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Settlement behavior of *Acropora palmata* planulae: Effects of biofilm age and crustose coralline algal cover

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Abstract. The role of crustose coralline algae (CCA) and bacterial biofilms in the settlement induction of *Acropora palmata* larvae was tested with ceramic tiles conditioned in reef waters for different time periods. Larval settlement varied among tiles by conditioning time (P<0.001), with low settlement (11%) on unconditioned tiles and high settlement (72-87%) on tiles conditioned for 2, 8 and 9 weeks. Tile surface texture and orientation also affected settlement (P<0.001). Larvae of *A. palmata* preferred the undersides of tiles as conditioned in the field (78% of total settlement), compared to upper surfaces (8%) or Petri dish surfaces (14%). CCA cover increased with conditioning time (P<0.001) and differed by tile orientation (P<0.005), revealing a positive correlation between settlement and CCA cover on tile bottoms, but not tile tops. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes revealed that biofilm age, tile surface and tile orientation affected microbial community structure. Further, biofilms that induced settlement were characterized by bacterial populations distinct from non-inductive biofilm communities. Thus, we present additional evidence of the involvement of CCA and bacterial biofilm communities in the process of coral larval settlement, suggesting that complex interactions among multiple cues are involved in larval settlement choices.

Key words: *Acropora*, settlement cues, microbial community, T-RFLP, crustose coralline algae

Introduction

Scleractinian corals are the foundational organisms that provide coral reefs with the habitat complexity that makes them amongst the most biologically diverse of marine ecosystems. For coral reefs to form, many species of corals must dominate the reef substrate and occupy space over long evolutionary time periods. This must involve both long-lived adult corals and persistent high rates of coral recruitment success on new substrates following disturbances.

Coral recruitment begins with the attachment of a coral planula larva to reef substrate and is successful when the environment into which the larva settles supports its long term survivorship. Conditions favoring coral recruitment success include low competition with faster growing algae and encrusting invertebrates, promoted by high rates of grazing (Hughes 1994; Carpenter 1997; Hixon 1997; Birrell et al. 2005). However, coral larvae are structurally simple and have limited ability to sense the environment, except through chemosensory means.

The current view of coral settlement is that when competent planulae explore the reef substrate, contact with certain chemical cues triggers them to stop swimming, attach to the substrate and develop into the primary polyp (reviewed in Harrison and Wallace 1990). These cues are thought to be chemicals associated with or secreted by crustose coralline algae (CCA) or microbes growing on the substrate. Chemical extracts from certain species of CCA have been demonstrated to induce coral settlement more strongly than others, indicating preference and specificity for cues associated with those species (Morse et al. 1988, 1996; Morse and Morse 1991, Heyward and Negri 1999, Harrington et al. 2004). However, settlement experiments conducted on *Montastraea faveolata* and *Acropora palmata* revealed that most larvae settled near, rather than on, CCA patches (Szmant and Miller 2005). A more recent, detailed study found that over 80% of the larvae from these coral species attached onto microbial films (Nugues and Szmant in prep.), suggesting that microbial communities may be responsible for generating the settlement signal. Indeed, studies have demonstrated the induction of larval settlement by bacteria, including a cultured isolate from CCA (*Pseudoalteromonas* sp. strain A3, Negri et al. 2001) and diverse, naturally-occurring reef biofilm communities (Webster et al. 2004).

Determining the settlement cues of coral larvae is crucial to reef conservation and restoration efforts, allowing for the identification and targeted preservation of high recruitment reef niches, as well as, the direct application of replicated cues to coral nursery programs requiring induction of larval metamorphosis under laboratory conditions.

In this study, we used an experimental approach similar to that described by Webster et al. (2004) to determine the role of CCA and microbial biofilms in larval settlement induction. New substrates (ceramic tiles) were 'conditioned' in seawater to develop natural reef biofilms. The complexity of biotic communities that develop on new substrates increases with field conditioning time, as does their attractiveness to coral larvae (Webster et al. 2004). Tiles were aged from 0 to 9 weeks in a back reef lagoon and used in settlement bioassays. Terminal restriction fragment length polymorphisms (T-RFLP) analysis, a popular culture-independent microbial profiling technique (Schütte et al. 2008), was used to characterize the community structure of microbial biofilms. We present here the correlations between settlement of the larvae of the Caribbean reef coral, *A. palmata*, and these microbial signatures, as well as, the amount of CCA colonizing the tiles.

