Mediterranean Marine Science

Vol 16, No 1 (2015)

Vol 16, No 1 (2015)

Biological variations in a long-term ex situ cultivation: a Mediterranean demosponge as model system

G. DI BARI, F. CARDONE, E. GAINO, G. M. LIUZZI, C. NONNIS MARZANO, F. SCOCCIA, G. CORRIERO

doi: 10.12681/mms.872

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/mms.872

Biological variations in a long-term ex situ cultivation: a Mediterranean demosponge as model system

G. DI BARI[1], F. CARDONE[2], E. GAINO[3], G.M. LIUZZI[1], C. NONNIS MARZANO[2], F. SCOCCIA[3] and G. CORRIERO[2]

[1] Università degli Studi di Bari, Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica, Via Orabona 4, 70125 Bari, Italy
[2] Università degli Studi di Bari, Dipartimento di Biologia, Via Orabona 4, 70125 Bari, Italy
[3] Università di Perugia, Dipartimento di Biologia Cellulare ed Ambientale, via Elce di sotto, 06123, Perugia, Italy

Corresponding author: carlotta.nonnismarzano@uniba.it

Handling Editor: Argyro Zenetos

Received: 15 April 2014; Accepted: 11 July 2014; Published on line: 28 November 2014

Abstract

The demosponge *Tethya citrina* Sarà & Melone was subjected to long-term aquarium farming, considering that several species belonging to this genus are a rich source of useful bioactive compounds, and in particular interesting bioactive proteins. The rearing method in the present research was mainly based on applying parameters taken from the current literature. The biological activity of the sponge was assessed in terms of survival and growth over time, status of filtering apparatus, and protein banding profiles. Farmed sponges showed a high survival rate and marked reduction in size. In addition, they were frequently affected by massive production of asexual buds. Histological and ultrastructural observations showed a gradual disorganization of the sponge choanocyte chambers, with a drastic reduction in choanocytes and a progressive increase in spherulous cells. Comparative electrophoretic analysis of the protein profiles of wild and reared specimens showed differences in protein composition and abundance between the tested groups. Such data are consistent with the increase in spherulous cells whose content was markedly electron-dense, a feature stressing the proteinaceous nature of these inclusions. Experimental tank-rearing of *Tethya* provided promising responses in terms of high survival rate of the species and easy reproduction, though the rearing protocol used led to profound morpho-functional changes in the sponge. This confirms the inadequate state of knowledge on farming techniques for this taxon, while also highlighting potential applications in biotechnology of *ex situ* breeding techniques, in order to modify the sponges' biological responses.

Keywords: *Tethya citrina*, *ex situ* rearing, bioactive compound, tissue reorganization, protein profile.

Introduction

Over the course of time, sponges have become target organisms because among all Metazoa they contain the largest number of bioactive substances (Proksch *et al.*, 2002; Leal *et al.*, 2012) utilized in drug development to fight several human diseases (Koopmans *et al.*, 2009). Nevertheless, sponge tissues usually bear only trace amounts of the bioactive compounds of pharmacological and industrial interest (Duckworth, 2009). On this account, a larger amount of sponge biomass than can be sustainably harvested from the seas is needed for the commercial production of these compounds (Koopmans *et al.*, 2009). This supply problem continues to hamper the development of many promising compounds from sponges and other marine invertebrates (Murray *et al.*, 2013).

Sponge aquaculture is one possible method that could supply sufficient and sustainable quantities of sponge compounds for drug development and manufacture (Duckworth, 2009; Murray *et al.*, 2013). Research on sponge farming consists of both *in situ* and *ex situ* experimentation (reviewed by Schippers *et al.*, 2012), the latter carried out in aquaria under controlled conditions. Although closed systems may ensure better control of environmental conditions and metabolite production, they are still far from paving the way to commercial production (Murray *et al.*, 2013). Indeed, the rearing performance of demosponges kept in aquaria is highly variable, with several cases of negative growth and considerable mortality in a few months (Brümmer & Nickel, 2003; Schippers *et al.*, 2012). Alterations in shape leading to a change in the normal appearance have also been observed (Brümmer & Nickel, 2003) although such a feature is not surprising owing to the high level of plasticity that allows sponges to remodel their body shape continuously (Bond, 1992; Gaino *et al.*, 1995). The high degree of dynamism is particularly evident after sponge handling associated with collection and transportation, which may induce tissue re-

gression accompanied by a reduction in the number of choanocyte chambers, as documented in *Ianthella basta* (Luter *et al*., 2011). In that species, it has been shown that tissue regression is a form of stress response because the sponges recovered after just a few days. Similarly, rapid tissue reduction and recovery has been observed in *Aplysinella* sp. (Thoms *et al*., 2008), thus confirming the ability of sponges to deal with different forms of environmental stress, such as predation (Ayling, 1983), physical disturbance (Wulff, 2010) and disease (Gaino & Pronzato, 1989).

Considering that bioactive metabolite biosynthesis by farmed sponges can be similar or greater than that found in natural populations (Klöppel *et al*., 2008) and that sponges can withstand stressful conditions even in a state characterized by the loss of choanocyte chambers, sponge farming may be a successful way of obtaining bioactive substances.

Among demosponges, different species belonging to the family Tethyidae are able to synthesize biologically active compounds, which can exert a broad range of biological activities. The first successful drugs derived from a sponge were the nucleosides spongothymidine and spongouridine, isolated from *Cryptotethya crypta* (=*Tectitethya crypta*) (Bergmann & Feeney, 1951). A derivative of these nucleosides, the Ara-C, is documented as the first marine-derived antitumor agent (Proksch *et al*., 2002), currently used for the treatment of leukaemia. Moreover, the family Tethyidae has proved to produce interesting bioactive proteins. Indeed, a strong cytotoxic protein with pronounced selectivity for certain tumour cell lines has been purified from *Tethya ingalli* (O'Keefe *et al*., 1999). This protein, which had an apparent molecular weight of 21 kDa, also caused human red blood cell lysis and was similar to a haemolysin previously isolated from the sponge *T. lyncurium* (=*T. aurantium*) (Mangel *et al*., 1992).

