed the revised version of the manuscript "**ULTRASTRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF MECHANICALLY ADAPTABLE COLLAGENOUS STRUCTURES IN THE EDIBLE SEA URCHIN *Paracentrotus lividus***".

by Barbaglio A, Tricarico S, Ribeiro AR, Di Benedetto CD, Barbato M, Dessi D, Fugnanesi V, Magni S, Mosca F, Sugni M, Bonasoro F, Barbosa MA, Wilkie IC, Candia Carnevali MD

considered for publication in Zoology.

This new version of the paper has been rearranged following in detail the reviewers' comments (*see the detailed answer to the reviewers*). Thanks to the reviewers' profitable suggestions, the paper now provides a more accurate and understandable analysis of the described results. In particular, the experimental design was better specified in the introduction section and main findings were clearly exposed in the abstract and discussion sections.
During the reviewing process our group published data on the biocompatibility for mammalian cell cultures of newly developed sea urchin collagen matrices. Consequently the following sentence (and the relative reference) was added in the discussion section (lines: 531-535): "Fibrils extracted with this method maintained their structural integrity. An update of this method including decellularization and filtration steps was just published by our group (Di Benedetto et al., 2014). Obtained fibrils were successfully used for producing 2D matrices that showed to be a biocompatibile substrate for mammalian cell cultures (Di Benedetto et al., 2014)."

I hope that you can approve these changes and that this new version of the paper can be now considered appropriate for publication.

Thank you very much in advance.

Yours sincerely,

Alice Barbaglio

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Response to reviewers

# ULTRASTRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF MECHANICALLY ADAPTABLE COLLAGENOUS STRUCTURES IN THE EDIBLE SEA URCHIN *Paracentrotus lividus*

Barbaglio A, Tricarico S, Ribeiro AR, Di Benedetto CD, Barbato M, Dessi D, Fugnanesi V, Magni S, Mosca F, Sugni M, Bonasoro F, Barbosa MA, Wilkie IC, Candia Carnevali MD

## Here you find a point by point response (in blue) to **Reviewer 1**:

Reviewer 1 did not suggest any further modification of the manuscript, as reported below.

Reviewer 1: In this new version Barbaglio and co-authors significantly improved their manuscript. The authors' efforts to improve the manuscript must be acknowledged especially concerning the reformulation of the statistical analysis and re-interpretation of the results. I agree with referee 2 in saying that this manuscript doesn't contain major novelties concerning MCTs and the authors themselves agree in this point; however, a huge amount of experiments and technical approaches were used that co-substantiate previously published results but also led to some new findings that open new doors for future experiments. Therefore, although this is not an outstanding manuscript, it is well executed and well-written, it is susceptible of raising interest in the MCTs community and in my opinion should be published in Zoology.

## Here you find a point by point response (in blue) to **Reviewer 2**:

The approach still needs to focus on clear hypotheses, and a more clear analysis of how the experimental work addresses those hypotheses. The paper would be much improved with such a focus. To this end, the introduction section should be revised substantially. At present, it just gives general background on the field, and suggests that a detailed analysis of the CDL and PM will find something interesting. This is too unfocused. The introduction section should clearly identify the major questions that they intend to address. Their overall goal is to determine what aspects of the connective tissue are responsible for mutability. Two alternatives are that the collagen changes or the GAG's change. The following sentence was added (lines 122-124): "*Specifically, this paper aimed to evaluate two aspects of PM and CDL that are candidate responsible for mutability: changes in collagen fibril ultrastructure and biochemistry and/or changes in the GAG content*."

They should then summarize published evidence for or against these alternative hypotheses. The introduction section needs to make it clear that the currently

published evidence is insufficient to form a final conclusion. Then it should state, as directly as possible, how their work will serve to answer the question with more certainty. It should be clear that their experimental design is sufficient to answer the questions. Once this is clear, the reader can more easily evaluate the possible significance of the work. Previous papers regarding GAGs and collagen modifications were cited and the importance of the present paper was explicated also with a detailed presentation of our experimental strategy and expected results (lines 124-153): *"MCT collagen was previously characterized in several models (Matsumura, 1974; Pucci-Minafra and Minafra, 1978; Smith et al., 1981; Hidaka and Takahashi, 1983; Burke et al., 1989; D'Alessio et al., 1990; Exposito et al., 1992; Wilkie et al., 1992; Trotter and Koob, 1994; Trotter et al., 1994; Cluzel et al., 2004). Nevertheless, data concerning biochemical changes associated with mutability rarely looked at collagen changes (Ribeiro et al., 2012). Namely, we expect to demonstrate if collagen bands on SDS-PAGE are different in the different mechanical states of the PM and CDL. A change in the intensity and/or position of collagen bands on SDS-PAGE would give a qualitative idea of possible changes in collagen protein structure, such as changes in the assembling of the α chains, higher/lower potential of common collagen denaturing agents (acetic acid and pepsin) of interchain crosslinks of collagen fibrils. Also when ultrastructure is concerned, previous literature analyzing in details collagen modifications (banding pattern of collagen fibrils, interfibrillar distances) associated with mutability is poor, mainly referring to the work of Hidaka and Takahashi (1983) on the sea urchin spine ligament. No other data are available related to ultrastructural measurements of MCT collagen in different mechanical states. Adding two new models (PM and CDL) to this scenario could help clarifying if collagen modifications are implied in the mutability process.*

*Whilst fibrillar collagen is obviously the dominant structural component of MCTs, there is consistent ultrastructural and biochemical evidence that GAGs are a component of interfibrillar linkages (Wilkie, 2005), although, in view of conflicting data from vertebrates, the contribution of GAGs to the tensile properties of MCTs is not predictable (see, e.g. Fessel and Snedeker, 2009; Ahmadzadeh et al., 2013; Rigozzi et al., 2013). Literature analyzing the role of GAGs on mutability are again referred to their characterization in only one mechanical state (Trotter and Koob, 1989; Erlinger et al., 1993; Kariya et al., 1997), rarely looking at changes in GAG content in different mechanical states (Kariya et al., 1990). Our recent investigation of GAG content in the CDL did not reveal major changes during viscoelastic changes (Ribeiro et al., 2012). Nevertheless, a high inter-individual variability could have masked some differences. In order to clarify this scenario we decided to increase the number of samples analyzed, to perform the analysis in order to detect GAGs with different sulphation degrees (using alcian blue solution at different pHs) and to compare CDL and PM results in order to verify if a common pattern of GAG modifications is present in the two models."*

Related to this, it is not clear how the SDS-PAGE and Western blotting results provide a test of a hypothesis involving mutability. The importance of SDS-PAGE data was explicated in the introduction (lines 129-134). Western blotting was not important for mutability. This analysis was performed only in order to further characterize MCT collagen. It is commonly said that MCT collagen resembles mammalian collagen, but its immunoreactivity against mammalian antibodies was not tested before. The intention to compare sea urchin and mammalian collagen was specified in the introduction (lines: 155-157): "*Finally, in order to further characterize similarities and differences between sea urchin and mammalian collagens we compared their respective SDS-PAGE running pattern as well as their immunoreactivity (western blot and immunofluorescence)*." In comparing biochemical data for CDL and PM collagens and mammalian collagen, what would constitute evidence in support of the hypothesis that collagen is involved in mutability, and what would constitute evidence leading one to reject it? Again, two different things were tested. First, CDL and PM similarities/differences in collagen biochemical pattern (SDS-PAGE bands) allowed to verify if two MCT models with a different spatial distribution of MCT components also displayed differences in the biochemical composition of collagen fibrils. Second, the comparison of CDL and PM collagen biochemical profile with that of mammalian collagen (SDS-PAGE and western blot) allowed to detect similarities between mammalian and sea urchin collagen and to hypothesize if collagen diversity could explain the loss of mutability in mammalian connective tissues. Once it is clear what hypotheses they are testing, and how their experiments will provide data that is relevant to testing these hypotheses, the discussion section can then focus on whether or not the evidence leads them to support or reject their hypotheses.

There are some flaws in their experimental logic. The authors detected variations in the D period in the CDL, but not the PM. They view this inconsistency as evidence that elastic change in collagen is not important. This should be viewed in the context of formal experimental logic. One hypothesis is that changes in collagen are responsible for mutability. The data from CDL offers some support for this hypothesis, but the data from PM leads them to reject the hypothesis. That's not strong enough to reject the hypothesis outright. Perhaps there are a variety of changes that give rise to mutability; several may work in concert, and every tissue does not have to behave exactly the same. In summary, they can conclude that collagen changes are not an absolute prerequisite for mutability in all tissues, but they cannot reject a role outright. Actually, we did not reject the hypothesis of an involvement of collagen ultrastructural changes, as stated in the discussion (lines: 467-469): "*Our finding that there was no significant difference in the D period of PM collagen fibril between different mechanical states implies that the change of fibril D period registered in CDL is not a prerogative for all MCTs, but is a characteristic of this particular MCT*") and in the abstract (lines: 55-57): "*provided further evidence that the mechanical adaptability of echinoderm connective tissues does not imply necessarily changes in the collagen fibrils themselves*").

A similar situation applies to their analysis of GAG's. Again, there were changes in the CDL, but not in the PM. In this case, unlike their analysis of the collagen data, they claim that this supported a role for GAG's in mutability. This should be addressed more formally. The hypothesis is that changes in GAG's are responsible for mutability. The evidence from the CDL supports this in some cases, but the evidence from the PM should lead them to reject the hypothesis. In the end, the situation is still unclear. To better clarify the main findings of our research work, the final sentence of the abstract was changed as follows: "*The possible involvement of glycosaminoglycans in the mutability phenomenon will need further clarification. Significant changes in glycosaminoglycan content were detected during the shift from a compliant to a standard condition only in the compass depressor ligament.*" Similarly, the discussion was implemented adding this sentence (lines 620-622): "*Anyway, the lack of significant changes in GAG content in the tree mechanical states of the PM suggested that these changes are not a prerequisite for mutability*."

Finally, the Abstract needs to summarize specific results, not just unspecified "similarities and differences" between collagens and "differences in GAG content". A more precise statement of results will allow readers to judge the potential value of the work. Similarities and differences between sea urchin and mammalian collagens were explained more precisely adding the following sentence (lines 62-66): "*Similarities in terms of ultrastructure (collagen fibrillar assembling) and biochemistry (two alpha chains) were found between the two models and mammalian collagen. Nevertheless differences in collagen immunoreactivity, alpha chain migration on SDS-PAGE and blast alignment highlighted the uniqueness of sea urchin collagen with respect to mammalian collagen*.". The sentence "differences in GAG content between the two models suggested a diverse role of these molecules in the two mutable collagenous tissues" was changed as follows: "*The higher GAG content registered in the peristomial membrane with respect to the compass depressor ligament suggested a diverse role of these molecules in the two mutable collagenous tissues.*"

# ULTRASTRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF MECHANICALLY ADAPTABLE COLLAGENOUS STRUCTURES IN THE EDIBLE SEA URCHIN *Paracentrotus lividus*

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**The present manuscript has not been published elsewhere and it has not been submitted simultaneously for publication elsewhere.**

***Declaration of interest:*** *The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.*

ABSTRACT

The viscoelastic properties of vertebrate connective tissues rarely undergo significant changes within physiological timescales, the only major exception being the reversible destiffening of the mammalian uterine cervix at the end of pregnancy. In contrast to this, the connective tissues of echinoderms (sea urchins, starfish, sea cucumbers etc.) can switch reversibly between stiff and compliant conditions in timescales of around a second to minutes. Elucidation of the molecular mechanism underlying such mutability has implications for the zoological, ecological and evolutionary field. Important information could raise also for veterinary and biomedical science, particularly regarding the pathological plasticization or stiffening of connective tissue structures. In this investigation we analyzed aspects of the ultrastructure and biochemistry in two representative models, the compass depressor ligament and the peristomial membrane of the edible sea urchin *Paracentrotus lividus*, compared in three different mechanical states. The results provided further evidence that the mechanical adaptability of echinoderm connective tissues does not imply necessarily changes in the collagen fibrils themselves. The higher GAG content registered in the peristomial membrane with respect to the compass depressor ligament suggested a diverse role of these molecules in the two mutable collagenous tissues. The possible involvement of glycosaminoglycans in the mutability phenomenon will need further clarification. Significant changes in glycosaminoglycan content were detected during the shift from a compliant to a standard condition only in the compass depressor ligament. Similarities in terms of ultrastructure (collagen fibrillar assembling) and biochemistry (two alpha chains) were found between the two models and mammalian collagen. Nevertheless differences in collagen immunoreactivity, alpha chain migration on SDS-PAGE and BLAST alignment highlighted the uniqueness of sea urchin collagen with respect to mammalian collagen.

KEYWORDS

Mutable collagenous tissue, echinoderm, collagen, glycosaminoglycan, sea urchin

[7]

[7] ABBREVIATIONS:
ECM = Extracellular Matrix
MCT = Mutable Collagenous Tissue
CDL = Compass Depressor Ligament
PM = Peristomial Membrane
GAG = Glycosaminoglycan
PP = Propylene phenoxetol
ACh = Acetylcholine
ASW= Artificial Sea Water
TEM = Transmission Electron Microscope
LM = Light Microscope
CSLM = Confocal Scanning Laser Microscope
SDS-PAGE = Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
WB = Western Blot
PBS = Phosphate Buffered Saline
EDTA = Ethylenediaminetetraacetic acid
TRITC = Tetramethylrhodamine-Isothiocyanate
DAPI = 4',6-diamidino-2-phenylindole
PTA = Phosphotungstic Acid
TBS = Tris Buffered Saline
HRP = Horseradish Peroxidase
FTIR = Fourier Transform Infrared Spectroscopy

## 1. INTRODUCTION

The term ‘mutability’ has been applied to the extreme and rapid changes in mechanical properties shown by certain connective tissue structures (Wilkie, 2005). An example of mutability is the reversible destiffening of the mammalian uterine cervix that allows delivery at the end of pregnancy: this phenomenon is under hormonal control and involves the degradation as well as the remodeling of the extracellular matrix (ECM) (Timmons et al., 2010). By way of contrast, in the mutable collagenous tissues (MCTs) of echinoderms (sea urchins, starfish, etc.) these changes are controlled by the nervous system (with different neuronal pathways for stiffening and softening mechanisms; Birenheide et al., 1998), and are accomplished by molecular mechanisms within the ECM that leave intact the main structural components (Ribeiro et al., 2012). As a consequence, the time scale of such processes in echinoderm MCTs is much faster (from under 1 s) than in the analogous tissues of other animals (hours or days in mammals) (Wilkie, 2005). Furthermore MCTs also display a wider range of expression of mutability potential: they can even completely lose their consistency, permitting the detachment of body parts (autotomy) that are then regenerated (Candia Carnevali and Bonasoro, 2001). MCTs show species- and tissue-specific characteristics (in terms of the amount and organization of different components and of response rate to specific agents), but they share the same main structural and cellular elements (collagen, microfibrils, juxtaligamental cells) and effector molecules (Santos et al., 2005).

