UNIVERSITÀ POLITECNICA DELLE MARCHE

DIPARTIMENTO DI SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale in Biologia Marina

Dinamiche del collagene viscoelastico di *Chondrosia reniformis* Nardo, 1847 (Porifera, Demospongiae) studiate tramite tecniche istologiche.

Viscoelastic collagen dynamics in *Chondrosia reniformis* Nardo, 1847 (Porifera, Demospongiae) studied by histology.

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Sessione Straordinaria
Anno Accademico 2021 – 2022

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Riassunto

Chondrosia reniformis Nardo, 1847 è una spugna tipica del Mar Mediterraneo in grado di filtrare 900 volte il suo volume corporeo in 1 ora di tempo, svolgendo un ruolo chiave nella catena alimentare costiera, regolando direttamente la produzione primaria e indirettamente la produzione secondaria.

La sua importanza è di duplice natura: ecologica ed economica. Infatti, tra gli invertebrati marini, viene considerata una valida fonte alternativa di collagene, in quanto possiede sia la tipologia fibrillare (tipo I) sia la non fibrillare (tipo IV), estratte e sfruttate in campo farmaceutico, cosmetico e biomedico. Si prevede infatti che solo nel settore sanitario, il mercato del collagene raggiungerà 8,8 miliardi di dollari entro il 2027.

Un'altra caratteristica peculiare che distingue *Chondrosia reniformis* dalle altre spugne è la sua plasticità morfologica che permette ad un organismo sessile, quale è la spugna, di "muoversi" e di cambiare forma. Questa plasticità dovuta alla sua componente viscoelastica, il collagene, conferisce capacità rigenerative uniche. Le principali componenti della spugna coinvolte nei processi di rigenerazione e fusione sono il cortex (area periferica composta da ectosoma e mesoila) e il coanosoma (zona più interna contenente

le camere coanocitarie). Il collagene di tipo I è stato osservato nella mesoila, mentre ectosoma e coanosoma contengono il tipo IV.

Gli obiettivi principali del nostro lavoro sono stati la 1) stima dei tempi di rigenerazione e fusione dei frammenti di spugna e 2) l'osservazione dei cambiamenti morfologici a cui andavano incontro le spugne durante le varie fasi dell'esperimento rivolgendo particolare attenzione alle dinamiche del collagene.

Gli esemplari di *C. reniformis* utilizzati in questo studio sono stati campionati lungo la Riviera del Conero (AN) e mantenuti in acquario in condizioni controllate per la conduzione dell'esperimento. Le spugne sono state tagliate per ottenere frammenti della stessa dimensione (3 cm^2), i quali sono stati poi appaiati facendo combaciare le superfici di taglio. Sono state considerate coppie di frammenti

1) appartenenti allo stesso individuo, con cortex integro (*genets* non decorticati)
2) appartenenti allo stesso individuo, privi di cortex (*ramets* decorticati)
3) appartenenti ad individui diversi, con cortex integro (*genets* non decorticati)
4) appartenenti ad individui diversi, privi di cortex (*ramets* decorticati)

Entro 12 giorni, si sono fusi il 30% dei frammenti con cortex integro e il 100% dei frammenti privi di cortex. Questi ultimi sono stati in grado di rigenerare il cortex in 20 giorni.

Nei genets non decorticati, la parte centrale delle due spugne (il coanoderma) si fonde, mentre il cortex comincia a crescere dalla superficie della spugna verso l'interno, formando un setto collagenoso incompleto tra i due frammenti. Qui il collagene assume un'organizzazione irregolare e, tramite l'istologia, è stato osservato un accumulo di cellule sferulose lungo il setto.

I frammenti derivanti da individui diversi non si fondono. I ramets privati del cortex si comportano come fluidi viscoelastici e si mescolano parzialmente. Alla fine dell'esperimento non è stato più possibile distinguere i due frammenti di partenza.

Da queste osservazioni emerge che lo strato esterno della spugna (cortex) svolge un ruolo "barriera" che contrasta la fusione tra frammenti e che le cellule sferulose, derivate dalla differenziazione degli archeociti, partecipano attivamente alla formazione del collagene. L'aspetto turbolento del collagene tra mesoila e coanoderma potrebbe essere dovuto alla formazione di nuovi canali collagenosi e dunque alla riorganizzazione del sistema acquifero.

Il tasso di rigenerazione dei frammenti decorticati era di 0,12 cm^2/giorno (± 0,012 SD), e quindi di 42 cm^2 l'anno. Questo dato indica che per poter pianificare la raccolta sostenibile di questa si dovrebbe tener conto sia dei tassi di rigenerazione (42 cm2) che di quelli di crescita riportati in letteratura (16 cm2 l'anno). Ad esempio, una spugna di 30 cm^2 di cui circa metà

verrebbe tagliata ($15cm^2$), impiegherebbe un anno e mezzo per rigenerarsi completamente.

1. Introduction

1.1 *Chondrosia reniformis*

Chondrosia reniformis Nardo, 1847 is a common marine Demosponge in the Mediterranean Sea, which usually lives on shallow rocky bottoms and owes its generic name to the cartilage-like consistency of its densely collagenous cortex (ectosome) (Garrone et al., 1975).

Like other sponges, this species has a simple level of organizations, and while it possesses several types of specialized cells, there are not organized in tissue or organs. Three regions can be observed, a cortical zone called ectosome, an internal zone called mesohyl and an inner zone, the choanosome (Figure 1), which constitutes the complex aquiferous system of these animals. (Bavestrello et al., 1998). The choanosome is a softer consistency and densely filled with choanocyte chambers. The ectosome is characterised by thin layer of flat exopinacocytes which surround dense bundles of collagen fibrils. The mesohyl is characterised by the high amount of collagen fibrils and a lower number of cells. (Nickel et al., 2003). Unlike the almost larger number of species belonging to the class of Demospongiae, *Chondrosia reniformis* is characterized by total absence of silica spicules and the support of the body is guaranteed from the closely associated collagen fibrils with sediment grains

and exogenous spicules which they actively incorporate (Bavestrello et al., 1998).

Chondrosia reniformis is object of study for a series of features which make it economically and ecologically important. This sponge attracted the attention of scientists for the capability to modulate its mechanical properties by acting on the collagen crosslinks (Bonasoro et al.,2001; Wilkie et al. 2006; Fassini et al., 2014) and, since its high content, as an alternative source of collagen (Swatschek et al. 2002; Heinemann et al., 2007; Silva et al. 2016).

Among lower invertebrates, and sponges in particular, *C. reniformis* has been considered an interesting source of alternative and biocompatible collagen given its high collagen content (Garrone et al., 1975) and for its interactions with silicic acids (Heinemann et al., 2007b). Moreover, among Porifera, *C. reniformis* is the reference model of dynamic collagenous tissues, which are characterized by the presence of labile and variable collagen crosslinks, and has been extensively investigated. (Wilkie et al.,2006; Fassini et al. 2012,2014)

Also, the ecological role is important: sponges like *Chondrosia reniformis* are suspension feeders (Riisgård et al., 2000) filtering up to 50,000 times their body volume per day (Weisz et al., 2008). This means that a sponge of 1 dm^3 can clean up a volume of water comparable to that of an aquarium of 900 L in

1 hour, thus acting as a biological filter in coastal waters by directly regulating primary production and indirectly secondary production (Gili & Coma, 1998). In fact, it has been demonstrated that sponges, besides feeding on nano- and picoplankton (Reiswig, 1971; Ribes et al., 1999), can intake large amounts of DOC and DOM (Yahel et al., 2003; de Goeij & van Duyl, 2007; Maldonado et al., 2012).

This efficient and versatile filtration makes sponges key drivers of the uptake, retention, and transfer of energy and nutrients within benthic ecosystems (Maldonado et al., 2012) and makes them interesting candidate species for the bioremediation of organic pollution, such as waste streams from aquaculture (Osinga et al., 2010; Gökalp et al., 2019).

