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Infestation biology of *Phallusia nigra* (Tunicata, Phlebobranchia) on hard corals in a subtropical bay

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ABSTRACT: Ascidians can settle and grow on corals and have become one of the main threats to hard corals in some areas of world, covering and smothering live coral tissues. We studied the biology of infestation of a solitary tunicate, *Phallusia nigra* Savigny, 1816, on hard corals in Chabahar Bay, a subtropical bay in the northern Gulf of Oman. Infested corals were bleached and eroded at epibiont attachment surfaces. The magnitude of infestation by the tunicate differed between *Pocillopora* and *Acropora* coral colonies, with more tunicates inhabiting *Pocillopora* colonies. This preference of *P. nigra* for *Pocillopora* may be attributed to differences in morphology (e.g. narrower branch spaces in *Pocillopora*) of the coral genera. Outbreaks of ascidians on coral reefs are usually associated with anthropogenic activities. *P. nigra* may have more localized damaging effects on hosts, in contrast to colonial ascidians that can kill entire coral colonies. These effects may be exaggerated by non-contact effects (e.g. decreased pH).

KEY WORDS: *Phallusia nigra* · Hard coral · Bleaching · Skeletal damage · pH

1. INTRODUCTION

Many sessile organisms in shallow marine habitats compete for light and hard surfaces on which to attach (Knowlton & Jackson 2001, McCook et al. 2001, Chadwick & Morrow 2011). Parts (or entire bodies) of hard-bottom sessile animals may thus be covered by fouling organisms. These epibionts may benefit from the availability of new spaces for attachment, growth, shelter, improved nutrition, water flow and light availability (Wahl 1989), but the host animal usually receives few benefits (e.g. camouflage, protection, facilitated energy or matter flow). Fouling may have several drawbacks for the host, including decreased elasticity, physical damage, increased susceptibility to predation, increased mechanical or chemical damage to the body or impaired nutrition, excretion or reproduction (Wahl 2009).

Corals may be fouled by a diverse array of organisms, including microbes (Koh 1997), algae (Coll et al. 1987), sponges (Beuck et al. 2007), polychaetes (Samimi Namin et al. 2010) and ascidians (Littler & Littler 1995, Li et al. 2016). An overgrowth of epibionts can cause skeletal damage/abnormalities, disease, bleaching, smothering and eventually the death of infested colonies (Littler & Littler 1995, Jompa & McCook 2003, Beuck et al. 2007, Li et al. 2016). However, corals have developed defenses against fouling. Non-scleractinian corals readily rely on chemical defensive strategies to cope with biofouling (Slattery et al. 1995). Chemical antifouling by scleractinian corals is mainly by antimicrobial defenses and may be inefficient for deterring macroinvertebrates (Bruno & Witman 1996).

Previous studies on infestations of scleractinian corals by tunicates have mainly emphasized colonial ascidians. For example, Littler & Littler (1995) found *Diplosoma simile* overgrowing corals of the Great Astrolabe Reef in tropical areas of the western Pacific Ocean, where they smothered and killed *Acropora* corals. Vargas-Ángel et al. (2009) later reported this

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animal spreading to American Samoa. Outbreaks of *Diplosoma* are also common on *Acropora* corals of the South China Sea (Li et al. 2016), and *Trididemnum solidum* has overgrown hard corals in the Caribbean Sea (Rodrıguez-Martınez et al. 2012). Coral mortalities are usually high when infested by tunicates (Li et al. 2016).

Solitary tunicates may also form dense aggregations in coastal environments, due to their high fecundity and low dispersal rates (Aldred & Clare 2014). *Phallusia nigra* Savigny, 1816 is a solitary tunicate that generally inhabits artificial substrata (Goodbody 1962, Dobretsov 2015), but it can also inhabit live, dying or dead corals (Goodbody 1962). *P. nigra* was originally thought to be distributed throughout tropical/subtropical areas of the Atlantic and Indo-Pacific Oceans, but a recent molecular study confirmed that it was native to the western Atlantic Ocean and the Red Sea (Vandepas et al. 2015). The animal has recently invaded other seas, probably via fouling of vessels (e.g. Çinar et al. 2006, Kondilatos et al. 2010). *P. nigra* can produce and store acids in its body (Hirose et al. 2001); so-called tunic acid is a fraction of sulfuric and hydrochloric acid that is stored in tunicate bladder cells. The pH of tunicates may decrease to < 2 when injured or stimulated (Hirose et al. 2001), but whether the production of acid by tunicates can drastically affect environmental pH or how acid production may affect interactions with benthic organisms (e.g. corals) is not clear.