Material and Methods

Settlement tiles: orientation and texture
Ceramic tiles (high-fired clay, Daltile®) attached to plastic racks on cement blocks were pre-conditioned in the field at 4 m for 2, 4, 6, 8 and 9 weeks. Tiles consisted of a glazed, flat surface and an unglazed, dimpled surface. For each time point, 24 tiles were deployed glazed-side up and 24 glazed-side down. Thus, there were 4 possible combinations of tile surface texture and orientation during field conditioning. Tiles were collected simultaneously in individual bags the day settlement experiments were conducted. Control tiles were soaked in 5µm filtered seawater (FSW) overnight.

Gamete collection and larval rearing
Gamete bundles were released by *A. palmata* colonies during the night of September 1, 2007. Spawn from multiple colonies were collected, cross-fertilized and reared to the planulae stage (Szmant and Miller 2005).

Settlement assay
Large Petri dishes (150 mm x 25 mm) were filled with 200 mL FSW at 29 °C and one tile of each orientation – glazed-side up and glazed-side down, as oriented in the field – were added to each dish. Twenty competent *A. palmata* larvae were added to each dish, with 5 replicate dishes per time treatment. After 48 hours, settled larvae were enumerated on each tile using a dissecting microscope. Following settlement counts, digital photographs were taken of each tile face and sterile cotton swabs were used to sample biofilm communities. Paired T-tests, a 2-way ANOVA and a nested ANOVA (tile texture and tile orientation nested with conditioning time) were run to determine the effects of conditioning time, tile texture and tile orientation on larval settlement using SigmaPlot 11.0 and Statistica (1998 version).

Crustose coralline algae quantification
Digital photographs were processed using ImageJ software (NIH). Quantification of CCA was only possible on glazed tile surfaces, where contrast was high between CCA patches and the tile background. Photographs were cropped to fit the tile surface, CCA patches were defined as regions of interest, and black and white mask images were created. CCA cover was calculated by measuring the dark area of the mask images. A 2-way ANOVA was run to determine the effects of conditioning time and tile orientation on CCA cover. Linear regression analyses were performed to compare CCA cover and larval settlement rates on each tile face (SigmaPlot 11.0).

DNA extraction and amplification
Community DNA was extracted from biofilm swabs preserved in RNAlater (Ambion) using the PUREGENE kit (Gentra Systems) and purified using the Wizard DNA Clean-Up System (Promega). Universal bacterial primers 8F-FAM (Reysenbach et al 1994) and 1522R (Martinez-Marcia et al 1995) were used for amplification of partial 16S rRNA sequences. Total PCR reaction volume was 50 µl, including 15 pmol of the forward primer, 10 pmol of the reverse primer, 25 µl GoTaq® Mastermix (Promega) and 2 µl DNA template. Thermocycler conditions consisted of an initial denaturing time of 5 min at 85 °C, then 35 cycles of 0.75 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C, with a final extension time of 10 min at 72 °C. For each sample, PCR products from 3 separate reactions were combined and gel-purified using the Wizard SV Gel Clean-Up System (Promega). PCR products were quantified using the Quant-iT Qubit® kit (Invitrogen).

Restriction enzyme digestion and T-RFLP analysis
Approximately 100 ng of purified PCR products were digested with the restriction endonucleases *HaeIII* and *MspI*. Total reaction volumes for *HaeIII* digests was 20 µl, including 17.3 µl PCR product, 0.2 µl BSA, 2 µl Buffer C and 0.5 µl enzyme (Promega). Total reaction volume for *MspI* digests was 20 µl, including 17.3 µl PCR product, 0.2 µl BSA, 2 µl NEBuffer 2 and 0.5 µl enzyme (New England Biolabs). All digests were incubated at 37 °C for 12 hours. Immediately following digestion, samples were precipitated with 75% isopropanol and dried (SpeedVac, LabConco). To each sample, 10 µl formamide and 0.5 µl ROX size standard (PE Applied Biosystems) were added. Samples were denatured at 94 °C for 2 min and immediately cooled on ice for 2

