UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Ecology of Coral-Microbe Interactions

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Kristen Laura Marhaver

Committee in charge:

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2010

The Dissertation of Kristen Laura Marhaver is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

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EPIGRAPH

WHEN I heard the learn'd astronomer;
When the proofs, the figures, were ranged in columns before me;
When I was shown the charts and diagrams, to add, divide, and
measure them;
When I, sitting, heard the astronomer, where he lectured with
much applause in the lecture-room;
How soon, unaccountable, I became tired and sick;
Till rising and gliding out, I wander'd off by myself;
In the mystical moist night-air, and from time to time,
Look'd up in perfect silence at the stars.

Walt Whitman

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ACKNOWLEDGEMENTS

Hurdles are difficult to clear before you know how to walk, how to run, and how to calm your nerves and gather your confidence at just the right moment. At the end of the academic steeplechase, I found myself thinking often of the teachers who helped me learn to walk and run. Simply by being passionate about their jobs and confident that young students can master difficult puzzles, many of those teachers unknowingly prepared me for life as a scientist.

At Tesseract, Steve Mintz taught me to give a public speech, to keep up with current events, to ski difficult terrain, and to be my own teacher. At Wichita Collegiate, Jerry Nixon taught me to write an essay, showed me there is room in scholarship for jokes, and allowed me to discover on my own that my calling was not as a historian. Sherri Shaake showed me that everything in the biological world is funny and served as my first mentor in Caribbean reef ecology. Kari Olson taught me how to clear real and metaphorical hurdles and the exhilaration of intense, all-encompassing focus on a goal. Don Funke taught me that long distances are worth traveling when you have great teammates, and that jokes work faster than ibuprofen. Gary Buettgenbach taught me that art is a noble endeavor and a reliable antidepressant.

At Georgia Tech, Amanda Gable taught me the science, art, and magic of grantwriting. Terry Snell taught me how to be a scientist and how to become one. When my graduate professors weren't looking, Pia Stern taught me to observe the world with a careful and mindful eye, and then to paint it in whatever way I pleased.

At Scripps, Nancy Knowlton and Jeremy Jackson graciously welcomed me into the first cohort of IGERT students. They selflessly shared their passion for coral reefs and deep knowledge of natural history and we each felt very lucky to be in the

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Stuart Sandin valiantly provided me with his brilliant project ideas and permission to run with them in unexpected directions. His ecological instincts are better than anyone else's I have encountered, and because of this, he legitimately deploys what would be considered unreasonable science optimism if wielded by any other person. Thank you for your attention to detail, your philosophies about being a scientist, and for occasionally rescuing me from the duty of making fun of Mark.

Each time I listed my Ph.D. committee on the final slide of a presentation, I was humbled thinking of the wisdom wielded by the group. Chris Wills enthusiastically encouraged me to go test for Janzen-Connell effects in the ocean, confident that it would be easier to study them in the ocean than on land. I would argue that this turned out to be true, as long as electrocutions sustained while filtering seawater are not included in the score. Doug Bartlett provided an expert microbial perspective as well as creative ideas and enthusiasm for new experiments. Dick Norris provided his encouragement when I decided to tell stories about coral evolution and he selflessly took on the task of troop leader for an increasingly large IGERT family. Eric Allen's sense of humor made academia seem far less imposing. He warmly welcomed my technical questions and gave advice on a number of different molecular methods. Forest Rohwer kindly suggested I should just skip molecular methods and go do more ecology. Forest provided encouragement as I traded the reliability of DNA sequencing for the insanity of underwater experiments and he generously imbued the San Diego Coral Club with brilliant ideas, reports of non-stop scientific progress, lots of wine, and chances to laugh at ourselves whether

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Studying coral reefs is the world's best job, but it comes with a burden to work your ass off while everyone else around you is on a Caribbean vacation. Such a decadent and scenic job is somehow also full of electrocutions, animal attacks, bleeding, underwater logistical problems, debt, and beauty disasters. Nevertheless, Mark Vermeij taught me to love this job and to endure its trials with tenacity and humor. Thank you for spending all those hours dissecting ideas and soaking them in coffee, Polar, Red Bull, Jack, and yet more ideas. Thank you for being unfazed by bad jokes and crazy scuba hair. No one has so eagerly embraced the Marhaver family motto nor has anyone else met the Marhaver family standard of dive buddy excellence by always having just as much air left as I do. Thank you for lending me your ear, and I agree, "It's not really electrocution."

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Chapter 1, in full, has been submitted for publication: Marhaver, K. L., M. J. A. Vermeij, F. Rohwer, and S. A. Sandin. *In review*. Microbes from adult corals cause

distance-dependent mortality in nearby juveniles. The dissertation author was the primary investigator and principal author of the manuscript.

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PUBLICATIONS

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Marhaver, K. L., M. J. A. Vermeij, F. Rohwer, and S. A. Sandin. *In review*. Microbes from adult corals cause distance-dependent mortality in nearby juveniles.

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Vermeij, M. J. A., K. L. Barott, A. E. Johnson, and K. L. Marhaver. 2010. Release of eggs from tentacles in a Caribbean coral. Coral Reefs **29**:411.

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ABSTRACT OF THE DISSERTATION

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At every moment, a tropical reef coral interacts with millions of microbial organisms from tens of thousands of different species. Among these viruses, bacteria, archaea, dinoflagellates, and protists are cooperative symbionts as well as pathogens and parasites. Each interaction between a coral and a microscopic organism has ecological consequences for the coral community.

In this dissertation, I show that juveniles of the Caribbean coral *Montastraea*

faveolata suffer distance-dependent mortality in the presence of adult corals of the same species which is caused by microbial communities near these adults. This previously undiscovered structuring force will affect the spatial patterning of reefs and appears strong enough to drive the evolution of habitat selection behavior by coral larvae.

The behavior of pre-settlement larvae differs based on both the microbial environment and the manner in which that environment is altered, indicating that water column microbes may be used as navigational information by dispersing corals. Sterilized seawater and penicillin both increase larval swimming rates, perhaps because larvae cannot detect the microbial "smell" of a suitable reef habitat, however only penicillin inhibits settlement and metamorphosis. Five antibiotics each induce a different pattern of abnormal behavior, indicating that the behavior of coral larvae could form the foundation of a new model system in toxicology. Furthermore, settlement failure on reefs may have a large behavioral component that has been underappreciated until now.

The coral-dinoflagellate symbiosis is the best-known coral-microbe interaction however coral bleaching remains enigmatic. Based on observations made after a tropical storm, I propose that corals evolved bleaching as an adaptive mechanism to readjust symbiont communities after storm damage moves corallites into new light regimes. In a survey of viral diversity in the coral *Diploria strigosa*, I demonstrate that coral-associated viruses likely infect all other cells in the coral holobiont, including symbionts and potential pathogens. I propose that interactions among these viral groups could help to stabilize mutualistic coral-microbe interactions.

Four additional natural history observations demonstrate the diversity of behaviors and interactions corals exhibit at the micron to millimeter scales and reveal that we have many more coral-microbe interactions left to discover.

INTRODUCTION

The Ecology of Coral-Microbe Interactions

K. L. Marhaver

This dissertation might have been called "The Microbial Ecology of Reef Corals." But if that were true, this would be a completely nonsensical way to start to the document. Far more than it is about microbes, this dissertation is about corals and the mysterious, complicated, and clever things they do in order to successfully live in one place for decades while building the habitat for millions of other species. Microbes are essentially a tool that corals—and this author—have employed to make their modest livings hanging out around other corals.

As humans, we are inherently poor at "thinking like a coral" (*sensu* N. Knowlton). Scleractinian reef corals have had 240 million years to figure out planet Earth and devise counters to her tricks and schemes. As a result, corals can slowly and steadily outwit fast-growing organisms in the stable conditions of nutrient-poor waters, resist microbial attack without an adaptive immune system, and simultaneously heal wounds and recalibrate their photophysiology when damaged and topped by periodic storms. That is all a testament to the amount of evolution that can take place in hundreds of millions of years, even if your generations happen to overlap a few hundred at a time. Meanwhile, we humans have had only a few thousand years to figure out Earth and only a few decades to figure out corals.

The goal of this dissertation is to convince the reader of two things. First, evolution has equipped corals with a bewildering array of adaptive strategies, most of which we have been too unimaginative to discover or perhaps just too large, short-lived, and terrestrial to observe. Second, corals have spent much of their evolutionary history devising ways to make microbial organisms perform tasks and provide information under their supervision and according to their rules.

Together, these two points run counter to the prevailing notion that corals are simple, fragile animals sitting around as hapless victims of their environments.

Indeed, most of the world's coral reefs are a rotted shadow of their former selves. But that is not because corals are fragile, it is because the rumble of the human footprint on planet Earth has changed the rules of every underwater game, including those games corals play with microbes.

While I conducted the research for this dissertation, the arrival of the field of coral microbiology was marked by a flood of review articles in a short time span. This nascent field introduced a number of brilliant new paradigms for the microbial ecology of corals. This also motivated a large number of people to look at microbial sequence data while I was underwater somewhere looking at corals. For making that trade, I am thankful for every microbial ecologist out there.

At a few points during this project, I could have similarly steered the work toward more sequencing and microscopy. The limiting factor was sometimes money but usually my captivation with natural history and larval behavior. In Chapter 9, I present a thorough vetting of two viral metagenomes to fill my sequence analysis quota, but in the remainder of this dissertation, microbes are usually discussed as whole communities and manipulated in bulk. (Admittedly, every time an experiment failed due to a tropical storm, 100% survivorship, catastrophic bleaching, or sheer coral trickery, I found myself jealous of PCR machines and ACTGs.)

In this dissertation, rather than describe the microbes themselves, I describe some of the ways corals make their living on a coral reef by using microscopic organisms: to structure their communities, provide information, perform metabolic functions, clean their habitats, fend off their pathogens, and stabilize their symbioses. Four of the main chapters are followed by short natural history observations in the tradition of "Reef Sites" from the journal *Coral Reefs*. These short papers present a novel observation and a brief commentary on its meaning for coral biology and reef ecology. Although these observations did not require months of fieldwork or

analysis, I am as excited to include these chapters as I am the main research chapters because they represent discoveries that would only be possible after spending thousands of hours watching corals underwater and above. I hope these short chapters give cnidarians a bit more craftiness credit where it is long overdue.

In Chapter 1, I present evidence that an ecological mechanism known from rainforests also operates on Caribbean coral reefs. In separate work in the 1970s, Dan Janzen and Joe Connell proposed that the distance- and density-dependent action of species-specific pathogens and predators structure the patterning of rainforest tree communities, and that this helps create and maintain high tree diversity. Using field and laboratory experiments, I show that a similar distance-dependent mortality phenomenon occurs in the Caribbean coral *Montastraea faveolata* and that this is attributable to the species-specific microbial communities in the waters surrounding conspecific adult colonies. This effect may be strong enough to help drive the spatial patterning of coral reefs. Based on these results, I propose that coral juveniles use the microbial landscape of the reef to select settlement habitats away from adult conspecifics in order to avoid high-mortality habitats. Differential mortality between reef habitats creates the selective pressure to drive the evolution of this habitat discrimination behavior.

While conducting the experiments for Chapter 1, I observed ciliates grazing at flocks of detritus and the outer edges of recently settled juvenile corals. This observation is presented in Chapter 2. Ciliates of this size have never been observed grazing on or near corals in the wild. Whether they pose a threat to the coral or provide a surface-cleaning service is unclear. What is true, however, is that we have failed to pay sufficient attention to this size fraction of cryptic organisms on coral reefs. Just as the action of nematodes in forest soils dramatically alters microbial dynamics and nutrient cycling, microscopic eukaryotes likely perform important

functions on benthic reef substrates, perhaps even benefitting corals by keeping surfaces relatively free of algal spores and potential pathogens.

Chapter 1 was motivated by theory from terrestrial plant ecology, however thinking about plants for too long gets you into trouble on a coral reef, because corals are very unlike plants when it comes to their sensory systems and behavior. In Chapter 3, I show that pre-settlement *M. faveolata* planulae exhibit a range of locomotory behaviors depending on the microbial environment in which they find themselves. I also show that penicillin causes abnormal swimming behavior and virtually eliminates settlement in culture. Based on these results, I propose that microbial communities in the water column act as a navigational cue for dispersing larvae.

In Chapter 4, I document an alternative but far less effective form of coral locomotion. In two different years, I observed post-metamorphosis coral polyps that had failed to produce skeletons or attach themselves to the substrate and were instead crawling and tumbling along the benthos. One of these animals insisted on traveling with its tentacles down, making the word "polyp" (from the Greek word "polypous," i.e., "many-footed") a particularly apt description. Rambling coral polyps are known from only one other species. This dispersal strategy may not be common, but it shows us how little we truly know about what corals do before they reach the photoquadrat-ready diameter of 0.5 cm.

The behavior of larval corals does not only change upon wholescale manipulation of microbial environments, but also in response to individual chemical compounds. In Chapter 5, I show that five different antibiotic compounds induce different behavioral profiles in pre-settlement *M. faveolata* planulae, and that these behaviors are different from those in untreated or filtered seawater. These results reveal the richness and diversity of locomotory behaviors in coral planulae and

suggest that abnormal behaviors may be mediated indirectly through changes to microbial communities and/or directly through altered neuronal signaling cascades. From these results, I propose that coral planula behaviors are informative response variables for future studies of toxicology.

In Chapter 6, I describe instances of abnormal morphological development in post-settlement corals treated with penicillin. These phenotypes were only observed in penicillin treatments, hinting that coral development may be interrupted by exogenous chemical compounds or by a loss of important microbial interactions. As reef restoration projects increasingly focus on sexual coral propagation, monitoring settlers for morphological abnormalities such as these will help researchers gauge whether rearing conditions are suitable and whether settlers are likely to survive being transplanted on the reef.

In 2008, I watched a tropical storm batter and kill many of the corals that participated in Chapters 1 through 3. On the shallow reefs of Curaçao's leeward side, massive *Montastraea* colonies were thrown sideways or inverted for the first time in decades, bringing their old skeletons suddenly above the sand to tell stories about their pasts. After toppling, many of these colonies also underwent partial bleaching in patterns consistent with the adaptive bleaching hypothesis. In Chapter 7, I use these observations to propose a new theory for the evolution of adaptive coral bleaching which attempts to resolve an ongoing debate in the coral literature about the flexibility of the coral-dinoflagellate symbiosis.

Although coral species can generally reset their symbiotic dinoflagellate communities after a bleaching event, bleaching becomes increasingly dangerous the longer it continues. Abnormally warm water caused a bleaching event in late October 2010, during which I observed colonies of *Colpophyllia natans* and *Montastraea faveolata* extending their mesenterial filaments into neighboring algal turfs. In Chapter 8, I

propose that this represents an improvisational feeding behavior by corals that are both heavily bleached and surrounded by algae. The coral's long mesenterial filaments, typically used for digestion within the body and aggression outside the body, appear to have become useful tools for long-distance consumption of much-needed photosynthate (or microbes on algal surfaces) as the bleaching event wore on. These observations highlight the fact that we humans know very little about the late night ecology—or nutritional biology—of reef corals.

Stresses such as bleaching affect the composition of coral mucus and therefore its antimicrobial properties. However, corals have many other strategies to maintain control of microbial communities in times of stress. In Chapter 9, I use metagenomic techniques to describe the viral communities associated with healthy and bleaching adult colonies of the Caribbean coral *Diploria strigosa*. These communities include viruses capable of infecting virtually every cell present in the coral holobiont: algal, microbial, and animal, including potential pathogens and parasites. Drawing a parallel to other animal-microbial symbioses, I propose that viruses help to stabilize the relationship between corals, dinoflagellates, and microbes by turning two-partner symbioses into multi-player relationships, which are far more difficult for a single species to unravel.

Once we have spent 240 million years or so thinking like a coral, we will be much better at understanding how their behaviors—as juveniles, in the open ocean, at night, and on the micron scale—affect the ecological processes of the reef communities that we observe during the day on the centimeter and meter scale. In the meantime, I hope the following nine stories convey the remarkably complex lives led by corals as they interact with the immense microbial diversity of the sea. I hope some of these ideas inspire readers to look at their own study organisms in a new way. Finally, I hope the theories I propose are quickly replaced with better ones. I feel

very blessed that this is my job. Given their creative strategies for making a living, there are far worse burdens than thinking like a coral.

CHAPTER 1

Microbes from adult corals cause distance-dependent mortality in nearby juveniles

K. L. Marhaver, M. J. A. Vermeij, F. Rohwer, and S. A. Sandin

ABSTRACT

Host-specific pathogens and predators promote the coexistence of tree species in tropical rainforests by causing distance-dependent and density-dependent mortality of seeds and seedlings. Tropical reef corals host species-specific microbial communities in their surface mucus layers that contain ubiquitous potential pathogens. Juvenile corals are particularly susceptible to microbial attack. Here we show that juvenile corals suffer distance-dependent mortality near adult corals due to host-specific microbial enemies. In field experiments with settled recruits of the dominant Caribbean coral *Montastraea faveolata*, juvenile survivorship was 2.3-3 times lower near conspecific adults than near two other coral species or in open areas of the reef. Survivorship of recruits was significantly higher when they were placed 96 or 192 cm up-current from conspecific adults than when they were placed within 6 cm of these colonies or in down-current locations. Microbial profiles of the seawater reveal that microbial abundance and juvenile survivorship vary around adult colonies at the same spatial scale. In four laboratory experiments, survivorship of swimming coral larvae was significantly lower in water collected near conspecific adults than near other coral species or further away. This effect was eliminated with filter-sterilization and/or antibiotics. The distribution of adult corals and their microbial halos across a coral reef will therefore affect the spatial pattern of juvenile survival and give rare species an advantage. Preserving coral species diversity should therefore help reefs build and maintain high coral cover. Furthermore, our results demonstrate that a diversity-promoting mechanism known from tropical forests can be generalized to a marine ecosystem.

INTRODUCTION

Ecologists have long sought to explain why groups such as trees, corals, plankton, and bacteria have remarkably high species diversity given that they occupy seemingly homogeneous habitats (Hutchinson 1961; Connell 1978; Dykhuizen 1998). To explain the maintenance and generation of diversity in tropical tree communities, Janzen (1970) and Connell (1971) proposed theories that together became known as the Janzen-Connell hypothesis: the action of species-specific herbivores, seed predators, and plant pathogens from adult trees creates a halo around each tree where juveniles of the same species are unlikely to survive. Janzen-Connell effects therefore promote the survival of rarer species and help maintain high tree diversity overall. The growing body of support for the Janzen-Connell hypothesis includes experimental evidence that species-specific plant pathogens cause distance-dependent mortality of both seeds and seedlings (Augspurger 1983; Gilbert et al. 1994; Packer and Clay 2000; Bell et al. 2006). Despite difficulties inherent to studying slow-growing species with myriad potential pathogens and predators, the Janzen-Connell hypothesis continues to provide a useful and popular framework for examining the forces contributing to terrestrial plant diversity.

Meanwhile, on tropical coral reefs, the creation and maintenance of coral species diversity have typically been considered separately by ecologists. *Speciation* of reef corals is often attributed to abiotic factors such as light, temperature, currents, and storm damage (Connell 1978; Veron 1995), and non-ecological factors such as polyploidization (Kenyon 1997). In one Caribbean species, predation on corals by parrotfish appears to be driving ecological speciation of a coral species along a depth gradient (Carlon and Budd 2002; D. Carlon, personal communication).

The *maintenance* of coral species diversity on the other hand, has more commonly been attributed to ecological factors. For example, competitive dominance networks in encrusting coral reef invertebrate communities provide an ecological mechanism for maintenance of species diversity by preventing any single competitor from overgrowing and outcompeting all other organisms (Jackson and Buss 1975; Buss and Jackson 1979). These interactions may be mediated by allelopathy (as in Buss and Jackson 1979) or by direct physical aggression, as was demonstrated in a group of three scleractinian corals by Lang (1973). Predation may also maintain species diversity in coral assemblages by favoring abundant species and thereby preventing competitive dominance. For example, the crown-of-thorns starfish is thought to affect coral species diversity through preferential predation on the abundant coral genus *Acropora* (Paine 1969; Porter 1972).

Importantly, coral species inventories have long suffered from a lack of sufficient taxonomic resolution and phylogenetic accuracy due to phenotypic plasticity and morphological convergence. More recently, studies of reproductive behavior (Van Moorsel 1983; Levitan et al. 2004), skeletal microstructure (Fukami et al. 2004), aggressive interactions (Knowlton et al. 1992), demographic parameters (Vermeij and Bak 2003), microhabitat use (Vermeij et al. 2007), and molecular genetics (Fukami et al. 2004) have improved coral taxonomy, revealing that corals are more speciose and their niches are more narrow than previously thought. Knowlton and Jackson (1994) argued that blurred species boundaries have traditionally prevented scientists from accurately identifying the habitat specializations of coral species. Thus, Connell himself concluded that niche diversification could not maintain coral species diversity due to broad species ranges and a lack of habitat specialization (1978). Instead, he argued that physical disturbance was responsible for maintaining

species diversity in coral assemblages by preventing communities from achieving an equilibrium state.

Despite the differences in their theoretical treatment, corals are the marine analogs of rainforest trees because they create three-dimensional habitats for thousands of other organisms, use photosynthesis to produce energy, compete with each other for space, and disperse as juveniles but live their adult lives in a fixed location. Given these similarities, Janzen proposed that his model of distance-dependent mortality for tree seedlings should also apply to long-lived sessile marine invertebrates that compete for space and suffer high mortality as juveniles (Janzen 1970). Janzen, however, did not mention corals, focusing instead on rocky intertidal organisms.

In sum, the idea that Janzen-Connell effects occur in scleractinian reef corals has never been tested experimentally. Furthermore, this idea has never been tested in an animal. (Admittedly, because the Janzen-Connell hypothesis invokes the spatially-explicit action of species-specific enemies over a timescale of many generations of the host, few animals would be suitable organisms for such an experiment.) Observational evidence from one coral species has shown that juveniles suffer decreased survivorship and recruitment as the local cover of conspecific adults increases. This effect occurs before all available space (and light) is occupied, suggesting that a waterborne factor that increases with adult cover causes mortality of juveniles (Vermeij 2005; Vermeij and Sandin 2008). These observations provided the motivation for the current study. The growing body of literature on coral-microbe interactions provided sufficient motivation to hypothesize that microbes could serve as the species-specific "enemies" of coral recruits in the reef environment.

Coral-associated microbial communities are diverse, largely host-specific, and generally stable over space and time (Rohwer et al. 2002; Koren and Rosenberg 2006;

Rosenberg et al. 2007). These communities include ubiquitous potential pathogens (Kline et al. 2006; Wegley et al. 2007), which are controlled in part by the coral's mucus chemistry (Brown and Bythell 2005; Ritchie 2006), coral-associated bacteriophage (Marhaver et al. 2008; Efrony et al. 2009), and/or other bacteria in the holobiont (Reshef et al. 2006; Ritchie 2006; Rypien et al. 2010). Stressed corals can lose control of their microbial communities; in such cases, potential pathogens residing on the host, outside pathogens, or general microbial community dysfunction can cause coral morbidity and mortality (Kline et al. 2006; Harvell et al. 2007; Rohwer and Youle 2010). Juvenile corals are particularly susceptible to microbial attack (Vermeij et al. 2009) due to their small size, immature immune systems (Frank et al. 1997), and lack of a fully developed protective microbial consortium (Apprill et al. 2009; Sharp et al. 2010).

Here we use the Caribbean coral *Montastraea faveolata* to show that juvenile corals suffer distance-dependent mortality near adults of the same species, and that this effect is mediated by microbes directly adjacent to the adult coral. Our findings highlight the generality of an ecological mechanism that promotes community diversity and represent the first demonstration of Janzen-Connell effects in the ocean.

RESULTS

Distance-dependent mortality of juvenile corals

We first tested whether juvenile corals suffer distance-dependent mortality by deploying newly settled, lab-reared coral recruits to a shallow reef with a consistent directional current. A total of 454 three-week-old recruits were placed on the reef at seven distances up-current and down-current from eight conspecific adult corals in an open sand flat (methods depicted in supplementary information Fig. 1.1B-D). In the up-current direction, the probability of survivorship was 15.4 times higher at the

furthest distance (192 cm) from the adult colonies than at the closest distance (3 cm), demonstrating *in situ* that coral recruits suffer distance-dependent mortality near conspecific adults (Fig. 1.2A). The probability of survivorship did not increase steadily with distance, however. Both relatively high and low survivorship probabilities were observed at intermediate distances. In the down-current direction, the probability of survivorship was not significantly different at the nearest and furthest distances, but survivorship at intermediate distances was significantly higher than survivorship close to the adult corals.

In the up-current direction, within 48 cm of the coral colonies, dramatic variation in survivorship was observed; importantly, this pattern was qualitatively similar between all seven replicates. This pattern may reflect the interactive factors of the coral-associated microbiota, water currents, the physical barrier of the coral colony, and other physical factors such as shading. It is therefore not immediately clear why the survivorship curve took this non-monotonic shape. The microbial profiles and laboratory experiments described below were conducted as a follow-up to this experiment to attempt to better resolve the relative strengths of these factors.

Overall, the probability of survivorship was significantly lower in the down-current direction than the up-current direction (Fig. 1.2A, $P = 0.004$). Therefore, the overall pattern of survivorship indicates that juvenile mortality is due to a factor that does not act symmetrically around the adult corals, but instead acts in a directional manner.

The replicates in this experiment had a range of starting juvenile densities; we therefore tested for effects of local recruit density on survivorship. Within an experimental distance, juveniles suffered increased mortality with increased density of recruits ($P = 0.02$). Janzen predicted this type of negative density-dependent survivorship would occur for seedlings (Janzen 1970) and this has been observed in

experimental manipulations of seedling density in the presence of a distance-dependent plant pathogen (Packer and Clay 2000). Notably, the density-dependent mortality we observed in coral recruits was stronger in locations down-current from the adult corals ($P = 0.02$). In sum, juvenile mortality is higher down-current from adult corals, and this effect is enhanced at higher densities of recruits. Given these observations, we hypothesized that the directional current of the reef water interacting with mucus-associated microbial communities on the adult coral colonies created heterogeneous microbial environments across the reef that could drive the survivorship patterns we observed.

Microbial profiles surrounding adult corals

Using fluorescent microscopy, we quantified the total abundance of microbes (Bacteria and Archaea) on the experimental reef at the same distances where recruit survivorship was measured (Fig. 1.2B). Microbial abundance varied predictably with distance and direction from the adult corals. As with the juvenile survivorship curves, this pattern was not symmetrical. The highest microbial abundance occurred immediately down-current from the coral heads (3 cm) and at 96 cm and 192 cm distances down-current from the coral heads; juvenile survivorship was also extremely low at these distances.

At intermediate distances down-current, microbial abundance was significantly reduced relative to both "near" (3 cm) and "far" (96-192 cm) distances. This pattern was qualitatively similar between replicate coral heads (Fig. 1.2B). Strikingly, this pattern is consistent with hydrodynamic models of flow around a cylinder. At certain Reynolds numbers, a cylinder in a unidirectional flow regime causes the formation of small vortices in the down-current direction, which ultimately interact to create a resonance pattern (e.g., König et al. 1993; Williamson

1996). The vortices begin to shed from the cylinder in an alternating fashion, creating a small eddy behind the cylinder in which flow is reduced. Because this eddy forms beyond the relatively thick boundary layer that exists behind the cylinder, any heat or material shed from the cylinder will occur at its lowest level at an intermediate distance. This is known as Von Karman vortex street.

In sum, microbial communities are highly structured in a consistent manner around adult coral colonies, and both microbial abundance and juvenile coral survivorship vary over similar spatial scales. In the down-current direction specifically, areas of lower microbial abundance generally exhibit higher recruit survivorship. Given this apparent correlation, we suspected that microbes moving from the adult corals in a directional manner contributed to low recruit survivorship down-current.

Species-specific mortality of juvenile corals

To test whether mortality near adult corals was species-specific, we placed newly settled coral recruits within 2 cm of adult colonies of three species and on open areas of sand on the same reef. (Methods are depicted in supplementary information Fig. 1.1E-G). As predicted by the Janzen-Connell hypothesis, survivorship was lowest for recruits placed next to conspecific adults (Fig. 1.3). The average probability of survivorship was 2.3-3 times higher when juveniles were placed near other coral species and 2.4 times higher in open areas of a sand flat. This result demonstrates *in situ* that the spatial mortality of coral recruits near adult colonies is constrained by the identity of the coral species nearby. Because recruits were out of reach of the tentacles of the adult corals, we suspect that this species-specific mortality was caused by a waterborne factor.

Effects of adult coral microbes on juvenile survivorship

We conducted four laboratory experiments to determine if a microbial component of the seawater surrounding adult coral colonies caused the patterns of juvenile mortality observed in the field experiments (Fig. 1.4A-D). We collected triplicate water samples from the experimental reef – up-current, down-current, and 1 cm above adult coral colonies. These water samples were used raw or treated with filter sterilization (0.22 μm Sterivex) and/or antibiotics (penicillin, 66 $\mu g\ ml^{-1}$) to reduce the number of microbes. In all four experiments, survivorship of *M. faveolata* larvae was significantly reduced in water collected near conspecific adults (Fig. 1.4A-D) and down-current (Fig. 1.4B) from these adults when compared to their survivorship in water collected elsewhere on the reef (Fig. 1.4A-D). When coral juveniles were exposed to water collected near other coral species, survivorship was significantly higher (Fig. 1.4B-C) in two experiments and suggestively higher in a third experiment (Fig. 1.4D).

Importantly, the experiment depicted in Fig. 1.4D was performed with larvae from a remote reef with low human pressure (Playa Kalki). Survivorship in all treatments was extremely high, providing anecdotal evidence of the importance of local reef health for juvenile coral performance. While a survivorship difference of <2% over seven days was not statistically significant, trends in survivorship occurred in the same direction as in the other three laboratory experiments. Furthermore, while such small difference in survivorship could not be resolved statistically, such a difference could still be expected to have ecological consequences for an organism that can survive for hundreds of years.

Survivorship in "near *M. faveolata*" treatments could be restored to control levels using filter sterilization (3 experiments, Fig. 1.4A-C), and/or antibiotic treatment (1 experiment, Fig. 1.4C). In total, these results demonstrate that mortality

of juvenile corals in close proximity to adult corals is caused by a microbial fraction of the water surrounding the adult colonies. This mortality is reduced with antibiotics and/or physical removal of bacteria, and this effect is specific to the adult coral species nearby. Species-specific microbes in the boundary layer near adult coral colonies therefore represent the marine analogues of the host-specific enemies responsible for driving Janzen-Connell survivorship effects in terrestrial systems.

In total, we have shown in six experiments that juveniles of the coral *Montastraea faveolata* suffer the highest mortality in close proximity to conspecific adults. Corals of rarer species are therefore expected to have a survivorship advantage over this highly abundant coral species at early life stages. This is a key to the creation and maintenance of biodiversity (Chesson 2000) and may help to explain why coral communities have high species diversity that has remained remarkably stable through geological time (Pandolfi and Jackson 2000; 2006).

Species-specificity of larval settlement

In one experiment (Fig. 1.4D), larvae were maintained in water collected near different coral species until differences in settlement rates became apparent (23 days, Fig. 1.5). Notably, the lowest settlement rate was observed in larvae exposed to water from conspecific corals. Settlement was 5.1-11.2 times higher in water collected near other species of corals and 11 times higher in water collected in the open reef flat. (Data are presented as the probability of a surviving larva settling in each treatment; the same qualitative pattern was also seen in the absolute number of settlers in each treatment). These data show that coral larvae avoid or delay settlement when in close proximity to conspecific adults.

As swimming planulae, corals employ a complex set of searching and tasting behaviors to choose a settlement habitat (Ritson-Williams et al. 2009) and they can

distinguish between individual molecules (Morse and Morse 1991) as well as biofilms formed at different depths (Baird et al. 2003). Our results suggest coral larvae are also able to distinguish between water column microbial environments near adult corals of different species. The differential mortality observed in our experiments would be strong enough to create positive selective pressure for the evolution of this behavior. Larvae of many marine species are known to preferentially choose low-mortality settlement habitats (Grosberg 1981), however our observations represent a previously undiscovered form of habitat discrimination in corals. This behavior—in addition to the survivorship patterns we report here—could structure the spatial patterning of coral communities and contribute to the overall maintenance of coral species diversity on tropical reefs.

DISCUSSION

Distance-dependent mortality in marine habitats

Our experiments and observations showed that microbial environments vary over short distances on reefs and influence coral juvenile survival on a scale of centimeters. Patterns of survivorship-with-distance were consistent in space (i.e., between experimental replicates) and time (i.e., between six experiments conducted over three years), but these patterns were not symmetrical in the up-current and down-current directions. Thus, for sessile marine organisms, patterns of spatial mortality caused by biotic factors are nevertheless influenced by physical oceanographic processes. (Similar asymmetric patterns of mortality may also be likely in terrestrial environments with strong physical forcing.)

Marine microbial populations are structured at a very fine spatial scale (Long and Azam 2001); on a reef, this is due to physical factors such as currents (Van Duyl 1985), water exchange through sediments (Hunter et al. 2006), and the effects of coral

colony shape on flow (Kaandorp et al. 2003) as well as biochemical factors including surfactant molecules in coral mucus (Deacon 1979), mucus shedding rate (Brown and Bythell 2005) and quantity (Johannes 1967), and the types of bacteria on the coral surface (Rohwer et al. 2002; Ritchie 2006). These factors must all be considered when predicting how patterns of survivorship-with-distance will take shape on a coral reef and how Janzen-Connell survivorship effects will ultimately structure a reef habitat.

Microbe-mediated, species-specific juvenile mortality

In both coral reefs and rainforests, many potential causes of propagule mortality cannot be easily observed or disentangled from one another through field observations or field experiments alone. In our field experiments, both physical factors and the behavior of animal predators may have varied with distance from the adult coral colonies. This makes our laboratory experiments a powerful complement because laboratory conditions excluded all physical (e.g., shading, water flow) and predatory (e.g., invertebrate predation/grazing, fish scraping/browsing) sources of mortality that might operate in a distance-dependent manner around a coral colony on a reef.

In water collected near adult corals, possible sources of mortality include microbes, viruses, and allelopathic chemicals. While all factors may affect coral survival at any given time, we were able to prevent mortality through the physical removal of bacteria and by using antibiotics; therefore microbes themselves are the most likely cause of mortality in our experiments.