P. nigra has recently been reported from Chabahar Bay, a relatively sheltered embayment in the northern Gulf of Oman (Khaleghi 2016). Relatively dense patches of *P. nigra* were observed on hard corals in the bay during January and February 2018. The animals mostly occupied live and dying hard corals. The present study was designed to investigate the biology of infestation of *P. nigra* on hard corals in Chabahar Bay. The main aims were to assess the effects of infestation on the host corals, to determine the levels of variation in infestation density/rate between 2 common coral genera and to identify possible causes of the differences.

2. MATERIALS AND METHODS

2.1. Study area

Coral reefs in Chabahar Bay include patches of both natural and transplanted colonies of hard corals, mainly on the eastern side of the bay at depths of ca. 5 m (25° 19' 9.2" N, 60° 37' 12.7" E, see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m626p135_supp.pdf). A previous study indicated that the bay contained representatives of 9 families of hard corals (Aminrad & Azini 2013). That study reported a mean percent coverage (PC) of ~28% for live hard corals in this area. An assessment of PCs for various hard-coral genera using a point-intercept transect indicated that *Pocillopora* (PC ≈ 45% of the total live coral cover) and *Acropora* (PC ≈ 34% of the total live coral cover) were the most abundant corals in the study area.

2.2. Data collection and experimental procedure

The field study included a photographic assessment of the magnitude of infestation, a colorimetric assessment of bleaching in infested corals, an assessment of bleaching recovery in infested corals and a 'simulated tunicate activity' experiment. Samples of infested and uninfested corals were collected and transferred to the laboratory for further analyses of infestation biology, including morphological and structural analyses, analyses of symbiont type and analyses of mineralogy (see Text S1 in the Supplement for details of the mineralogy study).

Approximately 60 coral colonies (n = 30 *Pocillopora* and 30 *Acropora*) corals were randomly selected while SCUBA diving along a 100 m transect to assess the magnitude of infestation. To date, 2 species of *Pocillopora* and 3 species of *Acropora* have been recorded from Chabahar Bay (Aminrad & Azini 2013). In the field, corals were identified only to genus level due to phenotypic plasticity in the morphology of *Acropora* and *Pocillopora* species (Richards et al. 2016, Johnston et al. 2017).

Variable fin kicks (2, 3 or 4, using a table of random numbers; distance traveled per kick ≈ 1 m) along the length of the transect were applied (Kuffner et al. 2006), and the closest *Pocillopora* or *Acropora* colony at each sampling point (perpendicular to the transect) was photographed at horizontal, vertical and oblique angles using a Hero4 digital underwater camera (GoPro). A scuba-diving weight was used as a scale for the digital photography. Photographs were analyzed using CPCe software (Kohler & Gill 2006), and counts of attached tunicates and the height, planar surface area, percentage degraded area (i.e. areas without visible corallite structures; Kramer & Lang 2003), terminal-branch density and interbranch distance (n = 4, measured at branch tips of the most central branches) of each coral colony were recorded (Gomez 2004, Lirman et al. 2014, Pereira & Munday 2016).

A coral-health chart was used to experimentally examine the effects of infestation on coral bleaching. The chart shows 24 color scores assigned to 4 categories; within each category, a color score of 1 represents severely bleached coral (Siebeck et al. 2006). A total of 6 infested and 6 uninfested *Acropora* colonies were randomly selected according to the above mentioned method, and infested and uninfested branches of the infested colonies and branches of the uninfested colonies were color matched. Animals attached to infested branches were carefully removed using a surgical blade and color matched 5 min after removal. Infested branches were then tagged and monitored for 30 d for signs of recovery (based on color changes).

Three fresh and untreated branches of infested *Acropora* (mean ± SD linear lengths of 14.7 ± 2.47 cm were scanned by computer tomography (CT) to determine the type of epibiont attachment. Sample branches were chosen from different colonies, and each branch had a single attached tunicate. The branches were scanned at 120 kV and 60 mA with an exposure time of 0.75 s. *Acropora* corals were also examined for skeletal damage. Two pairs of infested and uninfested branches, each from a different colony, were collected and transferred to the laboratory where they were retained for 48 h in 3% NaOCl to remove organic matter and then sonicated in deionized distilled water for 30 min at room temperature. The samples were first assessed for skeletal damage at attachment sites using a binocular stereo microscope at 7× magnification. They were then coated with gold and imaged by scanning electron microscopy (SEM) (FEI Quanta-200) at 2 kV for further microstructural observation.