min. Samples were analyzed on an automated sequencer (ABI377) with the program GeneScan (PE Applied Biosystems). The length of individual fluorescently-labeled terminal-restriction fragments (T-RFs) was determined by comparison with TAMRA size standards (Genescan™). Raw T-RFLP peak profiles were standardized using a proportional threshold of total fluorescence (Osborne et al 2006) and compared across samples using T-Align (Smith et al 2005). Peak profiles were standardized using relative abundance and peaks with areas <1% of total fluorescence were discarded as background noise.

Non-metric multi-dimensional scaling plots

To assess the similarity of bacterial community structures, Bray-Curtis similarity matrices were constructed using square root transformations of relative abundance T-RFLP data. To visually compare bacterial community similarity across samples and factors, non-metric multi-dimensional scaling (MDS) plots were constructed. Analyses of similarity (ANOSIMs) assessed the statistical significance of similarity among biofilm bacterial communities by the factors tile age, tile surface, tile orientation, and larval settlement. MDS plots and ANOSIM calculations were performed using PRIMER v5.1.2 (Plymouth Marine Laboratory, UK).

Results

Settlement assay

Larval settlement varied significantly over conditioning periods (P<0.001), with low settlement on unconditioned tiles, moderate settlement on tiles conditioned for 4 and 6 weeks, and high settlement on tiles conditioned for 8 and 9 weeks (Fig. 1). Notably, 2-week old biofilms induced settlement levels (72%) similar to the oldest biofilms (8 and 9 weeks), while settlement rates dropped in 4 and 6 week old biofilms.

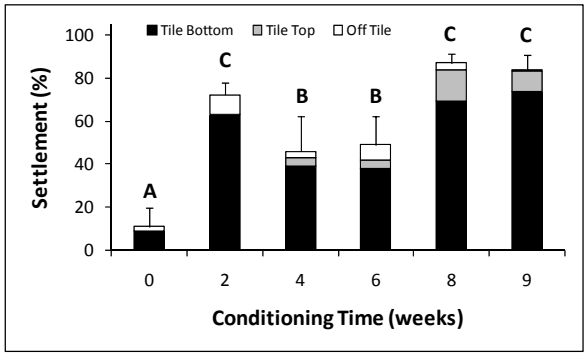

Figure 1. Effect of conditioning time on settlement of *A. palmata* planulae. Black bars represent tile surfaces facing upwards, gray bars tile surfaces facing downwards, and white bars non-tile substrate (Petri dish). Error bars represent 1SD and letters denote significant (P<0.05) differences in total settlement by treatments.

Within conditioning treatments, settlement rates were significantly affected by tile orientation (P<0.05) and tile surface (P<0.05). Total settlement of *A. palmata* larvae showed a clear preference for tile bottoms (78%) compared to tile tops (8%), as oriented in the field, with low settlement (14%) found off tiles.

Crustose coralline algal cover

CCA cover on settlement tiles increased significantly with longer conditioning periods (P<0.001); however, variation within treatments was large. Settlement tiles conditioned for greater than 4 weeks exhibited higher levels of CCA cover than control tiles and tiles conditioned for 2 weeks. CCA cover also varied significantly by tile orientation (P<0.005), with higher cover on tile tops compared to tile bottoms; however, pairwise comparisons within conditioning treatments were only significant for 6-week tiles (P<0.001; Fig. 2).