On these grounds, the present research aims to investigate the effects that long-term aquarium cultivation of *Tethya citrina* Sarà & Melone may have on its biological activity in terms of: i) sponge survival and growth over time; ii) histological and ultrastructural analyses of the choanosomal region, to ascertain any variations in their filter-feeding behaviour; iii) protein banding profiles for a comparison between wild and farmed specimens.

Materials and Methods

Sample collection and maintenance in aquarium

A total of 74 specimens of *Tethya citrina* Sarà & Melone 1965 were collected from the Adriatic coast of Apulia (Palese, Bari; N 41º 09' 39'' E 16º 45' 50''), on a rocky sea bed, around 2 m in depth, from February to March 2010.

The sponges were placed in closed tanks containing seawater, transported to the laboratory, tagged, photographed (see Cardone *et al*., 2010), and then reared in three 50-l tanks (stocking density: approximately 1 specimen per 0.5 l of water). Each tank was filled with raw untreated seawater (not filtered nor UV irradiated) maintained at a temperature of 21 (±1.2)°C and a salinity of 37 (±0.6). Dissolved oxygen and pH were monitored daily by means of a manual probe. Within the tank, sponges were disposed on a plastic grid located in the upper third of the water column and subjected to a recirculating water flow produced by an external filtering system (canister-type), PSU equipped with ceramic filtering material. This method has been demonstrated to be very suitable for the long-term maintenance of *T. citrina* post-buds, as reported by Cardone *et al.* (2008). In addition, the bottom of each tank was covered by 5 kg of live rocks collected from the sampling site, typically acting as a filtering substrate, as recommended by the "Berlin Method" (Fossa & Nilsen, 2000). In order to maintain water quality within acceptable values, and to provide sponges with their natural food source, about 10% of the tank water was replaced daily, using raw untreated seawater from the sampling site. In addition, the sponge diet was supplemented on a daily basis with 300 cc of solution containing the microalga *Nannochloropsis* sp. (about 2 µm in diameter) at a concentration of about 4×10^7 cells/cc.

Measurement of survival and growth

Taking into account the different number of specimens of the studied species, sponge survival and growth rate were measured monthly, at regular intervals, for one year.

Survival percentage (S) was estimated by considering the number of individuals at the beginning of the experiment (N_0) and the living specimens at successive steps of the rearing process (N_t): $S = (N_t/N_0) \times 100$.

In order to estimate sponge growth, each sponge was photographed monthly, using a Canon 400 D digicam, EF-S 60 mm f 2.8 Macro, appropriately set up on a metal support so as to take pictures at the same angle and distance. Data were plotted on a PC and elaborated using the AUTOCAD 2008 software (see Cardone *et al*., 2010). Considering each specimen as a sphere, both diameter and volume of the sponge were calculated. The volume of the specimens was then used to calculate the Specific Growth Rate (SGR), according to the formula:

$SGR = (\log_e V_{t2} - \log_e V_{t1}) / (t_2 - t_1) \times 100$

where V_{t2} and V_{t1} were the volume of each specimen calculated at time t_2 and t_1, respectively, while $(t_2 - t_1)$ was the range of time in days.

Histological and ultrastructural analyses

In order to estimate variations in the sponge aquiferous system, sponge samples were sacrificed soon after collection (wild specimens) and after every 2 months of rearing and then subjected to standard histological analysis. Each sponge was divided into fragments and fixed for 24 h in 4% formaldehyde in seawater. The selected material was then rinsed in seawater, dehydrated and embedded in paraffin. Tissue analysis was carried out on 7 µm thick histological sections of sponge choanosomal tissue, stained with toluidine blue. The sections were observed under light

microscope. Histological pictures were taken with a Nikon Eclipse 80i microscope equipped with ACT-2U software (Nikon UK, Kingston, UK). For each specimen, the condition of the aquiferous system was evaluated by counting the number of choanocytes per choanocyte chamber in 4 choanosomal fields measuring 0.07 mm^2 each.

For Transmission Electron Microscopy (TEM) investigations, fragments from wild specimens and from specimens reared for 4, 10 and 12 months were rinsed in artificial seawater, used as a buffer, and post-fixed in a solution of 1% osmium tetroxide in artificial seawater for 1 hour at room temperature. Subsequently, the samples were washed in the same buffer, dehydrated in an increasing alcohol series up to propylene oxide and embedded in an Epon-Araldite mixture. Ultrathin sections, obtained using a Leica DC 300 F Ultracut (Leica Microsystem AG, Rijswijk, The Netherlands), were contrasted with 5% uranyl acetate for 20 minutes and lead citrate for 5 minutes, and observed under a Philips EM 208 Transmission Electron Microscope (Philips, Eindhoven, The Netherlands).

Preparation of crude extracts

To study the effects of farming on the sponges at molecular level, crude extracts from wild-type and farmed specimens of *Tethya citrina* were prepared to evaluate their protein profiles. For this purpose, at 4, 10 and 12 months from the beginning of the experiment, 6 reared specimens were taken from the tank, sacrificed and pooled together to prepare crude extracts. The latter procedure was applied simultaneously to 6 wild specimens of *T. citrina* collected from the site at which reared sponges were originally sampled. Three replicates were performed for each experiment at each sampling time. In detail, each pool of wild and reared specimens at the indicated times was ground in sterile PBS at pH 7.0 (1:4 w/v) with mortar and pestle in an ice bath. The preparation was freeze-thawed 3 times for 20 minutes at -20°C. To avoid any microbial contamination due to symbiotic bacteria, penicillin/streptomycin ($5x10^3$ U/ml) was added to the homogenate, which was then centrifuged at 15000 g, 60 minutes at 4˚C. The clear supernatant from each pool was collected and filtered through a Millipore membrane with a diameter of 0.22 µm and indicated as crude extract. Total protein content of each extract pool was determined according to the Bradford procedure (Bradford, 1976). The yield of each extract was found to be approximately 50% compared to the corresponding homogenate. Extracts were then kept at -80°C until further analysis.

