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PRIMARY RESEARCH PAPER

Can artificial substrates enriched with crustose coralline algae enhance larval settlement and recruitment in the fluted giant clam (*Tridacna squamosa*)?

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Abstract Habitat recognition and selection can greatly increase the early-life survival of sessile reef organisms. This study describes the settlement and recruitment responses of the fluted giant clam, *Tridacna squamosa*, to concrete tablets and tiles containing different concentrations of crustose coralline algae covered coral rubble (CCACR). Crustose coralline algae is known to induce settlement in a variety benthic animals, but it has not been used previously as an aggregate in concrete—potentially a way of encouraging colonization of man-made structures erected on or near coral reefs. After being given the choice of small tablets made with 0%, 30% or 60% CCACR for 4 days, 11 days old larvae preferred the substrate containing the most CCACR. Recruitment responses of juvenile clams to larger tiles made with the same three CCACR concentrations were also tested. These tiles were further divided into rough and smooth surface textures. After 6 weeks, more juvenile clams had recruited to the rough surfaced tiles than the smooth ones, but no significant differences among the CCACR treatments were found. Thus, even though concrete made with CCACR is initially attractive to larvae, it has no effect on recruitment of juvenile *T. squamosa*.

Keywords Artificial substrates · CCA · Giant clam larvae · Recruitment · Settlement · *Tridacna squamosa* · Coral rubble · Singapore

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Introduction

Giant clams (Bivalvia: Tridacnidae), the largest bivalve molluscs in the world, inhabit the shallow coral reefs of the Indo-Pacific region (Rosewater, 1965). They derive nutrition by filter feeding (Klumpp et al., 1992), as well as via a symbiotic relationship with photosynthesizing dinoflagellate algae (zooxanthellae) (Yonge, 1936, 1975). Giant clams provide important ecological roles on coral reefs; for example, their shells act as nurseries to various other reef organisms (Mingoa-Licuanan & Gomez, 2002), however, they have been over-harvested for food and the live aquarium trade (Gomez & Mingoa-Licuanan, 2006). Because of this high demand, prior investigations into the biology of giant clams have focused on their mariculture (e.g. Beckvar, 1981; Heslinga et al., 1984; Copland & Lucas, 1988) and there exists limited

information regarding their early larval behaviour and settlement patterns (but see Jameson, 1976; Gwyther & Munro, 1981; Fitt et al., 1984; Alcala et al., 1986).

The majority of benthic marine invertebrates, including giant clams, display complex life-cycles that comprise of a planktonic larval period followed by bottom-dwelling juvenile and adult stages (Thorson, 1950; Watzin, 1986). Accordingly, there exists a wide range of processes that can affect populations at life cycle points such as settlement and recruitment (Eckman, 1996). These aspects are have been studied extensively in many bivalve species including oysters, *Crassostrea virginica* and *Crassostrea gigas*, dwarf surf clams, *Mulinia lateralis*, and mussels, *Mytilus galloprovincialis* (Luckenbach, 1984; Fitt & Coon, 1992), but remain poorly understood in giant clams. There is a distinction between settlement and recruitment, and it is important to differentiate them as separately occurring events. For benthic marine invertebrates with pelagic larvae, settlement is described as the inception of behavioural searching for a suitable substrate, attachment to the substrate and, finally, metamorphosis to the later life stages (Keough & Downes, 1982). There are several chemical, physical or biological factors that can exert positive settlement responses on marine invertebrate larvae. For example, epinephrine acts as a neurotransmitter and has been identified as an active inducer of settlement and metamorphosis for several bivalve molluscs (García-Lavandeira et al., 2005). Also, corals and many other sessile marine invertebrates (e.g. solitary ascidian, *Styela plicata* and barnacle, *Balanus* sp.) are known to settle preferentially on topologically complex surfaces (Harriott & Fisk, 1987). Recruitment, on the other hand, describes the newly settled individuals (recruits) that have survived a period of time to a specified size after settlement (Luckenbach, 1984; Roegner, 1991). These processes are fundamentally important at individual, population and community levels, as well as for mariculture and conservation (Rodŕiguez et al., 1993).