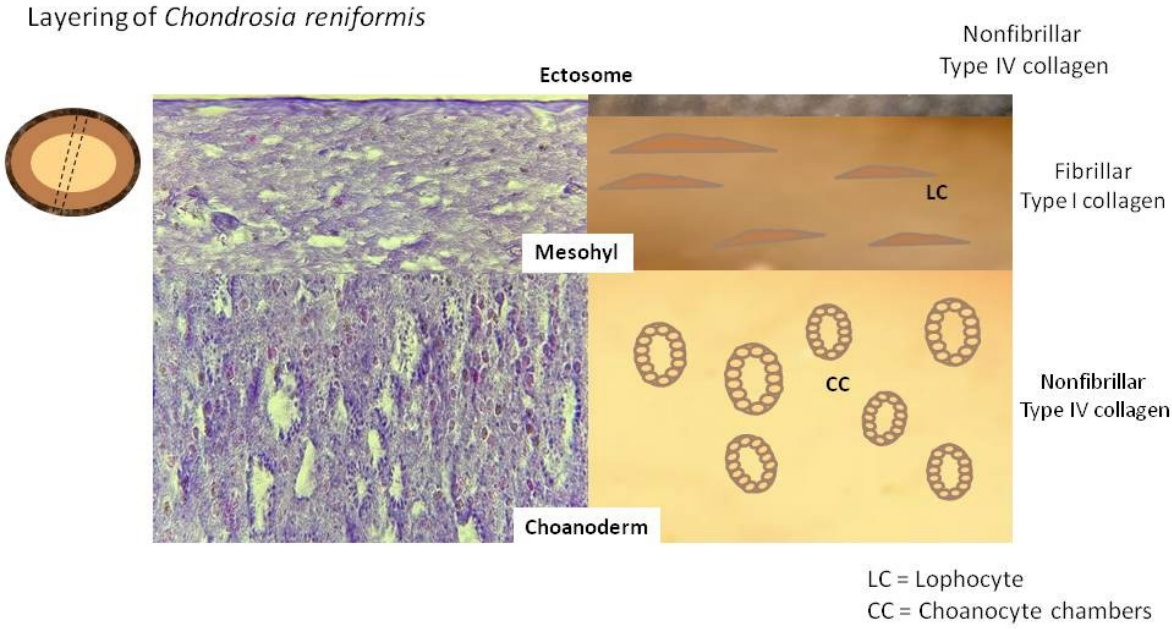

Figure 1 – Layering of *Chondrosia reniformis*

1.2 Collagen

Collagens are the most abundant high molecular weight proteins in both invertebrate and vertebrate organism, and possess mainly a structural role, existing different types according with their specific organization in distinct tissues. For years, cows and pigs were the primary sources for collagen production. Also, industry is constantly searching for new natural sources of collagen and upgraded methodologies for their production. (Silva et al., 2014). Its low immunogenicity and mechanical properties make this molecule a biomaterial that is extremely suitable for tissue engineering and regenerative medicine (TERM) strategies in human health issues (Pozzolini et al., 2018). Nowadays, collagen has a wide range of applications in the health-related sectors, namely in cosmetics, the pharmaceutical industry and in medical care (including plastic surgery, orthopedics, ophthalmology and dentistry) (Meena et al., 1999). Only considering healthcare industry, the collagen market was expected to reach $8.8 billion in 2027 at a compound annual growth rate (CAGR) of 9.1% (Global Collagen Report,2023).

As demonstrated by Pozzolini et al. (2018), collagen derived from marine sponges is suitable for biomedical applications, and to optimize the combined use of sponges as producers of natural products and bioremediators, we need to assess the biomass production and associated natural product content and

link these to the actual in situ filtration capacity of the sponge (Gökalp et al., 2020).

Sponges, the oldest metazoans living on our planet, are a rich source of collagen among the marine invertebrates, since, in many cases, as with *C. reniformis*, their skeleton and extracellular matrix are made mostly from collagen (Garrone et al., 1975; Ghosh et al.,2019).

Heinemann et al. 2007 used *C. reniformis*-derived collagen along with silica templating to produce hydrogels, which supported the adhesion, growth and differentiation of human mesenchymal stem cells into osteoblast cells. More studies have pointed that marine sponge collagen proved to be a good base for bone tissue engineering scaffolds (Lin, Z. Et al. 2011). Swatschek et al. (2002) evaluated the use of marine sponge *C. reniformis* as a source of collagen to be integrated as a moisturizer in cosmetic preparations. Collagen isolated from the marine sponge *C. reniformis* was also used to prepare microparticles by emulsifying and cross-linking, which were then incorporated in hydrogels and the effects on drug-stability and on dermal drug delivery were evaluated (Swatschek et al.,2002). It was shown that collagen from *C. reniformis* is effective for wound healing, and also for its antioxidant activity (Swatschek et al., 2002; Pozzolini et al., 2018).

In vertebrates, many different types of collagens have been described, which can be divided into several and genetically different subfamilies. Two main collagen groups are classified: (1) the fibril-forming collagen subfamilies, as types I, II, III, V, and XI, involved in the cross-striated fibril formation and characterized by uninterrupted repeats of the Gly–Xaa–Yaa triplet, and (2) the highly heterogenous nonfibrillar collagen subfamilies, which contain several interruptions in their collagenous domains (Exposito et al. 2002).

Collagens from *Chondrosia reniformis* have received attention due to their diversity (type I and IV, respectively fibrillar and nonfibrillar collagen) (Pozzolini et al., 2012). Type I collagen is the most extracted protein from marine animals, because it is a very important biomaterial in the field of tissue-engineering (Felician et al., 2018). Also type IV collagen extracted from *Chondrosia reniformis* is a potential biomaterial for tissue engineering (Heinemann et al., 2007; Pozzolini et al., 2012): used for potential collagen nanoparticles for drug delivery systems (Nicklas et al., 2009) and suitable for dry skin to cosmetic formulations (Swatschek et al., 2002).

The mesohyl is made of fibrillar collagen type I, while ectosome and above all the choanosome are made by nonfibrillar type IV collagen (Pozzolini et al.,2015). The collagen fibrils both mediate cell–matrix interactions via

membrane receptors and provide the structure of the extracellular matrix (Ehrlich et al.,2018)

Chondrosia has received attention also for interesting structural (Heinemann et al.,2012), physicochemical (Palmer et al., 2012) and ecophysiological properties (Bavestrello et al., 1996; Fassini et al., 2013, 2014; Pozzolini et al., 2016). For example, slices of fibrillar collagen incubated with collagenase are not modified even after 48h of incubation, and do not show any changes in the aspect, consistency and structure of fibrils (Ehrlich et al.,2018); Before only fibrillar collagen was used for biomedical purpose as an organic template for in vitro silification (Heinemann et al., 2007) but recently also collagen IV has been patented as a source of special membranes for biomedical applications. (Silva et al., 2016)

C. reniformis is the only sponge which has been experimentally proven to contain a dynamic collagenous mesohyl capable of stiffening upon being manipulated (Pozzolini et al., 2012) It was shown that the different physiological states recorded in laboratory experiments are expressions of the mechanical adaptability of the collagenous mesohyl of *C. reniformis*, and suggest that stiffness variability in this sponge is under cellular control (Heinemann et al., 2007).

1.3 Regeneration

Another aspect that has caught the attention of scientists is the plasticity of the tissue and ability to deform. Indeed, *Chondrosia reniformis* is characterized by the presence of labile interfibrillar crosslinks similarly to those described in the mutable collagenous tissues (MCTs) of echinoderms (Fassini et al. 2017, Bonasoro et al. 2000).

Thanks to the mesohyl plasticity, the sponge can change the shape of a certain area, which can elongate also thanks to gravity. The elongated portion may detach and reattach elsewhere (Cerrano et al., 2001). This phenomenon, called "creeping", has been interpreted as adaptive strategies related to environmental factors, asexual reproduction or localised locomotor phenomena (Bond and Harris., 1988). Bonasoro et al. (2000) investigated the histological modification during plasticisation and the results show that is limited and localised. The most significant structural changes involve mainly cytological features of specific cellular components characterised by granule inclusions (i.e. the spherulous cells) and the arrangement and density of the collagenous extracellular framework, though the integrity of the collagen fibrils themselves is not affected. The observations of paracrystalline or filamentous content in the spherulouse led some authors to suggest that these cells could be responsible for secreting collagen fibrils (Pavans de Ceccatty

and Thiney 1964a, b), the specific components of the extracellular organic matrix (Garrone 1978), or the spongin fibres in other sponges (Bretting et al. 1983).

The plasticity of mesohyl allows fast regenerative capacities of this sponge (Bavestrello et al.,1998; Nickel et al.,2001). Sponge fragments (fragmorphs) are easily cultured in aquaria to study regeneration processes in *Chondrosia* (Nickel and Brummer, 2003). In particular, Nickel and Brummer have seen that after fragmentation, the fragments have grown, especially the smaller ones, and during growth the reorganisation of tissue and cells depending on the region where the fragments were taken.

Given the ever-growing interest in these regeneration mechanisms, scientists conducted a series of experiment on self/non-self recognition system (Custodio et al., 2013) using different genetic conditions of specimens like allogeneic (specimens from different individuals but from the same species), autogeneic (specimens from same individual) or xenogeneic (specimens from different species) individuals (Smith, 1988) in order to understand rejection reactions such as the formation of barriers or cytotoxicity events (Saito., 2013).

1.4 Spherulous cells

According to Smith 1990 the spherulous cells have small, condensed, a-nucleolate nuclei that are located in the center of the cell. The cytoplasm is filled with large spherical inclusions (hence the name). There are three types of spherulous cells in *Callyspongia diffusa*, which are based on differences in spherule-staining properties (figure 1). However, the spherulouse cells do not divide but arise from archaeocytes, that are the totipotent cells at the basis of sponge regenerative capacities (Simpson, 1984).

Through the Hematossilina Eosina (HE) staining we can distinguish acidophilic, neutrophilic and mixed cells. The first are found scattered throughout the sponge and in clusters located just below the exopinacoderm where the mesohyl reaches the surface. While the neutrophilic are never found clustered in groups, nor do they appear to release their granules. The rare mixed spherulous cells are usually found on the endopinacoderm. (Cellular morphology of *Callyspongia diffusa*, Smith 1990).