One-dimension Sodium Dodecyl Sulphate (SDS) Poly-Acrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was carried out as described by Laemmli (1970). Sixty micrograms of total protein were mixed with the sample buffer containing 4% SDS/10% glycerol/10% β-mercaptoethanol/0.001% bromophenol blue and 0.5 M Tris-HCl at pH 6.8. Samples were heated at 100ºC for 2-3 minutes and then electrophorised on 12% (0.75 mm thickness) running gel overlaid with a 4% stacking gel.

Electrophoresis was performed at 4°C, 120 V constant voltage, using a mini-Protean II apparatus (Bio-Rad Laboratories). The molecular mass of the main proteins in the sponge extracts was estimated by comparison with the Bio-Rad standard protein kit. The protein bands were visualised by staining with 0.1% Coomassie brilliant blue R-250 and the gel was scanned using a Scan Maker 9800 XL-Microtek (Hsinchu, Taiwan) in a grey scale.

Statistical analysis

The results regarding the protein levels on SDS PAGE analysis are expressed as a percentage of CTRL±SD. Each experiment was repeated three times using 3 different pools of 6 wild specimens and 3 pools of 6 reared specimens at 4, 10 and 12 months from the beginning of the rearing experiment. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test (multiple comparison) to assess the statistical significance of the difference between wild and reared specimens for all experiments. A level of $p<0.05$ was considered statistically significant.

Results

Sponge survival and growth

At the end of the study period, the survival percentage of *Tethya citrina* was 81.1%.

A pronounced reduction in the mean volume of the reared specimens was observed during the study period. After 12 months, the volume had dropped by up to 46.5% (Fig. 1A). The size reduction affected all the reared specimens, but showed certain variability among specimens and over the period of study (Fig. 1A). Some pronounced negative peaks (1, 2 and 5 months after the beginning of the experiment) correspond to partially synchronized budding events involving several specimens, a process that was sometimes impressive due to the sheer number of buds produced (Fig. 2). The trend of volumetric changes was confirmed by SGR analysis, which mostly showed negative values (Fig. 1B).

Sponge aquiferous system

Choanocyte chambers were the dominant element in the choanosomal tissue of wild specimens (Fig. 3A). On histological analysis, the choanocyte chambers appeared closely distributed throughout the tissue, sub-spherical in shape, and surrounded by 15-25 choanocytes with flagella that were easily detectable under light microscope (Fig. 3A). This filtering tissue was illustrated better by the TEM images (Fig. 3B).

After 4 months (Fig. 3C), although in a few cases the choanosomal tissue of reared specimens still appeared

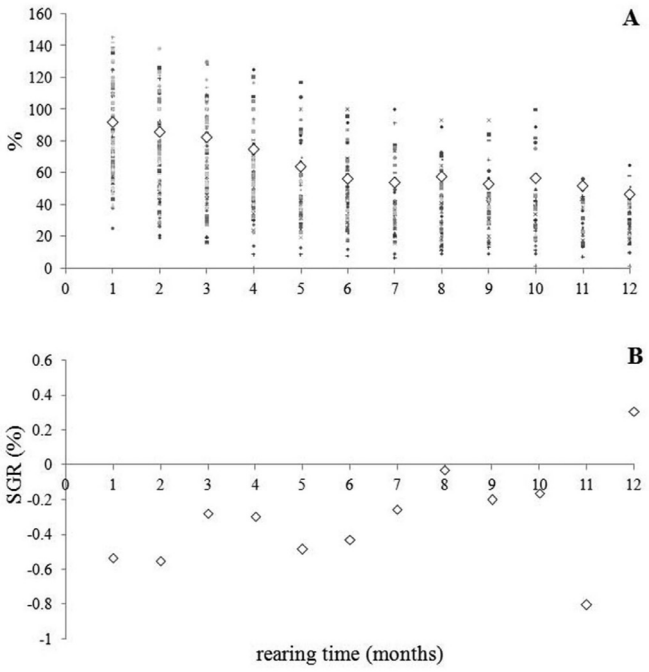

Fig. 1: Temporal variation in volume of reared *T. citrina* specimens**:** (A) mean volume variation and volume variation per specimen (% of initial volume); (B) Specific Growth Rate (SGR%) recorded during the observation period.

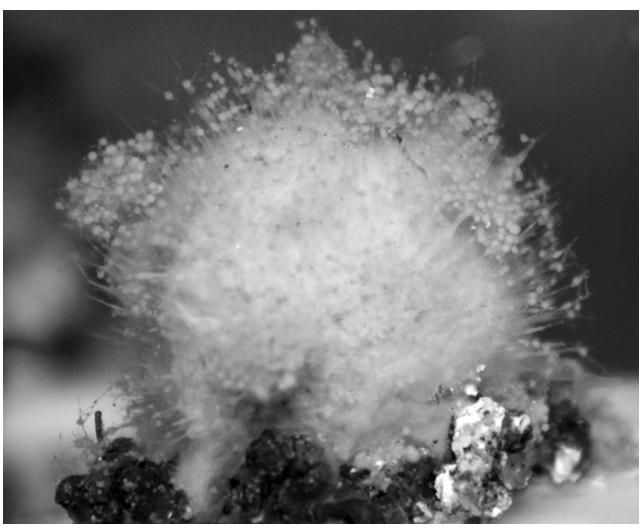

Fig. 2: A massive budding event in a reared specimen of *T. citrina.*

to be comparable to that of wild specimens, no pores or canals were evident, and in most samples the chambers were less structured, thus appearing irregular, disorganized and delimited by few choanocytes only, devoid of flagella and showing some remnants of the collar fringe (Fig. 3D).