MCT biology currently attracts considerable attention, because of its evolutionary, physiological and ecological implications, and because of its potential relevance for biomaterials engineering and biomedical science. Learning how to manipulate connective tissue mechanical properties could be of relevance to the cosmetic industry (*e.g.* anti-aging treatments), the pharmaceutical industry (*e.g.* drugs able to interfere with pathological alterations of connective tissue viscoelastic properties) and the biomaterials industry (*e.g.* dynamic cell culture substrates, mutable scaffolds for implants and surgical procedures).

The animal used in this investigation was the common sea-urchin *Paracentrotus lividus*, an appropriate species because it has established and well defined MCT structures at several anatomical locations (compass depressor ligaments, spine ligaments, peristomial membrane, etc.) and because, as an experimental model, it represents an economically and ecologically advantageous source of material: *P.lividus* has edible gonads, which are collected by the food industry, and the rest of the body, including the MCTs, is a waste product, which can be exploited for experimental procedures (*e.g.* collagen extraction). Here we present results derived from the compass depressor ligaments (CDLs) and peristomial membrane (PM). Each animal has ten CDLs, which both stabilize the position of the dental apparatus ("Aristotle's lantern") and contribute to its role as a respiratory pump (Wilkie et al., 1992). The PM is a flexible region of body wall, which supports the lantern and connects it to the hard calcified test (Wilkie et al., 1994). The PM consists of a three-dimensional network of collagen fibrils, microfibrils, fibroblast-like cells, phagocytes and juxtaligamental cells. The CDL has a simpler organization dominated by parallel collagen fibres (Wilkie et al., 1992; Ribeiro et al., 2011). Since the PM and CDLs are mutable collagenous structures that demonstrate organizational and functional differences, it was anticipated that a quantitative comparison of their ultrastructural organization and biochemical characteristics, might shed light on essential features underpinning mutability (Barbaglio et al., 2012). Specifically, the present paper aim to evaluate two aspects of PM and CDL that are candidate responsible for mutability: changes in collagen fibril ultrastructure and biochemistry and/or changes in the GAG content. MCT collagen was previously characterized in several models (Matsumura, 1974; Pucci-Minafra and Minafra, 1978; Smith et al., 1981; Hidaka and Takahashi, 1983; Burke et al., 1989; D'Alessio et al., 1990; Exposito et al., 1992; Wilkie et al., 1992; Trotter and Koob, 1994; Trotter et al., 1994; Cluzel et al., 2004). Nevertheless, data concerning biochemical changes associated with mutability rarely looked at collagen changes (Ribeiro et al., 2012). Namely, we expect to demonstrate if collagen bands on SDS-PAGE are different in the different mechanical states of the

PM and CDL. A change in the intensity and/or position of collagen bands on SDS-PAGE would give a qualitative idea of possible changes in collagen protein structure, such as changes in the assembling of the α chains, higher/lower potential of common collagen denaturing agents (acetic acid and pepsin) of interchain crosslinks of collagen fibrils. Also when ultrastructure is concerned, previous literature analyzing in details collagen modifications (banding pattern of collagen fibrils, interfibrillar distances) associated with mutability is poor, mainly referring to the work of Hidaka and Takahashi (1983) on the sea urchin spine ligament. No other data are available related to ultrastructural measurements of MCT collagen in different mechanical states. Adding two new models (PM and CDL) to this scenario could help clarifying if collagen modifications are implied in the mutability process.

Whilst fibrillar collagen is obviously the dominant structural component of MCTs, there is consistent ultrastructural and biochemical evidence that GAGs are a component of interfibrillar linkages (Wilkie, 2005), although, in view of conflicting data from vertebrates, the contribution of GAGs to the tensile properties of MCTs is not predictable (see, e.g. Fessel and Snedeker, 2009; Ahmadzadeh et al., 2013; Rigozzi et al., 2013). Literature analyzing the role of GAGs on mutability are again referred to their characterization in only one mechanical state (Trotter and Koob, 1989; Erlinger et al., 1993; Kariya et al., 1997), rarely looking at changes in GAG content in different mechanical states (Kariya et al., 1990). Our recent investigation of GAG content in the CDL did not reveal major changes during viscoelastic changes (Ribeiro et al., 2012). Nevertheless, a high inter-individual variability could have masked some differences. In order to clarify this scenario we decided to increase the number of samples analyzed, to perform the analysis in order to detect GAGs with different sulphation degrees (using Alcian blue solution at different pHs) and to compare CDL and PM results in order to verify if a common pattern of GAG modifications is present in the two models.

Finally, in order to further characterize similarities and differences between sea urchin and mammalian collagens we compared their respective SDS-PAGE running pattern as well as their immunoreactivity (western blot and immunofluorescence).

## 2. MATERIAL AND METHODS

### *2.1 Specimen collection*

Experimental animals (*Paracentrotus lividus*) were collected along the Ligurian coast of Italy according to national legislation (numbers and period of the year). They were kept in tanks of aerated artificial seawater (ASW: Instant Ocean, Aquarium Systems) at 18° C in the University of Milan and fed with commercial pellets (Wenger Manufacturing, Inc; patent n° 085115204).

### *2.2 Experimental treatments*

In order to compare CDLs and PMs in different functional states (compliant, standard and stiff), specimens were subjected to three different treatments, as described in (Wilkie et al., 1992). To obtain the "compliant" condition, the lower half of the experimental animals, which includes the dental apparatus, PM and CDLs, was immersed in 0.1% propylene phenoxetol (PP: Sigma Aldrich 484423) in ASW for 45 min. To obtain the "stiff" condition, the lower half of the experimental animals was submerged in 1 mM acetylcholine chloride (ACh; Sigma Aldrich 6625) in ASW for 15 min. To obtain the "standard" condition, half animals were kept in ASW alone for 45 min (standard 45', which served as controls against PP-treated animals) or 15 min (standard 15', which served as controls against ACh-treated animals). It should be noted that, as well as changing the passive mechanical properties of the PM and CDLs, PP and ACh also bring about dimensional changes in their collagenous frameworks through actions on muscular components of the lantern (as is explained further in the 'Results'). We chose to induce the different functional states in CDLs and

PM left *in situ*, rather than use excised tissues, in order to mirror as closely as possible the natural situation, where CDLs and PM tend to undergo simultaneous changes in dimension and stiffness. After the respective treatments, samples were processed for the different analyses described below.

*2.3 Light microscopy*

The oral halves of *P.lividus* specimens in different mechanical states were fixed with 4% paraformaldehyde in PBS, decalcified with EDTA (7% in 2% paraformaldehyde in PBS) and post-fixed with Bouin's fixative, washed with tap water and finally with distilled water. PM and CDLs were successively excised, dehydrated with an ethanol series (25-70-90-95-100%), washed in xylene and then with a 1:1 mixture of xylene and paraffin. Finally they were embedded in paraffin. Sections 10 μm thick were cut with a Reichert OmE microtome, stained with Milligan's Trichrome (Milligan, 1946) and observed with a Jenaval light microscope provided with a Panasonic GP-KR222 camera.

*2.4 Transmission electron microscopy*

The oral halves of *P.lividus* specimens in different mechanical states were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer and 1.4% NaCl for 2 hours, washed overnight with 0.1 M cacodylate buffer, post fixed with 1% $OsO_4$ in 0.1 M cacodylate buffer and washed in distilled water. PM and CDLs were excised and prestained with 2% uranyl acetate in 25% ethanol. Samples were then dehydrated with an ethanol series (25-70-90-95-100%), washed with a 1:1 mixture of propylene oxide and Epon Araldite 812 and finally embedded in Epon Araldite 812. Ultrathin sections (70-100 nm) were cut with a Reichert Ultracut E ultramicrotome; CDLs were cut transversely and longitudinally (parallel to the long CDL axis); PMs were cut parallel to the radius. The ultrathin sections were stained by conventional methods (uranyl acetate-lead citrate), carbon coated with a EMITECH K400X Carbon Coater and observed with a LEO /912AB transmission

electron microscope. Measurements of collagen fibril D period and diameter were performed with Adobe Photoshop CS3 software.

*2.5 Immunohistochemistry*

The oral halves of *P.lividus* specimens in different mechanical states were fixed with 100% acetone (1h) and washed with PBS (3 x 10 min). CDLs were excised, washed with PBS, dehydrated with an ethanol series (25%, 50% in PBS, 70% in distilled water) and maintained in ethanol 70% at 4°C until used. Rehydrated samples were permeabilized with PBTT (0.1% Tween20 and Triton X100 in PBS) and washed with PBS (3 x 10min). Then they were blocked (1.5 h) with 10% goat serum in PBTT (0.05% Tween20 and Triton X100 in PBS) and immunostained (1:50, overnight, 4°C) with a mouse anti-bovine collagen type I antibody (SIGMA C2456) in 5% goat serum in PBTT (0.05% Tween20 and Triton X100 in PBS). CDLs were successively washed with PBS (6 x 10min), with 1% BSA n PBTT (0.05% Tween20 and TritonX100 in PBS) and again with PBS (2x 10min). A goat anti-mouse TRITC antibody (SIGMA) in 5% goat serum in PBTT was applied (1:200, overnight, 4°C). Finally CDLs were washed with PBS (4 x 10 min), stained with DAPI (1:500 in PBS, 20 min), washed again in PBS (2 x 10min) and mounted on glass slides with glycerol 80% in PBS. Slides were observed with a CSLM Leica TCS-NT. Controls were samples from which the primary antibody was omitted.

*2.6 Collagen extraction*

The extraction of intact collagen fibrils was performed according to a modification of the protocol of Matsumura (1974) that implies the use of a reducing agent (β-mercaptoethanol). PM and CDL samples, excised from the oral halves of *P.lividus* specimens in different mechanical states, were weighed (wet weight) and immersed in a disaggregating solution of 0.5 M NaCl, 0.05 M EDTA-Na, 0.1 M Tris-HCl buffer (pH 8.0) and 0.2 M β-mercaptoethanol (1:20 wet weight/volume). The

suspension was stirred for 2 days at RT, filtered through Nylon gauze (PM samples only) and then centrifuged (10000 g; 1h; 4°C). The pellet was resuspended in a solution containing 0.5 M EDTA-Na (pH 8.0) and centrifuged (10000 g; 1h; 4°C). The pellet was resuspended in distilled water. For TEM analyses, an aliquot of the sample was deposited on Formvar-coated grids and stained with 0.5% K-PTA in distilled water (1 min) and observed with a JEOL SX100 TEM.

For biochemical analyses, the extracted collagen fibrils in distilled water were centrifuged (10000 g; 1h; 4°C) and the pellet was solubilized in a 1mg/ml solution of pepsin in 0.5 M acetic acid for 48 h at 4°C (1:20 wet weight/volume). Samples were then centrifuged (17000 g; 1h; 4°C). For TEM analyses, an aliquot of the supernatant was deposited on Formvar-coated grids and stained with 0.5% K-PTA in distilled water (1min) and observed with a JEOL SX100 TEM. The supernatant was collected, NaCl was added to make a final concentration of 5M and the mixture was shaken for 24h at 4°C. Then samples were centrifuged (16000g; 1h; 4°C) and the pellet resuspended in distilled water for direct analyses or in 0.5M acetic acid to preserve it at 4°C. In the last case, samples were dialyzed overnight against distilled water before performing SDS-PAGE analyses. Collagen extraction was performed also exposing directly CDL and PM samples to a 1mg/ml solution of pepsin in 0.5 M acetic acid for 48 h at 4°C (1:20 wet weight/volume). Samples were then centrifuged (17000 g; 1h; 4°C). The supernatant was collected, NaCl was added to make a final concentration of 5M and the mixture was shaken for 24h at 4°C. Then samples were centrifuged (16000g; 1h; 4°C) and the pellet resuspended in distilled water for SDS-PAGE analyses.

Collagen extraction was performed on CDL sets (10 CDLs from one sea urchin; one sea urchin per mechanical state) and on single PMs (1 PM from one sea urchin; one sea urchin per mechanical state).

The solubility in pepsin/acetic acid of the fibrillar collagen extracted with Matsumura's protocol was measured by the Sirius red method (Taşkiran et al., 1999). Briefly, collagen extracted with the

Matsumura's disaggregating solution and washed with EDTA and distilled water, was lyophilized, weighed (the percentage of dry with respect to the PM/CDL wet weight represents the insoluble collagen extraction efficiency) and resuspended in a 1 mg/ml solution of pepsin in 0.5 M acetic acid for 48 h at 4°C (1:20 dry weight/volume). Then it was centrifuged (17000g, 1h) and 50 µl of supernatant was mixed with 100 µl of 0.5M acetic acid and 1.35 ml of a 50 µM Sirius red solution in acetic acid 0.5 M. After 20 min at RT, absorbance was red at 528 nm. 0.5M acetic acid was used as a blank. A calibration curve was produced using collagen type I from rat tail (SIGMA C7661) at known concentrations mixed with the same Sirius red solution. The solubilization efficiency was calculated as the percentage of solubilized collagen with respect to the dry weight of the Matsumura extracted collagen.

*2.7 SDS-PAGE & WB analyses*

Collagen extracts (Matsumura's protocol + solubilization with acetic acid and pepsin) were quantified using the BCA protein assay (Thermo Scientific Pierce) before proceeding with SDS-PAGE (Laemmli, 1970). Following the quantification step, equal amounts of each extract were mixed with sample buffer (0.3 M Tris-HCl, pH 6.8, 13% glycerol, 1 % SDS, 0.005% bromophenol blue, 0.35 M β-mercaptoethanol), heated at 95 °C for 5 min and run over a 10% precast gel (Bio-Rad) at a constant voltage of 150 V. Rat tail (SIGMA C7661) as well as human collagen type III (Abcam ab7535) were run in parallel with CDL and PM collagen extracts. 10 µl of Precision *Plus Protein*™ Dual Color *Standards* (Bio-Rad) was used for molecular weight determination. The separated proteins were visualized by staining with Coomassie Blue R-250. Alternatively, transfer of proteins (250 mA for 2.5 h) from unstained gels to nitrocellulose membranes (Bio-Rad) for Western blot analysis was performed employing a Bio Rad blot module. Membranes were washed with milliQ water, TBST, blocked with milk 5% in TBST and immunostained with mouse anti-bovine collagen type I (1:125- 1:500; SIGMA C2456) or mouse-anti-human collagen type III

(1:250; SIGMA C7805) antibodies in 5% milk in TBST (overnight, 4°C). After a few washes in TBST, goat anti-mouse HRP secondary antibodies were applied (1:10000, 40 min, 4°C, SIGMA). Signal was detected exposing the membranes to ECL™ Western Blotting Detection Kit (GE Healthcare) and labeled proteins were visualized using Autoradiography (X-ray) films (UltraCruz sc-201696).