In *A. Cavernicola* are two principal morphotypes of spherulous cells occurs in the wound area of this sponge: larger cells with clear inclusions and smaller ones with dense inclusions. Indeed, these morphotypes represent different stages of the ontogenesis of spherulous cells (Vacelet, 1967). According to Vacelet (1967) in intact sponge “dense” small spherulous cells concentrate

mostly in the ectosome and around big exhalant canals, while “clear” larger ones are distributed in the mesohyl of endosome (Ereskovsky et al. 2020)

All spherulous cells, regardless of their appearance in the different types of tissue contained the lectins that may be involved in spongin production (in the choanosome of *Axinella polypoides*, Bretting et al. 1983)

Lectins are proteins with a unique capability to bind specific sugar moieties, for this they are widely applied in the medical research. (Lis and Sharon 1977; Goldstein and Hayes 1978; Pereira and Kabat 1979)

Vaith et al. 1979 speculated that lectins were involved in the defense mechanism of the sponge against pathogens.

Electron-microscopic studies by Bretting et al. 1983 revealed that the spherulous cells with small vescicles are derived from archaeocytes and transformes into spherulous cells with large vescicles, a process accompanied by the conversion of lectin II to lectin I (Figure 2).

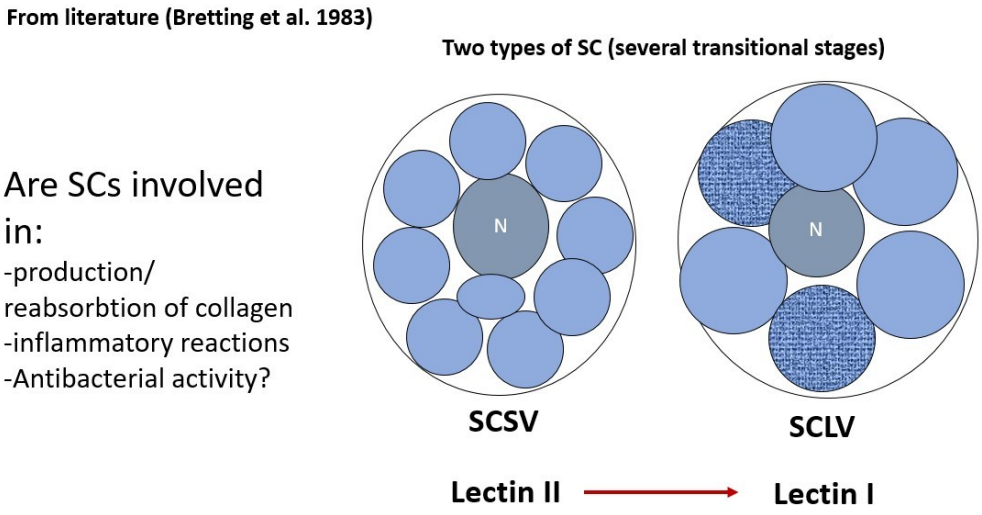

Figure 2 – Types and functions of spherulous cells (Bretting et al., 1983)

The spherulous cells of sponges were reported to participate in storage of various metabolites like brominated (Turon et al., 2000); they also produce defense metabolities in some species of sponges. Moreover play a role in immune response against non-symbiotic bacteria, or against fouling, predation (by release of attractive metabolites) (Thompson et al. 1983; Ternon et al. 2016) excretion (Vacelet,1967; Donadey 1978; Maldonado 2016; Ereskovsky et al. 2020), and mesohyl extracellular matrix synthesis and maintenance (Donadey and Vacelet 1977; Donadey 1982; Bretting et al. 1983; Smith and Hildemann 1990). About the metabolities, seems that sponges like *Crambe crambe* stored these compounds into spherulous cells and dispersed into the water column after release through the sponge exhaling channels, leading to a chemical shield surrounding the sponge. This mechanism of action called

“spherulization” may therefore contribute to the ecological success of encrusting sponges that need to extend their substrate cover to expand. (Ternon et al. 2016)

Furthermore, according to Maldonado et al., (2016) archaeocyte-like cells, known to have intense phagocytic activity, appear to be engaged in digestion and elimination of refractory leftovers, while spherulous and granular cells appear to be involved in excretion of metabolic by-products. Extrusion of spherulous cells through the epithelia of the aquiferous canals of *A. cavernicola* has previously been documented by Vacelet (1967), who first suggested that it could be a way to eliminate excretory products.

About defense of microbes in *A. cavernicola* the wound surface was free from invasive microbes from the first hours after injury up to the end of the regeneration. Such defence against invasive microbes is probably provided by the particular chemical substances in the spherulous cells. (Ereskovsky et al. 2020)

In sponge like *Geodia cydonium* most of spherulous cells are found in layers that are rich in ground substance, almost free of other cell types, rich in collagen fibers. For this spherulous cells could be involved in the formation of the amorphous substance of the second layer of the ectosome (Bretting et al. 1983).

2. Purpose of the thesis

Chondrosia reniformis has been reported as a rich source of biomedically interesting types of marine collagen (Pozzolini et al., 2018) usable in the cosmetic and medical industries. However, an intense exploitation would lead towards depletion of the resource. An indiscriminate collection would be not sustainable and would entail to an impoverishment of the habitat and loss of ecosystem services associated to an important filter feeder. Since the recovery times of the sponge are still unknown, it is crucial to investigate on regenerative processes in *Chondrosia reniformis*.

Sponges are also organisms' models reared to understand biological processes (Muller et al., 2013), to study symbiont transmission (Maldonado et al., 2022) and to inspire biotechnological applications (Ereskovsky et al., 2021). Marine collagen matrices can be used to produce resorbable scaffolds in tissue engineering and regenerative medicine (Pozzolini et al. 2018) thanks to their biocompatibility (Bermueller et al., 2013) with human tissues. Being *Chondrosia* a potential source of biomaterial, it is necessary to study the effects of manipulation and fragmentation in experimental modelling.

The main objectives of our work are i) to determine the regeneration timing of sponge fragments and ii) to monitor and to understand the changes in the organization of the sponge components (ectosome, mesohyl and choanosome)

during its recovery. Even if the experiments were conducted at laboratory, the obtained results could be a first benchmark towards a sustainable management of the resource in the wild and in mariculture plants.

Additionally, sponges may recover wounds or re-fuse after cut collection (Smith, 1986 ; Saito et al., 2013). Autografting (contact between fragments of conspecifics) experiments were conducted in rearing conditions to verify if genets (fragments from the same individual) and ramets (fragments from different individuals) had a different allorecognition mechanisms during forced contacting of the cut pieces. The self-nonself recognition system may affect the distribution and the organization of the sponge collagen, thus influencing the collagen quality/quantity (Heinemann et al., 2007). In Table 1 are summarized all our objectives and research questions of this study.

Issues	Objectives	Research questions	Hypothesis	Methods
Collagen is a protein that is particularly valuable in the cosmetic and pharmaceutical industries; this protein is extracted from both terrestrial and marine animals such as the sponge *Chondrosia reniformis*, that therefore should be considered an important commercial species. Furthermore this sponge has a high ecological importance: has been demonstrated that sponges, besides feeding on nano- and picoplankton (Reiswig, 1971; Ribes et al., 1999) , can intake large amounts of DOC and DOM (Yahel et al., 2003; de Goeij & van Duyl, 2007; Maldonado et al., 2010). Its ecological relevance could therefore be	To increase knowledge on the different type of collagen produced by the sponge *Chondrosia reniformis*	Where are the different type of collagen in *Chondrosia reniformis* located?	Ectosome, choanosome and regeneration/fusion zone have different forms of collagen	FTIR (future perspective)
	To enahnce the understanding of the regeneration and fusion processes in *Chondrosia reniformis*	During regeneration/fusion events how does the sponge rearrange cell and collagen distribution?	The amount of spherulose cells varies in the various compartments of the sponges undergoing regeneration/fusion	Morphological observation, histological approach

threatened by overexploitation.				
Chondrosia reniformis has an important morphological plasticity which allows it to regenerate its tissues and reorganize the collagen in a short time. Fusion events between genets and ramets have been observed both in the wild and in the aquarium. This capability may represent another potential application in the biomedical industry, in particular as regards the rejection of tissues.		What role do the spherulose cells play?		Microscopic count of histological sections, imageJ software
		What role does the ectosome play in the fusion events?	Genets without an ectosome are either unable to fuse or have different fusion times from ramets	Experiments in the aquarium: comparison between fusion processes in genetically identical portions (genets) and portions from different individuals (ramets)
				Time lapse
		How change, morphologically and histologically,the processes of recognition and fusion between genets and ramets?	The fusion processes between genets and between ramets take place differently…	Macroscopic observation in the aquarium, morphological observation, histological approach

3. Materials and methods

3.1 Ultrastructure of *Chondrosia reniformis*

Chondrosia reniformis it is made up of several layers and each have its own function. The ectosome is the general term for describing the peripheral zone of the sponge body, while the cortex is the superficial specialized reinforced part of the ectosome. It is characteristic for leuconoid sponges, such as *C. reniformis* (Ereskovsky et al., 2021). The cortex presumably acts as a protective layer (Vacelet 1971) and it occupies the area immediately below the exopinacoderm and consists of a specialized portion of the mesohyl (Ereskovsky et al., 2021). Below it there is the mesohyl, the inner space of the sponge body where is fibrillar type I collagen found. Besides collagens, the mesohyl includes organic ground substance, which encompasses all cellular elements. The mesohyl ground substance and collagen fibrils may play a crucial role in cell–cell or cell–matrix interactions, immune reaction, self/nonself recognition. (Ereskovsky et al., 2021). Moreover, the majority of cells demonstrate apparent motility and the ability to modify, making the structure of the mesohyl highly unstable (Ereskovsky et al., 2021).