To identify variation in dominant endosymbiotic microalgal symbionts between infested and uninfested *Acropora* corals, we sampled 5 pairs of unbleached and bleached branches, each from a randomly selected colony, and immediately placed them in 96% ethanol for transfer to the laboratory. A 5 cm segment of each branch (beginning 1 cm below the branch tip) was excised (Oladi et al. 2017), the tissue was removed by airbrushing, and genomic DNA was extracted from the homogenate of a CTAB protocol (Baker 1999). The complete ITS2 region (~320 bp) was amplified by PCR using the conditions and primer sets described by Hume et al. (2013). Three successfully amplified samples from each colony type were then directly Sanger sequenced by Macrogen, Inc. (South Korea). Sequences were assembled and edited using Geneious Pro 4.8.3 (Kearse et al. 2012) and were queried against the NCBI database using BLASTN to identify the most similar member of Symbiodiniaceae. To verify the BLASTN analysis, we grouped the sequences with a data set obtained from GenBank (Clark et al. 2016), aligned them with ClustalW implemented in MEGA6 (Tamura et al. 2013) and trimmed them to equal lengths. A maximum likelihood (ML) phylogram was constructed using RAxML v.7.2 (Stamatakis 2006) and the GTR + G model with 1000 pseudoreplicates.

A 'stimulated activity' experiment was applied to determine if the secretion of acid by *Phallusia nigra* could substantially affect environmental pH. For the this experiment, infested *Acropora* colonies (n = 8) were randomly selected in the field, and a marginally attached tunicate was subjected to a simulated injury by inserting a 0.8 × 40 mm hypodermic needle (simulating a fish bite), taking care not to insert the needle into the body cavity of the tunicate. Surrounding water (<1 cm and 10 cm from the tunicate) was collected in both inshore and offshore directions after 3 min using 10 ml plastic syringes. The water samples were immediately transferred to a boat where pH was measured using a portable pH meter (Xylem). The selected corals were ~2 m away from each other.

2.3. Data analysis

Differences in body shape parameters between coral genera as well as the pH variations in the stimulated activity experiment were assessed using independent 1-way ANOVAs. Data were assessed for assumptions of normality and homoscedasticity using Kolmogorov-Smirnov and Levene's tests, respectively. The pH data in the stimulated activity experiment were Box-Cox transformed to improve normality. Intensity (number of tunicates per colony) and density (number of tunicates per m^2) data were analyzed using generalized linear models (GLMs) with log-linear models assuming an over-dispersed Poisson distribution (Breslow 1984). Coral genus was treated as a fixed effect, and colony height, percentage degraded area, branch density and interbranch distance were treated as covariates. The data were checked for normality of deviance residuals and homogeneity of variances prior to the analyses (Manly et al. 1993). Pearson correlation coefficients were used to correlate tunicate density with the covariates. A chi-squared test was used to compare prevalence of the tunicates on examined coral genera. All statistical analyses were performed using SPSS v. 22.

3. RESULTS

3.1. Prevalence, mean intensity and density of tunicates

Phallusia nigra occupied branch tips and/or interbranch areas of both live and dead portions of both coral genera (Fig. 1), although it infested *Pocillopora* twice as frequently as *Acropora* (Fig. 2). The mean intensity and density of *P. nigra* were higher on *Pocillopora* than on *Acropora* (Fig. 2).

The mean density of terminal branches, interbranch distances and proportion of dead areas differed between the coral genera (Fig. 3). The intensity of infestation was moderately correlated with proportion of dead areas ($r = 0.64$, $p = 0.001$), number of terminal branches ($r = 0.46$, $p = 0.03$) and interbranch distances ($r = 0.31$, $p = 0.046$).