The pediveligers of giant clams, which develop after 7–10 days, are capable of some locomotion on bottom substrates (Yamaguchi, 1977). The emergence of an active foot indicates readiness for settlement and locomotion is achieved by attaching the tip of the foot to the substrate, which is then retracted to effect crawling (LaBarbera, 1975). The foot is also used for orientating the larvae into a vertical position (Jameson, 1976). Even though tridacnid clams demonstrate some searching behaviour (Alcazar & Solis, 1986; Courtois de Viscose, 2000) no empirical studies have examined the underlying cues that trigger substrate choice and settlement. As giant clams are found on coral reefs, it is possible that their larvae could respond to the same cues used by other reef marine invertebrates. Crustose coralline algae (CCA) is known to induce high rates of settlement and metamorphosis in a large diversity of marine invertebrate larvae, including agariciid corals, *Agaricia* sp. (Morse et al., 1988), starfish, *Acanthaster planci* (Johnson & Sutton, 1994), scleractinian corals, *Acropora* sp. and *Favia* sp., (Morse et al., 1996), and abalone, *Haliotis iris* (Roberts et al., 2004). The origins of these settlement cues could be from the algae itself or from CCA-associated bacteria (Morse & Morse, 1984; Johnson et al., 1991). Even though giant clam larvae are cultivated in large numbers in hatcheries, their substrate preferences remain relatively unknown.

To date, research on CCA-induced settlement has used living CCA or extracts of CCA. Here, for the first time, we incorporate ground CCACR into concrete to determine whether this will enhance *T. squamosa* settlement and recruitment. We hypothesize that concrete tiles containing CCACR will be more attractive to early stage (11 day old) clams, and also host more later stage (49 day old) clams, than tiles with no CCACR. As large concrete structures continue to be erected on or near coral reefs, encouraging colonization by using ground CCACR as an aggregate may mitigate against some of the negative effects often associated with such building projects. Furthermore, more knowledge of *T. squamosa* settlement and recruitment ecology can only benefit clam restocking and restoration efforts.

Materials and methods

General experimental procedures

All experiments were conducted at the marine aquaculture facility at the Tropical Marine Science Institute (TMSI) on St. John's Island, Singapore, from January 2007 to September 2007. There were

four stages to this study: spawning, rearing, settlement of larvae and recruitment of juveniles. Mature *T. squamosa* brood stock was first retrieved from a local reef (Raffles Lighthouse) located 13.7 km southwest of St. John's Island. The giant clams were induced to spawn in cubic tanks (length = 1.0 m, width = 1.0 m, height = 0.7 m) by injecting 2 ml of 20 μM concentration serotonin solution (crystalline 5-hydroxytryptamine, creatine sulfate complex, Sigma-Aldrich Pte Ltd, Product No. H7752-1G) into the gonads through the mantle tissue at the excurrent siphon region (Braley, 1985). After approximately 30 min, the clams spawned and the resultant sperm-water was collected in a 10 l bucket at a density of ~10,000 sperm ml^{-1} (determined with a Neubauer haemocytometer). The unfertilized eggs were then collected on a 22 μm plankton screen and washed with 1 μm-filtered and UV-treated seawater before being stored at a density of ~22 eggs ml^{-1} (counted on a Bogorov tray).

The eggs were fertilized with an egg-sperm ratio of 1:50 as this ratio greatly reduced the risk of polyspermy and self-fertilization (Neo et al., 2009). The developing larvae were placed in six 20 l funnel tanks (diameter = 0.5 m, depth = 1.1 m) containing lightly aerated 30 PSU, ~30°C, 1 μm-filtered and UV-treated seawater. From the veliger stage (days 4 and 6), the larvae were fed with a mixed-algal diet, *Tetraselmis suecica* + *Chaetoceros mulleri* + yeast, at a total concentration of ~10,000 cells ml^{-1}. At day five, zooxanthellae cells were introduced to the larvae. The zooxanthellae were acquired from a small piece of adult mantle tissue (400 mm^2), which was homogenized to release the cells and rinsed through a 25 μm plankton screen.

