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**Influence of spatial competitor on asexual reproduction of the marine sponge
Cinachyrella cf. cavernosa (Porifera, Demospongiae)**

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Abstract

Competition for resources, which often results in resource trade-offs, is intense among sessile benthic organisms. In this investigation, we tested the trade-offs between the primary and secondary functions by studying the influence of spatial competitor *Zoanthus sansibaricus* on the reproductive outcome of the sponge *Cinachyrella cf. cavernosa*. Field studies were carried out at a rocky intertidal beach at Anjuna, Goa, India, from October 2013 to September 2015. *C. cf. cavernosa* reproduces asexually via ‘budding’ that comprises three stages (Stage I- III) with distinctive morphological and skeletal characteristics. The budding frequency was positively regulated by temperature of the rock pool water and negatively by the substrate cover of *Z. sansibaricus*. In the absence of the spatial competitor *Z. sansibaricus*, there was a significant increase in budding frequency (10-30%), bud count per sponge (45-60%), bud settlement probability (60-80%) and physiological activity (RNA/DNA ratio) of buds (10-35%). Over-expression (75-80%) of heat shock protein 70 (stress response) was observed in the sponge during the competitive interaction. This investigation shows that spatial competition adversely affects asexual reproduction of *C. cf. cavernosa*, and it supports the earlier observations of tradeoffs between the primary and secondary functions.

Keywords: Porifera, Inter-tidal sponge; zoanths; asexual reproduction (Budding); competitive stress; physiological responses.

Introduction

A suitable substratum with adequate sunlight and influx of nutrients is required for the proper growth, settlement, and reproduction of sessile organisms such as sponges, corals, ascidians, bryozoans, barnacles, etc. (Connell et al. 2004; Wulff 2006; Chadwick and Morrow 2011). Competition for space is one of the major factors determining the structure of benthic communities and limit the population of sessile organisms in crowded environments (such as coral reefs and rocky intertidal regions) (Stebbing 1973; Buss and Jackson 1979; Karlson 1980; Turon et al. 1996; Bell and Barnes 2003; McLean and Yoshioka 2008; Chadwick and Morrow 2011). Sessile organisms have evolved different strategies to deal with interspecific competition. They protect their acquired space either by displaying direct aggressive behaviour (such as overgrowth or overtopping, digestion by sweeper tentacles, etc.) or indirect defensive behaviour (such as controlling larval settlement and growth of neighbours by allelochemicals) (Wulff 2006; Chadwick and Morrow 2011).

Sponges, which can inhabit all types of aquatic ecosystems (from marine to fresh water, from polar regions to temperate and tropical waters, and from the coastal area to deep oceans), are one of the most important components of the benthic ecosystem (Bell 2008). They are filter-feeders that play an important role in the benthic–pelagic coupling and nutrient recycling (Hadas et al. 2009; Riisgård and Larsen 2010). Their porous body structure provides a microhabitat for many other organisms (Henkel and Pawlik 2005; Hultgren and Duffy 2010). Sponges also participate in various ecological interactions, thereby determining the structure and function of benthic community (Suchanek et al. 1983b; Engel and Pawlik 2000). In addition, they produce several bioactive metabolites of biotechnological importance (Thakur and Müller 2004a; Sipkema et al. 2005). Siliceous sponges are thought to have an advantage over other calcareous organisms as they are less affected by ocean acidification and global warming (Bell et al. 2013; Vicente et al. 2015). Sponges serve as a great model system to understand chemically-mediated spatial competition because they are considered to be one of the longest living animals (McMurray et al. 2008), which make them persistent competitors for other sessile invertebrates. Moreover, their remarkable plasticity and ability to produce a variety of lethal or growth inhibitory compounds enable them to compete successfully with other fast-growing sessile organisms (Simmons et al. 2005).

Many studies on sponge- coral interactions have demonstrated that, despite the specialized aggressive defence structures (sweeper tentacles and stinging cells) of corals, sponges are the ultimate winners (Aerts and Van Soest 1997; López-Victoria et al. 2003; de Voogd et al. 2004; López-Victoria et al. 2006; Pawlik et al. 2007; Chaves-Fonnegra et al. 2008). On the other hand, corallimorpharians (also known as false corals) expand their territory by producing new polyps through longitudinal fission, marginal budding or inverse budding within a month (Chadwick-Furman and Spiegel 2000). Consequently, asexual reproduction results in a faster spread of multiple copies of well-adapted individuals, thus making them efficient competitors for space (Hall and Hughes 1996). However, the outcome of competitive interactions among sessile organisms is determined by the several intrinsic (age, size, morphology, growth rate, ontogeny, reproduction, and aggressive behaviour) and extrinsic factors (water parameters, water depth, weather conditions, habitat characteristics, predation, disturbance) (Alino et al. 1992; Genin et al. 1994; Kuffner and Paul 2001; Ninio and Meekan 2002). Thus, environmental factors can play a major role in reversing the competitive outcome by favouring growth and reproduction of weaker competitors.

During a spatial competition, the interacting organisms display a variety of responses at the morphological, physiological, and cellular level to defend or expand their occupied areas (Rossi and Snyder 2001). Adaptive traits such as fast growth rate, rapid asexual reproduction, and release of toxic chemicals have been reported in numbers of sessile marine invertebrates to maintain or protect their occupied space (Chadwick 1987; Langmead and Chadwick-Furman 1999; Cornell and Karlson 2000; Rossi and Snyder 2001; Connell et al. 2004). The maintenance of occupied space by overgrowth and/or producing toxic chemicals (allelochemicals) has been extensively studied during sponge-coral interactions (Suchanek et al. 1983a; McLean and Yoshioka 2008; Chadwick and Morrow 2011). However, the direct contact between interacting species generates stressful conditions that can subside primary functions (growth and/ or reproduction) of either one or both the competitors (Rabelo et al. 2013; Singh and Thakur 2015b). The competitive interactions demand a portion of an organism's total energy to be invested in defending the occupied area, thereby allocating fewer resources to other biological functions of primary importance (such as growth, regeneration, reproduction) (Zera and Harshman 2001). Only a few studies have examined the resource trade-offs during competitive

interactions, and its impact at the morphological, cellular, and biochemical level of the organisms (Rossi and Snyder 2001).

