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Field and laboratory investigations of the budding process of a tetillid sponge, *Cinachyrella cavernosa*

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Abstract. The budding process of the tetillid sponge *Cinachyrella cavernosa* was studied for one year in the subtidal rocky shore of Mhapan (15° 55' 27.48" N; 73° 33' 29.89" E), located along the central west coast of India. The sponges showed the highest budding frequency when the average water temperature of subtidal rock pools was $32.4 \pm 0.23^\circ\text{C}$ (February - March), followed by a significant decrease in budding efficiency (April - July) at $28.2 \pm 0.12^\circ\text{C}$ and no budding (August - November: i.e., early fall /winter) at $\leq 25.9 \pm 0.12^\circ\text{C}$. Stepwise multiple regression analysis of physico-chemical factors revealed temperature as the most prominent factor regulating the intensity of budding. Based on size and morphology, three stages of sponge buds were defined. The production of buds was found to be asynchronous, as adult sponges possessed buds from all three stages. Differences among these stages were examined at ultra-structural (in terms of spicules) and molecular (in terms of RNA/DNA) levels. Stage I (< 0.5 mm diameter) showed a complete absence of microscleres (sigma spires) whereas stage II (0.5-1mm) and stage III (> 1 mm) contained all spicules characteristic of the adult sponge. There was a significant increase in the RNA/DNA ratio from stage I to III, suggestive of a progressive increase in physiological activity during the developmental process. Additionally, we studied post-settlement bud growth under field and laboratory conditions. Newly settled buds displayed a lower average specific growth rate in the field, owing to variability in environmental conditions, but they showed more rapid growth under controlled conditions. This study highlights the role of abiotic factors in regulating the budding process and stresses the ecological significance of budding in maintaining natural sponge populations. Our data suggest that an increased budding frequency under stressful conditions, such as high temperature, is an advantageous adaptation for these sponges. Buds showed rapid development, as no metamorphosis is involved, and retained the genotype of the parents, yielding high reproductive outputs and survival rates.

Additional key words: marine sponge, subtidal rock pools, spicules, RNA/DNA, post-settlement bud growth.

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Marine invertebrates such as corals, sponges, sea urchins, sea cucumbers, sea anemones and barnacles can adopt different reproductive strategies (e.g., asexual and /or sexual) that maintain populations across variable environmental conditions. In general, asexual reproduction (in the form of resistant propagules) is often advantageous for organisms experiencing highly stressful conditions and frequent fluctuations in abiotic factors. Among various marine invertebrates, sponges are noteworthy because they have evolved different modes of propagation (both sexual and asexual) even though they lack specialized reproductive organs. Moreover, asexual reproduction can be particularly advantageous to sessile organisms such as sponges because adults are incapable of relocating to search for mates.

Sponges show various modes of asexual reproduction, including: a) fragmentation (Wulff 1991) b) gemmule formation (Simpson & Fell 1974) and c) budding (Simpson, 1984). Budding is the process of growth and differentiation of cellular masses that sprout from the tissue of the parent sponge (Hughes 1989). These buds have the ability to develop into fully functional, independent individuals. Sponges from all four classes [Calcarea (Connes 1964), Demospongiae (Chen et al. 1997), Hexactinellida (Schulze 1887) and Homoscleromorpha (Jackson & Sally 2010)] have been reported to produce external buds. The development of buds differs among species; for example, the formation of the aquiferous system can occur before buds are released from the parent sponge (Saller 1990; Gaino et al. 2009) or after detachment (Gaino et al. 2006; Ayling 1980; Battershill & Bergquist 1990). The rate of development from bud to a fully functional individual is also variable among sponge species (Corriero et al. 1996). However, budding follows a spatiotemporal morphogenetic pattern that is common to all sponge species. Hammel et al. (2009) demonstrated that the budding process occurs through a sequential series of well-defined morphological stages, which seem to be genetically controlled. Researchers have most often explored bud production at the population level (Ayling 1980; Corriero et al. 1996, 1998; Chen et al. 1997), rather than at the individual level (Cardone et al. 2010). There are only a few studies that provide detailed information about the morphology and cytology of buds (Ereskovsky 2007) and their production, dispersal and development in natural habitats (Ayling 1980; Battershill & Bergquist 1990; Cardone et al. 2010).

In sponges, budding seems to be positively correlated with the temporal and spatial stability of the environment. In unstable and stressed environmental conditions, asexual reproduction, like budding, has ecological significance in maintaining populations (Cardone et al. 2010). Asexual propagation is very efficient in sponges because of the high totipotency of sponge cells. Every sponge cell is capable of

differentiating into any of sponge's repertoire of cell types, enabling the sponge bud to grow into a whole new individual (Simpson 1984; Dawkins 2010). In addition, these new small individuals have the same genomic constitution as their parents and might therefore have higher rates of survival and proliferation than larval recruits (Jackson & Coates 1986), since sexual reproduction can result in the loss of an adaptive genotype through recombination (Smith 1992). Moreover, the structural organization of asexual propagules does not involve any metamorphosis in sponges, thus allowing maximum growth rates and functionality to be achieved in a short time. Asexual reproduction, being cost-effective (Smith 1992), often replaces sexual reproduction to increase dispersal efficiency and the chances of successful colonization (Jackson & Coates 1986; Maldonado & Uriz 1999). Changes in modes of reproduction between successive seasons have been reported in some sponge species (Corriero et al. 1996).

In addition to seasonal variations, intertidal sessile invertebrates are subjected to daily fluctuations in a variety of environmental factors including light, salinity, nutrients and temperature. However, organisms found in subtidal regions experience comparatively smaller fluctuations in environmental parameters. Temperature is one of the important factors for the regulation of sexual reproduction in both tropical and temperate sponges (Simpson 1984; Witte & Barthel 1994; Riesgo & Maldonado 2008). A number of other factors such as age, size, sex ratio and survival of offspring have been observed to influence reproductive cycles in other invertebrates, such as in corals (Hall & Hughes 1996). The contribution of abiotic and biotic components to spatiotemporal variation in the reproductive cycle of sponges has been studied in detail (Ayling 1980; Fromont & Bergquist 1994; Uriz et al. 1995; Whalan et al. 2007). Environmental factors like water temperature, salinity or nutrient availability are reported to regulate reproductive activity in sponges (Baldaconi et al. 2007; Ettinger-Epstein et al. 2007; Bautista et al. 2010.). However, the factors affecting the budding process in sponges are largely unknown. To date, few environmental parameters (e.g. temperature and salinity) have been studied in relation to the budding process and post-bud development. Of these, temperature was found to positively correlate with budding frequency (Corriero et al. 1998) and post bud growth (Cardone et al. 2010). However, salinity negatively influenced growth of newly settled buds (Cardone et al. 2010).