Linear regression analysis revealed that CCA cover on tile bottoms was positively correlated with larval

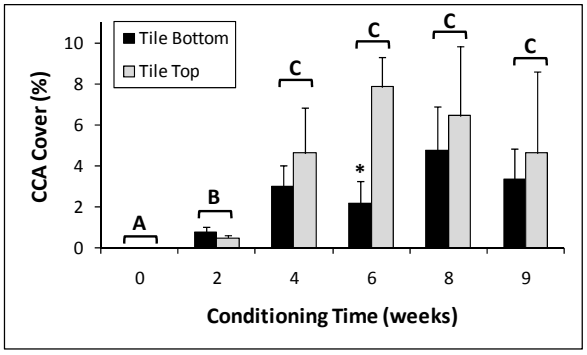

Figure 2. Effect of conditioning time and tile orientation on CCA cover. Black bars represent tile surfaces oriented downwards (bottom) and gray bars tile surfaces oriented upwards (top). Letters indicate significant (P<0.05) pairwise differences among conditioning treatments. Asterisks denote significant (P<0.05) differences among tile bottoms and tile tops within conditioning treatments. Error bars represent 1 SD.

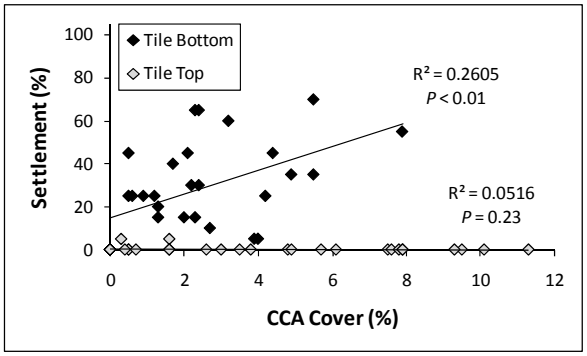

Figure 3. Effect of CCA cover and tile orientation on settlement of *A. palmata* planulae. Dark diamonds denote tile surfaces oriented downward (bottoms) and gray diamonds surfaces oriented upward (tops). Regression lines are shown for tile bottoms and tops.

settlement (P<0.01) and explained 26% of the variation in settlement by *A. palmata* larvae on tile bottoms. However, larval settlement on tile tops was not correlated with CCA cover (P=0.23), despite exhibiting a similar range of CCA cover found on tile bottoms (Fig. 3).

Microbial diversity and biofilm community structure

A total of 47 unique terminal restriction fragments (T-RFs) were identified from 27 T-RFLP profiles analyzed using the restriction endonuclease *MspI*; only 24 T-RFs were identified from 27 T-RFLP profiles using *HaeIII*. Lower resolution with *HaeIII* digestion is likely attributable to excessive digestion of 16S gene sequences, evidenced by short (<60bp), saturated peaks in chromatographs (data not shown).

ANOSIMs revealed distinctions among biofilm bacterial communities based on conditioning periods, tile surface, and tile orientation (Table 1). Distinct bacterial communities were also recovered from tiles inducing settlement compared to non-inducing tiles (*MspI* data). MDS plots exhibited low stress values, indicating congruence between MDS plot and similarity matrix distances. Samples clustered by factors with some overlap among factor levels, representative of the modest R-statistic values reported. MDS plots constructed from T-RFLP analysis with *MspI* showed clearer distinctions among factors compared to *HaeIII* (Fig. 4).

Table 1. ANOSIM summary statistics (R-statistic and *P*-value) for T-RFLP data recovered using *HaeIII* and *MspI*.

	HaeIII		***MspI***	
Factor	R-statistic	*P*-value	R-statistic	*P*-value
Age	0.217	0.002	0.192	0.009
Surface	0.069	0.046	0.210	0.003
Orientation	0.171	0.002	0.305	0.001
Settlement	0.087	0.099	0.186	0.013

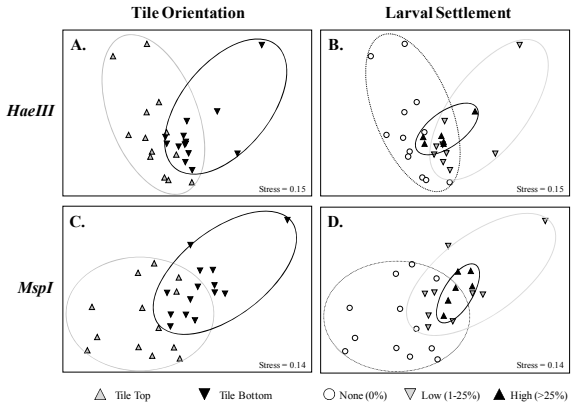

Figure 4. Non-metric multi-dimensional scaling ordination of biofilm bacterial communities recovered by T-RFLP analysis with the restriction endonucleases (A, B) *HaeIII* and (C, D) *MspI*. Data are grouped by (A, C) tile orientation and (B, D) larval settlement induction levels; circles encompass all samples by each factor level.