The composition of coral mucus and the bacterial assemblage it hosts are highly specific to each coral species and both will move into the water column around an adult coral (Allers et al. 2008; Wild et al. 2004; Crossland 1987; Brown and Bythell 2005; Ritchie 2006; Johnston and Rohwer 2007; Coffroth 1990). Therefore,

mortality of juveniles in our experiments may have been caused by the transfer of specific pathogenic microbes from adults to juveniles and/or the increased activity of water column or juvenile-associated microbes due to the presence of particulate organic carbon (i.e., mucus) from the adult corals. Nevertheless, under either mechanism, mortality is attributable to species-specific microbial activity caused by the presence of a conspecific adult.

Applying the Janzen-Connell model to coral reefs

The focal species of the current study is a mass-spawning coral capable of dispersing hundreds of kilometers during the larval phase. We therefore examined the survivorship of propagules at increasing distance from adult colonies that were *not* the parents of these juveniles. However, numerous coral species (and a wide range of other reef invertebrates) release brooded planulae that may disperse only millimeters or centimeters from the adult colony before settlement (Jackson 1986).

For every parent coral, a tradeoff exists between sending juveniles broadly to colonize new habitat and restricting dispersal to ensure juveniles find a habitat suitable for the species. The latter phenomenon (known as philopatry) is especially important for species in marginal or discontinuous habitats such as patch reefs and lagoons. Relative to the Pacific, the Caribbean itself is a lagoonal environment that hosts a relatively high proportion of brooding coral species. The importance of philopatry is therefore widely recognized in theoretical treatment of larval dispersal, however, neither brooding reproductive modes nor philopatry in general eliminate the potential for Janzen-Connell effects to occur in a coral species.

Importantly, the current study was motivated by observed density-dependent mortality in a brooding coral (*Siderastrea radians*) whose dispersal distances are so short that approximately 50% of settlers within a 0.5 m x 0.5 m quadrat are produced

by adults within the same quadrat (Vermeij 2005). The results from that study and its follow-up (Vermeij and Sandin 2008) showed that density-dependent and distance-dependent settlement and mortality in a *brooding* coral species occur at a scale of millimeters to centimeters. The results presented here demonstrate that distance-dependent mortality in a *spawning* coral species occurs at a scale of centimeters to meters. Thus, settlement of coral larvae to a habitat with a conspecific adult may be beneficial at one scale (as the presence of conspecifics indicates a habitat is suitable for the species) but detrimental at smaller scales (where nearness to conspecifics becomes detrimental to survival). The point at which staying "close to home" becomes dangerous for a coral larva will vary by species. It follows that some coral species may show clumped distributions due to brooding reproductive modes, yet these individuals may be *less clumped* than would be expected by chance (as is seen in some rainforest tree species; e.g., Wills et al. 2006) due to Janzen-Connell effects.

In sum, the assembly of coral communities is governed not only by larval supply on a scale of centimeters to kilometers, but also by species-specific dispersal and recruitment characteristics on the scale of millimeters to meters, and by species-specific microbial factors on a scale of microns to centimeters. Just as the distribution of seeds, seedlings, and adult trees provides clues to the processes maintaining forest diversity, the patterns of juvenile and adult coral colonies across a reef will reveal how the processes we describe here apply to other coral species, including those whose juveniles are difficult to study experimentally. Notably, because coral larvae can actively select their settlement locations, Janzen-Connell effects may be more apparent at early life history stages in coral communities than in tree communities. Coral reefs therefore provide ecologists with a setting in which to test the overarching generalities—and habitat-specificities—of ecological theories that explain the creation and maintenance of species diversity in tropical ecosystems.

MATERIALS AND METHODS

Study species

Montastraea faveolata planulae were reared from gametes collected during five mass spawning events on the island of Curaçao in the southern Caribbean. Gametes were collected by placing weighted nylon cones over spawning corals at 7-9 m depth at Snake Bay (September 2007 and October 2008, 12°13’90”N, 68°99’82”W), Playa Kalki (September 2008 and September 2009, 12°37’48”, 69°15’85”W), and the Water Factory (October 2009, 12°10’91”N, 68°95’49”W). Embryos were raised to the swimming planula stage using methods developed previously for mass spawning coral species (Vermeij et al. 2006; Vermeij et al. 2009). Glass microscope slides were placed at the bottom of the rearing containers prior to the onset of settlement. All field experiments, microbe counts, and water collections were conducted on the reef flat directly to the south of Carmabi Buoy Zero (12°12'35"N, 68°97'10"W) at depths of 5-6 m. The same adult coral colonies were used for all experiments and collections. Water current at the study site is extremely consistent in direction because it is driven by the constant easterly trade winds (Van Duyl 1985; Table 1.1).

Field experiments

Lab-reared *Montastraea faveolata* larvae were allowed to settle and metamorphose onto glass microscope slides (supplementary information Fig. 1.1A). The location of recruits on each slide was mapped before they were deployed to the field. Slides were secured underwater to small limestone tiles (2 cm x 4 cm) with plastic tie-wraps (supplementary information Fig. 1.1B). Tiles were held to PVC scaffolds using a plastic expansion plug. Tie-wraps touched only the edges of the

microscope slides and the limestone tile, but not the surface of the microscope slide holding the recruits.

Distance-dependence experiment: PVC spokes (15 cm tall, 2 m long) were installed extending up-current (n = 7) and down-current (n = 8) from adult *M. faveolata* coral heads (supplementary information Fig. 1.1B-D). A total of 454 recruits on 105 slides were deployed; slides were secured horizontally at distances of 3, 6, 12, 24, 48, 96, and 192 cm in each direction. Survivorship of each recruit was scored daily for six days.

Species-specificity experiment: PVC tees (15 cm tall, 12 cm wide) were installed next to boulder-shaped coral colonies of different species (n = 8 per treatment) to hold coral recruits vertically within 2 cm of the surface of the adult coral (supplementary information Fig. 1.1E-G). Tees were placed such that the PVC bar holding the recruits was in-line with the prevailing current. A total of 113 recruits were deployed. Treatments were *M. faveolata*, *Siderastrea siderea*, *Diploria strigosa* and a control (open area of a sand flat). Final survivorship was scored after four days.

Microbe counts on the reef

Water samples were collected at each experimental distance up- and down-current from three of the adult corals used in the distance-dependence field experiment and at the 96 cm distance up- and down-current from seven of the experimental corals. Samples were fixed with paraformaldehyde (final concentration 2% v/v) within 30 min of collection and microbes were visualized using standard methods for enumerating microbes and viruses from reef waters (Dinsdale et al. 2008). At least 20 fields of view were analyzed for each sample (NIH ImageJ).

Laboratory experiments

Four experiments were conducted over a three-year period using methods from previous studies of coral planula survivorship and behavior (Vermeij et al. 2006; Vermeij and Sandin 2008). Water collections for experiments were made in triplicate at the experimental reef within 2 cm of the surface of the coral colonies. Swimming larvae were pooled from multiple rearing containers, rinsed twice with sterile seawater (0.22 μm–filtered) to dislodge any loosely associated microbes, and allocated randomly into experimental replicates (15 cm Petri dishes). All experiments were arranged in a randomized block design. Surviving and settling larvae were counted at regular intervals. Settlers were considered those that had begun forming a calcified skeleton.

Statistical analysis

For all experiments, we used survivorship data taken over the course of each experiment to compare the relative fits of different demographic models. The proportion of juveniles surviving each time step was assumed to be determined by a single demographic parameter, the daily probability of juvenile mortality (μ), which is binomially distributed about the expected value. (For settlement data and the species-specificity field experiment, data were taken at a single time step but otherwise analyzed identically.) The best-fit values of μ under each experimental treatment were then compared using a likelihood approach to determine the most parsimonious number of parameters to describe the observed data (Hilborn and Mangel 1997).

Treatment effects were considered significant when the data were best explained by multiple parameters (i.e., multiple values of μ), given the penalty for added parameters. When two models had an equal number of parameters, the best

combination of parameters was selected based on an assumption of equal Bayesian prior expectations. For the distance-dependence field experiment, the number of starting coral recruits varied between experimental replicates. A density-dependence parameter was therefore included in the demographic models. The best-fit model was one that had separate density-dependent terms for the up-current versus down-current treatments.

For all analyses, the differences in likelihood values between statistically distinguishable treatment groups were used to calculate *P*-values using a Chi-square distribution with one degree of freedom. All *P*-values reported here represent the significance level of a comparison between the best-fit model and the next-best-fit model. Confidence intervals and standard error values were calculated within the likelihood model. All analyses were performed in R. Additional details of this statistical approach are described elsewhere (Vermeij and Sandin 2008; Vermeij et al. 2009).

AUTHOR CONTRIBUTIONS

All authors designed the research and each contributed a new method or analytical tool. K.L.M. and M.J.A.V. performed the research, K.L.M and S.A.S. analyzed the data, and K.L.M. wrote the paper.

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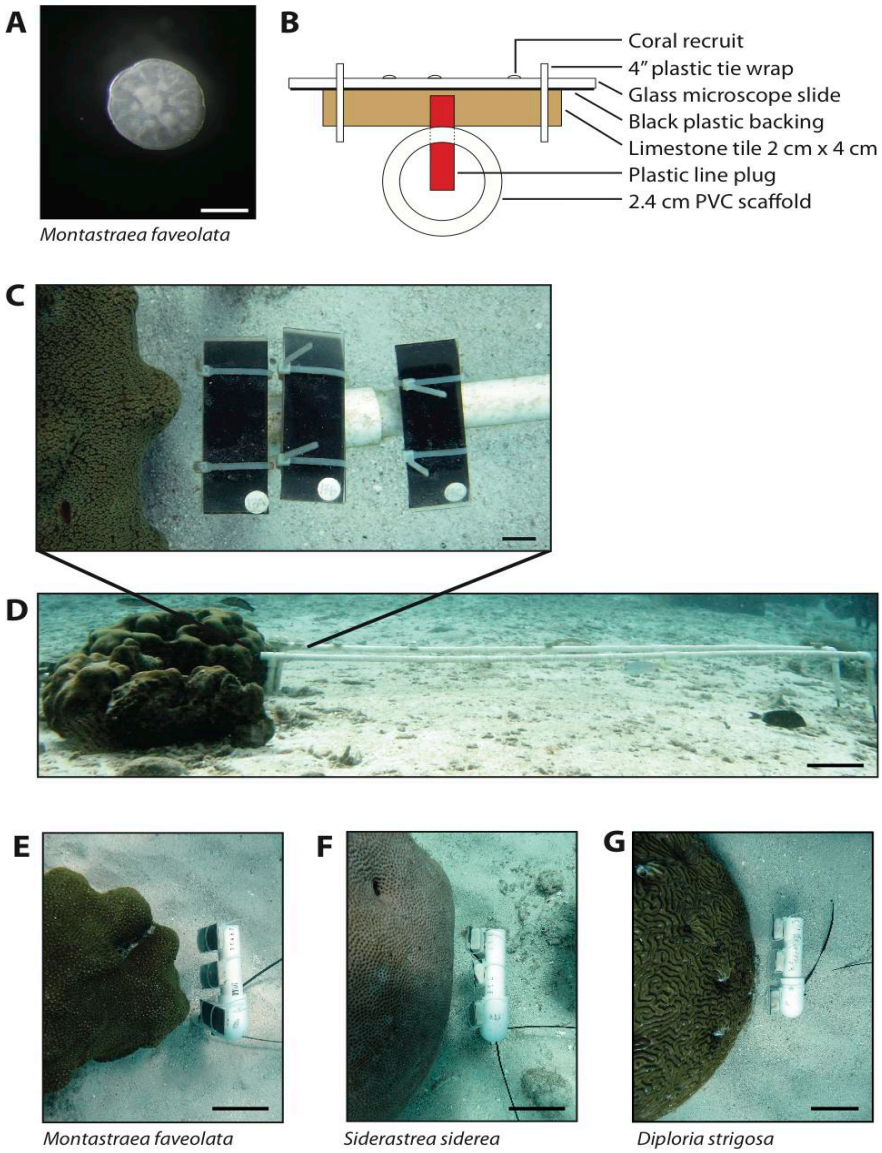

Figure 1.1. Experimental methods. Glass microscope slides were used to deploy settled coral recruits to different locations on the reef. (A) Newly-settled recruit of the study species *Montastraea faveolata* with mouth and tentacles visible. (B) Method used to deploy newly-settled recruits to experimental locations on the reef. (Plastic tie-wraps do not contact the front surface of the glass microscope slide.) (C) Close-up of distance-dependence field experiment showing microscope slides with coral recruits placed at increasing distances from adult colonies. (D) Side view of distance-dependence experiment. Total length of each PVC spoke is 2 m. (E-G), Species-specificity experiment. Each PVC tee with three microscope slides comprises a single experimental replicate. The current moves from the top to bottom of these photos. Scale bars lengths are 0.5 cm (A); 1 cm (C); 10 cm (D); 5 cm (E-G).

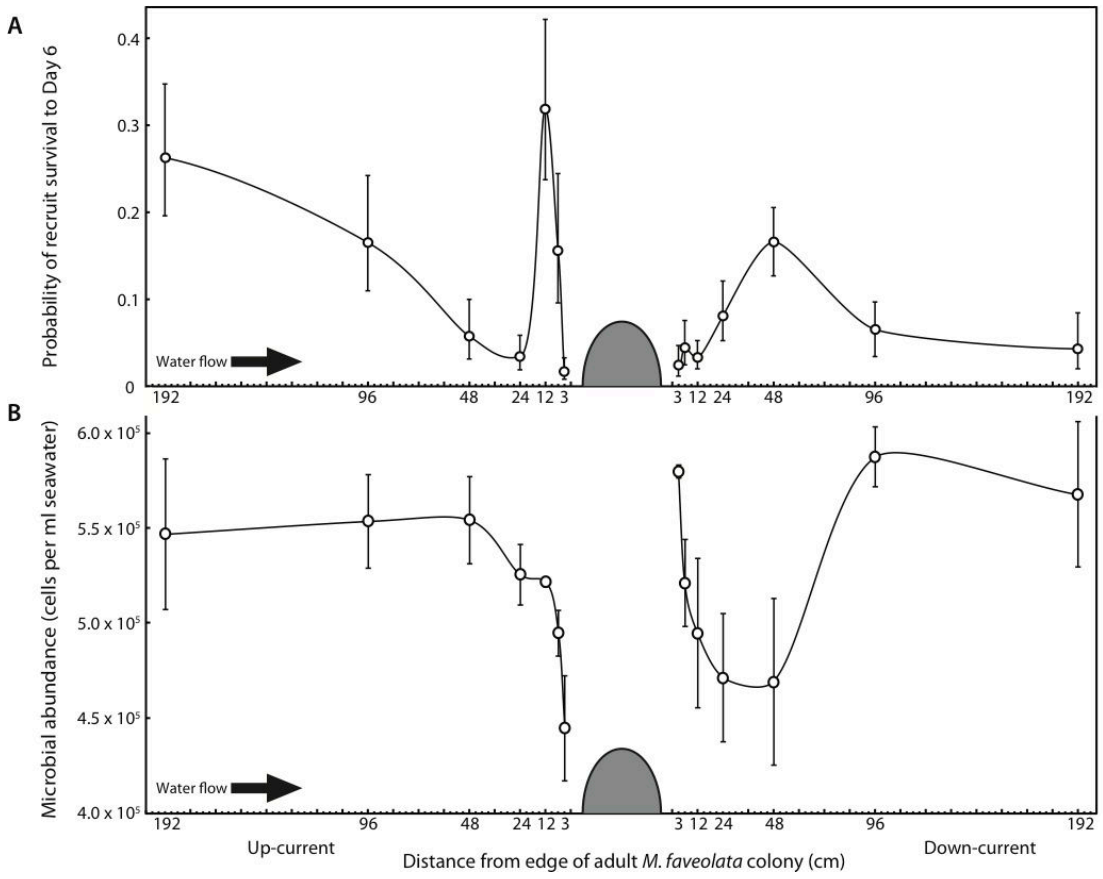

Figure 1.2. Survivorship of *Montastraea faveolata* coral recruits and seawater microbial abundance vary with distance from conspecific adults. (A) Probability of survival (± 1 S.E.M) of *M. faveolata* recruits placed at seven distances up- and down-current from adult *M. faveolata* colonies ($n = 7$ in the up-current direction, $n = 8$ in the down-current direction, 454 total recruits). Probability of survival was significantly lower down-current compared to up-current ($P = 0.004$). (B) Average microbial density (± 1 S.E.M.) up- and down-current from the same adult coral colonies depicted in A ($n = 3$ except at 96 cm distances, where $n = 7$). For both panels, the line is shown only to guide the eye.

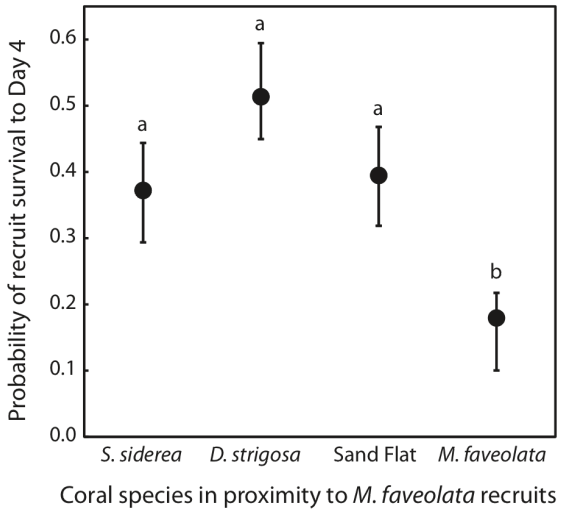

Figure 1.3. Survivorship of *M. faveolata* recruits is reduced in the presence of conspecific adults. Letters denote treatments with significantly different probabilities of mortality in a likelihood-based model ($P = 0.004$). Vertical bars = 95% C.I., $n = 8$.

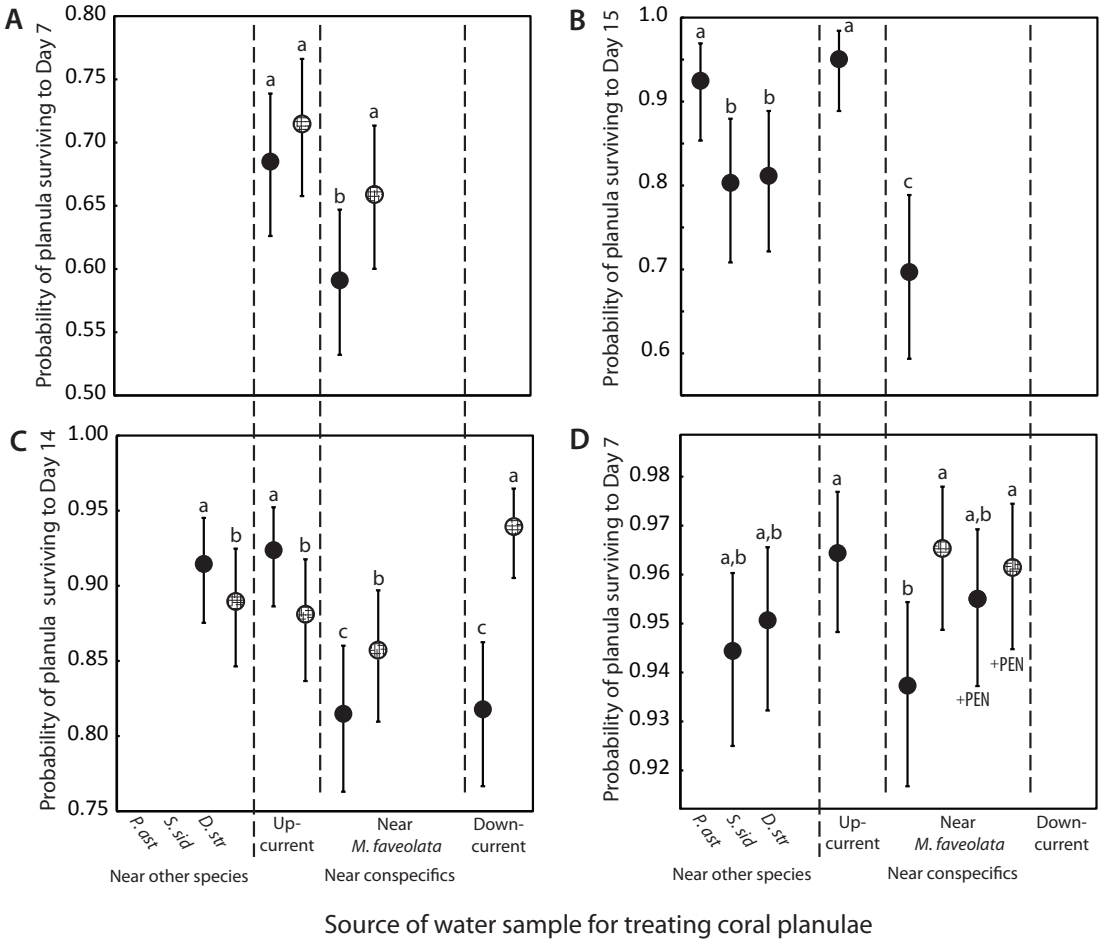

Figure 1.4. Survivorship of *M. faveolata* planula larvae is reduced in water collected near conspecific adults. Effects of distance (A-D), species (B-D), and microbes (A, C-D) on survivorship of juvenile corals near adult colonies. Black circles = raw water, Hatched circles = filter-sterilized water, "+PEN" = penicillin-treated water. *P. ast* = *Porites astreoides*, *S. sid* = *Siderastrea siderea*, *D. str* = *Diploria strigosa*. Lowercase letters denote treatments with significantly different probabilities of mortality. Vertical bars = 95% C.I. (A) 10 planulae per replicate, $n = 26$, $P = 0.004$. (B) 40 planulae per replicate, $n = 5$, $P < 0.001$. (C) 25 planulae per replicate, $n = 10$, $P = 0.001$. (D) 40 planulae per replicate, $n = 16$, $P = 0.04$.

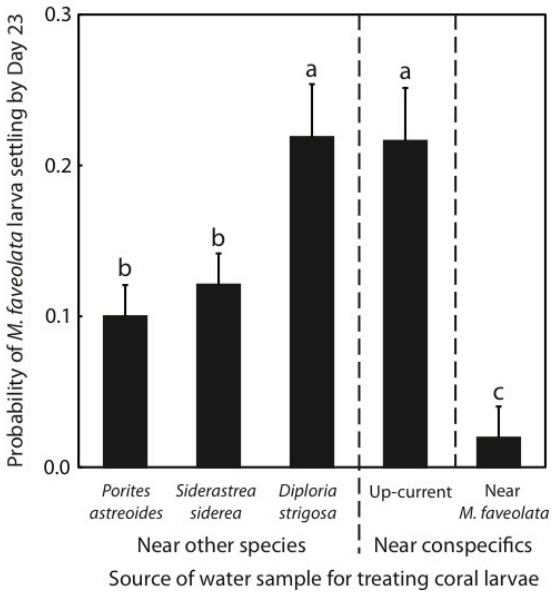

Figure 1.5. Settlement of surviving *M. faveolata* planulae is reduced in water collected near conspecific adult colonies. Settlement data were recorded in the experiment depicted in Fig. 1.4B. Letters denote treatments with significantly different probabilities of settlement in a likelihood-based model. Data are shown as the mean probability of survivorship and bars represent 1 S.E.M., 40 planulae per replicate, $n = 5$, $P = 0.027$.

Table 1.1. Wind speed, wind direction, and wave direction during distance-dependence field experiment with *M. faveolata* settlers. Data were obtained from the Meteorological Service of the Netherlands Antilles and Aruba. Data are for Willemstad, Curaçao, 4 km from the study site at CARMABI Buoy 0. Wind direction during the field experiment ranged from 8.5 to 16.9 knots from the E, ESE or ENE direction and never reversed direction, producing a consistent current on the shallow reef flat moving in a west-northwest direction.

Curaçao, Lat: 12.2, Lon: -68.97									
Time of Day →	**01h**	**04h**	**07h**	**10h**	**13h**	**16h**	**19h**	**22h**	**AVG**
Date	**Wind speed (knots)**								
22.09.2007	17	17	14	15	11	16	14	15	14.88
23.09.2007	14	14	13	12	7	14	13	12	12.38
24.09.2007	11	9	10	8	8	10	9	13	9.75
25.09.2007	13	13	12	9	11	17	19	18	14.00
26.09.2007	15	14	8	7	7	10	15	15	11.38
27.09.2007	15	17	15	15	10	15	16	17	15.00
28.09.2007	17	17	13	14	16	20	18	20	16.88
	Wind direction								
22.09.2007	ESE	ESE	ESE	E	E	ENE	ENE	E	
23.09.2007	ESE	ESE	ESE	ESE	ENE	NE	ENE	E	
24.09.2007	E	ESE	ESE	ESE	E	ENE	ENE	E	
25.09.2007	ESE	ESE	ESE	ESE	ENE	ENE	E	ESE	
26.09.2007	ESE	SE	SE	SE	ESE	E	E	ESE	
27.09.2007	ESE	ESE	ESE	ESE	E	ENE	E	E	
28.09.2007	ESE	ESE	ESE	E	ENE	E	E	ESE	
	Wave direction								
22.09.2007	E	E	E	E	E	E	E	E	
23.09.2007	E	E	E	E	E	E	E	ENE	
24.09.2007	E	E	ENE	ENE	ENE	ENE	ENE	E	
25.09.2007	E	E	E	E	E	E	E	E	
26.09.2007	E	E	E	E	ENE	ENE	ENE	E	
27.09.2007	E	E	E	E	E	E	E	E	
28.09.2007	E	E	E	ENE	ENE	ENE	ENE	E	

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CHAPTER 2

Observation of ciliates grazing around early post-settlement coral recruits

K. L. Marhaver

As part of a survivorship experiment, I deployed *Montastraea faveolata* settlers onto a shallow coral reef sand flat (Chapter 1 of this dissertation). These juvenile corals were reared during the fall mass coral spawning and were settled onto glass microscope slides, which were secured underwater to limestone supports and PVC scaffolds. When examined in the lab after five days on the reef, each slide hosted numerous red-brown ciliates, approximately 0.5 mm long (Fig. 2.1A and B).

Ciliates were actively grazing on the particulate debris that had accumulated on the surface of the slides during their incubation on the reef (Fig. 2.1B). Interestingly, they appeared to be grazing not only on the glass surfaces but also on the outer edges of the coral recruits themselves (Fig. 2.1C and D). The coral recruits were sufficiently well developed that their outer skeletal wall was relatively tall. Ciliates were observed picking repeatedly at the outside of these skeletons, however no ciliates were observed within the interiors of the corallite walls, regardless of whether the recruit was alive (Fig. 2.1D) or dead (Fig. 2.1C). The ciliates were present on all microscope slides examined (n = 20), at an abundance of 2-10 individuals per cm^2, indicating their grazing activity might significantly affect the abundance of particulate debris on these surfaces.

The ciliates I observed were only visible when viewed with a stereomicroscope using a white background and cast light. Typically, *M. faveolata* recruits are viewed against a dark background because they are translucent and colorless, therefore the presence of these ciliates may have been missed by other researchers in the past.

In general, very few coral-protozoan interactions have been reported (e.g., Kramarsky-Winter et al. 2006). Ciliates are known to infect adult corals of multiple species in the Caribbean (Croquer et al. 2006) and ciliates have been reported to cause

mortality of juvenile corals in captive settings (Cooper et al. 2008). Ciliates are also known to associate with the coral disease brown band syndrome in the Pacific (Bourne et al. 2008).

The observations here represent the first report of ciliates interacting with juvenile corals in the wild. In this case, no damage to the recruits was observed, despite the abundance of ciliates. It is therefore not clear whether these organisms positively or negatively affect the coral recruits nearby. Ciliates are important grazers of bacteria and detritus in other ecosystems (as in Gonzalez et al. 1990). Micro-scale grazing on coral reef surfaces might facilitate coral recruit survival by reducing the number of bacteria, particular organic matter, microalgae, microfungi, and/or algal spores on settlement surfaces. The high abundance of ciliates that recruited to these surfaces in a short period of time indicates that they could play a significant role in ecological processes occurring on the millimeter scale.

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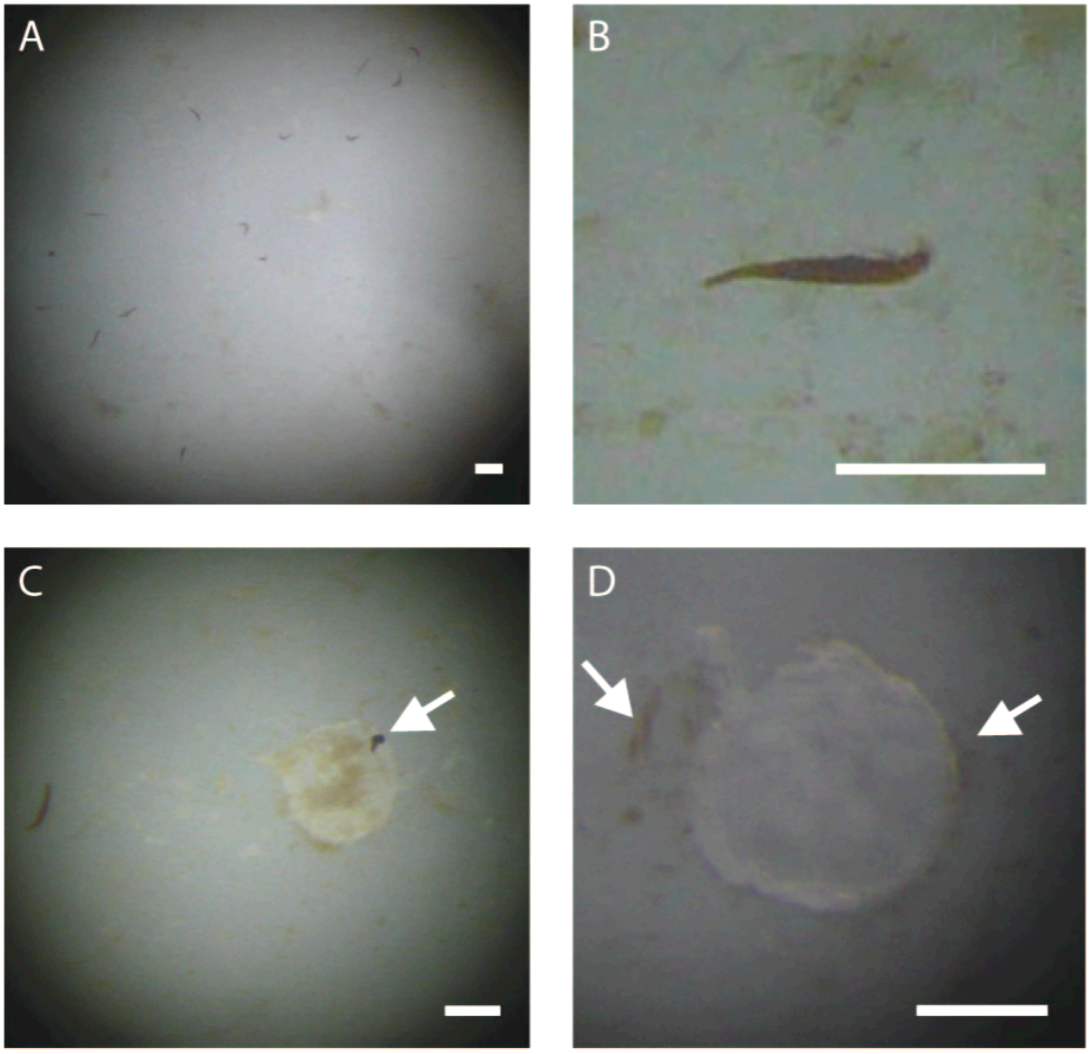

Figure 2.1. Ciliates grazing on the outer skeletal walls and in the close vicinity of coral settlers. Glass microscope slides were deployed onto the reef for five days and observed with a stereomicroscope. (A) Typical density of ciliates on glass microscope slides. (B) Close-up view of individual ciliate. (C) A single ciliate is visible on the outer surface of the skeleton from a dead corallite (white arrow). (D) Two ciliates (white arrows) approaching a live *M. faveolata* settler. Scale bars represent approximately 500 μm.

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CHAPTER 3

Effects of water column microbial environments and penicillin treatment on larval swimming and settlement behaviors in the Caribbean coral *Montastraea faveolata*

K. L. Marhaver, M. J. A. Vermeij, and S. A. Sandin

ABSTRACT

A crucial stage in the life of many marine organisms is the larval period, in which complex swimming and searching behaviors are used to locate appropriate settlement habitats. During dispersal, microbes (Bacteria and Archaea) in the water column and on settlement surfaces provide richly detailed, site-specific navigational information for pelagic larvae. A focus on settlement choice has left early dispersal behaviors—navigating the water column, swimming across the benthos, and sampling habitats—relatively understudied despite the fact that these locomotory behaviors often depend on microbial signals and serve as informative response variables for studies of ecology, evolution, and toxicology. Here we describe the locomotory and settlement behaviors of *Montastraea faveolata* planula larvae ("planulae") reared in a suite of microbial environments altered by water filtration and antibiotics. In filter-sterilized seawater, planulae increase the incidence of swimming, potentially to escape a habitat lacking navigational cues. In seawater treated with penicillin, planulae exhibit an abnormal, continuous swimming behavior and ultimately fail to settle and complete metamorphosis. This effect persists through time, meaning that early alterations in behavior can be predictive of settlement failure. Finally, behavioral differences observed between experiments suggest that behavior partially depends on the age and origin of coral planulae. The abnormal behaviors of planulae in sterile environments indicate that microbes (Bacteria and Archaea) serve an important role in signaling, development, and navigation of coral juveniles.

INTRODUCTION

The black box of larval behavior

The larvae of many marine organisms are capable of actively choosing their settlement habitats by responding to physical, chemical, and biological cues, however their small size and the cryptic nature of these behaviors make the study of dispersal and settlement processes difficult. Reef corals (Cnidaria: Scleractinia) produce swimming planula larvae ("planulae") that demonstrate remarkable discrimination in their choice of settlement substrate. The process of coral recruitment—swimming toward the reef, searching the benthos, settlement to the substrate, metamorphosis, initiation of skeletogenesis, and successful survival—is an extremely sensitive life history process in which the majority of mortality occurs, yet the recovery of coral reefs will depend heavily on the successful execution of this series of behaviors. In brooding coral species, the dispersal process typically involves traveling a far shorter distance than in spawning species (Jackson 1986), however both life history strategies involve navigation across the microbial landscape of the reef and a search for a suitable settlement surface. Most studies of larval behavior in aquatic ecosystems have examined this settlement choice exclusively. Therefore, navigation, swimming, and searching behaviors are largely unexamined, despite the fact that these behaviors occur over the majority of the distance covered and time elapsed during the larval dispersal phase (e.g., Graham et al. 2008).