We studied the tetillid sponge *Cinachyrella cavernosa* LAMARCK 1815, which is abundant in the subtidal habitats of Mhapan, India. This rocky shore is exposed during the lowest spring tides. During monthly field trips over two years (2011-2012), we observed that *C. cavernosa* with buds were present only from December to July. We designed a short-term field study to 1) examine the proportion

of *C. cavernosa* sponges with buds during the budding period (December to July); 2) determine whether variation in the budding of sponges is influenced by environmental factors; and 3) study progressive stages of buds in terms of their skeletal development and physiological activity. We investigated the presence/absence of characteristic spicules in buds of different size-ranges to account for skeletal changes during bud development. We estimated the physiological activity of buds by measuring their RNA to DNA ratio. The RNA to DNA ratio is based on the assumption that DNA content per cell does not vary with environmental condition or growth rate (Bulow 1970), whereas RNA content per cell determines the rate of protein synthesis. The RNA to DNA ratio has been reported as a good indicator of physiological condition for various marine organisms such as microbes, crustaceans and fishes (Robinson and Ware 1988; Anger & Hirche 1990; Chicharo et al. 1995; Clemmesen, 1996; Anil AC et al. 2001).

Methods

Study site and specimens

Sample collection and field studies were conducted in the rocky subtidal habitats of Mhapan, Maharashtra (15° 55' 27.48" N; 73° 33' 29.89" E), located along the central west coast of India. This nutrient-rich shore confines small rock pools in the subtidal zone. *Cinachyrella cavernosa* is abundant in the subtidal region, where it successfully colonizes hard rocky substrata. During the lowest spring tides, these sponges are covered by a continuous layer of sediment that prevents exposure to direct sunlight and desiccation. Literature on the life cycle and reproductive strategies of family Tetillidae is scant, especially for genus *Cinachyrella*. Reports on related species indicate the presence of both sexual (*Cinachyrella tarentina* = *Cinachyrella tarentina*) PULITZER-FINALI 1983 (Lepore et al. 2000) and asexual mode of reproduction [*Cinachyrella australiensis* (= *Cinachyrella australiensis*) CARTER 1886 (Chen et al. 1997)].

Physico-chemical parameters

For the duration of this study, the temperature, salinity, pH, and dissolved oxygen concentrations of seawater from the study area were measured on a monthly basis, using a multi parametric probe (Oakton PCD 650). Every month, water samples (n=3) were collected from this region and nutrient (phosphate, silicate, nitrate, nitrite and ammonia) analyses were carried out using a continuous flow

analyzer (San++, SKALAR, Holland). The rate of budding in the sponge changes slowly over the time. We assumed that our monthly physico-chemical data collection represented the annual pattern.

Bud frequency and count

The method described by Cardone et al. (2010) was adopted with slight modifications to study the budding process in the field. Ten permanent quadrats (0.15 m × 0.15 m), each containing a single adult sponge, were marked on rocky pools (**Fig. 1A**). Every month, during the lowest tide, these marked areas were photographed with a digital camera (Canon Power shot A650) set on a photo-quadrat stand to capture images at the same angle and distance. These images were analyzed to discover monthly changes in budding frequency by calculating the percentage of total individuals with buds. For bud count, ten adult sponge specimens were randomly collected each month (n = 120/ yr). Buds of varying ages/ sizes were picked from these adults using a sterile pointed forceps. The total number of the buds and their maximum and minimum diameter were calculated under the stereomicroscope (Olympus stereo microscope, SZX2-ILLD). Mean diameter was estimated for each sponge bud. Based on mean diameter, three groups of buds were established as: 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 a light microscope.

Skeletal investigations

To investigate the skeletal elements, spicule preparations were obtained from fragments of adult sponge and the representative buds of each stage (n=10 per stage). The samples were boiled in 60% nitric acid, rinsed with distilled water and dehydrated in a graded ethanol series. After being dried in an oven, the spicules were observed directly under a light microscope.

For SEM examination, live buds from all three stages were fixed in 4% formaldehyde prepared in filtered seawater. A few buds from each stage were cut longitudinally through the middle using a sharp blade. Bud sections were dehydrated gradually through ethanol series, treated with hexamethyldisilazane (HMDS, SRL Chemicals) and then allowed to dry, following the protocol by Nation (1983). All bud sections were mounted onto SEM stubs using double-sided tape. Buds were sputter-coated with gold (at a thickness of 200 Å) and were scanned and photographed using an SEM (JEOL 5800 LV) at a voltage of 20 kV.

RNA to DNA ratio

During the entire budding period, the bud samples of each stage were taken in replicates (n=10 per month) and weighed on a microbalance. Buds were preserved in absolute ethanol and nucleic acids were extracted using GenElute Mammalian Genomic DNA Minipreps (Sigma), following the manufacturer's instructions. The method described by Clemmesen (1988, 1993) was followed for estimation of RNA/ DNA ratio. Total nucleic acid content was determined using a NanoDrop Spectrophotometer (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. We 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.

Timing of bud development in the laboratory

Three budding sponges were collected along with their substratum during the peak budding month and maintained in an aquarium containing sea water collected from natural habitat. The sponges were kept under controlled temperature (30° C) and fed with mixed cultures of *Synechococcus* and *Prochlorococcus* sp. (Osinga 1999). The water was changed twice a week. Fixed quadrats (0.15 m × 0.15 m) were made around these sponges to keep them undisturbed (**Fig. 1B**). The buds attached to the adult sponges were monitored every day for increases in their size by taking images in the same way as described previously. The images were then analyzed in Image J software (Schneider et al. 2012) to find out increase in the diameter of buds and the time taken to pass through each stage.

Post settlement bud growth

The same ten marked quadrats containing adult sponges were studied to examine the rates of settlement and growth of buds in the enclosed area in the field (**Fig. 1C**). Every month, all marked areas were photographed in the same way as described previously. These images were analyzed with Image J software to estimate the number of settled buds and their monthly growth rate. The specific growth rate (SGR) of ten settled buds was estimated in the field for eight months (February 2013-September 2013) using the following equation described by Cardone et al. (2010): $SGR = (\log_e md_2 - \log_e md_1) / (t_2 - t_1) \times 100$ where \log_e are the natural logarithms, md_2 and md_1 the mean diameter at the two different times,

and $(t_2 - t_1)$ the time interval in months. We conducted growth studies on the tagged sponges until September 2013. Further field studies on the tagged sponges were not possible because of their loss due to high sedimentation.

The growth of settled buds around the three adult sponges maintained in aquaria was studied in a similar manner (**Fig. 1D**). The SGR was calculated for six settled buds for 11 months (February 2013-December 2013).