*2.8 GAG quantification*

The GAG quantification protocol was a modification of Björnsson's (1998). As for collagen extraction, each CDL sample comprised the complete set of 10 CDLs from one experimental animal. GAGs were extracted from CDLs and PM (excised from the oral halves of *P.lividus* specimens in different mechanical states) after immersion in 4 M guanidine-HCl for 15 min, at 4°C. Samples were centrifuged (12000 g, 2 min), then 100 µl of the supernatant were diluted with 100 µl of SAT-reagent (0.3% $H_2SO_4$ / 0.75 % Triton X – 100) for 15 min at 4°C. GAGs were then precipitated overnight at 4 °C with 1.5 ml of different Alcian blue reagents: pH 1.4 (0.1% Alcian blue in 0.1 % $H_2SO_4$/0.02 M guanidine-HCl/0.25% Triton X - 100), pH 0.2 (0.1% Alcian blue in 0.2 % $H_2SO_4$/0.02M guanidine-HCl/0.25% Triton X - 100), pH 5.6 (0.1% Alcian blue in 0.1mN NaOH/0.02 M guanidine-HCl/0.25% Triton X - 100). GAGs were collected by centrifugation (16000g, 20 min). The excess stain and contaminating proteins were removed by washing the pellet with 1 ml of 40 % DMSO - 0.05 M $MgCl_2$ for 15 min at 4°C. Samples were centrifuged (16000g, 20 min) and the pellets, containing the proteoglycan-Alcian blue complexes, were then immersed in guanidine-HCl/propanol (4 M guanidine-HCl/33% 1-propanol/0.255 Triton X – 100 / 0.1% Ficoll) until completely dissolved. After dissociation of the complex, the GAG concentration was determined spectrophotometrically (λ= 605 nm) using the corresponding calibration curve built with chondroitin sulfate sodium salt from shark cartilage (Sigma-Aldrich C4384). The amount of GAG/PGs is directly proportional to the Alcian blue concentration. The sulphated GAG

concentration was normalized against the wet tissue weight. Alcian blue at low pH (0.2) binds to highly sulfated GAGs, whereas at higher pH (1.4, 5.6) binds to medium and weakly sulfated GAGs (Hayat, 1993).

### *2.9 Statistics*

Statistical analyses were performed with Microsoft Excel ™ (Microsoft Corp., Redmond, Washington, United States), Graph Pad Prism 4 (Graph Pad Software, Inc., San Diego, CA, USA) and SPSS 15.0 (SPSS Inc,) softwares. Raw data were analyzed for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). If normality and homoscedasticity were verified, data were compared with ANOVA (more than two groups) /t-test (two groups). If normality and/or homoscedasticity were not verified, data were log-transformed, tested again for normality and homoscedasticity and, when both conditions were verified, compared with parametric tests: ANOVA (more than two groups) /t-test (two groups). If normality and/or homoscedasticity were not verified even for log transformed data, not parametric tests were applied: Kruskal-Wallis test (more than two groups)/Mann Whitney test (two groups).

If significant differences between individuals exposed to the same treatment were found, means per each individual were used when comparing different treatments instead of putting all together the measurement of different individuals exposed to the same treatment.

Data were expressed as mean ± standard deviation (SD).

## 3. RESULTS

### *3.1 General anatomy*

The general anatomy of the lantern is illustrated in Fig. 1. The CDLs connect the compass ossicles to the perignathic girdle. They are at their shortest length when the compass ossicles are fully

depressed, as when the lantern is treated with PP. They extend by a maximum of around 25% when the compasses are raised by contraction of the compass elevator muscles, as during treatment with ACh (Wilkie et al., 1992). On the other hand, the radial length of the PM is shortest in ACh and longest in PP. In the course of this investigation it was calculated that the PM radial length extends by a maximum of 24 % in PP with respect to ACh treated PM.

*3.2 Ultrastructure*

The histological structure of the CDL and PM is illustrated in Fig. 2 which emphasizes their typical collagenous matrices. Ultrathin sections were used to quantify the distribution of collagen fibril diameters (Figs. 3, 4) and the length of the fibril D period (repetitive banding pattern) in the different mechanical conditions (Figs. 3, 5).

Collagen fibril diameters were measured and subdivided by frequency distribution analysis (Graph Pad Prism 4 software) into 18 (CDL) and 37 (PM) "classes" (10 nm, 20 nm, etc.) plotted against the relative frequency (%) of fibrils lying in each class. It is clear from these plots (Fig. 4) that the diameter varied widely (CDL range: 11 – 184 nm; PM range: 20 – 410 nm) and had a unimodal distribution with a long right tail. The most represented was the 50 nm class for all the mechanical states of the CDL (Fig. 4A), whereas in the PM the most represented class varied between 60 nm and 90 nm depending on the mechanical state (Fig. 4B).

Inter-individual variability was analyzed comparing specimens exposed to the same treatment. Both considering CDL (N = 2 specimens per mechanical state; from 392 to 899 measurements per each specimen) and PM (N = 3 specimens per mechanical state; from 99 to 542 measurements per specimen), normality tests showed that diameter measurements were not normally distributed for all the specimens analyzed, even when log transformed (data not showed). Thus, a non parametric test was used to compare different specimens exposed to the same treatment. Kruskal-Wallis test revealed differences between specimens exposed to the same treatment (data not showed).

Consequently, when comparing different treatments, means for each specimen were used instead of putting altogether measurements of different specimens exposed to the same treatment.

Variances of CDL mean diameter values (table 1) resulted not homogeneous, thus a non parametric test was used in order to compare them. No statistically significant differences were registered between different mechanical states (Kruskal-Wallis test = 0.500, p = 0.919).

Means of PM samples (table 1) resulted normally distributed and their variances homogeneous, thus a parametric test was used to compare them. No statistically significant differences were registered between different mechanical states (ANOVA test = 1.226, p = 0.362).

In the CDL, the percentage of collagen fibrils in the high diameter classes was generally higher for compliant samples, whereas the percentage of collagen fibrils in the low diameter classes was higher for stiff samples. The contrary was registered in the PM (Fig. 4B).

The mean measured D period for the CDL and the PM are summarized in table 2. Inter-individual variability was analyzed comparing specimens exposed to the same treatment. Both considering CDL (N = 3-4 specimens per mechanical state, from 4 to 150 measurements per specimen) and PM (N = 3 specimens per mechanical state, from 3 to 46 measurements per specimen) D period values, normality tests showed that measurements were not normally distributed for all the specimens analyzed, even when log transformed (data not showed). Thus, a non parametric test was used to compare different specimens exposed to the same treatment. Kruskal-Wallis test revealed differences between specimens exposed to the same treatment (data not showed). Consequently, when comparing different treatments, means for each specimen were used instead of putting altogether measurements of different individuals exposed to the same treatment.

Means of CDL samples resulted normally distributed and their variances homogeneous, thus a parametric test was used to compare them. The analysis of D period length in the CDL (Fig. 5A) revealed a significantly lower value for this parameter in the compliant respect to the standard 45' state (ANOVA test: F = 4.560, p = 0.029, Tukey's multiple comparison test: p = 0.025).

Means of PM samples resulted normally distributed and their variances homogeneous, thus a parametric test was used to compare them. In the PM the analysis of D period length (Fig 5B) did not reveal significant differences in this parameter between the different experimental treatments (ANOVA test: $F = 1.243$, $p = 0.356$).

No statistically significant differences were found between PMs and CDLs D period in each of the four different mechanical states (data not showed).

Collagen fibril density was measured in the PM (Fig. 6) counting the number of collagen fibrils present in sectioned areas with fixed dimensions (1600 x 1600 nm) and fixed TEM magnification (8000x). Normality test showed that measurements were not normally distributed for all the specimens analyzed, even when log transformed (data not showed). Thus, a non parametric test was used to compare different specimens exposed to the same treatment. Kruskal-Wallis test revealed differences between specimens exposed to the same treatment (data not showed). Consequently, when comparing different treatments, means for each individual were used instead of putting altogether measurements of different individuals exposed to the same treatment.

Means of PM samples (N = 3 specimens per mechanical state; from 5 to 9 measured areas per specimen) resulted normally distributed and their variances homogeneous, thus a parametric test was used to compare them. No differences were detected between mechanical states (ANOVA test: $F = 0.111$, $p = 0.951$).

*3.3 Immunohistochemistry*

The antibody used for this analysis recognizes the native triple helix of bovine collagen type I. This antibody was chosen to verify cross-reactivity between mammalian and sea urchin collagen, taking the CDL as a representative collagenous tissue. Controls were not labeled. In treated samples positive labeling could be discerned in the form of parallel alignments of punctate structures (Fig. 7).

*3.4 Collagen extraction, SDS-page and WB analyses*

The collagen extracted with Matsumura's (1974) protocol was 51 ± 5.3 % of the CDL wet weight (range: 36%-64%, N=5). The remaining 49% of the CDL wet weight was mainly water and cell remnants plus other minor components. Acetic acid/pepsin solubilized 7.8% ± 1.5% of the dry weight of the collagen extracted with the Matsumura protocol (range: 5.1% -13.1%, N=5). No indication of CDL digestion was noted if pepsin was omitted. In the PM, the collagen extracted with Matsumura's (1974) protocol was 28 ± 2.6 % of the wet weight (range: 21%-35%, N=5). 50% of the PM wet weight was mainly water and cell remnants plus other minor components, whereas the remaining percentage was represented by calcareous material. Acetic acid/pepsin solubilized 0.36 ± 0.05 % of the dry weight of the collagen extracted with the Matsumura protocol (range: 0.23% - 0.49 %, N=5). No indication of PM degradation was noted if pepsin was omitted.

Extracted and solubilized collagen (Matsumura's protocol + acetic acid & pepsin solubilization) was observed in the TEM: a network of thin unbanded filaments ca. 30 nm in diameter could be observed (Fig. 8A). Insoluble collagen fibrils extracted with Matsumura's (1974) protocol (and not solubilized with acetic acid + pepsin) were observed in the TEM: fibrils were well preserved and a clear banding pattern could be detected (Fig.8B). The mean D period of these fibrils was 59.9 ± 4.25 nm for the CDL and 64 ± 2.8 nm for the PM.

CDL and PM collagen extracts run on SDS-PAGE showed two major bands at around 140 and 120 kDa, the former being almost double the latter in staining intensity (Fig. 9). Two more bands were detected at around 270 kDa and smaller bands were present at molecular weight ≤ 100 kDa, especially samples extracted directly with acetic acid/pepsin. These smaller bands are likely to be digested collagen fragments (Trotter et al., 1995; Robinson, 1997; Quinones et al., 2002). Rat tail

collagen type I showed bands at 130-115 kDa ($\alpha_1$ and $\alpha_2$ chains respectively), at 235-215 kDa ($\beta$ chains: $2\alpha_1$ and $\alpha_1 + \alpha_2$ chains respectively) and at 350 kDa ($2\alpha_1 + \alpha_2 = \gamma$ chain).

CDL and PM collagen extracted from animals treated with experimental solutions (PP, ACh, ASW) showed the same bands on SDS-PAGE (Fig. 10).

WB analysis performed with anti-bovine collagen type I antibody against CDL and PM collagen extracts showed a thick labeled band in the 140-120 kDa region and recognized $\alpha$, $\beta$, and $\gamma$ chains of rat tail collagen (Fig. 11A). WB analysis performed with anti-human collagen type III antibody against CDL and PM collagen extracts showed no labeled bands; rat tail collagen type I was also not recognized, whereas human type III collagen was labeled by the antibody (Fig. 11B).

*3.5 GAG quantification:*

GAG ‰ concentration data are summarized in Table 3 and plotted in Fig.12. Considering GAG concentration values of CDL and PM, statistical tests showed that measurements were not normally distributed and/or variances homogeneous for all experimental groups (pH or mechanical state), even when log transformed (data not showed). In this cases, non-parametric tests were used.

Statistically significant differences were detected in CDL sets between compliant and standard 45' samples using Alcian blue at pH 5.6 (Kruskal Wallis test = 13.33, $p = 0.004$, Dunn's multiple comparison test: $p < 0.01$) and between pH 1.4 and pH 5.6 in standard 45' samples (Kruskal Wallis test = 11.19, $p = 0.0037$, Dunn's multiple comparison test: $p < 0.01$) and stiff samples (ANOVA $_{\text{log-transformed data}}$: $F = 3.669$, $p = 0.047$, Tukey's multiple comparison test: $p = 0.039$).

No differences were detected in PMs at different pH or in a different mechanical state.

Comparing the PM and CDL it was evident that the PM had a higher GAG content than did the CDL. This difference became statistically significant at pH 0.2 between standard (45' and 15') PMs and CDLs (t-test: $t_{std45'} = 5.127$, $p_{std45'} = 0.001$, $t_{std15'}=5.278$, $p_{std15'}<0.001$), at pH 1.4 between compliant PMs and CDLs (t-test: $t = 2.293$, $p< 0.05$), at pH5.6 between standard (45' and 15') PMs

and CDLs (t-test $_{std45°}$: t = 5.442, p <0.001; t-test $_{std15°}$: t = 3.511, p = 0.003) as well as stiff PMs and CDLs (Mann Whitney test : $U_{stiff}$ = 7.000, $p_{stiff}$ =0.007).

## 4. DISCUSSION

### *4.1 Ultrastructure*

Our investigation explored the ultrastructural modifications by which echinoderm MCTs undergo both dimensional and tensile changes.