Specificity of coral settlement behavior

Studies of coral larval behavior traditionally have examined settlement preference, as this behavior is ecologically important and can be observed relatively easily at a single time point. UV irradiance (Gleason et al. 2006), sediments (Hodgson

1990), and some species of macroalgae (Kuffner et al. 2006; Birrell et al. 2008) can deter settlement. Vertical or cryptic surfaces, which may be covered by crustose coralline algae (CCA), are often preferred to horizontal, light-exposed surfaces (e.g., Vermeij et al. 2006; Price 2010). Chemical compounds isolated from CCA can induce settlement and metamorphosis in some species of coral (Morse and Morse 1991; Morse et al. 1994; Heyward and Negri 1999; Kitamura et al. 2009), but CCA is not the preferred substrate for all species (Lewis 1974; Golbuu and Richmond 2007), nor are all CCA types suitable settlement substrates (Ritson-Williams et al. 2010). Corals that settle to CCA demonstrate a preference for those CCA species on which they survive better (Harrington et al. 2004), though mortality is high even on preferred types of CCA (Ritson-Williams et al. 2010).

Settlement has also been attributed to bacteria isolated from CCA and to microbial biofilms in the absence of CCA (Negri et al. 2001; Webster et al. 2004), leaving the relative importance of algal versus microbial settlement cues a subject of ongoing debate. The importance of biofilms for settlement is widely appreciated for other organisms (Keough and Raimondi 1995), but the effects of biofilms—and microbes in general (i.e., the domains Bacteria and Archaea)—on coral larval biology have been studied significantly less than the effects of CCA. In one exception, a study of five coral species showed that planulae were able to distinguish between biofilms that were cured in shallow or deep habitats; each species settled preferentially to the biofilm from its adult habitat range (Baird et al. 2003). Corals can therefore use surface-bound microbial communities alone as navigational information. This is likely true for water column microbial communities as well.

Given the high specificity of planula settlement preferences, pre-settlement locomotory behaviors should be similarly sensitive to environmental variables, however few studies have examined the behaviors of coral planulae during the pre-

settlement phase. As with settlement choice, locomotory behaviors are expected to vary based on biological, chemical, and physical factors, but these behaviors are rarely studied. This is partially due to the difficulty inherent in rearing and maintaining spawned coral larvae.

Swimming behaviors of coral larvae

From a small number of studies, we know that abiotic factors such as pressure (Stake and Sammarco 2003) and salinity (Vermeij et al. 2006) affect the incidence of swimming in coral larvae. With collaborators, we also recently demonstrated for the first time that coral larvae swim toward a point source of coral reef sounds (Vermeij et al. 2010). Together, cues such as salinity, pressure, and sound likely help a navigating larva to locate a reef, at which point finer signal resolution is needed. Microbial communities are heterogeneously distributed across coral reefs and may vary at the milimeter scale, making them an additional source of potential navigational information for coral planulae in the water column (Chapter 1, this dissertation). In general, the role of microbes (Bacteria and Archaea) in animal behavior and ecology is becoming an increasingly important area of research because human activities have driven the increase in microbial abundance and pathogenic potential (i.e., the "microbialization") of ocean ecosystems (Dinsdale et al. 2008; Sandin et al. 2008).

Emerging paradigms in coral microbiology

Recent studies of coral microbiology have revealed that coral-microbe interactions structure ecosystems to a greater extent than we previously realized (Rohwer and Youle 2010). These studies provide evidence that coral-microbe

interactions during the larval stage are equally important for ecosystem assembly and functioning (Chapter 1, this dissertation).

As adults, corals host tremendous microbial diversity within their mucus, tissue and skeleton. The coral "holobiont" encompasses symbiotic dinoflagellates ("zooxanthellae"), bacteria (Knowlton and Rohwer 2003), archaea (Wegley et al. 2004), protists (Kramarsky-Winter et al. 2006), endolithic algae (Fine and Loya 2002), fungi (Bentis et al. 2000), and viruses (Marhaver et al. 2008; Vega Thurber et al. 2009). The coral holobiont may even encompass additional species of cnidarians (Pantos and Bythell 2010). Corals became a focus of microbial community characterization (reviewed in Rosenberg et al. 2009; Rohwer and Youle 2010) due to their ecological importance and the emergence of new coral diseases and disease-like conditions (Harvell et al. 2002; Harvell et al. 2007).

It is now widely accepted that microbial communities form stable, species-specific associations with their coral hosts (Rohwer et al. 2001; Rohwer 2002; Bourne and Munn 2005; Koren and Rosenberg 2006; Mouchka et al. 2010; Rohwer and Youle 2010). These communities may vary between a coral's mucus, skeleton, and tissue, and the surrounding seawater (Cooney et al. 2002), suggesting that microbes serve beneficial functions for the coral. Demonstrated functions of coral-associated bacteria include production of antibiotic compounds (Ritchie 2006) and nitrogen fixation (Lesser et al. 2004; Lesser et al. 2007). Likely additional functions include space occupation to prevent pathogen invasion and scavenging of vitamin compounds (Rohwer and Kelley 2004).

Changes in the coral microbial community may represent adaptive responses to environmental flux (Reshef et al. 2006; Rosenberg et al. 2007). Alternately, changes in the coral-associated microbiota may represent a loss of community function due to individual pathogens (Patterson et al. 2002; Ben-Haim et al. 2003; Barash et al. 2005;

Thompson et al. 2006; Denner et al. 2003) or an overall disruption to community stability (Kline et al. 2006; Kuntz et al. 2005; Smith et al. 2006; Rohwer and Youle 2010). The microbial consortium on a given coral is thought to contain potential pathogens at all times, which are normally kept in balance by other microbes (Ritchie 2006; Rypien et al. 2010) and or carbon limitation imposed by the coral itself (Ducklow and Mitchell 1979; Pascal and Vacelet 1981). The biochemistry, consistency, and shedding rate of coral mucus, which varies by species and with environments, affects the composition of the microbial consortium (Brown and Bythell 2005) and the coral's ability to avoid microbial pathogenesis (Ritchie 2006; Johnston and Rohwer 2007).

Coral-microbe interactions at the larval scale

Given the diversity of coral-microbe interactions and their importance for reef ecology, a handful of researchers have begun to examine interactions between microbes and coral planulae. Apprill et al. (2009) showed that the Pacific coral *Pocillopora meandrina* establishes associations with bacteria from the *Roseobacter* clade during the early days of the larval phase. The authors suggest these bacteria, localized within the endoderm, may play a role in the settlement. Meanwhile, Sharp et al. (2010) showed that six different mass-spawning Caribbean coral species, including *Montastraea faveolata*, do not establish microbial associations until after settlement and metamorphosis. This supports the notion that bacterial acquisition is mediated by the host itself and is not a consequence of passive colonization of the host by water column microbes.

In a survivorship experiment with planulae of the Pacific coral *Montipora capitata*, Vermeij et al. (2009) showed that the antibiotic penicillin prevented juvenile mortality when planulae were exposed to fragments of macroalgae. Microbial

communities derived from different reef microhabitats also differently affect survivorship and settlement rates of *M. faveolata* planulae (Chapter 1, this dissertation). Differences in settlement rates suggest that pre-settlement locomotory behaviors may also vary between microbial environments, but the effect of microbes on planula locomotion has not been examined previously.

Here I present evidence that microbial interactions affect not only the initiation of settlement and metamorphosis in coral planulae, but also the swimming and searching behaviors preceding settlement. The diversity of known interactions between adult corals and their microbial associates was a key motivation for examining the effects of microbes on coral planulae.

Focal species

Montastraea faveolata is massive reef-building coral, which—along with the closely related species *M. annularis* and *M. franksi*—builds a large proportion of the shallow framework of Caribbean reefs (Fig. 3.1A). *M. faveolata* is a hermaphrodite and reproduces by annual mass spawning (Fig. 3.1A), releasing egg-sperm bundles in synchrony approximately 6 to 8 days after the full moon in August, September, or October. While spawning times are highly predictable (Levitan et al. 2004), rearing the resultant planula larvae (Fig. 3.1B) can be extremely difficult as these larvae are very small and susceptible to early, catastrophic population crashes. Nevertheless, in recent years, improved techniques for rearing planulae (Fig. 3.1C) have allowed researchers to begin examining their physiology (Voolstra et al. 2009) and behavior (Vermeij et al. 2006).

RESULTS AND DISCUSSION

Two separate experiments were conducted to determine how water column microbial environments affect planula locomotory behavior. In experiment 1, microbial communities in the water were collected from different locations on the reef (near and far from adult coral colonies) and manipulated with filter sterilization. In experiment 2, the microbial communities were collected from similar locations and manipulated with antibiotic treatment, filter sterilization, or both. Behaviors were score by eye at regular time intervals and categorized into mutually exclusive categories as described below.

Experiment 1: Effects of the bulk microbial community on swimming behavior

Water was collected near and far from adult *M. faveolata* colonies and microbial communities were removed using filter-sterilization. Swimming behavior was scored on a daily basis for seven days. On day 1, the percentage of surviving planulae that were actively swimming (whether in the water column or on the bottom) was significantly higher in both filter-sterilized water treatments relative to the control treatments, regardless of where the water was collected (Figs. 3.2 and 3.3, $P < 0.0001$).

Prior to this experiment, only one study of swimming behavior had been conducted with *M. faveolata* planulae. Vermeij et al. (2006) showed that a decrease in salinity increased the incidence of swimming in *M. faveolata* planulae, which was coupled with a decrease in survivorship and a shortened larval duration (i.e., positive geotaxis began sooner). Based on these observations, the authors hypothesized that planulae increase the rate of swimming when they experience adverse conditions in order to escape these environments. The significantly smaller size of settlers surviving low-salinity treatments suggested that escaping adverse habitats carries

with it a significant energetic cost. In the results presented here, increased swimming in sterilized seawater may represent a similar attempt to avoid adverse pelagic conditions. Despite the fact that planula survivorship is typically expected to increase when microbes are removed, the lack of microbes entirely may be perceived by a dispersing larva to represent an unsuitable (or completely foreign) habitat because a completely microbe-free habitat would never be encountered in the ocean. These data represent the first observation that a biological factor affects the locomotory behavior of planulae while in the water column.

Experiment 2: Effects of antibiotics and water filtration on swimming behavior

A second experiment was conducted to determine whether different levels of water filtration affect planula behavior, and to determine whether inactivating microbes using penicillin would differentially affect behavior relative to filtration. Water was collected near adult *M. faveolata* colonies and subjected to four levels of filtration: control, 0.8 μm (which removes large particles and some large bacteria), 0.22 μm (which removes bacteria but few viruses) and 0.02 μm (which removes all bacteria and viruses, leaving only dissolved compounds to pass through the filter). Each filtration treatment was repeated with penicillin at a standard working concentration (66 μg/ml).

Profiles of planula behavior through time are shown in Fig. 3.4. Even in culture conditions lacking ideal settlement substrates, *M. faveolata* planulae exhibit a normal progression of behaviors, including settlement. First, a planula swims at the water surface for a few hours or days. It then transitions from swimming in the water column to swimming on the bottom, with occasional pauses. It will eventually attach itself loosely to the bottom. Shortly thereafter, it undergoes metamorphosis, attaches

permanently to the bottom, and begins calcification. This progression can be seen in Fig. 3.4. (upper left panel).

On day 1 of this experiment, the proportion of planulae swimming at the bottom of the containers was significantly higher in penicillin-treated water than in control treatments (Fig. 3.5, upper right panel). While this could be interpreted as normal behavior, this positive geotaxis occurred unusually early. Swimming on the water surface also varied significantly between each control and penicillin treatment within a filtration level, but penicillin did not induce this effect in a consistent direction (Fig. 3.5, upper left panel).

The inactivation of microbes by penicillin appears at first glance to cause a similar behavioral change to filter-sterilization in experiment 1, however in experiment 2, filter sterilization did not alter swimming behavior (Fig. 3.5, upper left panel, black circles under "Raw" and "0.22 um" treatments). This may be due to differences between the experiments. Experiment 1 was conducted with slightly older planulae that were reared from gametes collected from a less intact reef and planulae in this 1 were used at a slightly lower experimental density. Additionally, the behavioral difference between years may be partly due to the direct influence of parent colonies on juvenile behavior. Because these experiments were conducted in different years, each cohort of juvenile corals was drawn from a different set of adult colonies.

In experiment 2, penicillin had a much more dramatic effect on behavior than filter sterilization (Fig. 3.4). The effect of penicillin on swimming rate was not only immediate but also persisted through day 7 (Fig. 3.5, bottom right panel, $P = 0.002$). Mechanisms that may have mediated this effect are discussed below.

Effects of microbes and antibiotics on settlement behavior

Planulae had begun settlement and metamorphosis by day 7 of experiment 2 (Fig. 3.5). Interestingly, the method of microbe removal affected the rate of settlement. Filter-sterilization alone (0.22 μm/Raw) did not cause a difference in settlement relative to the unfiltered control, but penicillin alone eliminated settlement (Fig. 3.6, black bars under "Raw").

Furthermore, penicillin affected settlement regardless of filtration level. By day 7, planulae in all non-antibiotic treatments had begun to settle, but penicillin virtually eliminated settlement, regardless of filtration level (Fig. 3.6, $P < 0.0001$). Vermeij and colleagues observed a delay in settlement when raising *Montipora capitata* planulae with and without the antibiotic ampicillin (Vermeij et al. 2009). Therefore, larvae of two coral species treated with two different antibiotics exhibited similar abnormal behaviors.

In experiment 2, settlement in the absence of all viruses and microbes (0.02 μm filtration) was significantly higher than in the other filtration treatments. It is not clear why this effect occurred, however it suggests that planulae may somehow benefit from this high level of filtration. Filters with extremely small pore sizes are known to disrupt organic particles and this may have released useful dissolved organic compounds into the seawater.

Visualizing altered locomotory behaviors

The changes in settlement and swimming rate in penicillin treatments was also marked by abnormal locomotory behavior. In the laboratory, coral planulae typically spend a short period of time (1-2 days) at the water before beginning to swimming and search at the bottom of the Petri dish. This searching behavior includes brief pauses on the substrate followed by changes in direction and/or a takeoff back into the water column. In our experiments, planulae in all penicillin

treatments exhibited a continuous searching behavior with faster swimming, and fewer pauses or turns (Fig. 3.7). This abnormal behavior persisted for a full 30 days. The loss of resting behavior therefore appears to be predictive of a loss of settlement behavior. Importantly, this means that early alterations in larval behavior may be a metric researchers can use to determine whether a habitat is suitable for coral larvae. Furthermore, these results suggest that recruitment failure on coral reefs may have a significant behavioral component that has previously gone unrecognized.

Possible mechanisms of antibiotic-mediated behavioral change

Penicillin affected planula behavior differently than filter-sterilization of the treatment water by causing both early changes in behavior and long-term elimination of normal settlement behaviors. A number of potential mechanisms could mediate this effect.

First, penicillin may have eliminated key water column or surface-attached microbial communities needed for normal navigation and settlement behavior in culture. *M. faveolata* larvae will readily settle to artificial substrates including glass, limestone, and the polystyrene surfaces of Petri dishes. Perhaps a key microbial biofilm on these surfaces is required for adhesion and metamorphosis. To date, no studies have examined the influence of individual bacterial species on the pelagic behavior of coral planulae so it is not known whether the dysfunctional behavior of antibiotic-treated larvae would have resulted from the absence of individual bacterial cue species or a generic bacterial community cue.

Second, the penicillin compound itself may have interacted directly with the larval tissue to cause altered neuronal signaling and changes in behavior. Although penicillin is generally considered to be a low-toxicity compound, invertebrate larvae

are notoriously sensitive to exogenous compounds that may target neuronal and hormonal signaling pathways in unexpected ways.

Third, penicillin may have altered behavior by killing endosymbiotic bacteria or bacteria attached to the planula surface prior to the start of the experiment. Researchers have shown that *M. faveolata* planulae do not appear to be colonized by bacteria during the larval phase (Sharp et al. 2010), but it is possible that short-term interactions may have escaped detection in the previous study and this possibility is therefore presented here for thoroughness. Surface-attached or endosymbiotic microbes may help mediate environmental signaling cascades, they may modulate developmental pathways key for normal behavior later on, or they may perform important metabolic functions for coral-associated larvae. These phenomena are known in other model systems (Gilbert and Epel 2008) and may occur during the development of corals as well.

Microbes as information for corals, coral behavior as information for scientists

The settlement behavior of coral planulae represents the single most crucial animal behavior for the successful recovery of degraded coral reefs, yet the locomotory behaviors preceding settlement are rarely examined. The results presented here show that planula behavior is mediated by exogenous biological and chemical factors, and that behaviors are variable enough for researchers to detect by eye or by using videographic analysis. Microbial communities are heterogeneously distributed across reef environments and therefore represent a potential source of behavioral cues for navigation. The loss of normal navigation behaviors may be an early indicator that a habitat is unsuitable for settlement because it has either disrupted planula development or simply lacks the appropriate signaling molecules. In sum, the rich microbial environment experienced by a coral planula informs its

behavior, while the behavior of coral planulae in different environments provides information to us, the watching scientists, about the suitability of that habitat for settlement and survival.

MATERIALS AND METHODS

Rearing coral planulae

Montastraea faveolata planulae were reared from gamete bundles collected during mass spawning events on the island of Curaçao (former Netherlands Antilles) in the southern Caribbean. Gametes were collected at Snake Bay (September 2007, 12°13′90″N, 68°99′82″W) and Playa Kalki (September 2008, 12°37′48″, 69°15′85″W). On spawning nights, weighted nylon cones were placed over 25-30 adult *M. faveolata* colonies at depths of 7-9 m at least 1 h before spawning. At spawning, gamete bundles accumulated in inverted conical tubes atop the cones and were returned to shore, where they were mixed as the bundles began to break apart. Gametes were allowed to fertilize at high density for 2 h to ensure high fertilization rates. Minimizing the number of unfertilized eggs in each larval culture reduces the amount of lipid debris that accumulates in the rearing containers and helps to maximize health of the fertilized embryos. After fertilization, embryos were rinsed thoroughly in filtered seawater (Millepore 0.45 μm) to remove all traces of sperm (and prevent polyspermy). Embryos were then transferred to 2 L polystyrene containers containing 0.45 μm–filtered seawater. Larvae began swimming 19-21 h after fertilization and began to settle and metamorphose after five days. Debris and dead planulae were removed from each container and water changes were performed every 12 to 48 h.

Laboratory experiments

Larvae were rinsed twice with sterile seawater (0.22 μm–filtered) and allocated from a single combined stock container into experimental replicates. Only actively swimming larvae were used. All larvae were allocated randomly with respect to treatment. Dishes were arranged in a randomized block design. Survivorship and behavior were scored at regular intervals. Settlers were considered those that had begun forming a calcified skeleton.

Water for experiments was collected at the experimental reef using 60 ml plastic syringes. Water was returned to the lab and processed within 30 min of collection. Air temperature in the laboratory was held at 27 °C and laboratory lights were kept on from 0800 to 1900 hours each day. All laboratory experiments were conducted at or below a density of one planula per ml of seawater, because this density is known to prevent density-dependent mortality of pre-settlement coral juveniles while lending sufficient statistical power to detect treatment effects (Vermeij et al. 2009, and K. L. Marhaver, unpublished data).

For experiment 1, water was collected 2 cm and 2 m up-current from three adult *M. faveolata* colonies and half of each "near" and "far" sample was filter-sterilized (Sterivex 0.22 μm) to create four water treatments. 26 replicate Petri dishes per treatment each contained 10 swimming planulae and 20 ml of water. Larval survivorship and behavior were scored by eye daily at 19:00 h for seven days. Behaviors were scored for experiment 1 in four mutually exclusive categories: motionless in the petri dish (but not settled), swimming in the water column or at the water surface, swimming on the bottom on the Petri dish, and settled (i.e., completed metamorphosis, attached to the substrate). Only planulae that had begun to calcify were considered settlers. For statistical analysis, the number of planulae exhibiting each behavior was expressed as a proportion of surviving planulae. For analysis of

swimming rate, the total number of planulae swimming (regardless of location) was used.

For experiment 2, four filtration treatments were prepared: Control, 0.8 μm, 0.22 μm, and 0.02 μm. Each water filtration treatment was replicated with the broad-spectrum antibiotic penicillin (66 μg/ml). Each Petri dish contained 40 swimming planulae and 40 ml of water. Rearing planulae at this density eliminates density-dependent mortality due to bacterial accumulation (Vermeij et al. 2006). Planula behavior and larval settlement were scored daily for seven days. For experiment 2, behaviors were scored in five mutually exclusive categories: motionless in the water column or at the surface, moving in the water column or at the surface, moving on the bottom, resting motionlessly on the bottom, and settled. For statistical analyses, the number of planulae exhibiting each behavior was expressed as a proportion of the surviving number of planulae in each replicate.

Videographic analysis

To visualize locomotory behavior of swimming planulae, QuickTime movies were recorded using a digital movie camera. Image stacks were built from QuickTime movies using ImageJ. Each image stack represented 6.4 seconds of movement. The paths of individual larvae were tracked using the manual tracking plug-in from ImageJ (NIH). Distances were calibrated using the bottom surface of the Petri dish as a reference point.

Statistical analysis

Behaviors were scored in mutually exclusive categories which included various locomotory behaviors as well as settlement. Locomotory behaviors and

settlement rates were analyzed using different assumptions about the nature of the data.

Settlement data were analyzed as in previous experiments (Chapter 1, this dissertation) by assuming the proportion of surviving planulae that had settled was binomially distributed about the expected value. We then determined the number of different probabilities that best described the observed settlement data across treatments at a single time point. A likelihood ratio test was used to determine the degree of significance of added parameters, given a penalty for added parameters. From this test, we produced the most parsimonious number of parameters to describe the settlement data. Significantly different treatment groups are depicted in the graphs using lowercase letters. Standard error and confidence intervals were calculated within the likelihood framework. Significance levels were calculated by comparing the likelihood values of the best-fit model and the next-best-fit model using a Chi-square distribution with one degree of freedom.

For locomotory behavior data, the proportions of planulae exhibiting each behavior are not independent from one another. These data were therefore examined with a binomial test in a strictly exploratory approach to illustrate the relative occurrence of individual behaviors in different treatments. The proportion of planulae exhibiting a given locomotory behavior was analyzed in relation to the total number of survivors exhibiting all other behaviors (including settlement). These proportions were analyzed as described above for the settlement data, however due to the multinomial nature of the data, the confidence intervals associated with this binomial analysis are only shown to guide the eye and cannot be used for formal hypothesis testing (therefore P-values were not calculated for these data). Separate treatment groups identified in this exploratory approach are denoted with lowercase letters and were determined using an alpha-value of 0.05.

All analyses were conducted in R following the methods of Hilborn and Mangel (1997). This statistical approach is described in further detail in Vermeij and Sandin (2008) and Vermeij et al. (2009).

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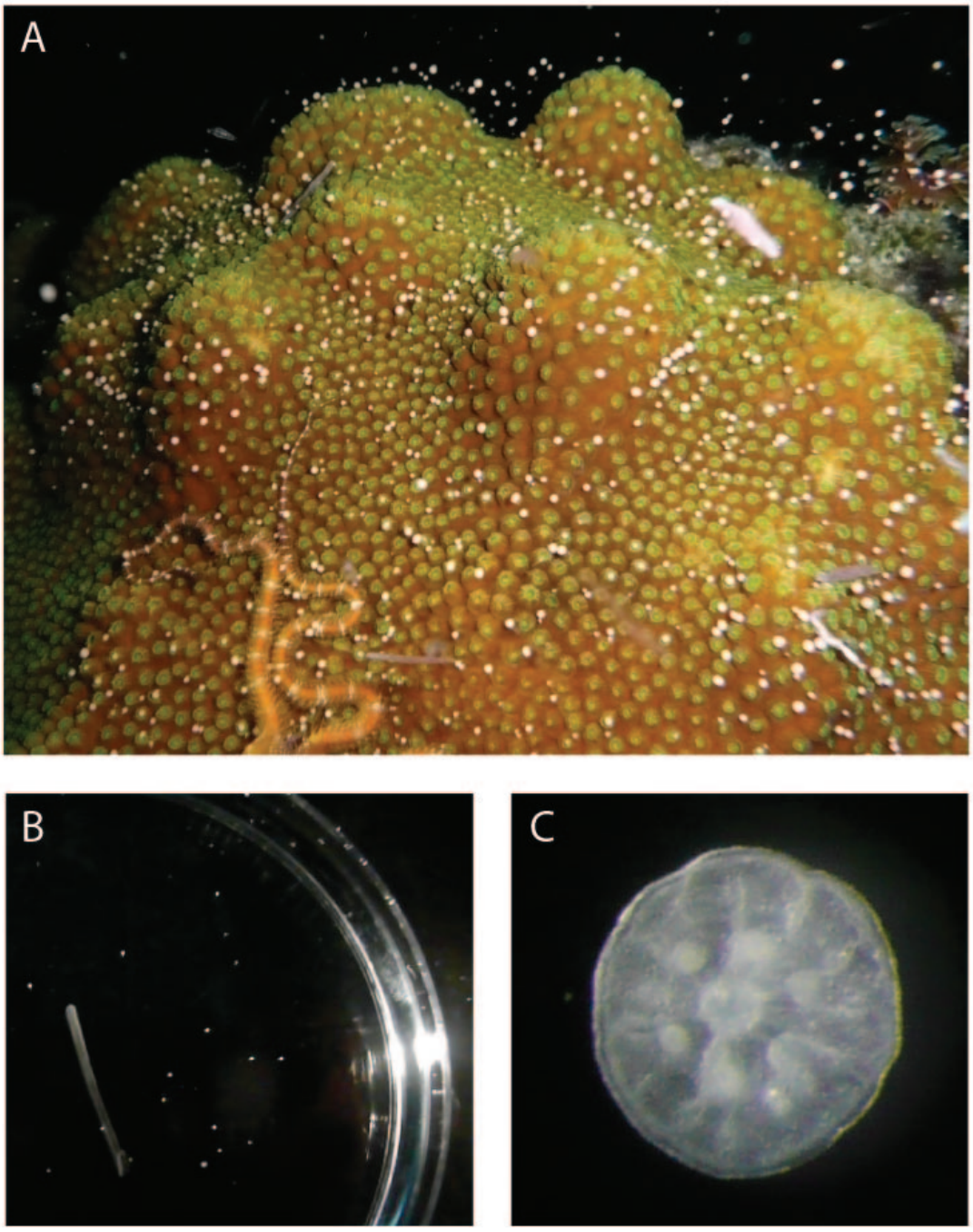

Figure 3.1. Life stages of the focal species *Montastraea faveolata.* (A) Adult *M. faveolata* colony releasing egg-sperm bundles on spawning night in Curaçao. (B) Swimming *M. faveolata* planulae in a standard 15 cm diameter Petri dish. (C) Newly-settled *M. faveolata* polyp with tentacles and mouth visible (approx. width = 1 mm).

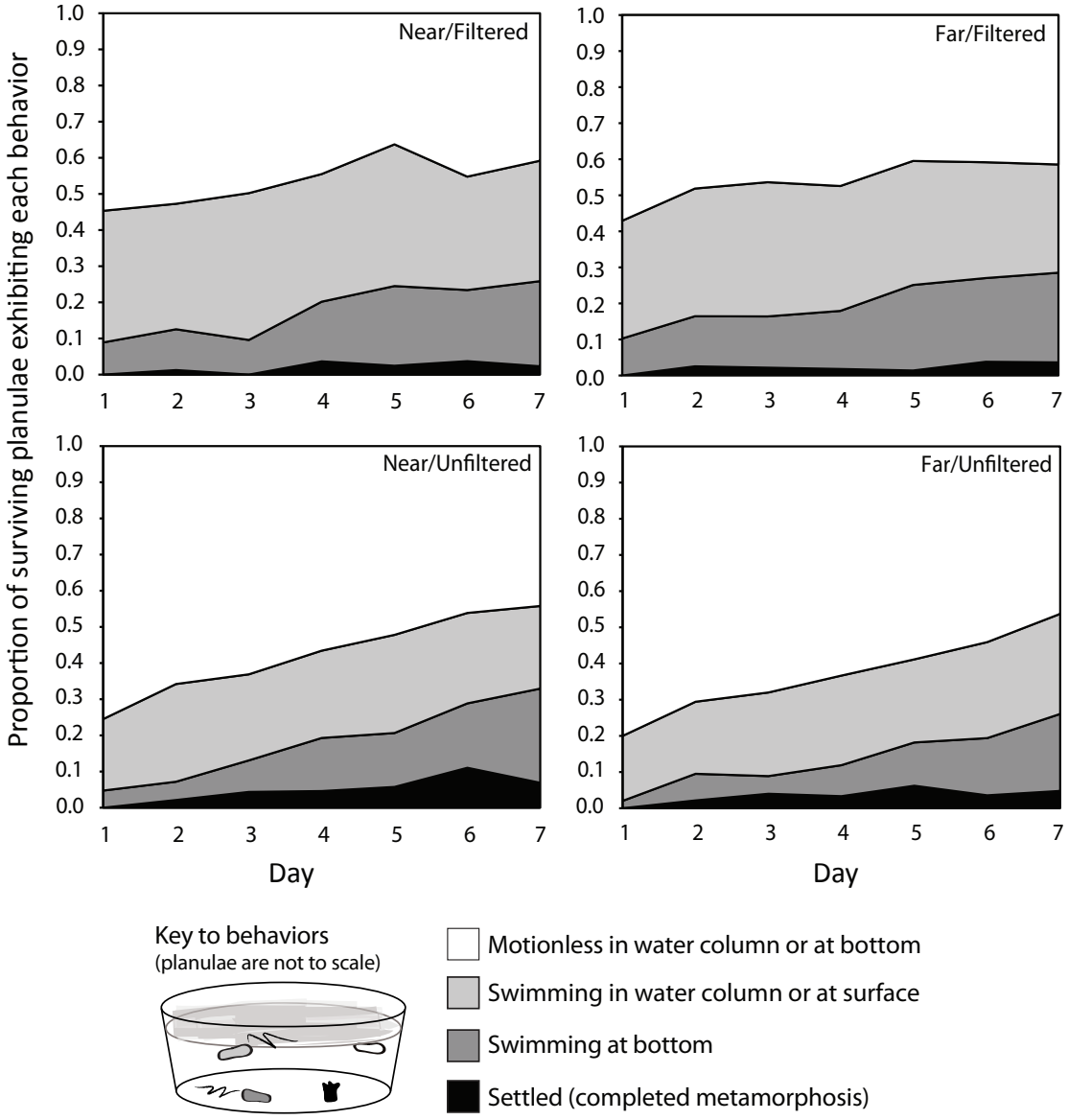

Figure 3.2. Swimming behavior of *M. faveolata* planulae in different microbial environments. "Near" = Water was collected near conspecific adult corals. "Far" = water was collected two meters up-current from conspecific adult colonies. "Unfiltered" = was used without filtration. "Filtered" = water was filter-sterilized (0.22 μm Sterivex) to remove microbes. For all treatments, n = 26, 10 planulae per replicate. For statistical comparisons, see Fig. 3.3.

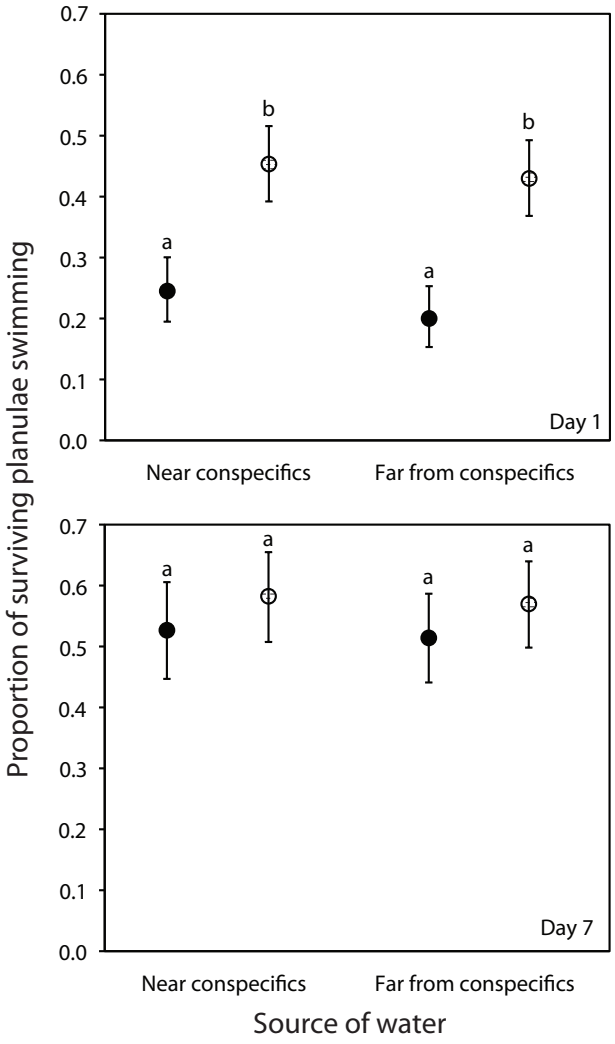

Figure 3.3. Proportion of surviving *M. faveolata* planulae swimming. Data are shown as mean proportion swimming. Bars represent 95% CI. Water was used unfiltered (solid circles) or filter-sterilized to remove all bacteria and large particles (open circles). Data are shown from experiment day 1 (upper panel) and day 7 (lower panel). Lowercase letters denote significance groups from a likelihood-based model. For all treatments, n = 26, 10 planulae per replicate.