After 10 months, the choanocyte chambers were not dissimilar (Fig. 3E) to those observed after 4 months, apart from a drastic increase in the cells with inclusions. In particular, in some images, these cells were evident, even entering into the chambers (Fig. 3F). After 12

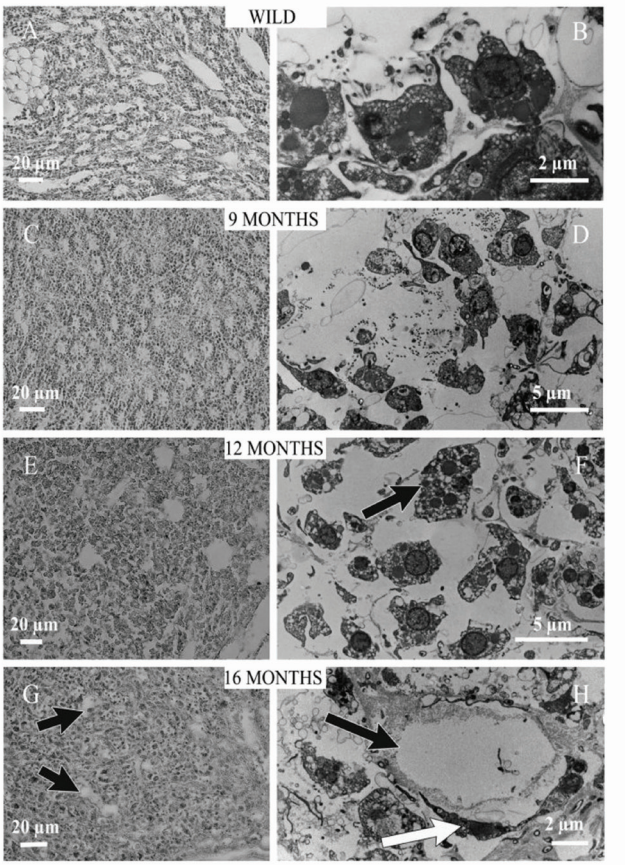

Fig. 3: *T. citrina*. Histological (A, C, E, G) and TEM (B, D, F, H) observations of the choanosomal tissue of wild specimens and of specimens reared for 9, 12, 16 months: (A) the choanosomal tissue of a wild specimen dominated by choanocyte chambers, spherical in shape and closely distributed through the tissue; (B) TEM detail of two choanocytes delimiting a chamber; (C) a reared specimen showing choanosomal tissue comparable to that of wild specimens; (D) less structured and disorganized choanocyte chambers; (E) persistence of the choanocyte chambers together with a drastic increase in spherulous cells with inclusions; (F) a spherulous cell (arrow) invading a choanocyte chamber; (G) the choanodermal region of a specimen showing apparent choanocyte chambers interspersed among lacunae (arrows); (H) lacuna (black arrow) delimited by elongated, pinacoderm-like cells (white arrow).

months, the choanodermal region only showed the presence of rudimentary choanocyte chambers interspersed among lacunae, as confirmed by TEM (Fig. 3G), delimited by pinacoderm-like cells (Fig. 3H).

Concomitantly with the gradual disorganization of the choanocyte chambers, the cells with inclusions also progressively increased in number (Fig. 4A). They differed in shape, size and in the electron-density of their inclusions that in numerous transmission electron micrographs showed the typical organization described for spherulous cells (Fig. 4B). They were gathered together or dispersed in the mesohyl matrix among other cell types, thus becoming the prevalent ones, because their density increased during the rearing process.

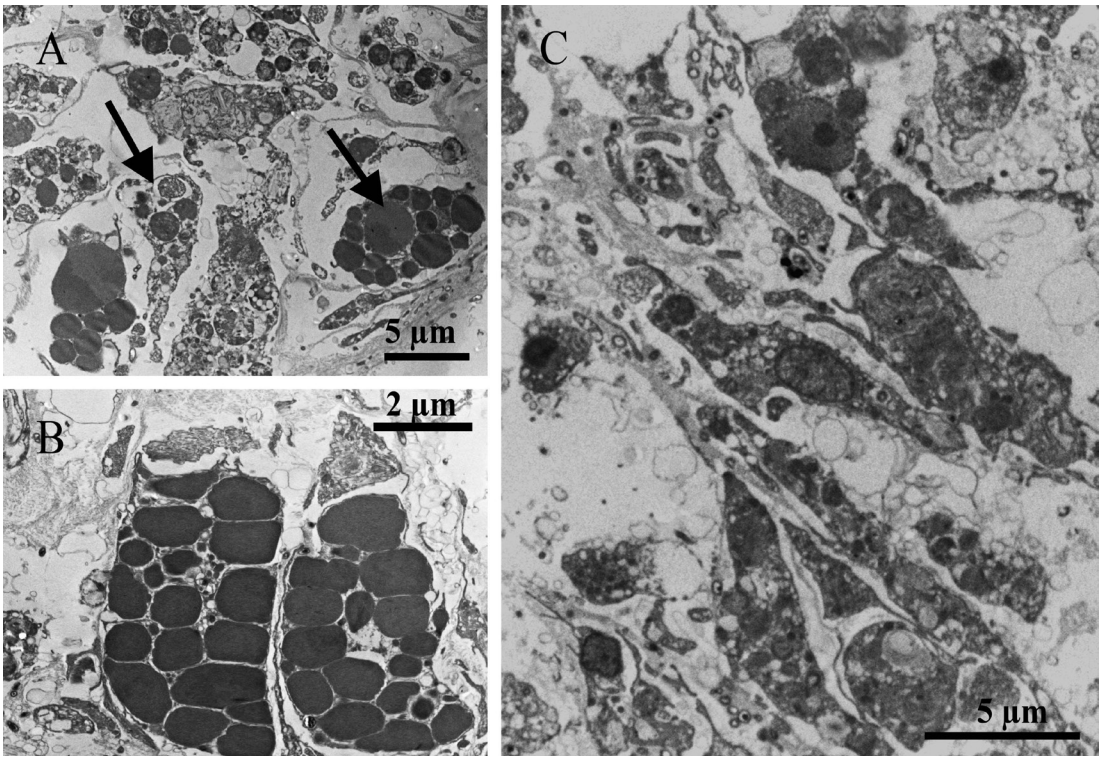

Fig. 4: *T. citrina.* TEM views of the choanosomal region of reared specimens: (A) the image shows some spherulous cells (arrows) that differ in shape, size and in the electron-density of their inclusions; (B) two spherulous cells with their typical morphology showing large and rounded electron-dense inclusions; (C) numerous elongated cells in the mesohyl matrix, whose morphology is consistent with their mobility.