Under is the choanoderm that consists only of choanocytes, which form the choanocyte chambers (Figure 3).

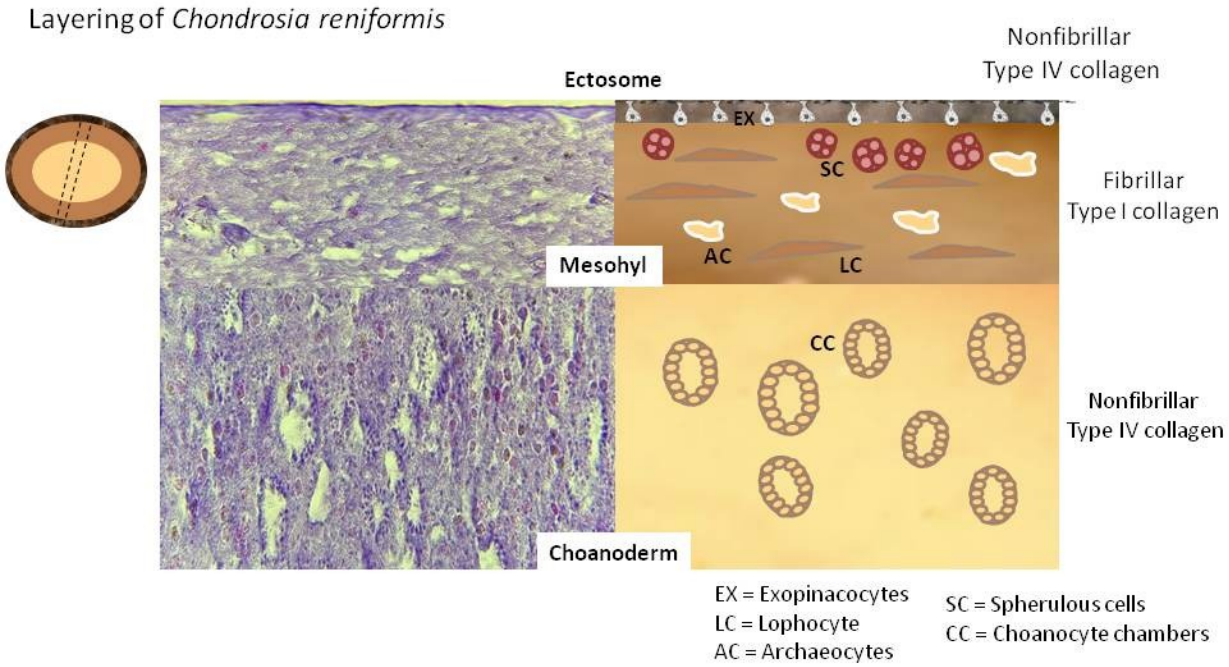

Figure 3 – Layering of *Chondrosia reniformis.*

In each layer there are different cell types, for their function and location, see the table 2.

Table 2

LOCALIZATION	CELL TYPE	FUNCTIONS
Ectosome	Exopinacocytes	The exopinacocytes can secrete components of the extracellular matrix and synthesize collagen (Garrone, 1978 ; Gaino et al., 1986)
	Melanocytes	Photoprotective role (Vijayan et al., 2017)
Mesohyl	Lophocytes	Cells participating in the secretion of collagen and formation of its fibrils (Ereskovsky et al., 2021)
	Spherulouse cells	Storage of various metabolites (Thompson et al. 1983; Uriz et al. 1996; Becerro et al. 1997), immune response against nonsymbiotic bacteria, defense against fouling, predation by release of metabolites (Thompson et al. 1983; Ternon et al. 2016), excretion (Maldonado 2016; Ereskovsky et al. 2020), and mesohyl extracellular matrix synthesis and maintenance (Bretting et al. 1983; Smith and Hildemann 1990).
Choanosome	Choanocytes	Flagellated cells that shapes choanocyte chambers, which are located between the inhalant and exhalant systems (Boury - Esnault and Rützler, 1997)

3.2 Sampling sites and transportation

In order to investigate about sponge regeneration and fusion processes, 29 specimens of *C. reniformis* were collected in May 2022 by scientific scuba

divers at Passetto (Ancona, Adriatic Sea) at depth of 6-8 where sea water was about 19-21 °C. m. This area is part of the Conero Promontory and is characterized by rocky cliffs surrounded by sand and mud seabed. The waters are highly eutrophic, with high rates of sedimentation and wide seasonal temperature fluctuations, ranging from 7°C in winter to 27°C in summer (Di Camillo et al., 2012). Due to the high availability of nutrients in this location, *Chondrosia reniformis* reaches considerable sizes with a wider reproductive period respect to specimens from other localities (Di Camillo et al., 2012).

The collected fragments were transported to the University of Ancona and, after an acclimatization period of 40 min., were transferred to aquaria.

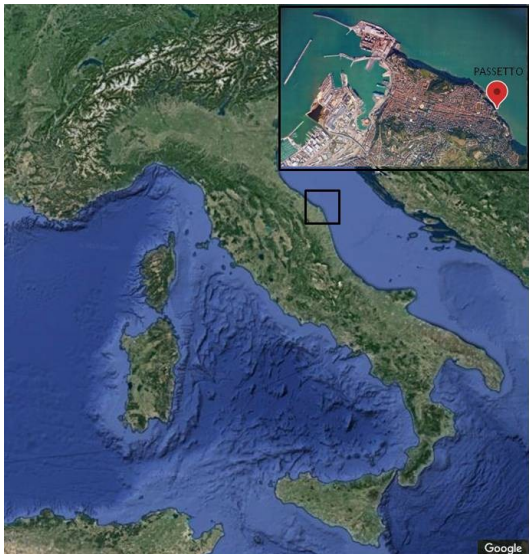

Figure 4 – Map of the study area in the North Adriatic Sea. The picture shows the sampling site (Passetto beach). (Google Earth).

3.3 Rearing conditions

During the experiments, the specimens of *Chondrosia reniformis* were kept in 6 aquaria (n. 4 fragments for each tank) of 30 liters capacity each. All the aquaria were filled with Artificial Sea Water (ASW) obtained by dissolving 38.2 g of Red Sea Salt into 1 liter of distilled water without silicates. Temperature in the tanks was kept constant around 19±2 °C using a chiller to limit the spreading of pathogens.

Nitrate levels were regularly monitored with a test kit, and water changes were carried out whenever they reached levels above 20 mg l^{-1} (in general, this happened every 15 days). During all trials the aquaria were kept fully in the dark, as to avoid algal proliferation and silicate depletion caused by diatoms, while the salinity was kept constant at 35psu and measured regularly with a refractometer. All sponges were fed with a basic food composed of marine phytoplankton gel in a mineral suspension (Easybooster 25). This mixture contains three species of microalgae and one diatom, respectively Nannocloropsis sp. (31%), Isochrysis sp. (33%), Tetraselmis sp. (18%) and Phaeodactylum sp. (18%), and was administered to our specimens after being diluted in aquarium water (1ml of phytoplankton gel per 100ml of water).

2.4 Fusion & Regeneration experiment

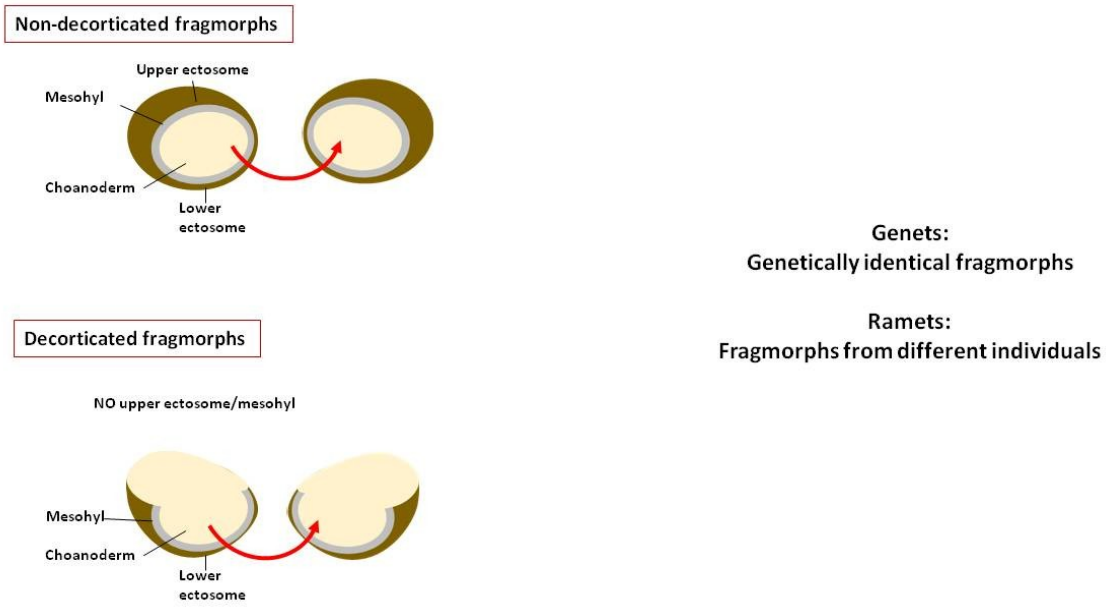

Figure 5 – Illustration of treatments on fragmorphs.