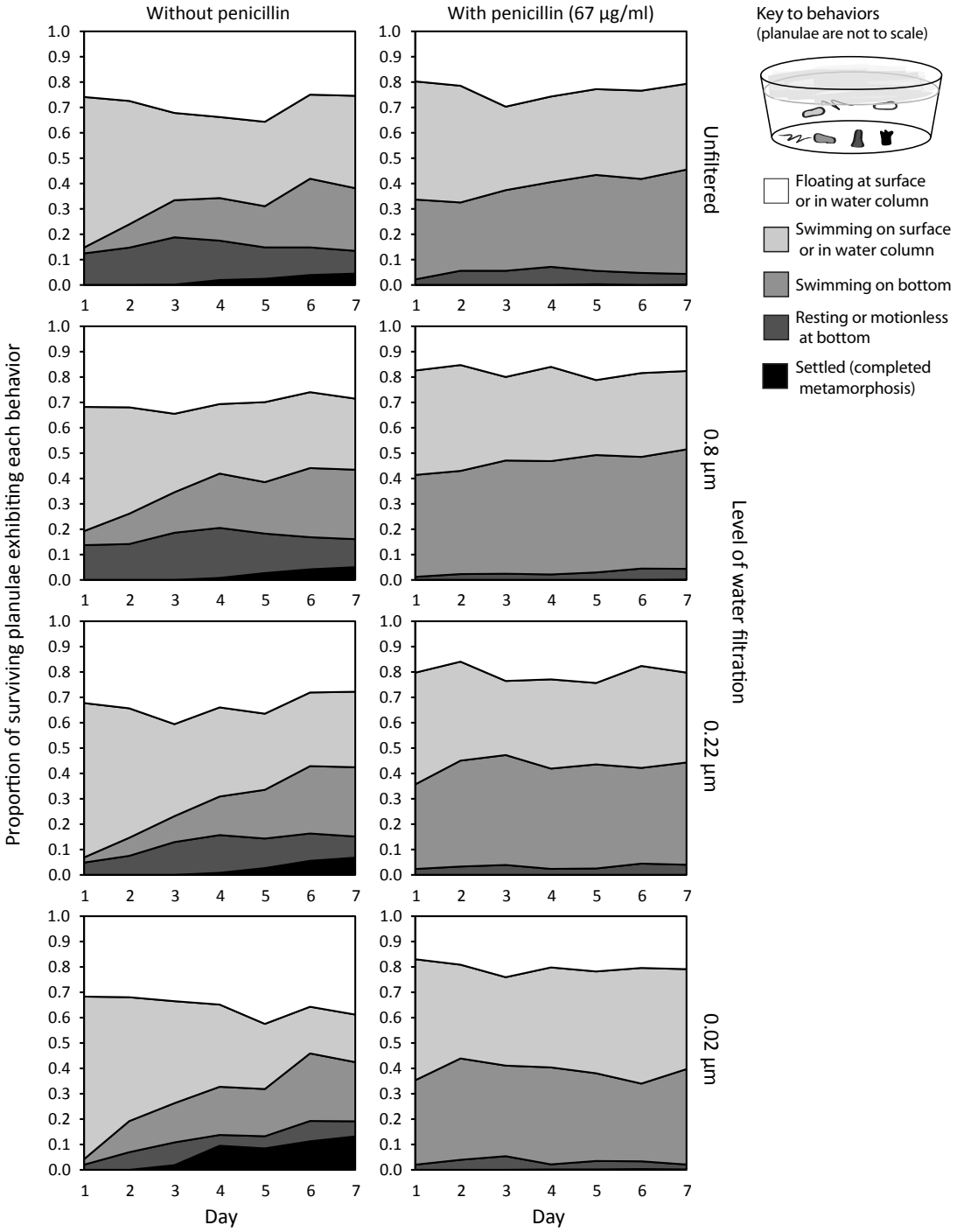

Figure 3.4. Behavioral profiles of *M. faveolata* planulae in water filtration and penicillin treatments. Water was collected near adult *M. faveolata* colonies and treated by filtration only (left column) or by filtration and with antibiotics (right column). For all treatments, n = 16, 40 planulae per replicate. The experiment was started 9 days after spawning. For statistical comparisons, see Fig. 3.5.

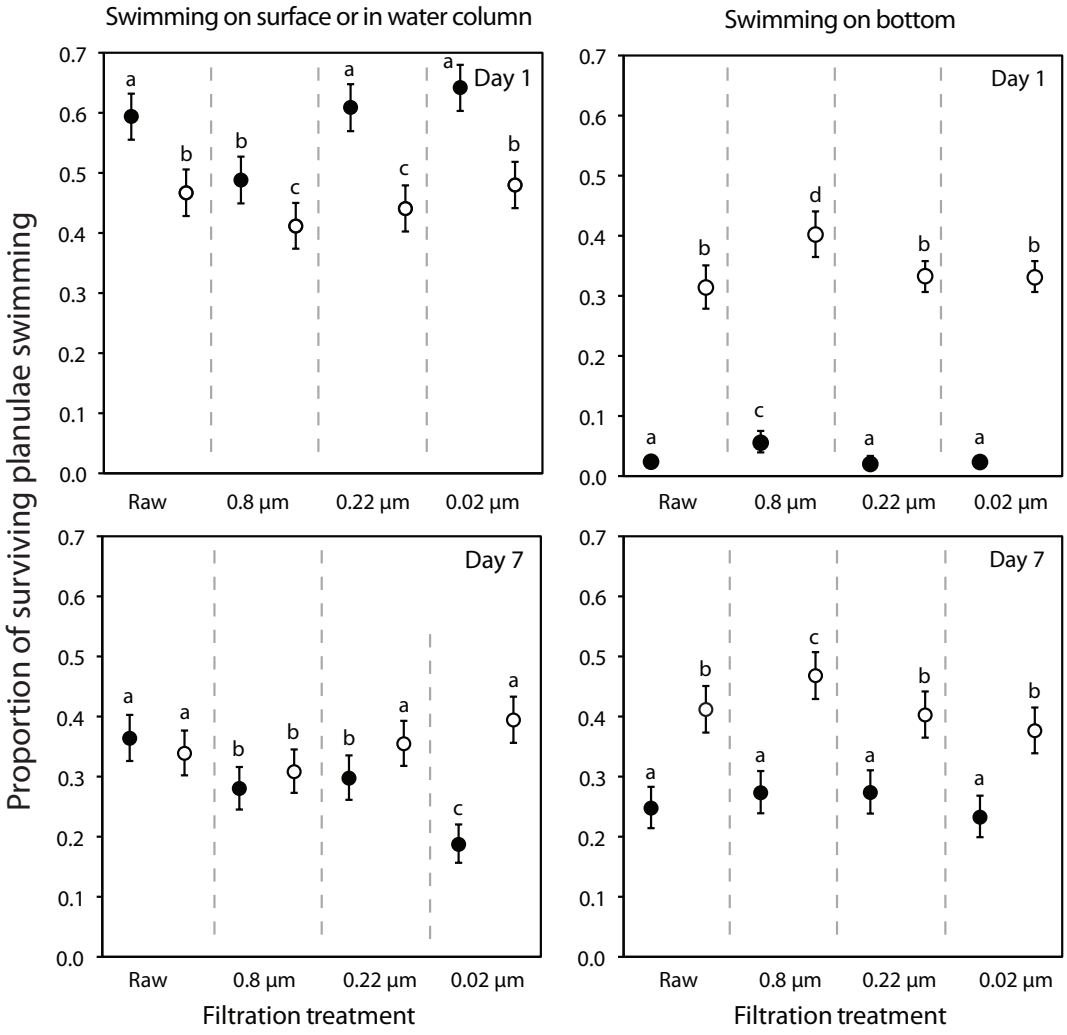

Figure 3.5. Effects of penicillin on rate and location of planula swimming behavior. Within various water fractions, penicillin (open circles) altered the location and incidence of swimming behavior relative to controls (black circles). Show is the mean proportion of planulae exhibiting each behavior. Bars represent 95% CI based on a binomial error structure and are not for hypothesis testing. Significance groups were identified using an alpha value of 0.05. The effects of penicillin were apparent on day 1 (left) and persisted through day 7 (right). Within each graph, letters denote significantly different treatment groups based on a likelihood model. For all treatments, n = 16, 40 planulae per replicate.

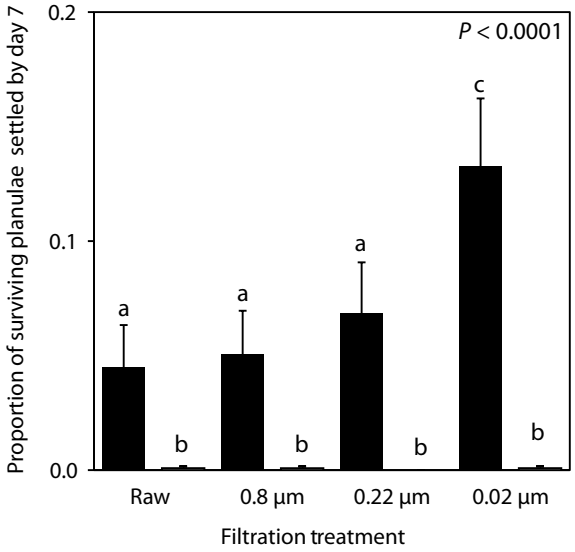

Figure 3.6. Inhibition of settlement by penicillin. Penicillin (bars marked "b") reduced settlement of *M. faveolata* planulae relative to control treatments (bars marked "a" or "c") regardless of water filtration level. Shown is the total proportion of surviving planulae that settled by day 7 and bars depict the 95% CI upper bound. Letters denote groups of significantly different treatments. For the 0.22 µm/penicillin treatment, all values were zero. For all treatments, n = 16, 40 planulae per replicate.

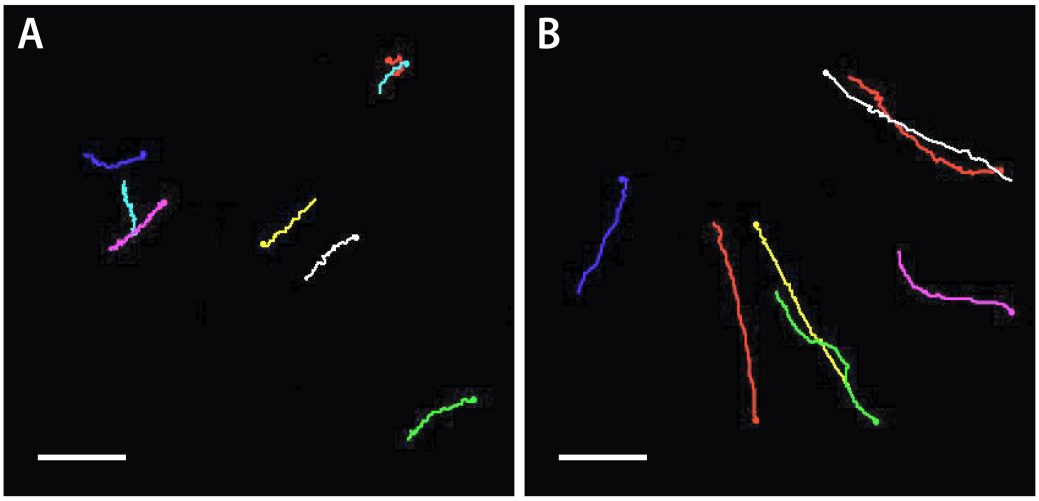

Figure 3.7. Swimming speed and pattern of *M. faveolata* larvae in control and penicillin treatments. Each track shows the path of an individual larva in the culture dish over a 6.4 second period. Image stacks were built from QuickTime movies and larvae were tracked frame-by-frame using the manual tracking plug-in from ImageJ (NIH). (A) 0.02 μm-filtered seawater without penicillin. (B) 0.02 μm-filtered seawater with penicillin (66 μg ml^{-1}). Scale bars = 1 cm. Planulae in the penicillin treatment exhibited a significantly faster mean swimming speed (0.14 cm/sec (SD 0.02) in FSW, 0.33 cm/sec (SD 0.05) in FSW+PEN, $P < 0.0001$).

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CHAPTER 4

Observation of post-metamorphosis *Montastraea faveolata* polyps moving across surfaces using their tentacles

K. L. Marhaver

In laboratory environments, planula larvae of the coral *Montastraea faveolata* typically settle to surfaces such as plastic and glass within a few days of spawning or within a few days of introduction to a new vessel (e.g., Vermeij et al. 2006). This occurs even in the absence of a preferred settlement surface (such as crustose coralline algae). *M. faveolata* planulae generally undergo metamorphosis, begin calcification, and attach themselves to the substrate simultaneously. The word "settlement" is therefore frequently used to describe these processes together as they rarely occur individually.

Here I present two separate observations of *Montastraea faveolata* polyps in which settlement, calcification, and metamorphosis did not occur in synchrony. On 26 October 2008, I observed two polyps of the Caribbean coral *Montastraea faveolata* that had undergone metamorphosis but lacked a skeleton. These polyps were not attached to the Petri dish surface. Instead, each polyp moved slowly across the bottom of the culture vessel, primarily by slowly spinning (Fig. 4.1A-B).

On 23 September 2009, I observed a polyp of *M. faveolata* in culture that also lacked a skeleton and was not attached to the substrate (Fig. 4.1C-D). This polyp was inverted, tentacles down (Fig. 4.1D), and was using these tentacles to move across the bottom surface of the Petri dish. When pushed onto its side, this coral polyp repeatedly rotated itself back to its former configuration with tentacles down toward the Petri dish bottom, after which it resumed "walking" across the Petri dish.

In the Caribbean coral *Madracis mirabilis*, early post-settlement polyps have been observed moving laterally across an artificial (glass) surface over a period of five days (Vermeij et al. 2002). This is the only previous report of "rambling" post-settlement coral polyps, but it demonstrated that such a phenomenon was possible. Other observations of decoupled settlement behaviors in corals include the "floating

corallites" that have been observed in laboratory conditions (Vermeij 2009). These corallites have undergone metamorphosis and developed a flattened, low-density skeleton, however they remain at the surface of the water column and concentrate zooxanthellae on the lower portion of the body (i.e., the surface closest to the overhead light source).

For dispersing larvae of sessile marine invertebrates, location of a final settlement surface often involves complex behaviors and highly specific settlement cues. The careful selection of a habitat both increases individual survivorship (Grosberg 1981) and leads to niche segregation within small patches of habitat (Vermeij 2006). It is unclear whether the settlers described here could readily reattach to the substrate at a later point, or whether their movements as post-metamorphosis polyps could be deliberate enough to locate a suitable habitat. However, these observations show that metamorphosis does not always require formation of a skeleton of permanent attachment, and that after "settling," some individuals of *M. faveolata* can still explore the reef for an extended period of time.

Among other members of the phylum Cnidaria, settlement and metamorphosis may be less tightly coupled than they are in reef corals. The estuarine anemone *Nematostella vectensis* undergoes metamorphosis prior to settlement and then spends a few days crawling across the benthos to find optimal sediments in which to live (Muller and Leitz 2002). Thus, the *M. faveolata* polyps observed here appeared to be exhibiting a behavior more common among their anthozoan relatives. This behavior would be almost impossible to observe in nature, but laboratory observations such as this expand our understanding of the developmental possibilities and dispersal capabilities of corals.

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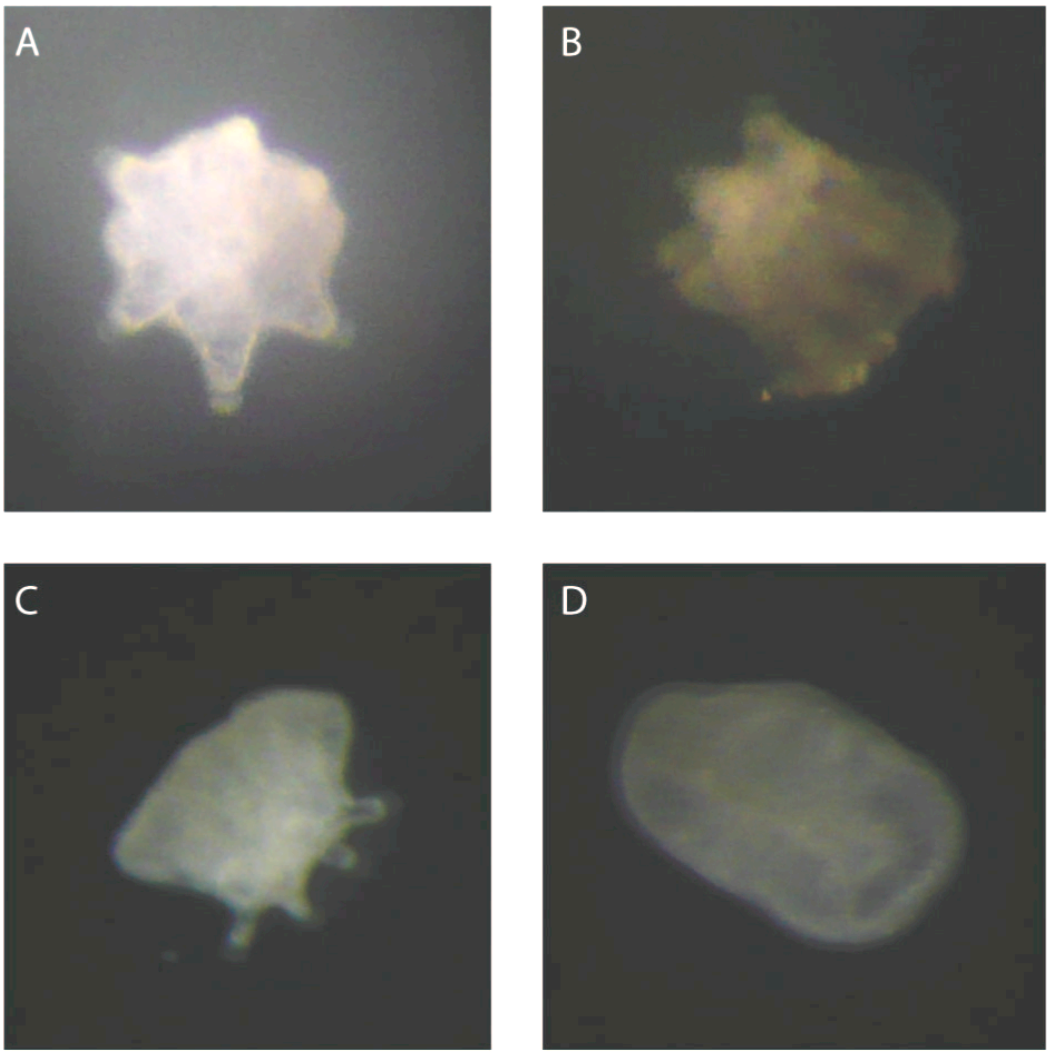

Figure 4.1. Movement of post-metamorphosis *M. faveolata* polyps. These polyps lacking skeletons were observed in 2008 and 2009. (A) and (B) Two polyps observed spinning slowly and moving across the surface of the Petri dish. Polyps were uncalcified and not attached to the dish. (C and D) Two views of a polyp observed inverted, traveling laterally on its tentacles in 2009. This polyp repeatedly resumed the position depicted (D) and moved slowly across the bottom of the Petri dish. "Rambling" behavior of post-metamorphosis polyps has only been observed in one other coral species.

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CHAPTER 5

Five different antibiotics enhance survivorship but cause abnormal behavioral profiles in planula larvae of the Caribbean coral *Montastraea faveolata*

K. L. Marhaver

ABSTRACT

In their larval phase, corals exhibit nuanced, deliberate locomotory behaviors, which allow them to select a settlement surface on which to live the rest of their lives. Bacteria are known to serve as settlement cues for dispersing planula larvae, however bacterial attack also causes a large proportion of juvenile coral mortality. The use of antibiotics to prevent bacterial pathogenesis has yielded anecdotes of abnormal planula behavior in some treatments, but this phenomenon has never been examined experimentally or studied in relation to survivorship. To test for and quantify antibiotic-mediated behavioral effects in juvenile corals, six-day-old *Montastraea faveolata* larvae were treated with five common antibiotics from a range of antibiotic classes. Behavior and survivorship in these treatments were compared to responses in filter-sterilized and untreated seawater. Differences in swimming and resting behavior between antibiotic treatments were observed after one day of exposure and behavioral differences persisted through time. While all antibiotic and filtration treatments increased survivorship relative to the control, antibiotics varied in their effectiveness and each produced evidence of behavioral toxicity. Compared to behavior in filtered and untreated seawater, the behaviors exhibited in the presence of different broad-spectrum antibiotics were maladaptive and varied between compounds. These results suggest that behaviors were not mediated entirely by the indirect effects of removing microbes (i.e., Bacteria and Archaea) from the water column but also by the direct action of these compounds on the physiology, development, or signaling processes of the coral planulae. These results show that, for purposes of research and restoration with juvenile corals, antibiotics should generally be avoided in favor of highly-filtered seawater. Furthermore, results presented here indicate that behavioral characteristics of coral planulae are

informative sub-lethal response variables for future studies of toxicology, bioactivity, and habitat suitability.

INTRODUCTION

In studies of coral life history processes, the settlement choices of coral larvae (known as "planulae") are a common research focus (reviewed in Ritson-Williams et al. 2009). This has left the locomotory behaviors exhibited prior to settlement largely unexamined. To date, only one study has quantified locomotory behavior in spawned coral planulae (Vermeij et al. 2006), and very few studies have examined locomotion in planulae of any coral species (Raimondi and Morse 2000; Stake and Sammarco 2003). Fortunately, difficulties inherent in rearing and studying the planulae of spawning coral species are becoming less burdensome as researchers improve methods for propagation and experimentation (Levitan et al. 2004; Vermeij et al. 2006; Vermeij and Sandin 2008; Voolstra et al. 2009).

The current study was motivated by (1) observations that planula swimming behavior may vary based on the physical environment (Vermeij et al. 2006) and (2) a small number of reports that antibiotics themselves appear to alter locomotory behaviors. First, the antibiotic ampicillin was shown to delay settlement of *Montipora capitata* planulae (Vermeij and Sandin 2008). Second, penicillin was observed to induce altered locomotory behaviors in *Montastraea faveolata* planulae (Chapter 3, this dissertation). Third, rifampicin was observed inducing a maladaptive *lack* of swimming behavior in planulae from a brooding coral species (K. Sharp, personal communication).

These anecdotes raised the possibility that some bacterial interactions may be important for normal larval function. Disruptions to the coral-associated microbial community (which comprises the domains Bacteria and Archaea) by pathogens or

environmental influences often increase coral mortality (e.g., Ben Haim et al. 2003; Rohwer and Kelley 2004; Barash 2005; Harvell 2007; Rosenberg 2009; Rohwer and Youle 2010) and bacteria do serve as a threat to coral planulae (Vermeij et al. 2009). However, bacteria may also serve as endosymbionts in planulae (Apprill et al. 2009) or as information for navigation and settlement (Negri et al. 2001; Webster et al. 2004, Chapter 1 of this dissertation). Therefore, this study sought to systematically test the effects of different antibiotic compounds on the survival *and* behavior of coral planulae and to increase the number of different behaviors quantified in studies of planula locomotion.

RESULTS

Antibiotics and water filtration both increase survivorship

On day 31, all filtration and antibiotic treatments had dramatically increased planula survivorship above control levels (Fig. 5.1). Survival trajectories through time were used to determine the daily probability of planula mortality in each treatment, revealing at a finer resolution the relative effectiveness of different treatments (Fig. 5.2). Although all antibiotics increased survivorship relative to the control, survivorship varied significantly between antibiotics. The daily probability of mortality in penicillin was significantly lower than in all other treatments (Fig. 5.2). Mortality in chloramphenicol was lower than in the remainder of the treatments. Mortality in ampicillin was not significantly different from mortality in 0.45 um-filtered seawater, while mortality in 0.22 μm-filtered seawater was slightly higher, but not significantly different from mortality in tetracycline or kanamycin. It is notable that planulae in filter-sterilized water achieved relatively high rates of survivorship on par with the effects achieved by various antibiotics. Together, these results suggest that microbial activity, mediated in large part by water column

microbes, is a primary cause of mortality in laboratory-reared coral planulae. Reducing microbial activity through two different approaches doubles the probability of survivorship.

Relationship between antibiotic toxicity and survivorship

Penicillin generally has low toxicity and low side effects in eukaryotes (Snavely and Hodges 1984; Anderson et al. 2001) and coral planulae survived best in this treatment. Kanamycin is known to have high toxicity effects and is no longer given to humans; survivorship of planulae in kanamycin was lower than in three of the other antibiotics. Known levels of toxicity did not always predict relative survivorship in this experiment, however. Chloramphenicol is highly toxic in humans (Anderson et al. 2001), but planula survivorship in this treatment was quite high. Based on survivorship rates alone, antibiotic use appears to benefit coral planulae, regardless of the compound used.

Planulae exhibit "normal" behaviors in raw and filtered seawater

Behavioral profiles for all treatments through time are depicted in Fig. 5.3. Although survivorship was the lowest in the untreated (raw) seawater, surviving planulae in this treatment exhibited a "normal" progression of behaviors. Planulae typically swim in the water column for a few days before becoming positively geotactic and moving down to the benthos (Vermeij et al. 2006; Chapter 3, this dissertation). Planulae on the benthos then swim, pause, turn, and make short hops in search of a settlement substrate. A planula will eventually stop moving and begin to calcify shortly thereafter, attaching its skeleton directly to the benthos. Therefore, resting motionlessly for more than a few seconds on the bottom of the Petri dish is typically considered to be a sign that a planula is in the process of settlement.

Planulae in filtered seawater treatments (0.45 μm and 0.22 μm, Fig. 5.3 and Fig. 5.4) behaved similarly to those in raw seawater, however they did tend to remain swimming in the water column slightly longer and settlement was slightly delayed (Fig. 5.3). This could be due to the lack of reef "smell" cuing settlement in filtered water, or the slower formation of a biofilm to which planulae may be attracted. However given high rates or mortality in raw seawater, filtered seawater treatments yielded a higher number of surviving settlers in total. When behavior and survivorship data are considered together, the most effective way to raise *Montastraea faveolata* larvae with high survivorship, normal swimming behavior, and normal settlement behavior is to filter their culture water with an impact filter that has a pore size of 0.45 μm.

Antibiotics cause abnormal behaviors

All antibiotics increased planula survivorship, but they differently affected planula behavior. Within one day of exposure, planulae in antibiotic treatments exhibited different behavioral patterns than those in filtered and untreated water (Fig. 5.3 and Fig. 5.4). On day 1, penicillin, ampicillin, and tetracycline had caused a significantly higher proportion of planulae to swim down to the Petri dish bottom relative to controls, while chloramphenicol and kanamycin induced significantly more planulae to move downward and rest motionlessly (Fig. 5.4). The majority of planulae in filtered and raw water meanwhile exhibited the "normal" behavior of remaining at the water surface and in the water column during the first day of the experiment.

While the removal of microbes from water using antibiotics might be expected to alter larval behavior by removing microbial cues, the different behaviors exhibited on day 1 indicate that there are compound-specific effects of antibiotics on behavior

and that these effects are different from that of removing microbes physically though filter-sterilization. This suggests that behavioral effects of antibiotics may not be entirely indirect (i.e., mediated by the removal of microbes) but may be partially or entirely induced by the direct effects of the compounds themselves on the planula tissue.

The experiment was continued for a full 31 days, during which behavioral profiles in different antibiotics remained distinct (Fig. 5.3 and 5.5). Interestingly, ampicillin induced a different behavioral profile than penicillin despite the fact that these antibiotics are both from the beta-lactam antibiotic class. Ampicillin induced planulae to swim downward more quickly than raw or filtered water, however the planulae remained swimming at the bottom of the Petri dishes far longer than in other treatments. Toward the end of the experiment, ampicillin-treated planulae began to sit motionlessly at the bottom of the Petri dishes in greater numbers, but ultimately very few settled (Fig. 5.3E). Kanamycin, meanwhile, induced planulae to swim at the water surface for an extended period of time, before transitioning to motionless rest at the bottom (Fig. 5.3H).

In the penicillin treatment, planulae initially began swimming downward in the Petri dishes, but over the course of the experiment, the large majority of survivors were found swimming in the water column or at the water surface rather than at the bottom of the Petri dish (Fig. 5.3D). This persisted for the full course of the experiment and very few planulae were ever observed resting on the bottom or settling in the Petri dishes. While penicillin treatment resulted in the highest survivorship, the behavior of planulae in this treatment was extremely abnormal and maladaptive, with only 1% of surviving larvae reaching settlement after 31 days (Fig. 5.5).

Resting on the bottom of the Petri dish is typically a sign of impending settlement, however this was not the case in the chloramphenicol treatment. For the duration of the experiment, chloramphenicol induced planulae to sit on the bottom of the Petri dish in abnormally high and increasingly large numbers (Fig. 5.3G). Despite this behavior, settlement was delayed relative to untreated seawater, indicating that in certain environments, motionless rest may be an indicator of stress or failed development. While chloramphenicol dramatically increased survivorship, it virtually eliminated normal swimming behavior and delayed settlement. Thus, overall, the two antibiotic treatments with the highest survivorship—chloramphenicol and penicillin—caused the most severely abnormal behaviors.

DISCUSSION

Significance of results for coral propagation

Antibiotics have occasionally been used by researchers to rear coral larvae for experiments and reef restoration, however this practice yielded anecdotal reports of abnormal behaviors such as delayed settlement (Vermeij et al. 2009), continuous swimming (Chapter 3, this dissertation), or a lack of locomotion entirely (K. Sharp, personal communication). The results presented here indicate that the specific type of antibiotic used is a primary driver of the behaviors observed. Two different mechanisms may contribute to abnormal behavior in antibiotic treatment: First, antibiotics may remove key behavioral cues from the microbial environment. Second, antibiotics may act directly on the coral tissue and alter signaling cascades that control behavior.

The results presented here show that water filtration using impact filters is a preferred method for increasing planula survivorship, as it results in a greater number of healthy settlers. However, antibiotics not tested in this study may have

lesser effects on behavior. Furthermore, a pulse-chase approach wherein antibiotics are removed shortly after they are used may reduce the occurrence of some of the abnormal behavior observed here. In any case, the use of antibiotics to manipulate bacterial numbers cannot be eliminated as a potential factor directly affecting response variables such as settlement, survivorship, and swimming behavior in studies of coral planulae.

Environmental cues for metamorphosis and behavior

Most of the antibiotics tested in this study are compounds originally isolated from microorganisms (Table 5.1). It is possible that these compounds affect marine animals such as corals by acting as signaling molecules, signal inhibitors, or toxins. Cnidarians have loosely-organized neuronal networks which mediate the signaling cascades involved in metamorphosis (Muller and Leitz 2002). For example, metamorphosis in *Hydractinia echinata* (a colonial marine hydroid) is triggered by a single neuropeptide (Schmich et al. 1998) or inhibited when this neuropeptide is antagonized by other molecules (Katsukura et al. 2003). A neuropeptide cue also induces settlement in planulae of the spawning coral *Acorpora palmata* (Erwin and Szmant 2010). Thus, the possibility exists that a variety of exogenous compounds also affect pre-metamorphosis behaviors, including locomotion, through direct effects on signaling cascades.

Coral planulae in toxicology studies

Classic toxicology model systems employ organisms that are easy to propagate and manipulate (e.g., Snell and Persoone 1989). Meanwhile, toxicology in corals is more typically studied through functional genomic analyses of adults (Edge et al. 2005, DeSalvo et al. 2008, DeSalvo et al. 2010) or planulae (Voolstra et al. 2009).

Coral larvae are far more difficult to work with than most model organisms, however it is becoming increasingly easy to rear healthy planulae in large numbers. Studying environmental toxicology in coral planulae (as in Markey et al. 2007) could therefore provide a valuable complement to model systems due to the ecological importance of corals and their basal position on the phylogenetic tree of metazoans.

Behavioral toxicology

In this study, antibiotics induced differences in planula behavior that were apparent within one day. Many of these differences persisted for weeks, while other behavioral difference emerged through time. Behavioral data could therefore serve as tool for measuring the toxicity of exogenous compounds on coral planulae, as behavioral changes may be a cue that a compound causes sub-lethal but deleterious effects on physiology.

Behavioral response variables are increasingly employed in toxicology studies in model organisms. For example, swimming behaviors are used as indicators of toxicity in studies of barnacles, rotifers, and *Artemia* (Faimali et al. 2006; Garaventa et al. 2010). Increasingly sophisticated videographic analyses of locomotion have been used to detect behavioral changes that results from toxin exposure or even genetic mutation (Pierce-Shimomura et al. 1999). These methods, while developed for model organisms, have direct applicability to marine ecology; for example, environmentally relevant concentrations of pharmaceuticals have been shown to cause altered predator avoidance behaviors in fish larvae (McGee et al. 2009; Painter et al. 2009).

In the past three decades, corals have suffered a dramatic collapse in recruitment in the Caribbean (Hughes and Tanner 2000). Working to understand the relationship between environmental signaling and coral planula behavior may help identify previously unknown drivers of this phenomenon. A greater understanding

of normal planula behavior and its sensitivity to external cues will also help researchers analyze habitat suitability and improve reef restoration and management approaches.

MATERIALS AND METHODS

Rearing coral larvae

Montastraea faveolata planulae were reared from gamete bundles collected during the September 2009 mass spawning event. Prior to spawning, nylon tents were placed over 25 adult *M. faveolata* colonies at depths of 6-10 m at Playa Kalki, Curaçao. The top of each nylon tent was fitted with a plastic funnel, which emptied into inverted 50 ml conical tubes. Positively buoyant gamete bundles accumulated in the conical tubes within a few minutes of release from the coral colony. Gamete bundles were returned to shore and mixed as they began to break apart (approximately 30 minutes after spawning).

After fertilization, gametes were rinsed in filtered seawater (0.45 μm Millepore) and transferred to polystyrene containers containing fresh 0.45 μm–filtered seawater. Embryos were further diluted within 24 hr to maintain embryos at a density below one larva per ml seawater. For the first 60 hours after fertilization, embryos were pipetted into fresh filtered seawater every 12 to 18 hours using clean glass Pasteur pipettes. Planulae began to swim at the water surface 21 hr after fertilization. Planulae were maintained in polystyrene containers thereafter and partial water changes were performed daily until the experiment was started six days after spawning. Debris and dead planulae was removed on a daily basis.

Experimental design

Six-day-old planulae were assigned to one of eight treatments arranged in a random block design. Water treatments were as follows: Raw water (unfiltered raw seawater), 0.45 μm (raw seawater passed through a 0.45 μm impact filter to remove most bacteria and any larger particles), 0.22 μm (raw seawater passed through a 0.22 μm Sterivex syringe filter to remove all bacteria and some large viruses), penicillin (penicillin sodium salt, 100 μm μl^{-1}), ampicillin (ampicillin sodium salt, 100 μm μl^{-1}), tetracycline (15 μm μl^{-1}), chloramphenicol (35 μm μl^{-1}), kanamycin (50 μm μl^{-1}). Antibiotics were chosen to represent a range of antibiotic classes, mechanisms of action, and toxicity levels. All were used at standard microbiology concentrations.