3.2. Effects of infestation on host bodies

The CT scans of the infested branches indicated peripheral contact between the tunicates and live coral tissue (Fig. 1). The tunicates and host bodies were separated by interstices, with no signs of tissue intrusion into the host bodies (Fig. 1). The SEM micrographs showed no signs of septal fractures at the attachment surface (Fig. 4). Radial bars and rods were also intact at this site. Infested surfaces, how-

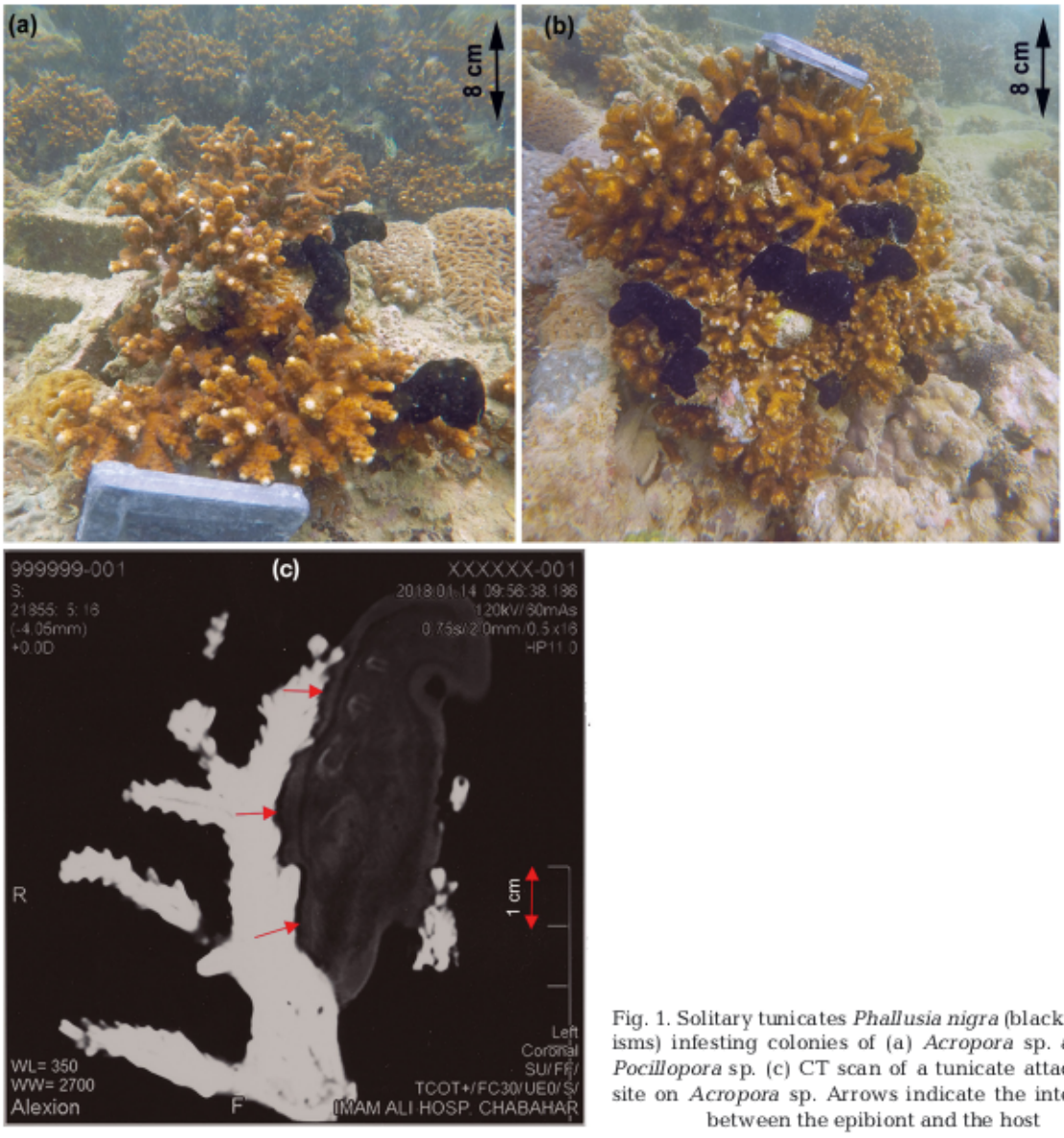

Fig. 1. Solitary tunicates *Phallusia nigra* (black organisms) infesting colonies of (a) *Acropora* sp. and (b) *Pocillopora* sp. (c) CT scan of a tunicate attachment site on *Acropora* sp. Arrows indicate the interstices between the epibiont and the host

