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

Università degli Studi di Bari  
Università degli Studi di Bari  
Università di Perugia  
Università degli Studi di Bari  
Università degli Studi di Bari

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

G. DI BARI1, F. CARDONE2, E. GAINO3, G.M. LIUZZI1, C. NONNIS MARZANO1, F. SCOCCIA2 and G. CORRIERO3

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

Corresponding author: carlotta.nonnismarzano@uniba.it
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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 cho-
anocyte chambers, as documented in Ianthella basta (Later
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 re-
duction 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 (Aylina, 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 character-
ized 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 biologi-
cally 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 (=Tec-
tithyta crypto) (Bergmann & Feeney, 1951). A derivative
of these nucleosides, the Ara-C, is documented as the first
marine-derived antitumor agent (Proksch et al., 2002), cur-
rently used for the treatment of leukaemia. Moreover, the
family Tethyidae has proved to produce interesting bioac-
tive proteins. Indeed, a strong cytotoxic protein with pro-
nounced selectivity for certain tumour cell lines has been
purified from Tethya ingalli (O'Keeffe et al., 1999). This
protein, which had an apparent molecular weight of 21
kDa, also caused human red blood cell lysis and was simi-
lar to a haemolysin previously isolated from the sponge T.
lyncurium (=T. aurantium) (Mangel et al., 1992).

On these grounds, the present research aims to inves-
tigate 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, photo-
graphed (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). Dis-
solved 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 mainte-
nance 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, typi-
cally acting as a filtering substrate, as recommended by the
“Berlin Method” (Fossa & Nilsen, 2000). In order to main-
tain 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 seawa-
ter from the sampling site. In addition, the sponge diet was
supplemented on a daily basis with 300 cc of solution con-
taining the microalga Namachloropsis sp. (about 2 µm in
diameter) at a concentration of about 4×107 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 consid-
ering the number of individuals at the beginning of the
experiment (N0) and the living specimens at successive
steps of the rearing process (Nt): S = (Nt/N0) x 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 dis-
tance. 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:

\[
SGR = \frac{\log V_t - \log V_0}{(t_t - t_0) \times 100}
\]

where V0 and Vt were the volume of each specimen cal-
culated at time t0 and tt, respectively, while (t_t - t_0) was
the range of time in days.

Histological and ultrastructural analyses

In order to estimate variations in the sponge aquifer-
ous system, sponge samples were sacrificed soon after col-
lection (wild specimens) and after every 2 months of rear-
ing 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 his-
tological 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 mm² 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 Microsystems 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 (5x10^4 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 100°C for 2-3 minutes and then electrophoresed 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-Protein 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
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 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.

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**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*.

**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).
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 choanocytes 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 choanocytes per choanocyte chamber). Thereafter, the percentage showed a steady pattern, with only slight variations (Fig. 5).

**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.
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-

**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).

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![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).](image-url)
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 a 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 hyra* - 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 (Rissgard 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 morpho-functional changes, it has provided promising responses in terms of high survival rate. Moreover, like most of its congeners, this species is able to reproduce asexually by budding, both in natural (Correro 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-

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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 individual 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.

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