To evaluate the influence of competitor on the reproductive output of opponent, we choose a model system comprising of an intertidal globular sponge *Cinachyrella cf. cavernosa* (Lamarck, 1815) and its aggressive neighbouring species, *Zoanthus sansibaricus* (Carlgren, 1900). Zoanthids such as *Palythoa caribaeorum*, *Zoanthus sociatus* and *Protopalythoa* sp. have a special ability to compete for space by means of faster growth and / or allelochemicals (Karlson 1980; Suchanek and Green 1981; Suchanek et al. 1983b; Sammarco et al. 1985; Karlson 1988; Bastidas and Bone 1996; Rabelo et al. 2013). The genus *Cinachyrella* is known to contain various unusual steroids and phospholipids (Aiello et al. 1991; Barnathan et al. 1992; Barnathan et al. 2003; Li et al. 2004; Xiao et al. 2005; Lakshmi et al. 2008; Wahidullah et al. 2015). The crude extract of *C. cf. cavernosa* showed high cytotoxicity when tested against the symbionts of zoanthids, thereby showing the involvement of allelochemical mediated interaction among them (A. Singh & N. L. Thakur, unpublished data). Although this is one of the most abundant sponges from the coastal regions of India, almost no data are available on its ecology (Thomas 1984). We selected the intertidal region Anjuna, located on the Indian west coast, where *C. cf. cavernosa* grows in close contact with *Z. sansibaricus*. The underlying hypothesis is that if competition exists, defence production costs should be reflected in trade-offs with other primary functions, such as reproduction. If production of allelochemical protects the substrate, and if there is a cost associated with it, then the sponges with low production of allelochemical are expected to have a higher fitness than those with high production in the less competitive environment. On the other hand, in the presence of tough competitor for space, the difference in their fitness is expected to be reversed, or at least reduced. We tested these predictions by comparing reproductive efficiency (budding) of sponge *Cinachyrella cf. cavernosa* with or without the competitor, *Zoanthus sansibaricus*. The influence of competitive stress on the budding process of *C. cf. cavernosa* was studied from October 2013 to September, 2015 through non-manipulative observation (tagged sponges with competitor) and manipulative experiment (tagged sponges without competitor) by scraping the competing *Z. sansibaricus* colonies from the vicinity of tagged sponges. This was followed by observing the changes in budding frequency, bud count, and settlement probability under the above two conditions. Additionally, to assess the effect of competitive stress at the physiological and molecular level, the protein synthetic capability (in

terms of RNA/DNA ratio) of buds and expression of stress-related proteins (HSP 70) in adult sponges with and without the competitor were compared. We assayed the expression of HSP 70 in sponges with and without the competitor to further validate our findings.

Method

Study site and specimens

This study was carried out in the intertidal region of Anjuna beach, Goa, India (15° 34' 34.986"N; 73° 44' 23.895" E), which is truly marine with no river discharges within its 8 km radius. Freshwater input is limited to precipitation. The mean tidal range of the study site is ~ 1.6 m. The climate in the region is hot and humid tropical with a seasonal pattern of heavy south-west monsoon (June to September) and a comparatively short dry winter (December to February). The coastal waters of Anjuna are subjected to a variety of anthropogenic activities contributing to eutrophication and reduction in diversity (Alkawri and Ramaiah 2010; Bhagat et al. 2012). The sponge *Cinachyrella* cf. *cavernosa* and the encrusting zoanthids, *Zoanthus sansibaricus* colonies are very abundant in the rock pools situated in the low inter-tidal region of this shore. *C. cf. cavernosa* sponges are distributed abundantly in lower intertidal rock pools while few were present in mid-intertidal rock pools. In contrast, the zoanthids are only present in lower intertidal regions. We selected one large rock pool containing a large population of both organisms in the lower intertidal region as the study site. Both these organisms were morphologically identified using taxonomical keys (Uchida and Soyama 2001; Hooper and Van Soest 2002; Ryland and Lancaster 2003), and representative samples of both species have been deposited at the Repository and Taxonomic Center of Council of Scientific and Innovative Research (CSIR)-National Institute of Oceanography (NIO), Goa, India.

Physicochemical parameters

During the entire study period (from October, 2013 to September, 2015), various physicochemical parameters (such as temperature, salinity, pH, and dissolved oxygen) of the rock pool water containing the tagged organisms were measured on monthly using a multi-parametric probe, Oakton PCD 650 (OAKTON Instruments, Vernon Hills, IL, USA). For analysis of nutrients (phosphate, silicate, nitrate, nitrite, and ammonia), water samples were collected in triplicates from the rock pool containing the study organisms and analysed using a continuous flow analyzer (San++, SKALAR, Holland). We tagged and examined the sponges

inside of a single large rock pool (about 10 metres wide) so as to minimize the effect of changing physicochemical parameters on their ecological interactions (such as competition for space).

Competitor removal experiment

The effect of the competitor *Z. sansibaricus* on the asexual reproduction (budding) of *C. cf. cavernosa* was analyzed by means of non-manipulative observation (sponge with competitor) and manipulative experiment (sponge without competitor). In the month of September 2013, a total of 10 quadrats (15 x 15 cm), each containing an adult sponge *C. cf. cavernosa* (mean diameter ~ 4 to 5 cm) surrounded by polyps of *Z. sansibaricus*, were marked with four stainless steel nails inside of the single large rock pool in the non-manipulative observation. In the manipulative experiment, an additional 10 quadrats were marked inside the same rock pool, and polyps of *Z. sansibaricus* surrounding the sponges were carefully removed using a scalpel (**Fig. 1**). Additional 5-7 sponges with and without the competitor *Z. sansibaricus* were marked in similar ways to be used for laboratory studies.

Effect of competition on reproductive efficiency of the sponge

a) Bud frequency and count: Non-destructive in-situ photography was used to study the budding process in *C. cf. cavernosa* with and without the competitor (Cardone et al. 2010; Singh and Thakur 2015a). During the entire study period, monthly photographs of the marked quadrats were taken using a digital camera (Canon Power shot A650) fixed on a photo-quadrat stand to capture images from the same angle and distance. These images were analyzed to calculate monthly budding frequency (i.e. the proportion of total individuals with buds). For bud counts, the images of surfaces of the marked sponges were taken in macro-mode, and a total number of buds per sponge (mean \pm SE) were calculated from these images.

b) Bud stages: During the peak budding months (February and March), the buds of varying ages/ sizes were removed from the additional tagged sponges (with and without competitor) ($n = 3$ sponges \times 2 conditions) using sterile pointed forceps. The mean diameter was calculated from the maximum and minimum diameter of each bud under the stereomicroscope (Olympus stereo microscope, SZX2-ILLD). Based on mean diameter, three groups of buds (all attached to mother sponges during their development) were categorized into stage I (< 0.5 mm), stage II (0.5 to 1 mm) and stage III (>1 mm). The external morphology of each bud was examined under stereo microscope. Buds from all three stages ($n = 20$ /stage) were fixed in 4% formaldehyde prepared

in artificial seawater and cut into longitudinal sections. Bud sections were dehydrated gradually through ethanol series and treated with hexamethyldisilazane (HMDS, SRL Chemicals) (Nation 1983). Finally, all sections were mounted onto SEM stubs using double-sided tape. Buds were sputter-coated with gold (at a thickness of 200 Å) to prevent charging the samples. The samples were then scanned and photographed using a SEM (JEOL 5800 LV) at a voltage of 20 kV.

c) **Bud settlement:** The tagged quadrants from both the non-manipulative (sponge with competitor) and the manipulative (sponge without competitor) experiments were checked for the probability of settlement of the mature buds (= stage III buds) near the mother sponge (i.e. within the defined boundaries of the marked quadrats). This was done to estimate the effect of the competitor on probability of successful settlement of buds. The probability of bud settlement was calculated as the final proportion of settled buds per quadrat at the end of budding cycle, out of total number of buds settled near the sponges throughout the budding cycle (December to July).

