The reaction of the sponge *Chondrosia reniformis* to mechanical stimulation is mediated by the outer epithelium and the release of stiffening factor(s)

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**A B S T R A C T**

Although sponges are still often considered to be simple, inactive animals, both larvae and adults of different species show clear coordination phenomena triggered by extrinsic and intrinsic stimuli. *Chondrosia reniformis*, a common Mediterranean demosponge, lacks both endogenous siliceous spicules and reinforcing spongin fibers and has a very conspicuous collagenous mesohyl. Although this species can stiffen its body in response to mechanical stimulation when handled, almost no quantitative data are available in the literature on this phenomenon. The present work was intended to quantify the dynamic response to mechanical stimulation both of intact animals and isolated tissue samples in order to evaluate: (i) the magnitude of stiffening; (ii) the relationship between the amount of stimulation and the magnitude of the stiffening response; (iii) the ability of the whole body to react to localized stimulation; (iv) the possible occurrence of a conduction mechanism and the role of the exopinacoderm (outer epithelium). Data on mesohyl tensity obtained with mechanical tests confirmed the difference between stimulated and non-stimulated isolated tissue samples, showing a significant relationship between ectosome stiffness and the amount of mechanical stimulation. Our experiments revealed a significant difference in tensity between undisturbed and maximally stiffened sponges and evidence of signal transmission that requires a continuous exopinacoderm. We also provide further evidence for the presence of a chemical factor that alters the interaction between collagen fibrils, thereby changing the mechanical properties of the mesohyl.

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1. Introduction

Although sponges (phylum Porifera) lack a nervous system, they show a range of behavioral responses (i.e. contraction of the whole body or part of it as well as alteration of the contraction rhythm and amplitude) that are affected by exogenously applied neuroactive compounds (for a review, see Nickel, 2010).

*Chondrosia reniformis* (Nardo, 1847) is a common Mediterranean demosponge that lives on shady rocky coasts at depths of up to 50 m. Unlike most sponges, *C. reniformis* lacks endogenous calcite or siliceous spicules or reinforcing spongin fibers. The tissue between the outer and inner epithelia, called the mesohyl, consists of two well-defined regions: the ectosome and the choanosome.

The ectosome is the outer region of the mesohyl; it contains small incipient canals and is composed mostly of collagen fibers and a few scattered cells (Bavestrello et al., 1998). In contrast, the inner region, known as the choanosome, is characterized by the presence of numerous choanocyte chambers and a tangled network of canals with a wide range of diameters. Collagen is less abundant in the choanosome than in the ectosome and differs also in including a nonfibrillar collagen (COLch; Pozzolini et al., 2012).

*C. reniformis* shows obvious contraction and expansion activity that involves the whole body as well as the osculum diameter (Nickel, personal communication); moreover, it can react to different stimuli by changing its tensive state: it can rapidly stiffen after mechanical or chemical stimulation (Bonasoro et al., 2001; Wilkie et al., 2006) or de-stiffen and, following accidental detachment of the substrate, produce long slender outgrowths (Parma et al., 2007; Fassini et al., 2012). These phenomena involve different responses induced by different external events, but both have been attributed...
to alteration, under cellular control, of the interactions between the collagen fibrils of the mesohyl (Bonasoro et al., 2001; Wilkie et al., 2006; Parma et al., 2007; Fassini et al., 2012). The ability to regulate the viscoelastic properties of the connective tissue matrix is a well-investigated phenomenon in other animal phyla, particularly the Echinodermata in which the mechanical, morphological and molecular aspects of mutable collagenous tissues have been extensively analyzed (Wilkie, 2005; Tamori et al., 2010; Yamada et al., 2010; Ribeiro et al., 2011, 2012a,b; Barbaglio et al., 2012; Sugni et al., 2014).

The main aims of the present work were (i) to quantify both the changes of the tensile properties and dimensional changes that occur when C. reniformis reacts to mechanical stimulation; (ii) to investigate the possible presence of a conduction mechanism and, in the light of evidence for the relevance of sponge epithelia in coordinated phenomena (see Nickel et al., 2011), the role of the exopinacoderm (outer epithelium) in any such conduction mechanism; and (iii) to provide more information on an endogenous stiffening factor that may be involved in the physiological control of stiffness in this sponge.