Data analysis

Images were processed to remove background and adjusted for contrast with the help of Adobe Photoshop CS3 Version 10.0. Pearson product moment correlation coefficients were determined to measure the strength and direction of the linear relationship of budding frequency (dependent variable) with each of the environmental factors (independent variables). We found multicollinearity in our data, as some environmental parameters showed low tolerance and high variance inflation factors. Therefore, a simple multiple regression approach was not used; instead, stepwise multiple regression analysis was used to examine the effect of abiotic factors on budding frequency. We used two approaches (e.g. forward selection and backward elimination) to select the predictive variables. The alternative models were compared using the analysis of variance (ANOVA) function of the R statistical software package. Factorial ANOVA was performed to test whether the RNA/DNA ratio significantly varied among three stages of bud development in different months. For significant ANOVA results, Tukey's post-hoc tests were used to examine pairwise differences among means. The analyses were completed using the Statistica 8 (StatSoft) and R (R Development core team 2010) software packages.

Results

Abiotic parameters

The seawater temperature of the rock pools varied from 32.4 °C in February to 24.1 °C in August. Although mean salinity was 33.42 ‰, salinity dropped dramatically to 16.42 ‰ – 20.1 ‰ during monsoon season (June-September) due to the influx of fresh water. Dissolved oxygen varied from 5.93 mg/l (December) to 3.07 mg/l (June), while the average pH value was 8.09. There was no significant difference in these physico-chemical parameters among different rock pools containing tagged sponges ($p = 0.09$). The budding frequency was positively correlated with temperature of the rock pools ($r = 0.810$, $p = 0.001$) and ammonia content of the seawater ($r = 0.659$, $p = 0.020$) (**Table 1**).

Multiple regression analysis (MRA) resulted in two significantly different models ($p = 0.035$). The forward selection MRA yielded a minimal model (Model A) comprised of two variables (temperature and ammonia) that were found to be the best predictors of budding frequency (**Table 2**). The backward elimination MRA removed the effect of salinity on budding frequency, yielding an alternative model (Model B) comprised of 6 significant variables affecting budding frequency (**Table 2**). Although Model B explained more variation in budding frequency, Model A used fewer parameters. In both models, temperature was the strongest single predictor of budding frequency.

Budding patterns under field conditions

Sponge specimens with buds were found from December 2012 to July 2013 during the study period. In the marked quadrats, the proportions of sponges with buds were recorded as 100% and 90% in February 2013 and March 2013 respectively, whereas from October 2012 to November 2012, the proportion of sponge specimens with buds was almost 0 % (**Fig. 2**). We also counted the total number of buds in 10 randomly collected individuals. A total of 3576 buds were counted from 10 sponges during the budding period (December 2012 to July 2013). The bud count corresponded to the frequency of budding sponges, with the maximum peak (128 ± 34 buds per sponge) in February 2013. A similar pattern was observed in 30 randomly tagged sponges for the next budding cycle. In February 2014, the highest number of budding sponges was found, which started declining after April 2014 (Singh & Thakur, unpubl. data). Budding sponges showed different size classes of buds for the entire cycle, demonstrating asynchronous production of buds. Buds were categorized into three different stages based on their mean diameter size and morphology. Stage I: In this initial phase of growth, buds were very small, varying from 0.1 mm to less than 0.5 mm in diameter. Stage II: At this stage, the spindle shaped bud changed to a globular form during growth. Buds measured between 0.5 to 1 mm in diameter. Stage III: Buds measured more than 1 mm in diameter (**Fig. 3**). In the month of February 2013, 80% of total buds were observed at stage III (**Fig. 4**).

Structural description of bud stages

The three stages, which were categorized on the basis of size, expressed a progressive level of skeletal arrangement. Stage I showed spicules protruding from the tapering regions. Spicules were not arranged radially and only few spicules were found. Stage I buds contained only few spicules that were arranged longitudinally. The skeleton in stage I buds consisted of a) long, fusiform shaped oxeads with their sharply pointed tips projecting beyond the surface of the bud and b) straight diaenes and triaenes

(spicules with one end pointed and the other end with short pointed rays termed clads). Anadiaenes / anatriaenes (with 2 or 3 clads, sharply curved backward) and prodiaenes / protriaenes (with 2 or 3 clads, sharply curved backward) were randomly distributed. The cladomes of all types of triaenes were directed outwards. Stage I contained all of the characteristic spicules of the adult specimen except sigmaspires (**Fig. 5 A-E**). However, sigmaspires started appearing in stage II buds and were found abundantly in stage III buds. The outer layer of each bud contained abundant sigmaspires (C- or S-shaped) and the extremities of the radial tracts of megascleres. Sigmaspires were also dispersed randomly throughout the tissue. Under SEM, stage I buds appeared elongated and spindle-shaped, each characteristically with a few megascleres positioned longitudinally (**Fig. 5 F**). Stage II and, III buds were considerably swollen in the middle and thus appeared roughly globular with megascleres intersecting from all angles. High magnification SEM of buds from all three stages showed no apparent signs of the formation of internal cavities or a canal system (**Fig. 5 F-H**). Cells in buds were compactly arranged, thus forming a solid mass. Abundant sigmaspires were apparent on the surfaces of stage II and III buds (**Fig. 5 G-H**).

RNA/DNA ratio

The DNA content in buds was homogenous at all three stages, without any significant difference ($p=0.15$). However, the RNA content showed a progressive increase with increasing buds size. Thus, the RNA/DNA ratio for each stages of *C. cavernosa* showed an overall increase with increasing size of buds. Stage II and III buds appeared to contain high RNA/DNA ratios, showing that advanced stages of buds are capable of high rates of protein synthesis, which is required for their rapid development (**Fig. 6**). Factorial ANOVA revealed significant differences among the RNA/DNA ratios of buds at all three stages in different months ($p < 0.05$). Tukey's post-hoc tests indicated the following trend in RNA/DNA ratio: stage III > stage II > stage I (**Table 3**).

Post settlement bud growth

A total of 732 buds attached to the adult sponges in aquarium were analyzed. The average time taken by an attached bud to traverse through each of these three stages was approximately 3 to 4 days. Stage I buds detached from the adult sponge after an average of 10 - 12 days under laboratory conditions.