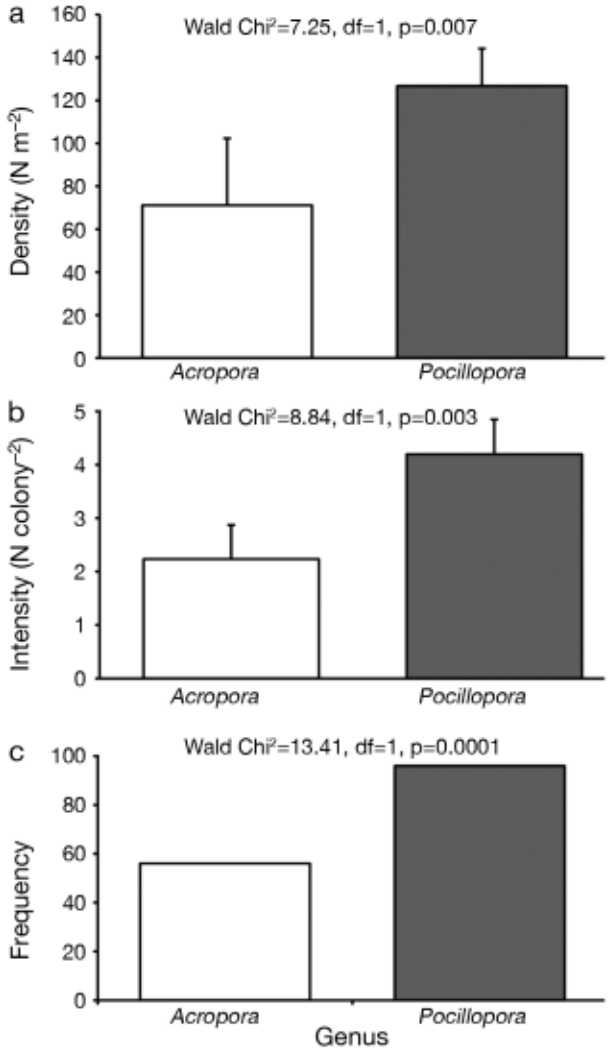

Fig. 2. Differences in mean (a) density, (b) intensity and (c) prevalence (%) of *Phallusia nigra* between *Acropora* and *Pocillopora* corals at Chabahar Bay, Gulf of Oman. Bars are standard error. Sample size = 30 colonies of each genus

ever, displayed erosive effects throughout the skeleton at higher resolutions, with moderately pitted surfaces and many bumps. The inner diameter of the corallites varied from 0.52 to 0.63 mm in healthy *Acropora* samples and from 0.38 to 0.89 mm in infested samples. Mean diameter did not vary between the healthy and infested samples.

Some parts of the corals covered by epibionts were nearly completely bleached (assigned to categories E1, E2, B1 or B2 of the coral-health chart; Siebeck et al. 2006), but uninfested branches of infested colonies and branches of uninfested corals were healthy (assigned to categories E5 or B4). No signs of color recovery were observed on infested branches during a period of 1 mo. Dead coral tissues were eventually occupied by suspended sediments and algae.

The BLASTN analysis indicated that dominant algal symbionts isolated from both infested and uninfested *Acropora* colonies were identical and belonged to the genus *Durusdinium*. The ML phylogram validated this result. The occurrence of a double peak in all chromatograms (base pair no. 439 displayed as Y in KJ019889), demonstrating the presence of D1 and D4 intragenomic variants, indicated that all specimens were *D. trenchii* (formerly D1–4 or D1a). Two sequences from each colony type have been deposited in GenBank (Fig. S2).

pH varied between treatments in the injury (simulated activity) experiment, with higher pH in offshore directions compared to surrounding environments with injuries (Fig. 5).

4. DISCUSSION

Our results suggest that infestations by *Phallusia nigra* may have both direct and indirect effects on hard corals. Coral tissue at the attachment surface became irreversibly bleached within 1 mo. Previous studies have reported different recovery times for bleached corals, including both short (e.g. 3–4 wk, Wesseling et al. 1999) and long (e.g. >5 yr, Smith et al. 2006) periods. Corals infested with *P. nigra*, however, may not recover even after epibiont removal, due to clogging by adhesive metabolites and further covering of sticky infested surfaces by particulate matter. In contrast, tunicates with a holdfast mechanism (e.g. *Botryllus*) may not cause bleaching of the underside of coral tissue (Shenkar et al. 2008).