2. Materials and methods

Specimens of C. reniformis were collected between October and May by SCUBA divers at two different locations on the Italian Ligurian coast: Bergeggi (44°14′37″N, 8°26′40″E) and Paraggi (44°18′40″N, 9°12′46″E). The sponges were transferred to 501 tanks filled with artificial seawater (ASW) (‘Instant Ocean’; Aquarium Systems, Sarrebourg, France) and kept at 14–16°C. All animals were left undisturbed for at least one night in the aquaria before being tested. All experiments were performed within 10 days of the collection date since over time C. reniformis tends to adapt to aquarium conditions by partial constriction of the oscula and inner canals (D.F., personal observation).

2.1. Testing mechanical properties: stimulation, response and signal transmission

2.1.1. Dimensional recovery

Whole sponges: Four sponges, after being manipulated to induce the maximally stiffened condition (MSC), were left undisturbed for 7 h. Using a Canon EOS 30D camera (resolution 2336 × 3506 pixels; Canon, Tokyo, Japan) with a Sigma EX 2.8/24–70 mm DG HSM objective (Sigma, Rödermark, Germany), two photographs (frontal and overhead views, Fig. 1A) of each specimen were taken at intervals of 1 h; the images were then used to measure changes in the three different axes, volume variations and the recovery pattern during the return to resting condition (RC; condition of an undisturbed sponge that has not been manipulated or stimulated for at least 4 h).

Isolated tissue samples: In order to determine the main factors contributing to volume change, we cut whole sponges vertically into slices about 2 mm thick. Each slice was placed in a Petri dish with ASW at room temperature. Images of the slices where taken soon after the excision and after 4 h. The images (Fig. 1B) were used to evaluate changes in the two dimensions of different regions of the sponges by measuring selected points using the Photoshop CS3 digital ruler (Adobe Systems, San Jose, CA, USA). We took into consideration the changes in thickness of both x- and y-axes of the ectosome, the choanosome, the small canals (inner diameters <1 mm) and the large canals. Changes were normalized by expressing length after 4 h as a proportion of the starting length. Starting lengths were considered to represent the maximally stiffened condition (MSC) and lengths at 4 h the partially de-stiffened condition (PDC).

2.1.2. Reaction to mechanical stimulation

Whole sponges:

(a) Biomechanical experiments were performed using a modified isotonic transducer (Fig. 1C) (Harvard Apparatus, Holliston, MA, USA) whose output was recorded using LabChart software (AD Instruments, Dunedin, New Zealand). One end of the lever was attached to a rod-shaped weight (contact surface area 10 mm², rod weight 18.9 g, generated pressure 18.52 kPa), which was applied to the sponge and induced a typical deformation curve (Fig. 1D). In order to define the mechanical state of the sponges the total amount of deformation, the speed of deformation in the constant phase and the magnitude and duration of the decelerating phase (Fig. 1D) were derived from the curves obtained from sponges in RC.

Data obtained from ten sponges in RC where plotted against the sponge thickness in the stimulated area in order to evaluate any possible relationship between the thickness and the other parameters. Thickness was calculated via images by using the Photoshop CS3 digital ruler.

(b) Sponges were collected from the aquaria and gently placed into a small glass container filled with ASW. After 2 h the specimens were placed on the bench and stimulated by hand pressure (pressure was about 463.75 ± 8.66 MPa) for 5 s. The animals were then replaced in the container and tested (as described in Section 2.1.2(a)) 20 s, 1 min or 3 min after stimulation. In control samples the animals were transferred onto the bench for the same period as the experimental specimens, but omitting stimulation; again measurement of the mechanical state took place 20 s, 1 min or 3 min after stimulation. Twelve individuals of different sizes were tested on different days; each day sponges were pooled in 4 groups of 3 specimens, the order of the six treatments (stimulated: 20 s, 1 min or 3 min; unstimulated: 20 s, 1 min or 3 min) was mixed in order to avoid pooling effects.