Under natural conditions, a total of 30 buds settled in the marked quadrats during between December 2012 and September 2013. The settled buds had mean diameter of 1.37 ± 0.16 mm ($n = 30$). Out of these 30 buds, 14 buds were missing within two months of their settlement. Under laboratory conditions, 21 buds settled on the substratum inside the marked quadrat. No mortality was observed among the settled buds in the aquarium. The average size of settled buds was 1.44 ± 0.22 mm ($n = 21$). The growth analysis was performed only on 10 buds that settled in the month of February in both field and laboratory conditions. The settled buds showed rapid increases in size in the beginning in both conditions. However, in their natural habitat, the buds showed a more inconsistent growth pattern (**Fig. 7A**). When comparing the average specific growth rate (SGR) of buds in the field and in the laboratory, we found that settled buds showed higher SGR under laboratory conditions compared to those in field (**Fig. 7A-B**). The mean SGR \pm SE was 0.39 ± 0.06 % month⁻¹ ($n = 10$) and 0.51 ± 0.09 % month⁻¹ ($n = 10$) under natural and laboratory conditions, respectively

Discussion

The present study reports the budding process in *Cinachyrella cavernosa* and its variation in relation to abiotic factors for the first time. To the best of our knowledge, this is also the first report that evaluates the physiological condition or protein synthetic ability (in terms of RNA to DNA ratio) of sponge buds at different developmental stages. In this investigation, *C. cavernosa* showed seasonal variation in the budding process. The sponges inhabited sub-tidal rocky shores, experiencing fewer fluctuations in the environmental parameters as compared to inter-tidal regions. The temperature of the rock pools was the most significant factor regulating the budding pattern: the proportion of sponges with buds was maximum at the highest average water temperature. The sponges showed a significant decrease in budding efficiency (April-July) at 28.2 ± 0.12 °C and no budding (August-November i.e. early fall / Winter) at or below 25.9 ± 0.12 °C. This trend was also observed for total bud counts in 10 randomly collected sponges. This effect of temperature on budding was supported by a study on *C. australiensis* from southern Taiwan, where the budding cycle started in late spring and the maximum number of budding individuals was found in warmer months and no budding was observed during winter (Chen et al. 1997). Seasonality in budding cycle was also found in *Tethya* spp. (Connes 1969; Corriero et al. 1996). Two species of *Tethya*, *T.citrina* and *T.aurantim*, from the stable, deep regions of the Stagnone di Marsala (North-west Sicily), which is subjected to less frequent temperature changes than coastal areas, showed increased bud production in winter (Corriero et al. 1996). However, in *T.*

citrina, inhabiting anoxic regions of Mar Piccolo di Taranto, North of the Gulf of Taranto, buds occurred throughout the year, with peak values in September, October and from February to April (Cardone et al. 2010). Besides temperature, the ammonia content of seawater was the second important source of variability in the budding frequency of the study sponge. However, the positive correlation between ammonia and the budding process of the sponge needs to be investigated thoroughly with long-term studies. Abiotic factors such as pH, dissolved oxygen, and nutrients such as phosphate and nitrite also affected the budding frequency to some extent. In contrast to *T. citrina*, where a drop in salinity increased post-bud growth (Cardone et al. 2010), no significant effect of salinity was obtained on budding process in the present study. These reports support the conclusions that 1) the budding process can change due to different geographical and environmental conditions, 2) budding frequency usually increases when environmental conditions are stressful, such as increased temperature and 3) budding can provide an adaptive advantage to sponge species inhabiting changing environmental conditions by maintaining their population size.

Environmental factors have been reported to influence the morphology, physiology and genetic makeup of sponges (Müller et al. 1995; Meroz-Fine et al. 2005). Under stressful conditions, sponges need to develop strategies to reduce the investment of energy in various physiological processes. In this context, we observed a progressive increase in complexity of skeletal organization with the advancement of developmental stages. Stage I buds completely lacked the sigmaspires, which are not the part of the supportive skeleton of the sponge. The sigmaspires developed only when sponge buds were ≥ 0.5 mm (mean diameter). Ereskovsky (2010) mentioned the presence of microscleres on the surface of buds of sponges belonging to order Hadromerida TOPSENT 1894. The prevalence of these spicules in buds of stage II and III, and the complete absence of these spicules in stage I buds, is an interesting observation, but this finding is difficult to explain here due to inadequate information about skeletal development during budding process. However, these observations clearly show that there is increase in skeletal complexity and development of accessory spicules (sigmaspires) at later stages of bud development.

Development of choanocyte chambers or a canal system in attached buds has been reported in sponges such as *Tethya seychellensis* WRIGHT 1881 (Gaino et al. 2009), *T. wilhelma* SARÀ, SARÀ, NICKEL & BRÜMMER 2001 (Hammel et al. 2009) and *Radiospongilla cerebellata* BOWERBANK 1863 (Saller 1990). However, sponges with unreleased buds having no such developed internal canal system are also known, which interestingly include two species of the genus *Tethya*, *T. citrina* and *T.*

aurantium (Gaino et al. 2006), and *Cinachyrella australiensis* (= *Cinachyra australiensis*) (Chen et al. 1997). The preliminary observations and SEM examination of attached buds of *Cinachyrella cavernosa* were performed to assess the internal structures of developing buds. The attached buds showed an absence of structures like choanocyte chambers or canal systems at all three stages of their development. Instead, the bud sections appeared like compact masses of cells forming a solid structure without any cavity, similar to the structure of buds from *C. australiensis*, *T. citrina* and *T. aurantium*. The compact structure of these buds can be speculated to be an adaptive strategy to reduce the cost of the budding process, thus making it energetically favored in a stressful environment.

The physiological conditions of buds at each stage were calculated in terms of the RNA/DNA ratio. The amount of DNA was found to be same among different stages of buds as DNA content remained unaffected by environmental conditions. As RNA is the main component in the protein synthesis, RNA content reflects the seasonal pattern in food supply and temperature. Hence, RNA to DNA ratio represents the protein synthetic capacity of the cell. The amount of RNA to DNA ratio was higher for stages II and III, indicating that there is increase in physical, biochemical and genetic processes during bud development. Presently, there is no report on the genetic regulation of budding in sponges. In *Hydra*, Wnt-pathway related genes were expressed during bud formation (Hobmayer et al. 2000). In the sponge *Oscarella* sp., formation of ostia was regulated by WNT/ β -Catenin signalling (Lapébie 2009). Hammel (2009) postulated the possible involvement of Wnt-pathway related genes in bud development and formation. Our knowledge of budding in sponges is incomplete and more studies are needed to understand the budding process and the environmental and genetic factors regulating it. Future studies on the expression of genes involved in developmental process such as cytoskeleton rearrangement, cellular organization, signal transduction, and protein synthesis/degradation in sponge buds under stressful conditions might provide valuable insights into the importance of environmental factors in controlling the budding process and bud development.

The buds were negatively buoyant and able to attach to the substrate immediately after detachment. This ability enabled buds to occupy a substrate quickly, reducing the risk of being transported to unfavorable environments, thus favoring survival in the local environment. Few buds were found attached to adult sponges and the surrounding substrate, suggesting that buds might have a possible role in locomotion (Nickel & Brümmer 2004). In contrast to the sponge *Thenaea abyssorum*, which did not show bud release under laboratory conditions (Witte 1996), the attached buds of *C.*

cavernosa were released and settled on the nearby substratum in laboratory, thus allowing us to conduct post-budding growth experiments and to compare growth under natural and laboratory conditions. There was a rapid increase in the size of new sponges soon after settlement under both conditions. However, the specific growth rate was more uniform and higher in the laboratory. The results reveal that the buds grow rapidly if the most prominent factor controlling the budding process, such as temperature, is kept constant. Moreover, the absence of any stress such as predators, exposure during low tides, and strong wave action aids in successful recruitment and survival of buds under laboratory conditions. Whereas, in field, the settled buds showed higher mortality and irregular, slower growth patterns, likely because of variability in environmental conditions.

