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Effects of environment and life history strategy on coral reproductive success

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

in

Marine Biology

by

Aaron C. Hartmann

Committee in charge:

Michael I. Latz, Chair
Lihini I. Aluwihare
Maarten J. Chrispeels
Dimitri D. Deheyn
Mark D. Ohman
Jennifer E. Smith

2014

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Chair

University of California San Diego

2014

DEDICATION

I dedicate this dissertation to my grandfather, Warren Ernst Hartmann. Little did he know that during those crisp fall days sitting on the Big Manistee River in his boat, *My Boat*, he was teaching me invaluable lessons about nature's resilience despite our terrifying ability to throw it off course.

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Chapter 3, in full, is currently being prepared for submission for publication of the material. Hartmann AC, KL Marhaver, MJA Vermeij. The dissertation author was the primary investigator and author of this paper.

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VITA

2007	Bachelor of Science, Biology, University of Vermont
2010	Master of Science, Oceanography, University of California San Diego
2007-2009	National Science Foundation IGERT Fellow
2009-2012	National Science Foundation Graduate Research Fellow
2012-2013	National Science Foundation GK12 Fellow
2014	Teaching Assistant, "Introduction to Biological Oceanography," University of California, San Diego
2014	Doctor of Philosophy, Marine Biology, University of California San Diego

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ABSTRACT OF THE DISSERTATION

Effects of environment and life history strategy on coral reproductive success

by

Aaron C. Hartmann

Doctor of Philosophy in Marine Biology

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Michael I. Latz, Chair

Coral reefs are threatened worldwide due to anthropogenic activities resulting in the loss of these ecosystems at an alarming rate. In this dissertation I build upon fundamental ecological theory related to early life history characters in order to identify

factors affecting coral reproductive success in response to environmental change. I found that apparently healthy corals on degraded reefs contained lower energetic lipid content than conspecifics on healthy reefs within the island of Curacao, which contains some of the Caribbean's healthiest coral reefs. This work also revealed that populations differed in the number of offspring produced, highlighting reef-specific differences in reproductive potential.

Motivated by observed variation in offspring size within and among species, I found that large size enhanced larval survival, yet unexpectedly did not afford larvae greater tolerance of harsh environmental conditions. Surprisingly, a species that produces large larvae and tolerates marginal environmental conditions as adults was particularly sensitive to environmental stress during the larval stage. I concluded that this difference arose due to the presence of symbiotic algae in larvae of this species.

I subsequently determined that the uptake of symbionts during the larval stage led to disadvantageous behaviors and gene expression patterns. Furthermore, symbionts did not provide energy to larvae, as they do in adults. These results led me to theorize that the timing of symbiont acquisition is most optimal when it occurs around the time larvae locate cues for metamorphosing into an adult body form.

To substantiate my theory in an evolutionary context, I performed a character trait reconstruction with the known phylogeny of reef corals. Evolutionary transition rates differed depending on whether a species transfers symbionts directly to larvae or obtains them from the environment. The loss of direct transmission correlated with transitions from high to low bleaching susceptibility in adult corals, suggesting a previously unknown evolutionary transition that favors environmental tolerance.

In summary, my work contributes to general understanding of how larval characters and early life history strategies affect survival and fitness in corals, and offers insights into the ecological and evolutionary mechanisms by which larval corals respond to human-induced environmental change.

Chapter 1.

INTRODUCTION

By examining layers of ecological complexity on scales of species, populations, and genetic relatives we can begin to understand competing and interactive factors that maintain balance in natural systems. To evaluate this complexity, many ecologists rely on the study of adult-adult interactions, and often specifically those interactions that result in positive and negative outcomes among participants. This dissertation considers ecological interactions on two different axes among stony corals: between partners in symbiosis and between generations.

Understanding how corals invest energy and pass symbioses to future generations is both intellectually stimulating and timely given their recent plight. Corals form an obligate symbiosis with photosynthetic protists that allow them to exploit the high light and low nutrient conditions of tropical seas (Muscatine and Porter 1977). While highly efficient, this symbiosis is sensitive to abiotic environmental changes (Lesser 1996, Lesser 1997). The warming of oceans in recent decades has led to higher incidence of failed symbioses in corals, termed "bleaching" because of the white coloration of the animals after losing its algal symbionts (Brown 1997). Due in large part to increasing bleaching events, corals are declining worldwide at an alarming rate (Hoegh-Guldberg et al. 2007, Carpenter et al. 2008). Thus, ocean warming has turned corals' greatest strength into their Achilles heel.

The sustainability of coral reefs depends both on the survival of existing populations as well as the success of sexual reproduction. In this dissertation, I aimed to better understand how life history strategies influence coral reproductive success in a

changing world. Through this work we may better understand the capabilities of corals to adapt to environmental change, as well as identify underlying ecological and evolutionary patterns that govern coral community composition as we see it today.

Coral as a holobiont

In his treatise *On the Origin of Species* (1859) Charles Darwin wrote: "I can understand how a flower and a bee might slowly become, either simultaneously or one after the other, modified and adapted in the most perfect manner to each other, by continued preservation of individuals presenting mutual and slightly favourable deviations of structure." Darwin was describing the concept of co-evolution, a process that undoubtedly played a role in the establishment of symbioses in tropical corals. Had Darwin known this, he would have been able to answer a question that befuddled him and later became known as 'Darwin's paradox:' How can highly productive ecosystems arise in nutrient poor environments?

The symbiotic partner harbored within corals would not be identified as a single celled alga until decades later (Klebbs 1884). In the following century, others began unraveling the complexity of corals and their associated fauna, identifying the extent to which the symbiont provided energy to the coral (Muscatine and Hand 1958) and delving into the complex trophic structure of coral reef ecosystems (Odum and Odum 1955). It is now known that most stony corals live in obligate symbiosis with endosymbiotic dinoflagellates of the genus *Symbiodinium*. These unicellular protists are capable of providing up to 100% of the host's energetic demand by translocating fixed carbon in the form of carbohydrates and lipids (Muscatine 1967, Muscatine and Weis 1992). In return,

the dinoflagellate receives protection and critical nutrients from coral waste products (Muscatine and Porter 1977). The association with *Symbiodinium* affords corals a mixotrophic lifestyle, allowing them to use photosynthesis and heterotrophy to obtain the nutrition they require for survival.

The coral holobiont extends well beyond *Symbiodinium*, and includes fungi (Golubic et al. 2005), skeleton-dwelling algae (Lukas 1974), boring sponges, bivalves, and worms (Holmes et al. 2000), bacteria, archaea (Wegley et al. 2004) and viruses (Marhaver et al. 2008). The nature of each relationship, and in particular that which corals share with *Symbiodinium,* is mediated by environmental conditions (e.g., light and temperature; Lesser 1996, Lesser 1997) that can dictate where the relationship falls on a scale of beneficial to harmful at any given point in time.

The coral lifestyle

Corals employ a complex life history that includes a mobile larval stage and a sessile adult stage. While corals provide little parental care, they vary in the extent to which they invest energy in offspring and pass symbionts across generations. Exceptionally long generation times and effective asexual reproduction via fragmentation lead to long-standing adult communities during periods of stable environment (Highsmith 1982). Yet, sexual reproduction is important for maintaining populations and communities, especially after periods of disruption such as storms or during persistent change such as ocean warming.

Stony corals employ a variety of strategies for sexual reproduction, all of which result in the production of a non-calcifying, mobile larva (Carlon 1999). Species can be

broadly categorized as those that fertilize eggs internally (brood) or externally (broadcast spawn). Brooding corals release clouds of sperm into the water column that fall on adjacent conspecifics. Fertilized eggs are subsequently held within the polyp and released as competent planula larvae that are able to rapidly metamorphose into a calcifying settler (Carlon and Olson 1993, Carlon 1999).

In contrast to brooders, broadcast spawning species use the external environment as the site of fertilization, simultaneously releasing eggs and sperm into the water column. This event is highly synchronized within single colonies and among conspecifics in a given area via temperature and light cues (Babcock et al. 1986, Carlon 1999). Despite lacking the ability to swim against currents, ciliated larvae generated by brooding or spawning can move from the water column to the benthos to search for suitable settlement substrate (Carlon and Olson 1993). While the vast majority of a coral's lifespan is spent in the adult stage, the larval period is the only time during which individuals can move, making the choice of settlement location critical to long-term success.

Coral larvae have a much higher proportional lipid content than adults (3-4 times) and have relatively high proportion of wax esters relative to other lipid classes (e.g. *Montipora capitata*; Padilla-Gamino et al. 2013). Wax esters are considered to be particularly useful as sources of long-term energy (Benson and Lee 1972), perhaps providing larvae the energy necessary for long dispersal. Lipid composition and laboratory observations suggest coral larvae are primarily non-feeding (lecithotrophic; Atoda 1951a, Atoda 1951b, Reed 1971, Graham et al. 2008, Alamaru et al. 2009) though

feeding has been observed in some species after the mouth has formed (Schwarz et al. 1999).

Evidence suggests *Symbiodinium* within larvae can fix and translocate fixed carbon, perhaps providing larvae with an additional energy source (Richmond 1987, Gaither and Rowan 2010). Yet, corals produce both symbiont-bearing (vertical transmission) and symbiont-free larvae (horizontal transmission), with brooders tending to use vertical transmission while spawners use horizontal (Baird et al. 2009). Spawned eggs are usually associated with smaller size (relative to brooded) and positive buoyancy, further highlighting potential differences in energy content, dispersal potential and competitive ability between brooded and spawned larvae.

Research motivations and overview

The diversity of reproductive life history strategies employed by tropical stony corals (e.g., size, dispersal period, symbiont transmission strategy) likely play a role in the dramatic ecological differences among species. Yet, little is known about how life history strategies influence reproductive success, in particular when integrated with assessments of environmental tolerance. In this dissertation, I examined how environmental change influences adult coral physiology, and how such experiences are passed on to offspring. Furthermore, I examined the ecological ramifications of continuous (e.g., size) or binary (vertical or horizontal transmission of *Symbiodinium*) early life history strategies among corals as a way to better understand their survival strategies.

In Chapter 2 of this dissertation I used stable isotopic records in coral skeletal cores to disentangle interactions between corals and multiple photosynthesizing organisms in the holobiont during times of environmental change. This technique, along with the ability to physically identify skeletal growth that occurred during symbiont loss and endolithic algae blooms, allowed me to test the predictions that photosynthetic activity within the coral holobiont is variable and that carbon fixation influences the internal pool of dissolved inorganic carbon, especially during times of stress such as bleaching.

In Chapter 3 I tested the prediction that apparently healthy corals living on degraded reefs contain less energetic lipid and produce fewer offspring. I further explored whether corals exhibited tradeoffs between the size and number of larvae they produce, as well as whether reproduction was driven by internal limitation (colony size or energy content) or external factors (depth or population). Additionally, I examined the extent to which offspring size, energy content, and number varied among species, between populations and within individuals.

Building off observed variation in offspring size within and among species, in Chapter 4 I tested whether large size increased larval survival. The importance of larval size in mediating harsh environmental conditions was also addressed experimentally in these experiments, and through this work I was motivated to assess how life history strategies can afford environmental tolerance across multiple early life stages in corals.

In Chapter 5 I determined whether "early" infection with *Symbiodinium* influenced fitness, energy use, and gene expression in a horizontally-transmitting coral species. This work built off observations in Chapter 4 that suggested the mode of

symbiont acquisition leads to inherent differences in how larvae interact with *Symbiodinium*.

Finally, in Chapter 6 I used an ancestral state reconstruction to test whether symbiont transmission strategies and reproductive modes are correlated on the coral tree of life, and to calculate the rates of evolution between states. Given the apparent differences in environmental tolerance between vertically- and horizontally-transmitting species, I also determined whether symbiont transmission strategy correlated with the susceptibility of adults to bleaching. This approach allowed me to assess the costs and benefits of symbiont transmission strategies I found through the investigations in Chapter 4 and Chapter 5 in an evolutionary framework.

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Chapter 2.

REPORT

Stable isotopic records of bleaching and endolithic algae blooms in the skeleton of the boulder forming coral *Montastraea faveolata*

A. C. Hartmann · J. E. Carilli · R. D. Norris · C. D. Charles · D. D. Deheyn

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Abstract Within boulder forming corals, fixation of dissolved inorganic carbon is performed by symbiotic dinoflagellates within the coral tissue and, to a lesser extent, endolithic algae within the coral skeleton. Endolithic algae produce distinctive green bands in the coral skeleton, and their origin may be related to periods of coral bleaching due to complete loss of dinoflagellate symbionts or "paling" in which symbiont populations are patchily reduced in coral tissue. Stable carbon isotopes were analyzed in coral skeletons across a known bleaching event and 12 blooms of endolithic algae to determine whether either of these types of changes in photosynthesis had a clear isotopic signature. Stable carbon isotopes tended to be enriched in the coral skeleton during the initiation of endolith blooms, consistent with enhanced photosynthesis by endoliths. In contrast, there were no consistent $\delta^{13}C$ patterns directly associated with bleaching, suggesting that there is no unique isotopic signature of bleaching. On the other hand, isotopic values after bleaching were lighter 92% of the time when compared to the bleaching interval. This marked drop in skeletal $\delta^{13}C$ may reflect increased kinetic fractionation and slow symbiont recolonization for several years after bleaching.

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A. C. Hartmann (✉) · J. E. Carilli · R. D. Norris · C. D. Charles · D. D. Deheyn
Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92037, USA
e-mail: achartma@ucsd.edu

Present Address:
J. E. Carilli
Australian Nuclear Science and Technology Organization, Lucas Heights, NSW 2234, Australia

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Introduction

Scleractinian corals, such as the *Montastraea faveolata* studied here, host an array of organisms, including symbiotic dinoflagellates (Klebbs 1884), boring endolithic algae (Lukas 1974; Shashar and Stambler 1992; Le Campion-Alsumard et al. 1995), boring animals (e.g., sipunculid worms, polychaetes, bivalves, and sponges; Holmes et al. 2000), fungi (Golubic et al. 2005), bacteria (Wegley et al. 2004), archaea (Wegley et al. 2004), and viruses (Marhaver et al. 2008). Of these organisms, the major photosynthesizers include symbiotic dinoflagellates, or zooxanthellae, that live in the coral tissue (Klebbs 1884) and endolithic algae that live in the skeleton of both live and dead coral (Lukas 1974).

Zooxanthellae fix carbon, which is translocated to their coral host, providing most, if not all, of the coral's metabolic requirements (Muscatine and Weis 1992). Despite its great success, the coral–zooxanthellae relationship can break down, commonly as a result of light and temperature stress (Glynn 1996). This often results in coral bleaching, recognized by decreased pigmentation or the direct expulsion of dinoflagellate symbionts (Glynn 1996). Bleaching can, but does not always, result in coral mortality.

Endolithic algae, commonly of the genus *Ostreobium*, can inhabit the skeletons of a variety of coral species (Lukas 1974; summarized in Shashar and Stambler 1992), often forming dense bands (Highsmith 1981). Entire communities of endolithic photoautotrophs (i.e., algae and cyanobacteria) can make up a large proportion of net primary productivity in heads of dead boulder forming corals (Tribollet et al. 2006). Yet, when shaded by live coral tissue,

endolithic algae receive less than 5% of ambient light (Rodriguez-Roman et al. 2006) limiting their productivity to <4% that of coral symbionts (Shashar and Stambler 1992). Despite this, endoliths can translocate fixed carbon to the tissue of their coral host, and both photosynthesis and translocation may be enhanced during endolith blooms (Schlichter et al. 1995; Fine and Loya 2002).

Dissolved inorganic carbon (DIC) plays a dual role in the coral–algal community, as it is not only fixed by symbionts for nutritional purposes, but it is also the carbon source for the coral's calcium carbonate skeleton. Even when sufficient DIC is available for both processes, changes in the relative amount of photosynthesis may be reflected in the isotopic signature of skeletal carbon (Swart 1983). During photosynthesis, symbionts preferentially utilize forms of DIC containing the light carbon isotope, ^{12}C, over the heavier, ^{13}C, termed metabolic fractionation (Swart 1983; McConnaughey 1989). The degree of metabolic fractionation is dependent on the amount of photosynthesis occurring at a given point in time, which is governed primarily by irradiance (Fairbanks and Dodge 1979; Pätzold 1984) and sea surface temperature (Fairbanks and Dodge 1979), both of which vary seasonally.

At approximately steady coral calcification rates, the metabolically fractionated $\delta^{13}C$ signal is passively recorded in the coral skeletal carbonate (McConnaughey 1989). Therefore, skeletal $\delta^{13}C$ measured along the skeletal growth axis may provide a record of changes in symbiont photosynthesis through time.

During bleaching events, when zooxanthellae photosynthesis is dramatically reduced or non-existent, the degree of metabolic fractionation is expected to drop precipitously, potentially resulting in a depleted $\delta^{13}C$ signal in the coral skeleton. Indeed, depletion of $\delta^{13}C$ across known bleaching events has been observed in multiple species such as *Montastraea annularis* (Porter et al. 1989), *Porites* spp. (Suzuki et al. 2003), *Montipora verrucosa* and *Porites compressa* (Grottoli et al. 2004), and *Montipora capitata* (Rodrigues and Grottoli 2006).

In theory, photosynthetically driven modulation of the DIC pool available for calcification can be affected by endolithic algae as well as zooxanthellae. When endolithic algae are in high enough abundance (i.e., dense green bands) and near to the site of calcification, they may influence the availability (and as a result the isotopic character) of DIC at the calcification site. This scenario, however, remains a working hypothesis, and the potential role of endolithic algae in modulating the DIC environment near the site of calcification in live coral tissues has yet to be addressed.

Accordingly, the two hypotheses addressed in this study are as follows: (1) The $\delta^{13}C$ of skeletal aragonite will be depleted beyond the seasonal periodicity within a compression band coincident with bleaching, either as a discrete change or a broad trend within the band; (2) The average $\delta^{13}C$ of skeletal aragonite will be significantly enriched across endolith bands when compared to non-endolith band areas of coral skeleton due to increased metabolic fractionation caused by increased endolith photosynthesis.

The samples used in the present study provide a unique natural experiment to address two similar hypotheses built on the assumption that the relative amount of photosynthesis drives the isotopic character of the internal DIC pool. In total, 92 coral cores were collected from four sites in the Mesoamerican reef tract between 2006 and 2007 (Carilli et al. 2009a, b). This area suffered widespread bleaching in 1998 (Mcfield 2000; Kramer and Kramer 2000), as did most coral reefs worldwide (Wilkinson 2000). This major bleaching event manifested clearly in the cores reported here as dense compression bands in skeletal X-radiographs, characterized by severely reduced extension rates and increased density: over 4 standard deviations outside the long-term mean (Carilli et al. 2009a, b). Overall, 95% of the cores contained compression bands coincident with the 1998 bleaching event (Carilli et al. 2009a). Endolithic algae are visually evident in these core samples as well, where they form green bands of variable intensity like those described by Lukas (1974) and Highsmith (1981), providing a unique opportunity to measure the isotopic signature of the coral skeleton during these anomalous periods.

Materials and methods

Coral collection

Cores of the scleractinian coral *Montastraea faveolata* were collected from three sites in the Mesoamerican Reef tract off the coasts of Belize and Honduras (Fig. 1). Divers used a pneumatic drill powered by a shipboard compressor to collect the cores, each approximately 5 cm in diameter and up to 1.3 m long. Samples were drilled vertically, parallel to the axis of maximum growth, at 5–6 m water depth on the fore-reef in spur-and-groove habitat. Specific drilling locations at each site were chosen away from lagoonal influence or human settlements in order to better represent regional conditions. One or two cores from each site were studied here, representing a small subset of the 92 total cores collected (Carilli et al. 2009a, b). Of the cores examined, Utila-1 was collected in July 2007, Utila-2 in June 2006, Cayos Cochinos in July 2006, and Frank's Caye in January 2006.

Dating and isotopic analysis

Slabs approximately 8 mm thick were cut through the center of each coral core parallel to the axis of growth

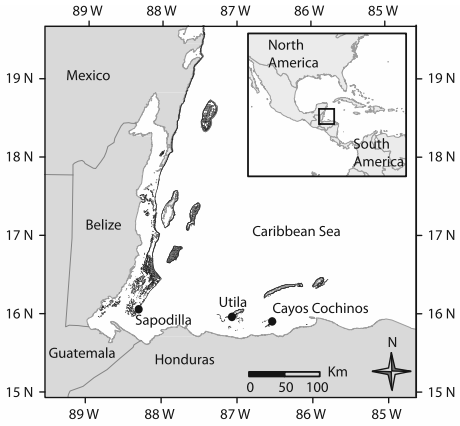


Fig. 1 Location of Utila, Cayos Cochinos, and Sapodilla Cayes sampling sites within the Mesoamerican Reef tract

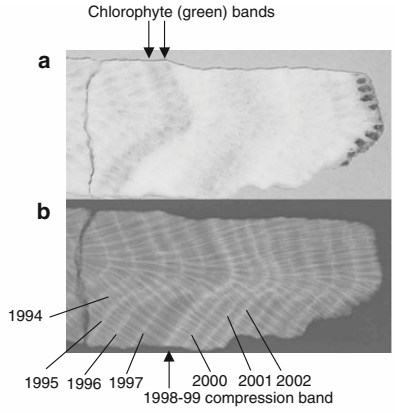


Fig. 2 Plain light photograph **(a)** and X-radiograph **(b)** of the same section of core collected from the Utila site in July 2007. The endolith bands in **a** are formed by endolithic algae and are indicated by *black arrows,* while the white bands in **b** are annual high-density skeletal bands. A compression band is observed here, coincident with the 1998 mass bleaching event

using a double-bladed carbide table saw. Each slab was X-rayed using a Siemens Polyphos 50 at a source-to-object distance of 40 inches and a setting of 63 kilovolts at 5 milliamps/s at Thornton Hospital, UCSD. To assign calendar dates to the cores, the initiation of a high-density band was assigned to be August of a given year coinciding with the observed beginning of high-density band formation in *M. faveolata* (Cruz-Pinon et al. 2003; Moses and Swart 2006). In the case of the exceptionally high-density, low-extension rate compression bands in these cores, cross-dating between multiple cores shows that the timing is coincident with the mass bleaching event of 1998 (see Fig. 2 for an example core). The initiation of a compression band may not reflect the exact onset of a bleaching event. However, the ubiquity of the compression bands (95% prevalence), as well as their consistent timing, makes them highly reliable estimators of the timing and duration of the event.

Single cores from Cayos Cochinos and Frank's Caye (in the Sapodilla Cayes) were analyzed along with two cores from Utila (Fig. 1). Approximately 200–300 μg of powdered calcium carbonate was sampled using a small drill press with a 0.5-mm bit at 0.5- or 1-mm increments down the growth axis of individual corallites, making sure to stay along corallite walls. Samples were not treated for removal of organic material prior to stable isotope analysis because such pretreatment has been shown to confound stable isotopic measurements, and it is an unnecessary precaution considering that organic matter is not a source of isotopic contamination in samples of coral skeletons (Grottoli et al. 2005).

In this study, endolithic algae showed morphological features under microscopic observations that strongly suggest they belong to the genus *Ostreobium*, which commonly inhabit coral skeletons. *Ostreobium* do not calcify (Lukas 1974), thus there is little concern that they contributed calcified material to these samples, or competed for DIC via a calcification process. It is also unlikely that bulk algal material contributed to δ^{13}C measurements. The consistent deviations across endolith bands reported here trend in the opposite direction of what would be expected if the isotopically light endolithic algae (−14.8 to −12.3‰; Titlyanov et al. 2008) were a source of contamination.

Endolithic algae are ubiquitous within coral skeletons and repeatedly rise from low background abundances, forming dense green bands like those studied here. Although the endoliths deep in coral skeletons are not living (e.g., Kanwischer and Wainwright 1967), organic material remains well preserved in massive coral skeletons and thus is clearly observable in these cores (Ingalls et al. 2003).

Multiple sample transects were collected across compression bands (aided by X-radiographs) and endolithic algal bands to assess changes in the isotopic composition of the coral aragonite associated with bleaching and increased endolith activity. Fine-scale isotopic transects were drilled from three cores: Utila-1, Utila-2, and the Cayos Cochinos core at 0.5-mm resolution. The length of these records varied from 10 to 14 years and each included a compression band associated with the 1998 mass bleaching event. Additionally, a long-term record (∼65 years) was drilled from a fourth core, Frank's Caye, at 1.0-mm resolution. Four short-term records were used to evaluate the bleaching effect: Utila-1, Utila-2, Cayos Cochinos, and a short portion of the Frank's Caye record.

Isotopic analyses were performed on a Finnigan MAT 252 stable isotope mass spectrometer attached to a Fairbanks carbonate preparation device. Samples were reacted in a common phosphoric acid bath at 90°C for 11 min, a quick reaction time during which organics do not readily hydrolyze. Isotopic ratios are reported in the per mil (‰) convention, are deviations from the PeeDee Belemnite (PDB) standard, and are reported as:

$$\delta^{13}\mathrm{C} = \left[\frac{{}^{13}\mathrm{C}/{}^{12}\mathrm{C}_{\mathrm{sample}} - {}^{13}\mathrm{C}/{}^{12}\mathrm{C}_{\mathrm{standard}}}{{}^{13}\mathrm{C}/{}^{12}\mathrm{C}_{\mathrm{standard}}}\right] \times 1000‰ \quad (1)$$

Each run of 40 randomly organized samples included 7 standards of ground *Porites* sp. coral as a quality control for instrumental precision. Samples were considered to represent before, after, or during the bleaching event or a given endolith bloom as identified by comparing X-radiographs as well as the actual sampled coral slab.

Statistical analyses

Compression bands

Linear regression models were fit to each of the four short-term records and the long-term record in order to detect factors significantly correlated with the skeletal δ^{13}C signal over the sampled sections. The model used the continuous variables *year*, *month* and *month*^{2}, and the categorical variable *compression* (within or outside of the compression band). The long-term trend (*year*) was removed from the δ^{13}C values used to evaluate the long-term record due to a significant progressive decline in skeletal δ^{13}C over the past 65 years in that core. To evaluate the bleaching effect on skeletal δ^{13}C in the four short-term records, no detrending was applied as the progressive isotopic decline was not consistently apparent in these shorter records.

To investigate the influence of bleaching, measured δ^{13}C values were pooled into those that were drilled before, during, and after the compression band. Values for each of these time horizons were further categorized by those occurring at isotopic maxima, minima, and all values. One-to-one comparisons were made for all possible time horizons and all data categorizations. In the case that multiple transects were measured from a single core, all data were considered as independent replicates for further analyses. Tests for significance were computed using permutation tests in R (R Development Core Team).

Cloudiness

The influence of irradiance on skeletal δ^{13}C (via light-dependent changes in zooxanthellate photosynthesis) was evaluated using cloud cover records from the globally gridded Comprehensive Ocean–Atmosphere Data Set maintained by the National Oceanic and Atmospheric Administration. The most robust and long-term cloud data available of the area from which these cores were taken (2 × 2-degree pixels centered on 17°N 87°W) were used to provide monthly average oktas, where oktas are measured as the number of 1/8ths of sky covered by clouds (i.e., scale ranges from 0 to 8). The coral δ^{13}C record was matched to the cloudiness record using Analyseries 2.0 (Paillard et al. 1996) to determine whether dating errors in the δ^{13}C record could be improved. To assess the magnitude of change in the coral record required for this matching, the difference between the original coral dating and the new dating was calculated. In all but Utila-2, the required shifts were, on average, less than 1 month and were as follows: Utila-1 = 0.9 months, Utila 2 = 2.6 months, Cayos Cochinos = 0.7 months, and Frank's Caye = 0.6 months. When considered, these shifts improved the linear correlation between the records from an average r^2 value of −0.13 to −0.30. The correlation between skeletal δ^{13}C and cloudiness was evaluated in Analyseries 2.0, and statistical significance was determined via a simple linear model in R.