The reduction in body size affected mesohyl cell density, which varied from region to region: from dispersion to major concentration, as evidenced by the close-knit arrangement of the elongated cells, whose morphology was consistent with that of a mobile condition (Fig. 4C).

In the reared specimens, the mean number of choanoytes per choanocyte chamber dropped markedly over one year when compared with the trend shown by wild specimens (Fig. 5). In particular, in the latter, the number of choanocytes presented small variations over time, varying from 15 to 25. By contrast, in reared specimens, choanocyte chambers lost their functionality within the first 2 months, due to the drastic reduction in the number of choanocytes (about 6 choanoytes per choanocyte chamber). Thereafter, the percentage showed a steady pattern, with only slight variations (Fig. 5).

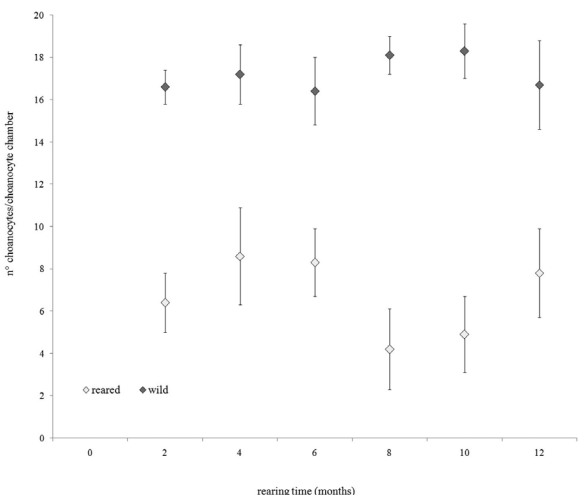

Fig. 5: *T. citrina.* Mean number of choanocytes per choanocyte chamber in wild and reared specimens.

Protein profile analysis by SDS-PAGE

In Figure 6A, a representative SDS-PAGE analysis of crude extracts from a pool of 6 wild specimens and from the pools of 6 reared specimens of *T. citrina* sampled at the indicated times is reported. Electrophoretic analysis showed that the protein profiles of the crude extracts from the reared specimens of *T. citrina* were similar to those of their wild conspecifics (Fig. 6A-B). Three common protein bands of 50, 36 and 27 kDa were present in the sponges (Fig. 6A-B). Independent comparative analysis performed with 3 different pools of 6 wild specimens and 3 pools of 6 specimens reared for 4, 10 and 12 months, respectively, indicated that the levels of the 50 kDa and 36 kDa protein bands, which were poorly represented in the wild specimens, increased statistically ($P<0.01$ by one-way ANOVA followed by the Tukey test) in the specimens reared for 4, 10 and 12 months (Fig. 6A-B). No variations in the levels of the other main proteins were observed in reared sponges compared to their wild conspecifics.

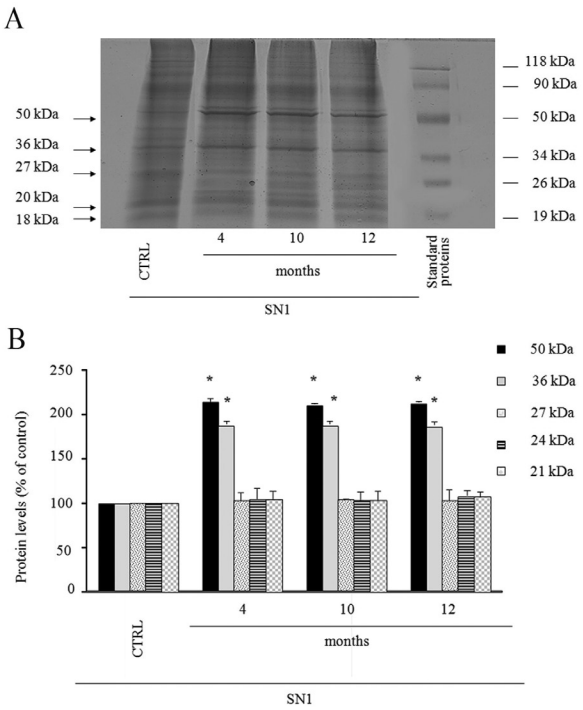

Fig. 6: *T. citrina*: SDS-PAGE comparative analysis of the protein profile of aqueous extracts (SN1) from wild (CTRL) and reared specimens. (A) A representative gel of the protein profiles is reported. Arrows indicate the major proteins present in the aqueous extracts. Months indicate the rearing period. Protein size standards are indicated on the right. (B) Histograms represent the results, expressed as percentage of CTRL±SD, of scanning densitometry and computerized analysis of gels from three pools of 6 wild specimens and three of 6 specimens reared until the indicated times (see Materials and Methods). Asterisks represent statistically significant ($P<0.05$) values in comparison to the crude extracts from wild specimens (one-way ANOVA followed by Tukey test).

Discussion

The production of bioactive metabolites in sponges is not stable through space and time, but rather subject to considerable qualitative and/or quantitative variation depending on various physico-chemical and biological factors (Koopmans *et al*., 2009; Tresa Remya *et al*., 2010). In this scenario, previous studies have investigated the possible influence of *ex situ* farming on sponge synthesis of bioactive metabolites, in most cases showing results comparable to those of wild specimens (de Caralt *et al*., 2003; Carballo *et al*., 2010). Moreover, some authors have observed greater metabolite production than that found in natural populations (Klöppel *et al*., 2008).

The possibility of rearing *Tethya citrina*, a member of the Tethyidae family, *ex situ* is a field of great interest given that several congeneric species have been utilized as producers of bioactive metabolites, with possible applications for the medical/pharmaceutical sector (Bergmann & Feeney, 1951; Mangel *et al*., 1992). It is well known that in most cases the original producers of the bioactive compounds of sponges are the symbiotic microorganisms harboured in their mesohyl (Newman & Hill, 2006; Taylor *et al*., 2007). However, as reported by Tresa Remya *et al.* (2010), no bioactive compounds have been reported from microbes associated with the family Tethyidae.