Previous work showed that the stretching of sea urchin spine ligaments results in tighter packing of the collagen fibrils but does not change the fibril banding pattern (Hidaka and Takahashi, 1983). In contrast, we showed here that in compliant CDLs the D period was significantly reduced. Differently from Hidaka and Takahashi (1983), we obtained these data from ligaments exposed to the experimental solutions *in situ,* thus more reliably mimicking natural conditions. Nonetheless, the D period shortening registered in compliant samples (compliant samples have a D period 6% shorter than stiffened samples) could not be held responsible for the total deformation of CDL (CDL elongates 25% of its length when stimulated with $K^+$; Wilkie et al., 1992) This means that, although an elastic deformation of collagen fibrils is present, this is not sufficient to explain the morphological modifications related to CDL mutability. Other mechanisms, mainly interfibrillar sliding, are primary candidates. Our finding that there was no significant difference in the D period of PM collagen fibril between different mechanical states implies that the change of fibril D period registered in CDL is not a prerogative for all MCTs, but is a characteristic of this particular MCT. The dimensional change undergone by all MCTs occurs more likely by interfibrillar slippage, as suggested by previous authors (Hidaka and Takahashi, 1983, Wilkie, 2005). In vertebrates, both D period deformation and inter-fibril sliding are available mechanisms for tendon stretching (Rigozzi et al., 2013). The 6% deformation of the D period is in accord with data published on mammals

(rats) where the triple helix could go through a 10% elastic deformation in stretched tendons (Silver, 2009).

Results obtained with the CDL of *P.lividus* confirmed a packing of collagen fibrils in stiffened samples (Ribeiro et al., 2011), as previously reported by Hidaka and Takahashi (1983). We found no significant differences in the fibril densities of PMs after the three different treatments. The denser packing of collagen fibrils in stretched CDLs was attributed to the movement of water out of the interfibrillar compartment, a process that could facilitate the strengthening of interfibrillar cohesion in CDLs (Ribeiro et al., 2012). PMs in different mechanical states showed no differences in fibril packing emphasizing the fact that fibril packing is not strictly necessary for mutability.

Both CDL and PM demonstrated a unimodal distribution of collagen fibril diameters, as was also documented in echinoid spine ligaments and holothurian dermis (Smith et al., 1981; Trotter et al., 1994). This contrasted with the usually bimodal distribution of collagen fibril diameters in mammalian tendons and ligaments. A unimodal distribution is typical of more compliant matrices like interstitial connective tissues of parenchymatous organs (Ottani et al, 2001). In echinoderms this could serve to increase the basal deformability of their connective tissues in comparison with mammalian tissues. The collagen fibril diameter range was wider in the PM than in the CDL. Larger fibrils may help to confer a higher tensile strength, whereas smaller fibrils would increase creep resistance because their higher surface/volume ratio would enhance the formation of interfibrillar bonds (Ottani et al, 2001). This fits with the different tensile stresses experienced by the PM and the CDL, the latter undergoing mainly creeping (uniaxial deformation), the first facing multidirectional stresses.

The mean collagen D period of standard CDLs (std 45’ = 62 nm, std 15’ = 57 nm) differed from the main D period reported for other sea urchin tissues (*P.lividus* Aristotle’s lantern, 64 nm, Pucci-Minafra and Minafra, 1978; *Strongylocentrotus purpuratus* peristomial membrane, 44 nm, Burke et

al., 1989), from that observed in other echinoderm MCTs (holothurian dermis, 67 nm, Matsumura, 1974; crinoid ligaments, 52.8 nm, Burke et al., 1989) and from the mammalian D period (64 -67 nm; Silver, 2009). The PM mean D period (std 45' = 64 nm, std 15' = 62 nm) is within the range of previously studied *P.lividus* tissues (Pucci-Minafra and Minafra, 1978) and of mammalian tissues (Silver, 2009). Differences in D period could be related to specific differences in the amino acid sequence of the collagen molecules present in different tissues of the same animal and of different animals: several collagen sequences have been described in sea urchins (Burke et al., 1989, Saitta et al., 1989; D'Alessio et al., 1990; Exposito et al., 1992; Aouacheria et al., 2004, Cluzel et al., 2004). The uniqueness of sea urchin collagens with respect to other echinoderms and vertebrates was confirmed by biochemical and immunological data. Concerning the latter, we detected aligned punctate structures in *P.lividus* CDL using an antibody against bovine type I collagen. This pattern of labeling could have resulted from the presence of molecules (glycosaminoglycans and proteoglycans) on the surface of the collagen fibrils, which partly masked the epitope. Alternatively, punctate staining may have arise from discrete epitope specificity of the monoclonal antibody used for this analysis. The effectiveness of the antibody on extracted and denatured sea urchin collagen cannot clarify which hypothesis is correct. Immunohistochemistry on GAG-depleted tissues will have to be performed in the future. The importance of collagen-related molecules will be discussed below.

At last, it is worth to notice that ultrastructural data revealed a high inter-individual variability. In fact, significant differences were detected between individuals exposed to the same treatment (*i.e.* in the same mechanical state). This is a common characteristics of MCTs (Wilkie, 2005, Ribeiro et al., 2011, 2012) that could mask some relevant physiological changes between different mechanical states. Nevertheless, the experimental strategy adopted in the present paper was chosen on the basis of previous experimental work showing its ability to induce a clear and reproducible physiological response in *P.lividus* CDLs (Wilkie et al., 1992; Wilkie, 2005; Ribeiro et al., 2011, 2012). The

variability registered by the ultrastructural analysis underlines the need to compare different types of data (physiological, morphological, biochemical) in order to get a reliable and comprehensive view of the mutability phenomenon.

*4.2 Collagen biochemistry*

In contrast to holothurian MCTs, from which collagen can be extracted by simply washing the tissue with EDTA and distilled water, the effective extraction of *P.lividus* collagen required β-mercaptoethanol, as reported for other sea urchins (Trotter et al., 1994). Fibrils extracted with this method maintained their structural integrity. An update of this method including decellularization and filtration steps was just published by our group (Di Benedetto et al., 2014). Obtained fibrils were successfully used for producing 2D matrices that showed to be a biocompatibile substrate for mammalian cell cultures (Di Benedetto et al., 2014). Previous studies showed that a high percentage of collagen is present in an acid-insoluble form in a *P.lividus* skeletal tissue (Aristotle's lantern; Pucci-Minafra and Minafra, 1978). This implied the presence of strong cross-links stabilizing the collagen matrix in *P.lividus* connective tissues. It is noticeable that PM insoluble collagen was less solubilized by the following exposure to pepsin than CDL collagen, suggesting that a higher number of cross-links is present in the PM. Also GAG-collagen interactions are thought to be involved in determining collagen solubility: in holothurians, once separated, both collagen and GAGs become acid-soluble (Trotter et al., 1995).

Concerning *P.lividus* collagen biochemistry, we confirmed the presence of two main chains in the CDL and PM, suggesting their collagen molecule is an heterotrimer in which there are two $\alpha_1$ – one $\alpha_2$ chains, as in vertebrate type I collagen (Brodsky and Persikov, 2005). The presence of two chains and their relative quantities was confirmed chromatographically in *Eucidaris tribuloides* by Trotter and Koob (1994). Despite the presence of two different chains in SDS-PAGE, the position of the $\alpha_1$ and $\alpha_2$ bands differed slightly between sea urchins and mammals. This was noted before in

other *P.lividus* tissues (Aristotle's lantern; Pucci-Minafra and Minafra, 1978) and in other sea urchins (*S.purpuratus* collagen chains, 120 and 63 kDa, Burke et al., 1989; *Hemicentrotus pulcherrimus* collagen chains, 140 kDa, 120 kDa, 90kDa, Tomita et al., 1994) and suggests differences in the respective amino acid sequences. Biomolecular analyses with BLAST and BLASTp tools (Table 4) revealed that homology between *P.lividus* collagen and mammalian type I collagen is not significantly higher than that observed between *P.lividus* collagen and other vertebrate collagens, as previously reported by Exposito et al. (2010). The same was noted for *H.pulcherrimus* collagen and underlines the uniqueness of sea urchin collagen (Tomita et al., 1994). Using Western blot analyses we showed that MCT collagen is recognized by an antibody against mammalian type I collagen, whereas no reactivity was detected with the antibody against collagen type III. This provides further confirmation that *P.lividus* collagen is in part similar to mammalian collagen type I, as evidenced by our recent findings (Ribeiro et al., 2012). Nevertheless, we previously observed small differences in some regions of the FT-IR spectra (CDL collagen - mammalian collagen type I) possibly due to the presence of more than one collagen type in CDL (Ribeiro et al., 2012). Collagen chains $\alpha_5$ (Cluzel et al., 2004) and $\alpha_3$ (Minafra et al., 1975) were identified in other *P.lividus* tissues. This is not unexpected: mature mammalian connective tissues contain heterotypic collagen fibrils consisting of more than one collagen type (Canty and Kadler, 2002; Shoulders and Raines, 2009). The uniqueness of sea urchin collagen is also indicated by the presence of a specific N-propeptide domain in the $\alpha_2$ chain (SURF domain; Exposito et al., 1992) identified in *S.purpuratus*. More generally, a high variability was registered also among the collagens of different echinoderm classes: antibodies against holothurian collagen did not react with sea urchin collagen and their collagen is a homotrimer (Trotter et al., 1995). Holothurian MCTs generally show a wider range of tensile states than do echinoid MCTs (Trotter and Koob, 1989). It might be relevant that human pathologies involving progressive weakness of connective tissues involve an increase in the presence of a homotrimeric collagen type I in place of the physiological

heterotrimer (Nicholls et al., 1984; Sasaki et al., 1987; Vouyouka et al., 2001). Whilst this might suggest that there could be a relationship between the molecular structure of collagen and the mutability phenomenon, it needs to be stressed again that MCTs are highly represented in both holothurians and echinoids, despite the differences in collagen biochemistry and immunoreactivity. Furthermore, this investigation found no differences in collagen biochemistry between different mechanical states and between different MCTs (PM and CDL), as we previously showed using Raman spectroscopy (Ribeiro et al., 2012). This suggests that the homotrimeric collagen of holothurians MCTs could explain their greater mechanical adaptability in comparison with those of sea urchins, but is not a prerequisite for the mutability process.

*4.3 GAGs*

Tensilin is a small protein characterized in holothurians that induces a shift from the compliant to the standard condition in holothurian dermis (Tipper et al., 2003). We previously isolated the sequence encoding a tensilin-like protein in *S.purpuratus* and a partial tensilin-like sequence in *P.lividus* (Tricarico et al., 2012). Tensilin effects viscoelastic changes only on MCT in which GAGs are not depleted (Tipper et al., 2003). Highly sulfated chondroitin sulfate GAGs regularly distributed along collagen fibrils were identified in sea urchin, crinoid and sea cucumber MCTs (Trotter and Koob, 1989; Erlinger et al., 1993; Kariya et al., 1990, 1997). A 77 kDa and a 49 kDa PG strongly bound to collagen were identified in the *P.lividus* dental apparatus, their amino acid composition and sugar content resembling those of keratan sulfate PGs (Minafra et al., 1980). Recently Ribeiro et al. (2012) detected a chondroitin sulfate peak in *P.lividus* CDL FT-IR spectra. Concerning the influence of GAGs on the viscoelastic changes, the spectrophotometric quantification of GAGs revealed that the PM had a higher GAG content than the CDL. This could be due to the different spatial arrangement of collagen fibrils in the two MCTs. In the CDL, collagen fibrils are all parallel, whereas in the PM collagen fibrils form a three-dimensional network

where they are likely to contact more adjacent fibrils than is the case in the CDL. Thus, in the PM a higher GAG content could be necessary to realize the higher number of interfibrillar bridges needed to maintain collagen fibrils in their reciprocal positions. Furthermore, the PM is subjected to deformation in different directions, whereas the CDL has to face mainly unidirectional tensile stresses. A higher GAG content could help the PM respond to multidirectional stresses, as has been suggested for mammalian models (Koob and Vogel, 1987). In comparison with the PM, the CDL showed greater variability in the GAG concentrations at different pH values and in different mechanical states. This may reflect the presence of a higher diversity of GAGs in the CDL. A general trend was detected towards a higher content of medium sulfated GAGs (quantified at pH1.4) in standard and stiff samples (significant in std 45' and stiff samples). Although not significant, an opposite trend was observed in compliant samples. There appeared to be relevant differences between the GAG concentrations of compliant and standard 45' CDLs at all three pH values, although only that at pH 5.6 was statistically significant. This suggests the possibility that in the CDL the compliant-standard shift, but not the standard-stiff shift, is accompanied by an alteration in GAG sulphation or composition, with the further implication that separate molecular mechanisms might be involved in the stiffening and destiffening processes in the CDL. However, our previous investigation of sulphated GAGs in *P. lividus* CDLs showed no evidence that their composition was affected by their tensile state, although high inter-individual variation may have masked any correlation (Ribeiro et al., 2012). As discussed above, the high variability registered also in our samples confirmed the possibility that significant differences could be partially masked by inter-individual variability. Furthermore, the use of only chondroitin sulfate for the construction of calibration curves could have hidden further important differences. Anyway, the lack of significant changes in GAG content in the tree mechanical states of the PM suggested that these changes are not a prerequisite for mutability.

The contribution of GAGs to vertebrate connective tissue mechanics is still under discussion. Bailey et al. (1982) suggested a lubricant role for GAGs in holothurian collagenous tissues where they would allow slippage of adjacent fibrils. In mammals changes in both the quality and quantity of proteoglycans have been demonstrated when a modification of connective tissue mechanics is required (*e.g.* at the level of the uterine cervix during pregnancy and labour; Westergren-Thorsson et al., 1998). We are currently conducting biomechanical, biochemical and ultrastructural analyses of CDL and PM depleted of sulfated and unsulfated GAGs in different mechanical states. We expect them to better clarify GAG role in MCT functioning.

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FIGURE LEGENDS

Figure 1

*P.lividus* oral half view. A) schematic drawing showing PM in red, CDLs in green. PG = perignathic girdle; AL= Aristotle's lantern. B) Section through the dental apparatus showing PM and one of the ten CDLs. Milligan's trichrome. Pr = Protractor muscle, PG = perignathic girdle, RM = retractor muscle, IM = interpyramidal muscles.

Figure 2

General organization of the CDL (A) and PM (B). LM histological sections. Milligan's trichrome staining. LM. A) Detail of a transverse sections of a CDL. B) Detail of a section parallel to the radius of a PM. ME = coelomic myoepithelium, CE = perivisceral coelomic epithelium, CM = collagen matrix, EP = epidermis.

Figure 3

TEM ultrathin sections of CDL (A-H) and PM (I-P). Longitudinal sections: A, C, E, G, I, K, M, O. Cross sections: B, D, F, H, J, L, N, P. The larger diameter of PM fibrils respect to CDL fibrils is evident. No clear differences could be detected looking at both fibril banding pattern and diameter comparing different mechanical states. A, B, I, J) compliant samples; C, D, K, L) standard samples (ASW 45'); E, F, M, N) standard samples (ASW 15'); G, H, O, P) stiff samples.

Figure 4

Collagen fibril diameter in CDL (A) and PM (B) in different mechanical states. No statistically significant differences were detected. PM showed a wider diameter distribution respect to CDL.