Resident microalgal symbionts in both infested and uninfested *Acropora* colonies were identified as *Durusdinium trenchii*. Hard corals in monsoonal environments and turbid water such as in Chabahar Bay most likely have symbiotic relationships with *Durusdinium*, particularly *D. trenchii*, due to their tolerance to harsh conditions and dispersal in turbid environments (LaJeunesse et al. 2018). The corals therefore not surprisingly continued their association with *D. trenchii*, because they already harbored most of the tolerant symbionts in this area (Oladi et al. 2019).

P. nigra usually produces acid as a defense (Hirose et al. 2001), and the pH of the basal part of the tunicate body does not exceed 4 during defensive responses (Hirose et al. 2001). The production of acid may thus cause skeletal damage around the underlying tissue. In our study, *P. nigra* was superficially connected to the coral tissue, causing no fractures to the underlying skeleton. Signs of erosion, however, were apparent throughout the attachment surfaces, which

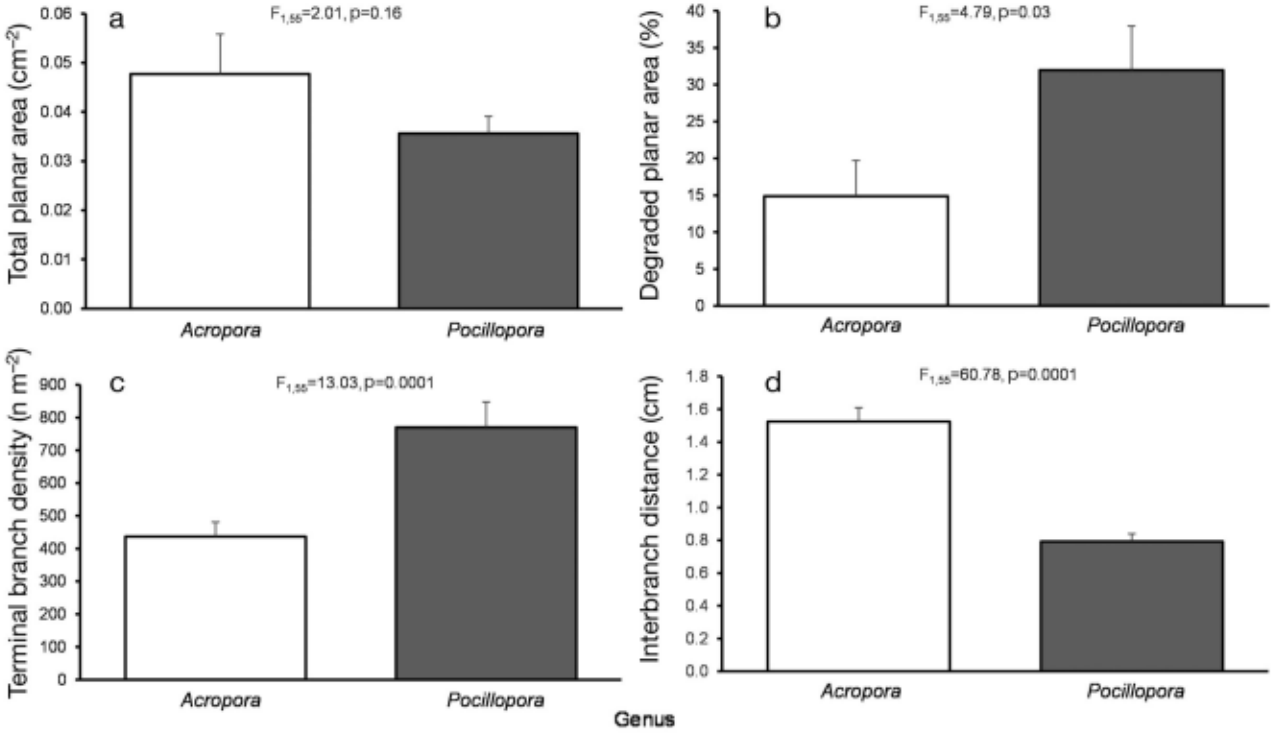

Fig. 3. Comparison of mean (a) total planar area, (b) percentage degraded area, (c) branch density and (d) interbranch distance between *Acropora* and *Pocillopora* corals at Chabahar Bay. Bars are standard error. Sample size = 30 colonies of each genus

were similar to skeletal deformities in corals subjected to decreased pH (Foster et al. 2016). In contrast, boring animals that are unable to produce acid may not erode attachment surfaces (Le Campion-Alsumard et al. 1995).