Endolith bands

Skeletal isotope values from the Frank's Caye core were used to evaluate the influence of endolith blooms on skeletal δ^{13}C and included 12 endolith bands spaced throughout the core, none of which exceeded 3 mm in width. Endolithic algae live just below the tissue in coral skeletons, growing upward with the coral (Lukas 1974), and driven by light limitation thresholds (Fine and Loya 2002; Magnusson et al. 2007). Endoliths in live *Porites* corals have been observed to grow no deeper than 5 mm below the tissue layer (Le Campion-Alsumard et al. 1995) and at a similar skeletal depth in *Montipora monasteriata* corals (Magnusson et al. 2007); it is assumed here that endoliths in *M. faveolata* have a similar limit.

Due to the nature of core samples, there was no way to determine exactly at what depth individual endolith bands grew in relation to the live tissue. Therefore, the influence of endolith photosynthesis on skeletal δ^{13}C values was evaluated over the 0–5 mm range of possible algal growth locations below the site of calcification. To do this, scenarios of endolith band locations from 0 to 5 mm below the calcification site were considered at 1-mm increments (Fig. 3a). Data were grouped by δ^{13}C values within the 'zone of influence', that is area of skeleton that was calcifying while an endolith band was forming below, and those outside of the zone of influence for each scenario. For example, the 4-mm scenario assumes that the site of calcification was four mm above the endolith band. Thus, the skeletal δ^{13}C values considered within the zone of

influence for this scenario began four mm above the bottom of the endolith band. The three mm width of the scenario corresponds to the width of the endolith band.

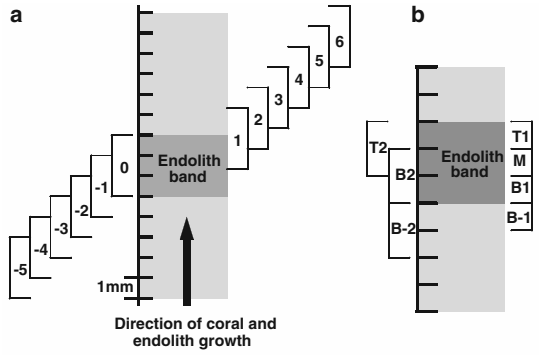


Fig. 3 **a** A schematic example of a section the Frank's Caye core that includes a 3–mm-wide endolith band. Each *bracket* represents a single scenario, or 'zone of influence', and its corresponding number denotes the distance between the endolith band and the site of calcification (e.g., 0-mm scenario assumed that endoliths grew adjacent to the coral tissue, 1-mm scenario assumed that endoliths lived 1 mm below the coral tissue and so on), and 0–5 represent possible endolith growth locations with respect to the live tissue, while 6 is unlikely because it is deeper than any published accounts of endolith growth locations, and −1 through −5 are impossible growth locations used as pseudo controls, since this would assume the algae were growing before the coral calcified. Thus, the skeletal δ^{13}C values compared within and outside the endolith band in each scenario include those that would have been influenced by endolith photosynthesis for one respective algal growth location. The width of each scenario and its corresponding endolith band are equal because it was assumed that the endolith band extended across an equal amount of skeletal growth. **b** The scenarios in **a** contain overlapping values and thus the application of the seven scenarios in **b** assists in differentiating between regions within the endolith band. Scenario "B-2" contains only the two points below each endolith band, scenario "B-1" contains only the single point below each band, "B1" only the bottom point within each band, "B2" the bottom two points within each band, "M" the middle point, "T2" the top two points within the band, and "T1" the single top point within the band

As pseudo controls, a 6-mm scenario, corresponding to a depth location at which the endoliths would likely die off, and five scenarios (−1 to −5) of skeleton calcified before the endolith band existed were included as well (Fig. 3a). Statistical significance of the influence of the endolithic bands was tested using a linear regression model that included the continuous variables *year* and *month*, as well as the categorical variables *compression* (within or outside of the compression band), and *endolith* (within or outside of an endolith band's zone of influence).

A limitation to using the above technique was that all of the endolith growth location scenarios intrinsically include point(s) that overlap with other scenarios. Thus, single and grouped skeletal δ^{13}C samples within and below the endolith bands (example for 0-mm scenario given in Fig. 3b) were analyzed as well in order to remove data overlap between scenarios. Each sample(s) within or below the endolith bands (e.g., Fig. 3b: B-2, B-1, B1.) was separately evaluated using a linear regression model that included the continuous variables *year* and *month*, as well as the categorical variables *compression* (within or outside of the compression band), and *endolith sample* (e.g., Fig. 3b: B-2).

Results

Compression bands

A significant ($P < 0.001$) long-term negative trend was evident in the Frank's Caye core (Fig. 4) and accounted for 21% of the isotopic variability in the record. In addition to the long-term trend, skeletal δ^{13}C values also correlated with the month of the year ($P < 0.01$), but explained very little of the variability ($\sim 1.4\%$).

Linear regressions of the short-term records showed that skeletal δ^{13}C values within the compression band in the

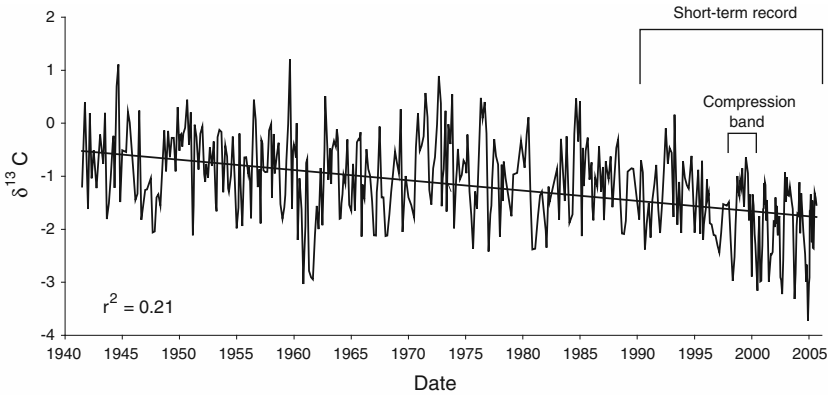


Fig. 4 δ^{13}C record from Frank's Caye Core from 1940 to 2006. Also shown is the short-term record sampled at higher frequency that was extracted from this record

Utila-1 record were heavier ($P < 0.001$) than those outside (Fig. 5a), δ^{13}C decreased ($P < 0.05$) through time (variable: *year*) in the Cayos Cochinos record (Fig. 5d), and no variable showed a significant correlation in the Utila-2 or Frank's Caye records (Fig. 5b, c, respectively).

Fine-scale analyses of the long-term Frank's Caye core detected no difference between isotopic values measured before and during the compression band, but the values after the compression band were significantly lighter than those before ($P < 0.001$). This depletion in δ^{13}C after the bleaching event was also found in comparisons using the four short-term records (Cayos Cochinos, Frank's Caye, Utila-1, and Utila-2) (Table 1). In the cores from Cayos Cochinos and Frank's Caye, maxima after the compression band were significantly lighter than maxima before the band ($P = 0.02$ and $P = 0.003$, respectively). In those two cores, as well as Utila-2, the minima of skeletal δ^{13}C after the compression band were also significantly lighter ($P = 0.04$, $P = 0.005$ and $P = 0.035$, respectively). When all isotopic values before and after the compression band were compared, Cayos Cochinos, Frank's Caye, and Utila-2 all show significant depletion after the bleaching event ($P = 0.01$, $P = 0.02$ and $P = 0.004$, respectively), but no significant differences during the event. Utila-1 was an anomaly within this group, with a significantly heavier signal inside ($P < 0.05$) the compression band and no differences before or after.

Heavier isotopic values were found at the beginning of compression bands in four transects (Fig. 5 b1, b2, c3, d), while lighter values were observed in three others (Fig. 5 a1, a2, c2). Of all comparisons of regions outside to those inside a compression band (Table 1), the compression band values were heavier 83% of the time, yet only 5 of 36 comparisons were statistically significant. In 83% of the comparisons, values after the compression band were lighter than those before, and 8 of 12 were significant. Additionally, isotopic values after the band were lighter

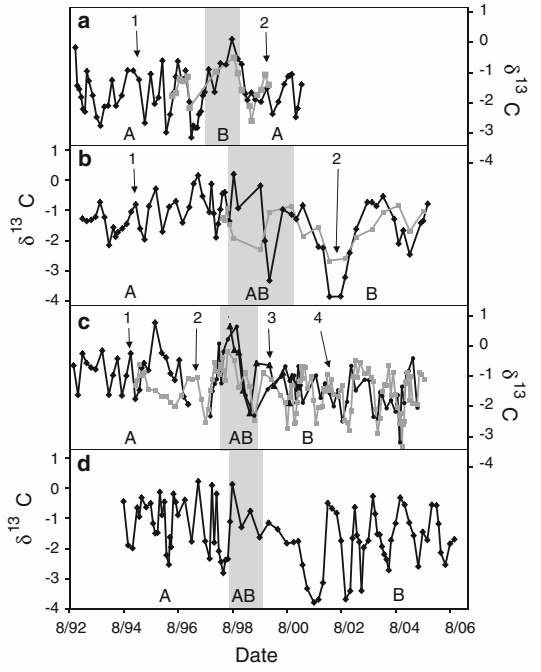


Fig. 5 δ^{13}C values across compression bands (*gray vertical bars*) due to the 1998 mass bleaching event from four cores taken from three different sites in the Mesoamerican Reef: **a** Utila-1, **b** Utila-2, **c** Frank's Caye, **d** Cayos Cochinos. Numbered arrows denote distinct measurement transects, which are shown in *gray* and *black* lines (**a**, **b**, **c**). In the Frank's Caye core (**c**), transects in addition to those included in the long-term record (Fig. 4) are shown. Capital letters (*A* and *B*) denote statistically significant differences between all isotopic values pooled for each time horizon (before compression band, within compression band, after compression band). For example, in **b**, there is a significant difference between all of the values before the compression band and all of the values after, but no difference between those before vs. within the compression band or those after vs. those within the compression band. These data are also shown in Table 1

Table 1 Pairwise comparisons of skeletal δ^{13}C values across the 1998 bleaching event utilizing values before ("Before"), during ("Compression"), and after ("After") the bleaching event, as well as before and after pooled ("Non-Compression")

	Compression vs. before	After vs. compression	After vs. before	Compression vs. non-compression
Difference in maximum δ^{13}C values (‰)				
Cayos	−0.23	−0.36	−0.59*	−0.95
Frank's	+0.39	−0.90*	−0.51*	+0.76*
Utila-1	+0.61	−0.99	−0.38	+0.61
Utila-2	+0.10	−0.37	−0.27	+0.29
Difference in minimum δ^{13}C values (‰)				
Cayos	+0.82	−1.65	−0.84*	+0.03
Frank's	−0.73	+0.01	−0.72*	−0.27
Utila-1	+0.97	−0.62	+0.34	+4.30
Utila-2	+0.77	−2.24	−1.46*	+0.77
Difference in all δ^{13}C values (‰)				
Cayos	+0.37	−0.91	−0.54*	+0.68
Frank's	+0.12	−0.43	−0.32*	+0.35
Utila-1	+1.09*	−0.92*	0.17	+1.01*
Utila-2	−0.05	−0.56	−0.61*	+0.15

Comparisons for each time period were performed three ways: (1) all isotopic maxima in the sinusoidal time-series; (2) all isotopic minima in the sinusoidal time-series; (3) all isotopic values. The data in the table represent the difference, on average, of skeletal δ^{13}C values of the first period when compared to the second (i.e., Compression vs. Before (‰) = −0.23 indicates that the (maximum) values in the compression band are on average 0.23‰ lighter than those before the compression band. *P*-values were calculated for each comparison using a permutation test and asterisks denote statistical significance ($P < 0.05$)

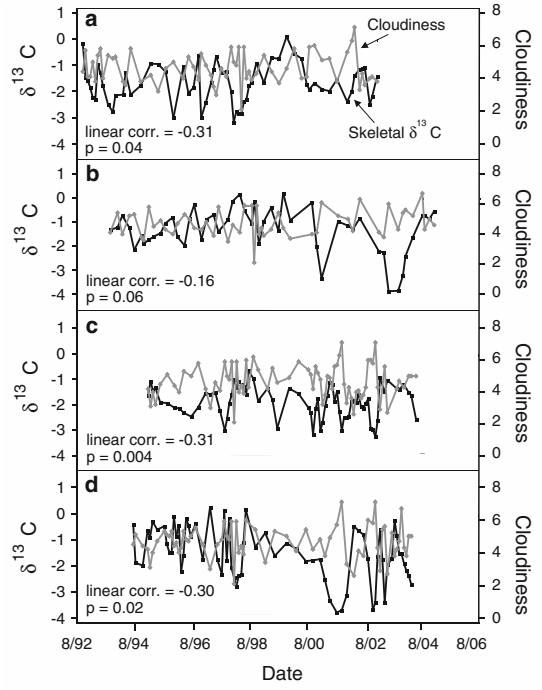


Fig. 6 Time series of monthly average cloudiness (as monthly averaged oktas) and skeletal δ^{13}C measurements from **a** Utila-1, **b** Utila-2, **c** Frank's Caye, and **d** Cayos Cochinos short-term record. The records in *black* are those of skeletal δ^{13}C measurements, while those in *gray* are standardized cloudiness. The linear correlation and associated *P*-values between the two records are reported for each record

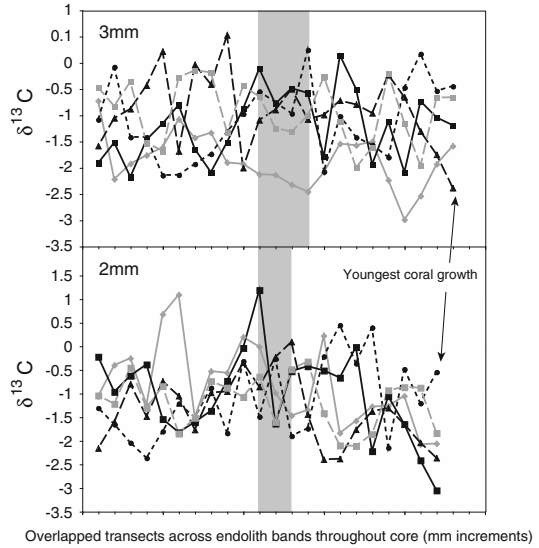


Fig. 7 Isotopic record across 10 endolith bands (*gray bars*) sampled throughout the core and overlaid on one another for comparison. Bands with widths of 3 mm (*top*) and 2 mm (*bottom*) are shown, each surrounded by 10 mm of measurements before and after the bands. The prevailing direction of coral growth is from left to right, with the youngest skeletal growth regions falling on the right as indicated

92% of the time when compared to values within the compression bands.

Cloudiness

Negative correlations between cloudiness and skeletal δ^{13}C were found in all cores examined here (Fig. 6) and three of the four records were significantly correlated ($P < 0.05$), while the fourth was marginally significant ($P = 0.06$).

Endolith bands

The endolith bands studied here followed the growth contours of the coral skeleton. No endolith band was more than 3 mm in width—less than half the $\sim$8 mm width of annual bands in these cores. Considering the narrow width of endolith bands, along with the observation that blooms appear no more than 5 mm below the tissue layer, it is likely that these blooms only lasted a few months.

The variables *year* and *month* correlated significantly with the skeletal δ^{13}C values ($P < 0.001$ and $P < 0.01$, respectively) in all of the endolith growth location scenarios outlined in Fig. 3. Conversely, only in the 0-mm endolith growth scenario (i.e., endolith band growing directly adjacent to the calcification site), was there a significant correlation between the presence of an endolith band and the skeletal δ^{13}C signal ($P < 0.05$), suggesting that endolith bands formed close to the underside of the coral tissue layer. δ^{13}C values within endolith bands in the 0-mm scenario were heavier than those outside (Fig. 7).

Fine-scale analysis of the individual points and groups of points within the endolith bands (Fig. 3b) found that in bands wider than 2 mm, only the lowest 1–2 mm of the band showed a significant ($P < 0.05$) correlation with skeletal δ^{13}C, the lowermost 1 mm being enriched by an average of 0.5‰, and the bottom 2 mm being enriched by 0.4‰ on average.

Discussion

Long-term trends

The long-term negative trend over time in the Frank's Caye core (Fig. 4) was at least partially driven by the Suess Effect, the progressive isotopic depletion of atmospheric CO_2 due to fossil fuel emissions, which are depleted in ^{13}C (Keeling 1979). δ^{13}C measurements from the calcium

carbonate of sclerosponges provide a reference for the $\delta^{13}C$ of seawater DIC because carbon isotope fractionation does not occur during calcium carbonate formation in this organism (Böhm et al. 1996). The sclerosponge record displays a steady decrease in $\delta^{13}C$ over the last decade in the Caribbean Sea and a total decrease of $\sim$0.5‰ from 1940 to 1990 (Böhm et al. 1996). In the Frank's Caye Core, skeletal $\delta^{13}C$ decreased by $\sim$0.7‰ over the same time period, suggesting that the coral skeleton was slightly depleted in the heavy isotope compared to the isotopic composition of seawater DIC, which is consistent with past reports (e.g., McConnaughey 1989).

The Suess Effect was not consistently observed in the four short-term records. Only in the Cayos Cochinos record, there was a significant decrease in isotopic values through time ($P < 0.05$). The Suess Effect is likely masked by other isotopic signals, such as those connected to bleaching, or may be obscured by the relatively short length of these records (15–20 years).

Hypothesis 1: $\delta^{13}C$ depletion upon the onset of compression bands

Isotopic profiles across compression bands coincident with the 1998 bleaching event did not consistently show depletions in $\delta^{13}C$, contrary to past studies (e.g., Porter et al. 1989; Carriquiry et al. 1994; Suzuki et al. 2003; Grottoli et al. 2004; Rodrigues and Grottoli 2006). Both enrichment and depletion of skeletal $\delta^{13}C$ occurred at the onset of compression bands in the four cores studied here (Fig. 5). Leder et al. (1991) observed similar inconsistencies in $\delta^{13}C$ across skeleton laid down following a bleaching event in *M. annularis*. However, the water depth of sample collection, known to influence the $\delta^{13}C$ signal (Weber et al. 1976; McConnaughey 1989), was not held constant in the Leder et al. (1991) study. In the present study, all coral cores were collected from the same water depth and similar observations to Leder et al. (1991) were made, suggesting that skeletal $\delta^{13}C$ is not a reliable bleaching proxy in *M. faveolata*.

Taken together, the data presented here show that isotopic values within compression bands were heavier than those before or after. Anomalously heavy values across compression bands reported by Leder et al. (1991) and in the present study may be explained by an abrupt decline in kinetic fractionation due to slowed calcification after bleaching. During calcification, $^{12}CO_2$ reacts more quickly than $^{13}CO_2$, a phenomenon termed kinetic isotope fractionation (McConnaughey 1989). Therefore, based on kinetic effects, the amount of ^{13}C precipitated should increase as the rate of calcification decreases. Indeed, enriched $\delta^{13}C$ values during periods of slow calcification were observed in *Porites lutea* collected in Thailand (Allison et al. 1996). The significantly reduced post-bleaching calcification of the corals studied here is consistent with reports of slow growth after bleaching in *Porites* spp. (Allison et al. 1996; Suzuki et al. 2003), *M. annularis* (Goreau and Macfarlane 1990), *Montipora capitata* and *Porites compressa* (Rodrigues and Grottoli 2006). While it is likely that isotopic depletion due to the metabolic effects of zooxanthellae loss would be evident if there was no interruption in growth rate post-bleaching, isotopic enrichments suggest that reduced kinetic fractionation due to slow calcification overwhelmed any metabolic modulation of skeletal $\delta^{13}C$.

The variability in post-bleaching isotopic signals (Fig. 5) likely reflects differential recovery of individual colonies after the event, primarily in terms of calcification and zooxanthellae repopulation rates. Changes in calcification and photosynthesis rates after bleaching may have shifted the degree to which the isotopic composition of DIC within a given coral head was driven by kinetic versus metabolic effects. Here, 93% of the transitions from the compression band back to "normal" calcification showed a decrease in $\delta^{13}C$, with three of the four cores having significant isotopic depletion after the band when compared to before. This suggests that kinetic fractionation increased again after the bleaching event, leading to less incorporation of ^{13}C into the skeleton. Authors have attempted to remove kinetic effects from skeletal $\delta^{13}C$ records through modeling (Heikoop et al. 2000; Adkins et al. 2003; Omata et al. 2005), but such an assessment was beyond the scope of this study.

In addition to kinetic and metabolic effects, other factors can drive the isotopic signal in coral skeletons. These include seasonal changes in DIC of the surrounding seawater (Swart et al. 1996); the isotopic composition of heterotrophically acquired energy sources (e.g., zooplankton; Grottoli 2002; Grottoli et al. 2006; Rodrigues and Grottoli 2006); and selective sequestration of ^{12}C in gametes (summarized by Swart et al. 1996). Relevant to the issue examined here is evidence that corals can respond to bleaching by increasing heterotrophic carbon acquisition (Grottoli et al. 2006). Increased heterotrophy on isotopically depleted food sources (e.g., zooplankton) can deplete the skeletal $\delta^{13}C$ signal, an effect that drives the skeletal DIC pool $\delta^{13}C$ in the same direction as the loss of metabolic fractionation by zooxanthellae (Grottoli 2002; Grottoli et al. 2006; Rodrigues and Grottoli 2006). Heterotrophic plasticity has not been examined in *M. faveolata*, but it is plausible that increased feeding alone or in synergy with loss of zooxanthellae can drive down the $\delta^{13}C$ signal during bleaching and recovery. Therefore, there could be at least two plausible explanations for why isotopic values were depleted after the compression band: increased kinetic fractionation which exposes decreased metabolic fractionation post-bleaching; and/or an increase in heterotrophic feeding to make up for the lack of fixed carbon from zooxanthellae.

Physical factors such as changes in temperature and light availability, as well as the biological responses to those factors, determine whether and when corals bleach, as well as the severity of the episode (Glynn 1996). Decreases in light intensity are thought to decrease photosynthesis as well as the degree of metabolic fractionation during calcification, driving positive correlations between irradiance and skeletal $\delta^{13}C$ (Heikoop et al. 2000 and references contained therein). Indeed, negative correlations between skeletal $\delta^{13}C$ and cloud cover (less irradiation) reported here suggest that metabolic fractionation by photoautotrophs within coral colonies is responsible for driving $\delta^{13}C$ to an appreciable extent. Yet, the marginal significance observed in one of the four cores, as well as the relatively low correlation coefficient in all four, further supports the notion that there are multiple factors influencing the skeletal $\delta^{13}C$ values measured here in addition to metabolic fractionation.

The degree to which each coral head initially bleached likely determined the relative influences of many factors influencing skeletal $\delta^{13}C$ values thereafter (e.g., kinetic and metabolic fractionation, heterotrophy), likely causing the variable isotopic responses between cores. Inconsistent reports of the $\delta^{13}C$ response to bleaching across genera suggest that species-specific responses may also explain why skeletal $\delta^{13}C$ depletions are not consistently observed (e.g., *Porites, Montipora*; Porter et al. 1989; Suzuki et al. 2003; Grottoli et al. 2004; Rodrigues and Grottoli 2006).

Overall, there is no clear isotopic signal of the severe and widespread bleaching event in *M. faveolata*. These results support past findings that metabolic effects are one of many influences on the $\delta^{13}C$ signal in coral skeletons, but the confounding effects of additional drivers reduce the utility of $\delta^{13}C$ as a bleaching proxy.

Hypothesis 2: $\delta^{13}C$ enrichment across endolith bands

The skeletal $\delta^{13}C$ values corresponding to endolith growth adjacent to the site of calcification (0-mm scenario in Fig. 3a) in the Frank's Caye core were significantly enriched. Finer-scale examination of $\delta^{13}C$ values within and around the endolith bands reveal that those values at the bottom of the endolith band showed the greatest enrichment. Taken together, these results suggest that endoliths in *M. faveolata* probably bloom adjacent to the coral tissue layer and enrich the coral's internal pool of DIC in ^{13}C. This is consistent with the hypothesis that the endoliths actively withdraw ^{12}C from the coral's internal DIC pool. Still it is unclear why a trend of enriched $\delta^{13}C$ was not observed throughout the entire band. It is speculated here that blooms (in particular during the intense growth phase) are ephemeral in time despite being several mm thick in certain places within the skeleton.

Endolithic algae residing in *M. faveolata* have been associated with high concentrations of brucite ($Mg(OH)_2$) crystals (Buster and Holmes 2006; Nothdurft et al. 2005). Brucite does not contain carbon and thus would not affect carbon fractionation during calcification or influence the measured carbon isotope signature in adjacent skeletal formations. While brucite precipitation may fractionate oxygen and therefore influence the character of $\delta^{18}O$ available for calcification, it is reasonable to conclude that brucite formation has little or no influence on skeletal $\delta^{13}C$ because it lacks carbon. Smith et al. (2006) found heterogeneity in Sr/Ca ratios across skeletal formations in *M. faveolata*, which, like $\delta^{13}C$, cannot necessarily be explained by brucite precipitation, but instead likely occurs due to post-deposition thickening of the skeleton, underscoring the need to sample accurately along corallite walls. However, the clear existence of annual cycles in the records described here (e.g., Fig. 4) indicates that the sampling did indeed proceed along the corallite walls and did not include significant amounts of younger material which may have masked this signal.

These results suggest that more attention should be paid to the influence of endolithic photosynthesizers on the skeletal $\delta^{13}C$ signal because of their potential effect on isotopic records. Furthermore, there is a need to better understand the ecological role of endolithic algae in the coral biome, as it appears that they may be a larger sink of internal DIC than previously recognized. Of particular importance is the interaction of the endolith blooming phenomena with coral bleaching or paling (Carilli et al. 2010). Endolith growth, photosynthesis, and translocation can increase after bleaching (Fine and Loya 2002; Rodriguez-Roman et al. 2006), though it is unclear if the algae provide a sufficient food source to increase the coral's survival after the loss of symbionts. Further work is needed to elucidate the drivers of endolith blooming as well as the potential for these organisms to provide fixed carbon to their host, both during "normal" coral growth and times of stress such as bleaching. A more comprehensive understanding of all potential mechanisms that confer bleaching resilience to corals will be instrumental in predicting the health of coral communities of the future under the lens of global climate change.

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Chapter 3.