Isolated tissue samples: Isolated samples were prepared as described by Wilkie et al. (2006) and were used to evaluate the responses to mechanical stimulation. Briefly, beam-shaped samples 2.5 mm × 2.5 mm × 15 mm in size were cut from both the ectosome and choanosome regions, using parallel-mounted razor blades. In each sample, two opposite long sides were roughly parallel to the external surface of the animal and the other two long sides, anatomically ‘lateral’, were orthogonal with respect to the external surface. Ectosome samples included no, or very little, exopinacoderm. Each sample was fixed to a glass coverslip using cyanoacrylate cement, with a ‘lateral’ surface in contact with the coverslip and with exactly 10 mm projecting from the edge of the coverslip. This orientation was chosen to minimize variability caused by the slow ‘spontaneous’ bending of ectosome samples, which occurs only in a plane orthogonal to the external surface (L.C.W., personal observation). The samples were transferred to and from test solutions by gripping the coverslip with forceps, never by gripping or touching the tissue itself. Each sample was left undisturbed for 6 h. The attached end was then stimulated mechanically (as described below) and the sample gently lifted from the stimulating apparatus (Fig. 1E) while a stopwatch was started; the coverslip was clamped horizontally with the sample in front of a 0.5 mm grid according to Wilkie et al. (2006). Samples usually bent under gravity and their deflection was recorded to the nearest 0.5 mm at specific times. The same procedure, omitting the stimulation, was performed on the control samples.

We used two different protocols:

(a) Both ectosome and choanosome samples were stimulated by dropping a load (41.4 g) three times from a height 5 cm above the attached end of the sample. Bending under gravity was recorded 15, 30 and 45 s after the stopwatch was started.
Fig. 1. (A) Views of the dorsal (above) and lateral (below) surfaces of a sponge during de-stiffening. White lines show the reference lengths that were measured every hour. (B) An isolated sponge tissue slice soon after excision (a) and after resting for 4 h (b). Definition of the lines: 1 = ectosome z-axis; 2 = ectosome y-axis; 3 = choanosome z-axis; 4 = canal sheath z-axis; 5 = canal sheath y-axis; 6 = canal diameter z-axis; 7 = canal diameter y-axis. (C) Schematic drawing of the apparatus used to stimulate sponges and record their responses. Abbreviations: a = rod-shaped weight; b = isotonic transducer; c = lever; d = blocking device; e = rails to keep the rod vertical; s = sponge specimen. (D) A typical curve obtained in the mechanical test and recorded with LabChart software. After releasing the locking device, the rod reached the sponge surface (x). The deformation could be divided into three phases: fast deformation phase (from point 1 to point 2); decelerating phase (from point 2 to point 3) and a subsequent period of relatively constant deformation speed (from point 3 to point 4). From the curve it was possible to extract different parameters: a = total induced deformation (TID); b = decelerating phase magnitude (DPM); c = decelerating phase duration (DPD); d = constant phase deformation speed (CPS). (E) Schematic drawing of the apparatus used to stimulate isolated mesohyl samples; the impact area is highlighted in red. Abbreviations: a = load used to stimulate the samples; b = mesohyl sample; c = locking devices to prevent slippage of d = glass coverslip; e = rails to keep the load vertical. (F) Photograph of the bending apparatus used to establish the mechanical state of isolated mesohyl samples. The white arrow indicates the glass coverslip to which the sample was glued using cyanoacrylate cement. Abbreviation: a = magnitude of deflection.
(b) Both ectosome and choanosome samples were stimulated as above but varying the number of impacts: samples were subjected to 0 (control), 3, 6 or 9 impacts. Bending under gravity was recorded 45 s after the stopwatch was started.

2.2.2. In vivo tests

Beam-shaped ectosome samples were produced as described above (Section 2.1.2).

(a) After de-stiffening for 6 h at 20 °C the samples were transferred to the bending apparatus and after 45 s each sample was photographed to record how much it had bent. Samples were then put in different solutions. In the control solution one part of dialyzing buffer was mixed with two parts of ASW; in the test solutions one part of each dialyzed protein extract was mixed with two parts of ASW. After 1 h each sample was gently transferred to the bending apparatus and its deflection after 45 s recorded photographically (Fig. 1f); this procedure was repeated after one more hour in the different solutions.

(b) The same experiment as in (a) was conducted on ectosome samples, but the de-stiffening time was reduced to 3 h and a third measurement was added 3 h after exposure to the different solutions.