To study allogeneic/autogeneic reactions between sponges' fragments, 29 fragmorphs (i. e., sponge fragments of approximately 1 cm^3) (Pozzolini et al., 2017) have been obtained from the collected sponges, 19 cut from genetically identical portion (genets) and 10 from different individuals (ramets) (Maldonado and Uriz, 1999) (Table 3). Then, 17 pairs of genets and 8 of ramets were transferred in 25 Petri dishes (each containing a couple of genets or a couple of ramets) placed on the bottom of the tanks. The maximum observation time was 20 days.

Table 3

TOT FRAGMORPHS	TYPE	TREATMENT
29	GENETS 19	DECORTICATED 13
		NO DECORTICATED 6
	RAMETS 10	DECORTICATED 5
		NO DECORTICATED 5

According to Nickel and Brummer (2002), cortical parts and pinacocytes seem to be necessary or at least auxiliary for the formation of reorganised fragments and since the cellular composition of the ectosome is the most important factor influencing wound healing and regeneration in *C. reniformis* (Nickel and Brummer , 2002), 5 pairs of genets and 4 pairs of ramets were deprived of the part of the upper ectosome (Figure 6) using a cutter.

The total area of each fragmorphs was 3cm^2 and the removed area was 1,5 cm^2. Given the thickness of ectosome of 0,2cm the total volume is 0,6 cm^3. To calculate regeneration rate per day, the cut area was divided for the total amount of days required to completely re-form the ectosome. The recovery

rate was the average ± SD of the rates calculated for each fragmorphs (11 in total), expressed both as regenerated surface (cm^2) and volume (cm^3).

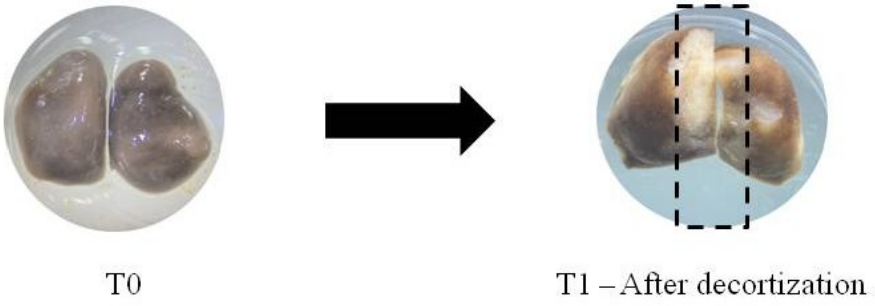

Figure 6 – Fragmorphs decortication explained.

Each week the pairs were photographed under a stereomicroscope in order to monitor the state of fusion progress. Before the embedding the fragmorphs were cut perpendicular to the fusion, observed and photographed under a stereomicroscope. The piece itself was reduced to proceed with the inclusion. Processes were conducted as carefully as possible and air exposure was minimized as well to avoid stresses that can lead to damage or necrosis of the individuals.

To improve the observation of the regeneration process, the specimens were monitored also using time - laps. To do this, another aquarium was set up under the microscope to which a camera (Z50 Nikkor ZDX 16-50mm) was mounted. The aquarium parameters were the same set for the aquarium used for the fusion experiment. The camera was set with 8 hours of continuous

shooting, with a picture taken every 10 minutes, reaching a total of 480 hours (20 days) per each monitored fragmorphs. 2 genets and 2 ramets were considered to carry out this experiment, both decorticated and no decorticated (Table 4).

Table 4 Schedule of *Chondrosia reniformis* experiment

Samples	Genets/Ramets	No decorticated/Decorticated	Merged time	Histology	Time laps
GND1	GENETS	No decorticated	8 Days	X	
GND2	GENETS	No decorticated	8 Days	X	
GND3	GENETS	No decorticated	8 Days	X	
GND4	GENETS	No decorticated	8 Days	X	
GND5	GENETS	No decorticated	Never	X	
GND6	GENETS	No decorticated	Never		
GND7	GENETS	No decorticated	Never		
GND8	GENETS	No decorticated	Never		
GND9	GENETS	No decorticated	Never		
GND10	GENETS	No decorticated	Never		
GND11	GENETS	No decorticated	Never		
GND12	GENETS	No decorticated	Never		
GND13	GENETS	No decorticated	Never		X
GD1	GENETS	Decorticated	12 Days	X	
GD2	GENETS	Decorticated	12 Days	X	
GD3	GENETS	Decorticated	12 Days	X	
GD4	GENETS	Decorticated	12 Days	X	
GD5	GENETS	Decorticated	12 Days	X	
GD6	GENETS	Decorticated	144 Hours		X
RND1	RAMETS	No decorticated	Never	X	
RND2	RAMETS	No decorticated	Never	X	
RND3	RAMETS	No decorticated	Never	X	
RND4	RAMETS	No decorticated	Never		
RND5	RAMETS	No decorticated	Never		X
RD1	RAMETS	Decorticated	12 Days	X	
RD2	RAMETS	Decorticated	12 Days	X	
RD3	RAMETS	Decorticated	15 Days		
RD4	RAMETS	Decorticated	15 Days		
RD5	RAMETS	Decorticated	240 Hours		X

3.5 Histology

Histological staining was done only on the specimens that had attached themselves. Genets and ramets were cut into fragments less than 1 cm^3 to insert into the tile. For histological preparation the sponges were cut in smaller pieces (about 1 cm^3 volume) and fixed in 4% formaldehyde on sea water. *Chondrosia reniformis* has not mineral skeleton but incorporates large quantities of foreign material, and displays a strong selectivity and polarization when doing so (Bavestrello et al., 1998). To prevent this material from interfering with the microtome cut, two washes were carried out, lasting two hour each: 1) 5% hydrofluoric acid for silica residue and 2) 4% solution of ethylenediaminetetraacetic acid, disodium salt (EDTA) to remove calcareous residue. Between the two steps, one wash in Phosphate Buffered Saline (PBS) was done. Then fixed tissues has been dehydrated through an ethanol series: 70% for 30 minutes, followed by 2 changes of 95% alcohol, 1 hour each, and finally 3 changes of 100% alcohol, 1 hour each). Clear the tissue through 2 changes of xylene, 1 hour each and finally embedded in paraffin overnight. The next day 2 changes of paraffine, 1 hour each, were performed. The final step consists in create paraffin block with plastic cages and embedding mold. The orientation of sponges in these block was chosen to be able to cut the junction in longitudinal section.

To have representative sections of the sample, 3 levels per samples were cut with a microtome (Leica RM2125 RTS), 3 sections per each level with a gap of 500 µm between levels. Sections, 7 µm-thick, were mounted on glass slides and stained, with hematoxylin and eosin. Since *Chondrosia* has a very porous tissue, changes have been made to the staining times protocol (10'' in diluted hematoxylin, 2' in eosin). With hematoxylin and eosin we wanted to highlight the cellular components near the contact point between the two fragmorphs, in order to identify cells involved in the fusion event.

To point out *Chondrosia*'s collagen, slides were also stained with Masson's trichrome stain (Bio – Optica). Also for this protocol some modifications were made in the staining due to the porosity of the tissues: the drops of the reagents have been reduced, and also the times of exposure.

To describe the sections at morphological level, they were observed with Zeiss microscope with camera (Axio imager m2) and photographed using the program Zen 2.6. They were taken at different magnifications in order to understand the structures.

3.6 Spherulous cells counting

Since the spherulous cells (Sc) appear to be involved during sponge tissue regeneration increasing their number during the different stages, particularly during the ectosome regeneration (Ereskovky et al., 2019), the density of the

cells was observed and measured to test the hypothesis that Sc concentration was different in the several sponge parts (mesohyl, choanoderm, ectosome, wounds). Spherulous cells were counted on images of hematoxylin – eosin sections of two decorticated and two no decorticated specimens. The images were obtained with Leica microscope at x40 magnification using Leica Application Suite X. For both decorticated and no decorticated organisms ten images, arising from two independent individuals, were used for counting. Spherulous cells were counted in the decorticated area. The number of spherulous cells were counted for an area of 4700 mm^2 for each image. Cell counting and area measuring were done with ImageJ software (National Institute of Health). For each condition mean and standard deviation were calculated.

4. Results

4.1 Fusion & Regeneration experiment

Fusion was not observed in any fragmorphs (Table 5).

One of our objectives was to verify if there were some differences in the fusion process between genets and ramets. Of 13 non-decorticated genets, only 4 were merged (about 30%) after 8 days. While all decorticated genets fused after about 12 days from the cut (Figure 7). Regarding ramets, only the decorticated ones (5/10, 50%) merged after 12-15 days (Figure 8).

About non-decorticated genets that never merged during the observation time, in particular at time T3, 18 days from T0, the fragmorphs changed their shape even without merging. While the decorticated genets between time T1 and T2 reformed ectosome and at time T3 merged changing the shape. Regarding the decorticated ramets at time T1 the shape begins to become irregular until it takes on a shape that does not allow the two fragmorphs to be distinguished at time T3.