ENERGY CONTENT AND REPRODUCTIVE OUTPUT IN CORALS ACROSS COMMUNITY, POPULATION, AND INDIVIDUAL SCALES

Aaron C Hartmann[1], Kristen L Marhaver[2,3], Mark JA Vermeij[3,4]

[1]Center for Marine Biodiversity and Conservation, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA, 92093, USA

[2]University of California, Merced, Merced, CA, 95343, USA

[3]CARMABI Foundation, Piscaderabaai z/n, Willemstad, Curaçao

[4]Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

Abstract. Protecting coral reefs is dependent upon the preservation of existing populations and the success of future generations of corals. Marine protected areas can facilitate both by preserving robust populations and healthy individuals, a tool often employed with fishes in mind. We determined whether similar benefits of protection occur in corals. Specifically, we predicted that corals living on quasi-pristine reefs would have higher energy reserves and higher reproductive output than conspecifics in degraded areas. In three of four species, we found that individuals in a quasi-pristine region indeed contained higher amounts of lipids critical for energy and reproduction. In two of three brooding species, fecundity was 350% and 200% higher in this region as well. Fecundity patterns were related to parental characters such as colony size and energy content, while the source of variation in larval size and energy content per larva was more difficult to constrain. Three coral species with apparently similar life histories showed dramatic differences in larval lipid content, buoyancy, and fecundity. Our results suggest high coral cover reefs can be disproportionately important sources of coral offspring as a result of larger population sizes as well as greater reproductive output per individual.

INTRODUCTION

Human impacts on marine ecosystems and the loss of associated flora and fauna continue to increase on a global scale (Hughes et al. 2003, Halpern et al. 2008). Attempts to mitigate losses of marine organisms have focused on the need to balance ecosystem sustainability with the demands of a growing human population. Marine protected areas, or MPAs, are perhaps the most widely employed tool to achieve this balance, at least in coastal ecosystems (Pauly et al. 2002, Halpern 2003, Gaines et al. 2010). Excluding or reducing human activities within the boundaries of an MPA can create refuge for organisms and protect entire ecosystems. Organisms within MPAs may also benefit from reductions in non-extractive activities, such as pollution, which harm target and non-target species alike.

A fundamental goal of many MPAs is to bolster fisheries by allowing populations to grow and then 'spillover' outside the reserve where fishes can be harvested (McClanahan and Mangi 2000, Harmelin-Vivien et al. 2008, Goñi et al. 2008). Given this goal, knowledge of reproductive life histories of target species is often used when choosing MPA locations (e.g., fish spawning aggregations; Sala et al. 2002, Gaylord et al. 2005). In principle, the same concept could be applied to non-target, but ecologically and economically important, organisms such as corals. Yet, while MPAs have been shown to help maintain coral populations (Selig and Bruno 2010) it is largely unknown whether protecting corals from human-induced harm enhances reproductive output or larval quality.

Coral reproductive patterns are complex and disparate among species (Carlon 1999). Thus, a better understanding of differences in coral reproductive behavior on

scales of individuals, populations, and species may identify whether coral-dominated reef ecosystems have higher reproductive output than expected based on population abundance. To assess this question, we drew on fundamental theories related to reproductive tradeoffs and applied them to tropical stony corals, with a particular interest in how environmental degradation influences coral energy content and reproductive behavior.

Across a wide range of taxa, large birth size can confer higher survival, suggesting that mothers must balance offspring size and number to maximize fitness, given a fixed amount of energy available for reproduction (Smith and Fretwell 1973, Vance 1974, Bernardo 1996). Optimal offspring size is not fixed or absolute for a given species, as the degree of advantage conferred by being a certain size is not consistent across environmental conditions (Bernardo 1996, Begon et al. 2006). Mothers may modify offspring size in response to external factors, such as harsh environmental conditions or high interspecific competition, in order to optimize offspring success (Mousseau and Fox 1998, Marshall and Uller 2007, Marshall and Keough 2008, Allen et al. 2008). As a form of 'maternal' or 'parental' effect, offspring size variation thus allows for adaptive responses to environmental change (*sensu* phenotypic plasticity rather than evolutionary change; Mousseau and Fox 1998, Marshall and Uller 2007).

Flexible reproductive investment by a given individual through time is not always adaptive (Marshall and Uller 2007). Environmental conditions that influence parental heath, such as degraded water quality, can lead to the production of fewer *and* smaller offspring (Van Veghel and Kahmann 1994, Ward and Harrison 2000, Cox and Ward 2002). This suggests that environmentally-induced changes in offspring size and number

can drive reproductive provisioning away from what is optimal for long-term survival (Uller 2008).

Despite increasing evidence that parental effects can mediate offspring survival, we have a poor understanding of the relative importance of parental state (energy reserves) versus environmental conditions in shaping offspring size-number tradeoffs across taxa (Martin et al. 2013). Understanding the relative importance of these intrinsic limitations and extrinsic pressures is a critical component missing from parental fitness optimization models, as well as our general understanding of population and species viability.

Reproductive investment can be limited by the total amount of energy available (e.g., lipids and carbohydrates). In organisms ranging from marine invertebrates to birds, reduced parental energy content is associated with decreased progeny size and survival (Marshall and Keough 2008, Gorman and Nager 2004), highlighting a direct link between parental nutrition and offspring success. For offspring, energetic content likely determines success more directly than size, making it likely the factor under greater selection (Moran and McAlister 2009). Despite direct links between parental energy content, offspring energy content, and offspring survival, surprisingly few studies have assessed these relationships.

We determined adult energy content and reproductive investment in corals living on degraded (Willemstad) and quasi-pristine (Oostpunt) regions of the island of Curaçao. Oostpunt reefs experience few human impacts and have high coral cover, while coral abundance has declined in the Willemstad region due to human activities such as pollution and overfishing (Bak et al. 2005, Vermeij 2012). Analogous to the idea relied

upon for fish stocks in MPAs, we sought to understand whether corals on coral-dominated reefs have the potential to act as source pools of larvae for other areas.

Lipids serve as energy reserves in adult corals, providing for reproduction, survival of stressful events, and basal metabolism (Glynn et al. 1985, Stimson 1987, Ward 1995, Villinski 2003, Grottoli et al. 2004, Rodrigues and Grottoli 2007, Rodrigues et al. 2008). Egg and larval lipid content can be 60-90% of their biomass, and declines with age, suggesting lipid content largely limits the duration of the larval period (Ben-David-Zaslow and Benayahu 2000, Harii et al. 2002, Harii et al. 2007, Alamaru et al. 2009, Harii et al. 2010, Figueiredo et al. 2012).

We predicted that corals living on quasi-pristine reefs at Oostpunt would have higher energetic lipid content and would invest more into reproduction (larval size and number) than conspecifics living on degraded reefs near Willemstad. Additionally, we expected that corals would exhibit a tradeoff between the number and energy content invested in offspring (i.e., produce few energy-rich offspring or many energy-deplete offspring). Our analyses also allowed us to determine whether intrinsic limitation and/or external factors related more strongly to reproductive investment, which may highlight whether these corals experienced physiological limitations or provisioned their larvae adaptively.

MATERIALS AND METHODS

Study location and species

All research was conducted on the leeward coast of the island of Curaçao in the southern Caribbean. We focused on the regions of Oostpunt, the undeveloped,

easternmost region of the island, which has little human impact on land and sits up-current from the rest of the island, in contrast to Willemstad, the urban center of the island, which has a population of 150,000 (Fig. 3.1).

We investigated five coral species were studied, two that broadcast spawn egg-sperm bundles once per year, and three species that release competent, brooded larvae throughout the year. Energetic lipid content was measured in both brooding and spawning species, while reproductive measures were made only in brooding species. The brooding species used in this study were: (1) *Agaricia humilis* (low-relief lettuce coral), a small (< 12 cm in diameter), encrusting to submassive, gonochoric stony coral that releases larvae on a semi-nightly basis (Van Moorsel 1983); (2) *Favia fragum* (golfball coral), a small (< 5 cm diameter), simultaneously hermaphroditic, submassive coral that releases larvae over 8-10 days per month coincident with the lunar cycle (Szmant-Froelich et al. 1985); (3) *Siderastrea radians* (lesser starlet coral), a small gonochoric encrusting species that releases larvae on a semi-nightly basis (Szmant 1986). The broadcast spawning species used in this study were: (1) *Orbicella annularis* (lobed star coral, previously *Montastraea annularis*), a dominant, massive coral and (2) *Acropora palmata* (elkhorn coral), a previously dominant, now endangered branching coral.

Assessing benthic community composition in the Oostpunt and Willemstad regions

The benthos at each reef site was surveyed on SCUBA with three 30 m transects with 10 m between each transect, at a depth of 10 m. On each transect, 20 random photoquadrat images of 0.90 x 0.55 m (0.5 m^2) were taken. The proportion of benthic groups (live coral, dead coral, macroalgae, turf algae, coralline algae, sand flat) in each

image was assessed using Coral Point Count with Excel Extensions (CPCe). This program displays a specified number of randomly distributed points on photoquadrat images. The benthic group lying under each point is enumerated for each image and then averaged at transect and site scales.

Coral collection in the Oostpunt and Willemstad regions

Samples of adult corals from four species were taken from each of three sites within the regions of Oostpunt and Willemstad (Fig. 3.1) within a one-month period in the fall of 2010. In the spawning species (*O. annularis* and *A. palmata*) a small fragment was collected using hammer and chisel. In the small brooding species (*A. humilis*, *S. radians*, and *F. fragum*) the entire colony was taken. Care was taken to maintain consistency of collection depth and all depths were recorded. Samples were put in plastic bags underwater and placed on ice when divers surfaced. Tissue was removed from the skeleton with an airbrush and 0.45 μm-filtered seawater (FSW) within two hours of collection. The coral tissue-seawater slurry was immediately frozen at -20° C and moved to -80° C upon return to San Diego for subsequent lipid extractions.

Reproductive investment measurements of brooding coral species

Brooding species were used to assess reproductive investment and tradeoffs because they allow for accurate measurements of whole colony size, offspring size, and offspring number. In addition, most hermaphroditic spawning species are self-incompatible (i.e., male and female gametes do not form a viable embryo) thus will gain a fertilization advantage from higher population numbers, while brooders have a high

degree of self-fertilization (Carlon 1999). Thus, the reproductive output of brooding corals may provide a more ecologically useful metric for comparing reproductive patterns among conspecifics living in different areas. Despite shorter dispersal distances than spawners, the brooding species studied here have high levels of local recruitment and are capable of dispersing on island-wide scales (*F. fragum*; Goodbody-Gringley et al. 2010).

Colonies of the three brooding species described above were collected from Site 6 in the Willemstad region ("Water Factory" site in Hartmann et al. 2013 and referred to here as "Willemstad"; 12°6'33" N, 68°57'15" W) and Site 2 near Lagun Blanku in the Oostpunt region (referred to here as "Oostpunt" 12°2'33" N, 68°46'49" W). Divers cautiously removed colonies from the reef with a hammer and chisel and placed them individually in plastic bags underwater. The depth of collection was recorded and the bags were brought to the surface where they were placed in a seawater-filled cooler and transported to the Carmabi Foundation. Within two hours of collection, corals were placed in 100 µm-filtered flow-through seawater in individual 1 L plastic tri-pour beakers. Each beaker was fitted with an outflow tube that released water into a semi-submerged nylon-mesh-lined containers (150 µm pore size), which allowed for continuous larval collection while maintaining constant seawater flow.

Full details of the dates of collection and number of colonies are recorded in Table 3.1. Briefly, corals were collected at Oostpunt and Willemstad in 2010 for *S. radians*, 2011 for *F. fragum,* and in 2010, 2011, and 2012 for *A. humilis.* The month and day of *A. humilis* collection in each of the three years was within a two-week period. *F. fragum* and *A. humilis* in 2011 and 2012 were collected on the same day at both sites. Collections of *S. radians* and *A. humilis* (2010 only) was separated by one week due to

laboratory space constraints. Neither species shows lunar periodicity in larval release (Van Moorsel 1983, Szmant-Froelich et al. 1985), thus we assumed this had no effect on inter-site larval release patterns.

Photographs were taken of adult colonies against a scale bar on the day of collection and at the end of the experiment. The two-dimensional surface area of each adult colony was measured from photographs using ImageJ (NIH, http://rsbweb.nih.gov/ij/). Colony size (cm^2) was used as a proxy for the number of polyps (i.e., reproductive potential). To ensure this was a reliable approximation we compared colony size and polyp number in a subset of ten colonies of each species. The slope of the best-fit line between colony size and number of polyps in *S. radians* was linear ($R^2 = 0.998$). In *A. humilis* and *F. fragum*, the relationship best fit a power function ($R^2 = 0.83$ and 0.56, respectively, as compared to a linear fit of $R^2 = 0.67$ and 0.51, respectively). The shape of the line was concave in the former and convex in the latter. Thus, by using colony size we may have slightly underestimated the reproductive potential of large colonies in *A. humilis* and slightly overestimated reproductive potential of large colonies in *F. fragum*. We concluded this was of marginal consequence because we found that *A. humilis* was the most fecund species and *F. fragum* the least (i.e., a signal arose despite the elevated risk of Type II error).

All three species release larvae during the night, therefore larval collections were made between 0800-1000 every day. Larvae were collected for 5-7 days for *A. humilis* and *S. radians* (constant larvae releasers) and for 12 days for *F. fragum* in order to ensure larvae were collected during the entire monthly peak in larval release (Szmant-Froelich et al. 1985). Upon collection, larvae were kept separate by parent colony in beakers

containing 0.45 µm-filtered seawater (FSW). The number of larvae released each day by each colony was counted. Up to five haphazardly-chosen larvae from each colony were photographed with a scale bar using a dissecting microscope from which the two-dimensional area of each larva was measured following the methods of van Moorsel (1983). While we took care to ensure that non-spheroid larvae were measured when the longitudinal axis was in plane with the camera, any slight deviations off-axis would cause an underestimation of size that would be mathematically amplified (with respect to population variance) by calculating larval volume based on a spheroid (Petersen et al. 2005b, Hartmann et al. 2013). Therefore, larval sizes are reported in two-dimensions (as area).

After larvae were photographed, 5-10 individuals were pipetted onto a 25 mm diameter glass fiber filter (Whatman, GF/F, Kent, UK), wrapped in aluminum foil, and frozen at -20°C for less than two weeks, then transported frozen to San Diego and stored at -80°C. At the end of the larval collection period, adult tissue was removed from the skeleton via airbrushing and the coral tissue-seawater slurry was frozen in the same manner.

Lipid Measurements:

Lipids were extracted from adult and larval tissues using the Bligh and Dyer (1959) protocol. Briefly, GF/F filters with larvae were submerged in a sequential 2:1:0, 2:2:0, 2:2:1.8 chloroform:methanol:water (v:v:v) solvent system. After separation of the polar and non-polar phases, the non-polar phase containing lipids was isolated and dried under a stream of N_2. For adult samples, the tissue-seawater slurry was homogenized

with a handheld electric homogenizer, after which a 1 mL aliquot was placed in a combusted aluminum weigh boat for tissue mass measurements and a 3 mL aliquot was placed into a combusted glass vial for lipid extraction**.** The sequential solvent system described above was adjusted to account for water already contained in the sample when lipids were extracted from adult tissue.

Total tissue of each adult sample was measured by drying samples at 70°C for at least 48 h, measuring mass before and after ashing samples at 450°C for 4 h. The difference in mass provided the ash free dry weight (AFDW) or total organic matter (i.e., tissue). We observed unexpectedly low values of lipids after standardizing to AFDW and thus assessed the potential for overestimation of AFDW due to salt, an issue identified by Moreno et al. (2001). We re-calculated AFDW of a subset of samples (~20%) after removing salt with isotonic ammonium formate. Indeed, AFDW had been overestimated by more that 400%. This discrepancy was corrected for in all samples using the equation of the best-fit regression line of AFDW of samples measured with and without the removal of salt.

To measure lipid class concentrations, each bulk lipid extract was resuspended in a known volume of chloroform and 1 µl was spotted onto each of three quartz Chromarods (S-III, Iatron Laboratories, Inc.). Lipid classes were then separated using thin layer chromatography in a two-solvent system. First the chromarods were placed in a mixture of hexane:diethyl ether:acetic acid (99:1:0.05, v:v:v) for 25 minutes, after which they were dried, then placed in hexane:diethyl ether:acetic acid (80:20:0.1, v:v:v) for 25 minutes, a protocol that has previously been used for coral lipids (Rodrigues et al. 2008). Separated lipid classes were immediately quantified using an Iatroscan TLC-FID MK-5

(Iatron Laboratories, Inc.) that pyrolized lipids along the entire length of each rod. The retention time and area of each peak were recorded with LabView software (National Instruments, Texas, USA) and lipid class identities and concentrations were determined based on retention times and calibration curves generated using the following standards: 5-α-cholestane for hydrocarbons (HC), palmitic acid palmityl ester for wax esters (WE), tripalmitin for triacylglycerols (TAG), stearic acid for free fatty acids (FFA), stigmastanol for sterols (ST) and L-α-phophatidylcholine for phospholipids (PL). Hereafter we define the combination of WE and TAG as "energetic lipids" due to their role as sources of energy in corals (Rodrigues et al. 2006).

Statistical analyses:

Differences in adult energetic lipid content within and between regions were determined using a nested analysis of variance (ANOVA) with independent variables: region and sites within region. Data of most types (e.g., fecundity in *S. radians*) were not normally distributed, thus differences in larval size, larval lipid content, and fecundity between sites were tested using a Wilcoxon test (non-parametric) applied to all in order to use a single analytical method. Regression analyses were employed to assess relationships between fecundity (larvae/day) and larval size (mm^2), larval lipid content (μg WE + μg TAG/larva), or larval condition (μg WE + μg TAG/mm^2*larva). The larval condition metric was used because larval size and energetic lipid content per larva were correlated in all species ($p < 0.0001$, $R^2 = 0.77$). When a high degree of heteroscedasticity was detected data were log transformed to reduce non-uniform variance.

For each brooding species, we used generalized linear models (GLM) to determine how one extrinsic and three intrinsic parental characters related to fecundity, larval size, and larval condition, in order to identify factors explaining differences in reproductive patterns between regions. The extrinsic character used was the depth at which each colony was collected. The intrinsic parental characters used were colony size, the per-colony energetic lipid content (μg WE + μg TAG/mg tissue), and, when larval size or larval condition was evaluated, the number of larvae produced per day. None of the predictor variables were correlated ($p > 0.05$ for all). The GLM approach allowed for the assessment of response variables with unique distributions (e.g., normal, Poisson) in a common analytical framework for each species. In *A. humilis,* four additional models were analyzed, one that included the additional metric of year of collection and three models that used year as units of replication.

Lipid content measurements from parent colonies (as opposed to those collected in the sampling at six sites, Fig. 3.1) were significantly lower than those measured when the sites and regions were surveyed. This was perhaps due to the use of substantially less tissue material for lipid extractions. Therefore, to confirm the results of GLMs that included the energetic lipid standardized to tissue mass, we ran GLMs using the ratio of (μg WE + μg TAG)/(μg ST+ μg PL), the ratios of which were equal across the two datasets. Using these ratios in the GLM yielded the same statistical conclusions as those obtained when tissue standardized lipids were used.

RESULTS

Regional scale differences in coral cover, lipid content, and reproductive investment

Live coral was 2.5 times higher and coralline algae cover was 3.6 times higher in the Oostpunt when compared to Willemstad (Fig. 3.1). In contrast, macroalgae, turf algae, and dead coral were more prevalent in the Willemstad region (3.8, 4.6, and 1.3 times, respectively), demonstrating that Oostpunt reefs were coral-dominated by comparison, while Willemstad reefs had higher abundance of coral competitors.

In *Acropora palmata*, *Orbicella annularis*, and *Agaricia humilis*, energetic lipid content was significantly different and higher at Oostpunt and equal among sites within each region (see Fig. 3.2 for statistical significance levels by species). *Siderastrea radians* colonies showed no difference in energetic lipid content between regions or among sites within each region ($p > 0.05$). Among species, the greatest difference in adult energetic lipid content between regions was found in *A. palmata*, in which energetic lipid content was nearly 40% higher among the Oostpunt sites. Overall, these differences highlight higher energetic lipid content of corals living at Oostpunt relative conspecifics near Willemstad.

Fecundity patterns among individuals, populations, and species

Fecundity, defined as the number of larvae released per day, was significantly different among brooding coral species ($F_{2,181} = 6.36$, $p < 0.01$). A Tukey's HSD post hoc test revealed that *A. humilis* colonies were significantly more fecund (11.6 larvae/day ± 4.0 (95% confidence interval), $n = 107$ colonies) than *F. fragum* (1.3 larvae/day ± 0.3, $n = 42$). Fecundity in *S. radians* (5.6 ± 3.7**,** $n = 35$) was statistically indistinguishable from

the other two species. One *S. radians* colony was nearly three times more fecund than the next most fecund individual (13.6 larvae/day versus 5.0 larvae/day). Data for this colony were left out of the graphical depiction in Fig. 3.3, though were not excluded from analyses because it had a Cook's D influence of less than 1, suggesting it did not have a disproportionately large effect on the variance in fecundity among colonies.

At the population level, over three times as many larvae per day were produced per colony by *A. humilis* at Oostpunt compared to Willemstad ($Z = -2.3$, $p < 0.05$; Fig. 3.3) and twice as many larvae were produced by *F. fragum* ($Z = -2.7$, $p < 0.01$). Fecundity in *S. radians* was almost twice as high at Willemstad ($Z = -2.1$, $p < 0.05$). Across years of collection in *A. humilis*, fecundity was equal between sites in 2010 ($p = 0.63$), 74% greater at Oostpunt in 2011 ($p < 0.05$), and 79% greater in 2012 ($p < 0.001$). There was also a significant difference in fecundity among years ($p < 0.01$), as *A. humilis* fecundity was lowest in 2010 (2.5 ± 1.5 larvae/day), highest in 2011 (17.7 ± 8.6) and intermediate in 2012 (14.2 ± 7.0).

Larval size patterns among individuals, populations, and species

Differences in offspring size were seen at the level of individuals and species, but did not differ between regions. *S. radians* produced the smallest larvae (0.200 mm^2 ± 0.014, $n = 306$; Fig. 3.3), *A. humilis* intermediate (0.271 mm^2 ± 0.005, $n = 1068$) and *F. fragum* the largest (0.478 mm^2 ± 0.014, $n = 451$, $p < 0.0001$ among species). In contrast to differences among species, there was no difference in larval size between sites in any species ($p > 0.05$ for each species; Fig. 3.3). Across three years of *A. humilis* sampling, there were no differences in larval size between sites, but there were differences in larval

size among years, which was consistently observed in *A. humilis* across three years of sampling ($p < 0.001$). Consistent with fecundity patterns, larval size was greatest in 2011 (0.310 $mm^2 \pm 0.008$, $n = 426$), intermediate in 2012 (0.261 $mm^2 \pm 0.007$, $n = 388$) and smallest in 2010 (0.220 $mm^2 \pm 0.010$, $n = 254$). There were no relationships between larval size and intrinsic factors in any species, though depth (an extrinsic factor) correlated positively with larval size in *A. humilis* and *F. fragum* (Table 3.3).

Offspring size and lipid class composition

Larval size is often used as a proxy for energetic content, which is more directly related to lipid content. Therefore, we assessed larval lipid content between sites, and determined whether there were relationships between lipids (by class) and larval size among and within species. Similar to our findings with respect to larval size, there was no difference in larval lipid content or larval condition between sites in any species.

As expected, larval size was positively correlated with PL in all species, as it is the primary constituent of larval membranes (Table 3.2). Of the energetic lipids, both WE and TAG increased with larval size in *S. radians* ($p < 0.01$ for both, $n = 17$) and WE but not TAG increased in larval size in *A. humilis* ($p < 0.0001$ and $p > 0.05$, respectively, $n = 29$). In contrast, neither WE nor TAG showed a relationship with larval size in *F. fragum* ($p > 0.05$, $n = 11$).

We determined the ratio of WE to TAG in larvae as these energetic lipids can perform different functions within larvae, and this metric has been applied to marine invertebrate larvae to assess differences in energy provisioning (Arai et al. 1993, Villinski et al. 2002, Figueiredo et al. 2012). All three species had statistically different WE/TAG

ratios ($p < 0.0001$). A Tukey's HSD test found that *F. fragum* had the highest mean WE/TAG ratio (6.7) followed by *A. humilis* (5.0) and *S. radians* (0.9; Fig. 3.4). WE/TAG increased with larval size in *A. humilis* ($p < 0.0001$; Fig. 3.4) and *F. fragum* ($p < 0.01$), but not in *S. radians* ($p = 0.55$), showing that larger larvae have more WE in both absolute and relative terms in the former two species. The significant relationship between WE/TAG and larval size is particularly noteworthy in *F. fragum* ($R^2 = 0.6$) because there were no individual patterns in WE or TAG in that species.

Relationships between fecundity and larval size/lipid content

In assessing hypothesized tradeoffs in offspring size and fecundity, we found that *S. radians* showed an expected negative linear relationship between larval size and number ($p < 0.001$), while *A. humilis* showed a significant positive relationship ($p < 0.01$) and *F. fragum* showed no association ($p = 0.51$; Fig. 3.5). We also assessed whether total lipid content/larva or larval condition (μg WE + μg TAG/mm^2 * larva) showed relationships that suggested a tradeoff with fecundity. In no species was there a significant relationship between larval lipid content and fecundity, demonstrating that larval size and larval lipid content did not share the same relationship with fecundity in *S. radians* and *A. humilis*. Additionally, while fecundity did not relate to larval condition in *S. radians* and *F. fragum*, *A. humilis* showed a significant negative linear relationship between larval condition and fecundity ($p < 0.001$) despite a positive relationship between larval size with fecundity.

Relationships between parental characters and offspring size, number, and condition

We used generalized linear models (GLM) to assess how larval characters related to intrinsic (colony size, colony lipid content, fecundity) and an extrinsic parental character (colony collection depth) in order to identify factors that may explain differences in reproductive output between regions. Depth was included in part because, while we attempted to keep depth constant, the mean depth where we were able to locate colonies was deeper for *F. fragum* and shallower for *S. radians* at Oostpunt ($p < 0.0001$ for both). In models examining larval size or larval condition, we included fecundity as another means to identify reproductive tradeoffs between larval condition and number in a multi-character framework.

Fecundity in *A. humilis* and *S. radians* showed strong statistical relationship with intrinsic factors. In both species, larger colonies had higher fecundity ($p < 0.0001$ and 0.001, respectively; Table 3.3), while fecundity decreased with parental energetic lipid content ($p < 0.001$ for both species). In *F. fragum*, no intrinsic factors were associated with fecundity, suggesting colony size and energy content did not related to the number of larvae produced in this species. Fecundity in *A. humilis* increased with depth ($p < 0.0001$), suggesting differences in larval production across habitats.

When year was included as a parental character in the *A. humilis* models, statistically significant relationships among previously identified factors persisted and year was also significant ($p < 0.001$; data not shown). In both years for which lipid data were collected (2010 and 2011), parental energetic lipid content had a significant negative relationship and colony size had a positive relationship with fecundity, consistent with the all-inclusive model. In contrast, depth showed significant and

opposite relationships with fecundity between years, suggesting it was not a reliable predictor of fecundity.

In *A. humilis*, larval condition decreased with fecundity ($p < 0.05$), implying a tradeoff between larval number and larval energy content. Larval condition did not show a significant relationship with parental energetic lipids or colony size in this species, perhaps highlighting greater limitation on the number of larvae produced rather than the condition of those larvae. *S. radians* also did not show a significant relationship between larval condition and any intrinsic factors. While fecundity was unaffected by intrinsic and extrinsic factors in *F. fragum*, larval condition increased with parental energetic lipid content ($P < 0.05$), implying that colonies with higher energetic lipid content produced more lipid-rich larvae.