Before being assigned to a treatment, all planulae were rinsed three times with sterile seawater to dislodge any loosely associated microbes from their surfaces. All planulae were allocated randomly with respect to treatment. Only healthy-looking and active planulae were used in the experiment. 40 swimming planulae were transferred to each treatment dish using new glass Pasteur pipettes. Planulae were kept at a density of one larva ml^{-1}, which falls below the threshold for density-dependent survivorship effects (Vermeij and Sandin 2008; K. L. Marhaver, unpublished data). The experiment was conducted in an air-conditioned laboratory. Diurnal cycles with artificial light were maintained in the lab, which also received filtered sunlight through north-facing windows.

Survivorship and behavior were scored every two days for one week and every four days for three weeks. Survivors were counted by eye using a hand clicker. In addition to the number of survivors, the following data were also recorded for each treatment: number of planulae motionless at the water surface or in the water column, number of planulae swimming on the upper surface of the water or in the

water column, number of planulae swimming along the bottom surface of the Petri dish, number of planulae resting motionlessly on the bottom of the Petri dish, number of planulae settled onto the bottoms of the Petri dish (or, occasionally, onto the sides of the Petri dish). Settlers were considered those that had attached, metamorphosed, and begun forming a calcified skeleton. The utility of this overall experimental approach has been demonstrated previously (Vermeij et al. 2006; Vermeij and Sandin 2008).

Statistical analysis

For survivorship data, planulae were assumed to exhibit a constant daily mortality probability and each survivorship point was assumed to be an independent draw from a binomial distribution. Using a likelihood approach (after Hilborn and Mangel 1997), the most likely mortality parameter (daily probability of mortality, μ) for each treatment and combination of treatments was determined. Multiple-parameter models were tested against each other; models were penalized for each additional parameter and the combination of models yielding the highest likelihood probability was accepted. Confidence intervals were calculated within the likelihood framework. P-values were calculated by comparing the likelihood values from the best-fit and next-best fit models using a Chi-square distribution with one degree of freedom.

Behavioral data were analyzed using the same binomial framework, however this was conducted for exploratory purposes only. The proportion of surviving planulae that exhibited a given behavior at a single time point were analyzed in relation to the total number of surviving planulae exhibiting all other behaviors (including settlement). These multinomial data were therefore collapsed into a single proportion for analysis in a binomial test. This approach allows difference in

behaviors to be visualized more easily, but the binomial error structure (shown as 95% CI) is presented only to guide the eye and is not appropriate for formal hypothesis testing because the multinomial behavior data are not independent. Groups of treatments were determined using an alpha-value of 0.05 and these treatment groups are denoted with lowercase letters in the graphs.

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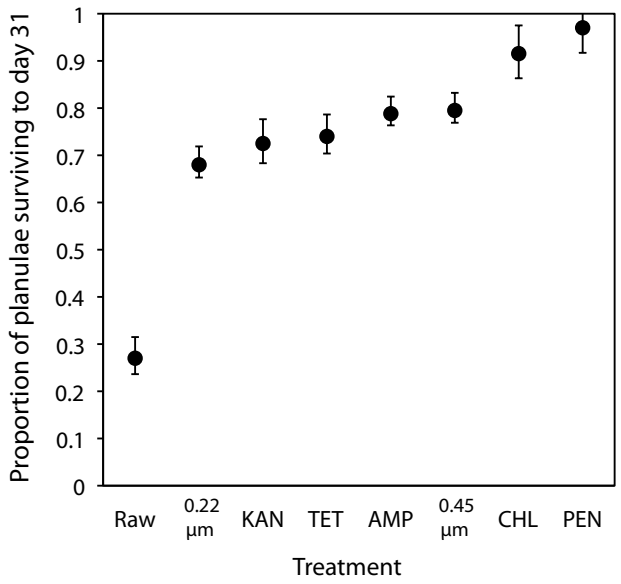

Figure 5.1. Survivorship of *Montastraea faveolata* planulae in three water filtration and five antibiotic treatments. Shown is the probability of a planula surviving to day 31. Bars represent 95% CI. For all treatments, n=5, 40 planulae per replicate. Raw = unfiltered seawater, 0.22 μm = 0.22 μm filtered seawater, KAN = kanamycin, TET = tetracycline, AMP = ampicillin, 0.45 μm = 0.45 μm filtered seawater, CHL = chloramphenicol, PEN = penicillin.

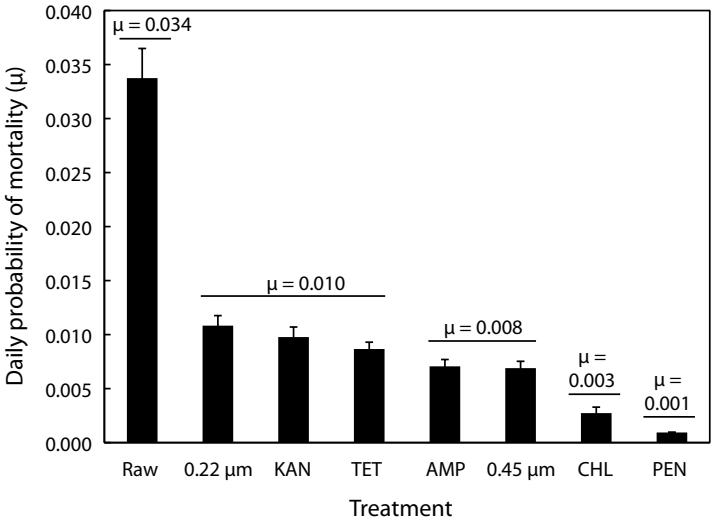

Figure 5.2. Daily probability of planula mortality in different water filtration and antibiotic treatments. Values shown are best-fit values from a likelihood model and bars represent the 95% CI upper bound. The best-fit likelihood model had five different mortality parameters, statistically significant treatment groups are marked with horizontal lines and the predicted value of μ is listed for each treatment group. Individual bars represent best-fit values for μ in an eight-parameter model. Raw = unfiltered seawater, 0.22 μm = 0.22 μm filtered seawater, KAN = kanamycin, TET = tetracycline, AMP = ampicillin, 0.45 μm = 0.45 μm filtered seawater, CHL = chloramphenicol, PEN = penicillin.

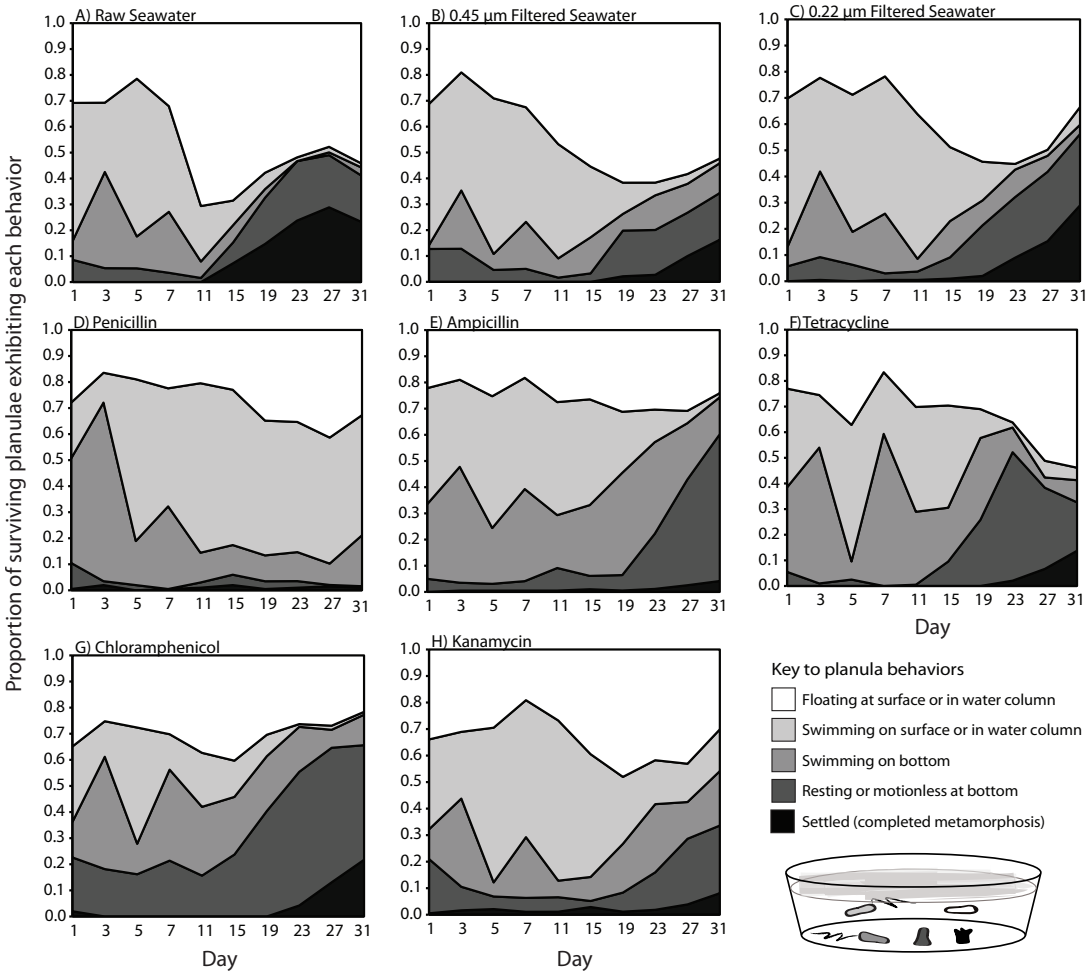

Figure 5.3. Behavioral profiles of *Montastraea faveolata* planulae in three water filtration and five antibiotic treatments. Shown is the proportion of surviving planulae exhibiting each behavior during a 31-day experiment. For each treatment, n = 5, 40 planulae per replicate. For statistical comparisons, see the following figures. Raw seawater = unfiltered, 0.45 µm = 0.45 µm filtered seawater, 0.22 µm = 0.22 µm filtered seawater, PEN = penicillin, AMP = ampicillin, TET = tetracycline, CHL = chloramphenicol, KAN = kanamycin.

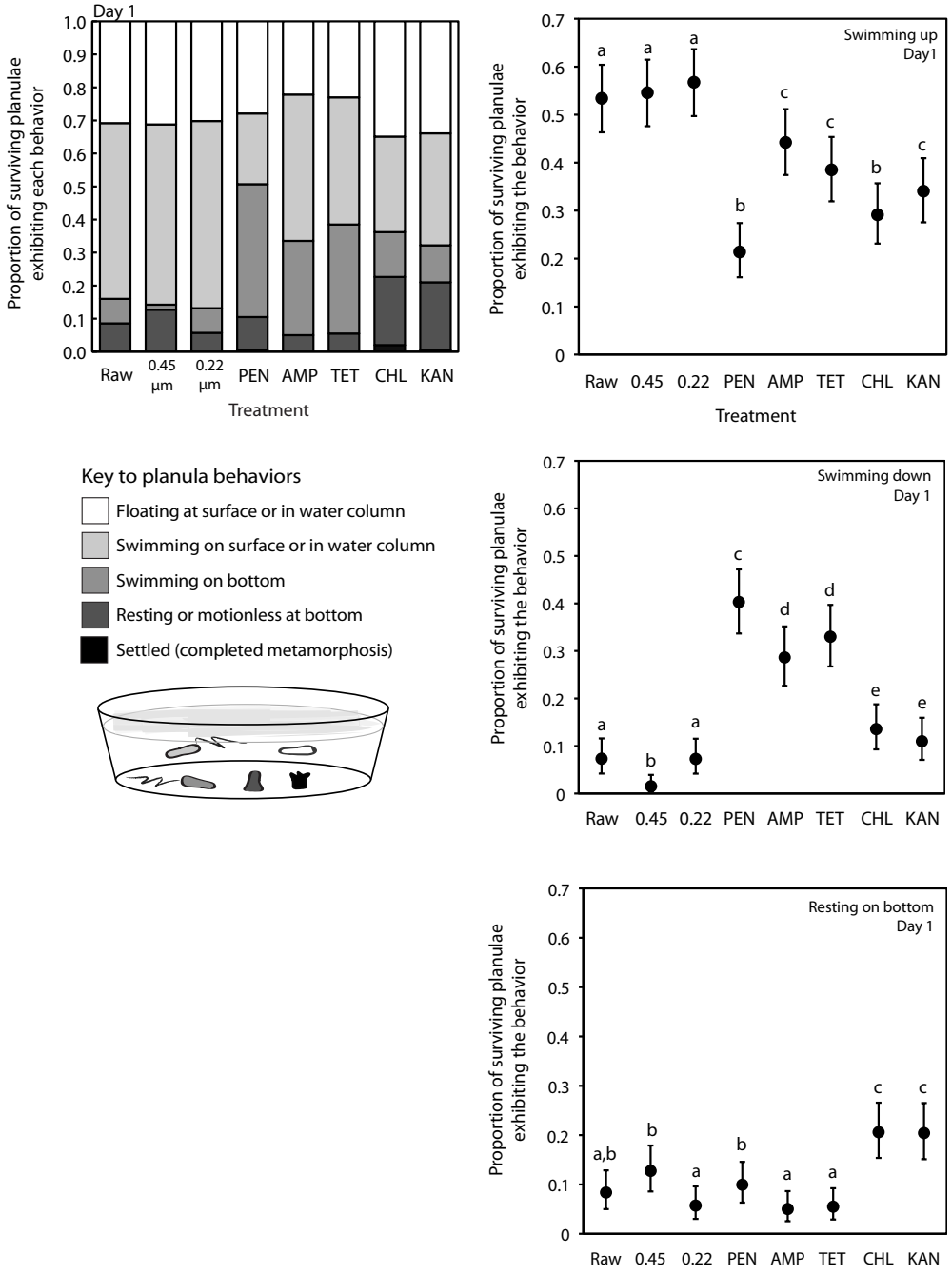

Figure 5.4. Behavioral profiles of surviving planulae on day 1. *Montastraea faveolata* planulae were treated with three water filtration and five antibiotic treatments. For each treatment, n = 5, 40 planulae per replicate. Bars represent 95% CI based on a binomial error structure and are not for hypothesis testing. Significance groups were identified using an alpha value of 0.05. Raw = unfiltered seawater, 0.45 µm = 0.45 µm filtered seawater, 0.22 µm = 0.22 µm filtered seawater, PEN = penicillin, AMP = ampicillin, TET = tetracycline, CHL = chloramphenicol, KAN = kanamycin.

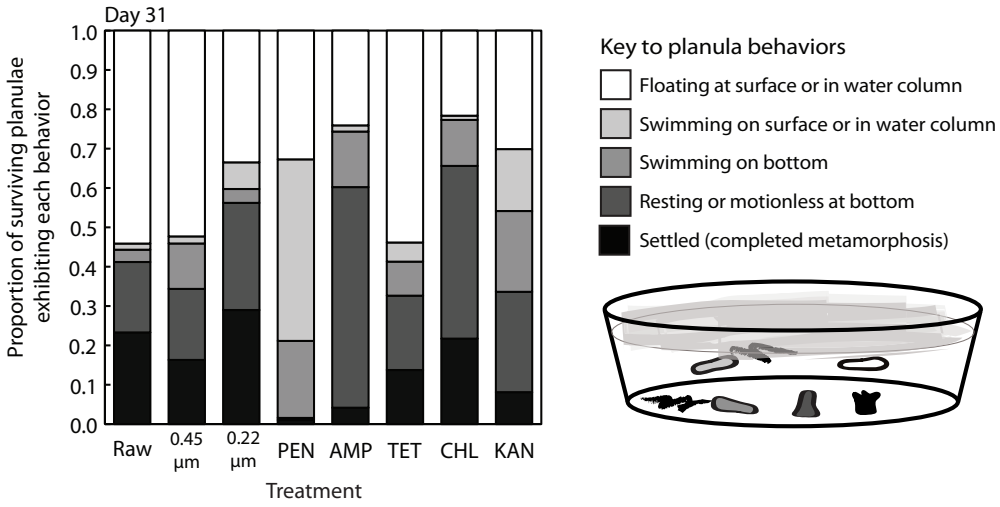

Figure 5.5. Behavioral profiles of surviving planulae on day 31. *Montastraea faveolata* planulae were exposed to one of three water filtration and five antibiotic treatments. For each treatment, n = 5, 40 planulae per replicate. Raw = unfiltered seawater, 0.22 μm = 0.22 μm filtered seawater, KAN = kanamycin, TET = tetracycline, AMP = ampicillin, 0.45 μm = 0.45 μm filtered seawater, CHL = chloramphenicol, PEN = penicillin.

Table 5.1. Mechanisms of action and toxicity of antibiotics used in the experiment.

Antibiotic	Antibiotic family	Specificity	Mechanism of action
Penicillin	beta-lactam antibiotic; from *Penicillium* sp.	Fairly broad-spectrum; targets primarily Gram-positive bacteria	inhibit formation of peptidoglycan cross-links, blocks cell division of organelles
Ampicillin	beta-lactam antibiotic; semisynthetic	Very broad-spectrum (Gram-negative and Gram-positive)	inhibits formation of peptidoglycan crosslinks in the bacterial cell wall
Kanamycin	Aminoglycoside antibiotic; from *Streptomyces kanamyceticus*	Broad-spectrum	inhibits protein synthesis by interacting w/ 30S ribosomal subunit
Chloram-phenicol	Chloramphenicol family antibiotic; from *Streptomyces venezuelae*	Very broad-spectrum	inhibits bacterial protein synthesis
Tetracycline	Polyketide antibiotic; from *Streptomyces* spp.	Fairly broad- spectrum	inhibits protein synthesis by binding the 30S ribosomal subunit

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CHAPTER 6

Abnormal morphologies of post-metamorphosis coral settlers exposed to the broad-spectrum antibiotic penicillin

K. L. Marhaver

Events that occur during the early life history stages of corals contribute disproportionately to their population dynamics. Juvenile corals are therefore becoming an important focus of research. Additionally, sexual coral propagation is an increasingly popular approach taken in reef restoration projects. Researchers and restoration programs typically gauge success by the proportion of planulae that successfully settle. Here I present examples of post-metamorphosis coral settlers that exhibit a variety of abnormal developmental outcomes in order to demonstrate various ways in which sublethal developmental problems manifest in early post-settlement juveniles.

As part of a survivorship experiment, I exposed *Montastraea faveolata* planulae to penicillin at a concentration of 100 μg ml^{-1}. Previous experiments showed that penicillin and the closely related compound ampicillin do not negatively affect planula survivorship (Vermeij et al. 2009; Chapter 3, this dissertation), however after one month of exposure to penicillin, many of the planulae that settled in penicillin treatments exhibited abnormal morphologies (Fig. 6.1).

Abnormalities included asymmetrical tissue development (Fig. 6.1A-B), asymmetrical skeletal morphologies (Fig. 6.1C), and the lack of a clearly developed mouth and tentacles (Fig. 6.1D-E). None of these morphologies were observed simultaneously in the control treatments.

Penicillin is a low-toxicity antibiotic, but antibiotics are known to delay settlement (Vermeij et al. 2009) and induce abnormal behaviors in swimming larvae (Chapters 3 and 5, this dissertation). Thus, it is possible that low-toxicity compounds have previously unreported developmental effects despite increasing overall survivorship.

Alternately, penicillin may have caused abnormal morphotypes indirectly, by removing key bacteria needed for successful development or metabolism. For example, in the squid *Euprymna scolopes*, normal postembryonic development requires the presence of the symbiotic bacterium *Vibrio fischeri* (Montgomery and McFall-Ngai 1994). *M. faveolata* is known to associate with a wide range of microbes after settlement (Sharp et al. 2010) and the coral microbial flora is known to be protective at later life stages (e.g., Richie et al. 2006, Rypien et al. 2010). Together, these studies provide evidence that coral-microbe associations on the planula surface or between the planula and water column microbes may be similarly important for normal development and physiology in early life stages of corals (Gilbert and Epel 2008).

In sum, it appears that coral development is altered either by a loss of normal microbial associations or by direct exposure to antibiotic compounds. Future studies of coral propagation methods and developmental biology should therefore measure not only survival and settlement, but also sublethal developmental changes such as those described here.

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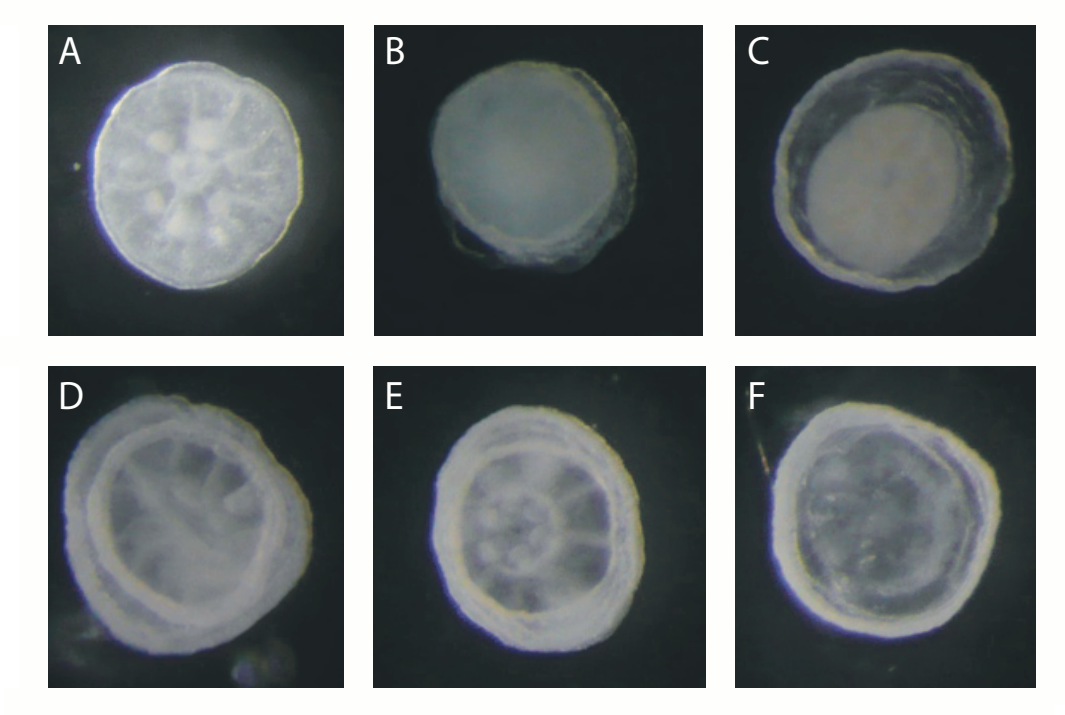

Figure 6.1. Abnormal morphologies observed among *M. faveolata* settlers in penicillin-treated water. (A) Normal *M. faveolata* settler with mouth, tentacles, and skeleton visible. The outer walls of the skeleton will thicken and grow vertically with time. (B) Post-settlement polyp apparently lacking a mouth or tentacles. (C) A post-settlement polyp with mouth visible shows abnormal calcification and no visible tentacles. Note symbiotic zooxanthellae can be seen within the tissues, indicating the mouth and gut retain function. (D-F) Settlers with irregular calcification. (D) Settler with irregularly-shaped mouth and thickened skeleton. (E) Settler with relatively short tentacles and a thickened skeleton. (F) Settler with disorganized calcification pattern. All settlers are approximately 1 mm in diameter and were photographed under a stereomicroscope.

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CHAPTER 7

Considering adaptive coral bleaching from a new angle

K. L. Marhaver

ABSTRACT

Following a bleaching event, some corals re-establish their symbiotic relationship with a different—and more environmentally appropriate—population of dinoflagellates, however other coral species apparently lack this capacity for "adaptive bleaching." In this Perspectives article, I propose that adaptive bleaching has evolved in only the subset of coral species for which symbiont switching would have been beneficial for surviving the selective events—such as periodic storms—that occurred regularly during the coral's lifespan and throughout the majority of the species' evolutionary history. To support this hypothesis, I document a bleaching event in *Montastraea faveolata*—a Caribbean species known to undergo symbiont switching—following a tropical storm on the shallow reefs of Curaçao. Tropical Storm Omar rotated and inverted *M. faveolata* colonies across the shallows, causing partial bleaching that coincided with known patterns of symbiont zonation and which was consistent with the adaptive bleaching hypothesis. The reorientation of these colonies often uncovered portions of skeleton that were previously buried in the sediment, revealing that they had been rotated by—and survived—previous storms of similar strength. Compared to long-term climate change, periodic colony rotation is a far stronger force for the evolution of the adaptive bleaching capability because it moves corallites more rapidly and more frequently into new light regimes. A coral species' lifespan, morphology, habitat type and depth range should therefore determine whether such selective pressure occurs within a coral's lifetime and whether the coral can survive it. This may explain why some coral species have the capacity for adaptive bleaching while others apparently do not.

INTRODUCTION

The Adaptive Bleaching Hypothesis (ABH) posits that when coral bleaching results from an environmental change, a coral may be repopulated by a different type of symbiotic dinoflagellates, potentially yielding a host-symbiont combination more suited to the new environment (Buddemeier and Fautin 1993). The ABH was originally proposed to explain two paradoxes of coral biology: 1) corals are seemingly fragile on a short time scale but robust over geological time, and 2) bleaching represents the breakdown of a supposedly beneficial mutualism. However, the ABH has become a puzzle of its own, because only a subset of coral species appear to have the ability to acclimate to new environments by switching symbiont types.

Evidence for symbiont switching

Pioneering work on the *Montastraea annularis* complex in the Caribbean highlighted the ability of polymorphic host-symbiont combinations to produce phenotypes with habitat-specific photoadaptation. *Montastraea annularis* and *M. faveolata* host multiple clades of *Symbiodinium* (Rowan and Knowlton 1995; Toller et al. 2001), which are structured both within each colony and across a depth gradient. Sub-habitats within a colony are differentially prone to bleaching (Rowan et al. 1997), and following a bleaching event, tissue is sometimes re-colonized by a different symbiont clade.

Later work confirmed that clades of symbiotic dinoflagellates are differentially heat-tolerant (Rowan 2004), although thermal tolerance also varies between subclades (Sampayo et al. 2008; Jones et al. 2008) and depends on the host in which it is found (Abrego et al. 2008; Baird et al. 2009). Despite this variability, Clade D is thought to be generally more thermally-tolerant. Because Clade D increased in abundance on reefs that suffered bleaching events (Toller et al. 2001; Glynn et al.

2001; Baker et al. 2004; van Oppen et al. 2005; Jones et al. 2008) and increases the thermal tolerance of individual corals (Berkelmans and van Oppen 2006), researchers posited that switching to more heat-tolerant symbiont types could help corals adapt to climate change and increasing sea surface temperatures (Baker 2001; Baker et al. 2004). In light of these findings, temperature change and climate change came to be the primary forms of "environmental change" thought to induce adaptive coral bleaching.

Evidence for symbiont specificity

Studies of additional coral species have revealed that the coral symbiosis is not always characterized by labile symbiont switching as predicted by the ABH. In some species, the coral-symbiont association is specific to one symbiont clade from the beginning of the larval stage (Weis et al. 2001) while in others species, the association is less specific in the larval stage but clade-specific shortly thereafter (Little et al. 2004). While sub-clades may vary in thermal tolerance, corals that survive a bleaching event may not change their symbiotic associations even at the sub-clade level (Sampayo et al. 2008). In a meta-analysis of scleractinian coral and octocoral species, Goulet concluded that 77% of the species examined thus far appear to host only a single symbiont clade, regardless of depth, relocation, stress or disease (Goulet 2006). Baker and Romanski (2007) countered that this was an artifact of insufficient taxonomic resolution and depth of study, but Goulet maintained that although most coral families and genera form associations with multiple symbiont clades, the minority of scleractinian coral *species* appear to have this ability (Goulet 2007).

Increased taxonomic resolution will help reveal whether symbiont switching at a very fine taxonomic level does indeed occur in all coral species and whether this contributes to local photoadaptation. However, given the adaptive bleaching

hypothesis, the lack of symbiont switching between clades appears to be a maladaptive trait in many coral species. If symbiont switching helps a coral adjust to environmental change, why are some corals able to switch between symbiont clades (Rowan et al. 1997) while others do not appear to switch even between subclades (Sampayo et al. 2008)? Wouldn't a broad capacity for symbiont switching benefit all coral species? I propose here that adaptive bleaching has evolved due to a different form of "environmental change" than we typically imagine, and that differences in a species' natural history explain why this environmental change affects the acclimation capability of each coral species differently.

Defining "environmental change"

The types of environmental change thought to induce adaptive bleaching, as described by Buddemeier and colleagues in their updated treatment of the Adaptive Bleaching Hypothesis (Buddemeier et al. 2004), fall along a continuum from "discrete changes in environmental state" such as "climate-driven shifts in major current patterns" and "alterations of lagoonal flushing and circulation during sea-level change" to "rapid oscillations around a stable baseline of environmental conditions" such as "enhanced ENSO regime(s)…imposing higher frequencies and intensities of extreme conditions." In general, temperature change is the environmental condition most often discussed in relation to adaptive bleaching, both in the original paper that proposed the ABH (Buddemeier and Fautin 1993) and the updated treatment (Buddemeier et al. 2004).

Indeed over the past few decades (i.e., over ecological time), temperature increases across entire reefs have been a major cause of coral bleaching (Hughes et al. 2003). However, over evolutionary time, corals have been subjected to a series of far more regular and dramatic environmental changes that have moved corallites from

shaded environments to high-UV exposure (and vice versa), and from shallow habitats to deep ones (and vice versa). Coral polyps experience these extreme environmental changes when damaged, moved, and fragmented by severe tropical storms. Storms move coral polyps into dramatically altered light and temperature regimes at a more localized scale, more quickly, and with more regularity than climate change or temperature rise, and this form of environmental change can rapidly trigger tissue bleaching. Storms have occurred on reefs since the beginning of coral evolution and differently affect corals based on life history, habitat, and morphology, resulting in different selective pressure on different coral species. To demonstrate how adaptive bleaching may have evolved as a response to periodic storm damage, and to show why species-specific life-history characteristics affect the strength of this selective pressure, I present here some observations of partial bleaching in a species known to switch symbionts, following a severe tropical storm.

OBSERVATIONS AND HYPOTHESES

Tropical storm Omar in Curaçao, southern Caribbean

On 14 and 15 October 2008, tropical storm Omar passed to the north of Aruba and the Netherlands Antilles (Curaçao and Bonaire) in the southern Caribbean. It became a category 1 hurricane on 15 October. Hurricane Omar reached category 3 on 16 October as it crossed the Greater Antilles before moving to the northeast and losing power over the Atlantic Ocean. Storms of this magnitude are unusual in Curaçao due to its extreme southern location in the Caribbean. Wind speed during Omar reached 130 km/hr and wave heights reached 7 m. The large wind field and slow movement of Omar's center produced strong wind and waves from the southwest, which battered Curaçao's shallow reefs more forcefully than did Hurricane Lenny in 1999 (Meteorological Service of the Netherlands Antilles and

Aruba 2010).

The underwater damage inflicted by Omar ranged from minor at the far ends of Curaçao to severe in the central regions of the island (K. Marhaver, pers. obs.). Shallow reefs that suffered the worst damage were most likely to have sandy bottoms and a large amount of loose rubble from past coral dieoffs (e.g., sites such as Carmabi Buoy Zero, Snake Bay, Porto Marie). On these reefs, coral colonies were lifted out of the sand and rotated, overturned, or thrown down the reef slope (e.g., Fig. 7.1a-b). Concurrently, loose coral rubble and flying coral colonies became projectiles with which other corals were beaten and scoured. Over half of the coral heads on these reefs suffered surface damage (A. Debrot, personal communication), with some corals missing up to half of their tissue (e.g., Fig. 7.1c) and other corals missing large chunks removed by single impacts (e.g., Fig. 7.1d).

On the shallow sand flats at Carmabi Buoy Zero and Snake Bay, a number of massive *Montastraea faveolata* colonies were lifted entirely out of the sand and overturned or thrown sideways (Figs. 7.1a, 7.2a-c and 7.3a-d). The partial colony bleaching that occurred afterward provided key insights into the evolution of this species and the environmental changes that cause it to bleach.

Whole-colony rotation causes "environmental change" for subsets of corallites

Many of the *M. faveolata* colonies moved by Omar were rotated 90 degrees as they were broken off of bioeroded bases or simply lifted up by storm surge and dropped back onto the reef (Fig. 7.1a, 7.2a-c, 7.3a-d). Two to three days after the hurricane, a subset of these colonies exhibited partial bleaching consistent with the adaptive bleaching hypothesis and known patterns of symbiont zonation within this species (Fig. 7.2a-d).

In some instances, bleaching occurred on the new side (former top) of a

colony (Fig. 7.2a-b). Corallites previously in high-irradiance habitats at the tops of colonies (where *Montastraea annularis* complex species tend to be dominated by Symbiodinium clades A and B, Rowan et al. 1997) suddenly found themselves in a lower-irradiance regime on the side of the colony, and underwent bleaching. This reaction is somewhat similar to "dark bleaching," i.e., the expulsion of zooxanthellae when a coral is subjected to partial or complete darkness (e.g., Franzisket 1970). Researchers often take advantage of this characteristic of some coral and anemone species, including *M. faveolata*, to create aposymbiotic specimens for studies of symbiont infection and physiology (e.g., Toller et al. 2001; McGill and Pomory 2008). The fact that bleaching in these colonies was partial and limited to newly-shaded corallites provides strong evidence that bleaching was not simply due to stress incurred by the hurricane but by a change in localized irradiance.

In other instances, bleaching occurred on the surface of the colony that was formerly a side and was now the top. These corallites were previously shaded by the majority of the colony but were now exposed to stronger and more direct irradiance. Importantly, bleaching occurred only in the valleys and not on the tops of the colony's peaks (Fig. 7.2c-d). The loss of symbionts in the valleys of *M. faveolata* was observed by Rowan and colleagues when they documented partial colony bleaching concurrent with symbiont zonation (Rowan et al. 1997); the more thermally-susceptible Clade C, which occupies the valleys and more shaded regions of *M. faveolata* in shallower water, is least robust to bleaching conditions and most likely to be expelled in a bleaching event. Thus, after colony rotation, *M. faveolata* appears to have expelled only its most thermally-susceptible symbionts in the tissues that underwent the most dramatic change in irradiance regime.