Figure 5

Collagen fibril banding pattern (D period) in CDL (A) and PM (B) in different mechanical states.

Figure 6

Collagen fibril density in PMs in different mechanical states. No differences were detected between mechanical states.

Figure 7

CSLM. Immunohistochemistry of CDL treated with anti-bovine collagen type I antibody. Spotted fibrous-like structures were labeled. Nuclei in blue.

Figure 8

Extracted CDL collagen fibrils. TEM. A) Acid-pepsin soluble collagen. A loose network of unbanded filamentous structures could be recognized. B) Insoluble collagen. Fibril periodicity remained unchanged respect to the *in vivo* situation.

Figure 9

SDS-PAGE. Comparison between rat tail collagen type I with CDL and PM collagen extracts.

Figure 10

SDS-PAGE. Comparison of CDL (A) and PM (B) collagen extracts derived by specimens in different mechanical states.

Figure 11

WB analyses. Anti-collagen type I immunolabeling of CDL and PM collagen extracts. B) Anti-collagen type III immunolabeling of CDL and PM collagen extracts..

Figure 12

GAG quantification (Alcian Blue) in CDLs and PMs in different mechanical states and at different Alcian blue pHs. Statistically significant differences are summarized in table 2.

**Table revised**

TABLES

| | | CDL | PM |
|---|---|---|---|
| **Mechanical state** | **compliant** | 59.36 ± 1.64 nm<br>(range: 14.00 – 153.1 nm)<br>N = 2 | 113.29 ± 22.29 nm<br>(range: 18.75 – 279.6 nm)<br>N = 3 |
| | **standard 45'** | 61.67 ± 7.37 nm<br>(range: 15.63 – 179.4 nm)<br>N = 2 | 120.96 ± 19.93 nm<br>(range: 15.63 – 364.6 nm)<br>N = 3 |
| | **standard 15'** | 57.74 ± 5.23 nm<br>(range: 15.63 – 145.3 nm)<br>N = 2 | 139.47 ± 21.85 nm<br>(range: 31.24 – 378.1 nm)<br>N = 3 |
| | **stiff** | 57.77 ± 6.28 nm<br>(range: 10.94 – 184.4 nm)<br>N = 2 | 140.13 ± 20.06 nm<br>(range: 20.37 – 408.3 nm)<br>N = 3 |

Table 1. Collagen fibril diameter in the CDL and in the PM in different mechanical states. Results are expressed as mean ± standard deviation.

| | | CDL | PM |
|---|---|---|---|
| **Mechanical state** | **compliant** | 51.98 ± 1.86 nm<br>(range: 37.39 - 62.95 nm)<br>N = 3 | 57.31 ± 4.66 nm<br>(range: 49.14 – 70.90 nm)<br>N = 3 |
| | **standard 45'** | 62.00 ± 2.54 nm<br>(range: 42.26 – 75.91 nm)<br>N = 3 | 63.68 ± 3.56 nm<br>(range: 49.91 – 74.54 nm)<br>N = 3 |
| | **standard 15'** | 57.06 ± 2.58 nm<br>(range: 39.67 – 77.89 nm)<br>N = 4 | 62.07 ± 3.05 nm<br>(range: 51.84 – 77.52 nm)<br>N = 3 |
| | **stiff** | 54.56 ± 5.25 nm<br>(range: 45.67 – 72.86 nm)<br>N = 4 | 60.65 ± 5.23 nm<br>(range: 37.90 – 71.69 nm)<br>N = 3 |

Table 2. Collagen fibril D period in the CDL and in the PM in different mechanical states. Results are expressed as mean ± standard deviation.

| | | | **Alcian blue solution pH** | | |
|---|---|---|---|---|---|
| | | | **0.2** | **1.4** | **5.6** |
| **Mechanical state** | **compliant** | CDL | 0.506 ± 0.365 (N = 5)[a] | 0.306 ± 0.166 (N = 7)[e] | 0.391 ± 0.194 (N = 5) |
| | | PM | 0.544 ± 0.102 (N = 5) | 0.560 ± 0.242 (N = 7)[e] | 0.522 ± 0.148 (N = 8) |
| | **standard 45'** | CDL | 0.249 ± 0.135 (N = 5)[a, b, c] | 0.583 ± 0.407 (N = 7) | 0.155 ± 0.081 (N = 8)[b, f] |
| | | PM | 0.600 ± 0.072 (N = 5)[c] | 0.577 ± 0.233 (N=7) | 0.558 ± 0.194 (N = 8)[f] |
| | **standard 15'** | CDL | 0.223 ± 0.070(N = 5)[d] | 0.682 ± 0.709(N = 7) | 0.306 ± 0.166 (N = 8)[g] |

| | | | | | |
|---|---|---|---|---|---|
| | | PM | 0.665 ± 0.174 (N = 5)[d] | 0.710 ± 0.283 (N = 7) | 0.601 ± 0.207 (N = 8)[g] |
| | **stiff** | CDL | 0.296 ± 0.143 (N = 5) | 0.624 ± 0.508 (N = 7)[h] | 0.227 ± 0.152 (N = 8)[h, i] |
| | | PM | 0.654 ± 0.485(N = 5) | 0.578 ± 0.206 (N = 7) | 0.576 ± 0.514 (N = 8)[h, i] |

Table 3. GAG quantification. Results are expressed as mean ‰ concentration (respect to wet weight) ± standard deviation. a-i = statistically significant differences between the two samples.

| QUERY ID | MAMMALIAN COLLAGEN WITH HIGHEST IDENTITY | | |
|---|---|---|---|
| | PROTEIN | QUERY COVERAGE | IDENTITY |
| alpha collagen type 1 precursor [*Paracentrotus lividus*] gi\|159958\|gb\|AAA29438.1\| | collagen alpha-1(**III**) chain [*Bos taurus*] DAA32863.1 | 97 % | 45 % |
| 2-alpha collagen precursor [*Paracentrotus lividus*] gi\|159962\|gb\|AAA29440.1\| | alpha2(**I**) collagen [*Homo sapiens*] AAB69977.1 | 100% | 36 % |
| | collagen alpha-2(**I**) chain precursor [*Rattus norvegicus*] NP_445808.1, [*Canis lupus familiaris*] NP_001003187.1, [*Bos taurus*] NP_776945.1 | 99 % | 38 % |
| collagen-like protein [*Paracentrotus lividus*] gi\|159960\|gb\|AAA29439.1\| | collagen alpha-1(**II**) chain isoform 2 precursor [*Homo sapiens*] NP_149162.2 | 100% | 59 % |
| | collagen alpha-2(**V**) chain precursor [*Homo sapiens*] AAL13166.1, [*Mus musculus*] NP_031763.2, [*Rattus norvegicus*] NP_445940.1 | 100% | 57 % |
| alpha-5 collagen [*Paracentrotus lividus*] gi\|38490686\|emb\|CAE53096.1 | collagen pro-alpha-1 type **I** chain [*Mus musculus*] AAA88912.1 | 49 % | 40 % |
| | prepro-alpha1(**I**) collagen [*Homo sapiens*] CAA98968.1 | 48 % | 38% |
| 6a fibrillar collagen [*Paracentrotus lividus*] gi\|53748114\|emb\|CAH10072.1 | procollagen type **V** alpha 1 [*Mus musculus*] CAM46251.1 | 98% | 38% |
| | collagen alpha-1(**XI**) chain isoform C preproprotein [*Homo sapiens*] | 72 % | 46% |
| 7a fibrillar collagen [*Paracentrotus lividus*] gi\|53748116\|emb\|CAH10073.1 | procollagen type **V** alpha 1 [*Mus musculus*] CAM46251.1 | 99 % | 30 % |
| | alpha-1 (type **XI**) collagen precursor [*Homo sapiens*] AAA51891.1 | 65 % | 34 % |

Table 4. BLASTp analysis of the similarities between identified *P.lividus* collagen sequences and mammalian collagen sequences.

Figure1

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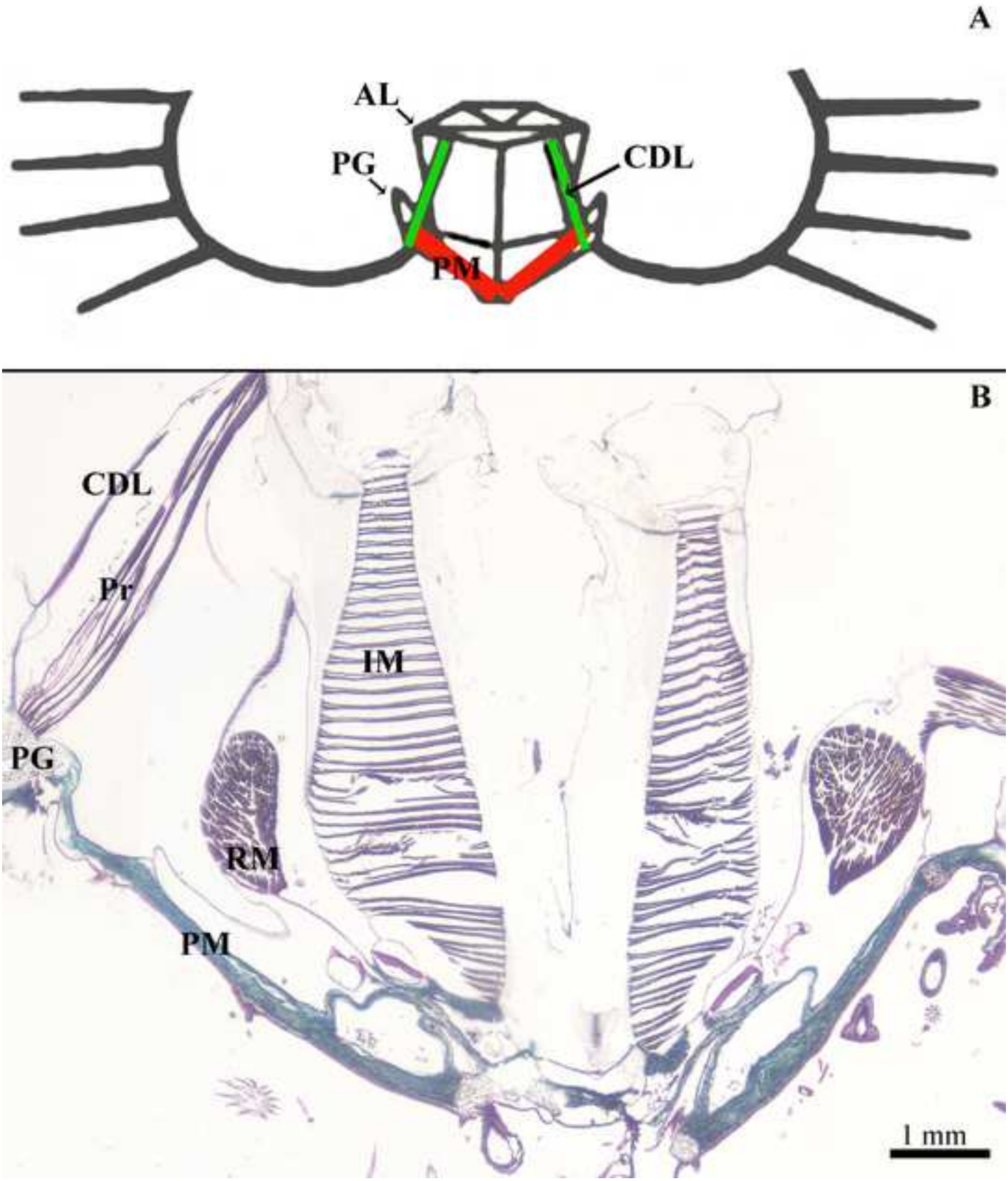

Figure1_B&W
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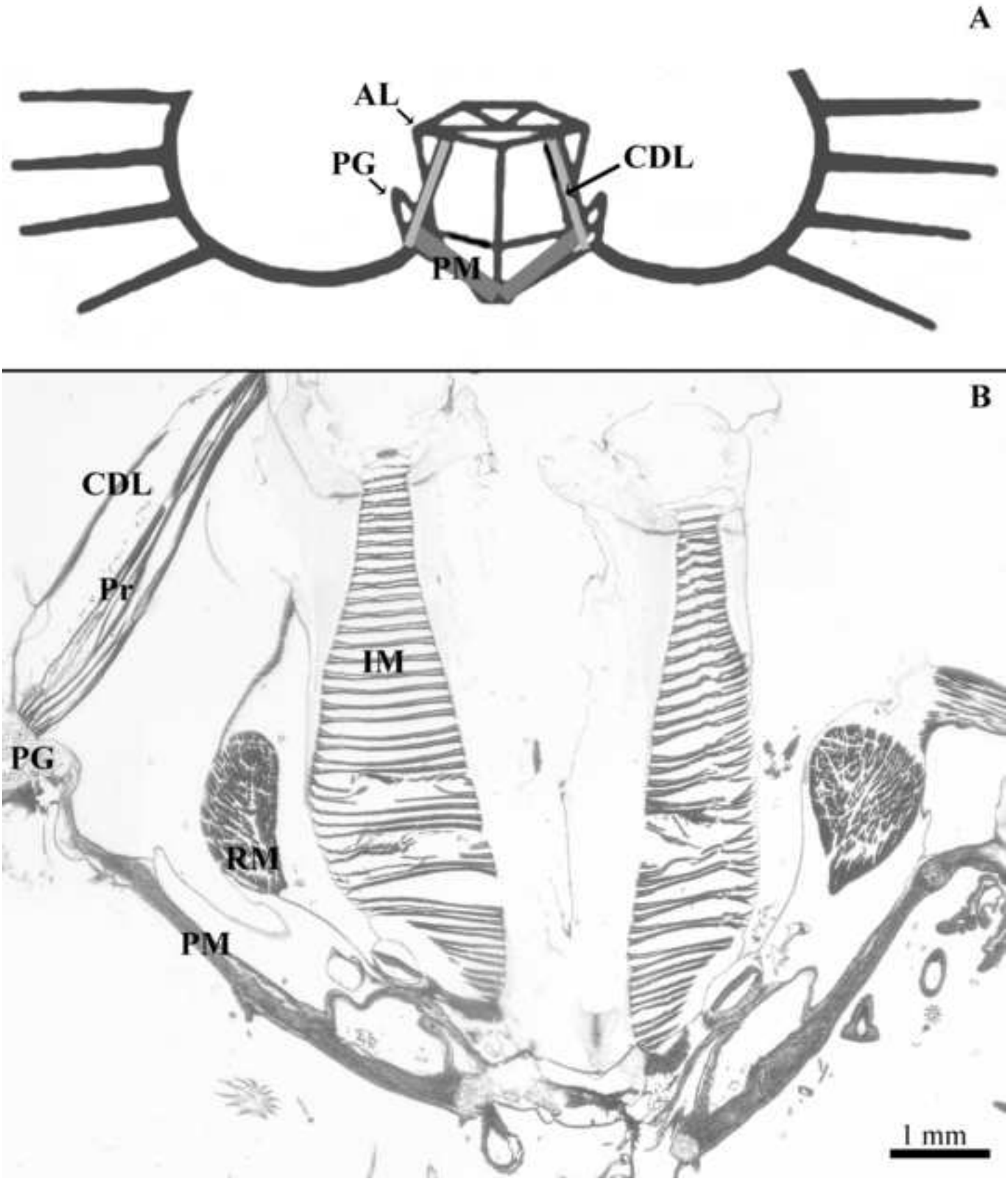