DISCUSSION

Reducing human activities that harm coastal marine ecosystems is important for preserving natural systems for future generations. Marine protected areas, or MPAs, offer one such method, as their enforcement excludes human activities within a given area. Many MPAs are designed to allow for a balance of ecosystem conservation and human needs, such as the harvesting of fishes and invertebrates (Gaines et al. 2010). As a result of stimulated population growth due to reduced fishing, most MPAs contain higher fish biomass relative to areas outside, which can migrate outside the MPA and be harvested (McClanahan and Mangi 2000, Harmelin-Vivien et al. 2008, Goñi et al. 2008). This ‘spillover’ effect may also occur among other important though non-target animals such as corals, whose populations can be enhanced within MPAs (Selig and Bruno 2010).

Corals are mobile during the larval stage, thus enhanced reproduction resulting from larger populations and perhaps more fecund individuals within MPAs may create larval source pools that seed degraded reefs outside the protected area. Yet, it is not known whether corals on highly functioning reefs are more reproductive than those on degraded reefs.

To determine how the state of a coral reef influences energy storage and reproductive output in corals, we compared energetic lipid content and larval release patterns of visually healthy corals living in quasi-pristine and degraded regions of the island of Curaçao. Benthic surveys confirmed that live coral cover was more than twice as high in the quasi-pristine region (Oostpunt) relative to degraded reefs near the urban center of Willemstad. Reefs near the urban center have experienced dramatic reductions in coral abundance in recent decades due to human activities such as industrial pollution (Bak et al. 2005, Vermeij 2012). Supporting our prediction, corals in the Oostpunt region had higher energetic lipid content relative to conspecifics in the degraded region, suggesting that apparently healthy corals on quasi-pristine reefs were more energetically robust.

Fecundity (larvae/day) in the brooding species *Favia fragum* and *Agarica humilis* was higher in the quasi-pristine region as well. Differences in fecundity did not show evidence consistent with a reproductive tradeoff at the population level, as there was no difference in larval size between regions. Instead, our data suggested that disparities in fecundity between regions were due to differences in the physiological capacity of corals in each population to reproduce that resulted in an overall depression of reproduction at Willemstad in *A. humilis* and *F. fragum*. Lower fecundity in response to environmental

stress has been predicted and experimentally demonstrated in corals (Van Veghel and Kahmann 1994, Ward 1995, Ward and Harrison 2000, Cox and Ward 2002), and here we find it among apparently healthy individuals living in an area that has progressively declined over decades.

In contrast to *A. humilis* and *F. fragum*, fecundity was higher in *S. radians* in the degraded region. *S. radians* shows a tolerance and perhaps preference for marginal habitats (Lewis 1989) such as inland bays on Curaçao (Vermeij et al. 2007). This may explain why this species was more fecund on the degraded reefs near Willemstad and exhibited no difference in energetic lipid content among adults between regions, in contrast to the three other species studied here.

Interspecific differences in lipid provisioning and larval dispersal potential

Wax ester lipids (WE) are believed to facilitate relatively long larval periods because they are slowly metabolized and particularly positively buoyant, while triacylglycerols (TAG) provide a more rapidly metabolized source of energy (Benson et al. 1972, Sargent et al. 1977, Harii et al. 2007, Figueiredo et al. 2012). The relatively high WE/TAG ratio in *A. humilis* and *F. fragum* larvae (Fig. 3.4) is consistent with their modest dispersal potential, such as that on intra-island scales (*F. fragum*; Goodbody-Gringley et al. 2010). Experimentally lowering WE content reduces larval buoyancy in echinoderm larvae (Villinski et al. 2002) and relatively low WE content and associated negative buoyancy of *S. radians* is expected to physically constrain dispersal.

Echinoderms evolved lecithotrophy (non-feeding larvae) from planktotrophy (feeding larvae) coincident with increases in larval WE/TAG ratios (lecithotrophic

species: WE/TAG > 2.5, planktotrophic species: WE/TAG < 1.0; Villinski et al. 2002). WE/TAG ratios are extremely high among long-dispersing spawning coral species (Arai et al. 1993, Figueiredo et al. 2012), and among the brooding species studied here WE/TAG was significantly higher in *F. fragum* (6.7) and *A. humilis* (5.0) than in *S. radians* (0.9). This suggests fundamental differences among species in proportioning of the energy available during dispersal. While long larval periods in these brooding species are likely uncommon in nature, proportionally high WE content in *A. humilis* and *F. fragum* may enhance survival after stochastic events such as storms that sweep larvae long distances (Allen et al. 2008, Marshall and Keough 2009) an advantage unlikely to be afforded to *S. radians*.

S. radians colonies tend to be found at high abundance and have a high likelihood of settling near natal colonies, suggesting limited dispersal (Vermeij and Sandin 2008). Low dispersal capacity may highlight a relatively high risk of local extinction in *S. radians*, perhaps explaining our inability to find the species at one Oostpunt site and its overall patchy distribution in Curaçao (Vermeij et al. 2007). Thus, low dispersal potential in *S. radians* suggests the higher fecundity observed at Willemstad likely only benefits that population.

Intraspecific variation in larval dispersal potential

Variation in the WE/TAG ratio was also evident within species. In *A. humilis* and *F. fragum,* WE/TAG increased with larval size, demonstrating that larger larvae have higher provisioning of a lipid type that may increase dispersal potential. Large birth size increases settlement and post-settlement survival of *A. humilis* (Hartmann et al. 2013).

Yet, mass-specific metabolic rates can be similar in echinoderm larvae of different sizes, which may negate the presumed energetic benefits of being large (Moran and Allen 2007). Therefore, the relatively high WE content of large larvae in *A. humilis* and *F. fragum* may provide a physiological explanation for the ability of large larvae to prolong dispersal and be more selective about settlement locations, thereby increasing success (Marshall and Keough 2003, Marshall and Bolton 2007).

High intercolony variation in larval size and energy content in *A. humilis* and *F. fragum* suggests colonies are producing larvae with variable dispersal potential (bet hedging strategy; Marshall et al. 2008, Crean and Marshall 2009). This variation may provide a means by which *A. humilis* and *F. fragum* colonies can produce highly dispersive larvae, a benefit when local conditions become degraded and a force that promotes connectivity (Marshall and Keough 2003).

Consistent current directions, such as that which runs along the leeward coast of Curaçao, can cause asymmetrical dispersal of pelagic larvae (Pringle et al. 2011). When this occurs, upstream populations can serve as disproportionately important sources of offspring for downstream locations, exacting a large effect on downstream population structure (Pringle et al. 2011). Therefore, higher fecundity, greater adult density, and an upstream location likely results in *F. fragum* and *A. humilis* larvae from Oostpunt contributing disproportionately large number of recruits to reefs in other regions of Curaçao.

Reproductive timing and the role of parental characters in fecundity

Differences in the timing of larval release among the brooding species studied here may explain species-specific relationships between fecundity and parental characters. *A. humilis* and *S. radians* release larvae nearly constantly and our data suggest fecundity in these species was limited by a colony's capacity for producing larvae (colony size) and its energetic lipid content. The negative relationship between colony energetic lipid content and fecundity in both *S. radians* and *A. humilis* suggests a high energetic cost of reproduction. In contrast to *A. humilis* and *S. radians*, *F. fragum* releases larvae over a few days each month and has low fecundity relative to the other two species. The production of fewer total offspring may then explain why fecundity was not associated with either physical or energetic constraints in *F. fragum*.

Reproductive tradeoffs and the relationship between larval size and lipid content

Models of reproductive investment assume a tradeoff between the size and number of offspring an individual produces (i.e., an increase in fecundity corresponds with a decrease in per offspring investment; Vance 1973, Smith and Fretwell 1974). Inherent to this tradeoff is the assumption that mothers have a finite amount of energy to invest in reproduction at any one time. While larval size has been extensively studied and modeled, energy content is the assumed mechanism underlying the benefits of size, for which size is an assumed proxy (Moran and McAlister 2009).

We found that larval size and total lipid content did not consistently show expected positive relationships in any species, thus larval size is not a ubiquitous indicator of energetic lipid content. Much like our investigations into larval size-

fecundity tradeoffs, we found little consistent evidence among species of relationships between fecundity and per larva lipid investment. Larval condition decreased with fecundity only in *A. humilis* though, interestingly, this relationship was opposite that of larvae size and fecundity. This demonstrates that the relationship between fecundity and larval condition rather than larval size may reveal the true reproductive tradeoff in this species, which was apparently masked in larval size-fecundity assessments. *A. humilis* was the most fecund of the three brooding species studied here and we conclude that it was therefore the most prone to reach a point of maximum energetic investment. Reaching maximum investment is likely necessary in order to find strong evidence of a tradeoff among individuals, perhaps explaining why a fecundity-larval condition relationship was not seen in either of the other two species.

Conclusions

Our data show a strong association between high coral community abundance and high energetic content and reproductive output of individuals. The combination of more numerous individuals and disproportionately high fecundity may have multiplicative benefits that lead to dramatic differences in reproductive potential between coral communities. Much like the fisheries benefits gained from MPAs, protecting highly-functioning coral reefs such as Oostpunt in Curaçao will be extremely important for growing local populations and providing source of new recruits to degraded communities on island-wide scales.

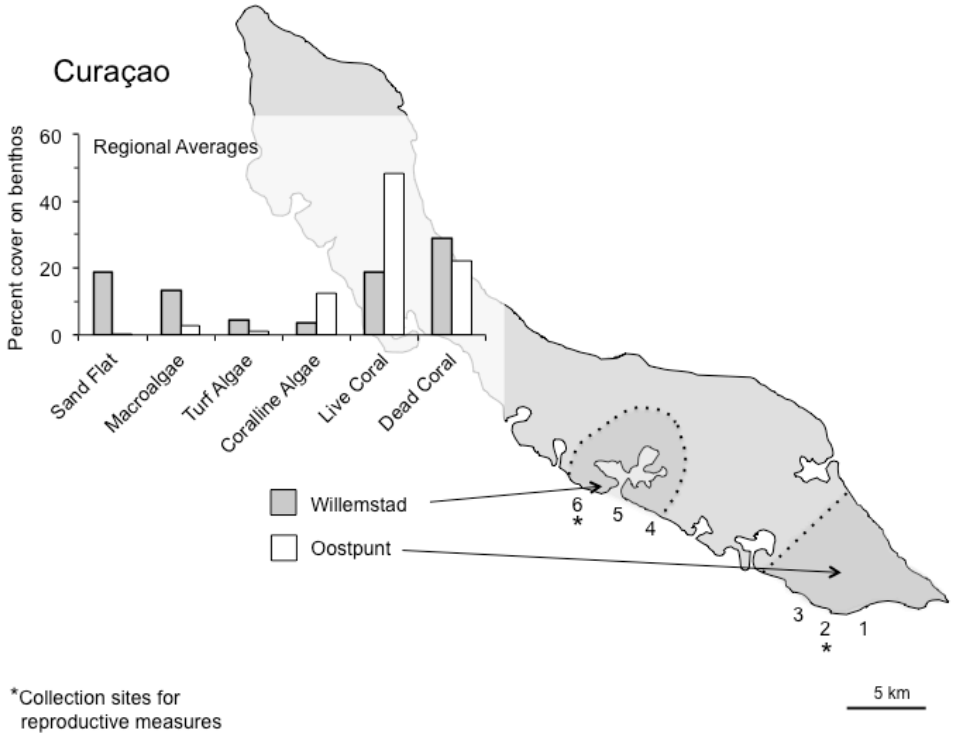


Figure 3.1: Locations and benthic composition of study sites in Curaçao. Sites 1-3 are within Oostpunt, the largest uninhabited area of Curaçao, and sites 4-6 are within Willemstad, the urban center. Community composition between the two regions is shown as the percentage of the benthos covered by sand flats, macroalgae, turf algae, coralline algae, live coral, and dead coral. Data represent the mean benthic coverage determined by surveys at the three sites within each region. Sites 1-6 are also the locations at which adult colonies were sampled for lipid class analysis. Additionally, three brooding species were collected at Sites 2 and 6 in order to assess reproductive output (shown with asterisks).

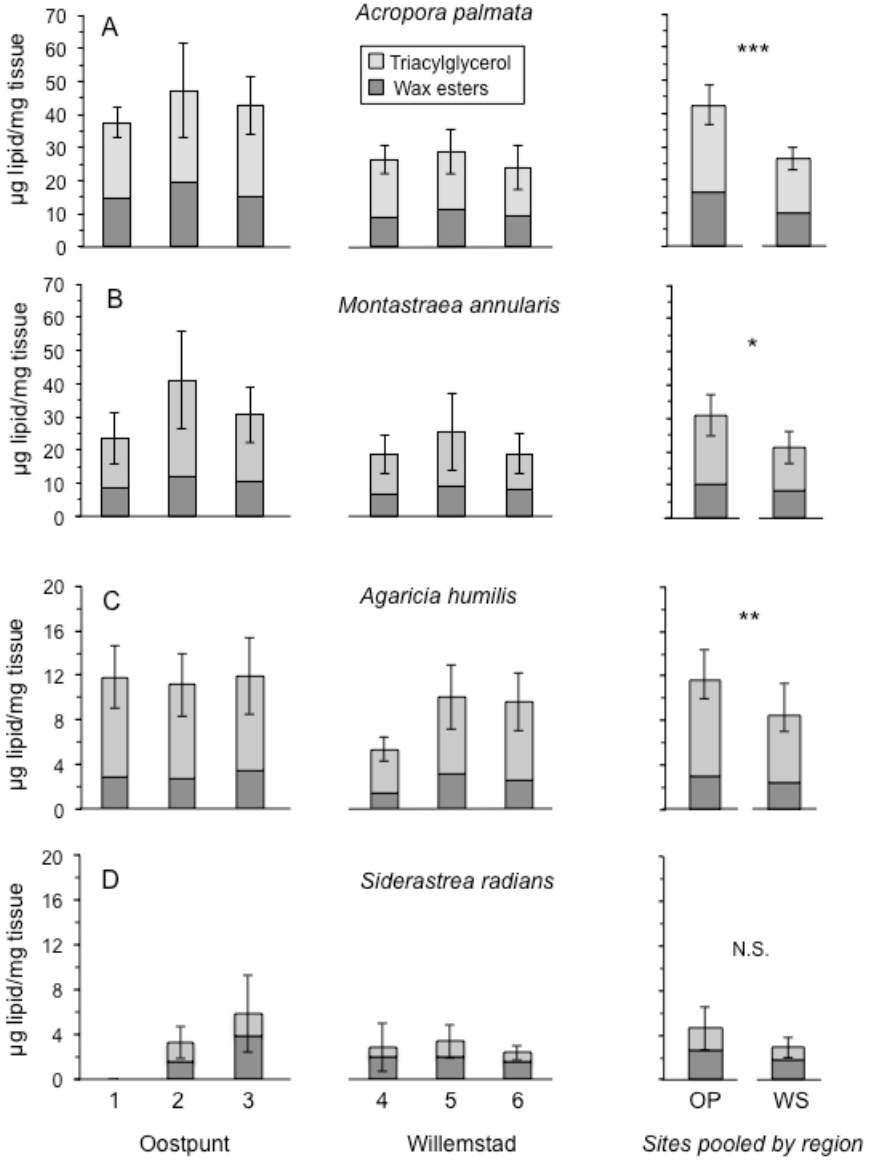


Figure 3.2: Energetic lipid content of adult coral tissue, measured as triacylglycerols and wax esters (µg lipid/mg tissue) sampled at the six sites shown in Fig. 3.1. Species include *Orbicella annularis* (A), *Acropora palmata* (B), *Agaricia humilis* (C), and *Siderastrea radians* (D). Data are shown by site (Oostpunt 1-3 and Willemstad 4-6) and by region with sites pooled. For each site, $n = 10$ colonies per species (i.e., $n = 30$ colonies/region for each species), except for *S. radians* at Oostpunt because the species was not found at one site ($n = 20$ *S. radians* colonies at Oostpunt). Bars represent 95% confidence intervals of the pooled values of both lipid classes. P-values were generated using a nested ANOVA of site nested within region and region alone. "NS" denotes that the difference is not statistically significant and asterisks denote the degree of statistical significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

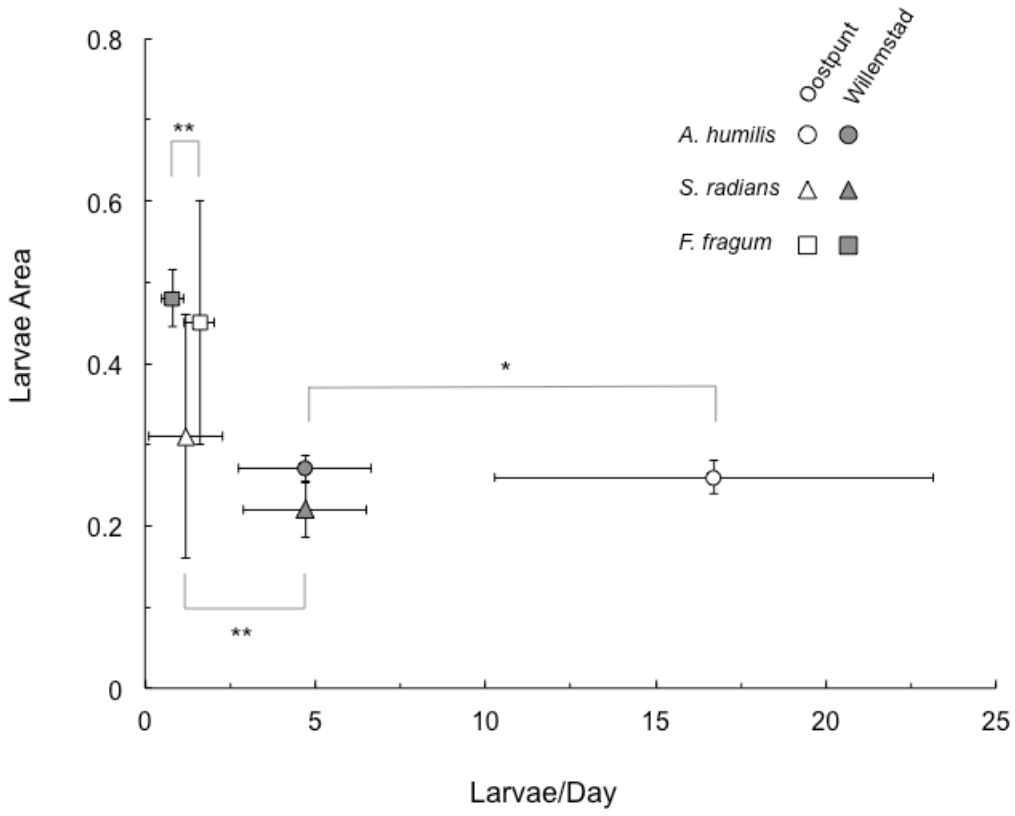


Figure 3.3: Mean and 95% confidence intervals of fecundity (larvae/day) and larval size by brooding species and region within Curaçao. For each species, significant differences in fecundity or larval size between the two regions are denoted with brackets. Asterisks depict the level of significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

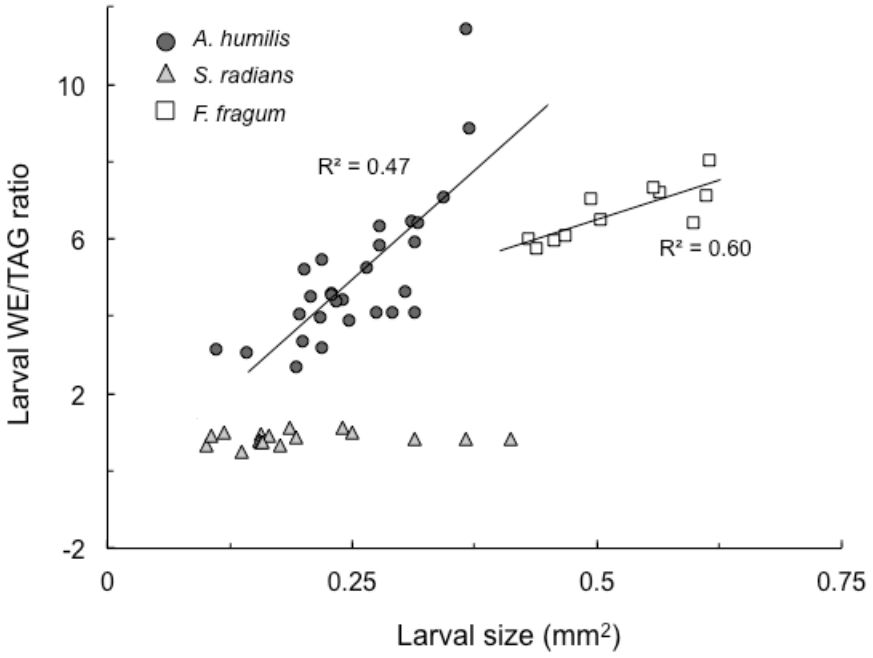


Figure 3.4: Relationship between larval size and the ratio between the two dominant energetic lipids (wax esters and triacylglycerols) in three brooding coral species. Each point represents the mean values of larvae produced by individual parent colonies collected at Oostpunt and Willemstad. Significant linear regressions and coefficients of determination are presented for *Agaricia humilis* and *Fragum fragum*. There was not a significant relationship exhibited by *Siderastrea radians*.

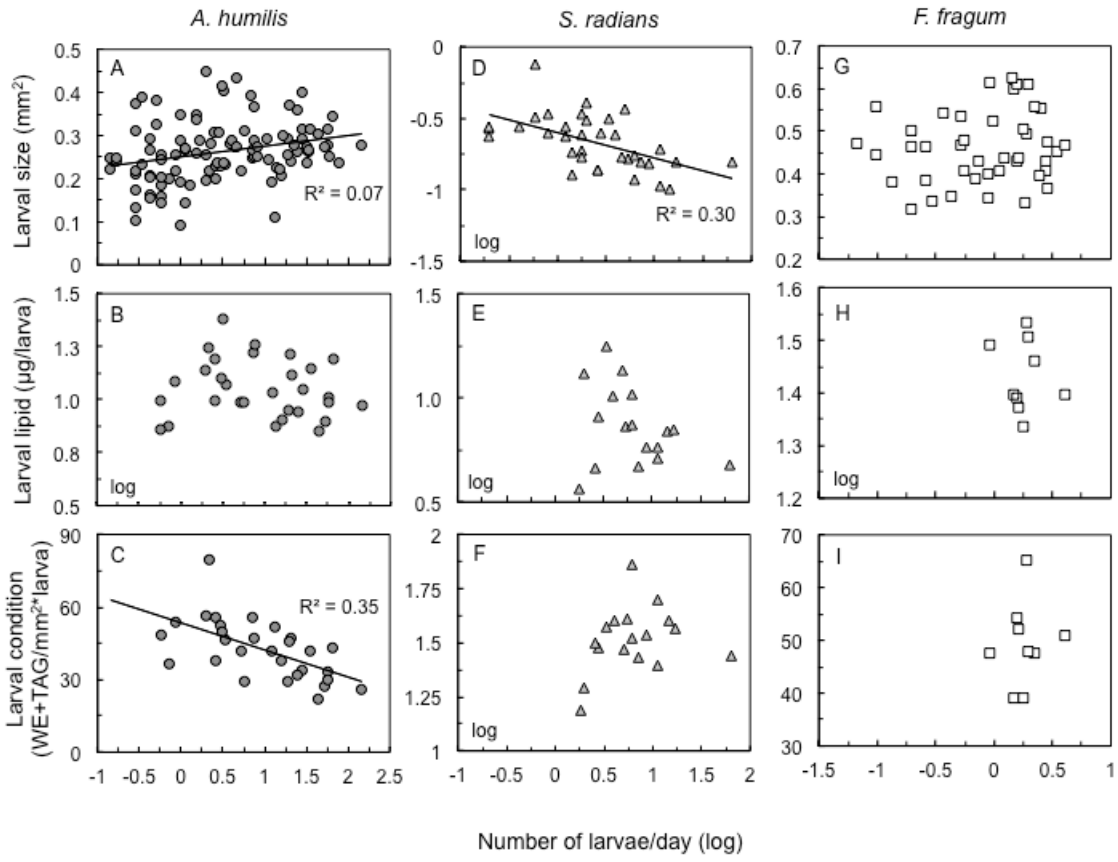


Figure 3.5: Evaluations of reproductive tradeoffs between the number of larvae produced per day per colony and larval size (A, D, G), larval lipid content (B, E, H), and larval condition (C, F, I). Data are shown for three brooding coral species: *Agaricia humilis* (A-C), *Siderastrea radians* (D-F), and *Favia fragum* (G-I). Larval condition was measured as the amount of energetic lipid (µg wax esters + µg triacylglycerol) per larva standardized to larval size (mm^2). The number of larvae produced per day was log transformed in all comparisons in order to meet the assumption of equal variance. When data for the y-axis variable (larval size, lipid content, condition) did not meet the assumption of equal variance, these data were transformed, as denoted by the word "log" in the corresponding plot. Linear relationships are shown on plots when the fit was statistically significant, along with the associated coefficient of determination (R^2).

Table 3.1: Details of the timing of adult colony collection, number of colonies collected, and length of larval collection period for the three brooding species *Agaricia humilis*, *Siderastrea radians*, and *Favia fragum*.

Species	**Adult collection date**	**Colonies collected (Oostpunt/Willemstad)**	**Days of larval collection**
A. humilis	9/2010	30/30	7
A. humilis	9/2011	27/27	6
A. humilis	9/2012	15/15	7
S. radians	9/2010	30/30	5
F. fragum	9/2011	27/27	12

Table 3.2: Correlation analyses between larval size (predictor variable) and wax esters (WE), triacylglycerols (TAG), phospholipids (PL) in three brooding coral species. Lipid class data were log transformed prior to analyses to account for allometric scaling and to meet assumptions of equal variance. Reported are the coefficient of determinations (R^2), t-ratios, and associated p-values. Significant correlations are shown in bold at $\alpha = 0.05$.

		Predictor: Larval size (mm^2)		
Species	Response (µg/larva)	R^2	*t-ratio*	*p-value*
A. humilis	WE	0.40	4.48	**0.000**
	TAG	-0.03	-0.14	0.889
	PL	0.19	2.77	**0.010**
S. radians	WE	0.36	3.14	**0.007**
	TAG	0.33	2.99	**0.009**
	PL	0.58	4.79	**0.000**
F. fragum	WE	0.18	1.81	0.104
	TAG	-0.08	0.46	0.659
	PL	0.35	2.51	**0.033**

Table 3.3: Results of generalized linear models predicting different measures of reproductive investment by parent colony character. Model results are depicted in three frames: by species (*S. radians*, *F. fragum*, *A. humilis*), by type of reproductive investment (larval size, larval condition, number of larvae/day), and by extrinsic (white: colony depth) and intrinsic (grey: colony size, colony larvae/day, colony energetic lipid content) parental characters. Significant predictors of each model are shown based on their parameter estimate, which indicates the strength and direction of the relationship that parameter has with the predictor (i.e., slope of line). The level of statistical significance is depicted with asterisks (P < 0.05*, 0.01**, 0.001***). Dashes represent non-significant relationships. Larval condition was calculated as the amount of energetic lipid per larva standardized to larval size.

	Response:	**Larval size:**	**Larval condition**	**Larvae/day**
Species	*Predictor*	*Parameter estimate*	*Parameter estimate*	*Parameter estimate*
A. humilis	Colony depth	**0.06*** **	**-6.02***	**1.48*** **
	Colony size	-	-	**0.09*** **
	Larvae/day	-	**-0.14***	N/A
	WE+TAG/mg	-	-	**-0.21*** **
S. radians	Colony depth	-	-	-
	Colony size	-	-	**0.17*** **
	Larvae/day	-	-	N/A
	WE+TAG/mg	-	-	**-0.35*** **
F. fragum	Colony depth	**0.15***	-	-
	Colony size	-	-	-
	Larvae/day	-	-	N/A
	WE+TAG/mg	-	**3.42****	-

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Chapter 4.