Effect of competition on physiology of the sponge

a) RNA to DNA ratio of buds

For the determination of physiological index or protein synthetic capacity (RNA/ DNA ratio), it is assumed that RNA synthesis, which is an important step in protein synthesis, varies with age, life-stage, organism size, and environmental conditions, whereas 'DNA' does not vary with environment or organisms (Bulow 1970). RNA/ DNA ratio has been established as an indicator of the physiological status of diverse organisms such as microbes, sponges, crustaceans, and fishes (Robinson and Ware 1988; Anger and Hirche 1990; Clemmesen 1996; Chícharo et al. 1998; Anil et al. 2001; Singh and Thakur 2015a). The buds of each stage were picked from additionally marked sponges with and without the competitor ($n = 10 \text{ buds} \times 3 \text{ sponges} \times 2 \text{ conditions} \times 3 \text{ stages}$) and weighed on a microbalance. Each bud was preserved in absolute ethanol, and nucleic acids were extracted from individual bud using GenElute Mammalian Genomic DNA Minipreps (Sigma), following the manufacturer's instructions. The spectroscopic method was followed for estimation of RNA/DNA ratio using EBproK (fluorophore: EB ethidium bromide and proteinase K) (Clemmesen 1996; Caldaroni et al. 2006). Total nucleic acid content was determined using a NanoDrop Spectro-photometer (ND-1000). Each sample was treated with ribonuclease A, incubated at 37°C for 30 minutes and then cooled to room temperature. The fluorescence due to total RNA was calculated as the difference of the

fluorescence before and after ribonuclease treatment. It was assumed that the fluorescence before ribonuclease A treatment was due to total concentration of nucleic acids (DNA and RNA), whereas the fluorescence after ribonuclease A was only due to DNA. The auto-fluorescence of ribonuclease A was also calculated and factored into final calculations.

b) Expression of heat shock protein 70 (HSP 70)

Stress proteins (such as HSP 70 family) have been used to quantify the intensity of spatial competition among sessile marine invertebrates (Rossi and Snyder 2001). Competitive interactions often lead to cell damage in the interacting organisms due to the involvement of allelochemicals (Jackson and Buss 1975; Porter and Targett 1988; Engel and Pawlik 2000), thereby causing accumulation of misfolded polypeptides within cells (Uriz et al. 1991; Rossi and Snyder 2001). Heat shock proteins are essential cell components that are expressed during stressful conditions and function as chaperones by reconfiguring the misfolded proteins (Sanders 1993; Gething 1997; Feder and Hofmann 1999; Hoffmann et al. 2003). The heat shock protein family comprises five different types of HSPs in eukaryotes, four are named on the basis of their molecular weight (HSP 90, HSP 70, HSP 58±60 and HSP 20±30), and the fifth one is ubiquitin (Schlesinger et al. 1982; Raboy et al. 1990). In October 2014, tissue from the marked sponges (with competitor, n = 3 and without competitor, n = 3) was frozen in liquid nitrogen and immediately stored at -80°C. Frozen tissue (1 g) from each sample was hand-homogenized in liquid nitrogen in 10 ml of tissue extraction reagent I (Invitrogen, Cat no. FNN0071), following the manufacturer's instructions. Protease inhibitor cocktail (Sigma, Cat. no P-2714) was added to tissue extraction reagent I prior to use. Homogenates were centrifuged at 10,000 RPM at 4°C for 5 min, and the supernatants were stored at -80°C till further analysis. Total protein content was determined by Total Protein Kit, Micro-Lowry, Onishi & Barr Modification (Sigma, Cat no. TP0200) with bovine serum albumin (BSA) (Sigma, Cat no. P 9369) as standard. Frozen supernatants were slowly thawed in an ice bath and then combined with SDS sample buffer (2X) (Invitrogen, Cat no. LC2676) and boiled for 5 min at 85°C. Samples of equal amounts of protein (40 µg) were loaded onto 10% polyacrylamide running gel with 5% stacking gel. 1 µg of HSP 70/72 (Human) (Enzo Life Sciences, Cat no. ADI-NSP-555) was loaded as the standard onto the gel. Gel was dry electro blotted onto iBlot® 2 transfer stacks, PVDF, regular size (Invitrogen, Cat no. IB24001) using the iBlot® 2 gel transfer device (Invitrogen, Cat no. IB21001), following manufacturers' instructions. The protein bands in each western blot were visualized by staining

with Ponceau S (Sigma). The membranes were then probed with a 1:500 dilution of mouse monoclonal anti-HSP 70 (Enzo Life Sciences, Cat no. ADI-SPA-822). This antibody was selected because it is reported to recognize HSP 70 of different target organisms such as human, mouse, rat, *Drosophila*, insect (housefly), rabbit, sponge, and yeast. Immunoblotting was performed using the WesternDot 625 Goat Anti-Mouse Western Blot Kit (Invitrogen, Cat no. W10132) according to the manufacturer's instructions. Blots were scanned using GeneSnap software (Syngene InGenius, Cambridge, UK), and the intensity of each immunopositive band was quantified using GeneTool Software (Syngene InGenius, Cambridge, UK). For each blot, the scanned intensity of the HSP was normalized against the intensities of the HSP 70/72 (Human) standard (Enzo Life Sciences, Cat no. ADI-NSP-555) from that blot; that is, the Syngene Image datum point was divided by the intensity of the HSP 70/72 (Human) standard. The relative intensity was calculated compared to the intensity of HSP 70 band at the time of maximal expression (i.e. HSP 70 protein expression in sponge with competitor; set at 100%).

***C. cf. cavernosa* versus *Zoanthus sansibaricus* cover:** The seasonal change in area cover of the sponge and the Zoanthids was determined from the monthly photographs of the marked quadrats (15×15 cm², N = 10) from the non-manipulative observation (sponge with competitor) using Image J software. The data was expressed as percentage of coverage.

Data Analysis

Images were processed in Adobe Photoshop CS3 Version 10.0. to adjust contrast. The relationship between the asexual reproduction (budding) in *C. cf. cavernosa* (dependent variable) with biotic (*Z. sansibaricus* cover) and abiotic parameters (temperature, pH, DO, salinity, and nutrients) (independent variables) was analyzed using stepwise multiple regression analysis to reduce the collinearity in the data. Factorial ANOVA was performed to determine if three stages of buds showed variation in RNA/DNA ratio in sponges with and without the competitor. For significant ANOVA results, Tukey's post-hoc tests were used to examine pair-wise differences among means. Unpaired student's t-test was performed to determine if significant differences in budding frequency, bud counts per sponge, bud settlement probability, and HSP 70 protein levels were present among sponges with and without the competitor. The monthly variation in sponge cover and zoanthid cover was estimated using Student's t-test, for single sample. The statistical 8 (StatSoft) software was used for these analyses.