Despite the fact that the *ex situ* cultivation of sponges has long been considered a task doomed to failure (Riisgard *et al*., 1993), recent studies suggest that it is possible to cultivate sponges in tanks (Schippers *et al*., 2012; Pérez López *et al.,* 2014). However, the experimental rearing of twenty-two Mediterranean sponges showed variable and generally low survival and poor growth performances (Brümmer & Nickel, 2003).

During the twelve months of the present study, the reared sponges showed good adaptability to tank conditions, as confirmed by the high survival values. In fact, despite the slight mortality recorded in *T. citrina*, our data are encouraging compared to those supplied by literature (Brümmer & Nickel, 2003), which indicated that only about 60% of the studied Mediterranean demosponges could be maintained in an aquarium for several months.

With respect to the monitoring of dimensional changes in sponges, different methods have been reported (Brümmer & Nickel, 2003). In this study, the growth rate of the reared specimens was calculated in terms of volume change, as reported in the methods section. The method chosen was minimally intrusive and did not cause stress to the animals. In addition, during rearing time, it allowed detection of minimal morphological and dimensional changes.

During the study, a decrease in volume was observed in reared sponges, even though there was considerable variability during different months. Poor growth performance and growth variability are well-known problems among researchers who have experimented with sponge cultivation. Moreover, previous studies on a wild population of *T. citrina* (Cardone *et al*., 2010) revealed that even *in situ* growth performance was highly variable among specimens and among seasons, and that they tended to decrease with sponge age. Indeed, eight out of ten young sponges (post-buds) monitored for thirteen months showed between one and three episodes of size decrease, while an older specimen of *T. citrina* underwent 4 decrease episodes and showed a flat overall growth trend in the same observation period (Cardone *et al*., 2010).

Literature data confirm that marine demosponges hardly grow at all under controlled conditions. In particular, for *T. aurantium*, Brümmer & Nickel (2003) did not observe any growth during their farming experience. The overall volume decrease could be due to inadequate nutrition, as observed in *Aplysina aerophoba* by Klöppel *et al*. (2008). Indeed, the trophic needs of sponges are not yet sufficiently known and thus hardly reproducible under controlled conditions (Mendola *et al*., 2007). The rearing method described in this paper is based on the application of parameters that have not been fully standardized, part-

ly taken from the literature (Brümmer & Nickel, 2003; Klöppel *et al*., 2008) and partly based on previous experience of the research group (Cardone *et al*., 2010). The size reduction appeared to be rearing time-dependent, reaching its greatest values at the end of the observation period. In this respect, histological analysis showed significant changes in the choanosomal tissue, with the reduction of the filtering system due to both a disorganization of choanocyte chambers and a marked numeric decrease in choanocytes per chamber. In addition, TEM images suggested that choanocytes were no longer functional: flagella were lacking, though the microvillar collars may have persisted. Concomitantly, a significant increase in archeocyte-type (spherulous) cells was evident.

Many sponge species can undergo anatomical rearrangements, considerably changing their morphology in response to environmental stress, due to their high regenerative capabilities (Wulff, 2010). In this study, the reduction in sponge size and the gradual disorganization of its choanocyte chambers appears to reflect an organizational model such as that found in carnivorous sponges, which have small dimensions and lack an aquiferous system, as documented for the first time in *Asbestopluma hypogea* (Vacelet & Boury-Esnault, 1995). Similarly, even though choanocyte chambers are still present in *Chondrocladia lyra* - a recently described carnivorous sponge -, their choanocytes are devoid of flagella and, therefore, the sponge has a macrophagous feeding habit (Lee *et al*., 2012). Indeed, the ability to take up nutrients through the pinacoderm layer has been proven even in sponges equipped with the typical aquiferous system (Willenz & Van de Vyver, 1982).

In the present research, the reduction in the aquiferous system did not contrast with sponge survival, and seemed to be a response to stressful conditions. This behaviour is consistent with sponge plasticity at cellular level, representing a very impressive trait of Porifera species. Many species of demosponges are able to remodel their shape continuously, even though the most evident cellular rearrangement takes place during reproduction, with the transformation of large portions of the filtering system into reproductive tissue (Gaino, 2011).

Generally, archeocyte-type cells are a minor component of the cell pattern in adult demosponges and their increase is linked to reproductive and reparative processes. According to the literature, archeocyte-type spherulous cells are commonly interpreted as storage cells (Gaino, 2011). A change in cell pattern with respect to wild specimens, with the spread of archeocyte and spherulous cells, has also been described in reared specimens of *Aplysinella* sp. (Thoms *et al*., 2008). In *Tethya* spp., the proliferation of archeocytes is generally associated with asexual reproduction, but this is usually limited to the cortical/upper layer of the sponge choanosome, and does not involve all choanosomal tissue (Gaino *et al*., 2009). In the present study, the proliferation of spherulous cells could be interpreted - consistently with the reduction in sponge size and aquiferous system - as a response by the sponge to unfavourable environmental conditions, probably resulting from long-term starvation in the aquarium.

Inadequate feeding of the reared specimens of *T. citrina* was the likely cause of the loss of the aquiferous system and of the consequent size reduction. This process had a marked impact on its metabolic activity, which was no longer sustained by the filter-feeding mechanism, and choanocytes were replaced by cells filled with stored material. In such a condition, water entered the sponge body only by diffusion, a process taking advantage of the reduction in size. In England, *Microciona prolifera* normally overwinters in a dormant form that lacks choanocyte chambers, canals and oscula (Simpson, 1968). Similarly, a dormant phase can also be induced by changes in salinity, which reduces the surface that comes into contact with the medium (Leamon & Fell, 1990).