Large birth size does not reduce negative latent effects of harsh environments across life stages in two coral species

Aaron C. Hartmann,[1,5] Kristen L. Marhaver,[2,3] Valérie F. Chamberland,[3,4] Stuart A. Sandin,[1] and Mark J. A. Vermeij[3,4]

[1]*Center for Marine Biodiversity and Conservation, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093 USA*
[2]*University of California, Merced, California 95343 USA*
[3]*CARMABI Foundation, Piscaderabaai z/n, Willemstad, Curaçao*
[4]*Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands*

Abstract. When juveniles must tolerate harsh environments early in life, the disproportionate success of certain phenotypes across multiple early life stages will dramatically influence adult community composition and dynamics. In many species, large offspring have a higher tolerance for stressful environments than do smaller conspecifics (parental effects). However, we have a poor understanding of whether the benefits of increased parental investment carry over after juveniles escape harsh environments or progress to later life stages (latent effects). To investigate whether parental effects and latent effects interactively influence offspring success, we determined the degree to which latent effects of harsh abiotic conditions are mediated by offspring size in two stony coral species. Larvae of both species were sorted by size class and exposed to relatively high-temperature or low-salinity conditions. Survivorship was quantified for six days in these stressful environments, after which surviving larvae were placed in ambient conditions and evaluated for their ability to settle and metamorphose. We subsequently assessed long-term post-settlement survival of one species in its natural environment. Following existing theory, we expected that, within and between species, larger offspring would have a higher tolerance for harsh environmental conditions than smaller offspring. We found that large size did enhance offspring performance in each species. However, large offspring size within a species did not reduce the proportional, negative latent effects of harsh larval environments. Furthermore, the coral species that produces larger offspring was more, not less, prone to negative latent effects. We conclude that, within species, large offspring size does not increase resistance to latent effects. Comparing between species, we conclude that larger offspring size does not inherently confer greater robustness, and we instead propose that other life history characteristics such as larval duration better predict the tolerance of offspring to harsh and variable abiotic conditions. Additionally, when considering how stressful environments influence offspring performance, studies that only evaluate direct effects may miss crucial downstream (latent) effects on juveniles that have significant consequences for long-term population dynamics.

Key words: Agaricia humilis; *latent effects; life history;* Montastraea faveolata; *offspring size theory; parental effects; scleractinian corals.*

Introduction

A major goal of ecology is to understand how species tolerate and acclimate to various abiotic conditions. This is particularly important in the earliest stages of life, when many organisms suffer high mortality due to an inability to tolerate environmentally induced stress (Thorson 1950, Begon et al. 2006). Reductions in offspring fitness can arise while individuals are in a stressful environment ("direct effects") and after they have left stressful environments or progressed to a later life stage ("latent effects"; sensu Pechenik 2006). While tolerance of offspring to a given environment can be determined by a number of factors including cellular defenses and adaptive responses (Hamdoun and Epel 2007), offspring size has long been considered an important mediator of environmental tolerance within species (Thorson 1950, Smith and Fretwell 1974, Parker and Begon 1986, Bernardo 1996).

Offspring size is primarily determined by maternal provisioning, and theory predicts that mothers have a finite pool of energy to invest in many small or few large offspring (Smith and Fretwell 1974, Parker and Begon 1986, Bernardo 1996). When mothers are able to influence the size and number of progeny they produce,

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[5] Present address: Scripps Institution of Oceanography, University of California at San Diego, 9500 Gilman Drive, Mail Code 0208, La Jolla, California 92093-0208 USA.
E-mail: achartma@ucsd.edu

this trade-off allows them to optimize offspring survival given their local environmental conditions ("parental effects"; Smith and Fretwell 1974, Bernardo 1996, Mousseau and Fox 1998). In poor-quality environments, large offspring tend to have higher success than smaller conspecifics (Parker and Begon 1986, Marshall et al. 2006, Allen et al. 2008), whereas size is of less or no importance in benign or extremely harsh environments (Moran and Emlet 2001, Begon et al. 2006).

Previous work has focused on the importance of offspring size while organisms are experiencing stressful conditions (i.e., direct effects; Allen et al. 2008, Marshall and Keough 2009, Burgess and Marshall 2011), leaving relatively little knowledge about whether size and experience can interactively influence offspring success after individuals move away from stressful conditions or progress to a later life stage (though see Crean et al. 2011). Despite this, a growing body of evidence is revealing that latent effects can be profound and important (reviewed in Pechenik 2006). For example, delaying larval metamorphosis can reduce subsequent fitness in salamanders and crabs (Semlitsch et al. 1988, Gebauer et al. 1999, respectively) and nutrient deprivation during early development can lead to stunted or abnormal growth in fish and birds (McCormick and Molony 1992, Merilä and Svensson 1997, respectively). Despite the pervasiveness of latent effects across many taxa, we do not know whether the benefits of extra parental provisioning can offset the negative latent effects that arise after offspring experience harsh abiotic conditions early in life.

The capacity of parental provisioning to buffer progeny from environmental stress depends intimately upon the source of stress. In most manipulative studies of the importance of parental provisioning, stresses have been generated via biotic factors such as inter- and intraspecific competition and dispersal duration (e.g., Emlet and Hoegh-Guldberg 1997, Allen et al. 2008, Marshall and Keough 2009, Burgess and Marshall 2011, Crean et al. 2011). In such situations, energy availability can provide inherent benefits to the organism (e.g., an increased ability to withstand delayed metamorphosis in non-feeding larvae). In contrast, we have a poor understanding of whether large size promotes tolerance to harsh and variable abiotic conditions such as high temperature and low salinity, despite the fact that mobile offspring commonly experience variable abiotic conditions during dispersal (Marshall and Morgan 2011). Therefore, in the present study, we determined whether offspring size influences susceptibility to latent effects of harsh abiotic conditions in two common Caribbean coral species.

Sessile marine invertebrates such as corals are particularly useful for exploring parental and latent effect theory because they produce offspring of variable size, progress through distinct early life stages, and commonly experience multiple environments prior to reaching adulthood (Pechenik 2006, Marshall and Morgan 2011). The two coral species studied here, *Agaricia humilis* and *Montastraea faveolata*, both produce larvae of different mean sizes with a high degree of size variability within offspring cohorts. This allowed us to test for interactions between parental effects and latent effects by measuring the performance of small and large larvae of each species after exposure to either high-temperature or low-salinity environments, two abiotic factors known to negatively impact coral offspring (Edmunds et al. 2001, Vermeij et al. 2006, Randall and Szmant 2009).

Based on offspring size theory and studies of direct effects, we predicted that for each species: (1) relatively large offspring would have higher pre-settlement survival, settlement, and post-settlement survival than smaller conspecifics (i.e., evidence of parental effects); (2) larvae exposed to harsh environments would have lower settlement and post-settlement survival after being moved to a "common garden" (i.e., evidence of negative latent effects of harsh environments); and (3) relatively large larvae would suffer the least reductions in settlement and post-settlement survival after experiencing harsh environmental conditions (i.e., evidence that large larval size buffers negative latent effects).

By measuring the performance of each species in the same manner, our experiment also allowed us to test whether these species differ in their degree of susceptibility to negative latent effects, and whether the species that produces larger larvae overall suffers less severe latent effects (i.e., whether absolute offspring size determines susceptibility to latent effects). Based on offspring size theory alone, one might predict that the species with larger progeny, *A. humilis*, would be less prone to negative latent effects than *M. faveolata*. However, *A. humilis* larvae disperse for a short period (hours to days), while *M. faveolata* larvae disperse for much longer (days to months). As a result, an alternative prediction can be generated based on life history theory. The short-dispersing *A. humilis* larvae might be expected to suffer more extreme latent effects when subjected to environments significantly different from the parental environment, while the long-dispersing *M. faveolata* larvae would be more tolerant of a variety of conditions like those experienced during their longer larval phase (sensu Uller 2008).

Materials and Methods

Collection of larvae from Agaricia humilis *and* Montastraea faveolata

Agaricia humilis (low-relief lettuce coral) is a small (<12 cm in diameter), encrusting to submassive stony coral found throughout the tropical West Atlantic (van Moorsel 1983). Eggs are fertilized internally (i.e., brooded) and relatively large spheroid larvae (~600 × 900 μm long) are released throughout the year. *A. humilis* larvae contain symbiotic dinoflagellates from birth and disperse for a short period (hours to days) before settling (van Moorsel 1983).

Twenty-seven colonies of *A. humilis* were collected on scuba from the southeast Oostpunt region of the leeward coast of Curaçao (12°2′33″ N, 68°46′49″ W). Within two hours of collection, corals were placed in individual 1-L beakers with a constant flow of 100-μm-filtered seawater. All larvae released on the first night after colony collection were pooled on the following day to create an experimental cohort of a single age ($n = 1235$). To generate large and small size classes in roughly equal numbers, larvae were gently passed through or retained above a submerged nylon mesh filter. A range of mesh sizes was tested; a mesh size of 430 μm was used for the final size class separation ($n = 712$ large, $n = 523$ small). Fourteen representative larvae from each size class were photographed with a scale bar using a dissecting microscope. From these photographs, the longitudinal and transverse axes were measured (NIH ImageJ; *available online*)[6] and used to calculate larval volume by assuming the volume of a spheroid (van Moorsel 1983).

Montastraea faveolata (mountainous star coral) is a dominant, massive reef-building stony coral that occurs throughout the tropical West Atlantic and can grow to 10 m in diameter (Szmant et al. 1997). *M. faveolata* is a simultaneous hermaphrodite that reproduces via broadcast spawning once per year in late summer and early fall by releasing bundles containing sperm and small spherical eggs (~200–300 μm in diameter). During their relatively long (days to months) dispersal period, the competent, swimming planula larvae can disperse tens to hundreds of kilometers prior to settlement (Vermeij et al. 2006).

Bundles of egg and sperm were collected from eight *M. faveolata* colonies during the annual mass spawning in Curaçao (Water Factory site, 12°6′33″ N, 68°57′15″ W). Gametes were pooled and allowed to fertilize for 120 minutes. Embryos were then sequentially diluted in 0.45 μm-filtered seawater (Millepore HA filter; EMD Millepore, Billerica, Massachusetts, USA) using methods adapted from Vermeij et al. (2006). After 24 h, swimming larvae were separated into two size classes using a nylon mesh (190 μm). A sample of larvae from each size class was stored in 5% volume/volume formaldehyde for later size measurements that were conducted in the same manner as those for *A. humilis*.

Experiment 1: Direct effects of exposure to harsh environmental treatments on survival

Larvae of each size class were placed into one of three environmental treatments: high-temperature (36 psu, 31°C), low-salinity (30 psu/85% of ambient salinity, 28.5°C), or ambient conditions (36 psu, 28.5°C). Temperatures were selected to correspond with average monthly mean water temperatures in Curaçao (ambient and low-salinity treatments) and 1.5°C above the bleaching threshold of adult corals in Curaçao (high-temperature treatment; data *available online*).[7] Salinity levels were selected to mimic ambient seawater conditions in Curaçao (ambient and high-temperature treatments), as well as conditions known to influence larval behavior after significant rainfall (low-salinity treatment; Vermeij et al. 2006). Salinity was manipulated by diluting seawater with sterile, deionized water. Nine replicates for each size/environmental treatment were generated by aliquoting 10 *A. humilis* or 30 *M. faveolata* larvae into 15-mL polystyrene conical tubes (BD Biosciences, San Jose, California, USA) that contained filter-sterilized seawater (0.22-μm-Sterivex-filtered; EMD Millepore, Billerica, Massachusetts, USA). Polystyrene surfaces and highly filtered seawater were used because they delay the formation of biofilms inside the tubes and thus encourage larvae to continue to swim as they would in the water column (Vermeij et al. 2009). The number of larvae per tube was chosen to minimize density effects after Vermeij et al. (2009). Tubes were closed and haphazardly assigned to test tube racks submerged in temperature-controlled water baths (after Edmunds et al. 2001). Three water baths were used for each temperature treatment and water baths were interspersed randomly on the laboratory bench. Temperature was recorded every five minutes (HOBO Pro V2 water temperature data loggers; Onset Computer Corporation, Bourne, Massachusetts, USA) for the duration of the experiment. For *A. humilis*, the average temperatures among all water baths over the six-day experiment were: high-temperature treatment, 30.9° ± 0.3°C (mean ± SD); ambient and low-salinity treatments, 28.4° ± 0.3°C. For *M. faveolata*, temperatures were: high-temperature treatment, 31.0° ± 0.5°C; ambient and low-salinity treatments, 28.7° ± 0.3°C. Larvae experienced a natural light cycle created by indirect sunlight and fluorescent laboratory lights that were on for ~12 hours per day.

Larval behavior was assessed by removing individual tubes from the water bath and examining them with a dim light in order to count the number of larvae alive and settled. Settlement was defined as the three-step sequence of attaching to a surface, undergoing metamorphosis, and beginning calcification. After counts, the tube cap was unscrewed for 5 s to allow for gas exchange, closed, and returned to the water bath. In total, tubes were outside of the water baths for <45 s each. Tubes within a given water bath were haphazardly rearranged in the test tube rack on each day of data collection. For *M. faveolata*, three repeat counts were taken for each tube and averaged due to the high number of larvae in each tube. On the final day of observation, larvae were counted using a pipette. Behavioral measures were recorded on days 1, 2, 3, 4, and 6.

[6] http://rsbweb.nih.gov/ij/

[7] http://coralreefwatch.noaa.gov/satellite/vs/caribbean.html

Because our experimental treatment tubes were closed and did not allow gas exchange, we evaluated whether larval respiration in the tubes led to decreased dissolved oxygen concentrations and thus created an additional source of larval stress. Using a Hach HQ40d oxygen probe (Hach, Loveland, Colorado, USA), we found that tubes with *A. humilis* larvae contained 2.4% less dissolved oxygen on average than an invigorated filter-sterilized seawater control (100% oxygen saturation) after three days. Tubes containing *M. faveolata* larvae had 0.5% less dissolved oxygen on average than an invigorated filter-sterilized seawater control after six days. Because these reductions in dissolved oxygen were small and less than measured diurnal variation in dissolved oxygen on a coral reef (Kinsey and Kinsey 1967), we concluded that it was unlikely larvae of either species experienced low-oxygen stress as a result of our experimental methods.

Experiment 2: Latent effects of exposure to harsh environmental treatments on settlement

After six days in the high-temperature, low-salinity, and ambient environmental treatments, all surviving larvae from a given size/environment were pooled in a single beaker containing filter-sterilized seawater. For *A. humilis*, 15 larvae per size/environment were dispensed into polystyrene cups with lids. Between three and five replicate cups were used per size/environment, the number of which was determined by the number of larvae that survived Experiment 1. Each cup contained 200 mL of 100-μm-filtered seawater and was maintained at 28.5°C. Into each cup was placed a limestone settlement tile ($4.8 \times 2.2 \times 1.0$ cm) that had been cured in the ocean offshore from the CARMABI Research Station for one month, as well as two small ($\sim$0.25 cm^2) chips of crustose coralline algae (CCA), which are known to induce settlement in this species (Morse et al. 1994, Raimondi and Morse 2000). The number of larvae that were alive or settled in each dish was recorded daily for four days. A blue light and yellow filter (NightSea LLC, Bedford, Massachusetts, USA) was used to visualize larvae and settlers via their fluorescent properties.

Concurrently, *A. humilis* larvae that were released on the same day that the settlement experiment began were placed in an analogous settlement experiment with 30 larvae per cup in three replicates to assess settlement immediately after release (i.e., these larvae were not subjected to six days in the environmental treatments). This experiment used no size separation and the number of larvae that were alive or settled in each cup was measured daily for four days. The duration of the larval period is a commonly studied causal agent of latent effects (Pechenik 2006); thus this experiment provided a secondary means for examining latent effects in this species that was independent of larval size and environmental treatment.

For *M. faveolata*, all surviving larvae from a given size/environment were pooled in a single beaker containing filter-sterilized seawater. Thirty larvae from each size/environment were allocated randomly in triplicate petri dishes that contained 30 mL of 0.45-μm-filtered seawater. For this species, settlement induction was encouraged by adding CCA "extract" (crushed CCA slurry filtered through a 0.22-μm Sterivex filter). These experiments were conducted with glass slides instead of limestone tiles because *M. faveolata* larvae will readily settle on glass slides and petri dishes, and because settlers are extremely difficult to observe on limestone (A. Hartmann, *personal observation*). The number of larvae that were alive or settled was recorded daily in each replicate for 14 days. The *M. faveolata* settlement experiment was run longer than the *A. humilis* experiment because of the relatively longer larval period of *M. faveolata* (van Moorsel 1983, Vermeij et al. 2006).

Experiment 3: Latent effects of exposure to harsh environmental treatments on in situ post-settlement survival

To measure post-settlement survival of *A. humilis*, all tiles with settlers from Experiment 2 were attached to a submerged PVC scaffold on the reef (n = 1–11 for settlers that experienced the six-day larval period, n = 13–23 for settlers with no larval period). The majority of settlers were oriented perpendicular relative to the sea floor to minimize smothering from sediments. The scaffold was arranged so that settlers were $\sim$0.5 m above the reef substrate at the average depth from which the parent colonies were collected ($\sim$3.5 m depth). The number of live settlers was counted on scuba using a blue light and yellow filter 7, 18, 25, 38, 78, and 145 days after out-planting. A comparable experiment was attempted for *M. faveolata* but two-thirds of the settlement dishes were lost shortly after out-planting due to strong surge. The limited data remaining were left out of this report.

Statistical analyses

A maximum-likelihood estimation approach was used to evaluate the best-fit model for survival and settlement (Hilborn and Mangel 1997). Briefly, a binomial error distribution was used to estimate the most supported probability of a given success (alive, settled) given a set number of trials (larvae in a replicate) for a group of size/environments. A suite of multiparameter models was generated for particular groupings of distinct size/environments (ranging from no difference among any size/environments to distinct parameters for each size/environment; see Appendix), and best-fit parameters were estimated using maximum likelihood. The relative fits of competing models were determined using one of two methods. When comparing models containing different numbers of parameters, we employed likelihood ratio tests (for nested models) or the Akaike Information Criterion (for non-nested models). When

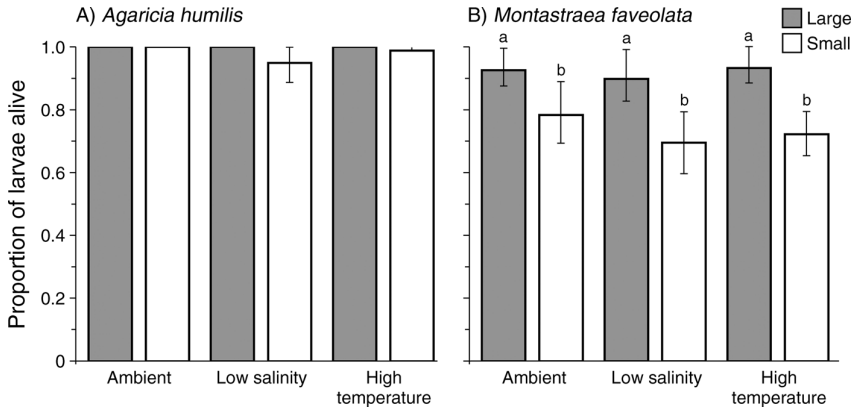


FIG. 1. The effect of parental investment and harsh environmental treatments on survival of (A) *Agaricia humilis* and (B) *Montastraea faveolata* coral larvae from colonies on the leeward coast of Curaçao. One day after birth, larvae were separated by size into large (gray bars) and small (white bars) larval cohorts and subsequently exposed to one of three environments: ambient, low-salinity, and high-temperature treatments. Values represent the mean proportion of larvae alive after six days (shown with arcsine square-root transformed 95% confidence intervals). Different lowercase letters above each bar represent statistically distinct groups as determined by maximum-likelihood estimation.

comparing models with equal numbers of parameters, assessment of relative fit was based on an assumption of equal Bayesian priors, from which statistical P values were generated (Vermeij and Sandin 2008, Vermeij et al. 2009, Marhaver et al. 2013 and Appendix: Table A1). When total mortality was observed within a replicate, that replicate was left out of maximum-likelihood model testing and data representations as the data violated the assumption of independence of individuals.

Tiles with settled *A. humilis* were placed on the reef scaffold with unequal numbers of individuals due to the fact that there were unequal numbers of settlers by size/environmental treatment. For statistical analyses we assumed that there were no density-dependent effects of settler number on survival on a given tile due to the extremely small surface area of tile covered by settlers (<3% on the densest tile).

RESULTS

Experiment 1: Direct effects of exposure to harsh environmental treatments on Agaricia humilis *larvae*

After they were separated into size classes for experiments, the mean volume of small *A. humilis* larvae (0.093 ± 0.022 mm^3, 95% CI; $n = 11$) was ~32% less than the volume of large larvae (0.137 ± 0.040 mm^3; $n = 10$; $P < 0.01$). The coefficient of variation equaled 0.34 among all larvae. After six days, the probability of larval survivorship was not equal across size/environmental treatments (null rejected; $P < 0.05$; Appendix: Fig. 1A and Table A2). Small larvae raised in harsh environments (i.e., low-salinity and high-temperature environmental treatments; data combined) had lower survival than all other size/environments (two-parameter model; $P < 0.05$), although this model could not be statistically differentiated from models where large larval size, large larvae in the ambient environmental treatment, and all larvae in the ambient environmental treatment experienced higher survival relative to all other size/environments ($P > 0.05$; Appendix: Table A2). In aggregate, the models show higher survival for large larvae and larvae in the ambient environmental treatment, and lower survival for small larvae and larvae in high-temperature or low-salinity environmental treatments. Survival was high overall, as 99% of large larvae and 94% of small larvae survived across environmental treatments. Negligible settlement (<4%) was observed during the six-day experiment. Total mortality was observed in 17% of the replicates. When the structure of these "crashes" was evaluated, it was found that total mortality was greatest for small larvae in the low-salinity environmental treatment (Fisher's exact test; $P < 0.05$).

Experiment 2: Latent effects of exposure to harsh environmental treatments on settlement of A. humilis *larvae*

When provided with cured substrate, newborn *A. humilis* larvae were nearly twice as likely to settle compared to the relatively older larvae that experienced our environmental manipulation experiment (no larval period vs. large and small larvae in the ambient environmental treatment combined; Fig. 2A; $P < 0.05$). Small larvae and those that had experienced high-temperature or low-salinity environmental treatments had significantly lower settlement than large larvae that had experienced the ambient environmental treatment ($P < 0.05$; Fig. 2A and Appendix: Table A3), suggesting there were both parental effects and latent effects of harsh larval environments in this species. The effects of larval size and environment on settlement were independent and multiplicative: regardless of environment, small larvae were 42% less likely to settle than large larvae, and regardless of size, larvae raised in high-

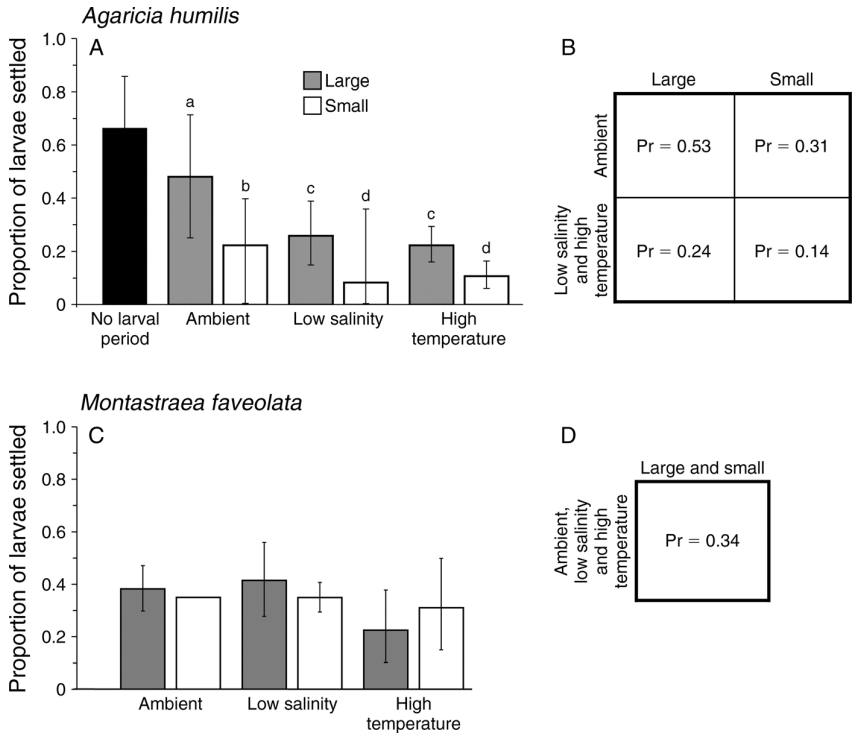


FIG. 2. The effect of parental investment and harsh environmental treatments on settlement success of (A) *A. humilis* and (C) *M. faveolata* larvae. After size separation and six days in ambient, low-salinity, and high-temperature treatments, larvae were placed in ambient conditions and provided with settlement substrate. Values represent the mean proportion of larvae settled after four days for *A. humilis* and 14 days for *M. faveolata* (shown with arcsine square-root transformed 95% confidence intervals) of individuals that survived the environmental treatment experiment. Different lowercase letters above each bar represent statistically distinct groups as determined by the best-fit maximum-likelihood estimators depicted in panel (B) for *A. humilis* and (D) for *M. faveolata*, where settlement probabilities (Pr) are also reported. The black bar in panel (A) denotes settlement of *A. humilis* larvae that were exposed to positive settlement cues the day after they were born (i.e., they did not experience six days of larval environmental treatments). These data were not included in statistical analyses.

temperature and low-salinity environments (pooled) were 54% less likely to settle than those raised in the ambient environmental treatment (Fig. 2B). Overall, 86% of all settlement occurred in the first 24 hours of the four-day experiment.

Experiment 3: Latent effects of exposure to harsh environmental treatments on in situ post-settlement survival of A. humilis *settlers*

After Experiment 2, all tiles with settled *A. humilis* were returned to the reef. A large die-off occurred between days 18 and 38, with only 24–64% of settlers surviving per tile (Fig. 3A). After this die-off, ~58% of the settlers from large larvae that experienced the ambient environmental treatment survived, while settlers from all other size/environments had survivorship of <20%. On day 78, survivorship was significantly different among size/environments (null rejected; $P < 0.05$; Appendix: Table A4), with consistent evidence that large size/ambient environment settlers survived better than small size/ambient environment settlers. On day 145, 28% of large size/ambient environment settlers remained alive compared to 3% on average for all other size/environmental treatments; the statistical conclusions for this time point were comparable to those for day 78. Of the newborn larvae that were allowed to settle immediately, 17% survived to day 145 compared to 16% of the settlers that were subject to the six-day larval experiment (both size classes combined, ambient environmental treatment only; Fig. 3B). This difference was not significant ($P > 0.05$).