Results

Abiotic and Biotic parameters

The mean and range of variation in abiotic and biotic parameters such as temperature, salinity, dissolved oxygen, pH, and nutrient (silicate, phosphate, nitrate, nitrite, and ammonia) of the study site have been discussed previously (Singh and Thakur 2015b). **Table 1a** shows the annual range of each of these parameters. The substrate cover of *Zoanthus sansibaricus*, which was calculated in percentage, was found to be the highest in October (65 to 78%) and the lowest in February and March (22 to 36%). Among various abiotic and biotic parameters studied, the temperature of the rock pool water containing tagged sponges and *Z. sansibaricus* cover explained 69% variability in the budding frequency (Model II; $p < 0.00001$). On the other hand, forward selection of the variables resulted in Model I; with four explanatory variables ($R^2 = 0.74$, $p < 0.00001$) (**Table 1b**).

Bud frequency, count, and settlement probability

The budding in *C. cf. cavernosa* showed a seasonal trend with the peak in a February and March. In the manipulative experiment with removal of competitor from the vicinity of tagged sponges, the budding frequency increased by 10-30 % whereas bud count rose by 45-60% (**Fig. 2**). The bud settlement probability (calculated in percentage) was only 0-10% in the tagged quadrants of the non- manipulative observation (sponge with competitor), which increased to 60-80% in manipulative experiment (sponge without competitor). Results from Student's t-tests showed a significant difference in budding frequency ($p = 0.0053$), bud count/ sponge ($p = 0.0289$), and bud settlement probability ($p = 0.00001$) of *C. cf. cavernosa* with and without the competitor, *Z. sansibaricus* (**Table 2**).

Bud stages: structural and skeletal investigations

The budding in *C. cf. cavernosa*, which was categorized into three stages on the basis of the size and morphology, showed the progressive level of skeletal arrangement. At stage I, buds (<0.5 mm diameter) were spindle shaped and contained only longitudinally arranged spicules. During stage II, the buds (0.5–1 mm diameter) became more globular in shape and contained few megascleres protruding from the middle part. Stage III buds looked similar to stage 2 but bigger in size (>1 mm) with a number of megascleres passing through at various angles. Under SEM,

Stage I buds showed the complete absence of accessory C- or S-shaped spicules (sigmaspires) whereas Stage II and Stage III buds contained abundant sigmaspires and the extremities of the radial tracts of megascleres. Buds of all stages demonstrated no visible difference at structural or skeletal level due to presence or absence of the competitor (**Fig. 3**).

RNA to DNA ratio of buds

The DNA concentration in buds was homogeneous at all three stages ($p = 0.25$). However, RNA content showed a progressive increase with increasing bud size. Thus, the RNA/DNA ratio showed an overall increase with increasing bud stage. The buds from all the stages showed significantly higher RNA/DNA ratio in absence of the competitor (**Fig. 4**). Factorial ANOVA revealed significant differences among RNA/DNA. Tukey's post hoc tests indicated the following trend in RNA to DNA ratio (**Table 3**):

$$\text{III (-)} > \text{II (-)} > \text{III(+)} > \text{II (+)} > \text{I (-)} > \text{I (+)}$$

Where I,II,III represents stages of buds and + or - represents presence or absence of the competitor.

HSP 70 expression in adult sponges

The competitive interaction between *C. cf. cavernosa* and *Z. sansibaricus* resulted in over expression of heat shock proteins (HSP 70). The relative intensities of the HSP 70 bands in non-manipulative observation (sponge with competitor) were set at 100%. As shown in **Fig 5**, in the manipulative experiment (removal of competitor), *C. cf. cavernosa* showed 75-80% decrease in the intensity of heat shock protein band (HSP 70) in Western blots using a monoclonal antibody against *C. cf. cavernosa* HSP 70. Results are presented as mean values (\pm SD) of three sponge individuals. Student's t- test showed a significant difference in expression of HSP 70 in the sponge with and without the competitor ($F_s = 4.28$, $p = 0.0001$).

Discussion

The proportion of budding sponges was the highest in February and March, when the average water temperature of rock pool water was between 30.9 to 31.2°C, whereas no budding was observed from August to November, when the temperature dropped to 27.6 - 29 °C. Seasonality in asexual reproduction has been reported for a number of other sponge species

(Corriero et al. 1996; Chen et al. 1997; Gaino et al. 2006; Cardone et al. 2010). Temperature acts as a positive regulator of asexual reproduction (budding) in many other sponges (Ayling 1980; Corriero et al. 1996; Chen et al. 1997; Cardone et al. 2010). Besides temperature, salinity, and nutrients have also been reported to affect the budding process and post-bud growth (Cardone et al. 2010; Di Bari et al. 2014; Singh and Thakur 2015a). Environmental factors such as water temperature, salinity, nutrient availability, and pollution are also reported to regulate sexual reproduction in sponges (Fromont and Bergquist 1994; Uriz et al. 1995; Baldaconi et al. 2007; Ettinger-Epstein et al. 2007; Whalan et al. 2007; Bautista-Guerrero et al. 2010; Chaves-Fonnegra et al. 2016; Nozawa et al. 2016; Wahab et al. 2017). The natural population of zoanthids also shrinks during the seasonal rise in seawater temperature, which might be due to partial or complete loss of their symbiotic zooxanthellae (Hibino et al. 2013). Our transplantation studies have shown that thermal stress owing to change of habitat (from lower intertidal regions to higher intertidal regions) can lead to bleaching in these zoanthids due to loss of symbionts (Thakur & Singh, unpubl. data).

To the best of our knowledge, this is the first long-term study on the influence of spatial competition on the asexual reproduction (budding) of *C. cf. cavernosa* in terms of physiological changes (decrease in bud frequency, count, and RNA/DNA ratio of buds) and stress generated responses (elevated expression of HSP 70). We tested the hypothesis of tradeoffs between the primary (reproduction) and secondary (spatial competition) functions of the sponge. The contact margins between *C. cf. cavernosa* and *Z. sansibaricus* showed no modification during the study period, which is suggestive of the involvement of allelochemicals for such "stand-off" interactions. The "stand-off" interactions are quite dynamic in nature and influenced by various abiotic and abiotic factors, thereby regulating the competitive abilities of interacting species (Aerts 2000). In a study on sponge/coral interactions in a coral reef Santa Marta, NE Colombia, the sponges *Scopalina ruetzleri*, *Rhaphidophylus venosus* and *Niphates erecta* mostly displayed standoff competitive strategy against the coral *Montastraea cavernosa* (Aerts 2000). However, in the same study, the sponge *R. venosus* quickly occupied vacant space left by dead coral polyps in about 54 % of such interactions; thereby showing the dynamic nature of these competitive interactions.