Table 5

TOT FRAGMORPHS	TYPE	TREATMENT	MERGED FRAGMORPH	N. DAYS	ULTRASTRUCTURE of the fusion
29	GENETS 19	NON-DECORTICATED 13	4/13 (30%)	8/20	Irregular organization of collagen at mesohyl level, close to the contact point between two fragmorph.
		DECORTICATED 6	6/6 (100%)	12/20	Both fragmorphs form again the ectosome. No evident separation between choanoderms of the two fragmorphs, while basal ectosomes remain distinct. Accumulation of spherulouse cells in the area where the ectosome was removed.
	RAMETS 10	NON-DECORTICATED 5	0/5 (0%)	0/20	Fusion was not observed.
		DECORTICATED 5	5/5 (100%)	14/20	Both fragmorphs form again the ectosome. Fusion occurs but the fragments mix their ‘layers’.

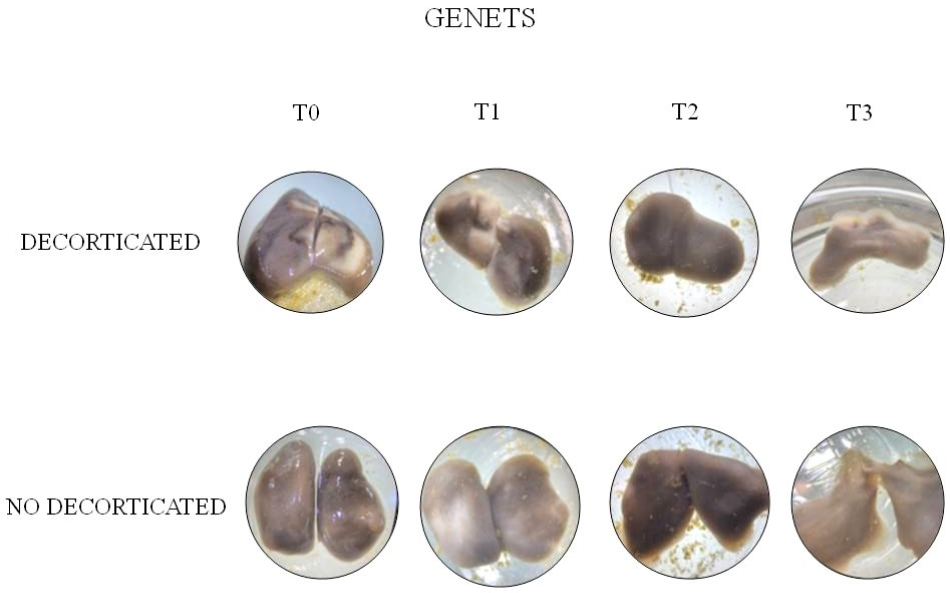

Figure 7 - Comparison between genets decorticated and non-decorticated. T1= after 6 days from T0, T2 = after 6 days from T1 and T3 after 6 days from T2.

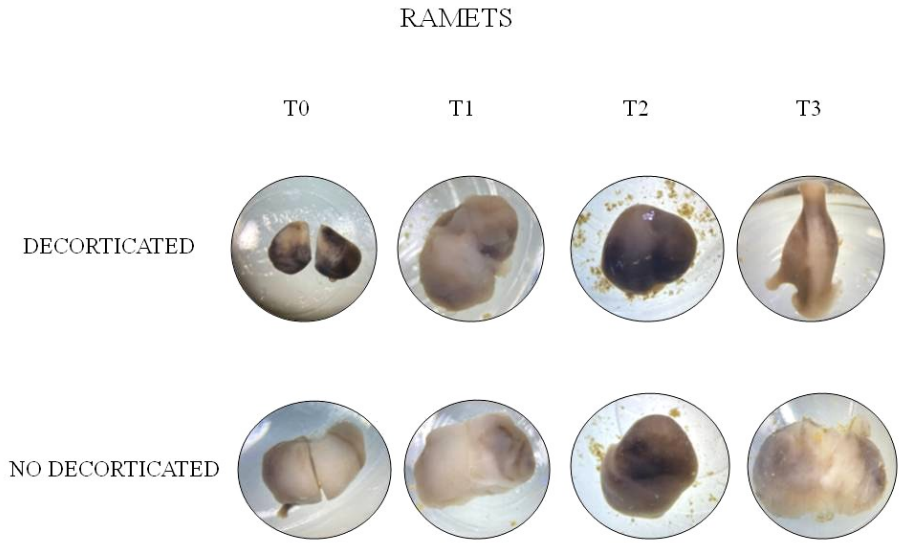

Figure 8 – Comparison between ramets decorticated and non-decorticated. T1= after 6 days from T0, T2 = after 6 days from T1 and T3 after 6 days from T2.

The contact points are identified, both in decorticated and non-decorticated genets, with a band of collagen which corresponds to the mesohyl, occurring in the merging areas, which allow us to distinguish between the two fragmorphs. The band was clearly evident with the Masson's trichrome stain (Bio – Optica). However, in decorticated genets the mesohyl reforms only in correspondence with the basal ectosome, because in the decorticated area the band will only form after the upper ectosome is rebuilt.

Regarding non-decorticated genets that have merged we were able to observe, a irregularity of a collagen close to the contact point, in the mesohyl. This irregularity was also observed in the histological sections, with Masson's trichrome staining (Figure 9). We can observe how the mesohyl collagen is inhomogeneous or turbulent and does not show its usual organized structure. Always with regard to non-decorticated genets, near the melting zone have been observed many and small channels. Also many choanocyte chambers have been observed. Lastly the choanoderms of two fragmorphs are recognise.

Figure 9 – Visual path of irregularity of collagen in non-decorticated genets.

During the observation time the decorticated genets reformed the ectosome and merged. In particular, the time to fuse was shorter than the time taken to reform the ectosome (48 hours). The point of contact show that the separation of the two fragmorphs is found at the level of the basal ectosome, while at the level of the choanoderms there is no evident separation (Figure 10).

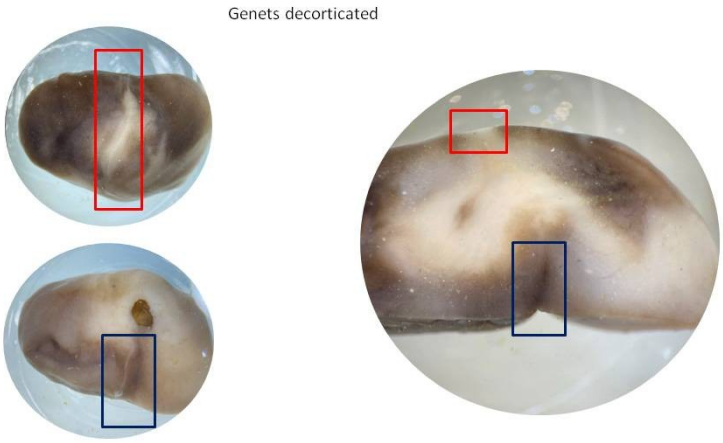

Figure 10 – Section to the binocular of decorticated genets.

Lastly in the decorticated genets an accumulation of eosinophilic cells (likely spherulose cells) has been observed both in the area where the ectosome was removed (Figure 11) and close to the fusion, in the mesohyl (Figure 12).

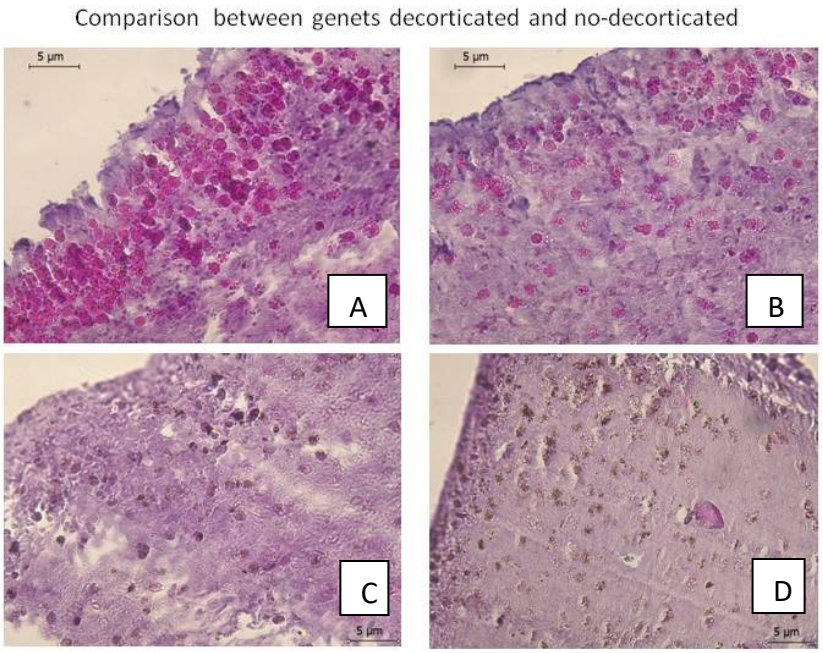

Figure 11 – Comparison between genets decorticated (A,B) and no-decorticated (C,D).