Artificial substrates

Tiles were made of washed and inert quartz sand (silicon dioxide), cement and fresh coral rubble (CR) that was encrusted with at least 50% CCA, collected from reef slopes around Singapore. The rubble was cleaned of any excess sediments and sessile organisms before being oven-dried for 24 h. The dried rubble was ground into ~1 mm grains in an industrial crusher. This aggregate we termed CCACR. The proportions of the component materials for each tile type were as follows: 0% CCACR = 4 parts sand + 1 part cement; 30% CCACR = 1.5 parts CCACR + 2.5 parts sand + 1 part cement; and 60% CCACR = 3 parts CCACR +1 part sand + 1 part cement. The mixtures were hydrated with tap water and shallow plastic trays were used as moulds. The smaller tablets used in the choice experiment were cut out of tiles made for the recruitment experiment, thus the substrates in both experiments were identical in composition.

Choice experiment

After the tiles were fully cured, six (two of each CCACR concentration) were selected haphazardly and cut into tablets (length = 15 mm, width = 10 mm, height = 5 mm), using a stone saw. All surfaces of these tablets were equally smooth. Each of 30 standard plastic Petri dishes was divided into three equal sectors by drawing on their undersides. Three tablets (one of each type: 0%, 30% and 60% CCACR) were distributed among these three sectors while ensuring every tablet touched the inside edge of the dish. The dishes were haphazardly pivoted around their centre before being laid out in a 6 × 5 Latin square in a culture facility (12:12 h LD photoperiod). All larvae used were 7 days old and came from the same stock (density 5.5 larvae ml^{-1}). To the centre of each Petri dish, 50 ml containing ~275 larvae was added. The Petri dishes were then covered to prevent evaporation, and the experiment was left undisturbed for 4 days. At termination, a three-way divider was inserted to prevent any movement of larvae among sectors while the Petri dish was transferred to a stereomicroscope. The number of live larvae in the 0% CCACR sector of 10 randomly selected Petri dishes was counted. Another 10 dishes were selected randomly for the 30% sectors and another 10 for the 60% sectors. Thus, all data were independent.

Recruitment experiment

Twenty-four tiles (length = 100 mm, width = 100 mm, height = 10 mm) of each of the three CCACR concentrations were used. When these were removed from the moulds, one large surface, the exposed side was rough whereas the opposite side, which had been in contact with the plastic mould, was glassy smooth. To quantify surface roughness, we cut five tiles of each type in half and traced the cross-sectional

profile of the rough and smooth surfaces using SigmaScan/Image (Jandel Scientific) measurement software. The mean ratio of smooth to rough distance traced was 1:1.08 (i.e. 100:108 mm). We used these surface textures as an additional factor in the experiment; thus, there were 12 rough-side-up and 12 smooth-side-up tiles of each CCACR concentration. These were positioned on the bottom of a large fibreglass tank (length = 243 cm, width = 122 cm, height = 77 cm) in a Latin square design. Approximately 10,000 7-day-old larvae were thoroughly mixed with 2,500 l of 30 PSU, 1 μm-filtered seawater, which was gently aerated throughout the experiment. After 7 weeks, all tiles were removed and examined under a stereomicroscope for juvenile clams.

Data analyses

A one-factor ANOVA and post hoc Tukey HSD tests were performed to determine differences in the mean number of live larvae found in each of the three Petri dish sectors. All data fulfilled assumptions of normality and homogeneity of variances. A two-factor ANOVA and post hoc Tukey HSD tests were performed to determine differences in the number of juvenile clams found on the tiles in the large tank; with surface texture as one factor and CCACR treatments as the other. For this test, data were log-transformed to fulfil assumptions of normality and homogeneity of variances. All statistical analyses were performed on Statistica (version 5.5).

Results

Choice experiment

The late D-veligers and pediveligers swam or crawled around the Petri dishes but, by the end of the experiment, no swimming larvae were observed. Pediveligers tended to lie on their sides when in close proximity to the tablets and significantly more larvae were found in the sectors with 60% CCACR tablets (Tukey HSD, $P < 0.01$; Table 1) than in sectors with 0% CCACR tablets. The mean number of larvae found in sectors with 30% CCACR tablets was not significantly different from sectors with 60% CCACR or 0% CCACR tablets (Fig. 1).