Competitive interactions demand additional energy to safeguard the limiting resources, thereby resulting in the allocation of less energy to other primary processes such as growth and

reproduction (Uriz et al. 1995; Turon et al. 1996; Wulff 2005). In our study, this trade-off was evident in the manipulative experiment involving complete removal of competitor. In such an environment, *C. cf. cavernosa* showed a significant increase in frequency, count, and settlement probability of the buds, which might be due to release from competitive stress. On the other hand, in non-manipulative experiment, the tagged *C. cf. cavernosa* specimens experienced constant competitive contact from *Z. sansibaricus*, which was reflected in their lower reproductive output. This suggests the possible resource trade-offs during spatial competition and reproduction in the studied sponge. This observation is also well supported by our chemical investigation on *C. cf. cavernosa* that showed variation in the production of cytotoxic compound 'β-sitosterol' depending on the abundance of its competitor *Z. sansibaricus*, thereby suggesting its potential role as an allelochemical to defend the occupied space from encroaching neighbours (Singh & Thakur 2016, unpubl. data).

Zoanthids, being fast growing organisms, can easily overgrow the sponge tissue (Rabelo et al. 2013) and/or control its growth by releasing toxic compounds (such as Palytoxin analogues) (Moore and Scheuer 1971; Hoffmann et al. 2008). Moreover, *C. cf. cavernosa* needed to invest a part of its total energy to defend its occupied substratum by preventing the overgrowth of aggressive neighbour (*Z. sansibaricus*), which might result in the decline in its reproductive output. The concept of resource allocation trade-offs have been well documented in terrestrial plants (Coley et al. 1985; Bazzaz and Grace 1997; Fine et al. 2004), marine algae (Dworjanyn et al. 2006), and marine invertebrates (Connell 1978; Turon et al. 1998; Aerts 1999; Walters and Pawlik 2005; Wulff 2005; Pawlik et al. 2008; Leong and Pawlik 2010a; Pawlik 2011). Optimal defence hypothesis, which proposes that defenses are costly and come at the expense of other functions such as growth, regeneration, and reproduction, is applicable to other marine organisms including sponges (Turon et al. 1998; Aerts 1999; Walters and Pawlik 2005; Wulff 2005; Pawlik et al. 2008; Leong and Pawlik 2010a; Leong and Pawlik 2010b; Leong and Pawlik 2011; Pawlik 2011). Various reports on Caribbean sponges have revealed that undefended sponges had faster growth, recruitment and/or healing rates (Walters and Pawlik 2005; Pawlik et al. 2008). However, the concept of resource allocation becomes complicated as there can be the trade-offs among various primary functions (growth, regeneration, and reproduction) as demonstrated in the case of terrestrial plants, and marine algae (Mole 1994; Dworjanyn et al. 2006). In competitive interactions, suppression of growth or reproduction of the opponent

appears to be one of the strategies to occupy the available space. The corallimorpharian *Corynactis californica* has been reported to reduce the abundance of its competitor, the hard coral *Balanophyllia elegans* by suppressing its reproductive output and successful larval settlement (Chadwick 1987; Chadwick 1991).

The comparative western blot analysis of the crude extracts from *C. cf. cavernosa* with and without the competitor depicted elevated expression of stress proteins (HSP70) in sponges with competitor. The expression of HSP 70 might be interpreted as a characteristic response of *C. cf. cavernosa* to the stress generated from the encroaching polyps of *Z. sansibaricus*. This observation is further supported by the experimental results of increased HSP 70 expression during competitive interactions between two cnidarians *Anthopleura elegantissima* and *Corynactis californica* (Rossi and Snyder 2001). There are reports of a variety of responses (such as elevated expression of genes related to stress proteins, cytoskeleton rearrangement, signalling pathways, protein synthesis, post-translational modifications, degradation, apoptosis, oxidative stress, and DNA damage and repair) in the sponges under stressful conditions such as elevated temperature, metal pollution, bleaching, change in natural habitat etc (Müller et al. 1995; Krusel et al. 1996; Koziol et al. 1997; Schröder et al. 1999; Lopez-Legentil et al. 2008; Wanick et al. 2013; Webster et al. 2013; Di Bari et al. 2014). These responses usually include up-regulation of specific proteins, such as heat shock proteins (such as HSP 70 family) that assist the sponges in maintaining homeostasis and surviving under stressful conditions. It is worth noting that *C. cf. cavernosa* with the competitor also showed significant decrease in its physiological activity (measured as RNA/DNA ratio), which implies that spatial competition adversely affect the physiological activity of the sponge by causing stressful conditions; and to overcome the cellular damage, the sponge expresses stress-related proteins (such as HSP 70). The physiological activity (measured as RNA/DNA ratio) is directly related to organisms' growth rate and nutritional state as shown for a variety of marine invertebrates (Wright and Martin 1985; Meesters et al. 2002; Vidal et al. 2006; Chícharo and Chícharo 2008). Since many of the epibenthic invertebrates (including sponges and soft corals) are efficient filter-feeders (Trench 1974; Peterson et al. 2006), *C. cf. cavernosa* growing in close vicinity of its competitor (*Z. sansibaricus*) might experience competition for food, which is evident from the comparatively lower RNA/DNA ratio in these specimens.

The “standoff” strategy displayed by *C. cf. cavernosa* and *Z. sansibaricus* permits them to create a balance in their use of space and appears to be one of their strategies to thrive successfully in highly diverse intertidal regions with fluctuating environmental conditions. Our results signify that in space-limited environment, the sponge allocates a part of its energy to maintain the occupied space and hinder overgrowth by competitor. This adversely affects its primary function such as reproduction (via budding). The buds of *C. cavernosa* are negatively buoyant with a tendency to settle near the mother sponge (Singh and Thakur 2015a). However, from our field observations and experimental data on probability of settled bud, we conclude that polyps of *Z. sansibaricus* do not allow the settlement of sponges’ buds on their surface, which could be due to the involvement of allelochemicals. Alternatively, reduction in bud settlement could also be caused due to occupancy of adjacent space by spatial competitors, such as zoanthids.

Understanding the factors affecting sponge budding is very important as this knowledge can help to optimize sponge aquaculture from buds, thereby 1) solving the biomass supply problem (Duckworth and Battershill 2003), and 2) promoting their sustainable use for several biotechnological applications (Thakur and Müller 2004b). Our knowledge of how the competitive interactions among sessile organisms can impact their life history processes holds a great promise for understanding the resource trade-offs involved in various biological functions. This can also provide a valuable insight into the role of sponges in structuring and functioning of the benthic community. Knowledge of sponge ecology is still limited to a few species, and additional studies are needed to improve our understanding of dynamic capabilities and competitive behaviour of these animals.