Infested corals may also encounter lower environmental pHs at sites other than the attachment site, reducing colony growth rates and bleaching larger areas. A superficial injury to epibiont tunicates in our study decreased mean environmental pH by nearly 0.06 at 10 cm from the tunicate. This small decrease in pH may not affect corals, because a decrease of 0.1 is needed to substantially affect calcification rates in corals (Marubini et al. 2008). Similarly, severe bleaching may not occur, because a minimum decrease in pH of 0.7 (e.g. from 8.40 to 7.70) would lead to up to 50% bleaching in corals within 8 wk (Anthony et al. 2008). Yet, dense colonization of corals infested by *P. nigra* on one side (e.g. 17 specimens attached to a single *Pocillopora* colony) and continued predation from the other side may increase secretion of acids by the animals, which would acidify the surrounding water beyond threshold levels and lead to growth anomalies or bleaching throughout the area of the infested colonies. *P. nigra* in our study formed more frequent and denser aggregations on *Pocillopora* than on *Acropora* corals. The ratios of dead area, branch density and inter-branch distance differed between *Pocillopora* and *Acropora*. Areas of dead coral colonies may provide new types of microhabitats for a wider array of settlers (Stella et al. 2010). The abundance of symbiotic assemblages will also decrease in the nonliving parts of corals, leaving free spaces for recolonization by more facultative nonsymbiotic animals such as tunicates (Coles 1980). Also, crevices in areas of dead coral provide darker areas preferred by invertebrate larvae (van Duyl et al. 1981, Hochberg et al. 2003). Tunicate larvae also prefer to settle on the dead parts of corals. Stoner (1994) reported that nearly 70% of *Diplosoma sirnilis* larvae that had time to examine the substrate only once before settling contacted dead corals. Live corals, however, may remove settled larvae during sedimentation by rejection, but stony corals with small polyps (e.g. *Acropora*, *Pocillopora*) usually have lower capability of active cleansing (Stafford-Smith 1993). The larvae that settle on live parts are also less susceptible to predation by the host, due to their defensive secondary metabolites (Lindquist & Hay 1995). *P. nigra* can thus occupy both living and dead coral tissue, but individuals would competitively prefer dead parts. The larger proportion of dead areas in the *Pocillopora* corals in our study may have been due to their higher susceptibility to bleaching (Marshall & Baird 2000).