These results have important implications for understanding the budding cycle and the effect of abiotic factors, especially temperature, in modifying the budding process. The importance of asexual modes of reproduction, such as budding and fragmentation in maintaining natural populations of sponges has been highlighted in several reports (Wulff 1991; Cardone 2010). The proportion of budding sponges increases with the seasonal rise in seawater temperature. In view of current climatic changes, the significance of budding becomes clear as these sponges might thrive under increasing temperatures (Bell 2013). The buds are exact replicas of the adult sponges, which mean that the buds retain the genotype of their parents. This observation suggests that asexual reproduction, i.e. budding in the sponge *C. cavernosa*, might provide an ecological advantage to thrive under stressful conditions and it might replace the sexual mode of reproduction (which was not investigated in present study). However, a decrease in genetic heterogeneity might reduce the resistance of the sponges to new changes in their environment and limit future adaptation. In such a situation, there is a possibility that alternative reproductive strategies will proliferate and survive under drastic environmental changes. It would be interesting to examine further how trade-offs between sexual and asexual modes of reproduction occur in this tetillid sponge in response to changing abiotic parameters.

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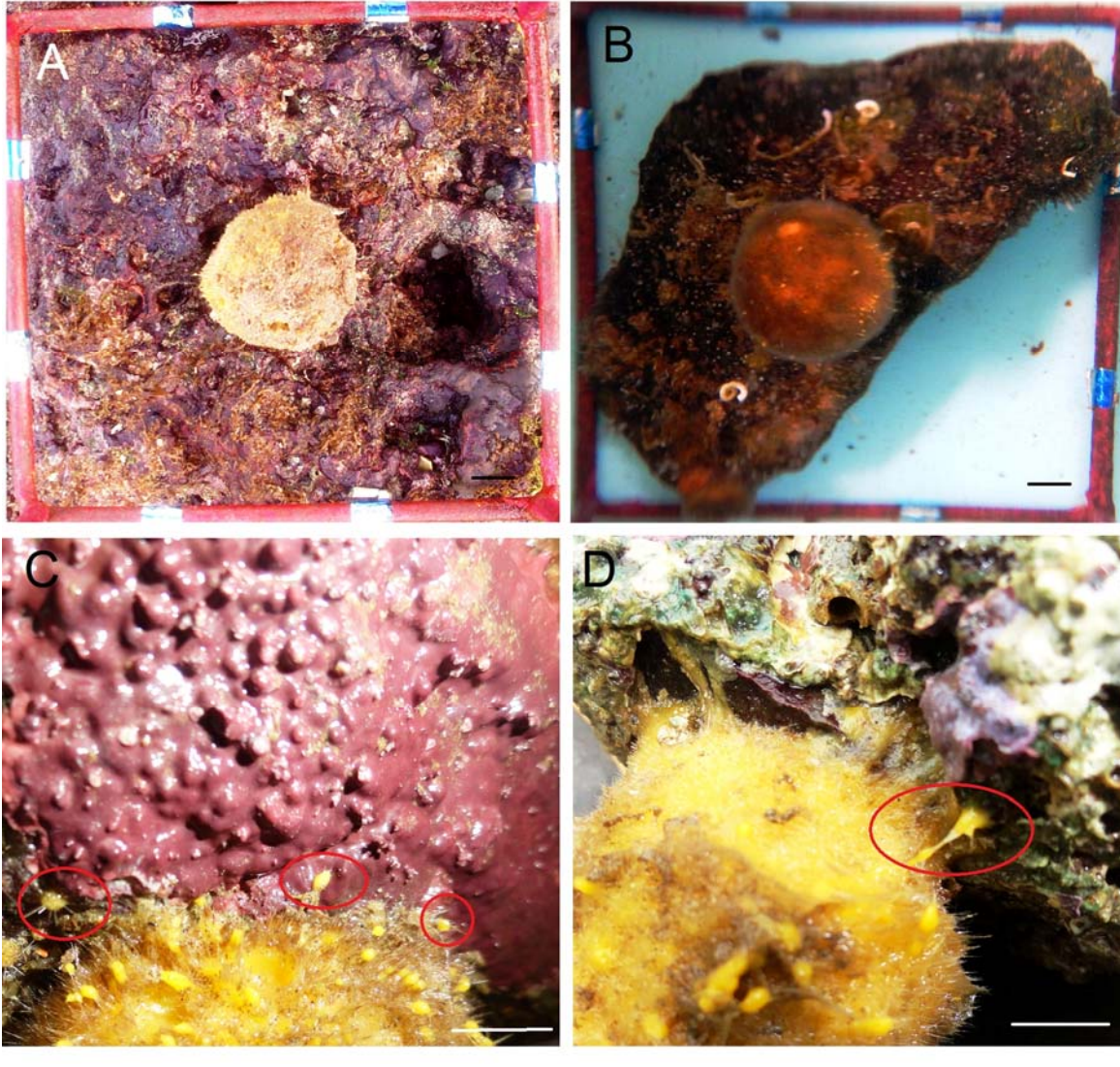


Fig. 1. *Cinachyrella cavernosa*. A. Photo-quadrate enclosing the adult sponge on field (Mhapan). B. Photo-quadrate enclosing the adult sponge in laboratory (under aquarium). C. Newly settled buds near the adult sponge on field. D. Newly settled bud in aquarium (Scale bar: 1cm).