In this work we also evaluated the protein composition and abundance in crude extracts prepared from reared specimens of *T. citrina* in comparison to the extracts from wild specimens of the same species. To this end, an SDS-PAGE comparative analysis of crude extracts from wild specimens and from specimens of the sponge reared for 4, 10 and 12 months, respectively, was carried out. The electrophoretic analysis of the crude extracts from wild and reared specimens of *T. citrina* showed increased levels of 36 and 50 kDa protein bands in specimens reared for up to 12 months. These data might be interpreted as a characteristic response of *T. citrina* to changes in environmental conditions. This hypothesis seems to be confirmed by the experimental evidence that many sponges, subjected to laboratory conditions, respond in a highly sensitive and specific manner and can exhibit a remarkable variety of responses to the stress caused by removal from their habitat (Riisgard *et al*., 1993; Schröder *et al*., 2006). These stress responses usually include the induction of the expression of specific proteins, such as heat shock proteins (hsp) that help the sponges adapt to the new environmental conditions (Koziol *et al*., 1997). It is worth noting that in reared sponges there is considerable metabolic activity leading to the synthesis of proteins, even during complex cell rearrangement. Therefore, we cannot exclude the hypothesis that the spherulous cells located in the former choanosomal region may sustain the metabolic activity involved in protein synthesis. Further experiments are being carried out to purify these increased proteins and to verify whether they possess a biological activity of pharmacological and/or industrial interest.

Even though during rearing time *T. citrina* has undergone profound morphofunctional changes, it has provided promising responses in terms of high survival rate. Moreover, like most of its congenerics, this species is able to reproduce asexually by budding, both in natural (Corriero *et al.,* 1996; Gaino *et al*., 2006; Cardone *et al*., 2010) and *ex situ* (Cardone *et al*., 2008) conditions. In natural environments, geographically distant *T. citrina* popula-

tions show different budding periodicity, from a clear seasonality (Marsala and Venice lagoons, Corriero *et al*., 1996; Gaino *et al*., 2006) to continuous budding all year round (SW Apulia, Cardone *et al*., 2010). In the present research, intense budding events took place during the first two months of rearing, after the increase of temperature at which the sponges were subjected when transported from their natural habitat to rearing tanks. This phenomenon was consistent with the observations of Cardone *et al*. (2008), who reported that in *T. citrina* reared *ex situ* budding can be experimentally induced by a drastic increase in water temperature. Notwithstanding the stability of the physico-chemical parameters of tank water, a third episode of budding occurred five months after the start of the experiment, probably due to the fact that the reared sponges belonged to a natural population showing almost continuous budding activity throughout the year (Corriero, personal observations). As reported by Cardone *et al.* (2008), buds develop into functional sponges within a few weeks. Therefore, the culture of such buds could be used to achieve higher biomass production, promisingly contributing to the mitigation of the environmental impact of wild sponge collection for seed supply purposes.

On the whole, our results underline a need for further investigation of rearing strategies for this taxon while confirming the potential of *ex situ* rearing techniques as regards biotechnological applications, in order to improve the biological response of sponges of commercial interest and thus increase profitability.

Acknowledgements

This research contributes to the RITMARE Project (SP2_WP4_AZ1_UO05) funded by the Italian Ministry of Education, University and Research.

References

Ayling, A.L., 1983. Growth and regeneration rates in thinly encrusting demospongiae from temperate waters. *Biological Bulletin*, 165, 343-352.

Bergmann, W., Feeney, R.J., 1951. Contributions to the study of marine products. XXXII. The nucleosides of sponges. I. *Journal of Organic Chemistry,* 16, 981-987.

Bond, C., 1992. Continuous cell movements rearrange anatomical structures in intact sponges. *The Journal of Experimental Biology*, 263, 284-302.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.

Brümmer, F., Nickel, M., 2003. Sustainable use of marine resources: cultivation of sponges. p. 143-162. In: *Marine Molecular Biotechnology, Sponges (Porifera)*. Müller, W.E.G. (Ed.). Springer-Verlag, Berlin.

Carballo, J.L., Yañez, B., Zubía, E., Ortega, M.J., Vega, C., 2010. Culture of explants from the sponge *Mycale cecilia* to obtain bioactive mycalazal-type metabolites. *Marine Biotechnology*, 12 (5), 516-525.

Cardone, F., Gaino, E., Corriero, G., 2010. The budding process in *Tethya citrina* Sarà & Melone (Porifera, Demospongiae) and the incidence of post-buds in sponge population maintenance. *Journal of Experimental Marine Biology and Ecology*, 389, 93-100.

Cardone, F., Nonnis Marzano, C., Spedicato, M.T., Lembo, G., Gaino, E. *et al*., 2008. Budding induction in a marine sponge: a cue for cultivation purposes. p. 81–85. In: *IFOAM Conference on Organic Aquaculture*, *Cattolica (RN), 18–20 June 2008*.

Corriero, G., Sarà, M., Vaccaro, P., 1996. Sexual and asexual reproduction in two species of *Tethya* (Porifera: Demospongiae) from a Mediterranean coastal lagoon. *Marine Biology*, 126, 175-181.

de Caralt, S., Agell, G., Uriz, M.J., 2003. Long-term culture of sponge explants: conditions enhancing survival and growth, and assessment of bioactivity. *Biomolecular Engineering*, 20, 339-347.

Duckworth, A., 2009. Farming sponges to supply bioactive metabolites and bath sponges: a review. *Marine Biotechnology,* 11, 669-679.

Fossa, S.A., Nilsen, A.J., 2000. *The modern coral reef aquarium Volume 3*. Birgit Schmettkamp Verlag, Bornheim, 448 pp.

Gaino, E., 2011. Overview of Porifera. p. 1-53. In: *Fauna d'Italia - Porifera I.* Pansini, M., Manconi, R., Pronzato, R. (Eds). Calderini Edizioni, Milano.

Gaino, E., Pronzato, R., 1989. Ultrastructural evidence of bacterial damage to *Spongia officinalis* fibers (Porifera, Demospongiae). *Diseases of Aquatic Organisms*, 6, 67-74.

Gaino, E., Manconi, R., Pronzato, R., 1995. Organizational plasticity as a successful conservative tactic in sponges. *Animal Biology*, 4, 31- 43.

Gaino, G., Mercurio, M., Sciscioli, M., Corriero, G., 2009. Choanocyte chambers in unreleased buds of *Tethya seychellensis* (Wright, 1881) (Porifera, Demospongiae). *Gamete Research*, 16, 259-266.