Figure2

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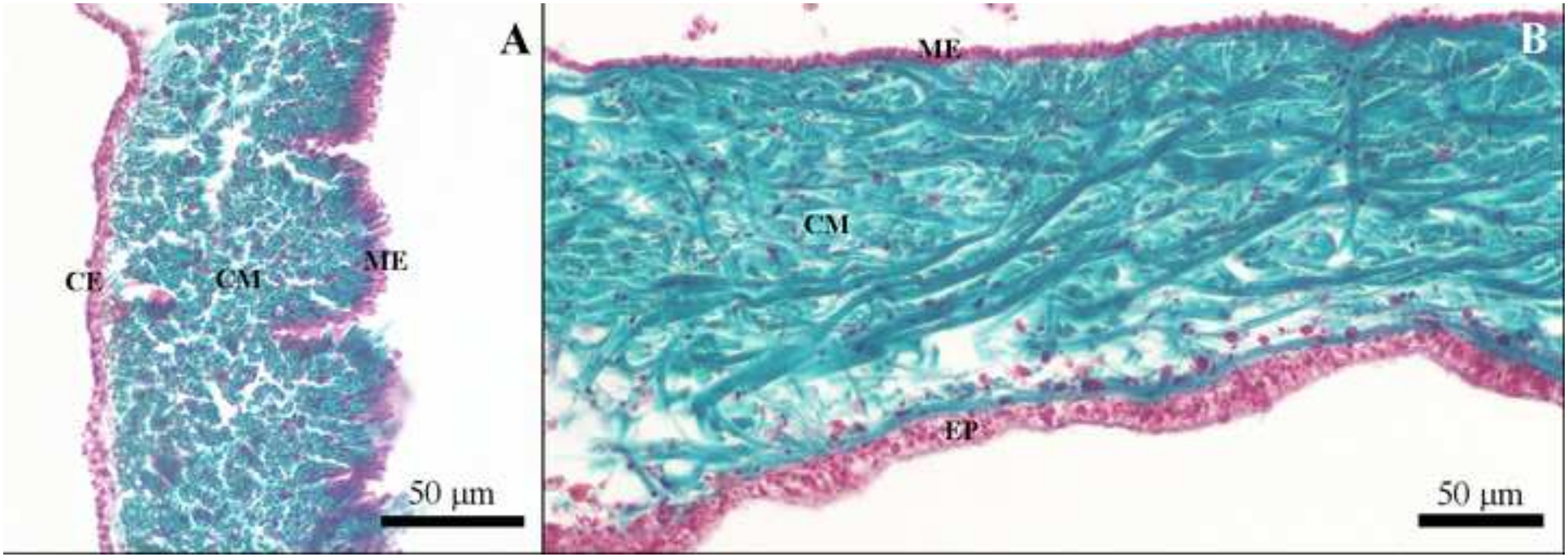

Figure2_B&W

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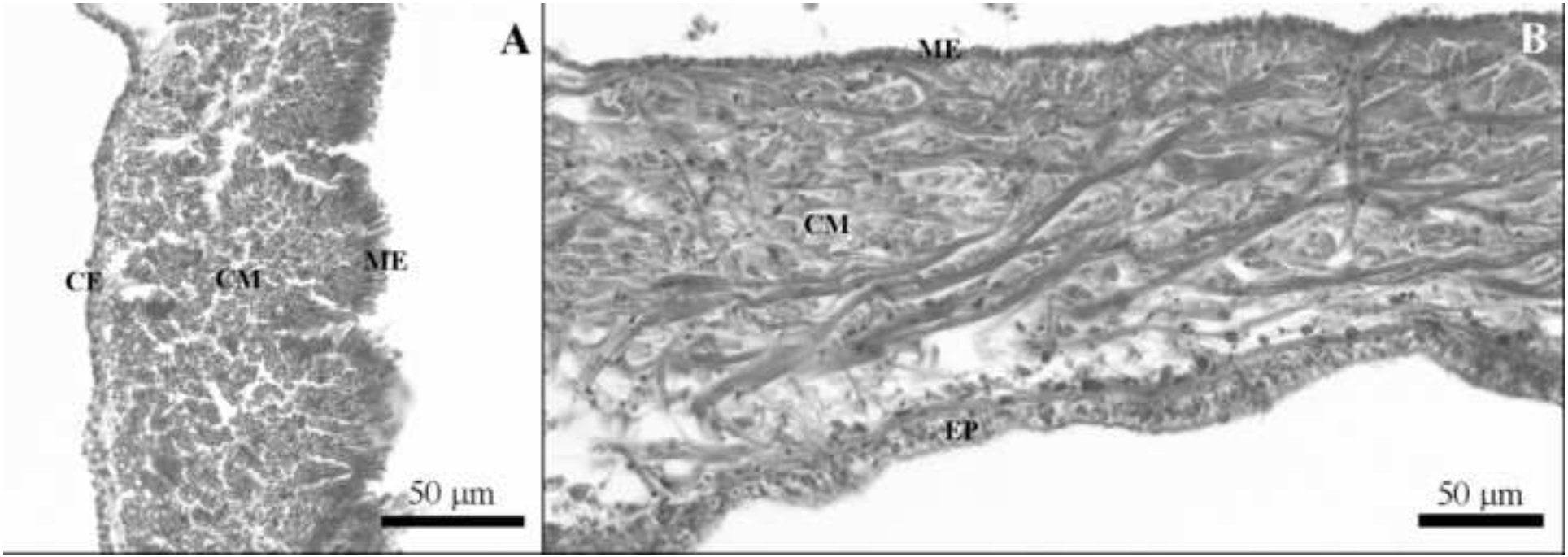

Figure3

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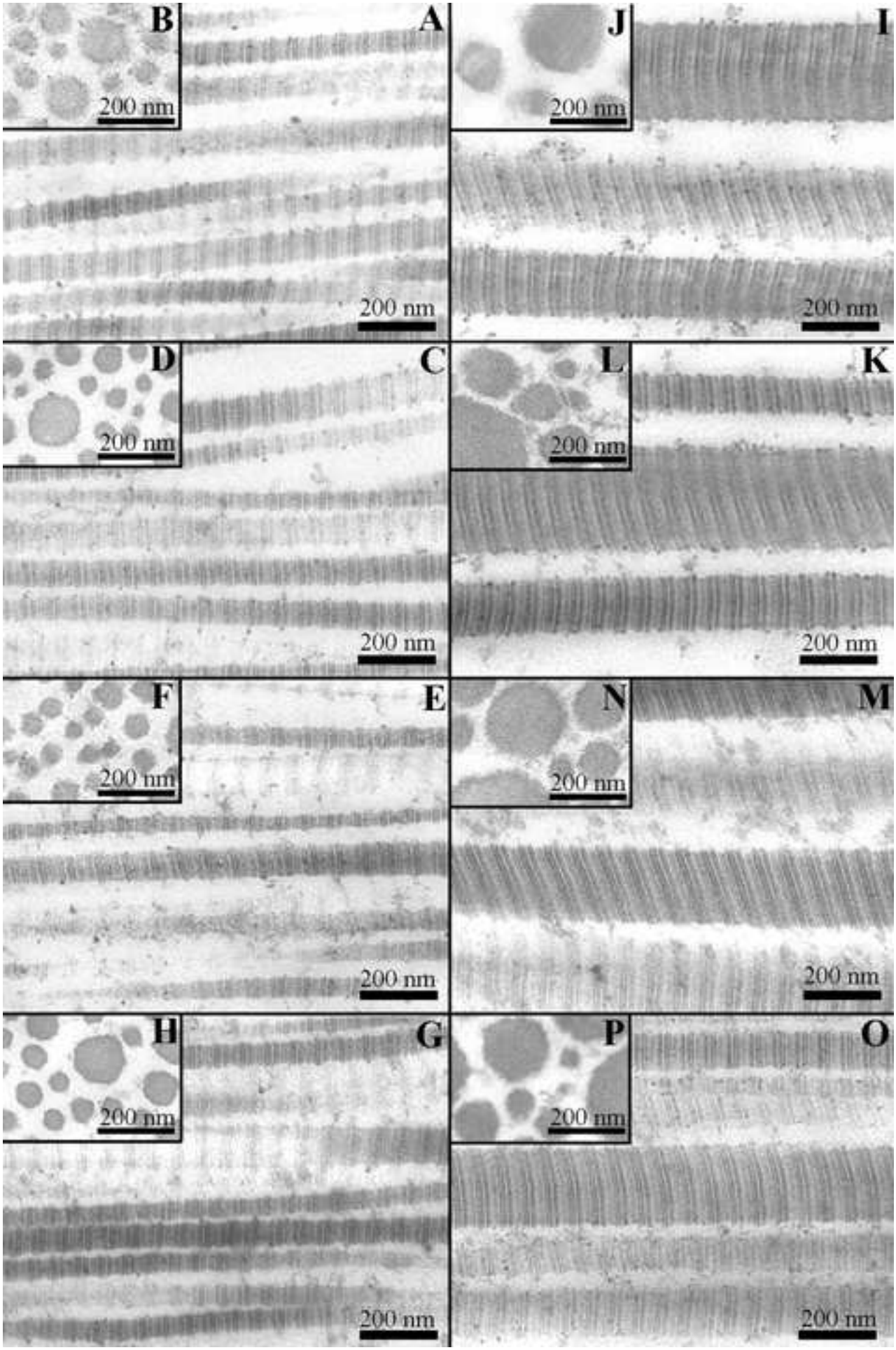

Figure4

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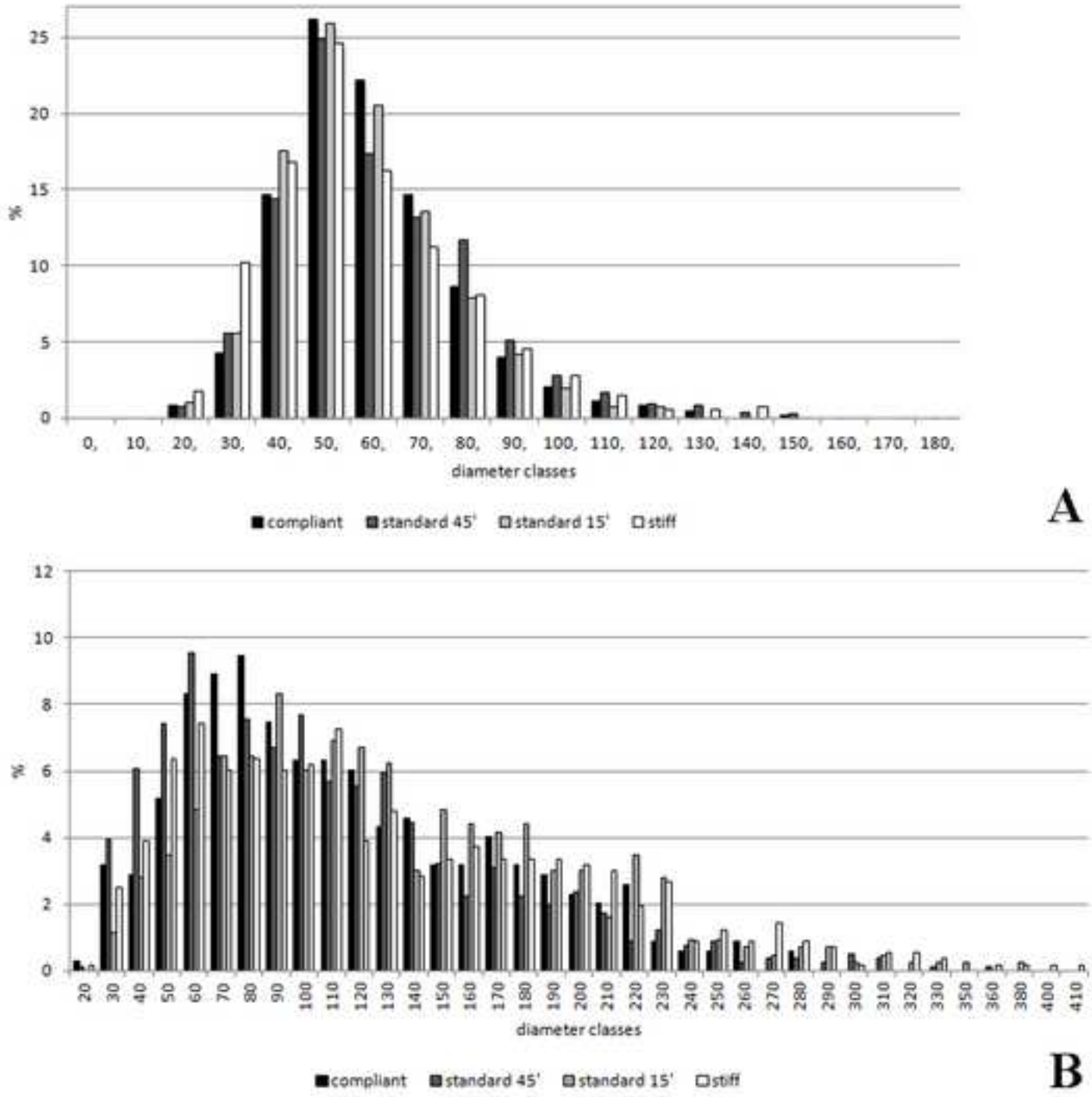