Notably, Rowan et al. experimentally "toppled" columns of the closely related species *M. annularis* and documented a shift in zooxanthellae clades over a six-month

period, with Clade C disappearing from former sides of columns and appearing in the new sides of the columns (Rowan et al. 1997). This elegantly demonstrated that zonation of symbiotic dinoflagellates in *Montastraea annularis* and *M. faveolata* is controlled by irradiance regimes, however the idea that toppling of a coral colony constitutes the type of "environmental change" that induces adaptive coral bleaching was not incorporated into the conversation about the ABH. Storm action was never mentioned as a probable cause of such change on ecological timescales (either in the Rowan paper or since) nor has it been suggested as a potential source of the selective pressure for the evolution of the ability to switch symbionts.

I observed both types of bleaching described above (i.e., bleaching of newly-shaded corallites and bleaching of newly-irradiated corallites) on the same reef at approximately the same depth. With the exception of small bleached spots on a small number of heavily-damaged (and therefore highly-stressed) coral colonies, bleaching was only observed in colonies that had been rotated. No fully-bleached corals were observed on any of five reefs examined in the week after the storm (K. L. Marhaver, personal observation). Together, these observations support the notion that toppling by storms (and not simply the stress of storm damage or changes in water temperature) can cause a sufficient "environmental change" for subsets of coral polyps to induce bleaching. This bleaching followed known patterns of symbiont zonation in *M. faveolata* in a manner consistent with the predictions of the ABH.

Corals record their own resilience from storms

When Omar overturned and uplifted coral colonies, it revealed portions of coral skeletons that had been buried during previous storms over the course of decades and centuries (Fig. 7.3a-d). These newly uncovered skeletons often had different growth axes than the living tissue. In many cases, it was apparent that a

coral's position after Omar represented at least its third orientation in space during its lifetime. Four examples of this are depicted in Figs. 7.3a-d.

For most coral species, including *M. faveolata*, tissue necrosis occurs within hours to days after a portion of the colony is buried in sediment. This sediment also prevents overgrowth by algae or fouling by worms and encrusting organisms, leaving the skeleton clean and the morphology easy to observe when it is later exposed. Combined with the characteristic growth forms of *M. faveolata*, years-to-decades of preservation in the sediment made the history of these newly uncovered skeletons easy to interpret. In some cases, bioerosion and fouling obscured the boundary between live and dead skeletal regions and the possibility remains that these dead *M. faveolata* colonies were simply colonized by a new *M. faveolata* recruit. However, based on the widths of live and dead regions and clear patterns of new growth that resulted from partial smothering of older regions of the colony, it was clear that many of these corals had undergone and survived multiple rotations during their lifetimes. Therefore, the ecological history of these coral colonies reveals that storm damage was the likely source of selective pressure for the evolution of the adaptive bleaching capability.

Other colonies moved by Omar revealed an "ice cream cone" structure (e.g., note the bottom of colony depicted in Fig. 7.3d), revealing that the coral had suffered repeated sedimentation events that buried 1-2 cm of tissue around the colony's lower ridge, while the rest of the colony continued growing above the sediment. Importantly, this increasingly top-heavy colony structure (observed for both *M. faveolata* and the boulder-shaped coral *Diploria strigosa*) also makes a colony increasingly prone to rotation or inversion in subsequent storms. Thus, while top-heavy colonies may be increasingly likely to fall down a reef slope, they may also be more likely to fall over in flat, sandy reef habitats.

Tropical storms place stochastic but strong selective pressure on corals

M. faveolata and its sibling species can live for hundreds of years (e.g., Van Veghel and Bosscher 1995); therefore, we should expect them to survive extreme storm events that occur on the scale of decades, and we should expect these events to shape the species' evolution. A colony reoriented by a storm which can quickly adapt to dramatically different light regimes by reconfiguring its symbiont cohort could potentially reproduce for hundreds of additional years during its lifetime. Thus, while selective pressure on species with numerous overlapping generations may be generally weaker than in organisms with non-overlapping generations (Hughes et al. 1992), such strong selective events could still provide sufficient pressure over millions of years to drive the evolution of strategies to adapt to these events.

Polymorphic symbioses provide slow-growing, long-lived species with a mechanism by which to *adjust* to environmental change on a timespan shorter than the average individual's lifespan, but in order for a coral to truly *adapt* to frequent environmental change, selective events must occur often enough for selection to repeatedly favor individuals who can survive one change after another. A colony of *M. faveolata* may suffer reorientation and partial mortality through dozens of massive storms during its lifetime, and the surviving polyps will continue to reproduce, passing the winning genotypes onto hundreds of future generations. Of the "environmental changes" thought to cause adaptive coral bleaching (e.g., changes in currents, increased sea surface temperature, and increasing ENSO strength), none occurs with the frequency of tropical storms. (While ENSO cycles occur every few years, these cycles change strength and/or frequency on far longer time scales). Therefore it seems tropical storms that cause "environmental change" by reorienting coral colonies have provided sufficiently strong and frequent pressure to select for

the adaptive bleaching ability of *some* corals species, and this ability has simply become convenient for corals faced by human-induced environmental assaults in the modern era.

Life history characteristics will affect the evolution of adaptive bleaching

If periodic storm damage throughout evolutionary time causes some coral species (but not others) to evolve the capacity for adaptive bleaching, what characteristics of a species might favor its evolution? Loya (2001) described characteristics of coral species that best predict the ability to *survive* bleaching, including massive/encrusting morphologies and thicker tissue layers. Goulet (2006) categorized corals as those which can and cannot host multiple symbiont types, either in series or in parallel, but did not consider did species morphology or life history. In proposing those corals that would be expected to exhibit adaptive bleaching, I use the word "types" in the following paragraphs to indicate that these theories may apply at the symbiont clade, subclade, or strain level.

Coral species that can host multiple symbiont types are expected to 1) have colony morphologies that create sub-habitats with different irradiance levels or 2) exist over a wide range of depths and therefore over a wide range of irradiance levels. Species that form large columns, large branches, or massive boulders have corallites that simultaneously exist in a variety of irradiance regimes, and colony fitness may be higher when these corallites are able to associate with different symbiont types spatially or temporally. These colony shapes are more likely to survive being overturned or fragmented with large pieces of live tissue remaining intact, but in a new irradiance regime. These species also live long enough for selection events to occur regularly. Thus, while storm damage may be periodic, it will occur often enough that the strength of positive selection *for* the adaptive bleaching

ability counteracts selection for reducing energetic costs by maintaining highly specific symbiont associations.

Species that occupy a wide depth distribution may be more likely to exhibit adaptive bleaching than those with a narrow depth range because individual colonies survive across a variety of irradiance regimes. However, the ability to switch symbionts after damage/reorientation may depend on the extent to which the symbiotic relationship remains plastic throughout a colony's lifespan. A wide depth range alone is therefore not a guarantee that adaptive bleaching should evolve in a species.

For smaller, shorter-lived species with sub-massive, encrusting, or plating morphologies, each corallite is more likely to exist in a relatively homogeneous irradiance environment. Phenotypic plasticity is energetically expensive, and species with these morphologies may have higher fitness by associating exclusively and consistently with a single symbiont type. (This may also explain why many "weedy" coral species transmit symbionts vertically to their offspring.) These species are less likely to survive being moved or damaged by a storm, as they are more likely to be killed completely, smothered, or thrown onshore or down a reef slope. A shorter lifespan also means multiple reorientation events are not a strong selective force that small, sub-massive coral species must typically survive in order to persist. Surviving repeated storms is a less important aspect of their life history compared to recruiting to newly cleared surfaces after a disturbance.

CONCLUSIONS

Buddemeier and colleagues noted the importance of determining the "fundamental limits to symbiosis-based adaptation and acclimatization." Here I have proposed some reasons why not all coral symbioses appear to readily recalibrate

themselves to environmental change. If symbiont switching were not very costly to a coral, then perhaps all coral species would benefit from the ability to perform adaptive bleaching. But thus far, only some species appear to have undergone sufficient selective pressure to achieve this trait. The coral reefs of the future may be dominated by coral species that exhibit regular adaptive bleaching, either because corals evolve this ability in the coming millennia, or because those species that cannot regularly reconfigure their symbioses to withstand modern day environmental change simply go extinct.

The observations and ideas presented here are meant to provide a new perspective on the evolution of the adaptive bleaching capability shown by some coral species. The robustness of corals over geological time is a testament to their ability to deal with nearly any natural assault, in some cases by undergoing the tremendous additional stress of dismantling and then re-establishing their dinoflagellate symbioses. But only coral species that have evolved this ability are able to employ it now. If adaptive bleaching is restricted to a few coral species for which rapid environmental change was a periodic driver of their evolution, humans should not assume their myriad and massive alterations to planet Earth can be handled to the same degree as occasional events that have occurred on coral reefs on the time scale of years to decades. Corals are incredibly resilient to single stressors, but only when they are otherwise healthy. In a time of pervasive and rapid global change, we are again reminded of the importance of local protection from the (human) elements (Knowlton and Jackson 2008).

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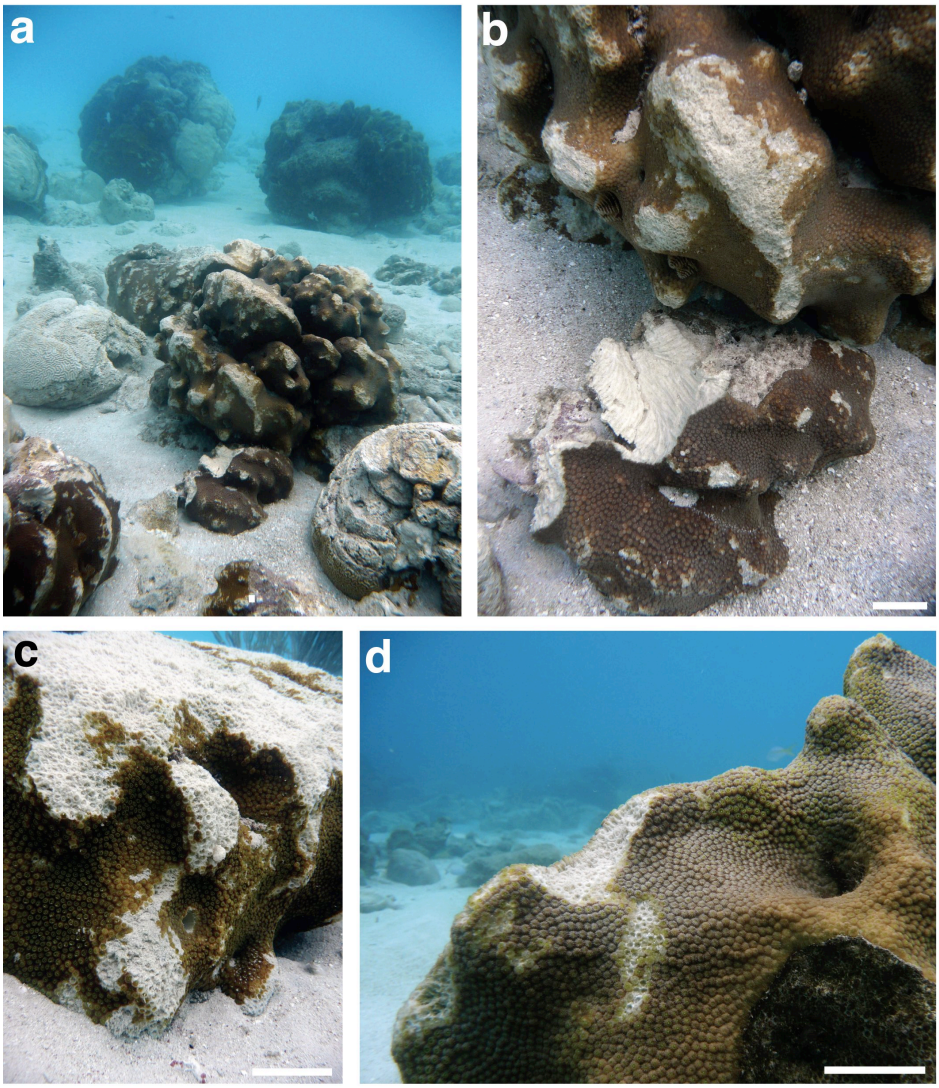

Figure 7.1. *Montastraea faveolata* colonies damaged and/or moved by wave action and flying rubble demonstrate the force of Tropical Storm Omar. Shallow reefs suffered the worst damage from the storm. Here, corals photographed on the shallow reef inshore from Carmabi Buoy Zero on 18 October 2008 demonstrate the type and extent of damage typical on shallow reefs, where rubble from previous coral dieoffs damaged live coral colonies. All scale bars represent approx. 3 cm and apply to the foreground object in the picture. (a) Reefscape showing *M. faveolata* colonies that have been rotated, flipped, and/or broken apart by the storm. (b) Close-up of the *M. faveolata* colony in the foreground of part a, showing that the colony that has split lengthwise down its growth column. (c) An *M. faveolata* colony that suffered multiple impacts from flying rubble; the tissue and skeleton were entirely removed from protruding surfaces but tissue survived in crevices protected from the impact. (d) In some colonies, large chunks of skeleton were apparently removed by a single impact from flying corals and debris.

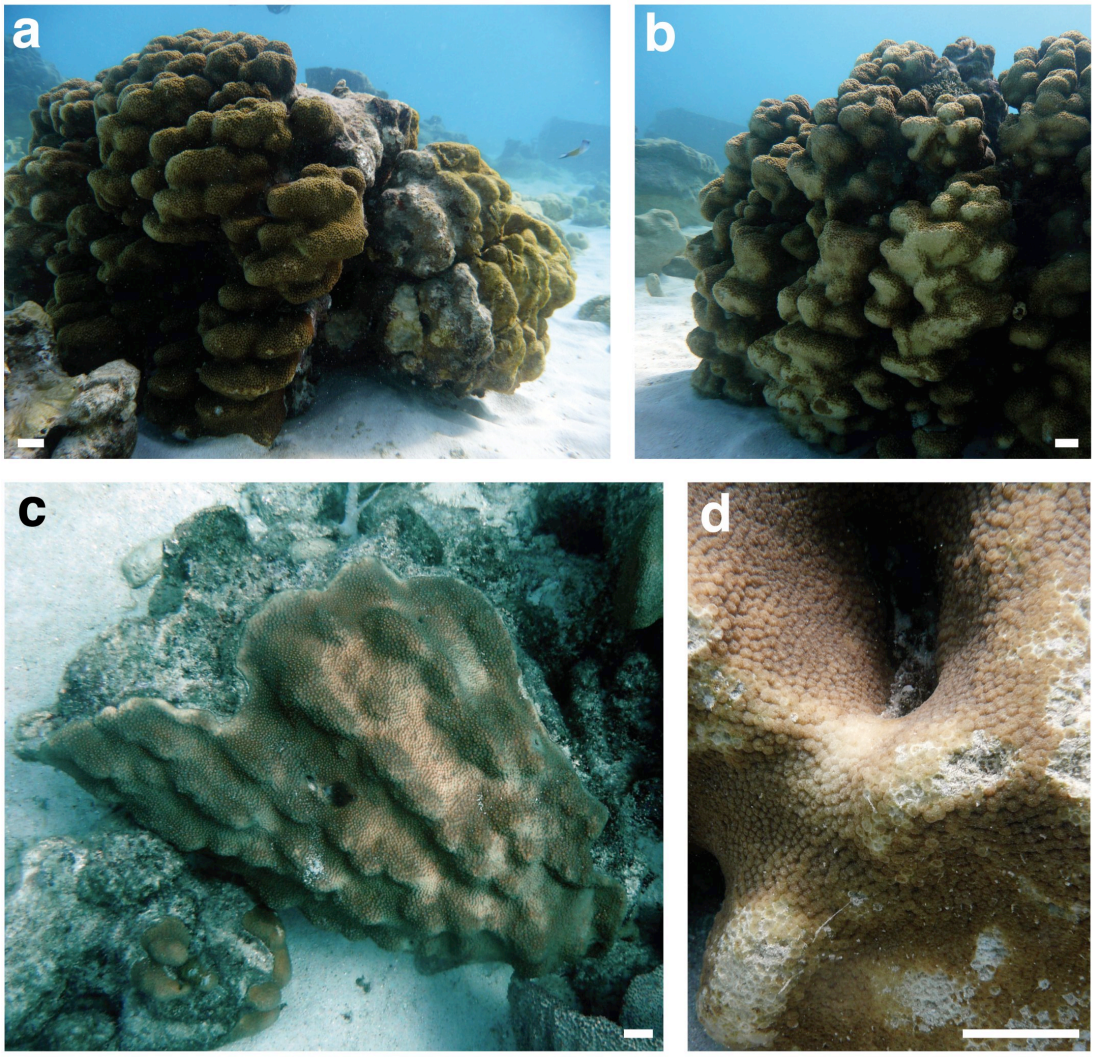

Figure 7.2. Colonies exhibit partial bleaching after being reoriented by wave action. *Montastraea faveolata* colonies were photographed at Snake Bay on 18 October 2008, three days after Tropical Storm Omar. Shown are colonies that underwent obvious rotations and exhibited partial bleaching in tissue areas that were moved into a new light regime. All scale bars represent approx. 3 cm and apply to the closest corallites in the photograph. (a) *M. faveolata* colony shown from the side to demonstrate 90-degree rotation of the colony by the storm and the exposed skeleton which was formerly buried in the sediment (b) Side-view of colony from (a) showing partial bleaching among corallites that were previously on top of the colony and were moved in a lower-irradiance light regime on the new side of the colony. (c) *M. faveolata* colony that has rotated 90 degrees and now shows partial bleaching in areas of the colony that were previously on the side and are now on the top surface. (d) Close-up of partial bleaching of *M. faveolata* in a valley of a larger colony that was also damaged by impacts from flying debris and rubble.

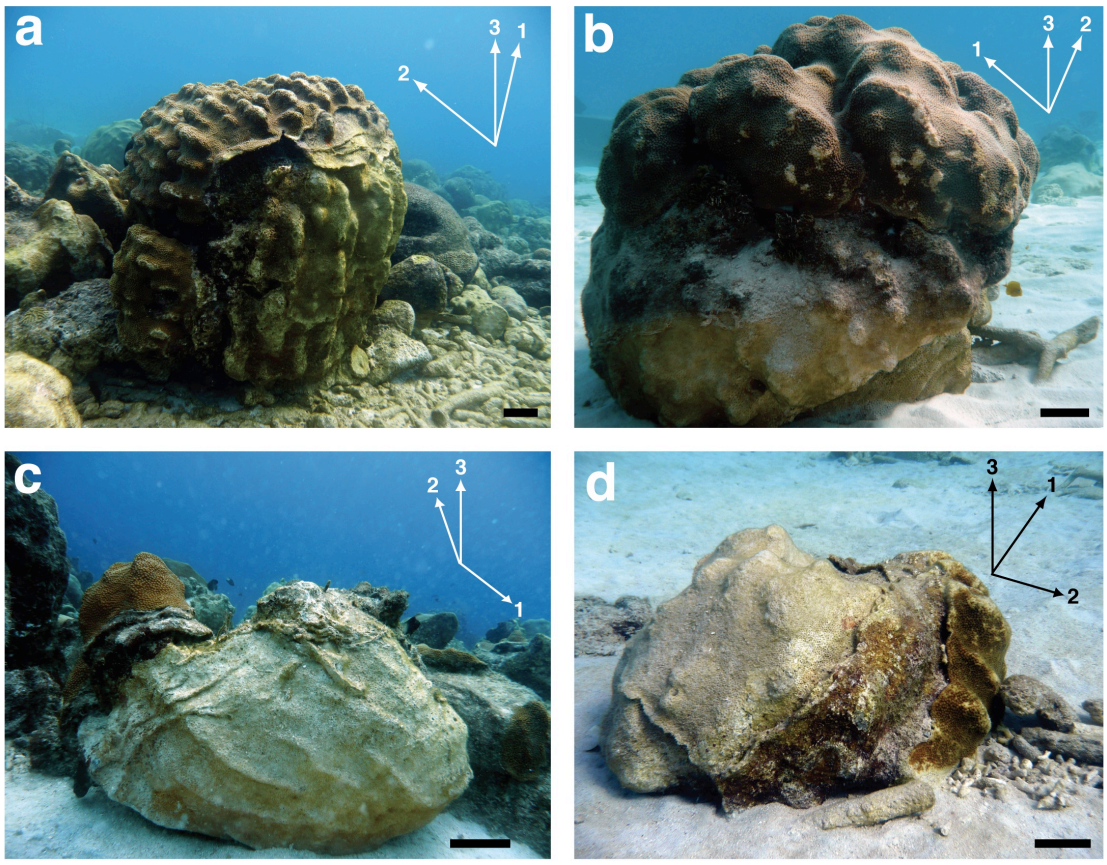

Figure 7.3. Examples of *M. faveolata* colonies that survived whole-colony rotation in the past. *Montastraea faveolata* colonies were photographed at Snake Bay on 18 and 21 October 2008. Scale bars represent approximately 10 cm and apply to the nearest corallites in each photograph. Arrows indicate the approximate direction of the vertical axis of each colony through time, numbered from the oldest orientation (1), to the pre-storm orientation of the colony's live tissue (2), to the current orientation of the colony after Omar (3). (A) *M. faveolata* colony that has undergone two rotations of nearly ninety degrees. (B) *M. faveolata* colony that has undergone one large rotations and one less dramatic rotation. Note the appearance of the "ice cream cone" structure on the oldest portion of the colony at the bottom. (C) *M. faveolata* colony that has suffered a rotation of nearly 180 degrees and was most recently lifted and flipped sideways nearly 90 degrees. (D) *M. faveolata* colony that was rotated approximately 90 degrees, then approximately 120 degrees.

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CHAPTER 8

Observation of bleached corals extending mesenterial filaments into nearby algal turfs

K. L. Marhaver

The mutualism between corals and their symbiotic dinoflagellates is the best-known animal-microbe interaction that occurs on coral reefs. The transfer of energy from symbiont to host underpins the creation of the reef structure itself as corals obtain the vast majority of their daily energy requirements from their symbiotic dinoflagellates.

Photosynthetically fixed carbon drives a large proportion of respiration in the coral animal (Edmunds and Davies 1986) and in some species, including *Montastraea annularis*, symbiont photosynthesis can produce over 100% of the daily energy requirements of the animal host (Davies 1977). In fact, primary production by zooxanthellae is often so great that half of all assimilated carbon may be shed by the coral as mucus (Davies 1984, Crossland 1980).

During a bleaching event, corals draw down their lipid reserves in the absence of the primary fuel for respiration and mucus production (Grottoli et al. 2004, Yamashiro et al. 2005). Corals can also obtain photosynthate from endolithic algae (Schlichter et al. 1995), and one coral species has been shown to increase its reliance on this alternative source of energy during a bleaching event (Fine and Loya 2002). This allows the coral more time to re-establish its population of symbiotic dinoflagellates. Coral species vary in their ability to switch to heterotrophically acquired carbon during bleaching events (Grottoli et al. 2006), making alternative sources of photosynthate potentially more valuable than hetreorophically obtained energy.

On the nights of October 27 to 29, 2010, between 20:00 and 23:00 hours, I observed five bleaching colonies of *Colpophyllia natans* with their mesenterial filaments extruded from 1 to 4 cm. In every case, mesenteries were extended into neighboring algal turfs (Fig. 8.1A-B). All of these colonies were heavily bleached.

I also observed this behavior in a single colony of *M. faveolata* on October 29, 2010 (Fig. 8.1C). Importantly, in over 30 hours of night diving and over 100 total hours of diving on this reef (Water Factory site, leeward side of Curaçao), I only observed this behavior during the 2010 bleaching event. Mesenterial filaments were only observed to be interacting with algal turfs, and not with any neighboring corals. Thus, it appeared that these corals were feeding from the nearby turf algae or on the epifaunal organisms (e.g., amphipods, copoepods, nematodes) sheltered therein.

The most parsimonious explanation for this phenomenon is that during a bleaching event, the coral lacks its regular supply of photosynthate (primarily glucose). Macroalgae, including turf algae, are known to exude glucose and other photosynthetic products into the water (Haas et al. 2010). Thus, these coral colonies may have used neighboring algae as a fast source of photosynthate. Their extended mesenteries may have been feeding only on the dissolved organic carbon released by the turfs, or they could have been actively digesting heterotrophic bacteria associated with the algal surfaces (Barott et al., *In press*). *Colpophyllia natans* is known to be a fairly aggressive coral, one that can fight neighboring corals using its relatively long mesenterial filaments. However, neither this coral nor *M. faveolata* is known to "fight" neighboring algal turfs in the same manner.

Although many coral species are able to alter their relative use of autotrophically- and heterotrophically-obtained carbon (Anthony and Fabricius 2000), evidence from studies of anemones suggests these carbon sources are used at different rates and for different purposes (Bachar et al. 2007). Autotrophically-acquired carbon is more likely to be used more quickly for immediate respiration and lipid replenishment, while heterotrophically-acquired carbon is used more slowly and more evenly for respiration, growth, and maintenance. Thus, during an extended bleaching event, ready sources of photosynthate may be preferred over plankton as a

carbon source. The apparent feeding behavior described here represents a previously unreported subsistence strategy for a bleaching coral.

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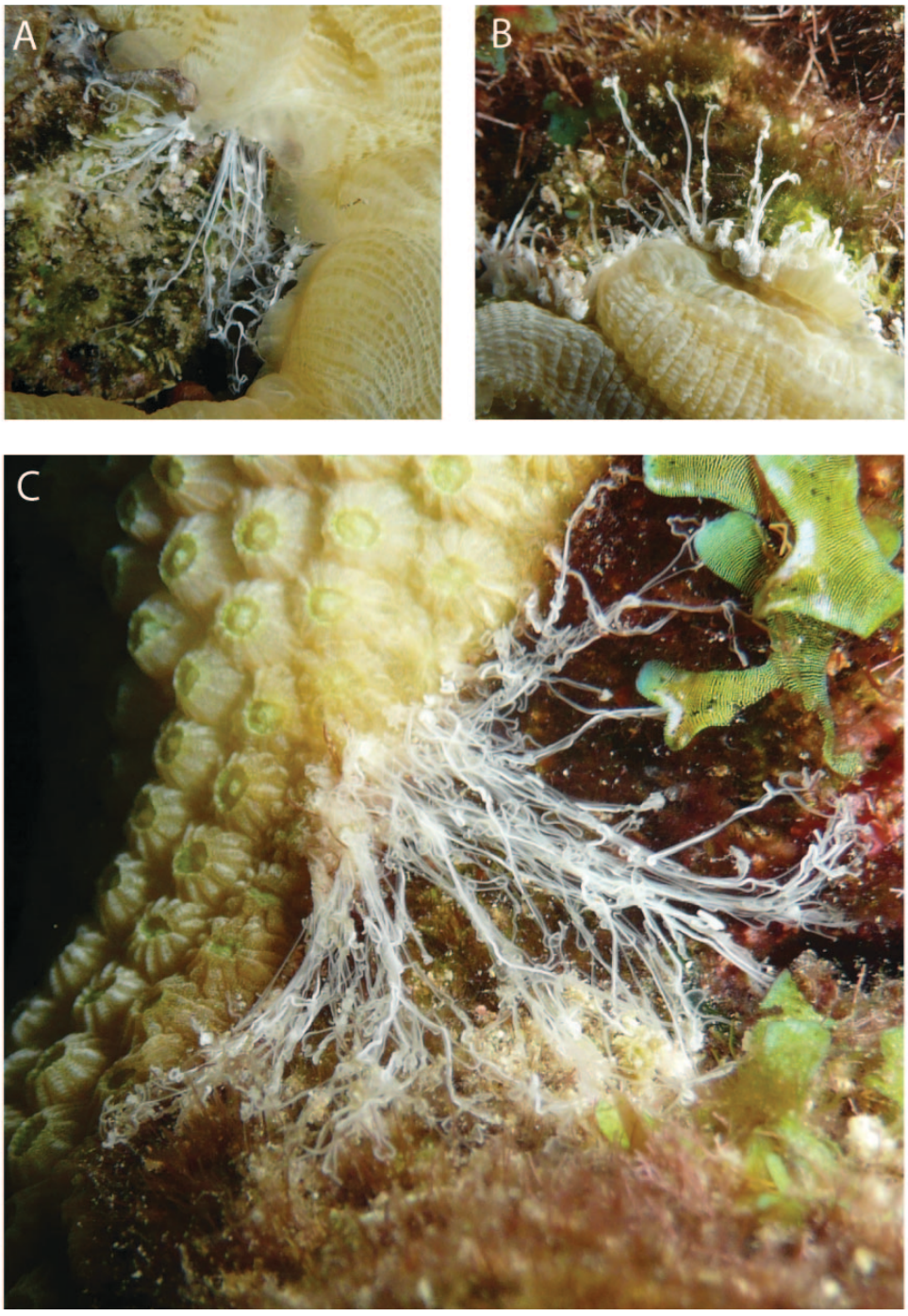

Figure 8.1. Bleached corals with mesenterial filaments extended into nearby algal turfs. (A-B) Partially-bleached colonies of *Colpophyllia natans*. (C) Partially-bleached colony of *Montastraea faveolata*. All colonies were observed between 27 and 29 October 2010 at depths of 7 to 10 m.

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CHAPTER 9

Viral communities associated with healthy and bleaching corals

K. L. Marhaver, R. A. Edwards, and F. Rohwer

ABSTRACT

The coral holobiont is the integrated assemblage of the coral animal, its symbiotic algae, protists, fungi, and a diverse consortium of Bacteria and Archaea. Corals are a model system for the study of symbiosis, the breakdown of which can result in disease and mortality. Little is known, however, about viruses that infect corals and their symbionts. Here we present metagenomic analyses of the viral communities associated with healthy and partially bleached specimens of the Caribbean reef-building coral *Diploria strigosa*. Surprisingly, herpes-like sequences accounted for 4-8% of the total sequences in each metagenome; this abundance of herpes-like sequences is unprecedented in other marine viral metagenomes. Viruses similar to those that infect algae and plants were also present in the coral viral assemblage. Amongst the phage identified, cyanophages were abundant in both healthy and bleaching corals and vibriophages were also present. Therefore, coral-associated viruses could potentially infect *all* components of the holobiont—coral, algal, and microbial. Thus, we expect viruses to figure prominently in the preservation and breakdown of coral health.

INTRODUCTION

Within a coral's skeleton, tissue, and mucus, there exists a diverse assemblage of Bacteria, Archaea, algae, fungi, and protists (Knowlton and Rohwer, 2003). Endosymbiotic algae, called zooxanthellae, and some Bacteria form relatively stable and species-specific associations with corals (Rohwer et al. 2002; Goulet 2006). It has been hypothesized that the coral animal can adapt to differing ecological niches by "switching" its algal and microbial associates. In the case of corals and zooxanthellae,

this so-called adaptive bleaching may allow the coral animal to adjust to changing water temperatures (Buddemeier et al. 2004). Coral-associated Bacteria can serve as a food source for corals (Sorokin 1973; Bak et al. 1998) and provide beneficial metabolic capabilities such as nitrogen fixation in at least one coral species (Lesser et al. 2004; Lesser et al. 2007). It has been hypothesized that changes in microbe-coral associations will facilitate the survival of corals under future environmental changes (Reshef et al. 2006).

The least-studied constituents in the coral holobiont are the viruses. No cnidarian viruses have been isolated to sufficient purity to be identified genetically prior to this study, although viruses have been observed visually in association with corals and other cnidarians. An observation of virus-like particles (VLPs) in the zooxanthellae of anemones first implicated viruses in coral bleaching (Chapman 1974; Wilson and Chapman 2001). VLPs were later observed in the tissues of heat-shocked and control specimens of the scleractinian coral *Pavona danai* (Wilson et al. 2005) and in the tissue and zooxanthellae of three coral species and one species of zoanthid, all under thermal stress (Davy et al. 2006). The origin of these VLPs was not known. A recent study demonstrated that UV stress induced one type of latent virus in cultures of coral zooxanthellae (Lohr et al. 2007). In sum, observations of VLPs in corals have generally been made under the impression that their presence is an indicator of coral stress or disease (Wilson et al. 2005; Davy et al. 2006). However, given the abundance and diversity of coral-associated microbes, it is expected that these virus populations will consist of abundant and diverse bacteriophages in addition to viruses suspected to target Eukaryotic cells, and that viruses will consistently be found in association with corals.

Viral genetic diversity is difficult to characterize because viruses share no single conserved sequence that can be used in a manner analogous to the sequencing of ribosomal RNA from cellular organisms (Rohwer and Edwards 2002). Individual viruses contain extremely small amounts of DNA (Steward et al. 2000) and often use modified bases, making cloning difficult (Warren 1980). Viruses also carry genes toxic to bacterial cloning hosts (Wang et al. 2000). Thus, in order to characterize an entire community of coral-associated viruses genetically, the viruses must be physically isolated from bacterial, archaeal, algal and host cells, as well as free DNA, *prior* to DNA extraction and cloning (Rohwer et al. 2001a).

Here, a homogenization and centrifugation technique was developed to purify viruses from the tissues of healthy and partially bleached specimens of the Caribbean coral *Diploria strigosa*. Shotgun sequencing and metagenomic analyses were then used to determine the genetic content and diversity of these two viral communities. Our results show that coral-associated viruses are extraordinarily diverse and potentially infect all members of the coral holobiont.

RESULTS AND DISCUSSION

Isolation and sequencing of coral-associated viruses

Viral particles were physically isolated prior to DNA extraction and shotgun cloning. Fragments of healthy and partially bleached *Diploria strigosa* colonies were collected in triplicate. Bleaching corals were visually identified as those that had lost 40 to 60% of their normal pigmentation. Tissue was removed from coral skeletons using an airbrush. Tissue in each set of triplicate samples was pooled to create two metagenomic libraries, DsH and DsB. ("Ds" in the sample name represents the coral

species, *Diploria strigosa*, while "H" and "B" indicate the corals' healthy or bleaching condition.) Pooling was not necessary to obtain sufficient viral material. However, we chose to pool samples to reduce the sample-to-sample variation associated with metagenomic datasets (Angly et al. 2006). Pooling samples also allowed us to examine a broader diversity of coral-associated viruses, though it concealed any variability between the individual corals collected. The raw coral tissue blastate was too viscous for processing, so power homogenization was used to liquefy it. Virus counts before and after homogenization showed no significant difference in virus-like particle (VLP) abundance (Table 9.3; $P = 0.88$). Centrifugation in cesium chloride density gradients was used to separate VLPs from cellular debris. Removal of microbial and Eukaryotic cells was confirmed with epifluorescence microscopy (Fig. 9.1). To ensure that all microbial DNA was removed, the viral fraction from the cesium chloride gradient was treated with chloroform to lyse mitochondria. DNase I was then used to degrade the exposed DNA (Breitbart and Rohwer 2005).