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Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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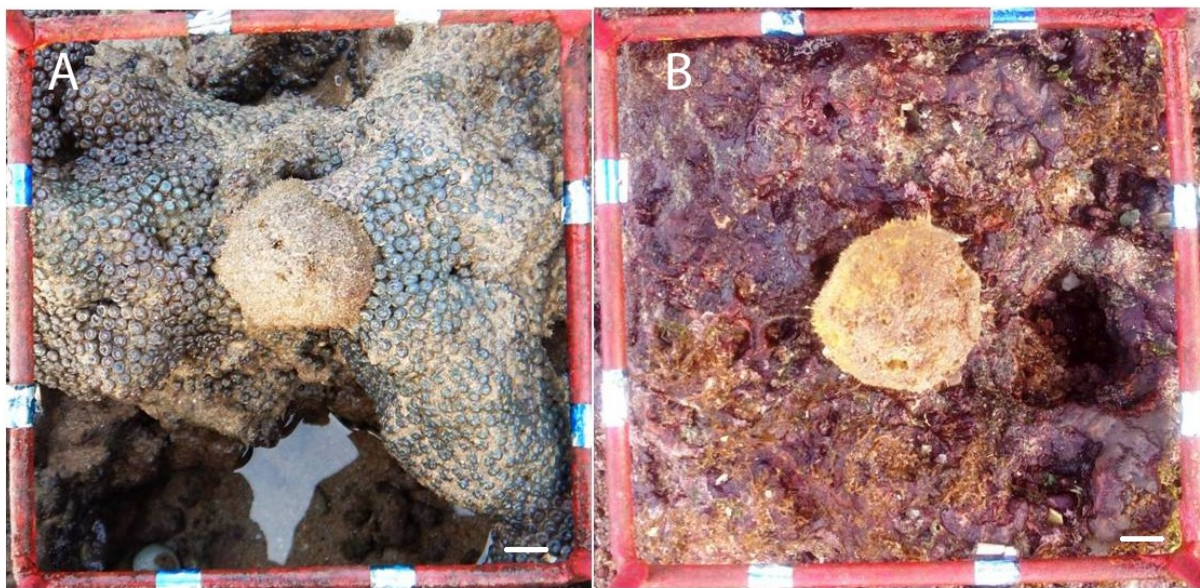


Fig.1 A. Tagged *C. cf. cavernosa* with competitor *Z. sansibaricus* polyps B. Tagged *C. cf. cavernosa* without the competitor *Z. sansibaricus* polyps (Scale = 1 cm)

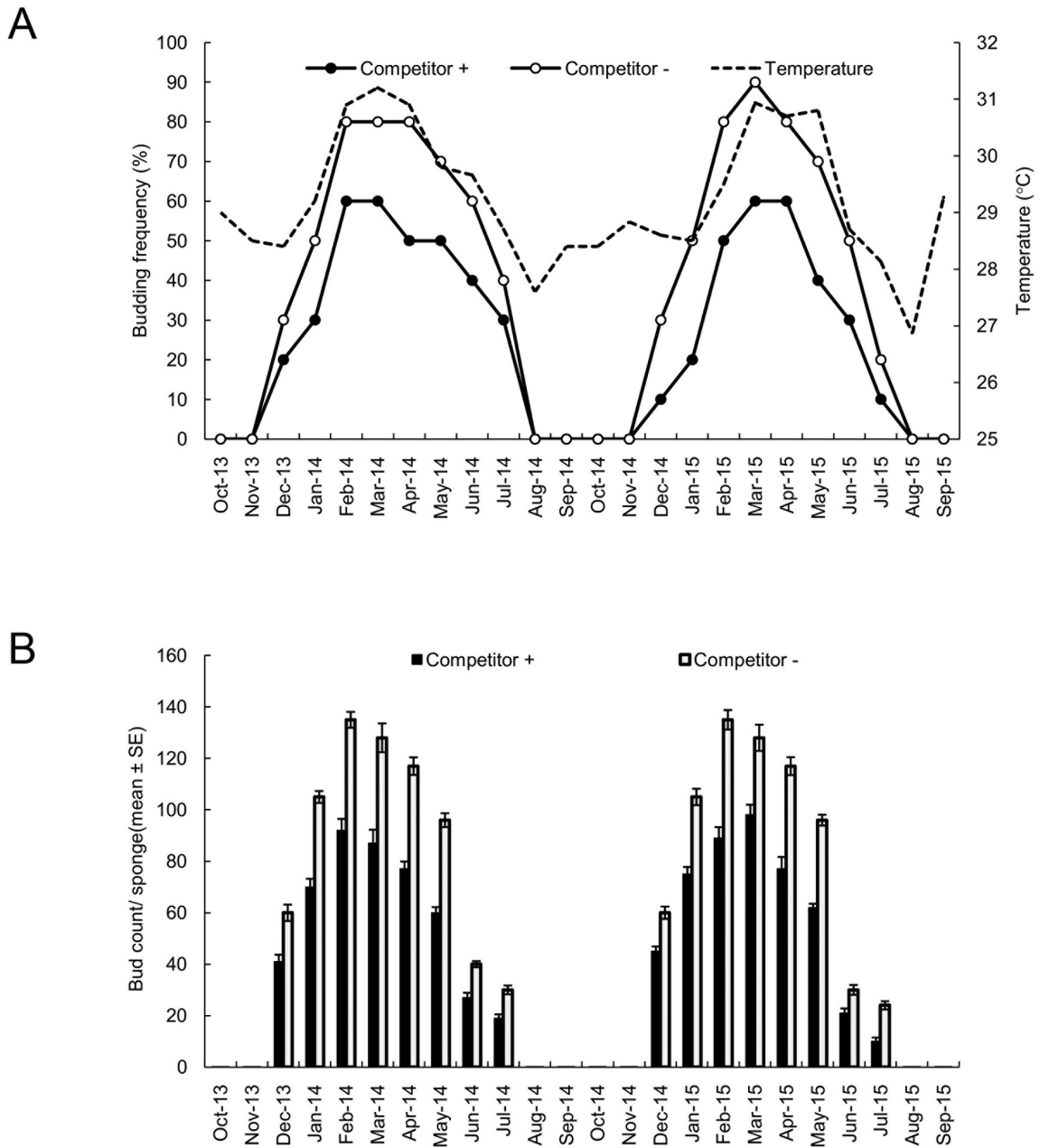


Fig. 2 A. Budding frequency and **B.** Bud count in *C. cf. cavernosa* with (+) and without (-) competitor.