Table 1 One-way analysis of variance comparing mean number of live larvae found in the three Petri dish sectors (each sector containing a tablet with a different concentration of CCACR)

Source of variation	*d.f.*	MS	*F*-ratio	*P*-value
Treatment	2	172.2333	7.2729	0.0030
Residual	27	23.6815		
Total	29			

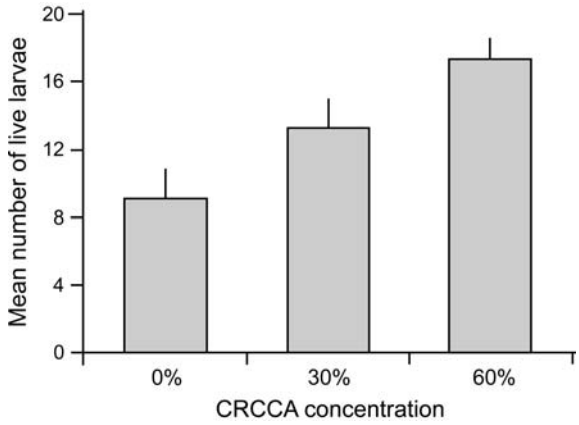

Fig. 1 The mean number of live larvae found in the three Petri dish sectors (each sector containing a tablet with a different concentration of CCACR). Error bars indicate standard error

Recruitment experiment

After 42 days (the termination of the experiment), many juvenile clams were visible to the naked eye, with sizes ranging from ~1 to 3 mm shell length. The juvenile clams were firmly attached to the surfaces of the tiles with byssal threads. ANOVA results indicated no differences in the mean number of clams among the three concentrations of ground CCACR. Significantly more clams, however, were found on rough surfaces than on smooth surfaces (Tukey HSD, $P < 0.05$; Table 2, Fig. 2).

Table 2 Two-way analysis of variance comparing mean number of juvenile clams found on the six different tile types (two surfaces × three CCACR concentrations)

Source of variation	*d.f.*	MS	*F*-ratio	*P*-value
Surface	1	0.2898	5.3502	0.0239
Concentration	2	0.0522	0.9643	0.3866
Surface × Concentration	2	0.0077	0.1429	0.8671

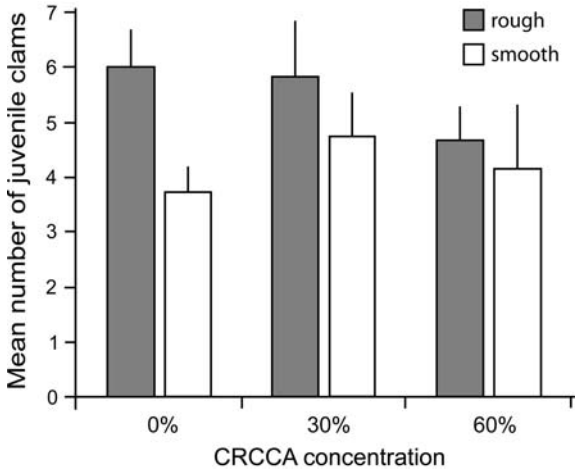

Fig. 2 The mean number of live juvenile clams found on the six different tile types (two surfaces × three CCACR concentrations). Error bars indicate standard error

Discussion

Choice experiment

The selection of settlement site is critical for the subsequent survival of benthic marine invertebrates as their choice will largely determine the environmental conditions experienced by later life stages (Keough & Downes, 1982; Rodŕiguez et al., 1993). When presented with concrete tablets enriched with three different percentages of CCACR, more *T. squamosa* larvae were attracted to the tablets containing the highest concentration. Many studies have shown that a wide diversity of marine invertebrate larvae have a strong settlement response to live CCA, which also rapidly induces metamorphosis (Kaspar, 1992; Webster et al., 2004). For giant clam larvae, we know only that *Hippopus hippopus* (Alcala et al., 1986) and *T. maxima* (Alcazar & Solis, 1986) larvae prefer coral fragments and rubble compared to stones or pebbles, and this substrate preference may be related to the presence of CCA. However, unlike other marine invertebrates such as the starfish, *Acanthaster planci* (Johnson & Sutton, 1994) and abalone, *Haliotis rufescens* (Morse & Morse, 1984), the *T. squamosa* larvae observed in the present study were not induced to settle by direct contact with CCA surfaces.