DNA was extracted from the isolated viruses and cloned using the Linker Amplified Shotgun Library (LASL) method. In total, 1,580 sequences from the DsH library and 930 sequences from the DsB library were obtained. When compared to the GenBank non-redundant (NR) database using TBLASTX, 44% of DsH sequences and 59% of DsB sequences had significant hits to known sequences (E-value < 0.001; Table 9.1). When compared to the environmental sequence database (ENV), 60% of DsH and 77% of DsB sequences had significant hits (E-value < 0.001; Table 9.1). When NR and ENV hits were compiled, 35% of DsH and 18% of DsB sequences were entirely novel (i.e., they had no hits to either database). This percentage of novel sequences is lower than that observed in other viral metagenomes (Breitbart et al. 2002; Breitbart et

al. 2003; Breitbart et al. 2004; Angly et al. 2006) but there still remains a large fraction of coral-associated viruses with no significant similarity to any known sequences.

Animal viruses associated with corals

Remarkably, 4.3% of all DsH sequences and 7.6% of all DsB sequences had significant hits to herpesviruses when compared to the NR database (Table 9.1). This represents 69% and 84% of the hits to Eukaryote-specific viruses in the DsH and DsB libraries, respectively. The most common herpesvirus hits were cercopithecine herpesvirus 2 sequences in the DsH library and cercopithecine herpesvirus 1 sequences in the DsB library (summarized in Table 9.2; listed in full in Table 9.4). These are both alphaherpesviruses, but significant hits to all subfamilies of the family *Herpesviridae* were observed.

Two features of the TBLASTX hits indicate that the coral viral community did not contain known herpesviruses, but rather "herpes-like" viruses. First, sequence similarity was rarely above 70% amino acid identity. Second, many sequences hit simple repeats in complete herpesvirus genomes. These repeats are characteristic of herpesviruses (McGeoch et al. 2006), but not diagnostic given the level of sequence divergence. To better visualize these herpes-like sequences, we used the location of the best TBLASTX hit for each sequence to plot sequence hit along the most commonly identified herpesvirus genomes (Fig. 9.2). These hits were distributed fairly evenly across the target genomes, indicating that there are many regions of similarity. This supports the identification of these viruses as "herpes-like".

To better characterize sequences functionally, DsH and DsB were compared to a database of complete, annotated genomes from Eukaryote-specific viruses using

BLASTX (*E*-value < 0.001). Eight sequences from each metagenome had a significant hit to a herpesvirus gene (Table 9.5). This included a total of four hits to genes involved in nucleotide metabolism (i.e., ribonucleoside-phosphate reductase), four hits to predicted or known glycoprotein genes, and four hits to genes for latency-associated nuclear antigens. The small number of hits demonstrates the high degree of sequence novelty in the coral-associated viral communities and further supports the notion that the "herpes-like" viruses associated with corals are highly divergent from any previously studied viruses. Nevertheless, the TBLASTX and BLASTX results together show that a subset of coral-associated viruses is more similar to herpesviruses than to anything else known.

Herpes-like sequences did not comprise a large percentage of hits in previously published viral metagenomes from nearshore seawater, global ocean seawater, marine sediment, or human feces (Breitbart et al. 2002; Breitbart et al. 2003; Breitbart et al. 2004; Angly et al. 2006). The occurrence of these sequences at such high abundance is therefore novel to corals. This should expand the scope of research on coral disease, but more work is needed before the potential pathogenicity of these viruses is known.

Algae and plant viruses associated with corals

Hits to viruses that infect algae and plants (family *Phycodnaviridae*) were also observed in the TBLASTX comparison to the NR database (summarized in Table 9.2, listed in full in Table 9.4). The DsH library contained one hit to *Ectocarpus siliculosus* virus, a virus of brown algae. The DsH and DsB libraries included three and four hits, respectively, to chlorella viruses, which infect green algae. Each library also contained four hits to *Emiliania huxleyi* virus 86, a virus of coccolithophores. The presence of

phycodnavirus genes in the "bleaching" DsB sequences is consistent with the fact that this metagenome was created from corals that had only partially bleached when they were collected. This tissue therefore still contained large numbers of symbiotic algae, which could serve as targets for phycodnaviruses.

When compared to the database of virus genomes using BLASTX, 30 DsH sequences and 22 DsB sequences had significant hits to phycodnavirus genes (Table 9.6; *E*-value < 0.001). Nearly all of these genes coded for hypothetical or putative proteins. Most functional predictions, when available, involved nucleotide metabolism (e.g., thymidylate synthase and ribonucleoside-triphosphate reductase). Although **t**he phycodnavirus hits comprised fewer than ten percent of hits to Eukaryote-specific viruses in both TBLASTX and BLASTX searches, their presence is notable because some phycodnaviruses are known to infect the symbiotic microalgae of hydra (Van Etten et al. 1982). This collection of hits suggests that a subset of coral-associated viruses may target algal cells in the coral holobiont. Thus, the potential remains for coral viruses to contribute to coral bleaching by directly infecting zooxanthellae, as researchers have previously posited (Wilson et al. 2005).

Coral-associated phages

When compared to the NR database with TBLASTX, 29% of the phage hits in DsH and 44% of the phage hits in DsB were to cyanophage sequences (summarized in Table 9.2; full list in Table 9.4). These phages represent a guild that is known to infect cyanobacteria rather than a taxonomic group of phages. For example, cyanophages P-SSP7 and P60 are podoviruses, but cyanophages P-SSM2 and P-SSM4 are myoviruses. DsH and DsB both contained sequences with high similarity to all four of these cyanophages.

To further characterize the phage hits, DsH and DsB were compared to a database containing only phage genes (the Phage Sequence Databank) using BLASTX (*E*-value < 0.001). A total of 173 DsH sequences and 153 DsB sequences had significant hits to cyanophage genes. These hits represented nearly all functional categories of cyanophage genes, including tail and capsid components, nucleotide metabolism, DNA replication, DNA repair, and protein translation (Table 9.7). Interestingly, there were extremely strong hits in both libraries to the core photosystem II reaction center protein encoded by the PsbA gene. To ensure that these photosystem hits were not derived from coral zooxanthellae symbionts (*Symbiodinium* spp.), which also have PsbA genes, each sequence was compared to the GenBank NR database using BLASTN (*E*-value < 0.001). The NR database contains PsbA genes from four different *Symbiondinium* clades, however there were no significant hits to these sequences in any of the comparisons. The core photosystem II reaction center proteins encoded by the PsbA gene were recently shown to be present and functional in cyanophage genomes (Sullivan et al. 2006). Their presence here lends supports to the identification of these coral-associated viruses as cyanophages.

The discovery of cyanophages in the coral viral assemblage is not unexpected. Cyanobacteria were previously identified in one of four 16S rDNA clone libraries from *Diploria strigosa*-associated Bacteria (Rohwer et al. 2002) and as endosymbionts in the Caribbean coral *Montastraea cavernosa* (Lesser et al. 2004). The cyanobacteria in *M. cavernosa* belong to the Order Chroococcales. This Order also contains *Prochlorococcus* spp. and *Synechococcus* spp., which are the targets of the cyanophages identified in the DsH and DsB libraries.

It has been proposed that cyanobacterial symbionts of corals perform nitrogen fixation within either the coral skeleton or tissue (Shashar et al. 1994; Lesser et al. 2004). Nitrogen fixation within the tissue has now been demonstrated in *M. cavernosa* (Lesser et al. 2007). Zooxanthellae are typically thought to be nitrogen limited, but the authors of this study showed that zooxanthellae could acquire fixed nitrogen directly from endosymbiotic cyanobacteria. Thus, the infection of cyanobacteria by cyanophages could determine the ability of zooxanthellae to acquire fixed nitrogen within the coral holobiont.

Vibriophages made up 3.7% and 6.0% of DsH and DsB phage hits, respectively, to the NR database (summary in Table 9.2, full list in Table 9.4). When compared to the Phage Sequence Databank using BLASTX, DsH and DsB contained 67 and 80 hits, respectively, to vibriophage genes, including those involved in tail construction, protein translation, nucleotide metabolism, and DNA packaging, replication, and repair (Table 9.8).

A subset of *Vibrio* spp. known to cause coral bleaching and disease has become the basis for model systems used to examine the interactive effects of microbes and temperature on coral physiology (Kushmaro et al. 2001; Ben-Haim et al. 2003). Recently, phage therapy with cultured vibriophages was shown to prevent tissue necrosis and bleaching in *Pocillopora damicornis* specimens experimentally infected with the bacterium *Vibrio coralliilyticus* (Efrony et al. 2007). Vibriophages in the coral holobiont could similarly infect bacterial pathogens and therefore benefit the coral, however their presence could be detrimental if they disrupt the coral holobiont (e.g., by releasing toxins or clearing space for more virulent strains). Further investigations should be conducted to determine the degree of influence that

vibriophages have on their target populations in the complex environment of the coral holobiont.

The abundance of cyanophages and vibriophages in these sequence libraries should not be taken to represent precise phage abundances in nature. These two groups are well studied and well represented in GenBank. As other microbial symbionts of corals are identified and their phages are studied, the relative abundances of cyanophages and vibriophages identified in coral viral metagenomes will decrease. The functional capacities of these two groups, however, will remain an important focus of attention.

Statistical comparison of phage communities

UniFrac (Lozupone et al. 2006), a metric of the unique phylogenetic distance between communities (Lozupone and Knight, 2005), was used to compare the phage populations in our coral virus metagenomes to each other and to two previously obtained phage sequence libraries: Reef (from water collected at four coral reefs) and Ocean (a pooling of sequences from 4 oceanic provinces; Angly et al. 2006). There was no significant difference between the DsB and DsH samples in this analysis ($P = 0.94$). A clustering of environments based on the UniFrac metric showed that these communities were more similar to each other than to the Ocean or Reef communities, with 100% jackknife support for all nodes. Because this method compares metagenome sequences to a phylogeny of known phages, it cannot reveal differences in phages that are undescribed. Because we chose to pool tissue samples prior to DNA extraction, this method also cannot reveal variability between individuals. In the context of known phage families, the coral phage communities do not have significant differences from each other, however, they do cluster together when

compared to other marine phage communities, which suggests that the coral holobiont is a distinct environment from that of the surrounding seawater. This falls in line with previous studies demonstrating that coral bacterial communities differ from those in the surrounding seawater (Rohwer et al. 2001b; Frias-Lopez et al. 2002; Ritchie 2006).

Viral community structure

To characterize the sequence diversity of coral-associated viral communities as a whole, metagenome sequences were assembled into groups of contiguous sequences (contigs). The number of sequences in each contig was tallied and the frequencies of contigs of each size were used to predict characteristics of the viral communities (Breitbart et al. 2002; Angly et al. 2005) using PHACCS (Angly et al. 2005). The DsH metagenome contained 1523 singletons, 22 contigs of two sequences, three contigs of three sequences, and one contig of four sequences (thus, the contig spectrum was [1523, 22, 3, 1]; Table 9.9). In the DsB metagenome, contig construction yielded only singletons and two-sequence contigs, which are not considered sufficient for accurate modeling. Based on its contig spectrum, the DsH community was predicted to have a total of 28,600 viral types and a Shannon-Weiner index of 8.96. The most dominant genotype was predicted to comprise 2.6% of the total viral community. This is extraordinary diversity, comparable to that of viral communities in soil (Fierer et al. 2007) and more diverse than viral communities in seawater (Breitbart et al. 2002).

Implications for the coral holobiont

Interest in coral microbiology has surged with the recognition that coral-associated microbes may serve as pathogens or as mutualists that perform nitrogen fixation, vitamin and nutrient scavenging, antimicrobial production, or space filling. The relative importance of each potential role has yet to be elucidated, however bacteriophages should be expected to affect these microbial communities and their functionality. Phages are responsible for 50% of bacterial death in the ocean (Wilcox and Fuhrman 1994) but may be responsible for a much higher percentage in the coral holobiont if the mobility of protist predators is restricted by the coral's tissue and mucus. Thus, the differential infection of bacterial groups by phages might serve as a "top-down" control on the diversity of the coral-associated microbial consortium and its ability to fill the various roles described above.

The coral animal itself is also expected to regulate its microbial communities. It was demonstrated that sterilized coral mucus acts as a selective agent, promoting the growth of non-pathogenic and antibiotic-producing bacterial strains over known pathogens; this selectivity was not observed when mucus was collected during a bleaching event (Ritchie 2006). Changes in coral mucus chemistry over long (evolutionary) or short (ecological) timescales can therefore provide "bottom-up" control over a community of microbial associates. The relative extent to which a coral or its phage population is able to exert such control on a microbial community is not yet known, but this study shows that further research is needed to understand the role of phages in structuring these communities.

Microbial abundances in coral tissues are approximately 10^7 per cm^2 (Wegley et al. 2004). In most environments, there are 10 VLPs for every microbial cell, the majority of which are phage. If this held true for the coral holobiont

microenvironment, we would expect corals to have 10^8 VLPs per cm^2. Considering the large number of hits to Eukaryote- and algae-specific viruses in our metagenomic libraries, the typical viral abundance on corals might be much greater. While the degree of natural variability in coral virus abundance remains to be determined, we propose that observations of extraordinary numbers of viruses in apparently healthy corals may represent a diversity of functions rather than a severity of infection.

Studies of coral-associated viruses often describe these communities as a latent pathogen reservoir, susceptible to induction by environmental stressors. The presence of Eukaryote-specific viruses is demonstrated by the sequence data presented here and it is indeed likely that some coral pathologies are caused by viral vectors. However, it appears that corals are chronically infected by thousands of viral strains. The DNA used to create our metagenomes was extracted from viral particles, thus these sequence libraries represent not latent viral sequence embedded within Eukaryote genomes but viral particles present in the coral tissue at the time of collection. The presence alone of viruses is therefore not indicative of disease.

Furthermore, the observed genetic diversity of viruses in the coral holobiont casts the very nature of coral symbiosis in a new light. Lesser et al. showed that while coral-associated cyanobacteria could provide fixed nitrogen to zooxanthellae, zooxanthellae did not appear to depend on this source of nitrogen (Lesser et al. 2007). Therefore, the mechanism maintaining coral-zooxanthellae symbiosis was not fully understood. Villarreal (2007) has suggested that viruses can serve as a stabilizing force for symbioses by establishing addiction systems within a host. For example, zooxanthellae infected with a latent virus are resistant to lysis by VLPs (Wilson and Chapman 2001). Phages may also stabilize symbioses. For example, a phage specific to *Hamiltonella defensa*, a bacterial symbiont of the pea aphid, produces a toxin that

appears to protect host aphids from Eukaryotic parasites (Moran et al. 2005). The diversity of constituents in the coral holobiont elevates the potential for these types of viral functions to exist therein. In fact, the stability of the holobiont itself may ultimately depend on the action of viruses. The study of viruses within the coral holobiont will shed new light on the basic biology of symbiosis, but it will also be particularly important as corals face ever-increasing threats to their health and habitats.

Here we have described the complexity of an under-studied facet of the coral holobiont. Herpes-like viruses occur in both healthy and bleaching corals. This should be a focus for future research on coral holobiont complexity, symbiosis, and immunology. The largest identified functional group of coral-associated viruses, cyanophages, may affect the population structure of symbiotic cyanobacteria and endolitic algae, while vibriophages present in coral tissue may affect the pathogenesis of coral-associated *Vibrio* spp. While these are important structuring forces for the coral holobiont, the prediction that up to 28,600 viral types occur in a healthy coral's viral community indicates that there are myriad functions and interactions still unidentified in this viral assemblage. When compared in the framework of a phage phylogenetic tree, coral-associated phage communities from bleaching and healthy corals are not significantly different from each other, but the coral holobiont as a phage environment is distinct from that of coral reef and oceanic waters. Thus, it appears that a diverse community of viruses continuously occupies coral tissues. With the potential to target animal, algal, and microbial cells, viruses are likely to be crucial in maintaining the overall function of the coral holobiont.

MATERIALS AND METHODS

Sample collection and preservation

Coral fragments were collected on March 15, 2005 from the fringing reef at Mount Irvine Bay (GPS coordinates: 11°11'45" N; 60°47'54" W) in Buccoo, Tobago. Corals were collected close to shore at a depth of 3.7 m using a hammer and chisel on SCUBA. Bleaching colonies were identified visually as those that had lost 40 to 60% of their normal pigmentation and lacked any apparent disease or tissue loss. Individual fragments (approx. 5 cm diameter) were placed in plastic bags and transported to the laboratory. For each sample, coral fragments were collected in triplicate from different colonies and blastate was pooled after airbrushing (see below).

Within 1 hour of collection, coral tissue was processed and preserved. Corals were rinsed with filtered, autoclaved seawater (FASW) seawater to dislodge any loosely associated microbes, viruses and sediments. Coral tissue, mucus and microbes were then removed from the coral skeleton with an airbrush (40 psi/2.8 bar) and filtered, autoclaved seawater. Coral blastate was collected in plastic bags and transferred to 50 ml conical tubes. Chloroform was added (approximately 5 ml per 40 ml blastate) to kill all cells. Samples were stored at 4 °C.

Microscopy of coral virus-like particles

Samples were diluted in FASW, fixed with paraformaldehyde (final concentration 4% v/v), and filtered onto 0.02 mm glass filters (Anodisc; Whatman Inc., Clifton, NJ) using vacuum suction (8 psi/0.6 bar). Filters were incubated with 1X SYBR Gold fluorescent nucleic acid stain (10 min; Invitrogen, Carlsbad, CA) and

mounted onto glass slides. Filters were viewed using an epifluorescence microscope with a FITC filter. Images were captured and viral particles were quantified with Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

Isolation of viral DNA from coral blastates

Coral blastates were liquefied using a handheld homogenizer (PowerGen, 5000 rpm, Fisher Scientific, Pittsburg, PA) and centrifuged at 3000 rpm for 15 minutes to remove sediments and large debris. Supernatant was transferred to sterile glass Corex tubes and centrifuged at 10,000 rpm for 15 minutes to pellet the majority of microbial cells (SS-24 rotor, 12,000 xg). Cleared coral blastates were loaded onto cesium chloride (CsCl) step gradients (1.35, 1.5, 1.7 g ml^{-1} CsCl in FASW) in polycarbonate tubes and spun in an ultracentrifuge at 22,000 rpm for 2 hours at 4°C (SW-41 rotor, 60,0000 xg). The viral fraction, at the junction of 1.35 and 1.5 g ml^{-1} density fractions, was collected using a sterile syringe as described by Steward et al. (Steward et al. 2000).

To eliminate any remaining microbes, mitochondria, and free DNA, 150 ml of chloroform was added to each isolated phage fraction and samples were agitated. To digest resulting free microbial DNA, samples were incubated with DNase I (1 unit per 100 ml sample) at 37°C for 2 hours. This method was previously employed to isolate purified viruses from human blood and fecal samples (Breitbart and Rohwer 2005; Zhang et al. 2006). Aliquots were prepared for microscopy after each isolation step to ensure retention of viral particles.

DNase I was inactivated by the addition of EDTA at the beginning of the viral lysis procedure (final concentration 0.025 M). Viral capsids were disrupted with a

formamide extraction. DNA was purified with isopropanol precipitation and a CTAB (hexadecyltrimethylammonium bromide) extraction (Sambrook et al. 1989).

Shotgun library construction

Viral DNA was amplified and cloned by constructing linker-amplified shotgun libraries (LASLs, Lucigen Corp, Middleton, WI). DNA was mechanically sheared using a HydroShear (GenMachine, San Carlos, CA) and resultant fragments were ligated to common-sequence linkers. Primers specific to the linkers were used to randomly amplify the entire DNA population with Vent DNA polymerase. Amplified DNA was cloned into pSMART vectors and electroporated into MC12 cells. This method has been shown to amplify sequences randomly from total environmental DNA (Rohwer et al. 2001a).

Sequence processing

Plasmids containing viral DNA inserts were sequenced on capillary sequencers (Agencourt Biosciences, Beverly, MA and SymBio Corp, Menlo Park, CA). PHRED was used to call bases and to trim vector and adapter sequences (Ewing and Green 1998; Ewing et al. 1998). Base quality was scored using PHRAP (Green 1994). Bases with PHRAP scores <20 were masked with an N. Sequences were trimmed further to remove ends containing fewer than 50 unambiguous bases by using FastGroupII (Yu et al. 2006). Trimmed sequences shorter than 100 bp were eliminated from analyses.

Sequences were assembled into contigs using TIGR Assembler (Pop and Kosack, 2004). Chromatograms from all contigs were visually inspected for the

occurrence of identical sequences, which result from sequencing a bacterial clone that has grown in two neighboring culture wells. Duplicate sequences of this kind were removed from analyses. The DsH library yielded a total of 1580 high-quality sequences; the DsB library yielded 930. From sequence assemblies, contig spectra were determined and were used to model viral community structure with the PHACCS (PHAge Communities from Contig Spectrum) online tool (Angly et al. 2005).

Sequence comparison to GenBank and specialized databases

Sequences were compared to the GenBank non-redundant nucleotide (NR) and NCBI environmental (ENV) databases using TBLASTX (Altschul et al. 1990; Altschul et al. 1997). Significant hits were defined as matches with *E*-values less than 0.001. For summary statistics, sequences were sorted based on hits to each of the two databases. For analysis of viral types, sequences were categorized based on the top viral hit and further sorted based on viral host (microbe or Eukaryote).

Sequences were then compared to two smaller databases using BLASTX (*E*-value < 0.001). First, all sequences were compared to a database of complete genomes from Eukaryote-specific viruses. These genome sequences are curated by RefSeq and were downloaded from the NCBI Viral Genomes Resource on August 20, 2007. The resulting database of 1,974 viral genomes contained a total of 28,456 annotated proteins. Second, sequences were compared to the Phage Sequence Databank, which contains 510 complete phage genomes as well as manually curated phage and prophage sequences. A list of BLASTX hits and a list of accession numbers used in the Eukaryote-specific virus database are available from the authors. To visualize the distribution of sequence hits across viral genomes, sequences were compared to the

same two databases using TBLASTX (*E*-value cutoff = 0.001) and the best hit was used to plot each sequence onto a viral genome.

UniFrac test of community similarity

Sequence libraries were compared to a database of all complete phage genomes with BLASTN (significance cutoff of E < 0.01). Significant hits were transposed onto a previously constructed multi-protein phylogenetic tree of the phage genomes (Rohwer and Edwards 2002). The tree topology and the distribution of hits along this tree were uploaded to the UniFrac online computational platform (Lozupone and Knight 2005; Lozupone et al. 2006). The Cluster Environments analysis was used to group environments based on the similarity of the viral lineages contained within each (100 jackknife permutations, Use abundance weights = True). The UniFrac Significance Test was used to compare pairs of viral communities (100 permutations, Use abundance weights = True).

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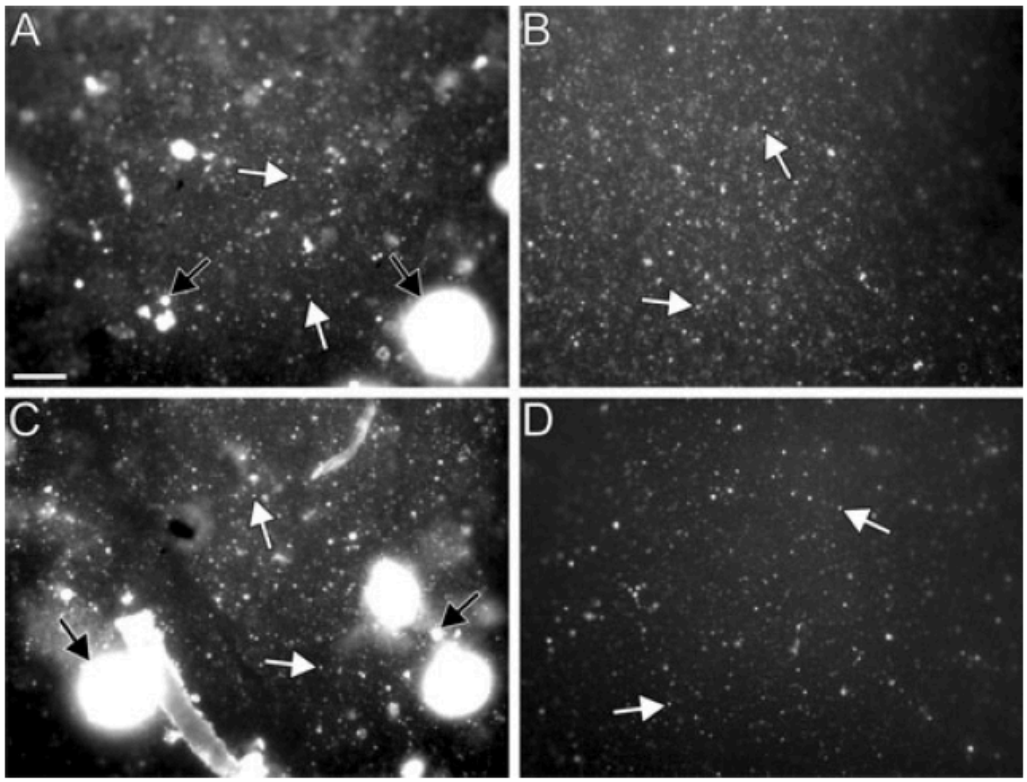

Figure 9.1. Epifluorescent micrographs of samples before and after viral particle isolation. DsH = *Diploria strigosa* - Healthy, DsB = *Diploria strigosa* - Bleaching. Samples were stained with SYBR Gold nucleic acid stain and visualized under epifluorescence at 1000X. (A) and (C). Whole coral blastate from (A) DsH and (C) DsB. Visible are abundant virus-like particles (VLPs, white arrows), as well as intact microbial cells and auto-fluorescent zooxanthellae (black arrows). (B) and (D). Purified viruses from (B) DsH and (D) DsB. VLPs are visible (white arrows) in addition to auto-fluorescence of coral GFP-like proteins. Scale bar represents approximately 5 μm and applies to all four panels.

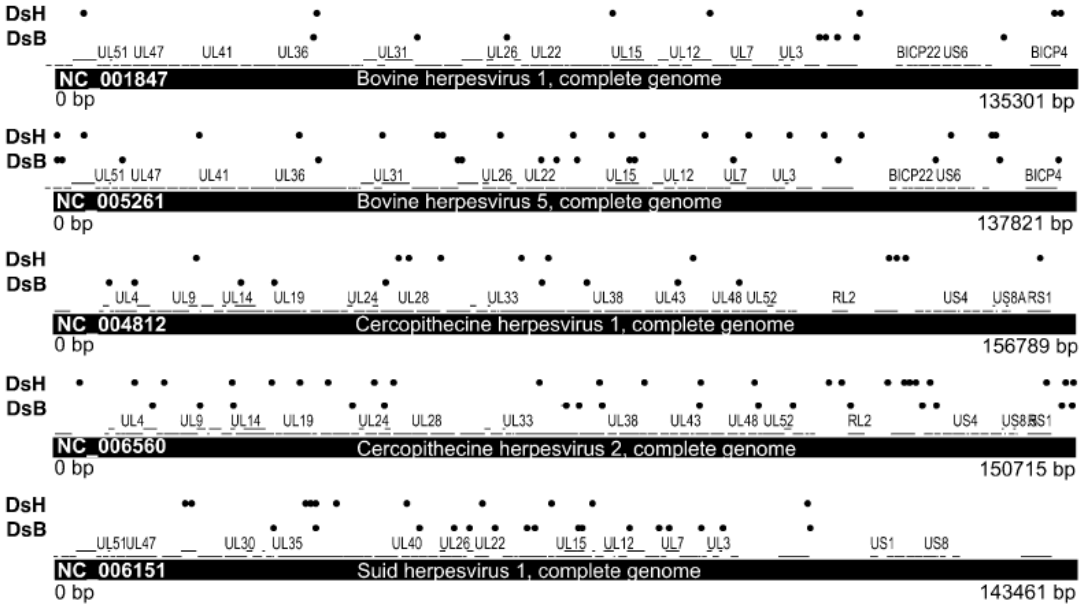

Figure 9.2. Alignment of coral virus sequences to alphaherpesvirus genomes. Each dot represents the location of an individual metagenome sequence based on its best TBLASTX hit (*E*-value < 0.001) to the virus genome. Shown are the five alphaherpesviruses with the most hits.

Table 9.1. Summary of TBLASTX hits to GenBank NR and ENV databases. Percentages are calculated from the total sequences in each metagenome. Virus hits are categorized based on the top hit to a virus sequence.

	DsH Hits		DsB Hits	
By Database	Number of hits	% of all sequences	Number of hits	% of all sequences
None	557	35.3	172	18.5
Environmental DB Only	324	20.2	208	22.4
GenBank + Environmental DB	626	39.6	508	54.6
GenBank Only	73	4.6	42	4.5
Total sequences	1580		930	
By Virus Type				
Eukaryote-specific Viruses	98	6.2	85	9.1
Herpesviruses	68	4.3	71	7.6
Phages	188	12	147	16
Cyanophages	55	3.5	64	6.9
Vibriophages	7	0.4	9	1.0
Total Virus Hits	286	18	232	25

Table 9.2. Summary of TBLASTX hits to GenBank NR database. Listed are the most commonly hit viruses in four categories: herpesviruses, algae viruses, cyanophages, and vibriophages (*E*-value < 0.001). Herpesviruses are classified by subfamily, other viruses by family. Full TBLASTX hits are presented in Table 9.5.

		Number of Hits	
Family/Subfamily	Virus name	DsH	DsB
Alphaherpesvirinae	Cercopithecine herpesvirus 2	11	7
Alphaherpesvirinae	Bovine herpesvirus 5	10	8
Gammaherpesvirinae	Saimiriine herpesvirus 2	8	7
Alphaherpesvirinae	Cercopithecine herpesvirus 1	6	13
Alphaherpesvirinae	Suid herpesvirus 1	5	7
Alphaherpesvirinae	Ateline herpesvirus 3	4	2
Alphaherpesvirinae	Human herpesvirus 1	4	0
Alphaherpesvirinae	Bovine herpesvirus 1	3	5
Alphaherpesvirinae	Human herpesvirus 2	3	4
Gammaherpesvirinae	Human herpesvirus 8	3	7
Phycodnaviridae	*Emiliania huxleyi* virus 86 isolate EhV86	4	4
Phycodnaviridae	*Ectocarpus siliculosus* virus	1	0
Phycodnaviridae	*Paramecium bursaria* Chlorella virus 1	3	3
Phycodnaviridae	*Chlorella* virus	1	0
Podophage	Cyanophage P-SSP7	18	41
Podophage	Cyanophage P60	15	7
Myophage	Cyanophage P-SSM2	13	13
Myophage	Cyanophage P-SSM4	4	2
Podophage	Vibriophage VP2	4	1
Podophage	Vibriophage VP5	1	3
Myophage	*Vibrio harveyi* bacteriophage VHML	0	3

Table 9.3. Viral particle abundance in raw and homogenized DsH tissue blastate. Viral particles were counted in homogenized and un-homogenized coral blastate using epifluorescence microscopy to determine whether mechanical homogenization resulted in shearing and loss of viral particles.

Treatment	Control (Not homogenized)	Homogenized (5000 rpm)
Number of fields analyzed	6	5
Average viral particles per field	295	305.2
Two-sample T-TEST (2-tailed, equal variance): $P = 0.88$		

Table 9.4. TBLASTX hits to virus sequences in GenBank. Sequence libraries DsH and DsB were compared with the GenBank NR database using an *E*-value cut-off of 0.001. The top virus sequence hit for each metagenome sequence was used to calculate totals. Percentages are calculated from the total hits in each of two categories: sequences from Eukaryote-specific viruses and sequences from phages. Hits to herpesvirus sequences are sorted according to their subfamily within the family Herpesviridae; all other virus hits are sorted according to virus family, if such classification exists. Hits to phage sequences are categorized by family according to the Phage Proteomic Tree and further sorted by phage morphology. ND = no classification data available.

Eukaryote-Specific Viruses			DsH Hits		DsB Hits	
Family / Subfamily	Genus	Virus name / Common name	#	%	#	%
Subfamily within *Herpesviridae*						
Alphaherpesvirinae	*Simplexvirus*	Cercopithecine herpesvirus 2	11	11%	7	8.2%
Alphaherpesvirinae	*Simplexvirus*	Cercopithecine herpesvirus 1/Monkey B virus	6	6.1%	13	15%
Alphaherpesvirinae	*Simplexvirus*	Ateline herpesvirus 3	4	4.1%	2	2.4%
Alphaherpesvirinae	*Simplexvirus*	Human herpesvirus 2/Herpes simplex virus type 2	3	3.1%	4	4.7%
Alphaherpesvirinae	*Simplexvirus*	Human herpesvirus 1/Herpes simplex virus 1	4	4.1%		
Alphaherpesvirinae	*Varicellovirus*	Bovine herpesvirus 5	10	10%	8	9.4%
Alphaherpesvirinae	*Varicellovirus*	Suid herpesvirus 1/Pseudorabies virus	5	5.1%	7	8.2%
Alphaherpesvirinae	*Varicellovirus*	Bovine herpesvirus 1	3	3.1%	5	5.9%
Alphaherpesvirinae	*Varicellovirus*	Equid herpesvirus 1/Equine herpesvirus 1	1	1.0%	2	2.4%
Alphaherpesvirinae	*Varicellovirus*	Equid herpesvirus 4	1	1.0%		
Alphaherpesvirinae	*Varicellovirus*	Human herpesvirus 3/Varicella zoster virus	1	1.0%		
Betaherpesvirinae	*Cytomegalovirus*	Human herpesvirus 5/Human cytomegalovirus	1	1.0%		
Betaherpesvirinae	*Muromegalovirus*	Murid herpesvirus 1/Mouse cytomegalovirus 1	2	2.0%		
Betaherpesvirinae	*Muromegalovirus*	Murid herpesvirus 2/Rat cytomegalovirus Maastricht			2	2.4%
Betaherpesvirinae	Uncl. *Betaherpesvirinae*	Tupaiid herpesvirus 1	2	2.0%	1	1.2%
Gammaherpesvirinae	*Rhadinovirus*	Saimiriine herpesvirus 2/Herpesvirus saimiri	8	8.2%	7	8.2%
Gammaherpesvirinae	*Rhadinovirus*	Human herpesvirus 8/Kaposi's sarcoma-associated HV	3	3.1%	7	8.2%
Gammaherpesvirinae	*Rhadinovirus*	Equid herpesvirus 2	1	1.0%		
Gammaherpesvirinae	*Rhadinovirus*	Alcelaphine herpesvirus 1/Wildebeest herpesvirus	1	1.0%		
Gammaherpesvirinae	*Lymphocryptovirus*	Human herpesvirus 4/Epstein-Barr virus	1	1.0%	2	2.4%
Uncl. *Herpesviridae*	ND	Stealth virus 1			4	4.7%
		Total hits to herpesviruses	68	69%	71	84%

Table 9.4. TBLASTX hits to virus sequences in GenBank, Continued.