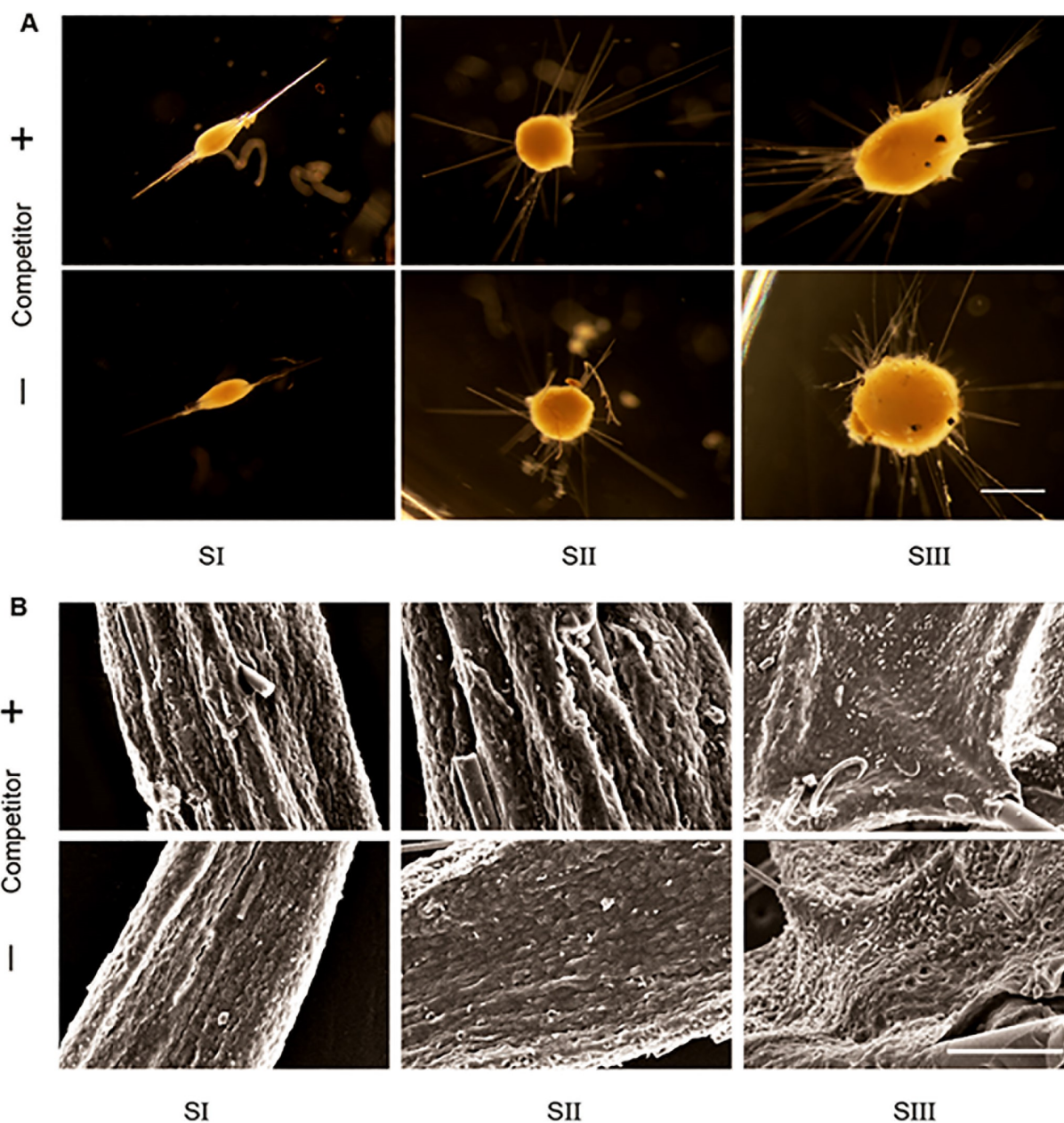


Fig. 3 A. Stereomicroscopic images showing bud stages of *C. cf. cavernosa* with (+) and without (-) competitor. Stage I buds are elongate, with megascleres positioned longitudinally. Stage II buds are globular, with few megascleres intersecting in the middle whereas Stage III buds are comparatively large, with a number of megascleres passing through at various angles (scale bar = 1 mm) **B.** SEM images showing bud sections of *C. cf. cavernosa* with and without competitor. Stage I buds showed compact arrangement of cells and complete absence of sigmaspires whereas stage II and stage III bud showed large numbers of sigmaspires (scale = 100 μ m).

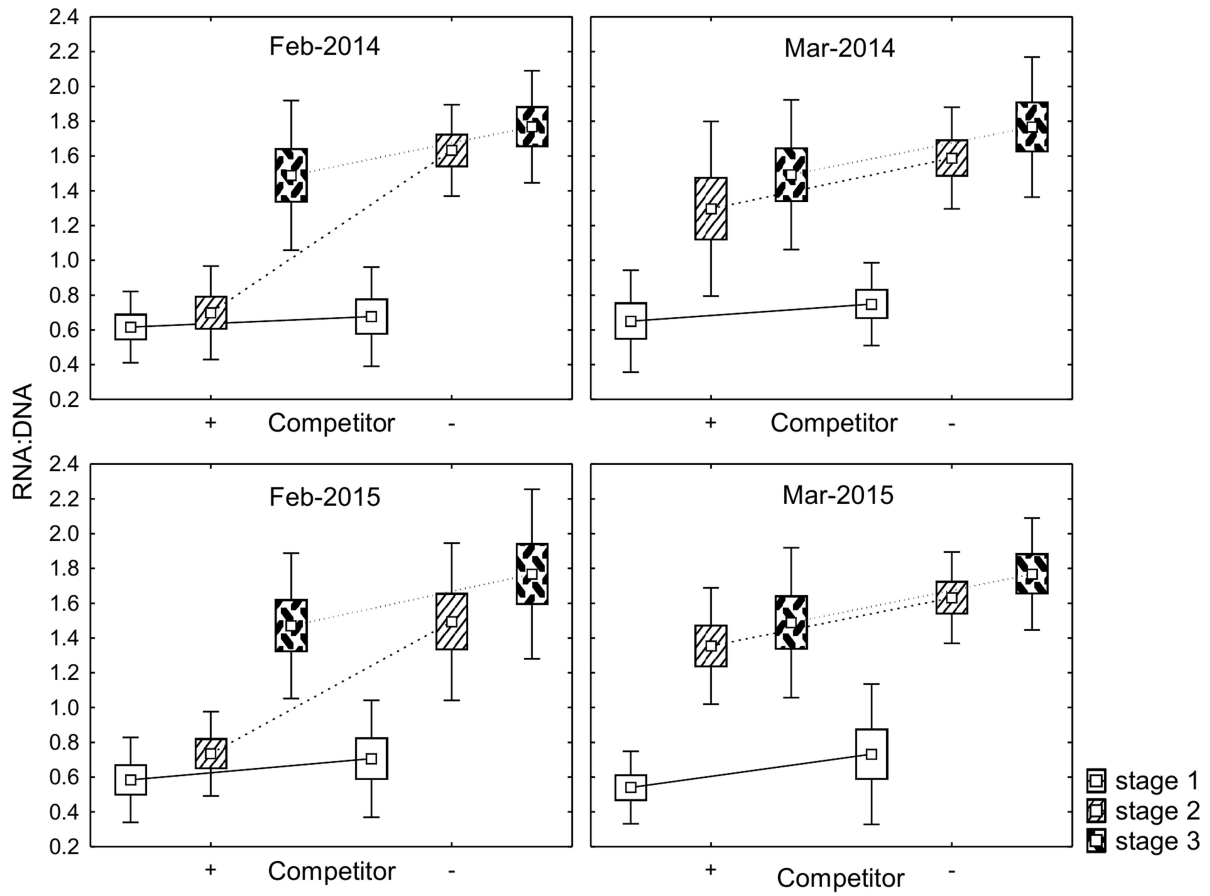


Fig. 4. RNA/ DNA ratio in buds of *C. cf. cavernosa* with (+) and without (-) competitor, during peak budding months (February and March)