Experiments 1 and 2: Direct and latent effects of exposure to harsh environmental treatments on Montastraea faveolata *larvae*

After they were separated into size classes for experiments, the mean volume of small *M. faveolata* larvae (0.006 ± 0.001 mm^3, 95% CI; $n = 9$) was ~65% less than the mean volume of large larvae (0.017 ± 0.004 mm^3; $n = 10$; $P < 0.001$). The coefficient of variation equaled 0.60 among all larvae. After six days in each of the three environmental treatments, large larvae had significantly higher survival than small larvae (88.3% and 70.4%, respectively) regardless of environment ($P <$

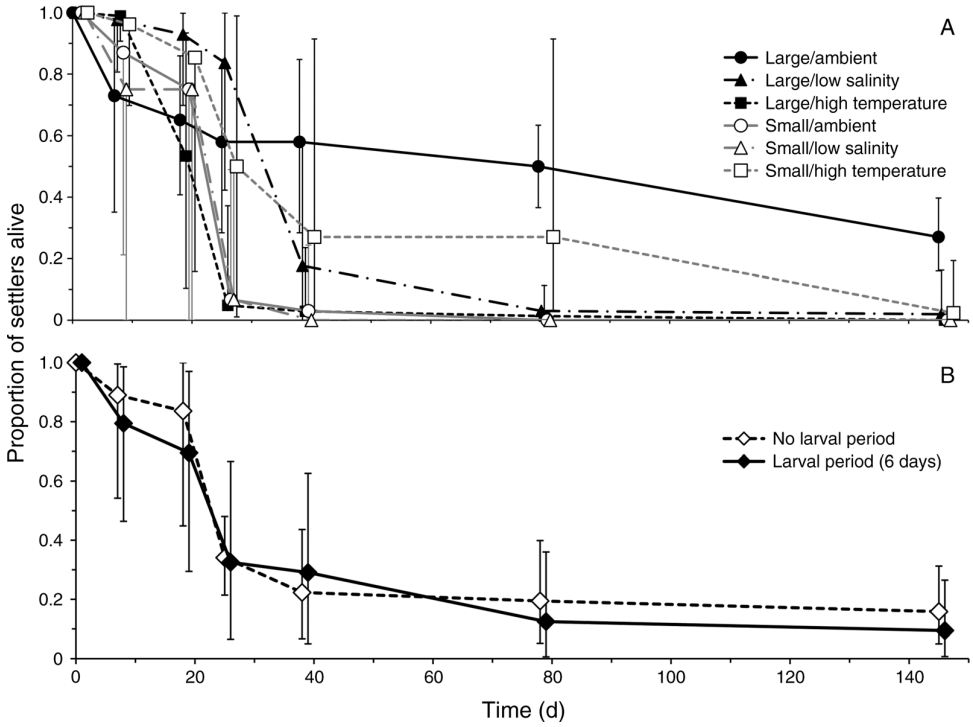


FIG. 3. The effect of parental investment, harsh environmental treatments, and larval duration on in situ post-settlement survival of *Agaricia humilis*. Settlers were outplanted on the reef at approximately the same depth from which parent colonies were collected. Values represent mean proportions of original settlers that were alive (shown with arcsine square-root transformed 95% confidence intervals) over a 145-day time period based on (A) their birth size and larval environment or (B) the length of the larval period they experienced.

0.001; Fig. 1B and Appendix: Table A2), consistent with expectations based on parental effects. Fewer than 7% of replicates experienced total mortality and these crashes were left out of maximum-likelihood estimations. Incidence of total mortality was greatest in the low-salinity environmental treatment ($P < 0.01$).

After larvae were returned to ambient conditions and exposed to CCA extract, settlement of large *M. faveolata* larvae was significantly greater than settlement of small larvae after six days ($P < 0.001$), although settlement was equal across size classes by day 14 ($P > 0.05$; Fig. 2C, D and Appendix: Table A3). At no point did larvae incubated in the high-temperature, low-salinity, or ambient environmental treatments show different settlement patterns, cumulatively demonstrating no evidence for parental effects or latent effects of harsh environmental treatments on settlement in *M. faveolata*.

DISCUSSION

Latent effects of harsh larval environments were more pronounced in Agaricia humilis

We saw no direct effects of harsh environments on larval survival in *M. faveolata* and found weak statistical support in *A. humilis*. In the latter, larval survival was $\geq$94% in all size/environmental treatments. In contrast, the latent effects of harsh larval environments were more notable and therefore have larger ecological significance. Settlement of *A. humilis* was, on average, reduced by 55% in both harsh environments, and post-settlement survival after five months was 28% for large larvae raised in the ambient environmental treatment compared to 3% among all other size/environments. This suggests that the effect of experiencing harsh environments during the larval stage was acute months later for *A. humilis*, well beyond the typical time horizon for most studies of larval performance. In contrast, exposure to harsh pre-settlement environmental treatments did not reduce subsequent settlement of *M. faveolata* once placed in ambient conditions (post-settlement survival measurements were not successful), suggesting that this species does not suffer negative latent effects of harsh larval environments on settlement.

The latent effects we observed due to the low-salinity environmental treatment are particularly interesting as they highlight distinct responses to abiotic stressors in closely related marine organisms. In the barnacle *Balanus amphitrite*, low-salinity stress during the larval stage reduced post-metamorphic growth rates (Thiyagarajan et al. 2007). In contrast, low salinity caused

negative direct effects but no post-metamorphic latent effects in three gastropod species (Diederich et al. 2011). In our study of two coral species, *M. faveolata* showed no latent effects of the low-salinity treatment on settlement, whereas *A. humilis* showed decreased settlement and post-settlement survival. Our results further demonstrate that the latent effects of low-salinity conditions on marine larvae can differ both within and among taxa.

We conclude that *A. humilis* larvae are less tolerant of harsh environments than *M. faveolata* larvae. The latter were surprisingly robust to the harsh environments in our experiments. This is particularly interesting because *A. humilis* larvae are perceived to be more resilient than *M. faveolata* larvae due to a higher degree of parental care (brooded rather than broadcast spawned), larger larval size, and a higher propensity of adults to tolerate marginal environments such as bays and areas with high human disturbance (Knowlton 2001). Despite this, our observation of negative latent effects of harsh environments in *A. humilis* larvae is consistent with past studies showing that larvae of the small brooding coral *Favia fragum* had lower total metamorphosis after exposure to elevated temperatures (Randall and Szmant 2009), and that settlers of the brooding coral *Porites asteroides* had lower post-settlement survival after experiencing elevated temperatures prior to metamorphosis (Ross et al. 2012).

A majority of past studies of latent effects have examined the role of biotic factors (e.g., swimming behavior, delayed metamorphosis, reduced feeding), rather than abiotic factors as the causal agents of latent effects (reviewed in Pechenik 2006). In these studies, the most common treatment was delayed metamorphosis. In our initial experiment, in which larvae were subjected to harsh environmental treatments and denied settlement cues or substrates, we delayed settlement in all size classes and environmental treatments in order to examine the latent effects of abiotic environments. However, we were also able to determine whether delayed settlement itself caused negative latent effects in *A. humilis*. We found that delaying settlement by six days reduced final settlement by 48% but had no effect on post-settlement survival, suggesting that *A. humilis* larvae suffer negative latent effects of delayed settlement as a reduced ability to settle, but not in the form of reduced post-settlement longevity. Interestingly, our study also revealed that high-temperature and low-salinity environments are comparable, if not stronger, drivers of negative latent effects (on settlement) than delayed settlement in this species (55% vs. 48% reduction, respectively). Consistent with our observations, cyprid larvae of the barnacle *Balanus amphitrite* exhibited negative latent effects of low salinity on post-metamorphic growth that were as severe as latent effects caused by delayed metamorphosis (Thiyagarajan et al. 2007).

The role of life history in determining species-specific tolerance of harsh environments

Expectations based on offspring size theory were not observed across species, as harsh environments caused negative latent effects in larvae of *A. humilis*, which are relatively large, but not in larvae of *M. faveolata*, which are relatively small. Instead, this distinction is supported by life history theory based on the different reproductive biology and dispersal characteristics of each species. The physiological plasticity necessary to tolerate a range of environments is energetically costly and will be selected against in a homogenous environment, such as that experienced by a species with short-distance dispersal like *A. humilis* (Parsons 1998). During their long larval period, *M. faveolata* have a greater likelihood of encountering a higher variety of environments than do short-dispersing brooded larvae; thus selection should favor more robust tolerance of a wider range of larval environments in *M. faveolata* (Strathmann et al. 2002, Uller 2008).

Parental effects that increase per-offspring success, such as large offspring size, are strongly selected for when parents can "predict" the environment their offspring will experience. Thus, selection for increased parental investment per offspring can be particularly strong in short-distance dispersers (Uller 2008, Crean and Marshall 2009). The *A. humilis* parent colonies used in this study did not experience the harsh environments that their larvae did, and thus their offspring were exposed to an environmental "surprise." *M. faveolata* parent colonies, on the other hand, are expected to produce larvae that can survive environments unlike their own, given the species' greater dispersal period and distance. This could explain why, while *A. humilis* larvae are much larger in size, our experiments showed that their tolerance of stressful environments was much lower than larvae of *M. faveolata*.

The propensity for latent effects in *A. humilis* offspring has the potential to create a ratcheting-down effect where experiencing harsh larval conditions carries over into later life stages, even after the condition has been escaped or has reverted to a less-stressful state. In contrast, the recruitment of new individuals of *M. faveolata* appears to depend more heavily on the quality of larvae, as offspring were unaffected by the harsh larval environments used here. This suggests that the health of adult *M. faveolata* in a given source population plays a greater role in determining the survival of offspring than do the effects of environmental conditions during dispersal.

Parental effects were evident in both coral species

We observed evidence of parental effects in both coral species, the magnitude of which differed between species and among life stages. Large *M. faveolata* larvae had ~25% higher survival than small larvae during the pre-settlement period, while survival of large *A. humilis* larvae was only 5% greater than small larvae during the

same period. At the start of the settlement phase, small *M. faveolata* larvae exhibited initial delays in settlement, but eventually settled at the same rate as larger conspecifics, while total settlement by small *A. humilis* larvae was significantly lower than settlement by large larvae. Thus, our data suggest that offspring size is of greater importance for planktonic survival in *M. faveolata* and for settlement ability in *A. humilis*.

Energy exhaustion can reduce settlement success in a number of marine taxa and may explain the reduced settlement we observed in small *A. humilis* larvae (Emlet and Hoegh-Guldberg 1997, Pechenik 2006). Metamorphosis in marine invertebrates can be energetically taxing (Videla et al. 1998) and proportionally more so for small larvae within a species (Wendt 2000). Thus energy reserves in small *A. humilis* larvae might have been insufficient for the completion of metamorphosis despite high survival during the pre-metamorphic period ("living dead" hypothesis; Raimondi and Morse 2000). The fact that newborn coral larvae contain greater energy reserves (as storage lipids) than week-old conspecifics (Harii et al. 2007, Figueiredo et al. 2012) can explain why newborn *A. humilis* offspring that were given metamorphosis cues immediately after being released had approximately double the total settlement of larvae that experienced six days of conditioning in our study.

In contrast, *M. faveolata* settlement may not have been limited by energy availability in our experiments, given the relatively long dispersal period of this species. Upon exposure to settlement cues after six days of larval conditioning, 86% of all observed settlement by *A. humilis* occurred within one day, while only 13% of all observed settlement by *M. faveolata* occurred within the first six days of the 14-day experiment, demonstrating that the latter species can delay settlement even when conditions favor it. Interestingly, when *A. humilis* larvae were exposed to settlement cues on the day they were born, 97% of all observed settlement occurred within two days. Thus, individuals that experienced a six-day larval period (i.e., a relatively long larval period for the species) were no more desperate to settle than newborn larvae, though newborn larvae had higher settlement overall. This suggests that *A. humilis* larvae settle rapidly when they encounter settlement cues and this response is independent of age, while *M. faveolata* larvae delay settlement even in the presence of positive cues.

Offspring size did not mediate latent effects of harsh environments

Contrary to our predictions, offspring size did not influence the magnitude of negative latent effects of harsh larval environments. Small *M. faveolata* larvae demonstrated delayed settlement compared to large, but no overall reduction in settlement after experiencing harsh environments. *A. humilis* experienced both parental and latent effects, yet they were independent and additive rather than synergistic. Furthermore, small larval size and harsh larval environments reduced settlement probability in nearly equal amounts in *A. humilis*, suggesting their approximately equal importance in determining offspring success.

Begon et al. (2006) proposed that environments can be classified as "offspring size-sensitive" in which large offspring have higher survival or "offspring size-insensitive," in which offspring size is of little consequence. Because the environment experienced by parent colonies in our study was benign in nature, it is likely that individuals provisioned and otherwise influenced their larvae as they would in an offspring size-insensitive habitat. As a result, the responses we observed to harsh larval environments likely reflected the inherent role of offspring size in buffering harsh conditions, rather than benefits to larvae that were gained from adaptive parental provisioning. Because this inherent benefit of size was independent of the response to harsh environmental conditions, this suggests that large size per se does not increase the ability of these coral progeny to withstand harsh abiotic environments.

In highly variable environments, some species increase offspring size variability as an adaptive strategy to optimize the likelihood that some individuals survive (Crean and Marshall 2009). This is analogous to the long dispersal period of *M. faveolata*, and we would therefore expect less selection for a specific/consistent larval size in this species and greater overall variability in size. We did in fact observe this, as the coefficient of variation of *M. faveolata* larval volume was nearly double that of *A. humilis* (0.60 vs. 0.34, respectively).

The importance of life history and environmental tolerance for community dynamics

The observed benefits of large birth size in both coral species studied here suggest that these progeny will be overrepresented in settler/recruit populations compared to initial larval cohorts. Overall, the parental effects we observed demonstrate that, in addition to larval abundance, offspring size partially determines the probability of recruitment success in a given area for each species. Therefore, assuming a static relationship between adult abundance, fecundity, and recruitment success does not account for crucial larval size-dependent factors that will affect population structure and dynamics of settlers and recruits.

Our results showed that species-specific responses to harsh environments are independent of offspring size in these corals. Extending our results to other species we theorize that organisms with high local recruitment resulting from short larval periods are more susceptible to latent effects after experiencing harsh (i.e., locally unique) environments, while species with long dispersal periods are less prone to such forms of latent effects. In order to test whether dispersal distance per se affects susceptibility to negative latent effects, one would need to evaluate species with similar life histories (e.g., compare among multiple brooding species).

Our findings are interesting given that, due to anthropogenic disturbance, many coral reefs are transitioning from adult communities dominated by mass-spawning, slow-growing species like *M. faveolata* to communities dominated by smaller, brooding species like *A. humilis* (Green et al. 2008, Burman et al. 2012). While these transitions are largely driven by die-off of adults, it is unclear if and to what extent recruitment failure affects or accelerates these changes in community structure (Szmant 1991, Hughes and Tanner 2000, Vermeij et al. 2011). The life history strategy of species like *M. faveolata* (wide dispersal, low per-offspring survival) is likely to result in extremely low recruitment, yet with increasing environmental variability or persistent change, there may be an increase in recruitment opportunities due to chance (sensu Chesson and Warner 1981), or perhaps due to greater tolerance of stressful and variable environments. Indeed, while overall recruitment has declined on the island of Curaçao, where our study took place, the relative representation of settlers of spawning species vs. brooding species has increased in the last 40 years (Vermeij et al. 2011). This suggests a discordance whereby spawning adult population numbers are declining more rapidly than adult brooders, while recruitment of spawned offspring has not declined as much as recruitment of brooded offspring. As environmental variability and harsh conditions become more common due to human disturbance, future coral community demographics and dynamics, and the overall success of a particular life history strategy, will depend on the robustness of each species to this new stress regime during its vulnerable early life stages.

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Supplemental Material

Appendix

Parameter estimations and maximum-likelihood values for experiments depicted in Figs. 1–3 (*Ecological Archives* E094-179-A1).

Supplemental Appendix A: Parameter estimation and maximum likelihood values for experiments depicted in Figures 1-3.

Table A1 – The suite of multi-parameter models used to determine the best fit for all larval survivorship and settlement data. A broader suite of models was used from which it was determined that in no instances did high temperature or low salinity larval environmental treatments elicit statistically distinct responses, therefore these treatments were combined into a harsh environmental treatment group (H) for comparison against the ambient environmental treatment (A). Large and small larval size classes are denoted as (L) and (S), respectively. The models include: a single-parameter (null) model, single deviant two-parameter models in which a single size/environment is compared against all others, single factor two-parameter models independently comparing size and larval environmental treatments, a multiplicative three-parameter factorial model, and a saturated four-parameter model.

Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$
Null	1	a_o	a_o	a_o	a_o
Single Deviant (Large/Ambient)	2	a_1	a_2	a_2	a_2
Single Deviant (Small/Ambient)	2	a_2	a_1	a_2	a_2
Single Deviant (Large/Harsh)	2	a_2	a_2	a_1	a_2
Single Deviant (Small/Harsh)	2	a_2	a_2	a_2	a_1
Single Factor (Size)	2	a_1	a_2	a_1	a_2
Single Factor (Environment)	2	a_1	a_1	a_2	a_2
Factorial	3	a_o	a_oa_1	a_oa_2	$a_oa_1a_2$
Saturated	4	a_1	a_2	a_3	a_4

Table A2 – Parameter estimation of the probability that *A. humilis* and *M. faveolata* larvae survived the six-day larval period. The parameters and maximum likelihood (ML) of the best-fit model are in bold and non-statistically distinct models with an equal number of parameters are italicized. Graphical representations of the raw data are presented in Figure 1.

A. humilis

Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$	**ML**
Null	1	0.993	0.993	0.993	0.993	11.11
Large/Ambient	2	0.999	0.992	0.992	0.992	10.53
Small/Ambient	2	0.992	0.999	0.992	0.992	10.68
Large/Harsh	2	*0.989*	*0.989*	*0.999*	*0.989*	*9.85*
Small/Harsh	2	**0.999**	**0.999**	**0.999**	**0.975**	**7.45**
Size	2	*0.999*	*0.984*	*0.999*	*0.984*	*8.77*
Environment	2	*0.999*	*0.999*	*0.990*	*0.990*	*9.95*
Factorial	3	0.999	0.985	0.998	0.984	8.87
Saturated	4	0.999	0.999	0.999	0.975	7.45

M. faveolata

Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$	**ML**
Null	1	0.800	0.800	0.800	0.800	162.33
Large/Ambient	2	0.889	0.779	0.779	0.779	153.16
Small/Ambient	2	0.811	0.752	0.811	0.811	160.01
Large/Harsh	2	0.756	0.756	0.880	0.756	145.32
Small/Harsh	2	0.850	0.850	0.850	0.667	134.70
Size	2	**0.883**	**0.702**	**0.883**	**0.702**	**125.04**
Environment	2	0.820	0.820	0.788	0.788	161.19
Factorial	3	0.901	0.716	0.872	0.693	124.06
Saturated	4	0.889	0.752	0.880	0.667	122.18

Table A3 – Parameter estimation of the probability that *A. humilis* and *M. faveolata* larvae had settled after four days (*A. humilis*) and fourteen days (*M. faveolata*). The parameters and maximum likelihood (ML) of the best-fit model are in bold and non-statistically distinct models with an equal number of parameters are italicized. Graphical representations of the raw data are presented in Figure 2.

				A. humilis		
Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$	**ML**
Null	1	0.257	0.257	0.257	0.257	69.31
Large/Ambient	2	0.483	0.212	0.212	0.212	60.58
Small/Ambient	2	0.248	0.300	0.248	0.248	68.97
Large/Harsh	2	0.264	0.264	0.244	0.264	69.22
Small/Harsh	2	0.314	0.314	0.314	0.122	61.24
Size	2	0.318	0.186	0.318	0.186	65.11
Environment	2	0.392	0.392	0.190	0.190	61.08
Factorial	3	**0.496**	**0.282**	**0.236**	**0.134**	**56.08**
Saturated	4	0.483	0.300	0.244	0.122	55.91

				M. faveolata		
Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$	**ML**
Null	1	0.064	0.064	0.064	0.064	34.21
Large/Ambient	2	0.117	0.053	0.053	0.053	32.77
Small/Ambient	2	0.077	0.001	0.077	0.077	29.92
Large/Harsh	2	0.042	0.042	0.108	0.042	31.43
Small/Harsh	2	0.083	0.083	0.083	0.025	31.56
Size	2	**0.318**	**0.186**	**0.318**	**0.186**	**26.74**
Environment	2	0.058	0.058	0.067	0.067	34.16
Factorial	3	0.103	0.015	0.115	0.017	26.70
Saturated	4	0.117	0.001	0.108	0.025	25.56

Table A4 – Parameter estimation of the probability that *A. humilis* settlers survived after 78 and 145 days *in sit*u. The parameters and maximum likelihood (ML) of the best-fit model are in bold and non-statistically distinct models with an equal number of parameters are italicized. Graphical representations of the raw data are presented in Figure 3.

Day 78

Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$	**ML**
Null	1	0.175	0.175	0.175	0.175	22.16
Large/Ambient	2	**0.370**	**0.075**	**0.075**	**0.075**	**17.04**
Small/Ambient	2	*0.215*	*0.001*	*0.215*	*0.215*	*18.95*
Large/Harsh	2	0.222	0.222	0.077	0.222	20.72
Small/Harsh	2	0.177	0.177	0.177	0.167	22.16
Size	2	0.226	0.074	0.226	0.074	20.55
Environment	2	0.238	0.238	0.105	0.105	20.90
Factorial	3	0.331	0.097	0.131	0.038	18.83
Saturated	4	0.370	0.001	0.077	0.167	15.34

Day 145

Model	**Params.**	$\mathbf{a_{LA}}$	$\mathbf{a_{SA}}$	$\mathbf{a_{LH}}$	$\mathbf{a_{SH}}$	**ML**
Null	1	0.113	0.113	0.113	0.113	14.81
Large/Ambient	2	**0.259**	**0.038**	**0.038**	**0.038**	**10.64**
Small/Ambient	2	*0.138*	*0.001*	*0.138*	*0.138*	*12.83*
Large/Harsh	2	*0.148*	*0.148*	*0.039*	*0.148*	*13.57*
Small/Harsh	2	0.118	0.118	0.118	0.083	14.75
Size	2	*0.151*	*0.037*	*0.151*	*0.037*	*13.44*
Environment	2	*0.167*	*0.167*	*0.053*	*0.053*	*13.43*
Factorial	3	0.236	0.053	0.068	0.015	11.78
Saturated	4	0.259	0.001	0.039	0.083	9.82

Chapter 4, in full, is a reprint of the previously published material as it appears in Ecology 94(9): 1966-1976, 2013, Hartmann AC, KL Marhaver, VF Chamberland, SA Sandin, MJA Vermeij. The dissertation author was the primary investigator and author of this paper.

Chapter 5.

MECHANISMS MAINTAINING HORIZONTAL SYMBIONT TRANSMISSION IN SPECIES WITH COMPLEX LIFE HISTORIES

Aaron C Hartmann[1,6], Kristen L Marhaver[2,3], Anke Klueter[4], Collin J Closek[4], Erika Diaz[4], Valérie F Chamberland[3,5], Mark JA Vermeij[3,5], Mónica Medina[4]

[1]Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA, 92093, USA

[2]University of California Merced, Merced, CA, 95343, USA

[3]CARMABI Foundation, Piscaderabaai z/n, Willemstad, Curaçao

[4]Penn State University, State College, PA, 16801, USA

[5]Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands

Abstract. Symbioses that allow hosts to exploit otherwise unavailable resources permit species to proliferate in nutrient-poor environments. Despite their necessity, symbionts must be acquired from the environment in a surprising number of host species (horizontal transmission) even when theory suggests the direct transmission of symbionts from parents to eggs (vertical transmission) is advantageous and evolutionarily stable. In a series of studies, we found that early acquisition of endosymbionts by larvae of a horizontally-transmitting coral species induced a mild molecular stress response and higher mortality, likely due to common processes associated with preparedness to metamorphose into an adult form. Despite molecular responses suggesting preparation to metamorphose, infected larvae largely failed to do so in the absence of positive external cues. Based on these patterns, we hypothesize that offspring survival in horizontally-transmitting species is optimized when symbiont acquisition occurs just prior to locating cues for metamorphosis. This suggests the timing of symbiont acquisition is a critical and often overlooked component of the host's life history. The reduced survival that occurs when horizontally-transmitting species obtain symbionts well before metamorphosing likely selects for populations bearing appropriate symbionts for their local environment and helps maintain this theoretically suboptimal symbiont transmission strategy.

INTRODUCTON

Symbioses between animal hosts and autotrophic endosymbionts allow each to overcome the limitations of nutrient-poor environments. Despite their critical importance, the strategies by which symbionts are passed across generations are remarkably distinct among closely related taxa. For many host species, symbionts are directly sequestered in eggs (vertical transmission), presumably providing offspring with an immediate nutrition source (Trench 1987). Yet, numerous species whose survival requires symbionts during adulthood (obligate symbiosis) produce aposymbiotic offspring, thereby forcing progeny to acquire their symbionts from the surrounding environment (horizontal transmission). The existence of such a strategy demonstrates that an autotrophic symbiont is not mandatory for survival early in life, even when adults form an obligate symbiosis.

Symbioses that provide hosts with forms of energy (e.g., carbohydrates) commonly occur in animals with complex life histories, such as corals, molluscs, and annelids. In these marine organisms, individuals progress through a mobile larval stage, followed by a sessile adult stage on the seafloor. During the mobile larval stage, individuals can experience environmental conditions, threats, and demands that differ from those of adults, leading to life stage-dependent selection pressures (Strathmann et al. 2002, Marshall and Morgan 2011). While this difference in habitat may provide a general explanation of why species employ the apparently risky strategy of producing non-symbiotic offspring, the mechanisms leading to and maintaining horizontal symbiont transmission strategies are not well understood (Wilkinson and Sherratt 2001, Knowlton and Rohwer 2003).

The duration of the larval period in non-feeding larvae is largely limited by energy exhaustion (Morgan 1995). It has been theorized that harboring symbionts throughout the larval period (vertical transmission) lengthens offspring competency periods by providing an additional, and possibly inexhaustible, source of energy (Richmond 1987, Raimondi and Morse 2000, Harii et al. 2002, Harii et al. 2010). This may be particularly beneficial for marine invertebrate larvae because they require an external cue to initiate metamorphosis into an adult form, in contrast to many insects and amphibians (reviewed in Hadfield 2000 and Hadfield 2011). An inability to locate positive external cues can lead to failed metamorphosis due to loss of competency, while settlement in poor locations can reduce survival (Raimondi and Morse 2000). Therefore, mechanisms to extend competency may provide additional time to find the most optimal locations for metamorphosis.

Theory suggests vertical symbiont transmission is evolutionarily stable due to reduced competition among competing symbiont types and guaranteed transmission success across generations (Wilkinson and Sherratt 2001). If harboring symbionts during the larval period extends competency, vertical symbiont transmission should be particularly favorable in species with long larval periods (i.e., in species expected to have a relatively high absolute energetic demand). Despite the theorized benefits of vertical transmission during a prolonged larval period, many species with long larval periods use horizontal symbiont transmission, including molluscs (Fitt and Trench 1981, Won et al. 2003, Ramos and Banaszak 2013), annelids (Nussbaumer et al. 2006) and stony corals (Baird et al. 2009). In hydrothermal vent tubeworms, for example, obligate chemoautrophic bacterial symbionts have high site fidelity for the patchy and highly

distinct vent environments at which they are found (Di Meo et al. 2000). As a result, it is critical for each new generation of tubeworms to obtain locally appropriate symbionts after weeks to months of dispersal to a non-natal vent (Nussbaumer et al. 2006).