Discussion

Settlement behavior of *A. palmata* planulae was significantly affected by the conditioning periods of settlement tiles. Both CCA cover and biofilm bacterial communities varied significantly across conditioning treatments, but neither could consistently explain the observed larval settlement preferences. CCA cover correlated with settlement, but only on tiles undersides and accounted for only 26% of settlement variability. Previous work has also reported preferential settlement of coral larvae on the undersides of field conditioned tiles (Babcock and Mundy 1996; Raimondi and Morse 2000; Szmant and Miller 2005; Szmant in prep.). Other studies have reported little effect of CCA presence on larval settlement (Petersen et al. 2005) and settlement of coral planulae in the absence of CCA cues (Negri et al. 2001; Webster et al. 2004). While CCA and non-coralline crustose red algae have been implicated in the induction of coral larval settlement (e.g., Heyward & Negri 1999), settlement cues of crustose algae vary with the algal species and settlement response with different coral species (Golbuu and Richmond 2007). In this study, CCA that colonized settlement tiles may not have represented 'inductive' algal species for *A. palmata* larvae.

Another hypothesis is that the CCA function as part of a multi-cue cascade that leads planulae to settle and metamorphose, in which physical conditions (e.g., low light intensity) or other chemical cues initially attracted larvae to tile undersides, where close or direct contact with CCA surfaces then signals an attractive habitat to the planulae. Indeed, phototactic behavior by coral larvae and increased settlement on tile bottoms under high light intensities has been reported (Mundy and Babcock 1996). In addition, evidence for synergistic interactions among multiple chemical cues derived from CCA that enhance coral larval settlement has been recently reported (Kitamura et al 2007). Clearly, some attribute of tile undersides proved a stronger larval attractant than CCA alone, as CCA were similarly abundant on tile tops where very little to no settlement occurred.

Microbial biofilms play a key role in the settlement induction of many cnidarian larvae (Müller and Leitz 2002), including scleractinian corals (Negri et al. 2001). In fact, 'conditioning' of settlement tiles for microbial biofilms is routinely performed prior to coral larvae bioassays and usually required to achieve high larval settlement. Despite the crucial role of biofilms in larval settlement, few studies have investigated the bacterial community structures established by 'conditioning' (Webster et al. 2004). One recent comprehensive study employed culture-independent molecular and electron microscopy methods to characterize biofilm communities

inhabiting conditioned slides used in settlement assays (Webster et al. 2004). The authors reported no differences in relative densities of major bacterial lineages between biofilms that induced settlement and those that did not, and suggested metamorphic cues may be associated with particular bacteria species (Webster et al. 2004). Similarly, T-RFLP analysis of biofilm bacteria in this study revealed community-level differences among microbial films based on conditioning periods, tile surfaces and tile orientations. Further, distinct biofilm communities were recovered from tiles that induced settlement compared to those that did not. These results further highlight the complex nature of larvae-bacteria interactions and suggest multiple sensory cues from biofilm communities affect settlement choices by coral larvae.

Determining the environmental cues that dictate coral larvae settlement is essential to understanding how reefs will respond to and recover from reef degradation and is crucial for successful reef restoration programs. Loss of critical settlement cues from reef biota will result in continued, precipitous ecosystem declines by reducing coral recruitment at impacted sites. Identification of settlement cues may offer insight into preservation of biotic components that enhance reef recovery and aid restoration efforts, by providing a stock of metamorphic cues to enhance larval settlement under artificial conditions and the production of juvenile corals for transplantation to degraded reefs. The roles of some reef organisms (e.g., CCA) have been commonly targeted; however, other key organisms (e.g., bacteria) require further investigation. Additional studies targeting the role of coral reef microbial communities in larval settlement induction are required to fully elucidate the cues that attract new recruits and thus sustain reef ecosystems.

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