2.2.3. In vitro tests

Collagen fibrils were extracted using 3 g frozen mesohyl in 15 ml disaggregating solution according to the procedure of Matsumura (1974). The suspension was then centrifuged for 30 min at 4500 rcf and the supernatant dialyzed overnight against distilled water. Aggregation tests (following Tipper et al., 2003) were performed in 24 multi-well plates. Briefly, 1 ml of collagen fibril suspension was diluted with 4 ml of distilled water and mechanically rinsed. 250 μl of diluted suspension was placed in each well and different amounts (400, 200, 100 μl) of fraction P40 were added. We tried P40 dialyzed against either ASW or distilled water. The control test was done by adding different volumes (400, 200, 100 μl) of distilled water or ASW to 250 μl of diluted suspension.

2.3. Statistics

Statistical tests were done using StatPlus version 5.8.3.8 (AnalystSoft Inc., Alexandria, VA, USA) (Student’s t-test: normality: Kolmogorov–Smirnov; correlation: Pearson test; linear regression: ANOVA) and SPSS version 16.0 (IBM, Armonk, NY, USA) (Bonferroni post hoc test).

We used paired samples Student’s t-tests to compare the differences between means in the experiments described in Sections 2.1.1 and 2.1.3(a). Two-tailed t-tests were used to compare values obtained from experiments described in Sections 2.1.2 for whole sponges (b), 2.1.3(b), and 2.2. The Pearson test was used to evaluate the presence of any possible correlations between the impact number and the stiffening responses described in Section 2.1.2(b) and between sponge thickness and the variables extrapolated from the curves as described in Section 2.1.2(a); all the variables were tested for normality with the Kolmogorov–Smirnov test; linear regression and ANOVA were used to test the relationship between sponge thickness and the stiffening response in Section 2.1.2(b). Differences in the means obtained in the experiments described in Section 2.1.2 for isolated tissue samples (a and b) were evaluated using ANOVA and the Bonferroni post hoc test.

Further details are presented in the figure captions.
3. Results

3.1. Dimensional recovery

3.1.1. Whole sponges

After intense mechanical stimulation, sponges shrank along all three axes, the biggest changes occurring along the x-axis (Fig. 2).

Partial recovery of shape and volume was evident after 1 h (Fig. 3). Although the volume gain seemed to continue in the following hours, after 3 h the variations were not statistically different.

3.1.2. Isolated tissue samples

Cutting sponges during the preparation of slices resulted in significant shrinkage along the z-axis of ectosome and choanosome, of small and large canals, and also of the surrounding canal sheath (a well-defined whitish region where most of the sponge actinocytes are located) (Figs. 3B and 4). The x-axis of the large canals changed little.

3.2. Reaction to mechanical stimulation

3.2.1. Whole sponges

Deformation of samples subjected to the pressure induced by the rod occurred in three phases: (1) fast deformation; (2) decelerating deformation; and (3) slow deformation at a constant rate (Fig. 1D). No relationships were found between sponge thickness and any of the parameters (Fig. 5). The speed of deformation in the constant phase, which can be easily extracted from the curves, was used to describe the mechanical state of the sponge. Sponge stiffness was inversely proportional to the speed of deformation in the constant phase.

The method used stiffened both control and stimulated sponges (Fig. 6). However, the stiffening response faded quickly in the
control sponges, whereas in the stimulated sponges the stiffened state was maintained for at least 3 min after the stimulation (Fig. 6). At 1 and 3 min the control animals were significantly softer than the stimulated animals. The mechanical states of the controls at 1 and 3 min were significantly different with respect to the values obtained at 20 s after stimulation. There were no statistically significant differences between the stimulated sponges measured at different endpoints, although, comparing the values at 20 s with those at 1 and 3 min, a small decrease in the mean value (representing an increase in viscosity) was recorded.

3.2.2. Isolated tissue samples

Beam-shaped samples always reacted (at least after 30 s) to mechanical stimulation (3 impacts) by stiffening (decreasing the bending in a fixed time); this effect was statistically significant after 15 s only for ectosome samples (Fig. 7). There was a significant positive relationship between the number of impacts and the magnitude of the stiffening response, which was more evident in ectosome samples (Fig. 8).