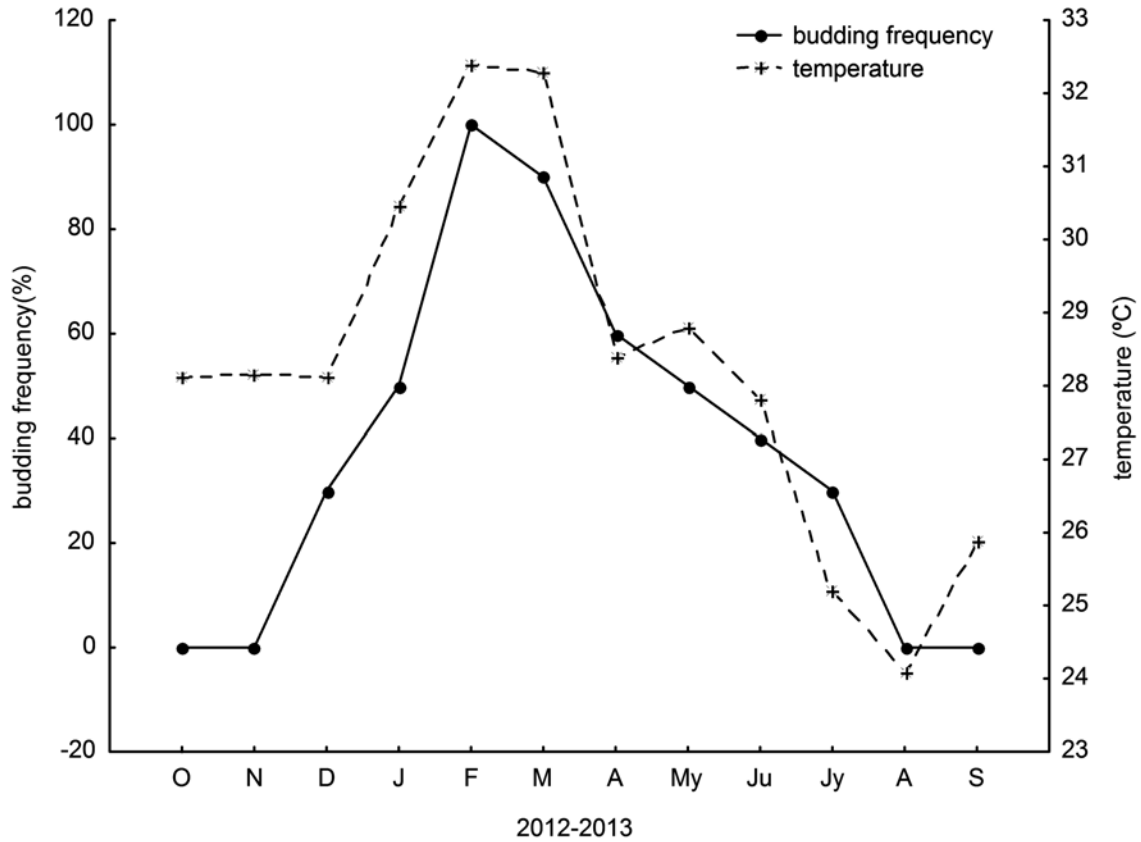


Fig. 2. Occurrence of budding (% frequency) in *C. cavernosa* with monthly variation in temperature. Peak in budding was observed in February.

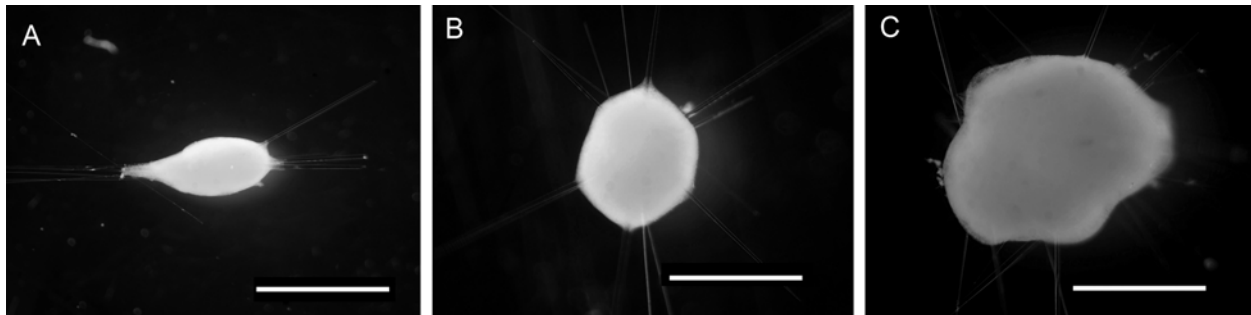


Fig. 3. Photographs of buds of *Cinachyrella cavernosa*. A. Stage I bud (<0.5 mm in diameter), elongated bud with megascleres positioned longitudinally. B. Stage II bud (0.5-1 mm in diameter), somewhat globular with few megascleres intersecting in the middle. C. Stage III bud (>1mm in diameter), comparatively large with number of megascleres passing through at various angles (Scale bar: 1 mm).

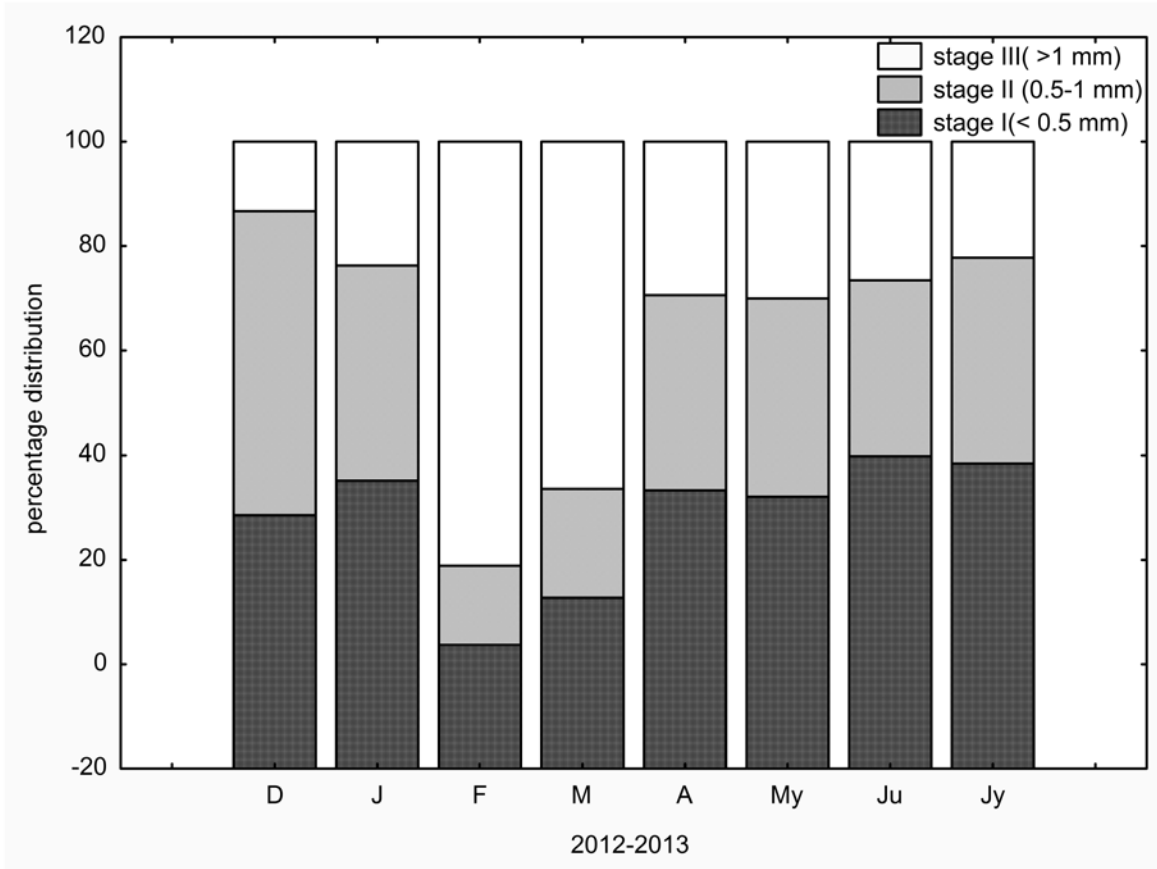


Fig. 4. Relative % of buds of *C.cavernosa* under different size classes. The sponge showed all three stages of buds for entire budding season, demonstrating that at individual level, budding in *C. cavernosa* is asynchronous and rapid.