Larvae responded to ground CCACR that had been oven-dried and used as an aggregate in concrete, as opposed to live CCA as used in previous studies on other species (e.g. Morse & Morse, 1984; Harrington et al., 2004). These results suggest that whatever the larvae were attracted to was water-soluble and capable of being emitted from a concrete matrix. This is not wholly unexpected as, for instance, water-soluble cues from prey can induce activity responses that affect the subsequent settlement patterns of larvae (Krug & Manzi, 1999; Hadfield & Koehl, 2004) and chemicals leaching from concrete is a well-studied phenomenon (e.g. Jo et al., 2007). Clam larvae seemed to use their feet to sense their surroundings before propelling themselves towards the direction of their chosen substrate. Similar behaviour has been documented by Jameson (1976) where giant clam pediveligers crawled until a settling spot that provided maximum protection was found. The ciliated structures on the foot (LaBarbera, 1975) could possibly be the sensory mechanism which aids the detection of CCA-associated signals.

Recruitment experiment

The results of the choice experiment indicated that high concentrations of ground CCACR attracted 11-day-old *T. squamosa* larvae (after 4 days of exposure to the tablets). The recruitment study, however, showed that CCACR had no effect on the number of 49-day-old juvenile clams found on the tiles (after 42 days of exposure to the tiles). There are a number of potential explanations for this result.

1. Whatever was attracting the larvae in the choice experiment was missing in the recruitment experiment; possibly because the CCACR was no longer emitting chemical morphogens and/or there was a time-associated reduction in leaching from the concrete tiles. Although no studies have examined temporal patterns of morphogen release from dead CCA, it is known that leaching decreases with time for other chemicals (e.g. Zbigniew & Król, 2008) and thus a similar reduction in CCA emissions is expected.
2. The water-soluble cues emitted could have been degraded by biofilm on the tiles. Biofilm bacteria easily colonize coralline surfaces (Lewis et al., 1985; Johnson et al., 1991) and they may affect the larval settlement of *T. squamosa* larvae by producing and/or degrading settlement-inducing cues (Kaspar et al., 1989; Johnson et al., 1991).

3. The function of CCACR might be concentration-dependent, i.e. the diffusate was diluted by the large volume of water and hence failed to present a directional cue to the swimming larvae. Even in still water, morphogen concentration will decrease rapidly with increasing distance from the source and it is generally thought that invertebrate larvae must be very near the emitting substrate in order to detect inducing chemicals (Pawlik, 1992).
4. The chemical morphogens were present but, as the larvae continued to grow and metamorphose, they had limited ability to swim or move across the tank in search of a suitable substrate. This is unlikely, however, as locomotory abilities in *T. squamosa* continue into juvenile, and even adult, life stages (Huang et al., 2007).
5. When the larvae had metamorphosed to juveniles with larger body forms, their settlement site requirements could have changed towards locating protection-providing refuges (Walters & Wethey, 1991, 1996).

This latter point may explain why the tiles with rough surfaces attracted more *T. squamosa* larvae compared to the smooth-surfaced tiles. Studies on coral recruitment patterns have also shown that rough surfaces such as dead coral skeletons are more attractive to larvae than substrate with smooth surfaces—glazed tiles for example (Carleton & Sammarco, 1987; Harriott & Fisk, 1987). It is known that *T. maxima* larvae tend to move to the corners of tanks and to the edges of plastic panels (Jameson, 1976; Gwyther & Munro, 1981) and, like corals, clam larvae may prefer to settle on substrates which provide shelter via grooves, pits and crevices (Petersen et al., 2005a, b).

Conclusions and potential future research

Whereas the water-soluble cues from CCACR attracted larvae in the choice experiment, there was no overall effect on recruitment, i.e. the selection of settlement substrates by giant clam larvae appears to be life-stage dependent. Further experimentation, however, is required to confirm this. For instance, 11-day-old *T. squamosa* larvae could be given the choice of three CCACR concentrations, but only after the tablets have been 'aged' in seawater for 6 weeks. Correspondingly, 49-day-old juvenile clams could be presented with freshly made tablets to see whether CCACR really has no effect on substrate choice at this life-stage. Nevertheless, overall, the findings presented here suggest that CCA-enriched concrete would not enhance recruitment on man-made subtidal structures. In fact, simply ensuring the surfaces are rough may be a more effective way to encourage colonization.

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