Gaino, E., Scalera-Liaci, L., Sciscioli, M., Corriero, G., 2006. Investigation of the budding process in *Tethya citrina* and *Tethya aurantium* (Porifera, Demospongiae). *Zoomorphology*, 125, 87-97.

Klöppel, A., Pfannkuchen, M., Putz, A., Proksch,P., Brümmer, F., 2008. *Ex situ* cultivation of *Aplysina aerophoba* close to *in situ* conditions: ecological, biochemical and histological aspects. *Marine Ecology*, 29 (2), 259-272.

Koopmans, M., Martens, D., Wijffels, R.H., 2009. Towards commercial production of sponge medicines. *Marine Drugs*, 7, 787-802.

Koziol, C., Scheffer, U., Pancer, Z., Krasko, A., Müller, W.E.G., 1997. Sponges as biomarkers of the aquatic environment: application of molecular probes. p.121-132. In: *Sponge science.* Watanabe, Y., Fusetani, N. (Eds). Springer-Verlag, Tokyo.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Leal, M.C., Puga, J., Serôdio, J., Gomes, N.C.M., Calado, R., 2012. Trends in the discovery of new marine natural products from invertebrates over the last two decades – where and what are we bioprospecting? *Plos One*, 7 (1), 1-15.

Leamon, J., Fell, P.E., 1990. Upper salinity tolerance of and salinity-induced tissue regression in the estuarine sponge *Microciona prolifera. Transactions of the American Microscopical Society*, 109, 265-272.

Lee, W.L., Reiswig, H.M., Austin, W.C., Lundste, L., 2012. An extraordinary new carnivorous sponge, *Chondrocladia lyra*, in the new subgenus *Symmetrocladia* (Demospongiae, Cladorhizida), from off of northern California, USA. *Invertebrate Biology*, 131, 259-284.

Luter, H.M., Whalan, S., Webster, N.S., 2011. The marine sponge *Ianthella basta* can recover from stress-induced tissue regression. *Hydrobiologia*, 687 (1), 227-235.

Mangel, A, Leitão, J.M., Batel, R., Zimmermann, H., Müller, W.E. *et al*., 1992. Purification and characterization of a pore-forming protein from the marine sponge *Tethya lyncurium*. *European Journal of Biochemistry*, 210 (2), 499-507.

Mendola, D., van den Boogaart, J.G.M., van Leeuwen, J.L., Wijffels, R.H., 2007. Re-plumbing in a Mediterranean sponge. *Biology Letters*, 3, 595-598.

Murray, P.M., Moane, S., Collins, C., Beletskaya, T., Thomas, O.P. *et al*., 2013. Sustainable production of biologically active molecules of marine based origin. *New Biotechnology*, 30 (6), 839-850.

Newman, D.J., Hill, R.T., 2006. New drugs from marine microbes: the tide is turning. *Journal of Industrial Microbiology and Biotechnology*, 33, 539-544.

O'Keefe, B.R., Beutler, G.A., Cardellina, J.H. II, Prather, T.R., Schoemaker, R.H. *et al*., 1999. Isolation of a novel Kunitz family protease inhibitor in association with tethya hemolysin from the sponge *Tethya ingalli*. *Journal of Natural Products*, 60 (11), 1094-1094.

Perez-Lopez, P., Ternon, E., Gonzalez-Garcia, S., Genta-Jouve, G., Feijoo, G. *et al*., 2014. Environmental solutions for the sustainable production of bioactive natural products from the marine sponge *Crambe crambe*. *Science of Total Environment*, 475, 71-82.

Proksch, P., Edrada, R., Ebe, R, 2002. Drugs from the seas - current status and microbiological implications. *Applied Microbiology and Biotechnology*, 59 (2-3), 125-134.

Riisgard, H.U., Thomassen, S., Jakobsen, H., Weeks, J.M., Larsen, P.S., 1993. Suspension feeding in marine sponges *Halichondria panicea* and *Haliclona urceolus*-effects of temperature on filtration-rate and energy cost of pumping. *Marine Ecology Progress Series*, 96, 177-188.

Sarà, M., Melone, N., 1965. Una nuova specie del genere *Tethya*, *T. citrina* sp. n. del Mediterraneo (Porifera, Demospongiae). *Atti della Società Peloritana di Scienze Fisiche, Matematiche e Naturali*, 16, 123-138.

Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A. *et al*., 2012. Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Advances in Marine Biology*, 62, 273-337.

Schröder, H.C., Efremova, S.M., Margulis, B.A., Guzhova, I.V., Itskovich, V.B. *et al*., 2006. Stress response in Baikalian sponges exposed to pollutants. *Hydrobiologia*, 568 (1S), 277-287.

Simpson T.L., 1968. The biology of the marine sponge *Microciona prolifera* (Ellis & Sollander). II. Temperature related, annual changes in functional and reproductive elements with a description of larval metamorphosis. *Journal of Experimental Marine Biology and Ecology,* 2, 252-277.

Taylor, M.W., Radax, R., Steger, D., Wagner, M., 2007. Sponge associated microorganisms: Evolution, ecology and biotechnological potential. *Microbiology and Molecular Biology Reviews*, 71, 295-347.

Thoms, C., Hentschel, U., Schmitt, S., Schupp, P., 2008. Rapid tissue reduction and recovery in the sponge *Aplysinella* sp. *Marine Biology*, 156, 141-153.

Tresa Remya, A.T., Devanand, P.K., Ponnapakkam, A.L., 2010. Marine drugs from sponge-microbe association-a review. *Marine Drugs*, 8, 1417-1468.

Vacelet, J., Boury-Esnault, N., 1995. Carnivorous sponges. *Nature*, 373, 333-335.

Willenz, P., Van de Vyver, G., 1982. Endocytosis of latex beads by the exopynacoderm in the fresh water sponge *Ephydatia fluviatilis*: an in vitro and in situ study in SEM and TEM. *Journal of Ultrastructure and Molecular Structure Research*, 79, 294-306.

Wulff, J., 2010. Regeneration of sponges in ecological context: is regeneration an integral part of life history and morphological strategies? *Integrative and Comparative Biology*, 50 (4), 494-505.