**Figure 5 revised**

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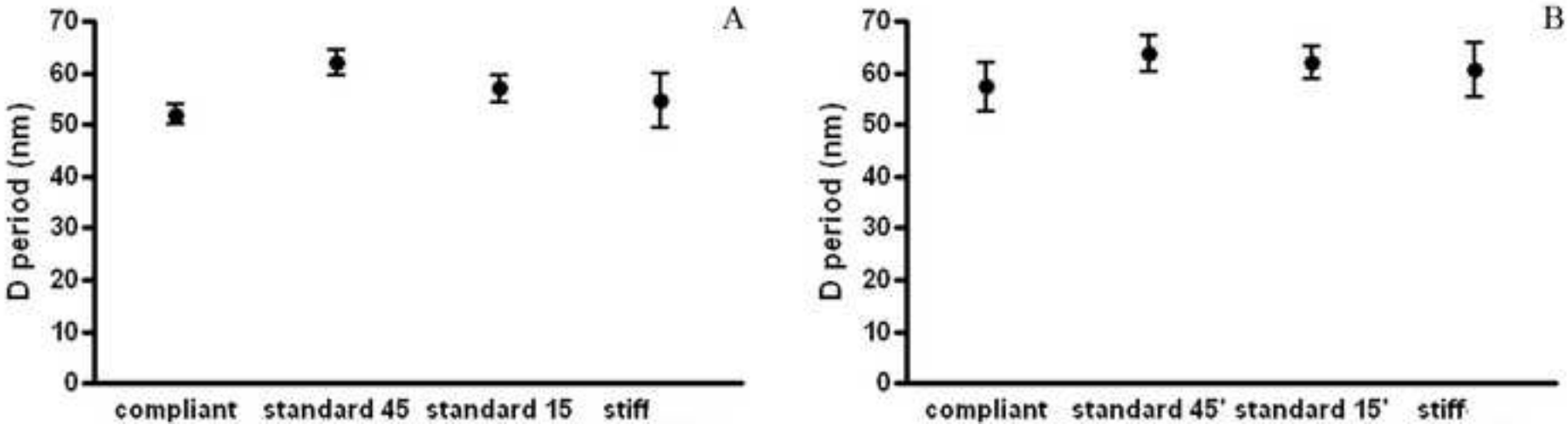

**Figure 6 revised**

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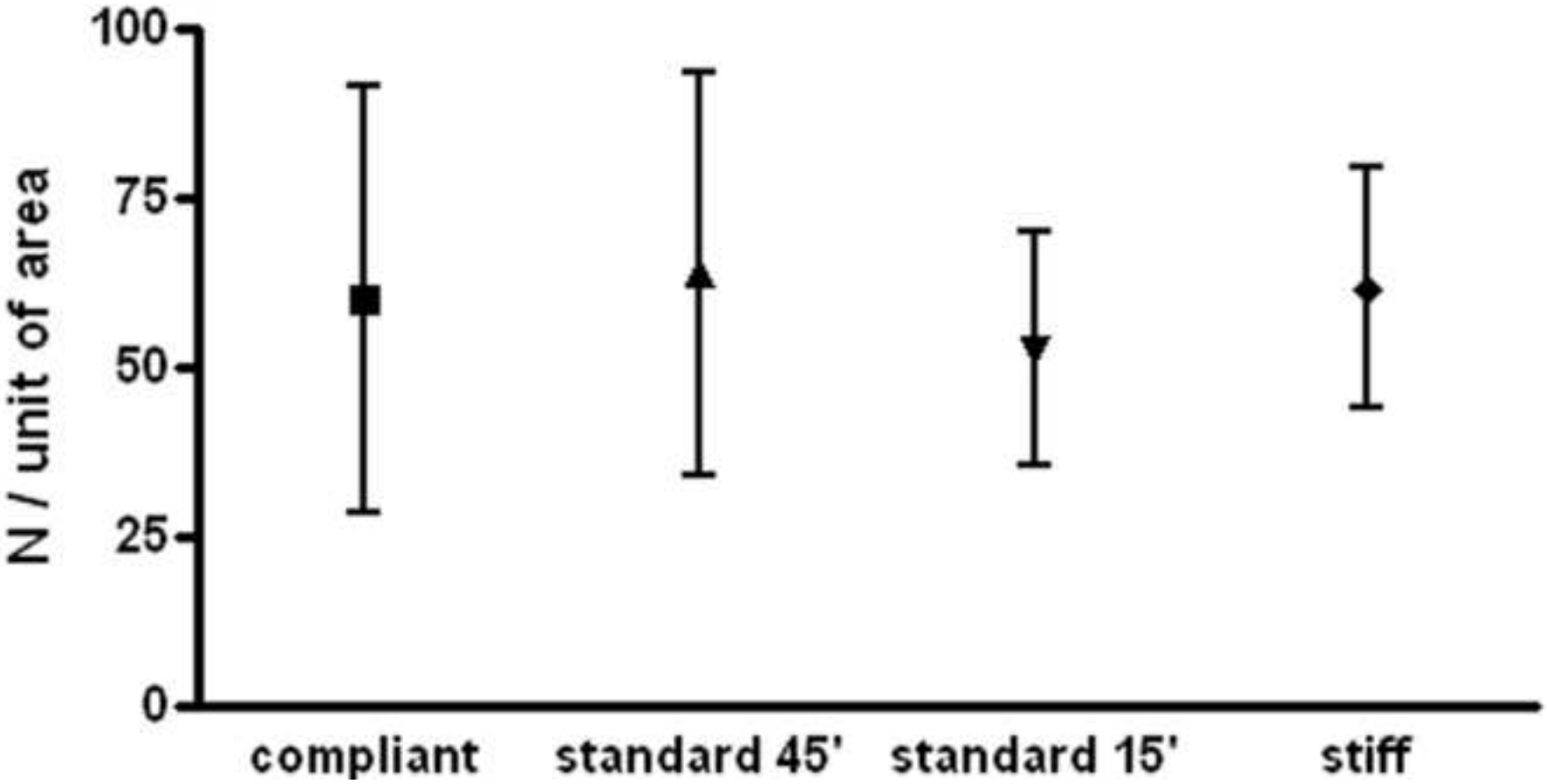

**Figure7**

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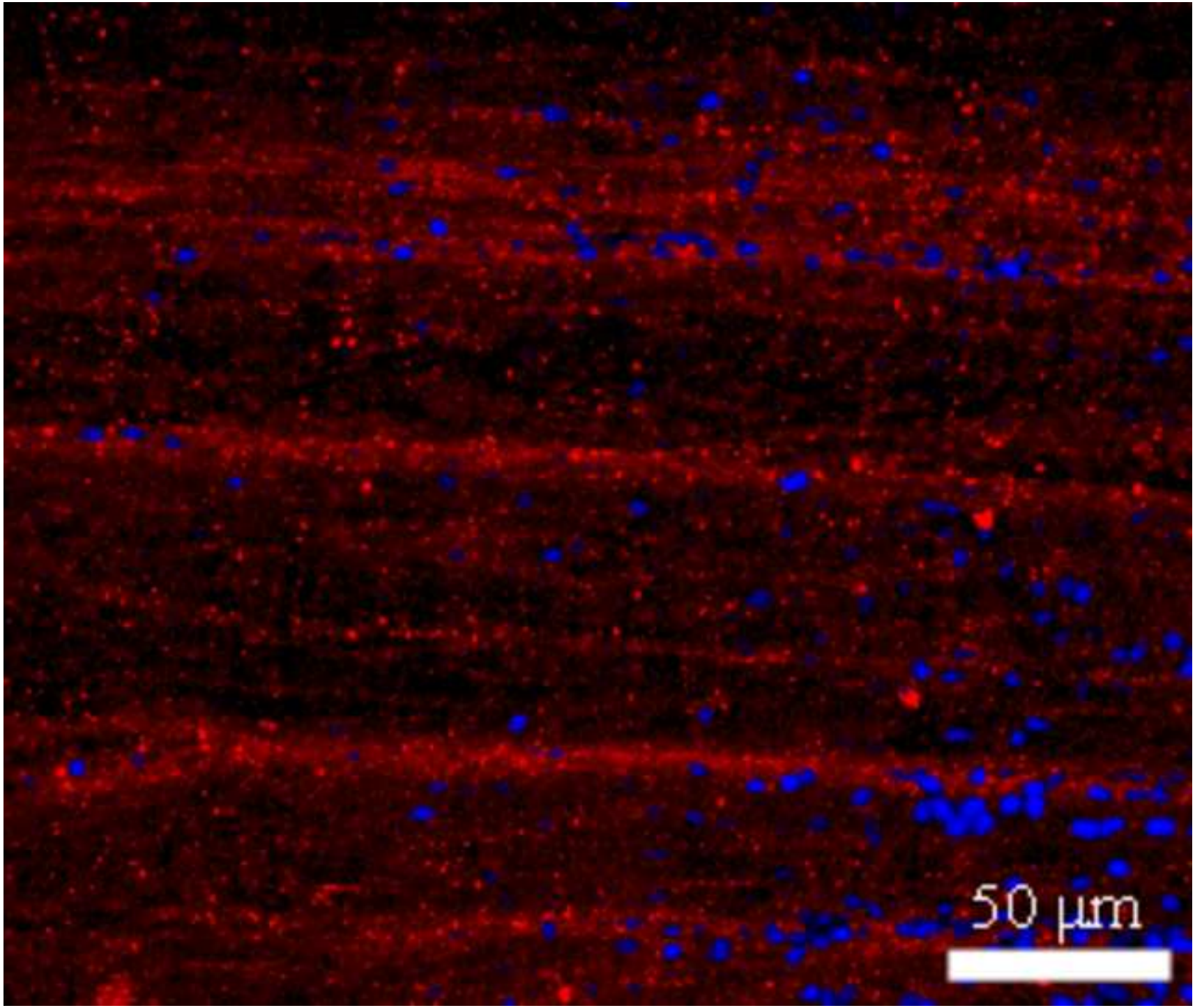

Figure7_B&W

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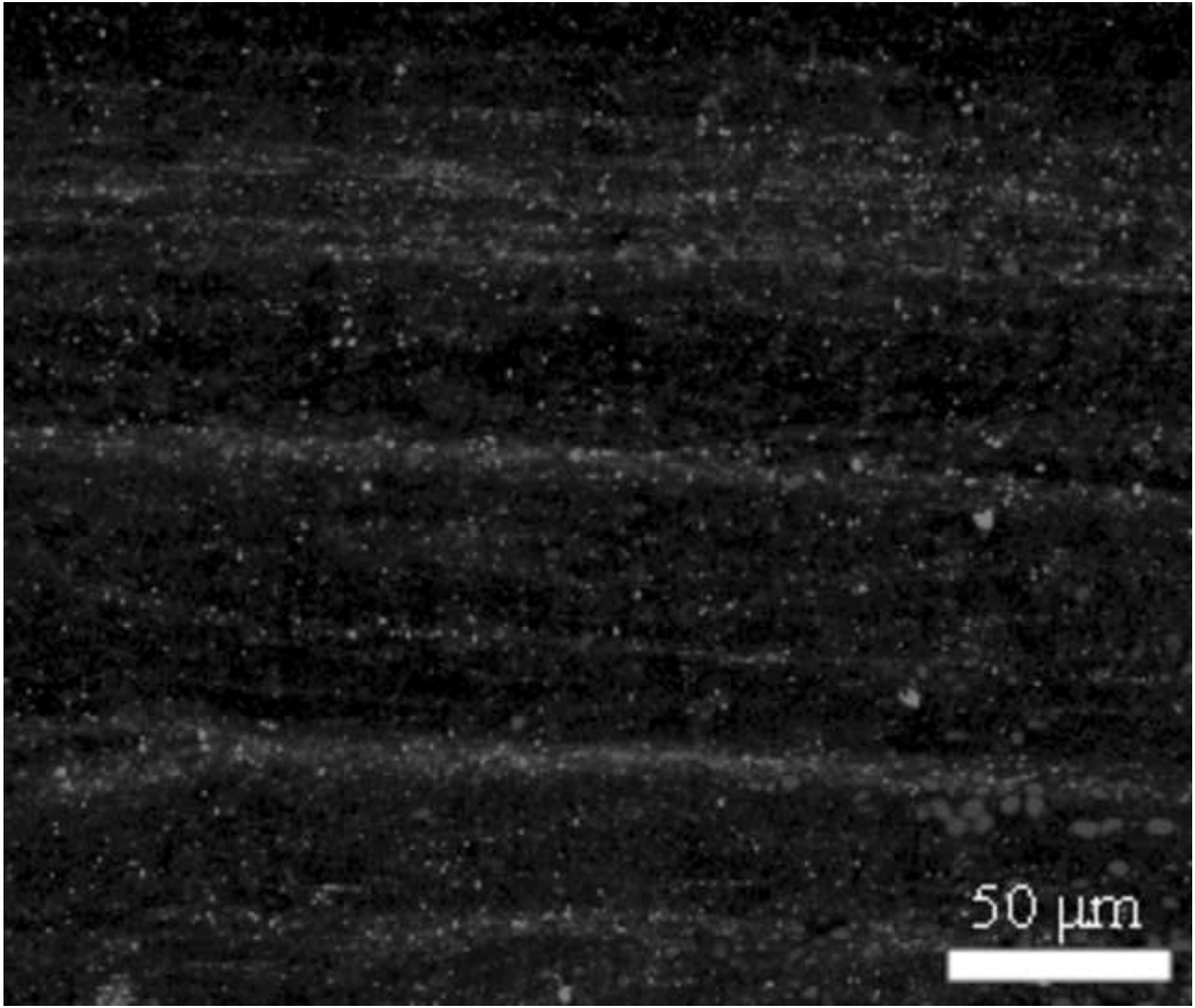

Figure8

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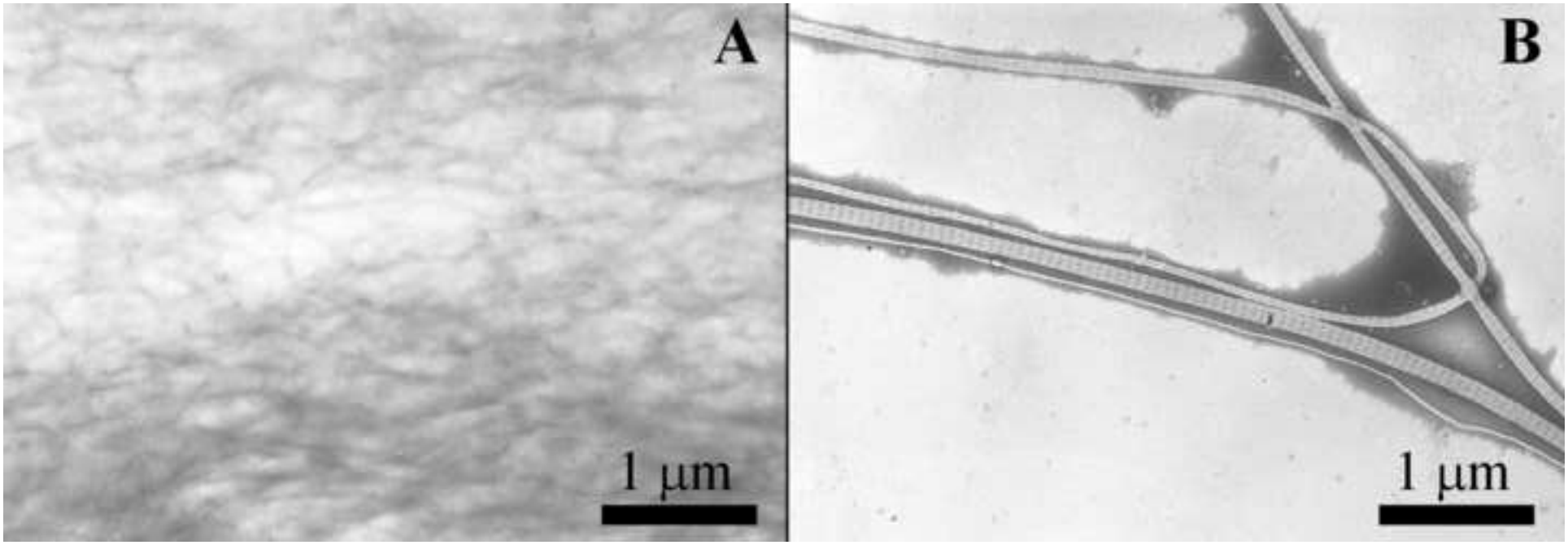