3.3. Signal transmission

The sponges reacted to stimulation by stiffening the mesohyl (with sponge stiffness inversely proportional to the speed of deformation in the constant phase). Fig. 9 shows that point A’ is significantly stiffer than point A both in the individuals in which the exopinacoderm integrity was preserved and in those animals in which the exopinacoderm was disrupted. With this protocol we observed no significant differences between the stiffening responses at points A and B in both treatments. In contrast, we observed a significant increase of viscosity at point C only in the samples where the exopinacoderm was intact (Fig. 9A).

Fig. 10 emphasizes the pronounced stiffening effect of strong mechanical stimulation, since mean deformation speeds at point E in the constant phase were significantly different from all others.
Stimulation in the form of three impacts also had a significant stiffening effect (compare A and B); after three more impacts at point B we did not record any statistically significant effect at 4 cm distance (compare A and C), though we measured a significant stiffening effect at 2 cm (compare A and D).

### 3.4. Stiffening factors

#### 3.4.1. In vivo tests

Of the samples that had rested for 6 h before the experiment and had been treated with the different extract solutions for 1 or 2 h, those treated with protein fractions P40, P50, P60 and P70 were all significantly stiffer than the controls and the samples treated with fraction P80 (Fig. 11A). P40 was the fraction that led to the strongest stiffening response after 2 h. In contrast, samples that had rested for only 3 h showed no statistically significant stiffening response when exposed to fractions P40 and P50 (Fig. 11B). Only after an exposure to P40 and P50 for 3 h did the samples show significantly lower values compared with the controls.

The method employed here resulted in only partial separation of proteins: many bands were common to different fractions, though varying quantitatively (see Fig. S2 in the online Appendix).

#### 3.4.2. In vitro tests

Collagen fibril suspensions mixed with P40 dialyzed against ASW aggregated quickly (within a few seconds when shaken). On the other hand, mixing the suspension with ASW, distilled water alone or P40 dialyzed against distilled water showed no aggregation.

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**Fig. 7.** Mean deflection after 15 s, 30 s and 45 s of stimulated (white) and unstimulated (gray) samples (n = 10) of (A) ectosome and (B) choanosome. Stimulated ectosome samples were always significantly stiffer than controls; stimulated choanosome samples were significantly stiffer only after 30 s and 45 s. Vertical bars represent standard deviation and asterisks indicate statistically significant differences: *P < 0.05; **P < 0.001.

**Fig. 8.** Relationship between number of impacts and mean deflection of (A) isolated beam-shaped ectosome and (B) choanosome samples. A strong linear relationship could only be found between the number of impacts and ectosome stiffness (y = 7.5 – 0.7833x; R = 0.932, P < 0.001, n = 20); no correlation was found for choanosome samples (R = 0.39, P = 0.08). Statistical comparison is between unstimulated and stimulated samples. Asterisks indicate statistically significant differences: ***P < 0.001, N = 5.

**Fig. 9.** Mean deformation speed in the constant phase of sponges (n = 6) in which the exopinacoderm was (A) intact and (B) disrupted. A second measurement (A′), taken after stimulation at point A, revealed that in both conditions the sponges showed a significant decrease in the speed of deformation. In both conditions there was no significant response at 1 cm (point B). At 2 cm (point C) there was a significant decrease of the speed of deformation only in the samples with intact exopinacoderm. Vertical bars represent standard deviation and asterisks indicate statistically significant differences: *P < 0.05.
even after 24 h, suggesting that the presence of both P40 and certain ions is needed for the reaction. We should emphasize that we have evidence, to be published elsewhere, that distilled water does not denature P40. The qualitative dose-dependent assay suggests that the ability to aggregate collagen fibrils is dose-dependent. We did not observe any changes in the speed of the aggregation; on the other hand, adding 200 µL of P40 appeared to be enough to aggregate all the fibrils (see Fig. S3 in the online Appendix).

4. Discussion

4.1. Mechanical aspects: stimulation, response and signal transmission

The ability of C. reniformis individuals to stiffen in response to mechanical stimulation is obvious when they are being handled, although until now this was detected only by feeling that the animal was softer the first time it was touched than on subsequent occasions and by demonstrating stiffening through bending tests on tissue samples immediately after excision (Wilkie et al., 2006). Although these methods left no doubt that the phenomenon exists, the former was too subjective and the latter too unnatural, and neither directly demonstrated the true range of stiffening.