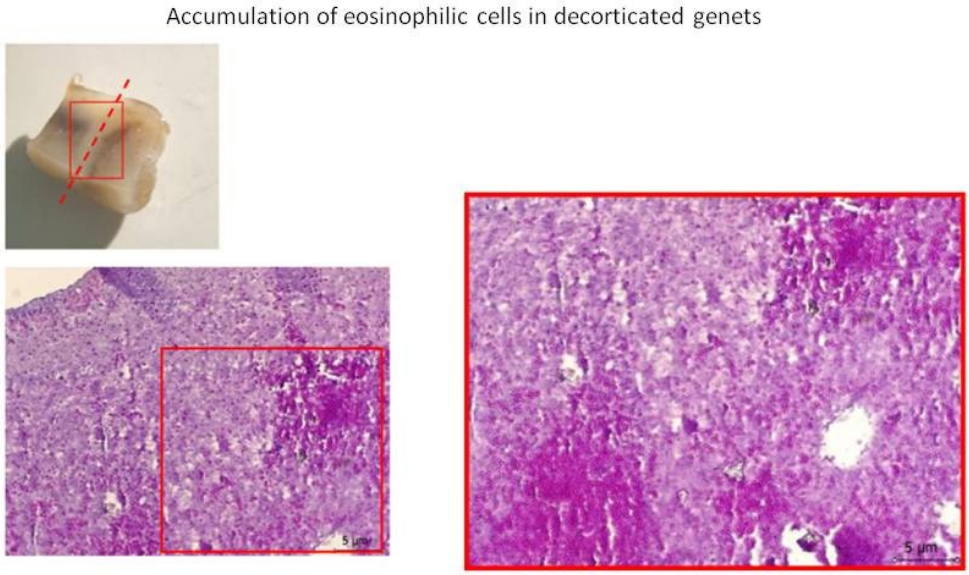

Figure 12 – Accumulation of eosinophilic cells close to the fusion in decorticated genets.

The comparison between decorticated and non-decorticated specimens, in terms of density of spherulous cells (i. e., n. of SC per cm^2) showed that decorticated specimens had significantly more spherulous cells in the mesohyl than non-decorticated ones (Figure 13)

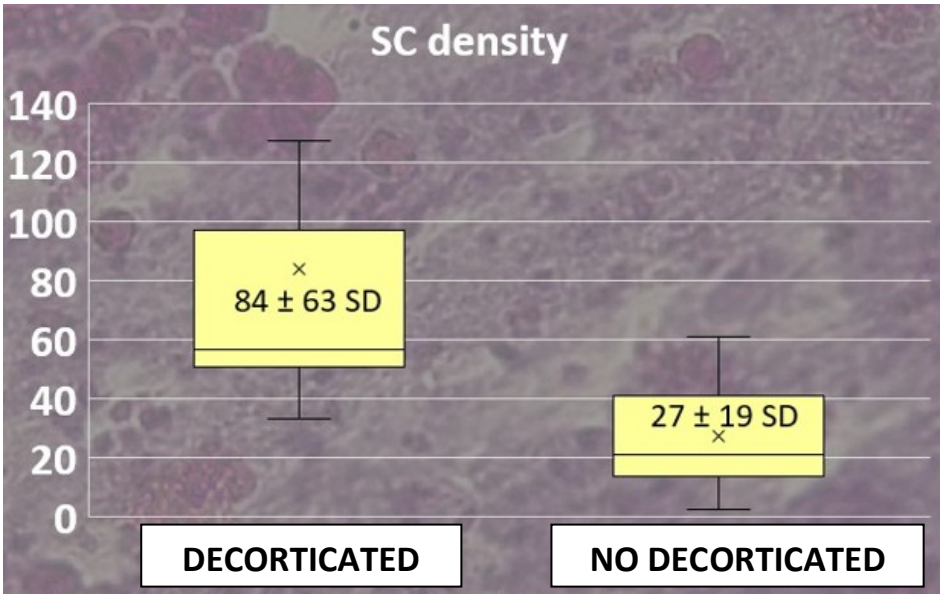

Figure 13 – Graphic that shows difference of spherulouse cell density in genets decorticated and non-decorticated. (SC/cm^2)

If in the genets we observed the collagen band that allowed us to distinguish the two fragmorphs, in the decorticated ramets the merger causes a reorganization of the collagen (likely mesohyl collagen) that does not allow the distinction of the two fragmorphs. In particular the ultrastructure of the fragmorphs changes and a mix of viscoelastic material is formed where the mesohyl becomes abundant while the choanoderm shrinks. Consequently the choanocyte chambers are few or absent. At last the density of spherulouse

cells in decorticated ramets is high throughout the fragmorphs, with accumulation zones (Figure 14).

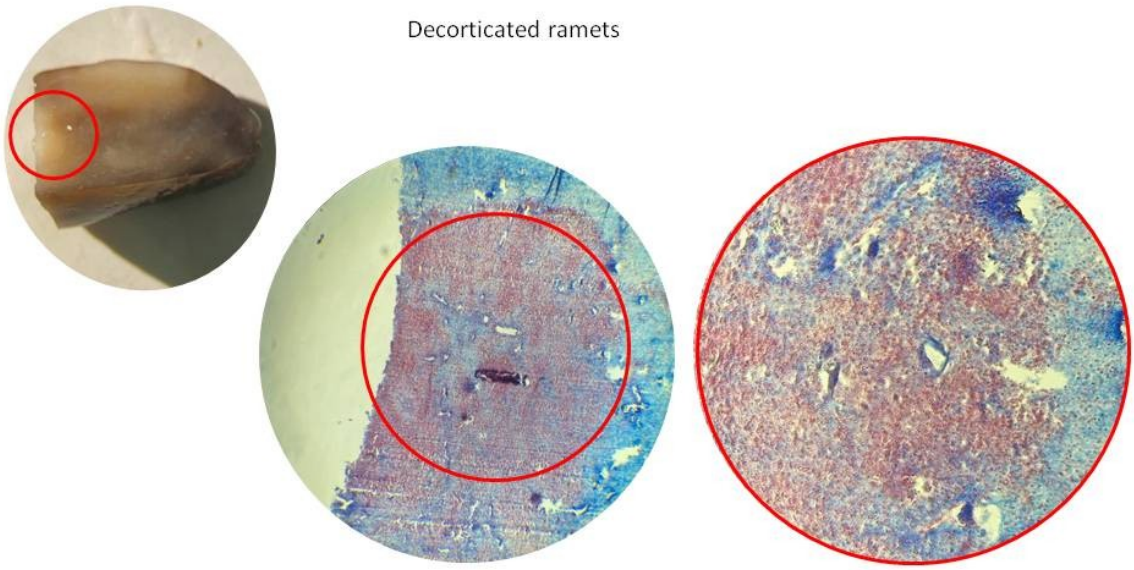

Figure 14 – Accumulation zone of spherulouse cells in decorticated ramets.

Time-lapse technique unrevels that complete fusion of genets and ramets decorticated occurred in 144 and 240 hours respectively. In particular they joined quickly, but ectosome regeneration occurs more slowly over time (336 hours).

The recovery rate of fragmorphs was the average 0,116cm^2/day (± 0,012 SD) or 0,046 cm^3/day (±0,005 SD).

The non-decorticated specimens never stuck to each other or to the substrate.

In the end, in all fragmorphs analyzed with histology, different stages of the spherulouse cells have been observed (Figure 15).

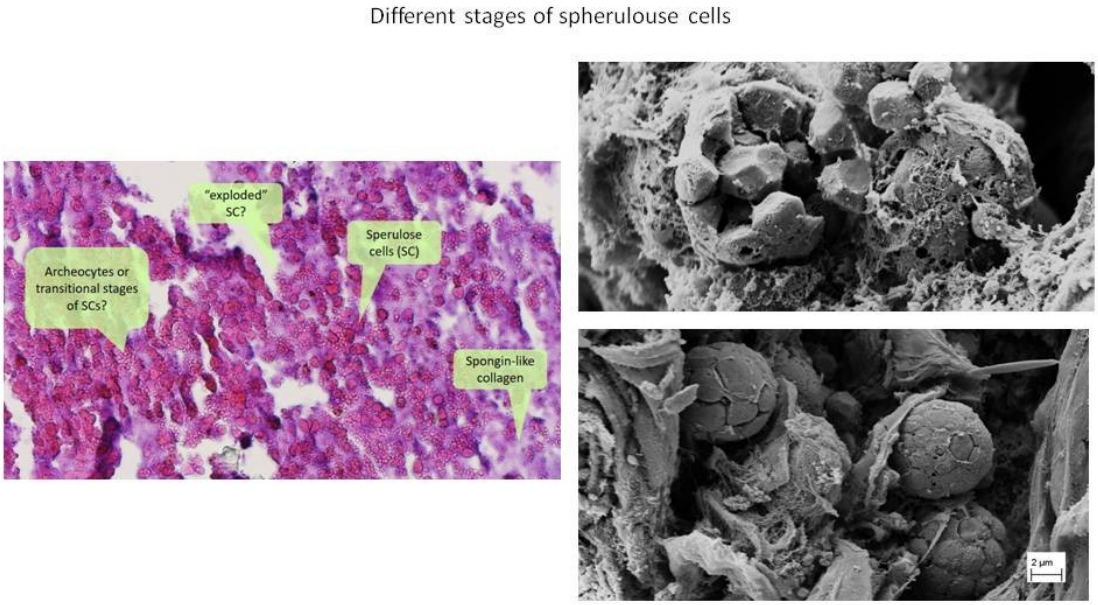

Figure 15 – Different stages of spherulous cells.