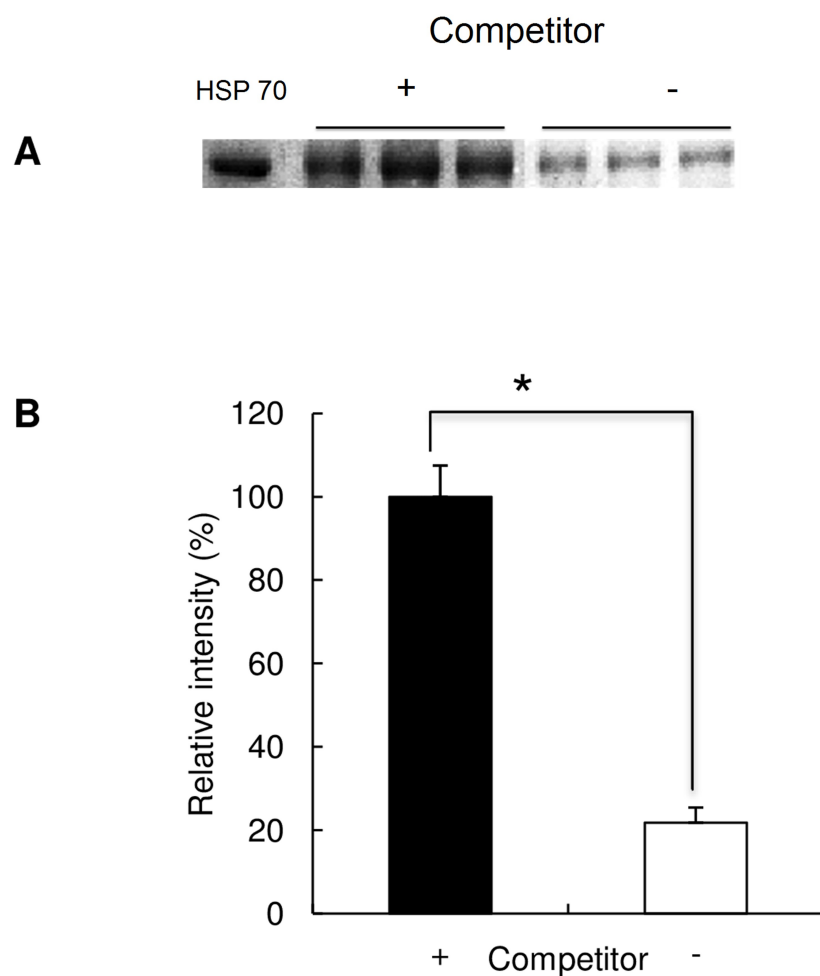


Fig. 5 A. Immunoblot showing HSP70 expression in *C. cf. cavernosa* with and without competitor. **B.** The relative intensities (%) of HSP70 bands in *C. cf. cavernosa* with (S+Z) and without competitor (S-Z). Results are mean values (\pm SD) of three sponge individuals. Asterisks indicate significant differences between means ($P \leq 0.005$), unpaired Student's t-test.

Table 1a. Physicochemical parameter of seawater in the rock pool.

	Mean	Range
pH	7.92	6.6 - 8.4
Temperature(°C)	29.28	27.6 - 31.20
Salinity (‰)	33.06	26.82 - 34.91
DO (mg L ⁻¹)	4.50	2.19 - 5.76
Silicate (μM)	8.09	2.76 - 19.12
Phosphate (μM)	1.57	0.62 - 2.7
Nitrate (μM)	5.91	3.04 - 11.16
Nitrite (μM)	0.81	0.40 - 1.6
Ammonia (μM)	2.78	0.97 - 5.13

Table 1b. Estimated coefficients, standard errors, and p-values of multiple regression models of “asexual reproduction (budding)” as predicted by abiotic and biotic environmental variables using a forward stepwise selection procedure (Model A, four explanatory variables, Multiple R = 0.88, R² = 0.77, adjusted R² = 0.74, p < 0.00001) and a backward stepwise elimination procedure (Model B, two explanatory variables, R = 0.84, R² = 0.70, Adjusted R² = 0.69, p < 0.00001).

	Estimate	Standard Error	p
Model I (Forward Selection)			
Intercept	-552.60	59.57	0.0000
Temperature	20.53	1.99	0.0000
zoanthus	-0.02	0.01	0.0032
Ammonia	-3.73	1.83	0.0479
Silicate	1.15	0.64	0.0788
Nitrate	0.36	0.14	0.0170
Nitrite	-13.15	7.84	0.1011
Model II (Backward Elimination)			
Intercept	-513.11	63.41	0.0000
Temperature	18.93	2.15	0.0000
zoanthus	-0.02	0.01	0.0018

Table 2. Student's t-test for a) budding frequency b) bud count/ sponge and c) bud settlement probability in *C. cf. cavernosa* with and without competitor (*Z. sansibaricus*).

	Mean	SD	Fs	p
a) Budding frequency				
+	38.75	17.84	1.51	0.0053
-	60.00	21.91		
b) Bud count				
+	59.38	28.65	2.01	0.0289
-	87.88	40.58		
c) Bud settlement probability				
+	0.42	0.78	17.20	0.0000
-	5.21	3.22		

Table 3. Analysis of variance of RNA to DNA ratio of buds of *C. cf. cavernosa* with and without competitor (*Z. sansibaricus*) in peak budding months (Feb/Mar).

	df	SS	MS	Fs	p	Tukey post hoc test
Intercept	1	171.47	171.47	2184.83	0.00	
months	3	0.43	0.14	1.81	0.15	
competitor(+/-)	1	3.12	3.12	39.70	0.00	competitor (-) > competitor (+)
Stages (I, II, III)	2	19.50	9.75	124.21	0.00	III > II > I
months * competitor(+/-)	3	0.23	0.08	0.99	0.40	
month*stages	6	0.70	0.12	1.49	0.19	
competitor(+/-)*stages	2	1.03	0.51	6.55	0.00	III(-) > II (-) > III(+) > II (+) > I(-) > I (+)
month*competitor(+/-)*stages	6	0.62	0.10	1.32	0.26	
Error	96	7.53	0.08			
Total	119	33.15				

df- degree of freedom; s-sum of the squares; ms-mean of squares; Fs-Fischer constant; I, II,III – stages of buds r; +/- represents with and without competitor (*Zoanthus sansibaricus*).