Regarding sponge dimensional recovery after MSC was obtained by mechanical stimulation we can infer the following. Manipulation of the whole sponge resulted in stiffening of the mesohyl and in shrinkage along all three axes. After 3 h sponges in MSC returned to their probable original size and possibly their original mechanical state in RC (Fig. 3). The major dimensional changes occurred along the axis parallel to the line of action of the applied pressure. Whole animals used for the test were handled mainly along the x-axis (corresponding to the major force generated by grasping the animal with the fingers), whereas isolated samples showed major changes in the z-axis, which is the axis compressed by the method used to produce the slices. The small reduction in the x-axis of large canals suggested that these structures may show active contraction. These observations, together with the data related to the dimensional changes occurring in isolated tissue (Sugni et al., 2014) during the return to RC, suggest that the stiffening process is accompanied by shrinkage of both canals and mesohyl. This phenomenon therefore differs from the contractile activity described in Tethya wilhelma where the contraction of the canals may be accompanied by a slight passive expansion of the mesohyl (Nickel et al., 2011).

Our experiments provided quantitative evidence that C. reniformis stiffens in response to mechanical stimulation. In particular, as previously shown by Parma (2007) in preliminary experiments, it was observed that any attempt to measure tissue stiffness itself affected tissue tensility (Fig. 9). For this reason it is still not possible to obtain a measurement of the absolute mechanical properties of the sponge mesohyl, but we need to compare samples with different stimulation magnitudes. From the same experiment Parma (2007) reported an increase of stiffness in the entire region close to the stimulated area (at a distance of less than 0.5 cm). This suggested the presence of a functional conduction system.

Our present experiments provided evidence for signal transmission within 2 min at 2 cm from the first stimulations spots (Figs. 9 and 10). Animals in which the exopinacoderm was disrupted showed a strong decrease in the ability to stiffen, which suggests that the exopinacoderm itself could play a role in the conduction mechanism.

Regarding the hypothetical conduction system, recent work (Ellwanger and Nickel, 2006; Leys et al., 2009) suggested the presence, in sponges, of an integration system involved in signal transmission and it was proposed that the endopinacocytes of the internal epithelium (endopinacoderm) could be the effectors of such a system (Nickel, 2010; Nickel et al., 2011). Morphological observations on C. reniformis demonstrated the presence of long, irregular cell processes in the mesohyl forming a loose network of cytoplasmic processes interlaced with, and juxtaposed against, each other in short tracts (Bonasoro et al., 2001). These processes probably belong to endopinacocytes and are often in close contact with granulocytes. It is thus possible that in C. reniformis endopinacocytes are also components (perhaps effector elements) of the integrative system responsible for the stiffening response. Indeed, data related to the reaction induced by mechanical stimulation of isolated tissue samples deprived of the exopinacoderm revealed that both the ectosome and the choanosome have the capacity to stiffen. In the light of the signaling pathway hypothesized by
Ellwanger and Nickel (2006) for the sponge *T. wilhelma*, we suggest a similar pathway regulates the stiffening reaction of *C. reniformis*. The exopinacoderm may thus be responsible for the “horizontal” signaling and the endopinacoderm for the “vertical” signaling that seems to act via a paracrine mechanism. The ectosome displays a much more predictable reaction to stimulation than does the choanosome. Moreover, the ectosome shows a stronger tendency to modulate the response with respect to stimulation magnitude (Fig. 8). The greater responsiveness and the stronger relationship between the number of impacts and stiffness shown by the ectosome compared with the choanosome samples may be due to the histological and functional differences between these two regions. The ectosome is the outer part of the sponge and is densely collagenous with abundant granule-containing cells, which may be involved in the release of a stiffening factor (Bonasoro et al., 2001). Furthermore, the ectosome has been considered to dominate the mechanical properties of the whole animal and to protect the choanosome region, which is responsible for the sponge's pumping activity, generating the water flow through the entire aquiferous system, facilitating food uptake, catabolite elimination, gamete expulsion and possibly communication functions via a pseudo-endocrine signaling (Ellwanger and Nickel, 2006). Since the choanosome is characterized by a less densely collagenous extracellular matrix (ECM), its minor contribution to the mechanical properties of the whole animal is not surprising. The important protective function of the ectosome region may explain the significant differences between the two regions in terms of ECM organization and their responses to mechanical disturbance.