Increasing evidence suggests that environmental specificity is a common trait of many symbionts (Rowan and Knowlton 1995, Di Meo et al. 2000, Baker 2003, LaJeunesse et al. 2004). This specificity suggests the most optimal symbiotic partner for a given host depends in part on local environmental conditions. Thus, long-dispersing, horizontally-transmitting species may benefit from obtaining a symbiotic partner that is adapted to local conditions (and unlike those of the natal environment). Horizontal transmission also allows larvae to avoid symbiont-induced stress caused by harsh conditions experienced to a particularly high degree during the larval stage (e.g., high irradiance at the sea surface, Yakovleva et al. 2009). In contrast, short-dispersing, vertically-transmitting species are well suited for the natal environment due to obtaining locally-appropriate symbionts from their parents and rapidly metamorphosing (Schwarz et al. 1999, LaJeunesse et al. 2004).

Nearly all stony corals form an obligate, energy-deriving symbiosis with one or more clades or sub-clades of photosynthetic dinoflagellates from the genus *Symbiodinium*. Coral species use either vertical or horizontal symbiont transmission (Baird et al. 2009) and there are numerous species exhibiting both of these strategies, making corals an optimal study system for testing theories related to endosymbiont transmission (Trench 1987, Baird et al. 2009). A vast majority of coral species that broadcast spawn gametes into the water column transmit *Symbiodinium* horizontally, such that larvae lack symbionts. In contrast, most species that brood eggs internally and

release competent larvae transmit *Symbiodinium* vertically (Baird et al. 2009). Spawned larvae have longer pre-adult periods and greater average dispersal distances than brooded larvae (Sammarco and Andrews 1989, Graham et al. 2008, Underwood et al. 2009), which demonstrates that horizontal transmission tends to be associated with long larval periods in corals.

Coral larvae obtain energy during dispersal primarily from parentally-provisioned lipids, which comprise 60-90% of their biomass (Harii et al. 2007, Figueiredo et al. 2012), though *Symbiodinium* have been shown to fix and translocate carbon in larvae of some vertically-transmitting species (Richmond 1987, Alamaru et al. 2009, Harii et al. 2010, Gaither and Rowan 2010). In such species, larvae with active *Symbiodinium* (rather than inactivated due to darkness) use energetic lipid reserves more slowly and can have higher survival, suggesting energy gained from *Symbiodinium* increases larval success (Alamaru et al. 2009, Harii et al. 2010).

In contrast to the apparent benefits larvae gain from harboring symbionts, oxidative stress, DNA damage, and heightened mortality can occur when larvae of horizontally-transmitting species are infected with *Symbiodinium* and exposed to high temperature or light conditions (Yakovleva et al. 2009, Nesa et al. 2012). These responses are similar to the bleaching response of adult corals (Lesser 1996, Brown 1997) and suggest that certain environmental conditions put *Symbiodinium*-bearing larvae at a disadvantage. High irradiance in particular, is most often experienced when larvae are at the surface of the ocean, as they are during the obligate period of development in most spawning species, perhaps leading to differences in the degree of mortality induced by harboring symbionts in brooding and spawning coral species.

Energetic "gains" and stress-induced "losses" suggest both beneficial and harmful roles of *Symbiodinium* in coral larvae, while the ability to gain locally appropriate symbionts offers a life history-based explanation of interspecific differences in symbiont transmission strategies. To examine the relative strength of these competing roles of symbionts, we measured behavior, lipid use, and gene expression patterns in larvae of a horizontally-transmitting species after they took up *Symbiodinium*. Larval survival and settlement patterns provided metrics of reproductive success, while lipid use and molecular responses allowed us to examine changes in metabolism due to infection. The transcriptomic response of larvae to *Symbiodinium* acquisition also allowed us to identify whether harboring symbionts induced cellular stress. Our study addressed the null hypothesis that *Symbiodinium* infection (1) has no effect on larval survival or the duration of the larval period, and considered the alternative hypotheses that *Symbiodinium* infection (2) enhances larval survival and lengthens the larval period by providing nutrition to larvae; or (3) reduces larval survival and shortens the larval period due to inducing molecular stress.

MATERIALS AND METHODS

Experiment 1: Gene expression and lipid content changes in response to Symbiodinium *infection*

Bundles of symbiont-free eggs and sperm were collected *in situ* from *Orbicella faveolata* (formerly *Montastraea faveolata*; Budd et al. 2012) colonies during the annual mass spawning on the leeward coast of Curaçao in September 2011. After fertilization, planula larvae were reared as described in Hartmann et al. (2013). On day 7 after

spawning, approximately 1500 actively swimming larvae were placed into each of twenty bins each containing 300 mL of 0.45 μm-filtered seawater, hereafter referred to as FSW (Millepore HA filter; EMD Millepore, Billerica, Massachusetts, USA). Cultured *Symbiodinium minutum* (*Symbiodinium* taxon B184, LaJeunesse et al. 2012), hereafter referred to as *Symbiodinium* B184, was added to ten randomly assigned bins to a density of 1000 cells/mL, while a seawater control was added to the other ten (Fig. 5.1). Additional *Symbiodinium* cells were added on days 13 and 16 to ensure that a substantial infection of treated larvae occurred and a final concentration of 5000 cells/mL was achieved. *Symbiodinium* B184 was originally obtained from a conspecific adult in the Florida Keys by M.A. Coffroth. Infection rate was monitored through time by examining flattened larvae under a light microscope.

After ten days a high level of infection was achieved (> 50 cells/larva). Larvae from each bin were rinsed in FSW to remove cells that were not taken up. Larvae remained with their infection cohort and were assigned to a 2 x 2 experimental design that included treatments of: (A) infected and non-infected ("*Symb*" and "No*Symb*", respectively), and (B) exposed to a light-dark cycle (12 h:12 h) or constant darkness ("Light" and "Dark", respectively) under two fluorescent aquarium lights. Light levels were measured using a pulse amplitude modulated handheld fluorometer (WALZ Mess und Regeltechnik, Germany). Light intensity equaled 50-60 μEinsteins m^{-2} s^{-1}, which is low relative to intensities of >1000 μEinsteins m^{-2} s^{-1} that are typical of tropical surface waters.

Before starting the experiment, two batches of larvae were removed from each bin for lipid class and carbon fixation analyses. For lipid class analysis, 50 larvae were placed

on a pre-combusted glass fiber filter (GF/F; Whatman; Maidstone, Kent, UK) and frozen at -20°C (time = 0). In order to determine the extent to which *Symbiodinium* in larvae were fixing carbon during the experiment, 55 larvae were placed in a petri dish with 30 mL of FSW adjusted to 2.3 mM $NaH^{13}CO_3$ (99% ^{13}C, BD Biosciences). The locations of the 20 bins and 20 petri dishes in the experiment were randomly assigned to locations under the light source. Bins and petri dishes in the Dark treatment were covered using aluminum foil.

The experiment was conducted for 48 h. At its conclusion, all surviving larvae in each petri dish with ^{13}C-enriched sodium bicarbonate were placed on a combusted glass fiber filter and frozen as described above prior to lipid class analysis (t = 48 h). The remaining larvae in each bin were consolidated on a 50-µm nylon mesh filter and placed in a 2 mL cryovial (Symport Scientific, Beloeil, QC, Canada) containing RNAlater (Qiagen Sciences, Germantown, MD, USA). These samples were kept at 4°C for 18 h and then placed at -20°C prior to moving to -80°C within days, where they remained until analyzed.

Symbiodinium *quantification, lipid class content, and stable isotope analysis*

The number of *Symbiodinium* infected per larva was measured by imaging 10 larvae per filter (20% of total, $NaH^{13}CO_3$-enriched filters) with an epifluorescence microscope. The number of *Symbiodinium* cells per larva was counted visually in ImageJ (NIH) from which the mean cells/larva was calculated for each replicate.

The bulk lipid fraction was extracted from larvae on GF/F filters taken at times 0 and 48 h using the Bligh and Dyer (1959) method. Once isolated, the bulk lipid fraction

was dried under a stream of N_2 and resuspended in 50 µl of chloroform for lipid class separation and quantification. Lipid classes were separated chromatographically by spotting 1 µl of crude extract on a quartz Chromarod (S-III, Iatron Laboratories, Inc., Tokyo, Japan) in triplicate per sample, then eluting the rods in a two solvent system of hexane:diethyl ether:acetic acid (99:1:0.05, v:v:v for 25 minutes) followed by hexane:diethyl ether:acetic acid (80:20:0.1, v:v:v 25 minutes).

Lipid classes were quantified via thin layer chromatography with flame ionization detection on an Iatroscan TLC-FID MK-5 (Iatron Laboratories, Inc.). Lipids were pyrolized the entire length of the Chromarod and the retention time and area of each peak was recorded with LabView software (National Instruments; Austin, Texas, USA). The identity and concentration of each lipid class was determined by the retention times and calibration curves generated from known standards. The standards used were: 5-α-cholestane for hydrocarbons, palmitic acid palmityl ester for wax esters, tripalmitin for triacylglycerols, stearic acid for free fatty acids, stigmastanol for sterols and L-α-phophatidylcholine for phospholipids (Carilli et al. 2012).

To determine differences in lipid use among treatments, we performed a two-factor ANOVA of change in lipid content through time for each lipid class (e.g., $\Delta WE = WEt_{48} - WEt_0$) with the independent variables *Symbiodinium* status and light status. If the change in lipid content was not normally distributed we performed the ANOVA on log-transformed data (e.g., $\Delta WE = \log WEt_{48} - \log WEt_0$). In addition, we determined whether changes in lipid content were equal to zero (null) for each lipid class and treatment group using a paired t-test. When data were not normally distributed, they were log transformed data prior to analysis.

Samples containing larvae exposed to $NaH^{13}CO_3$ were acidified for six hours via fumigation in a desiccator containing a small open beaker of concentrated HCl (12M) to remove residual $NaH^{13}CO_3$. Filters were dried at 70°C and immediately placed in tin boats for total C and $\delta^{13}C$ analysis at the UC Davis Stable Isotope Facility. Samples were analyzed using a Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) and a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Values were compared against standards of peach leaves, nylon, and glutamic acid (USGS-41). Mean $\delta^{13}C$ isotope enrichment were compared among *Symb*-Light, *Symb*-Dark, and No*Symb*-Dark treatments using a single-factor ANOVA.

Application of microarray technology to follow gene expression

For construction of cDNA probes, total RNA was extracted from ~1400 frozen (-80^{o} C) larvae per experimental replicate using QIAzol Lysis Reagent and an RNeasy Kit (Qiagen Sciences, Germantown, MD, USA). The standard QIAzol protocol was used with a few minor modifications for coral larvae and polyps (refer to http://medinalab.org/new/protocols/). Following extraction, total RNA was purified using MEGAclear Kit (Ambion Life Technologies, Carlsbad, CA, USA). Total RNA was amplified (2 rounds) using MessageAmp™II aRNA Kit RNA Amplification for Array Analysis (Ambion Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Before and after purification, the integrity and quality of total RNA was assessed using a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and Bioanalyzer (Agilent Technology, San Diego, CA, USA) methods.

Synthesis of cDNA probes was attained from 3 µg aRNA (refer to

http://medinalab.org/new/protocols/). Briefly, samples amplified using 3.5 nmol of random pentadecamer primers per reaction (pdN15; 10 min at 70°C). A reverse transcription (RT) was performed using SuperScriptIII Reverse Transcriptase (2 hr at 50^{o} C; Invitrogen Life Technologies, Carlsbad, CA, USA). The RT master mix contained a 4:1 ratio of aminoallyldUTP to TTP. RT reaction products were purified using a MinElute Cleanup kit (Qiagen Sciences, Germantown, MD, USA). To prepare the cDNA probes, fluorescent dyes Cy3 and Cy5 (GE Healthcare, USA) were dissolved in 18 µl DMSO. These coupling reactions were incubated for 2 hr at room temperature in darkness. Following the coupling reaction, dye-coupled cDNA probes were again purified using a MinElute Cleanup kit (Qiagen Sciences, Germantown, MD, USA). The incorporation of each dye (Cy3 and Cy5) in each of the coupling reactions was assessed using a Nano-Drop spectrophotometer. The relative proportions of probes to be used for the microarrays were assigned according to these dye incorporation rates.

Prior to hybridization, microarrays were post-processed in four steps: 1) UV crosslinking at 60 mJ, 2) washing (3x SSC, 0.2% SDS at 65°C, 3) blocking with 5.5 g succinic anhydride / 335 mL 1-methyl-2-pyrrolidinone and 15 mL sodium borate and 4) drying by centrifugation. Mixed Cy3/Cy5 cDNA probe preparations were buffered in 0.25% SDS, 25 mM HEPES, and 3x SSC. These hybridization mixes were boiled at 99° C for 2 min for denaturation and then returned to room temperature for 5 min. Hybridization mixes were then applied to array slides. Arrays were placed into Corning hybridization chambers (Corning Inc., Corning, NY, USA) and hybridization at 63° C for 16 h. Following hybridization, arrays were washed twice in 0.6 x SSC and 0.01% SDS followed by a brief rinse in 0.06x SSC and dried by centrifugation. Slides were scanned

immediately using a GenePix 4000B microarray scanner (AXON Instruments, USA). Image acquisition was performed using GenePix Pro 5.0 software.

The microarrays used for this study contain 10,930 PCR-amplified cDNA targets, spotted in duplicate, yielding a total of 21,860 features. The array is referenced as Mfav-G2 (Mfav for *Montastraea faveolata*) and further detailed in Aranda et al. (2011). The cDNA targets spotted to the arrays were chosen from EST libraries described partially in a previous paper by Schwarz et al. (2008). Genes were annotated using tBLASTx and BLASTx (E-value cut-off $1e^{-5}$) against the Swissprot, Uniprot, and GenBank nonredundant DNA and protein databases. At the time this array was designed, 52% of the 10,930 cDNA sequences were found to have significant similarity to known genes. The experimental setup for the microarrays followed a reference design, i.e. all samples were hybridized against a reference pool, which was made up of equal amounts of RNA from all samples. The reference samples were labeled with Cy3 dye and the treatment samples with Cy5. Each of the four treatments were replicated five times.

Statistical analyses of gene expression data

Extraction of spot intensity and correction was implemented on the median data by subtracting background signal using TIGR Spotfinder 2.2.4 (Saeed et al. 2003). Data were normalized using Locfit LOWESS in TIGR MIDAS 2.22 in TM4 http://www.tm4.org/midas.html. Customized R scripts were used to edit and merge files as well as to log transform and filter the data.

Differentially expressed genes were identified using Significance Analysis of Microarrays (SAM; Tusher et al. 2001) in MultiExperiment Viewer (MeV; Saeed et al.

2003) adjusted to a False Discovery Rate of 5%. Only genes that hybridized in at least three of five experimental replicates per treatment group were included in analyses. Comparisons to identify differentially-expressed genes were carried out in two ways: (1) pooling samples within a treatment (e.g., comparing *Symb* vs. No*Symb* after pooling Light and Dark) and (2) analyzing separately between treatments (e.g., *Symb*-Light vs. No*Symb*-Light). An additional comparison was made between *Symb*-Light and the other three treatment groups in order to investigate the specific differences unique to the only treatment in which *Symbiodinium* were photosynthetically active. Up-regulated genes identified in a given comparison were defined as those with associated fold changes > 1, while down-regulated genes were defined as those with associated fold changes < 1. Because down-regulated genes cannot change expression more than one-fold, the fold-change for down-regulated genes was normalized to -1/FC. To narrow our focus to genes with the most dramatic changes in expression, we further reduced our list of differentially-expressed genes to only those with an absolute fold change >1.5.

Over-represented gene ontology terms were identified using all differentially expressed genes for a given comparison in GOEAST (Zheng and Wang 2008). An alpha threshold of 0.1 and the Alexa et al. (2006) scoring correction was used, similar to the methods of DeSalvo et al. (2010). We then reduced redundancies, identified unique terms, and further winnowed the long list of over-represented gene ontology (GO) terms using REVIGO, a program that accounts for nestedness and sibling relationships of GO terms (Supek et al. 2011). A semantic similarity score of 0.5 (“small”) was employed to define the threshold for inclusion of a GO term, following the methods of Polato et al. (2013).

Based on the hypotheses outlined in the Introduction, we focused on specific gene functions identified via GO terms. Genes that encode for pathways of oxidative stress, cell death, and lipid use in corals are not fully resolved, therefore we relied on GO terms to identify genes related to each of these functions within the array (response to oxidative stress – GO:0006979), apoptotic processes –GO:0006915, and lipid catabolism – GO:0016046). The mean expression levels by infection status and light treatment were compared using a two-factor ANOVA at $\alpha = 0.05$. Therefore, we acknowledge that 5% of genes would incorrectly pass the threshold for significance (i.e., Type I error) and we regard our array results with appropriate caution.

Experiment 2: Behavioral response to infection by two different Symbiodinium *strains*

We determined how larval behavior responded to infection with *Symbiodinium* B184 and *S. microadriaticum* (*Symbiodinium* taxon A194, LaJeunesse, 2001), hereafter referred to as *Symbiodinium* A194, which was originally isolated from *Cassiopea* spp. (upside-down jellyfish) in Kaneohe Bay, HI by R.A. Kinzie III. *Symbiodinium* cultures were added to six-day old larvae from the same spawning event as the larvae used in Experiment 1 (Fig. 5.1). Five replicates each of 40 larvae in 40 mL FSW were made for three treatment groups: (1) non-infected (control); (2) *Symbiodinium* B184; and (3) *Symbiodinium* A194. Larvae were exposed to a pseudo-natural daily cycles of 12 hours of light and 12 hours of dark cycle with the same fluorescent laboratory lights as used in Experiment 1. The number of larvae alive, swimming, and settled was counted on days 10, 16, 17, and 33 after spawning. The degree of *Symbiodinium* infection was not

measured. Larvae were allowed to be in the presence of free-living *Symbiodinium* for the duration of the experiment.

Survival, settlement, and swimming data were analyzed separately by day, primarily because sampling dates were not regularly spaced in time. A binomial error distribution was used to estimate the most likely probability of survival, swimming, and settlement at each time point for each treatment. A suite of models describing unique groupings of treatments was used to identify treatment groups with significantly different means for each factor (Statistical Appendix A and B; see Hartmann et al. 2013 for a detailed description of this method). Survival was calculated as the proportion of larvae alive each day relative to the initial population, settlement as the proportion of settled larvae relative to those alive on that respective day, and swimming as the proportion of larvae swimming relative to those alive on that respective day. The best-fit probabilities for a given set of treatment groupings was estimated using maximum likelihood and models were compared with likelihood ratio tests or AIC depending on nestedness. There were negligible differences between data collected when the larvae were 16 and 17 days old, therefore data from day 17 is not presented here.

Experiment 3: Behavioral responses to Symbiodinium *infection and light environment*

In 2013, *O. faveolata* gametes were collected at the same site as in 2011 and reared as described above for Experiment 1 and 2. Consistent with Experiment 1, seven-day-old larvae were exposed to *Symbiodinium* for ten days in order to achieve a high level of infection. Infection was monitored with an epifluorescence microscope (Leica Microsystems Orthoplan, GmbH, Germany), which was also used to image larvae to

determine the number of *Symbiodinium* per larva in the same manner as described in Experiment 1. The *Symbiodinium* used for infection in Experiment 3 were isolated from five samples of conspecific adults collected on the previous day from the same reef tract (~500 m away). Symbionts were isolated from adult tissue on the same day that infection was started. On the eleventh-day after *Symbiodinium* exposure, all larvae were rinsed in FSW and placed in a 2 x 2 experiment of treatments in the same manner as Experiment 1 with: (A) infected and non-infected ("*Symb*" and "No*Symb*-", respectively), and (B) exposed to a diurnal light-dark cycle (12h:12h) or constant darkness ("Light" and "Dark", respectively).

Each of the four treatments was replicated in five petri dishes and each contained 40 actively-swimming larvae in a total of 40 mL of FSW. All petri dishes were placed under the light fixture used in Experiment 1 and 2. Dark treatment petri dishes were covered with a double layer of aluminum foil. The number of larvae alive, swimming, and settled was counted on days 19, 20, 21, 22, 23, 24, 25, and 33 after spawning.

Data were analyzed in two ways. First, analogous to Hartmann et al. (2013), we used the per day maximum likelihood approach described for Experiment 2 for survival, settlement, and swimming behavior. Second, because we collected daily behavior for the first week, we determined whether there were differences in rates of settlement or swimming behavior among treatments. To do this, we calculated the proportion of larvae either swimming or settling at each time point relative to the pool of larvae that could have performed that behavior based on the previous time step (e.g., settled larvae at time t_1 cannot settle again nor swim at t_2). After the proportional data were arcsine square root transformed, we performed a repeated measures two-factor ANOVA to compare means

of larvae performing a behavior by symbiont infection status and light treatment. This analysis was performed using days 19-25 as the repeated measures response variable. Day 33 was analyzed separately as a single time point due to inconsistency of sampling relative to the daily sampling.

RESULTS

Experiment 1: Lipid use and gene expression in response to infection with Symbiodinium *B184*

Infection was equal between Light and Dark treatments (t_6 = -0.372; p = 0.72). After 10 days, larvae in the Light treatment contained 81.2 ± 14.2 *Symbiodinium* cells/individual (mean ± SE) and larvae in the Dark treatment contained 100.2 ± 49.0 cells/individual. There was a significant difference in mean $\delta^{13}C$ enrichment among treatments ($F_{2,8}$ = 15.48; $p < 0.01$) and Tukey's HSD test revealed that enrichment was significantly greater in *Symb*-Light than *Symb*-Dark and No*Symb*-Dark ($p < 0.01$). Enrichment was not significantly different in the two latter treatments (p = 0.972) demonstrating that dark conditions suspended *Symbiodinium* photosynthesis in infected larvae.

Energetic lipids declined in non-infected larvae

After the 48 h treatment period, wax ester (WE) content was significantly different and lower than at the beginning of the experiment in the No*Symb*-Light (t_4 = -4.31; $p < 0.05$; Fig. 5.2) and No*Symb*-Dark (t_4 = -4.04; $p < 0.05$) treatments, but did not change in both *Symb*-Light (t_4 = -1.34; p = 0.25) and *Symb*-Dark (t_4 = -0.97; p = 0.39).

Triacylglycerol (TAG) content was significantly different and lower at the end of the experiment in No*Symb*-Dark (t_4 = -3.64; $p < 0.05$) with no significant change in the other three treatments ($p > 0.05$). Taken together this shows that energetic lipids declined in non-infected but not in infected larvae during the 48-hour experiment.

When the magnitude of change in lipid content among treatment groups was compared, there was a significant effect of *Symbiodinium* presence on TAG ($F_1 = 6.7$; $p < 0.05$; Fig. 5.2); the decline in TAG was greater in non-infected larvae regardless of light environment. In contrast, there was no difference in WE content among treatments. There was also no difference in phospholipids (PL) among treatments, nor a change in PL through time in any treatment, highlighting a relative constancy of these structural lipids during the experiment.

Differential gene expression in response to infection

Larvae showed a large transcriptomic response to infection with *Symbiodinium* and little response to light environment (Table 5.1). In infected larvae, 245 genes were up-regulated (43% annotated) and 156 were down-regulated compared to non-infected larvae (26% annotated) regardless of light environment. No genes were differentially expressed between Light and Dark environments when *Symb* and No*Symb* treatments were pooled.

Over-represented functional groups that were primarily up-regulated in *Symb* included calcium ion binding (GO:0005509) endopeptidase activity (GO:0004175), positive regulation of JUN kinase activity (GO:0043507), alcohol dehydrogenase (NADP) activity (GO:0008106) and activation of cysteine-type endopeptidase activity

involved in apoptotic process (GO:0006919; Fig. 5.3, Table 5.1). Infected larvae also showed up-regulation of particular cellular processes that are known to be a response to stress, including ubiquitin conjugating factor (E2), oxidative stress growth inhibitor 1, and rhamnose binding lectin.

All four genes associated with nucleosome assembly (GO:0006334) were down-regulated in infected larvae. Similarly, genes associated with the GO terms determination of adult lifespan (GO:0008340) were down-regulated, suggesting higher levels of pro-apoptosis activity. Genes related to energetic pathways were down-regulated as well, including NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 2, ATP synthase subunit beta (mitochondrial), and glyoxylate reductase. Among them, glyoxylate reductase was one of the most down-regulated annotated genes identified (FC = -4.24).

Differences in gene expression between infection status in Light treatments

Many differentially expressed genes were identified when *Symb* and No*Symb* were compared after separating Light and Dark replicates (Table 5.1B and C, respectively), which is most likely a result of the fact that our microarray approach employed a pooled reference design. Among the Light treatment replicates, 17 genes were up-regulated in infected larvae (24% annotated) while 67 genes were down-regulated (22% annotated). In contrast, in the Dark treatments nearly all differentially-expressed genes (33 of 36) were up-regulated in infected larvae (45% and 0% annotated, respectively).

In Light (but not Dark), genes associated with the regulation of lipid metabolic processes (GO:0019216) and lipid transporter activity (GO:0005319) were over-

represented. Down-regulation of two glutathione-s-transferases led to the over-representation of genes categorized under the GO term determination of adult lifespan (GO:0008340), and among the notable unassociated genes represented only in Light were the up-regulation of aldose reductase and down-regulation of glyoxylate reductase, both of which are involved in energy metabolism.

Genes associated with the GO terms proteolysis (GO:0006508), negative regulation of cell growth (GO:0030308), calcium ion binding (GO:0005509), oxioreductase activity (GO:0016702), and alcohol dehydrogenase (GO:0008106) activity were all up-regulated in Dark treatments, while no functional groups were down-regulated. To isolate only those larvae with actively photosynthesizing *Symbiodinium*, we compared expression in *Symb*-Light to the three other treatment groups (Table 5.1D). Five genes were up-regulated in *Symb*-Light and none were down-regulated. The two annotated genes among the five were aldose reductase and oxidative-stress induced growth inhibitor 1, leading to over-representation of genes associated with the carbohydrate metabolic process (GO:0005975) and negative regulation of cell growth (GO:0030308), respectively.

Function-focused analysis: Lipid catabolic process (GO:0016042)

Of the genes associated with lipid catabolic processes (GO:0016042), 23% showed differential expression with respect to infection status ($\alpha = 0.05$) and 3% to Light. In infected larvae, five lipid catabolism genes were up-regulated and three were down-regulated. A number of lipid catabolism genes had a negative linear relationship with per larva *Symbiodinium* abundance, while none showed a positive relationship. Among them

were four mitochondrial acyl CoA dehydrogenases, which catalyze the first step in β-oxidation of fatty acids for energy use via the TCA cycle ($p < 0.05$ for all, adjusted R^2 = 0.64, 0.63, 0.61, 0.45).

Function-focused analysis: Apoptotic process (GO:0006915)

Among the 106 genes in the array associated with apoptotic processes (GO:0006915), 20% showed a differential response based on *Symbiodinium* presence and 5% showed a differential response to light conditions. Of particular interest were caspases, as they act as cell executioners in the apoptotic processes and have recently been implicated in the coral bleaching response (Ainsworth et al. 2011, Kvitt et al. 2011, Tchernov et al. 2011). An ANOVA implied that infected larvae had higher mean expression of caspase-3, the only caspase gene represented in our dataset ($F(1,15) = 5.40$; $p < 0.05$). Among the caspase-regulating Bcl-2 family genes, an inhibitor of the pro-apoptosis Bax gene (Probable Bax inhibitor 1) was down-regulated in infected larvae ($F(1,14) = 9.62$; $p < 0.01$).