4. Discussion

Chondrosia reniformis is an ideal organism to investigate the regeneration and fusion processes: its morphological plasticity allows to regenerate its components and to reorganize the collagen in a short time. First objective of this work was to enhance the understanding of regeneration and fusion processes. The results of the different trials conducted in this study shed some light on times and modalities of wound healing in *C. reniformis*.

4.1 Fusion & Regeneration experiment

When two genetically identical fragmorphs with intact ectosome (i. e., non-decorticated genets) were put side-by-side along a cut, both the upper and the lower ectosome start regenerating the cortex (mesohyl+ectosome) along the contact area, likely to heal the wound, to establish again a barrier between the two fragmorphs and to restore the individuality of each fragmorph. It remains unclear if both fragmorphs contributed to the renewal of the cortex and if it was synchronous or asynchronous. The collagen fibrils between the newly recovered mesohyl and the choanosome are more irregular than in areas far from the wound, likely due to the reorganization of the aquiferous system.

The same observation was made by Cerrano et al (2001) in a study focusing on the mesohyl dynamics in *Chondrosia reniformis,* from Portofino: during

creeping, the aquiferous system of the elongated part was clearly reduced. It was also observed a characteristic process of rearrangement of extracellular matrix components.

The mesohyl "tissues" are able to migrate freely into new growing areas without following a coordinated sequence of events. In *C. reniformis* this implies the continuous transformation of the cortex into proper mesohyl tissue and the consequent development of a new cortex. This allows (or requires) constant morphogenetic remodelling rather than a step-by-step series of interrelated processes typical of development of most metazoan (Cerrano et al., 2001).

The formation of a collagenous barrier at the contact area has been observed for several species of sponges (Smith, 1988) when allogeneic fragments (i. e., from different individuals but from the same species) were forced to merge (Saito, 2013).

As observed by Smith (1986), the fragments were cut cross-wise and allowed 24 hours to heal before joining; the mesohyl cells such as archaeocytes, acidophilic and neutrophilic spherulous migrated towards the contact zone. After 48-96 hours from the contact, an evident rejection occurred, as deduced from large mesohyl tracts, impinging upon the graft interface, appeared to supply spherulouse and archaeocyte cells to these large tissue bridges. Small

isolated areas of collagen deposition were present within the infiltrated mesohyl cells just on either side of the line of allogeneic contact. As the rejection progressed, the spherulouse and archaeocytes cells began travelling in mesohyl tracts that were oriented perpendicularly or obliquely to the graft interface. Small deposits of sponge collagen were located within regions of archaeocytes and spherulouse cells accumulations and just to one side or the other of the graft interface (Smith 1986). It is likely that these cells regenerate the wound as Smith (1986) observed that mesohyl cells migrate and accumulate to form opposing fronts of cells at the allogeneic interface. However, the trigger of accumulation of mesohyl cells and the cells secreting fibrous materials should also be clarified (Saito, 2013).

As noted by Gaino et al. (1999), during autograft fusion (genets in our experiments) the two ectosomes come into contact and fuse together without the distinction of the two ectosomes being seen anymore. The same happened for our non-decorticated genets.

In our treatments, decorticated fragmorphs (both genets and ramets) regenerated the upper cortex in a few days after decortication. Similar results have been reported from Nickel and Brummer (2002) in an experiment of fragment culture.

When the upper cortex is missing, the choanoderm of paired decorticated genets coalesces and it is not possible distinguish the two fragmorphs.

The archaeocytes, stem cells present in mesohyl and in the choanoderm, differentiate in spherulouse cells and migrate where necessary to form again the ectosome. However, the function of spherulouse cells type is not clear. According to Bretting and Konigsmann (1979) some cells with similar characteristics are known to possess lectin in their vacuolar contents (Bretting and Konigsmann 1979; Simpson 1984). Details on the role of lectins in sponges are still scarce, but speculation exists that they are involved in the elimination of non-self material and in defense, by accumulating bioactive substances (Bretting and Konigsmann 1979; Arason 1996). In a study done by Custodio et al. (2003) spherulouse cells are roughly associated with initial tissue reorganization. During this process, an increase in the direct contact with allogeneic material could be expected, and the proliferation of this cell type could be triggered by this event. Other possible function that could explain the accumulation of spherulouse cells close to the contact point is the mesohyl extracellular matrix synthesis and maintenance (Bretting et al. 1983; Smith and Hildemann 1990). Also according to Amaroli et al. (2023) the extensive and fast deposition of new extracellular matrix components, such as fibrillar collagen, is essential for the tissue regeneration of *C. reniformis*.

Regarding the regeneration of the ectosome, in a study done by Ereskovsky et al. (2019) the number of spherulous cells in the regenerated ectosome significantly increases.

In the decorticated fragmorphs, the mesohyl is recovered first, as can be inferred by the whitish colour of the healing wounds; then, the sponge becomes darker as the ectosome is re-built. This leading us to think that stem cells from the choanoderm give rise to spherulose cells which are in charge to recover the mesohyl. Successively, a high number of spherulose cells in the mesohyl contribute to recover the ectosome. The reconstruction of the ectosome is likely fundamental in *C. reniformis* to protect the sponge from pathogens and predators. 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 signalling (Ellwanger and Nickel, 2006).

Ramets with ectosome did not merge. The same happened with the species *Halicondria japonica* during an experiment where the fusion never occurred between two individuals from different populations (Saito, 2013). According

to Custodio et al. (2003), the adhesion of allogeneic pairs is limited and the paired fragmorphs remain distinguishable over time.

Decorticated ramets in our treatments behave as mixing viscoelastic fluids that give rise to a new fragmorph where the original ones are not more distinguishable. Further experiments should be performed to know the fate of the 'chimeric' sponge and to find such mixing ramets in the natural environment. Moreover, choanocyte chambers in the choanoderm of the chimeric sponge were reduced or absent. Besides the archaeocytes, choanocytes also perform transdifferentiation and could play an important role in regenerative processes as they are totipotent cells (Funayama, 2012) And then again, in a study of Cerrano et al. (2001) the dynamic structure of the mesohyl of *Chondrosia reniformis* was observed and in particular the viscoelastic changes during the elongation process. From their observation the overall development of the aquiferous system was clearly reduced but not completely absent, as in our ramets. Whatever their adaptive significance, all these complex problems of physiological malleability seem to be solved, at least in *C. reniformis*, by employing mechanisms of temporary plasticisation of the cortex. This phenomenon appears to be less surprising when it is remembered that sponge structural organisation is highly dynamic and that the arrangement of the functional elements is in a constant state of flux (Hartman

and Reiswig 1973), which makes it particularly difficult to define a standard model of sponge morphology.

Fusion or fragmentation of genets (Maldonado et al 2006, Di Camillo et al. 2012) often occur in sponges in relation to biotic/abiotic factors (water movement, food availability (Teixido et al., 2009). In natural conditions, integer genets are also able to merge, suggesting that the fusion may take place only after reabsorption of the cortex. After 2 or 3 weeks the major reorganisation was finished and the fragments were round or ovoid (Nickel and Brummer, 2002).

The results of this study will contribute to understand the regeneration times of a sponge important for commercial purpose.

Sponges, and in particular, demosponges are generally known as slow growing, long-living animals characterized by low adult mortality, and only smaller individuals show higher growth rates (Sara`,1970; Ayling,1983; Garrabou & Zabala, 2001). Considering that, the fragmorphs show an average recovery rate of 0,116 cm^2/day (± 0,012 SD), the estimated annual recovery rate is around 42 cm^2. The regeneration time rate is therefore quite fast it should be considered the growth rate which is very slow. In fact, as written by Di Camillo and Cerrano (2015) the annual growth rate of Chondrosia reniformis is 10cm^2 per year. Even if regeneration rate are higher than the

growth rate, it must be taken into account that if I have a sponge of 30cm^2 and half is removed, the sponge will take 1 year and half to completely regenerate. On the other hand, another important aspect which guided our investigation is the production of collagen of commercial interest for bioapplication. In particular we have observed that the fibrillar collagen of the mesohyl is of the type I that is the most extracted protein from marine animals, and a very important biomaterial in the field of tissue-engineering (Felician et al., 2018). Moreover, a study conducted by Heinemann et al. (2007) shows that collagen derived from sponge exhibited FT-IRAS spectra very similar to those from calf skin collagen. This aspect is important because marine collagen is easy to extract, has low production cost, has bioactive properties, and is biocompatible.

We noticed that decortication triggers the hyperproduction of spherulose cells and collagen in fragmorphs; however, quantity and quality of collagen maybe lower in stressed sponges. More sophisticated techniques would be needed for a more thorough investigation.

Being the regeneration rates higher than the growth rates, a sponge of 30 cm^2 where about half is cut (15 cm^2), would take one year and half to completely regenerate. This should be considered to plan a sustainable collection in a marine sponge farm.

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