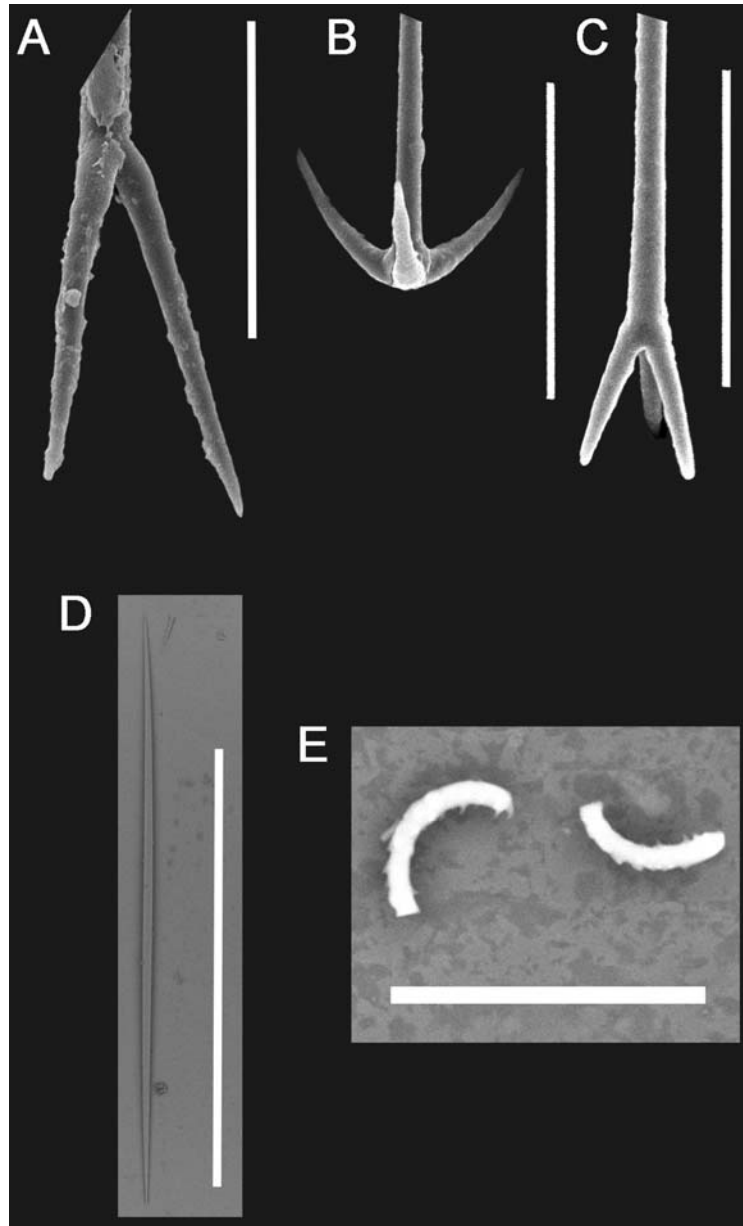


Fig. 5. Skeletal characters of *Cinachyrella cavernosa* A. Prodiaene. B. Anatriaene. C. Protriaene. D. Oxea. E. Sigmaspire. Same spicules were observed in adult sponge and its buds of stages II and III (Scale bar: A - C= 90 μ m; D = 1 mm; E = 15 μ m). F-H. SEM images of the buds of *Cinachyrella cavernosa* (magnification X500) .F. Stage I bud showed complete absence of sigmaspire, note the compactness of cells. G. Stage II bud, no choanocyte chamber or canal formation is apparent. H. Surface of stage III bud showing large number of sigmaspires (Scale-bar: 100 μ m).

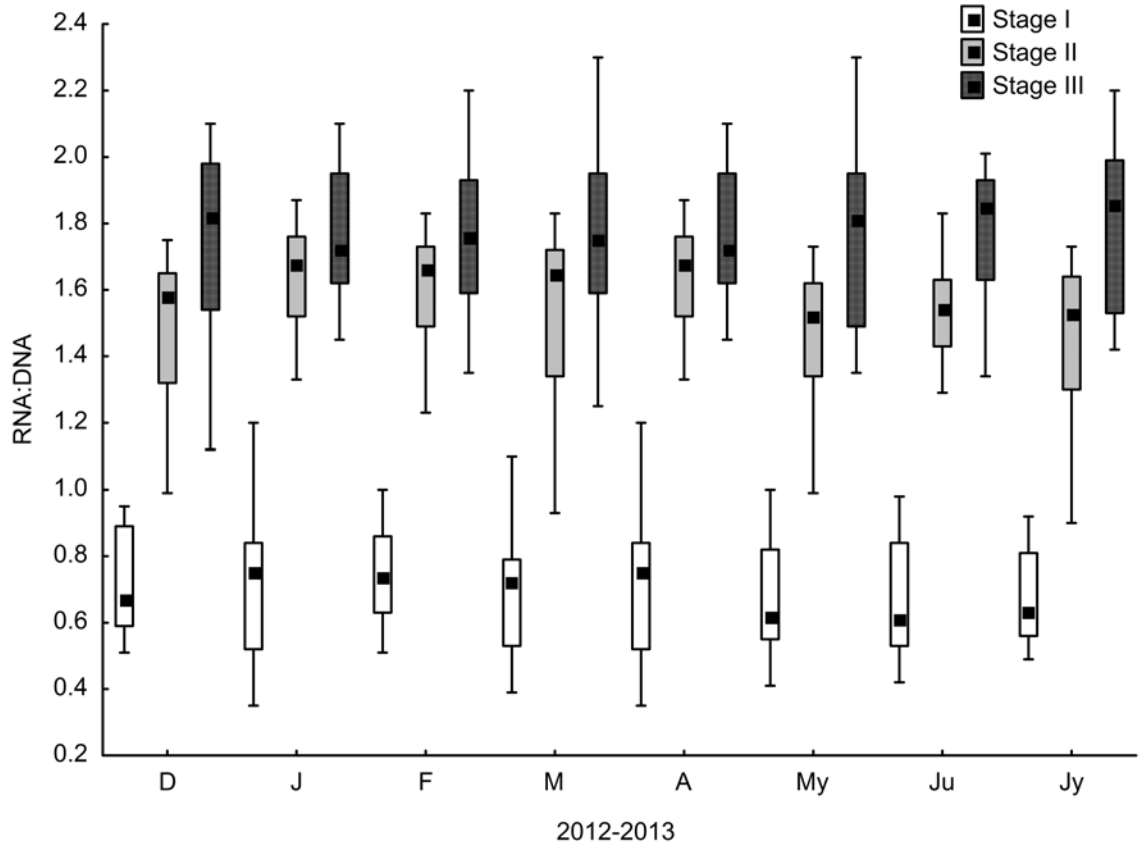


Fig. 6. Box and whisker plot for monthly variation in RNA: DNA ratio at three different stages of bud. Stages II and III showed significantly higher RNA: DNA ratio as compared to stage I.