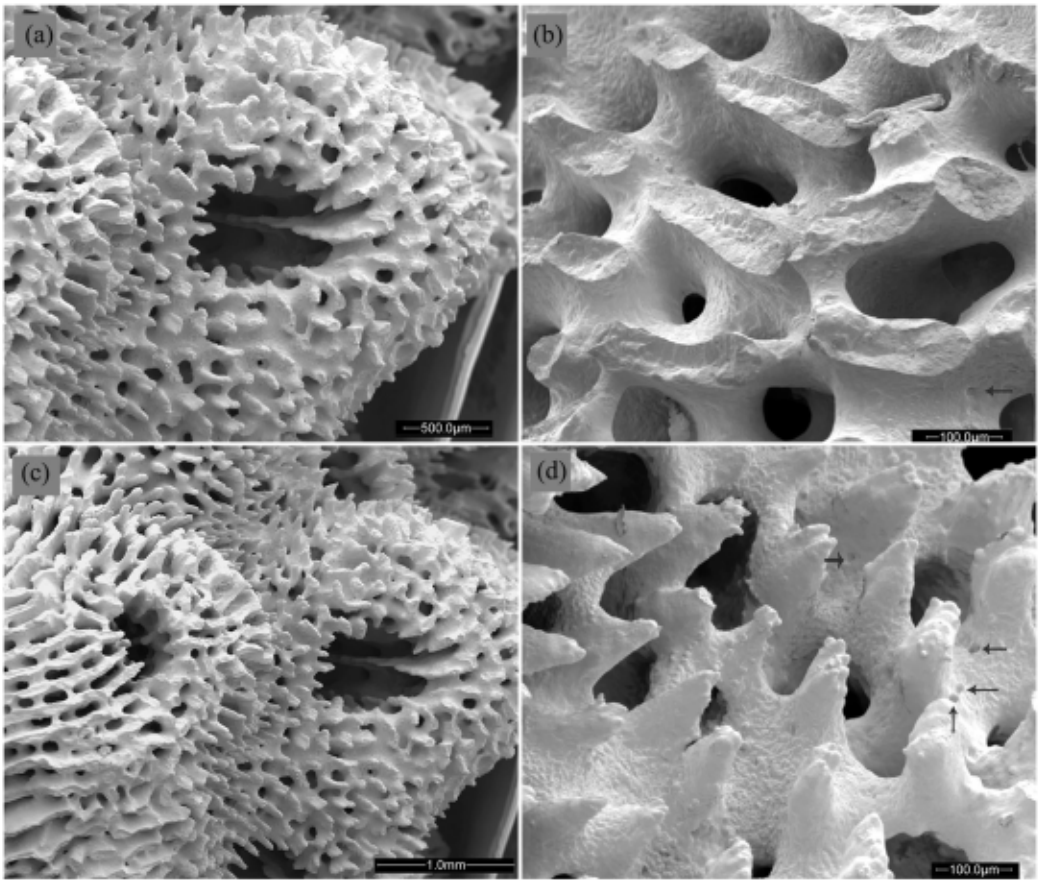

Fig. 4. SEM micrograph of (a,b) healthy and (c,d) tunicate-infested *Acropora*. Note the bumps and pits (indicated by arrows in panel d) on the surface of the infested coral

Pocillopora corals generally have thicker and more branches per unit area than *Acropora* corals and so have fewer inter-branch spaces (Doszpot et al. 2019). Motile fauna will benefit from niche segregation and the protection provided by narrower spaces (Vytopil & Willis 2001). Larvae and juveniles of sessile organisms such as *P. nigra* may also take advantage of obtaining more food from the smaller inter-branch spaces of *Pocillopora* corals (even at high water flows during monsoons) due to the fewer disruptions of benthic boundary layers (Sebens et al. 1997, Vytopil & Willis 2001). The narrower branch spaces of *Pocillopora* corals may not provide antipredatory advantages for adult *P. nigra*, probably because the animals use chemical defenses against predators (Hirose et al. 2001).

P. nigra may prefer elevated substrates (i.e. taller corals) to nearly horizontal planes (e.g. *Acropora* and *Pocillopora* corals in this study) to be able to cope with smothering, leading to intraspecific competition during settlement and growth. Except for differences in the morphological attributes of a host, host heterogeneity may also increase colonization by epibionts,

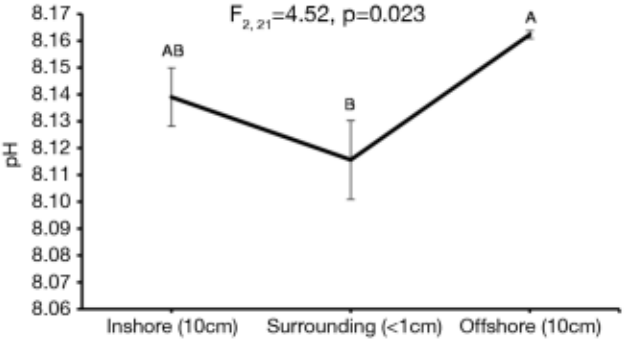

Fig. 5. Changes in environmental pH (mean ± SE; n = 8) brought about by stimulated activity of the tunicate *Phallusia nigra* at Chabahar Bay. Inshore, surrounding and offshore indicate distances and directions from the simulated injury, which was inflicted to determine whether the secretion of acid by *P. nigra* could substantially affect environmental pH. Different letters indicate differences at $p < 0.05$

probably due to the availability of broader spectra of habitat sizes and ecological niches (Kovalenko et al. 2012). The estimated branch densities and ratios of dead areas in our study were more variable (i.e. had

higher standard deviations) for *Pocillopora* than *Acropora* corals, which may also account for the preference of substrate selection of *P. nigra*.

Outbreaks of ascidians on coral reefs are usually associated with anthropogenic activities (Rodriguez-Martinez et al. 2012). *P. nigra* may have more localized damaging effects on hosts, in contrast to colonial ascidians that can kill entire coral colonies. These effects may be exaggerated by non-contact effects (e.g. decreased pH). *P. nigra* primarily relies on reproductive cycles for dispersal, in which it produces larvae that settle from the plankton onto corals (da Rocha et al. 1999), so its substrate preference may be more dynamic (Monniot et al. 1991) and may thus change over time.

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