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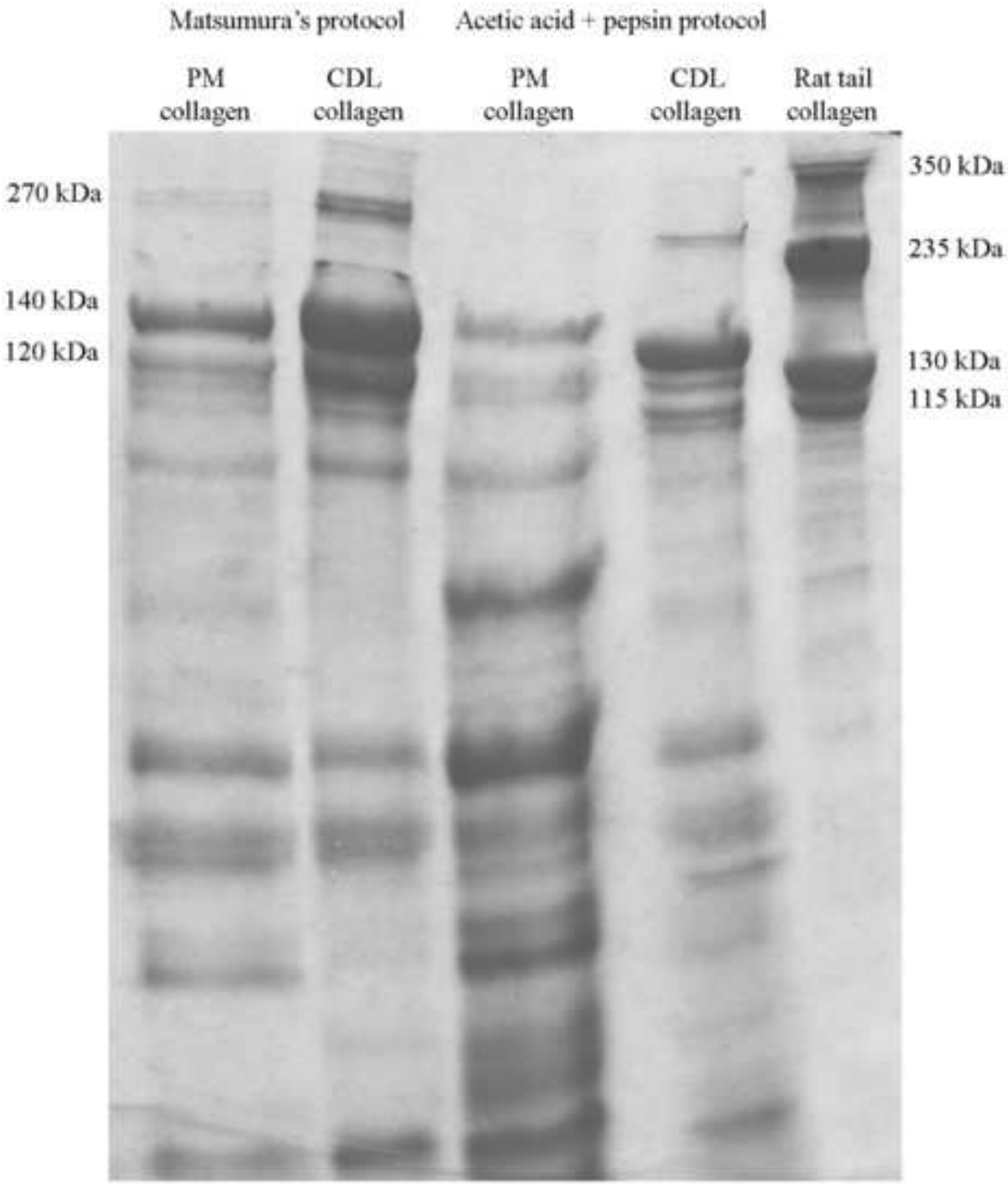

Figure10
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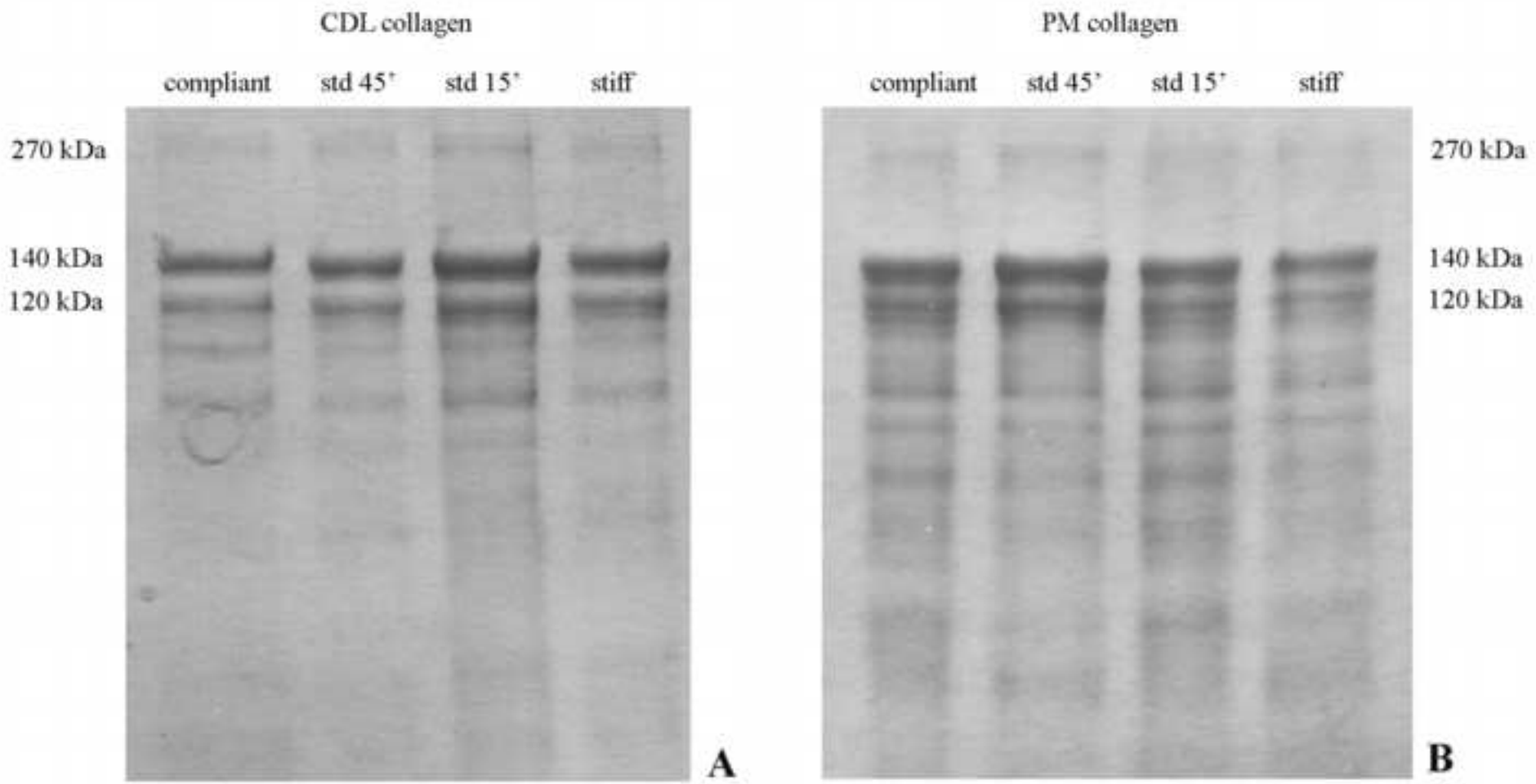

Figure11

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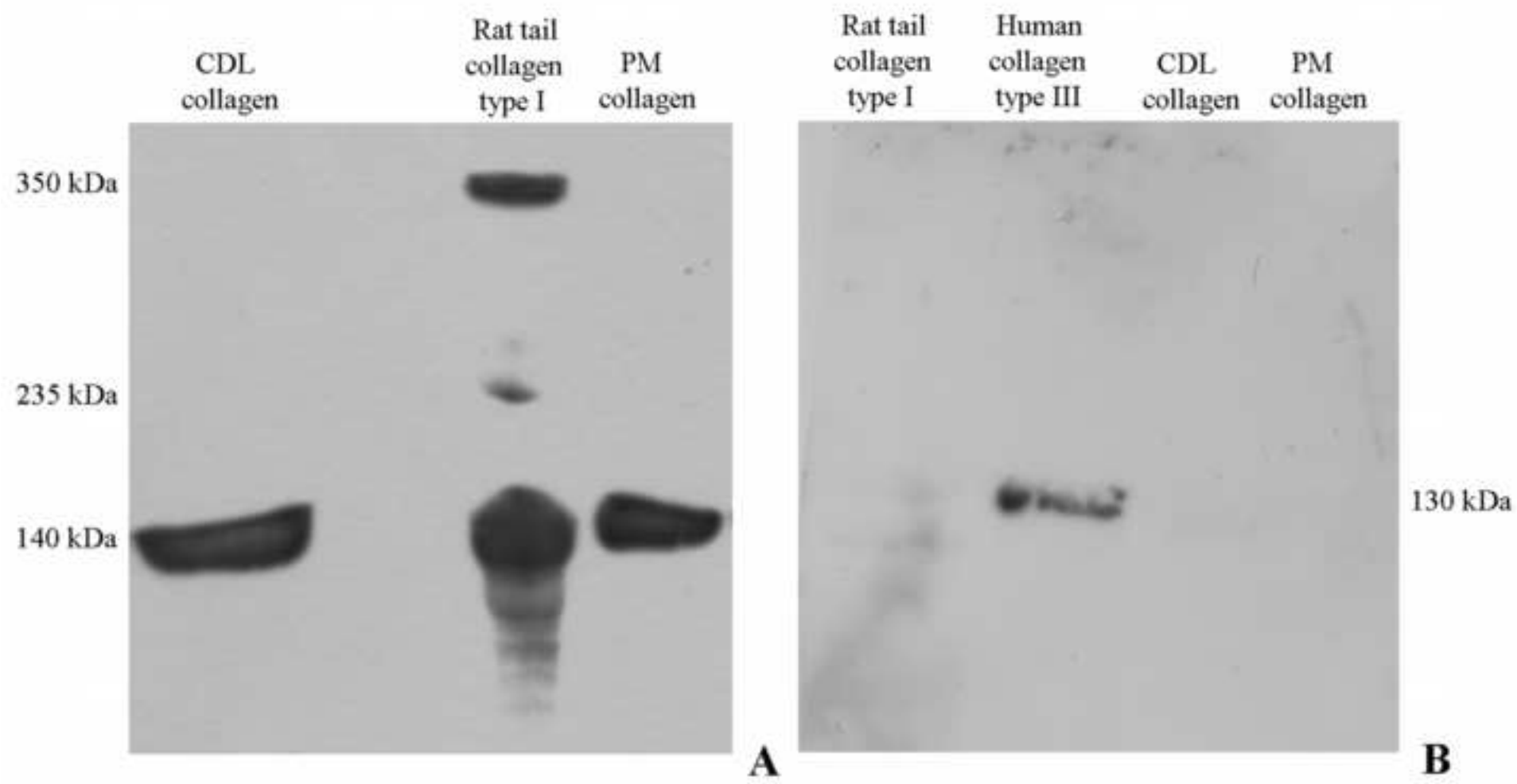

**Figure 12 revised**

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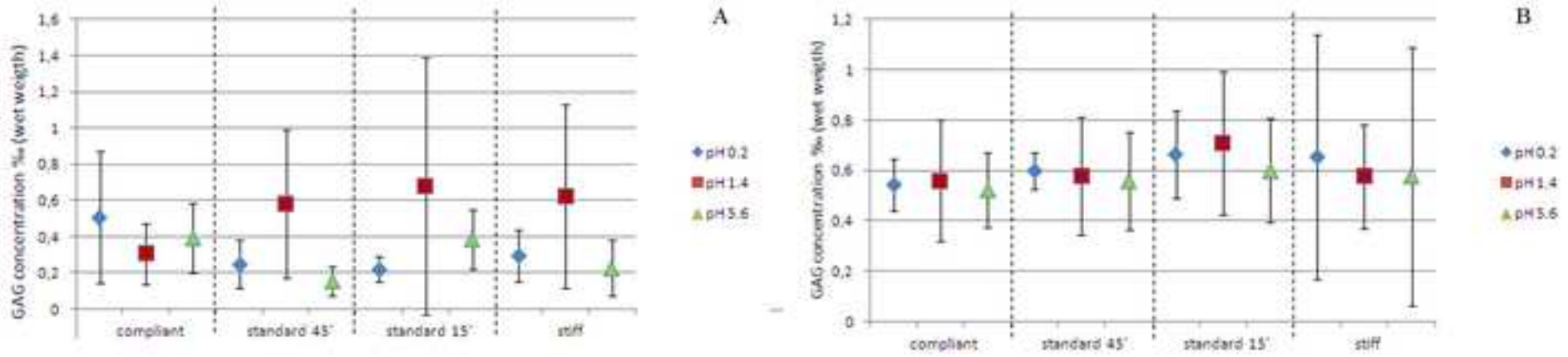

**Figure 12 revised B&W**

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