It is interesting that data on the return to RC of isolated beam-shaped samples revealed that the ectosome is more affected by temperature than the choanosome (Sugi et al., 2014). This could also explain the seasonality of the so-called “creeping” phenomenon (Fassini et al., 2012). The creeping phenomenon has been regarded as an example of opportunistic asexual reproduction (Fassini et al., 2012) and in this light the possibility that the sponge can accelerate or inhibit the formation of propagules depending on external circumstances makes sense.

4.2. Presence and role of stiffening factor(s)

The present results appear to confirm the previous hypothesis (Bonasoro et al., 2001; Wilkie et al., 2006) that stiffening of the mesohyl is mediated by presumptive stiffening factors. According to our experiments at least one protein is able to stiffen isolated ectosome samples in vivo. This protein is weakly hydrophilic and directly affects collagen fibril interactions, as demonstrated by the aggregation test. At the moment we can tentatively exclude the possibility that this factor’s action is enzymatic, since the speed of aggregation was unaffected by the amount of partially isolated protein mixture that was added to the collagen solutions (see supplementary Fig. S3). However, it could be hypothesized that some enzymes are involved in the removal of the stiffening factor, because, when isolated ectosome samples are passing from MSC to RC, the effect of P40 is less evident and tends to retard the process, which could be due to enzymes still present in the ECM. The presence of a softening factor, softenin, has been recently detected in the dermis of the sea cucumber *Stichopus chloronotus* (Takehana et al., 2014). These authors suggested that this protein interacts with the tensilin binding sites thus eliminating the bonds between the collagen fibrils. Our data suggest that in the sponge *C. reniformis* the mechanism is different and that any presumptive softening factor would be an enzyme.

In our in vitro experiments on isolated fibrils, the stiffening factor appeared to require the presence of ions. Since it has been demonstrated in vitro that divalent cations are directly involved in the cohesion of the mesohyl ECM (Wilkie et al., 2006), it is reasonable to hypothesize that calcium ions are involved in bond formation.

Another unknown is the site of production and storage of the presumptive sponge stiffening factor. One hypothesis is that in sponges, as in echinoderms, the elements responsible for storing and secreting stiffening molecules are specific cell types. The weaker stiffening response of the choanosome in comparison with the ectosome, together with morphological evidence that cells with granular inclusions (represented mainly by spheroid cells and gray cells) are more abundant in the ectosome than in the choanosome (Bonasoro et al., 2001), suggest that the stiffening factor could be stored in one type of these granulocytes. This indicates another intriguing similarity to echinoderm mutable collagenous tissues, in which the presence of a regulatory factor has been localized to a specific population of granule-containing cells (juxtaligamental cells), which are considered to be the effector cells regulating changes in ECM viscoelasticity (Wilkie, 2005).

5. Conclusions

Our data support the view that the stiffening of *C. reniformis* involves a combination of phenomena including dimensional compression of the sponge body and modification of the interactions between the collagen fibrils of the mesohyl mediated by a secreted stiffening factor. In particular the mechanical reaction after different types of mechanical stimulation implies:

- an initial passive response due to externally imposed compression, followed by an active response consisting of an increase in mesohyl stiffness;
- a rather slow propagation of the stiffening response through the surrounding stimulated region at a signal transmission speed of 1 cm/min;
- a presumptive specific role of the exopinacoderm in signal transmission.

The biological significance of the stiffening phenomenon remains unclear. The physiological control of mesohyl stiffness may be related in part to regulation of the release of reproductive propagules. However, the adaptive advantage of the stiffening response to touch is more difficult to understand. Although some aspects related to the reaction of *C. reniformis* to mechanical stimulation have been resolved, many fundamental problems remain. In particular the molecular mechanisms that are the basis of the dynamic phenomena exhibited by sponge mesohyl need to be explored in detail. The ongoing characterization of the sponge stiffening factor(s) could throw more light on the evolutionary relationship between the mechanically adaptable collagenous tissues of sponges and echinoderms.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.zool.2014.03.003.
References