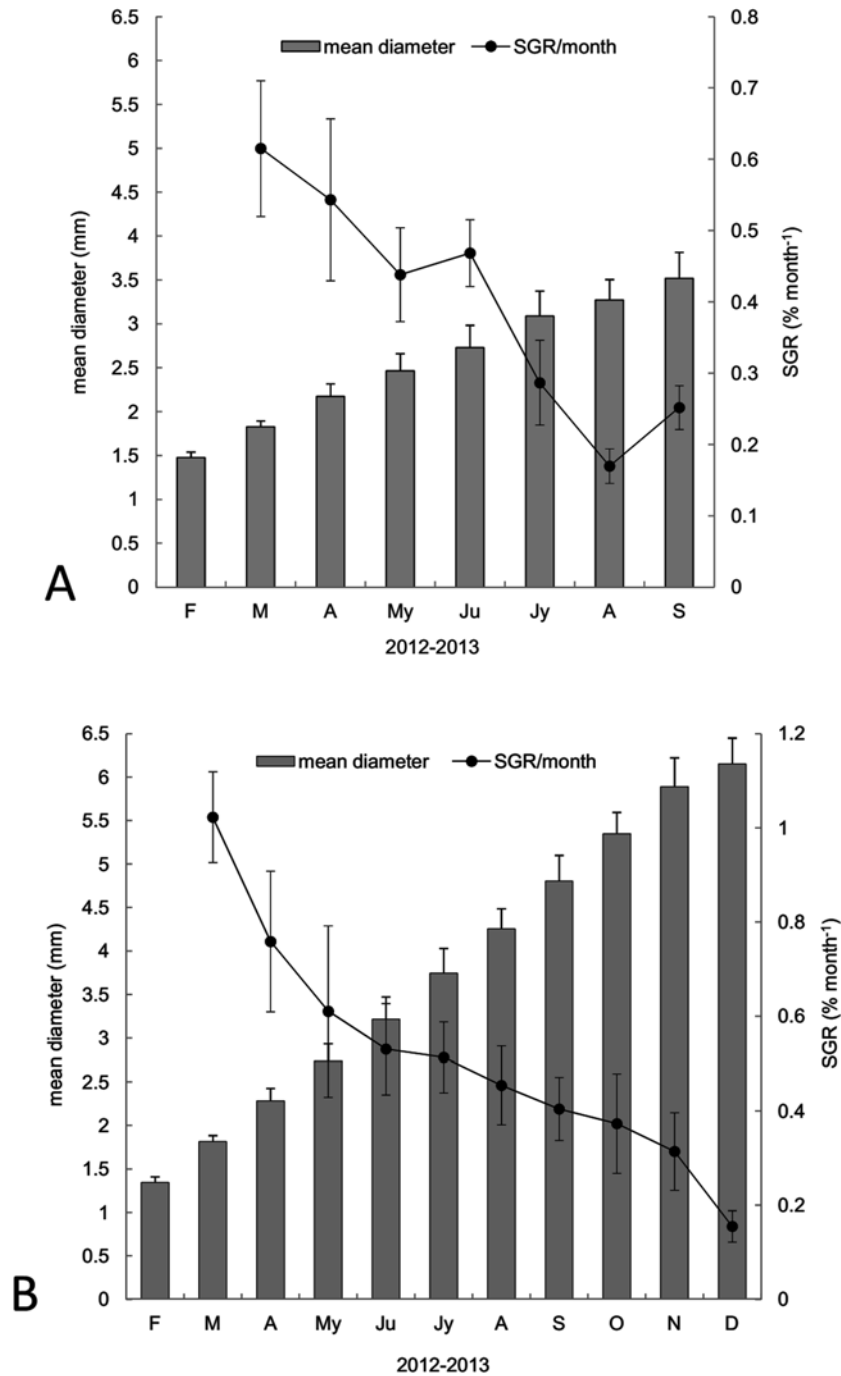


Fig. 7. Comparison of growth performance of ten settled buds under **A.** field and **B.** laboratory condition. Monthly increase in mean diameter \pm Standard error (SE) and specific growth rate (SGR) \pm Standard error (SE) was higher for buds reared under laboratory conditions.

Table 1. Pearson correlation of individual environmental variables with budding frequency.

Variables	Correlation	P
pH	0.472	0.122
Temp.	0.810	0.001
Salinity	0.457	0.135
DO	-0.299	0.345
SiO ⁴	-0.111	0.731
PO ₄ ³	0.062	0.848
NO ₂ ⁻	0.372	0.234
NH ₃	0.659	0.020
NO ₃	-0.294	0.353

Table 2. Estimated coefficients, standard errors, and P-values of multiple regression models of “budding frequency” as predicted by environmental variables using a forward stepwise selection procedure (Model A, 2 environmental variables, $R^2 = 0.757$, $P < 0.001$) and a backward stepwise elimination procedure (Model B, 6 environmental variables, $R^2 = 0.970$, $P < 0.005$)

Model A	Estimate	Standard Error	P
Intercept	-246.83	58.65	0.002
Temperature	8.93	2.19	0.002
NH ₃	7.56	2.94	0.030

Model B	Estimate	Standard Error	P
Intercept	-251.71	80.03	0.051
pH	-66.88	13.17	0.015
Temp.	23.44	2.74	0.003
DO	27.77	8.64	0.049
SiO ₄ ⁴⁻	-1.69	0.71	0.097
PO ₄ ³⁻	46.47	10.69	0.022
NO ₂ ⁻	-138.13	37.32	0.034
NH ₃	9.30	2.47	0.033
NO ₃	3.18	2.31	0.261

Table 3. Analysis of variance of RNA/DNA ratios among developmental stages of buds in different months of the year

Effect	<i>df</i>	SS	MS	F _s	P	Tukey's HSD post hoc test
Intercept	1	212.92	212.92	2606.77	0.000	
Months	7	0.19	0.03	0.34	0.932	
Stages	2	24.49	12.25	149.95	0.000	Stage III>Stage II >Stage I
Months × Stages	14	0.15	0.01	0.13	0.999	
Error	96	7.84	0.08			

degree of freedom; s-sum of the squares; ms-mean of squares; F_s-Fischer constant