Family (non-herpesvirus)						
Nimaviridae	*Whispovirus*	Shrimp white spot syndrome virus	1	1.0%		
Baculoviridae	*Nucleopolyhedrovirus*	*Autographa californica* nucleopolyhedrovirus	1	1.0%		
Baculoviridae	*Nucleopolyhedrovirus*	*Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus	1	1.0%		
Poxviridae	*Parapoxvirus*	Bovine papular stomatitis virus	2	2.0%	3	3.5%
Poxviridae	*Parapoxvirus*	Orf virus/Parapoxvirus ovis	2	2.0%	1	1.2%
Iridoviridae	*Ranavirus*	Frog virus 3	2	2.0%		
Iridoviridae	*Ranavirus*	*Rana tigrina* ranavirus	1	1.0%		
Iridoviridae	*Ranavirus*	Singapore grouper iridovirus	1	1.0%		
Iridoviridae	*Lymphocystisvirus*	Lymphocystis disease virus 1	1	1.0%		
Iridoviridae	*Coccolithovirus*	Chilo iridescent virus			2	2.4%
Phycodnaviridae	*Coccolithovirus*	*Emiliania huxleyi* virus 86 isolate EhV86	4	4.1%	4	4.7%
Phycodnaviridae	*Phaeovirus*	*Ectocarpus siliculosus* virus	1	1.0%		
Phycodnaviridae	*Chlorovirus*	*Paramecium bursaria Chlorella* virus 1	3	3.1%	3	3.5%
Phycodnaviridae	*Chlorovirus*	*Chlorella* virus/*Paramecium bursarium Chlorella* virus	1	1.0%		
Picornaviridae	*Cardiovirus*	Encephalomyocarditis virus	1	1.0%		
Tymoviridae	*Maculavirus*	Grapevine fleck virus	3	3.1%	1	1.2%
Retroviridae	*Alpharetrovirus*	Avian musculoaponeurotic fibrosarcoma virus	1	1.0%		
ND	ND	*Pan troglodytes* endogenous retrovirus 1	1	1.0%		
Retroviridae	*Mimivirus*	*Acanthamoeba polyphaga* mimivirus	2	2.0%		
ND	ND	*Heliothis zea* virus 1	1	1.0%		
		Total hits to Eukaryote-specific non-herpes viruses	30	31%	14	16%
		Total Eukaryote-specific virus hits	98		85	

Phages		DsH Hits		DsB Hits	
Phage Name	Family on Phage Proteomic Tree	# of Hits	% of Total	# of Hits	% of Total
Cyanophage P-SSP7	T7-like podophage	18	9.6%	41	27%
Cyanophage P60	T7-like podophage	15	8.0%	7	4.6%
Enterobacteria phage T7	T7-like podophage	15	8.0%	1	0.7%
Roseophage SIO1	T7-like podophage	7	3.7%	5	3.3%
Pseudomonas aeruginosa phage PaP3	T7-like podophage	4	2.1%	5	3.3%
Vibriophage VP2	Unclassified podophage	4	2.1%	1	0.7%
Bordetella phage BIP-1	Unclassified podophage	2	1.1%	0	0.0%
Burkholderia cepacia phage Bcep22	Unclassified podophage	2	1.1%	1	0.7%
Enterobacteria phage epsilon15	P22-like podophage	1	0.5%	1	0.7%
Streptococcus phage C1	Phi29-like podophage	1	0.5%	0	0.0%
Bacteriophage SPP1	PZA-like podophage	1	0.5%	1	0.7%
Enterobacteria phage K1-5	T7-like podophage	1	0.5%	0	0.0%
Pseudomonas phage gh-1	T7-like podophage	1	0.5%	0	0.0%
Uncultured T7-like podovirus	T7-like podophage	1	0.5%	0	0.0%
Vibriophage VP4	T7-like podophage	1	0.5%	0	0.0%
Yersinia pestis phage phiA1122	T7-like podophage	1	0.5%	1	0.7%
Acyrthosiphon pisum bacteriophage APSE-1	Unclassified podophage	1	0.5%	0	0.0%

Table 9.4. TBLASTX hits to virus sequences in GenBank, Continued.

Phages		DsH Hits		DsB Hits	
Phage Name	Family on Phage Proteomic Tree	# of Hits	% of Total	# of Hits	% of Total
Acyrthosiphon pisum bacteriophage APSE-2	Unclassified podophage	1	0.5%	0	0.0%
Podovirus SOG	Unclassified podophage	1	0.5%	0	0.0%
Vibriophage VP5	Unclassified podophage	1	0.5%	3	2.0%
S. typhimurium bacteriophage ES18	Unclassified podophage	1	0.5%	0	0.0%
Enterobacteria phage P1	PZA-like podophage	0	0.0%	2	1.3%
Bacteriophage T3	T7-like podophage	0	0.0%	2	1.3%
Enterobacteria phage SP6	T7-like podophage	0	0.0%	2	1.3%
Bordtella phage BMP-1	Unclassified podophage	0	0.0%	1	0.7%
Bacteriophage phiKMV	T7-like podophage	0	0.0%	1	0.7%
Vibriophage VpV262	T7-like podophage	0	0.0%	1	0.7%
Podovirus GOM	Unclassified podophage	0	0.0%	1	0.7%
	Total podophage	80	43%	77	51%
Cyanophage P-SSM2	T4-like myophage	13	6.9%	13	8.6%
Bacteriophage KVP40	T4-like myophage	4	2.1%	0	0.0%
Bacteriophage S-PM2	T4-like myophage	4	2.1%	0	0.0%
Cyanophage P-SSM4	T4-like myophage	4	2.1%	2	1.3%
Bacteriophage S-RSM2	Unclassified myophage	3	1.6%	1	0.7%
Mycobacteriophage Bxz1	Unclassified myophage	3	1.6%	0	0.0%
H. salinarum virus phiH	PhiH-like myophage	2	1.1%	1	0.7%
Bacteriophage Aaphi23	Unclassified myophage	2	1.1%	3	2.0%
Bacteriophage S-WHM1	Unclassified myophage	2	1.1%	1	0.7%
Burkholderia cenocepacia phage Bcep1	Unclassified myophage	2	1.1%	0	0.0%
Enterobacteriophage RB43	T4-like myophage	2	1.1%	1	0.7%
Burkholderia cenocepacia phage BcepMu	Mu-like myophage	1	0.5%	0	0.0%
Enterobacteria phage Mu	Mu-like myophage	1	0.5%	0	0.0%
Vibrio parahaemolyticus phage VP16C	P2-like myophage	1	0.5%	1	0.7%
Bacteriophage Aeh1	T4-like myophage	1	0.5%	0	0.0%
Bacteriophage EJ-1	Unclassified myophage	1	0.5%	0	0.0%
Burkholderia cepacia phage Bcep781	Unclassified myophage	1	0.5%	0	0.0%
Cyanophage S-RSM88	Unclassified myophage	1	0.5%	0	0.0%
Listeria bacteriophage P100	Unclassified myophage	1	0.5%	1	0.7%
Bacteriophage LP65	SPO1-like myophage	0	0.0%	1	0.7%
Cyanophage S-BnM1	Unclassified myophage	0	0.0%	1	0.7%
Bacteriophage phi CTX	P2-like myophage	0	0.0%	1	0.7%
Haemophilus phage HP2	P2-like myophage	0	0.0%	1	0.7%
Vibrio harveyi bacteriophage VHML	P2-like myophage	0	0.0%	3	2.0%
Enterobacteria phage T6	T4-like myophage	0	0.0%	1	0.7%
	Total myophage	49	26%	32	21%
Bacteriophage lambda	Lambda-like siphophage	3	1.6%	0	0.0%
Bacteriophage phiE125	Lambda-like siphophage	3	1.6%	5	3.3%
Bacteriophage T5	T5-like siphophage	3	1.6%	2	1.3%
Burkholderia cepacia phage Bcep176	Unclassified siphophage	3	1.6%	2	1.3%
Pseudomonas phage D3	D3-like siphophage	2	1.1%	0	0.0%
Bacteriophage D3112	Lambda-like siphophage	2	1.1%	0	0.0%
Bacteriophage N15	Lambda-like siphophage	2	1.1%	1	0.7%
Enterobacteria phage HK022	Lambda-like siphophage	2	1.1%	1	0.7%
Phage BP-4795	Lambda-like siphophage	2	1.1%	0	0.0%
Bacteriophage phi JL001	Unclassified siphophage	2	1.1%	0	0.0%
Mycobacteriophage CJW1	Cordog-like siphophage	1	0.5%	1	0.7%
Bacteriophage HK97	Lambda-like siphophage	1	0.5%	0	0.0%
Enterobacteria phage P22	Lambda-like siphophage	1	0.5%	0	0.0%
Bacteriophage phiKO2	Unclassified siphophage	1	0.5%	0	0.0%

Table 9.4. TBLASTX hits to virus sequences in GenBank, Continued.

Phages		DsH Hits		DsB Hits	
Phage Name	Family on Phage Proteomic Tree	# of Hits	% of Total	# of Hits	% of Total
Mycobacteriophage PG1	Unclassified siphophage	1	0.5%	0	0.0%
Bacteriophage Mx8	Cordog-like siphophage	0	0.0%	2	1.3%
Mycobacteriophage Che8	Cordog-like siphophage	0	0.0%	1	0.7%
Mycobacteriophage TM4	Cordog-like siphophage	0	0.0%	3	2.0%
Bacteriophage bIL285	D3-like siphophage	0	0.0%	1	0.7%
Bacteriophage phi3626	D3-like siphophage	0	0.0%	1	0.7%
Shigella flexneri bacteriophage V	D3-like siphophage	0	0.0%	1	0.7%
Bacteriophage phi1026b	Lambda-like siphophage	0	0.0%	1	0.7%
Bacteriophage PY54	Unclassified siphophage	0	0.0%	1	0.7%
Methanobacterium phage psiM2	Unclassified siphophage	0	0.0%	1	0.7%
	Total siphophage	29	15%	24	16%
Actinoplanes phage phiAsp2	Unclassified	4	2.1%	1	0.7%
uncultured cyanophage	Unclassified	3	1.6%	1	0.7%
Bacteriophage VP882	Unclassified	3	1.6%	2	1.3%
Bacteriophage CP-1639	Unclassified	2	1.1%	0	0.0%
Bacteriophage WO	Unclassified	2	1.1%	2	1.3%
Bacteriophage WOcauB1	Unclassified	2	1.1%	1	0.7%
Bacillus clarkii bacteriophage BCJA1c	Unclassified	1	0.5%	0	0.0%
Bacteriophage Rho11s	Unclassified	1	0.5%	0	0.0%
Coliphage K1F	Unclassified	1	0.5%	1	0.7%
Mycobacterium phage DS6A	Unclassified	1	0.5%	0	0.0%
Neisseria meningitidis phage 2120	Unclassified	1	0.5%	1	0.7%
Lactobacillus reuteri phage	Unclassified	1	0.5%	0	0.0%
Wolbachia Wkue bacteriophage	Unclassified	0	0.0%	1	0.7%
Uncultured cyanophage clone Bac9 D04	Unclassified	1	0.5%	1	0.7%
Burkholderia cepacia complex BcepC6B	Unclassified	2	1.1%	3	2.0%
Bacteriophage 187	Unclassified	1	0.5%	0	0.0%
Bacteriophage Felix 01	Unclassified	1	0.5%	1	0.7%
Bacteriophage KS7	Unclassified	1	0.5%	0	0.0%
Sinorhizobium meliloti phage PBC5	Unclassified	1	0.5%	0	0.0%
Xanthomonas campestris pv. *Pelargonii* Xp15	Unclassified	1	0.5%	0	0.0%
Bacteriophage 16-3	Unclassified	0	0.0%	1	0.7%
Xanthomonas oryzae bacteriophage Xp10	Unclassified	0	0.0%	1	0.7%
	Total unclassified phage	30	16%	17	11%
Bacteriophage SfX	Unclassified inophage	0	0.0%	1	0.7%
	Total phage hits	188		151	

Table 9.5. BLASTX hits to herpesvirus genes. Sequences were compared with a database of all complete genomes from Eukaryote-specific viruses. *E*-value cut-off = 0.001. Only the top hit for each metagenome sequence is listed.

Sequence	Similar To	Herpesvirus Genome	Function	Alternate Function	Percent Identity	Hit Length	Gaps
DSH_001_C05	YP_656628.1	Ranid herpesvirus 2	DNA (cytosine-5-)-methyltransferase	similar to RaHV-1 ORF86	36.36	55	0
DSH_002_B07	YP_001033968.1	Gallid herpesvirus 2	ribonucleotide reductase subunit 1	none listed	26.9	145	3
DSH_003_E10	YP_001096176.1	Koi herpesvirus	ORF141	ribonucleotide reductase large subunit	26.6	188	4
DSH_008_C12	YP_067966.1	Cercopithecine herpesvirus 15	EBNA-3B	similar to Epstein-Barr virus EBNA-3B (nuclear antigen)	28.16	103	1
DSH_012_D02	YP_001096176.1	Koi herpesvirus	ORF141	ribonucleotide reductase large subunit	29.47	95	3
DSH_012_D05	YP_001096176.1	Koi herpesvirus	ORF141	ribonucleotide reductase large subunit	33.68	95	4
DSH_013_E08	YP_068003.1	Cercopithecine herpesvirus 15	LF3	similar to Epstein-Barr virus LF3	35.09	114	6
DSH_014_H08	NP_570820.1	Cercopithecine herpesvirus 17, genome.	latent nuclear antigen	ORF 73; similar to Kaposi's sarcoma-associated herpesvirus ORF 73	36.17	94	4
DSH_025_A07	YP_001129431.1	Human herpesvirus 8	LANA	latent nuclear antigen; component of latency-associated nuclear antigen; LANA; LNA; ORF73	28.7	108	2
DSB_002_J20	NP_044888.1	Murid herpesvirus 4	glycoprotein 150	none listed	30.11	186	8
DSB_002_E20	YP_001096184.1	Koi herpesvirus	ORF149	predicted membrane glycoprotein; member of ORF25 gene family	30.87	149	5
DSB_003_D05	YP_001096103.1	Koi herpesvirus	ORF68	similar to myosin and related proteins, and also to IcHV-1 ORF22	23.4	141	3
DSB_002_M07	NP_045288.1	Equid herpesvirus 4	envelope glycoprotein J	type 1 membrane protein; contains a signal peptide	26.27	217	1
DSB_001_G04	YP_067991.1	Cercopithecine herpesvirus 15	BDLF3	similar to Epstein-Barr virus BDLF3 (glycoprotein gp150)	27.33	161	3
DSB_002_F04	YP_656634.1	Ranid herpesvirus 2	ORF126	similar to RaHV-1 ORF56 family; similar to RaHV-1 ORF89 and IcHV-1 ORF22; contains myosin-like domain	19.43	283	8
DSB_001_O03	YP_001129431.1	Human herpesvirus 8	LANA	latent nuclear antigen; component of latency-associated nuclear antigen; LANA; LNA; ORF73	25.19	131	2
DSB_003_L19	YP_001033965.1	Gallid herpesvirus 2	large tegument protein	complexed with tegument protein UL37; ubiquitin-specific protease (N-terminal region)	36.62	71	2

Table 9.6. BLASTX hits to genes from algae viruses (family Phycodnaviridae). Sequences were compared with a database of all complete, annotated genomes from Eukaryote-specific viruses. *E*-value cut-off = 0.001. Only the top hit for each sequence is listed.

Sequence	Similar To	Virus Genome	Gene Function	Alternate Function	Percent Identity	Hit Length	Gaps
DSH_002_E02	YP_001427299.1	Chlorella virus ATCV-1	hypothetical protein	thymidylate synthase X	66.0	47	0
DSH_002_H06	YP_001426874.1	Chlorella virus ATCV-1	hypothetical protein		31.6	57	0
DSH_008_H02	YP_001427299.1	Chlorella virus ATCV-1	hypothetical protein	thymidylate synthase X	47.8	90	2
DSH_009_F11	YP_001427319.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleoside-triphosphate reductase	34.2	111	2
DSH_011_E06	YP_001427319.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleoside-triphosphate reductase	65.5	84	0
DSH_011_F10	YP_001427028.1	Chlorella virus ATCV-1	hypothetical protein		50	60	1
DSH_012_D02	YP_001426582.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleotide reductase (large subunit)	25.2	119	3
DSH_013_F11	YP_001427028.1	Chlorella virus ATCV-1	hypothetical protein		38.7	62	2
DSH_014_A06	YP_001427028.1	Chlorella virus ATCV-1	hypothetical protein		51.7	60	2
DSH_017_B09	YP_001427299.1	Chlorella virus ATCV-1	hypothetical protein	thymidylate synthase X	31.9	119	2
DSH_022_E11	YP_001426582.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleotide reductase (large subunit)	34.2	120	0
DSH_025_F12	YP_001426761.1	Chlorella virus ATCV-1	hypothetical protein		66.7	27	0
DSH_002_B02	YP_001381854.1	Chlorella virus FR483	hypothetical protein N277L	putative SWI/SNF helicase	29.8	124	2
DSH_009_G12	YP_001381961.1	Chlorella virus FR483	hypothetical protein N535L		27.7	202	7
DSH_015_C04	YP_001381961.1	Chlorella virus FR483	hypothetical protein N535L		35.7	112	2
DSH_002_D10	YP_001381963.1	Chlorella virus FR483	hypothetical protein N542L		50	30	0
DSH_001_A07	YP_001381971.1	Chlorella virus FR483	hypothetical protein N565L		31.3	112	3
DSH_003_E10	YP_001382055.1	Chlorella virus FR483	hypothetical protein N766L	putative large subunit of ribonucleotide reductase	30	207	4
DSH_013_E01	NP_077667.1	*Ectocarpus siliculosus* virus	EsV-1-182	replication factor C small subunit	34.6	110	1
DSH_012_D05	YP_294186.1	*Emiliania huxleyi* virus 86	putative ribonucleoside-diphosphate reductase protein	Similar to the N-terminal region of Homo sapiens ribonucleoside-diphosphate reductase M1 chain	29.9	87	1
DSH_002_B07	NP_048985.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	similar to *Schizosaccharomyces* ribonucleotide reductase M1 chain	26.9	134	2
DSH_004_A05	NP_049030.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	similar to *Synechocystis* ORF s111635	52.8	178	1

Table 9.6. BLASTX hits to genes from algae viruses (family Phycodnaviridae), Continued.

Metagenome Sequence	Similar To	Virus Genome	Gene Function	Alternate Function	Percent Identity	Hit Length	Gaps
DSH_009_D05	NP_049030.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	similar to *Synechocystis* ORF s111635	63.6	33	0
DSH_013_A11	NP_048466.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	PBCV-1 GDP-D-mannose dehydratase	36.2	58	0
DSH_014_F05	NP_048711.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein		29.47	95	3
DSH_021_B01	NP_048933.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein		45	40	2
DSH_021_G03	NP_048532.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	PBVC-1 DNA polymerase	34.1	88	2
DSH_024_H01	NP_048758.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein		34.8	155	4
DSH_025_A06	NP_048779.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	similar to Bacteriophage SP01 gene 31 intron	42.6	61	1
DSH_026_A12	NP_049013.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	GDQGE (5x)	59.4	32	0
DSB_001_A04	YP_001426973.1	Chlorella virus ATCV-1	hypothetical protein		33.3	219	7
DSB_001_O09	YP_001426973.1	Chlorella virus ATCV-1	hypothetical protein		33.5	212	5
DSB_002_L03	YP_001427319.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleoside-triphosphate reductase	51.9	185	4
DSB_002_O10	YP_001427319.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleoside-triphosphate reductase	52.7	201	3
DSB_003_A01	YP_001426596.1	Chlorella virus ATCV-1	hypothetical protein		40.1	142	4
DSB_003_C09	YP_001427148.1	Chlorella virus ATCV-1	hypothetical protein	glycosyltransferase	27.3	161	5
DSB_003_C13	YP_001427299.1	Chlorella virus ATCV-1	hypothetical protein	thymidylate synthase X	28	143	2
DSB_003_E09	YP_001426668.1	Chlorella virus ATCV-1	hypothetical protein	ATP-dependent DNA ligase	26.8	153	5
DSB_003_H14	YP_001426973.1	Chlorella virus ATCV-1	hypothetical protein		36.1	72	1
DSB_003_I04	YP_001427299.1	Chlorella virus ATCV-1	hypothetical protein	thymidylate synthase X	53	168	0
DSB_003_K15	YP_001427028.1	Chlorella virus ATCV-1	hypothetical protein		51.6	62	1
DSB_003_O06	YP_001427319.1	Chlorella virus ATCV-1	hypothetical protein	ribonucleoside-triphosphate reductase	56.1	230	2
DSB_001_M11	YP_001381961.1	Chlorella virus FR483	hypothetical protein N535L		25.1	199	9
DSB_002_N03	NP_077550.1	*Ectocarpus siliculosus* virus	EsV-1-65	viral hybrid histidine kinase	34.6	179	5
DSB_002_E20	YP_294122.1	*Emiliania huxleyi* virus 86	hypothetical protein	repeat regions to several proline-rich proteins, for example,*Chlamydomonasincerta* SAG1 plus agglutinin	30.9	139	4

Table 9.6. BLASTX hits to genes from algae viruses (family Phycodnaviridae), Continued.

Metagenome Sequence	Similar To	Virus Genome	Gene Function	Alternate Function	Percent Identity	Hit Length	Gaps
DSB_001_G04	NP_048366.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	Asn/Thr/Ser/Ile rich protein; similar to *Rickettsia* cell surface antigen	25.9	135	5
DSB_001_K15	NP_048448.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	PBCV-1 glucosamine synthetase	31.9	72	2
DSB_002_D15	NP_048952.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	similar to *Vibrio fischeri* dCMP deaminase	38.6	140	4
DSB_002_H01	NP_048466.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	PBCV-1 GDP-D-mannose dehydratase	29.8	181	4
DSB_002_M07	NP_048362.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	Asn/Thr/Ser/Val rich protein	22.8	202	5
DSB_003_G06	NP_048904.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	similar to *Caenorhabditis* transcription activator	34.6	81	0
DSB_003_L19	NP_048405.1	*Paramecium bursaria* Chlorella virus 1	hypothetical protein	contains Pro-rich Px motifs: SPKPP (20X), PEPPA (9X)	43.3	60	2

Table 9.7. BLASTX hits to cyanophage genes in the Phage Sequence Databank (*E*-value < 0.001). All metagenome sequences with best hits to cyanophage genes were compiled. Listed are the 15 genes with the lowest *E*-value in each search and any gene with four or more hits. BLASTX statistics are listed for the best hit to each gene. Hits to hypothetical and unknown targets are not shown.

		DsH Hits			
Cyanophage Genome	Gene Function	Top Hit % ID	Top Hit Length	Top Hit *E*-value	# Hits total
P-SSP7	DNA maturase beta subunit	78.1	169	8.00E-73	3
P60	DNA polymerase	40.4	166	9.00E-31	5
P-SSM2	glycosyltransferase family 25	66.9	151	3.00E-63	1
P-SSM2	glycosyltransferase family 6	77.8	135	4.00E-62	1
P-SSM2	NrdA	93.0	129	7.00E-56	4
P-SSM4	NrdA	90.6	127	3.00E-53	3
P-SSM2	NrdB	66.7	165	1.00E-54	1
P-SSM2	phage tail fiber-like protein	50.6	158	4.00E-37	10
S-PM2	photosystem II D1 protein	94.8	134	2.00E-67	4
P-SSM2	possible endonuclease	46.0	100	2.00E-20	9
P-SSM2	PurM	85.4	123	2.00E-60	1
P-SSM2	RegA	78.1	128	2.00E-57	1
P-SSP7	ribonucleotide reductase domain	71.8	149	7.00E-61	8
P-SSM2	T4-like tail sheath protein	74.2	128	7.00E-50	3
P-SSP7	T7-like capsid protein	71.3	143	1.00E-57	3
P-SSP7	T7-like head-to-tail connector	69.7	201	1.00E-78	6
P-SSP7	T7-like internal core protein	57.0	100	4.00E-27	4
P-SSP7	T7-like primase/helicase	69.1	220	5.00E-86	5
P-SSP7	T7-like tail tubular protein B	39.5	205	2.00E-35	7
S-PM2	thymidilate synthase	55.6	180	4.00E-51	5
		DsB Hits			
P-SSM2	core ps II reaction center protein	91.6	214	2.00E-111	1
P-SSP7	DNA maturase beta subunit	88.9	260	7.00E-134	7
P60	DNA polymerase	44.3	185	4.00E-38	5
P-SSM4	fiber	29.9	204	1.00E-10	5
P-SSM2	phage tail fiber-like protein	38.2	212	7.00E-29	9
S-PM2	photosystem II D1 protein	90.4	177	6.00E-92	1
P-SSP7	ribonucleotide reductase domain	73.7	167	4.00E-75	6
P-SSM2	T4-like capsid assembly protein	67.9	237	3.00E-96	2
P-SSM2	T4-like DNA pkg lg subunit terminase	55.5	220	6.00E-69	1
P-SSM2	T4-like major capsid protein	65.2	276	5.00E-88	2
P-SSP7	T7-like capsid protein	69.6	250	1.00E-91	4
P-SSP7	T7-like exonuclease	66.0	156	3.00E-59	4
P-SSP7	T7-like head-to-tail connector	63.5	271	5.00E-96	4
P-SSP7	T7-like internal core protein	47.2	263	2.00E-61	5

Table 9.7. BLASTX hits to cyanophage genes in the Phage Sequence Databank (*E*-value < 0.001), Continued.

		DsH Hits			
Cyanophage Genome	Gene Function	Top Hit % ID	Top Hit Length	Top Hit *E*-value	# Hits total
P-SSP7	T7-like RNA polymerase	70.2	238	8.00E-90	7
P-SSP7	T7-like ssDNA binding protein	56.4	188	3.00E-54	2
P-SSP7	T7-like tail fiber	43.3	231	7.00E-39	9
P-SSP7	T7-like tail tubular protein B	44.1	229	3.00E-59	6

Table 9.8. BLASTX hits to vibriophage genes. Sequence libraries were compared with the Phage Sequence Databank using an *E*-value cut-off of 0.001. All metagenome sequences with best hits to vibriophage genes were compiled. Listed are the 10 genes with the lowest *E*-value in each search, as well as any gene with three or more hits. BLASTX statistics are listed for the best hit to each gene. Hits to hypothetical and unknown targets are not shown.

		DsH Hits			
Vibriophage Genome	Gene Function	Top Hit % ID	Top Hit Length	Top Hit *E*-value	# Hits total
KVP40	aerobic ribonucleoside diphosphate reductase large subunit	50.0	124	2.00E-26	4
VP4	DNA packaging protein B	55.1	207	5.00E-57	4
VP4	DNA polymerase	35.3	170	8.00E-19	4
VP4	DNA primase/helicase	39.2	189	4.00E-29	5
KVP40	gp44	65.8	38	1.00E-07	3
VP4	head-to-tail joining protein	36.0	211	2.00E-29	3
KVP40	NMN adenylyl tranferase	30.2	265	3.00E-25	1
VHML	ORF22	37.3	225	1.00E-34	1
VP16C	putative tail protein	49.3	201	1.00E-46	1
KVP40	RegA	50.4	119	3.00E-30	1
VP2	superfamily II DNA/RNA helicase	39.7	174	5.00E-29	4
KVP40	tail sheath protein	61.4	132	2.00E-41	2
VP4	tail tubular protein B	32.7	98	2.00E-08	4
		DsB Hits			
Vibrophage Genome	Function	Top Hit % ID	Top Hit Length	Top Hit *E*-value	# Hits total
VP4	DNA packaging protein B	51.5	266	6.00E-66	9
VP4	DNA polymerase	28.4	271	8.00E-21	5
VP4	endonuclease	52.1	119	2.00E-26	2
VP4	exonuclease	38.6	145	1.00E-22	5
KVP40	gp23	45.1	213	1.00E-41	2
VP4	head-to-tail joining protein	38.1	278	1.00E-46	4
VHML	ORF22	55.1	176	4.00E-54	5
VHML	ORF23	32.6	239	1.00E-23	2
KVP40	portal vertex protein of head	45.1	235	1.00E-52	2
VP4	RNA polymerase	31.8	277	2.00E-25	8
VP4	tail tubular protein B	31.8	214	1.00E-24	3
VP2	terminase	32.7	153	1.00E-15	3
KVP40	terminase DNA packaging enzyme large subunit	40.9	220	2.00E-42	1

Table 9.9. Diversity and structure of the viral community in healthy *Diploria strigosa* tissues. Contig spectrum was tallied from metagenome sequence overlaps and used to predict aspects of viral community structure and diversity using the PHACCS online tool.

Sample	DsH
Number of sequences	1580
Contig spectrum	1523 22 3 1 0 0
Richness	28600
Evenness	0.873
Most Abundant Genotype	2.63%
Shannon-Weiner Index	8.96
Average sequence length (bp)	461

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CONCLUSIONS

The Ecology of Coral-Microbe Interactions

K. L. Marhaver

I was once told that a Ph.D. was a success if you got to the end of the process and could identify what it was that you *should* have studied during your Ph.D. I definitely should have studied corals. This project raised more intriguing questions than I could have ever imagined at the outset. The chance to answer just a few more of those questions has somehow mysteriously clouded my memories of all the electrocutions it took to get this far.

Microbes and reef ecology

In testing whether Janzen-Connell effects occur on coral reefs, I was surprised to find that packets of reef water alone could so dramatically affect the survivorship of coral larvae. In fact, these water packets could potentially contain both the microbial communities that kill small corals as well as the information those corals use to avoid deadly habitats (Chapter 1). Larvae of many marine organisms can preferentially select low-mortality habitats (Grosberg 1981) and we should certainly give corals more credit for this capability as well. Future studies will reveal the extent to which distance-dependent mortality promotes the survival of rare species and structures the patterning of coral communities as it does in tropical forests (Wills et al. 2006). It is certain that interactions between small-scale physical processes and microbial communities create habitat heterogeneity that a dispersing planula experiences during every approach to the reef. We now have excellent motivation to examine this structure more closely.

Microbes and behavior

While putting together lab experiments to test the Janzen-Connell hypothesis in corals, I often paused to watch planulae scoot, turn, hop, and perform the occasional non-stop death spiral. Only after hours spent observing these behaviors

for sport did I begin to realize what they might mean for the evolution and ecology of coral species. Because they are so small, and liable to die a spectacular exploding death at any moment, coral planulae lure us into thinking they are simple animals. But larval corals have thousands of cells, thousands of genes, and as many sensory abilities as we do: they respond to ultraviolet light (Gleason et al. 2006), pressure (Stake and Sammarco 2003), biological cues such as the metabolites bound to crustose coralline algae (Morse and Morse 1996), abiotic cues such as water column salinity (Vermeij et al. 2006), biofilms from different depths (Baird et al. 2003), and even the sound of the reef itself (Vermeij et al. 2010).

Based on the data presented in Chapters 3 and 5, it is clear that the locomotory behaviors of coral planulae are highly sensitive to outside influences. We know with great certainty that planulae may use just a single surface-attached molecule when initiating settlement (Morse and Morse 1996), but we are only just beginning to understand how a planula might navigate the transition from open ocean to reef habitat and the transition from the overlying reef waters to the benthos. The habitat with the ideal combination of depth, light, salinity, pH, and symbionts is there somewhere. Based on the number of senses they have, it is certain that coral larvae have dozens of signaling cascades that allow them to detect these preferred microhabitats. Microbial communities associated with reef organisms add yet another layer of navigational information, which may be particularly useful during the transition from the overlying reef waters to the benthos.

Microbes and symbioses

Corals interact with millions of other organisms at any given moment. In the midst of the metagenomic revolution, we can look to studies of other species to predict what we will likely discover about corals in the near future. Coral-associated

viruses are tremendously abundant and diverse (Chapter 9; Vega Thurber et al. 2009) and not all viruses pose a threat. In other animal hosts, viruses are known to carry useful genes that can fend off eukaryotic parasites and harmful bacteria, thereby helping to stabilize interactions between hosts and symbionts (Moran et al. 2005; Villarreal 2007). These same phenomena are surely happening within the coral animal.

Like the coral virome, the coral-associated bacterial community is spectacularly diverse (reviewed in the pages of Rohwer and Youle 2010 that are not spent making fun of other members of this dissertation committee). Coral-associated microbes are known to perform metabolic services for coral hosts (e.g., Lesser et al. 2007), however we have just begun to use the barrage of metagenomic data to detect and study these individual functions. Furthermore, because coral-associated bacteria are performing thousands of metabolic tasks in their relationships with coral hosts, they are certainly also mediating developmental processes and altering phenotypes as they do in other animals (Gilbert and Epel 2008; Li et al. 2008).

Coral-scientist interactions

Coral reefs started dying very quickly when humans began eating and polluting them (Pandolfi et al. 2003). In the ensuing panic to diagnose coral ills, many researchers forgot (or funding agencies ignored) the fact that corals are still absolutely fascinating animals in their own right. Happily, many of the gems buried by physiologists decades ago are now being dug up and returned to prominence while sophisticated tools from other systems are increasingly being used to study corals (Mackie 2002). We can look to almost any other study system—terrestrial ecology, development, microbial metagenomics, animal behavior, toxicology—for paradigms to test and methods to deploy in the study of corals. Juvenile corals are no

longer too difficult to raise or too boring to study. Instead, they are the perfect new model system, an animal in which almost anything is possible and on which the world's most beautiful ecosystem depends.

Kurt Vonnegut once said in an interview that he was astounded anyone sits down to read anything, given that it requires such immense effort on the part of the reader. It is therefore humbling than anyone would read these words. If I have made it to the end of this Ph.D. so that others might think like a coral, then I suppose it has been a success.

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