There were significant linear relationships between expression of apoptotic inhibitors and promoters in *Symb* but not No*Symb* larvae. Expression of a Bax inhibitor (transmembrane Bax inhibitor motif-containing protein 4) was strongly positively correlated with expression of the Bax gene ($F(1,8) = 79.04$, $p < 0.0001$, adjusted R^2 = 0.90). The same Bax inhibitor also showed a negative relationship with expression of the anti-apoptotic Bcl-X gene ($F(1,8) = 12.04$, $p < 0.01$, adjusted $R^2 = 0.55$), highlighting greater regulation of apoptosis in infected larvae.

Function-focused analysis: Oxidative stress (GO:0006979)

Among genes associated with oxidative stress, 28% were differentially expressed in response to infection status: six were up-regulated and five were down-regulated in *Symb* larvae. Among them, catalase was up-regulated ($F(1,16) = 5.46$, $p < 0.05$) consistent with a common response to oxidative stress in corals. In contrast, two peroxidasins (peroxidasin-6 and peroxidasin-like protein) and a superoxide dismutase (Mn) gene, all of which are associated with response to oxidative stress in corals, were down-regulated in infected larvae.

Experiment 2: Larval behavior in response to infection with Symbiodinium *B184 and A194*

Mortality was similar across larvae infected with *Symbiodinium* B184, A194, and larvae that were not infected ($p > 0.05$; Fig. 5.4), demonstrating that there was no effect of *Symbiodinium* infection or type on survival in this experiment. We similarly detected few clear settlement patterns among treatments. Most notably, on day 33 after spawning, larvae infected with *Symbiodinium* A194 consistently had higher settlement probabilities while *Symbiodinium* B184-infected larvae had lower settlement probabilities. One replicate in the non-infected treatment had extremely high settlement and low swimming activity on day 33, which were more than three times outside the standard deviation. Therefore, we tested differences among treatments in swimming and settlement with and without those data. When this replicate was included, settlement of *Symbiodinium* B184 larvae was significantly lower than in the other two treatments (two-parameter model; $p < 0.01$).

Differences in swimming activity among treatments were more evident than differences in survival or settlement. On day 10 after spawning, each treatment group had had different proportions of larvae swimming (three-factor model; $p < 0.05$; Fig. 5.4). Swimming probability was 0.82 for *Symbiodinium* A194, 0.59 for *Symbiodinium* B184, and 0.41 for non-infected larvae. On day 16, swimming activity was different in each treatment ($p < 0.05$), but the relative order changed, as *Symbiodinium* B184 were the most active (0.74), non-infected were intermediate (0.58), and *Symbiodinium* A194 were the least actively swimming (0.20). Consistent with this response, *Symbiodinium* A194-infected larvae had lower swimming activity (0.10) than the other two treatments (0.78; $p < 0.0001$). This result was the same with and without the outlier described above. These results demonstrate that as the experiment progressed, *Symbiodinium* A194 infected larvae either settled or stopped moving, while non-infected and *Symbiodinium* B184 larvae continued to swim.

Experiment 3: Larval behavior in response to infection and light environment

Larvae infected with *Symbiodinium* isolated from local conspecific colonies contained 172.9 ± 36.2 algal cells per individual (mean ± SE) after 10 days of infection. At the end of the subsequent 15-day experiment, 72 ± 7% (mean ± SD) and 67 ± 12% of non-infected larvae survived in the Light and Dark treatments, respectively, compared to survival of 57 ± 15% and 58 ± 18%, respectively, in infected larvae (Fig. 5.5). On day 26 after spawning, the probability of survival was highest in No*Symb*-Light (0.91), lower in No*Symb*-Dark (0.84), and lowest in *Symb* larvae of both groups (0.77). These differences in survival among groups could not be statistically distinguished from two other three-

parameter models, though all three models show that survival of *Symb* larvae was lower than survival of No*Symb* larvae. The same survival patterns were found on day 33 after spawning, at which time survival was higher in No*Symb*-Light (0.72) than in No*Symb*-Dark (0.67), and was lowest in *Symb* larvae in both light conditions (0.57), suggesting that *Symbiodinium* infection reduced survival in this experiment.

On day 26 after spawning, settlement in No*Symb*-Light was significantly different (two-parameter model; $p < 0.05$; Fig. 5.5A) and lower than in the other three groups, while on day 33 settlement for all four groups was similar ($p > 0.05$). These results suggest that individuals in the No*Symb*-Light group delayed settlement relative to the other three groups. When the proportion of larvae settling per day was assessed for days 19-26, there was no effect of treatment group on settlement, which is consistent with the above results from day 33. There was a significant effect of infection status on swimming activity ($F_{1,16} = 6.38$; $p < 0.05$). Of the larvae in each replicate not settled (or dead), an average of 50% of larvae in non-infected replicates and 39% of larvae in *Symbiodinium* infection replicates were observed actively swimming during Experiment 3.

DISCUSSION

Animals that live in obligate, energy-deriving symbioses with autotrophs show marked differences in the way their symbiotic partners are passed across generations. Symbionts may be transmitted directly to offspring in the egg (vertical transmission) or must be acquired from the external environment (horizontal transmission). It is theorized that vertical transmission is favorable because symbionts provide energy to offspring, which may lengthen the larval competency period (Richmond 1987, Baird et al. 2009,

Harii et al. 2010), reduce inter-symbiont competition, and guarantee successful transmission of appropriate symbiont types (Wilkinson and Sherratt 2001). Despite its apparent benefits, there is a remarkable number of species exhibiting long larval periods and horizontal symbiont transmission, contrary to the notion that symbiont-derived energy extends larval competency.

One possible explanation for this contrast is that vertical symbiont transmission is disadvantageous in certain environmental conditions, such as those that induce metabolic or immunological stress in symbionts, which is subsequently transferred to the host (e.g., high light and temperature, Yakovleva et al. 2009, Nesa et al. 2012). This result highlights how life history characters (e.g., dispersal patterns) and environmental context determine the costs and benefits of a given symbiont transmission strategy.

Symbiont infection and energy use: no evidence of energetic subsidy

While *Symbiodinium*-infected larvae consumed less energetic lipid than non-infected conspecifics, this was not the result of an energetic subsidy gained from *Symbiodinium.* The use of energetic lipids in infected larvae was equal between a light:dark cycle and complete darkness, in which photosynthesis by *Symbiodinium* was halted. Therefore, the difference in lipid content between infected and non-infected larvae could not have been the result of translocation of fixed carbon. Instead, this difference likely arose because non-infected larvae were more active swimmers than their infected counterparts. The energy required for active swimming in marine invertebrate larvae constitutes a substantial portion of their energy budget (Wendt 2000, Bennett and Marshall 2005) and can come directly from lipid catabolism (Lucas et al. 1979).

Gene expression related to energy metabolism differed between infection states as well, most notably with regard to glucose and glyoxylate metabolism. Aldose reductase, which performs the first step in glucose oxidation, was up-regulated in infected larvae in light treatments. Up-regulation of aldose reductase may have been a response to obtaining glucose from photosynthetically-active *Symbiodinium*. In infected larvae in both light environments, there was a large down-regulation of glyoxylate reductase (FC = -4.24). This suggests that infected larvae were maintaining glyoxylate levels (rather than reducing glyoxylate to glycolate) compared to uninfected counterparts. Glyoxylate is used in the glyoxylate cycle, an anabolic process that produces carbohydrates. It was recently found that genes associated with the glyoxylate cycle were up-regulated in response to high-temperature stress in larvae of another Caribbean coral (Polato et al. 2013).

While it is difficult to ascertain from these gene expression patterns whether infected larvae experienced higher or lower energetic demand, gene expression patterns do suggest fundamental differences in energy metabolism between symbiont-bearing and symbiont-free larvae. Such patterns are to be expected given that the energy-deriving nature of the coral-*Symbiodinium* association, though it is intriguing that the majority of metabolic differences among larval treatments occurred in response to infection, independent of *Symbiodinium* photosynthetic activity. Thus, infection itself, rather than a response to increased concentrations of photosynthetic products, appears to lead to shifts in larval energy metabolism.

Up-regulation of apoptotic processes and its role in metamorphosis and mortality

Programmed cell death, or apoptosis, is a fundamental process during development as well as a means to remove damaged or degraded cells. Apoptosis is thought to be a primary cause of coral mortality resulting from cellular stress associated with bleaching (Tchernov et al. 2011). The apoptotic process involves numerous upstream regulators, and the susceptibility of a given coral species to bleaching-induced mortality is dependent on its ability to curb apoptotic processes when cellular stress occurs (Tchernov et al. 2011, Kvitt et a. 2011, Ainsworth et al. 2011).

In coral larvae, the metamorphic process involves significant tissue remodeling (Hirose et al. 2000, Clode and Marshall 2008, Grasso et al. 2011). Extensive work in a hydrozoan reveals that metamorphosis is mediated by apoptosis (Seipp et al. 2001, Seipp et al. 2006, Wittig et al. 2011), and *Symbiodinium* infection alone can lead to apoptosis as well (Dunn and Weis 2009, Voolstra et al. 2009). Multiple lines of evidence suggest that *Symbiodinium* infection activated competing pathways for apoptosis in the coral larvae studied here, consistent with patterns indicating preparation for metamorphosis and mild stress associated with immune responses (Dunn and Weis 2009, Voolstra et al. 2009, O'Rourke 2011). Similar to the need for adults to curb apoptotic responses to cellular stresses associated with bleaching, we conclude that slightly higher mortality in infected larvae may have been due to the inability of individuals to regulate apoptotic processes after infection.

While not identified as differentially expressed in our analysis of the entire array, our targeted analysis of genes associated with the apoptotic process (GO:0006915) found that caspase-3 was up-regulated in infected larvae. Metamorphosis in a hydrozoan is

dependent on increased activity of the caspase-3 enzyme and may be halted by its inhibition (Seipp et al. 2006). We found evidence of tighter regulation of caspase-mediated apoptotic processes in infected larvae, in particular with respect to the Bcl family of genes that regulate caspase-3. Occurring only in infected larvae, there was a strong positive correlation ($R^2 = 0.9$) between expression of the pro-apoptosis Bax gene and the expression of a Bax gene inhibitor (transmembrane Bax inhibitor motif-containing protein 4). This Bax inhibitor was also negatively correlated with expression of the anti-apoptotic Bcl-X gene in infected larvae only, showing a negative relationship between two genes associated with similar functions. These patterns demonstrate fundamental, infection-dependent differences in apoptotic processes and suggest that apoptosis was more tightly regulated in infected larvae.

The activity of ubiquitin and proteases, which mark and denature proteins, respectively, plays an important role in apoptotic processes in addition to Bcl genes (Glickman and Ciechanover 2002, Jesenberger & Jentsch 2002). The ubiquitin-protease pathway can have pro- and anti-apoptotic roles depending on the protein it destroys (Jesenberger & Jentsch 2002). Increasing evidence suggests that cell death is avoided by the ubiquitination of caspases, as well as targeting of mediators of caspases such as the Bcl-2 family of proteins (Jesenberger & Jentsch 2002).

In numerous marine invertebrates, increased ubiquitin-protease activity has been associated with cellular, tissue, and even whole organism death in the case of colonial ascidians (Mykles 1998, Jesenberger & Jentsch 2002). In corals, increased ubiquitin concentration is a common response to stress (Brown et al. 2002, Downs et al. 2005, Barshis et al. 2010). In our experiment, infected larvae up-regulated ubiquitin conjugating

factor (E2), which is responsible for ubiquitylating proteins for degradation, as well as ubiquitin carboxyl-terminal hydrolase isozyme L5, which removes ubiquitin after protein degradation (Jesenberger & Jentsch 2002). Two subunits of the proteasome, which carries out protein denaturation, were up-regulated as well (proteasome subunit alpha type-1 and proteasome subunit alpha type-7), further indicating elevated ubiquitin-protease activity in infected larvae.

Genes associated with positive regulation of JUN kinase activity (JNK pathway), comprised of stress-stimulated kinases that initiate apoptotic pathways, were over-represented and up-regulated in infected larvae. Furthermore, two glutathione-S-transferase genes were down-regulated in infected larvae, the protein product of which inhibits apoptosis through interactions with kinases early in the JNK pathway (Laborde 2010). The up-regulation of similar pathways (MAPK) was identified previously after infection of *O. faveolata* with B184 strain, which was associated with an immune response to infection (Voolstra et al. 2009).

The above evidence suggests that *Symbiodinium* infection initiated numerous competing pathways of apoptosis. We suggest that any failure to appropriately regulate/halt apoptosis in larvae likely led to cellular death and may have caused the demise of entire individuals. Increased mortality due to symbiont infection, even when small, is likely to have evolutionary consequences when borne out over many generations, especially if larvae do not settle soon after or fail to form a competent symbiosis (Voolstra et al. 2009, O'Rourke 2011). This apparent multi-pathway competition between pro-and anti-apoptotic gene expression also highlights the need to measure concentrations their protein products. By assessing concentrations of, for

example, Bcl-2 to BAX proteins we may begin to understand the true extent to which apoptosis may be inducing mortality.

Oxidative stress and the effect of light on larvae

Two genes related to stress, oxidative stress-induced growth inhibitor 1 and aldose reductase, were up-regulated in larvae containing photosynthetically-active symbionts (*Symb*-Light). The former decreases cell division during oxidative stress, while the latter is involved in glucose metabolism and is up-regulated during copper stress in adults of a closely-related species (*O. franksi*; Schwarz et al. 2013). Behavioral differences between infected and non-infected larvae were most acute in the Light treatment, particularly in days 21-24. During this time, infected larvae had higher mortality, lower swimming activity and more rapid settlement.

While these patterns offer some evidence of oxidative stress due to photosynthetic activity, the majority of molecular stress responses we identified were attributable to infection regardless of the light environment experienced. Oxidative stress in larvae can occur for numerous reasons beyond being a stress response to high light and temperature (e.g., ß-oxidation of fatty acids). Given that temperature was not manipulated in Experiment 1 and light intensity was much lower than at the surface of the ocean it is not surprising that we detected little oxidative stress in response to the environmental conditions.

Prothrombin, which is associated with responses to inflammation was highly up-regulated in infected larvae (FC = 4.2), as was rhamnose binding lectin, a gene associated with an immune response in a number of marine invertebrates (Watanabe et al. 2009).

Genes associated with nucleosome assembly were all down-regulated in infected larvae, similarly to the stress-induced bleaching of adults *O. faveolata* (DeSalvo et al. 2008), highlighting both oxidative and immune-related stress responses due to *Symbiodinium* infection. Immune responses, even when larvae form a competent symbiosis with a given strain, appear to be a common feature of the infection process (Voolstra et al. 2009, Dunn and Weis 2009, O'Rourke 2011).

Infection and settlement: molecular priming but no enhancement of settlement

In addition to tighter regulation of apoptosis, patterns of gene expression suggest that symbiont infection led to heightened preparation to metamorphose and settle. Calcium binding activity was highly up-regulated in infected larvae, a pattern associated with the initiation of metamorphosis in *O. faveolata* and other coral species (Reyes-Bermudez et al. 2009, Grasso et al. 2011). Infected larvae also up-regulated calmodulin, which is theorized to regulate the differentiation of membrane tissues responsible for calcium carbonate secretion in corals. Furthermore, two metalloproteases were up-regulated, both of which are likely involved in developmental remodeling and perhaps the secretion of organic matrix in corals (Reyes-Bermudez et al. 2009).

Despite the apparent priming of larvae for metamorphosis, *Symbiodinium* infection had only a small influence on the timing or magnitude of settlement, which requires an external cue in corals (Grasso et al. 2011, Tebben et al. 2011, Siboni et al. 2012). Recent work by Hartmann et al. (2013) provides a useful comparison of settlement rates because experiments were conducted using the same cohort of *O. faveolata* larvae in the same laboratory and over the same time horizon studied here. In that study, larvae

were provided a crustose coralline algae cue known to stimulate settlement (Morse et al. 1994). When the cue was present, larvae had a 39% probability of settlement by day 22 after spawning (the last day of data collection). In contrast, settlement was no greater than 7% on day 16 and 20% on day 33 in the same cohort of larvae (Experiment 2), and was 10% in infected larvae on day 22 of Experiment 3, which used a cohort of larvae collected from the same reef two years later. This shows that settlement of larvae was substantially lower when cues were not available, regardless of infection status or light environment.

Symbiont acquisition: timing and type

To understand the ecological consequences of the patterns identified here it is important to consider the timing of *Symbiodinium* uptake. In laboratory conditions, coral larvae are capable of taking up symbionts as soon as the mouth has formed, though uptake may be delayed until after metamorphosis, demonstrating that the acquisition of symbionts is not limited to a certain life stage (Schwarz et al. 1999). The acquisition of symbionts during the larval stage is apparently common among species (Schwarz et al. 1999, Harii et al. 2009) and taking up symbionts just prior to settlement enhances post-settlement survival (Suzuki et al. 2013). In the latter study, larvae that did not acquire *Symbiodinium* prior to settling suffered higher post-settlement mortality, even when they successfully established symbiosis after settling and metamorphosing.

Coral larvae most likely acquire symbionts after making contact with the seafloor (Adams et al. 2009, Cumbo et al. 2013). The abundance of free-living or cysting *Symbiodinium* can be more than an order of magnitude greater in sediments compared to

the water column, which likely allows for the entrainment and maintenance of stable and location-specific *Symbiodinium* communities (Littman et al. 2008). Most coral species examined to date acquire multiple *Symbiodinium* types and thus initially reflect local *Symbiodinium* communities (summarized in Cumbo et al. 2013 Table 4). In a choice experiment, aposymbiotic *O. faveolata* larvae chose to settle on substrate with *Symbiodinium* present, while brooded, symbiont bearing larvae showed no preference for *Symbiodinium* (Vermeij et al. *in press*). This difference in behavioral patterns suggests that larvae of horizontally-transmitting species can sense *Symbiodinium* and will settle near them, while larvae already containing symbionts show no preference for associating with free-living symbiont populations.

While the ability to harbor multiple symbiont clades/subclades may be common among coral species, including *O. faveolata* (Rowan and Knowlton 2005, Baker 2003), we found that behavior of *O. faveolata* larvae depended on the *Symbiodinium* strain to which they are exposed. When provided a strain isolated from conspecific adults and known to form a competent symbiosis (*Symbiodinium* B184; Voolstra et al. 2009) larvae showed behavioral patterns similar to non-infected conspecifics, which was typified by high levels of swimming and low levels of settlement in the absence of positive settlement cues. Yet, when exposed to a strain known to associate with the Caribbean coral *Acropora palmata* (*Symbiodinium* A194), larvae showed an apparent behavioral dysfunction; the vast majority of individuals stopped moving as the experiment progressed, but did not proceed through metamorphosis and settlement. Consistent with this symbiont-specific response, when *O. faveolata* larvae are infected with a *Symbiodinium* strain with which they do not form a competent symbiosis, gene

expression patterns show higher stress responses than those that occur after infection with B184, both during the larval stage (Voolstra et al. 2009) and after settlement (O'Rourke 2011). Apoptotic and immune responses, in particular, are up-regulated when infected larvae take up an incompetent strain, perhaps in an attempt to remove cells infected with that symbiont (Dunn and Weis 2009, Voolstra et al. 2009).

Behavioral and metabolic dysfunction as a result of taking up poorly-associating *Symbiodinium* strains suggests selection should favor regulation of the type of *Symbiodinium* taken up at the point of infection, though no such mechanism has yet been identified in *O. faveolata*. Despite the high likelihood of taking up multiple available strains (Cumbo et al. 2013) some horizontally-transmitting species show rapid winnowing of *Symbiodinium* communities after settlement (Suzuki et al. 2013) and perhaps even an ability to discern between symbiont types at the point of or within hours of infection in larvae (Weis et al. 2001, Dunn and Weis 2009, Bay et al. 2011).

Our observations that *O. faveolata* larvae continued to swim for days to weeks after infection with *Symbiodinium* further demonstrates that infection alone does not stimulate settlement. *O. faveolata* larvae can be particularly discerning towards settlement cues (Vermeij et al. 2006, Ritson-Williams et al. 2014), perhaps heightening the risk of acquiring symbionts in one area then moving to another area where such symbionts are unsuitable. The high degree of specificity for settlement cues, coupled with the metabolic load of harboring symbionts and apparent behavioral dysfunction from taking up certain strains, suggests that temporal coupling of symbiont acquisition and the detection of suitable settlement substrate is critical. If early acquisition of symbionts causes a decrease in fitness (settlement and post-settlement survivorship rates) because

larvae either host symbionts that do not suit their settlement habitat or suffer such stress from early symbiosis that settlement habitats are never encountered, this mortality creates selection for individuals that acquire appropriate symbionts at appropriate times during the dispersal phase.

The symbiont-settlement coupling hypothesis

We hypothesize that survival is greatest in horizontally-transmitting species when the timing of symbiont acquisition and the detection of appropriate settlement substrate (cues) is coupled (Fig. 5.6). Therefore, the timing of symbiont acquisition will be a critical component of the host's life history. Decreased fitness due to early acquisition of symbionts may occur because of (1) increased apoptotic activity with no induction of metamorphosis, (2) molecular and physiological shifts towards preparation to metamorphose that impinge upon "normal" larvae functions (e.g., searching patterns), (3) heightened risk of larvae experiencing environmental conditions (e.g., high irradiance) that lead to symbiotic breakdown, and (4) heightened risk that larvae take up *Symbiodinium* that are inappropriate for the environment in which they settle.

The final two may be exacerbated when symbionts or hosts have a high degree of specificity (Fabina et al. 2012) or when habitats are patchy or heterogeneous (e.g., hydrothermal vents Fig. 5.6B). The length of the pre-adult period can increase the likelihood that offspring encounter symbionts or environments different from those near the parent, analogous to experiencing a heterogeneous environment (Fig. 6B). This may explain the co-occurrence of long larval periods and horizontal symbiont transmission,

though conclusions regarding selection on these characters require a critical assessment of the evolutionary relationships between them (Knowlton and Rohwer 2003).

Physiological benefits, such as reduced metabolic stress during the pelagic stage and the prevention of symbiont-environment mismatches, may have selected for the lack of symbionts in gametes and larvae in some species, thereby enabling increased larval dispersal distances. Conversely, the benefits of long-distance dispersal per se, such as the subsequent increases in genetic diversity (larger population of potential mates, larger effective population size, larger species range and therefore lower likelihood of local extinction leading to species extinction) may select for any characters that allow longer pelagic phases, one of these characters being the elimination of symbionts from gametes.

In the following chapter, we evaluate character transition rates between symbiont transmission strategies and reproductive modes (as a proxy for the duration of the larval period) to determine the extent to which each trait is evolutionarily constrained. We also consider how vertical transmission, by allowing for uninterrupted symbiosis and co-evolution of partners, may lead to symbioses that are entirely distinct from those in horizontally-transmitting species (LaJeunesse et al. 2004). Through a character trait reconstruction, we make estimates of the evolutionary rates of these co-occurring characters and make inferences as to how they may influence species-specific tolerance to environmental perturbations during the earliest and most sensitive stages of life.

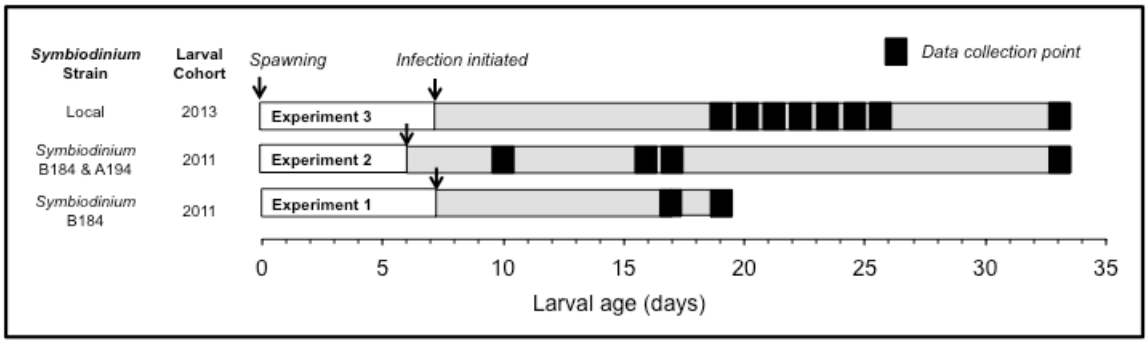


Figure 5.1: Timeline of Experiments 1, 2, and 3 relative to the date of spawning. Variables shown are the strain of *Symbiodinium* used for infection, the larval cohort used for the experiment, the day *Symbiodinium* infection began, and the days on which data were collected. In Experiment 1, we measured how energetic lipid content changed and gene expression patterns responded to infection, both when photosynthesis was active and inactive (via constant darkness), using the cultured Mf1.05b *Symbiodinium* strain that was cultured from conspecific adults. In Experiment 2, we measured larval survival, swimming activity and settlement in response to infection with the cultured *Symbiodinium* B184 and *Symbiodinium* A194. In Experiment 3, we measured larval behavior, survival, and settlement in response to infection with a mixed population of *Symbiodinium* (extracted from local conspecific adult corals) both when photosynthesis was active and inactive (via constant darkness).

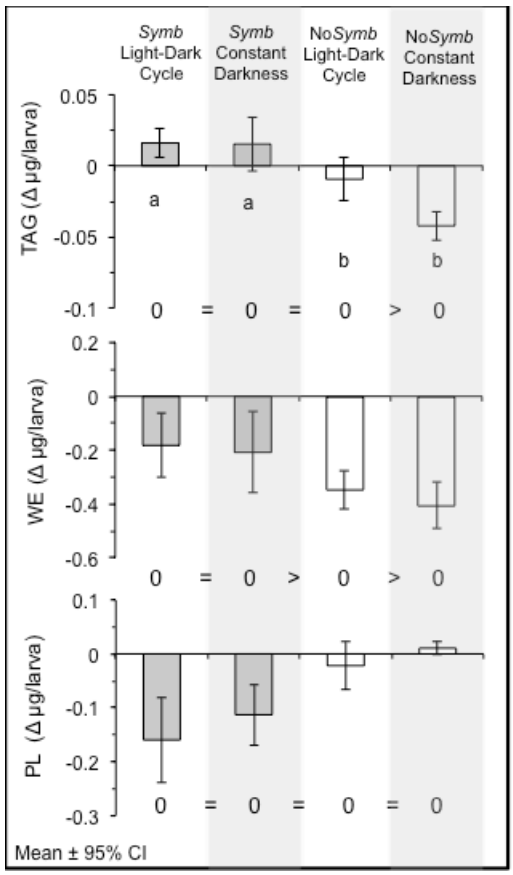


Figure 5.2: Changes in energetic and structural lipid content from time 0 to time 48 h during Experiment 1. In a factorial design, four experimental treatments were used in which larvae were infected ("*Symb*") or not infected ("No*Symb*") with *Symbiodinium* B184. Larvae were exposed to a light:dark cycle ("Light-Dark Cycle") or constant darkness ("Constant Darkness"). Energetic lipid content was measured as µg lipid/larva and is shown for the energetic lipids triacylglycerols (TAG) and wax esters (WE), as well as the structural phospholipids (PL). Values represent means with 95% confidence intervals. The relationships among 0 values indicate whether lipid content per larva changed over the 48 hours for a given lipid class. Letters denote significant differences in the change in lipid content between treatment groups.

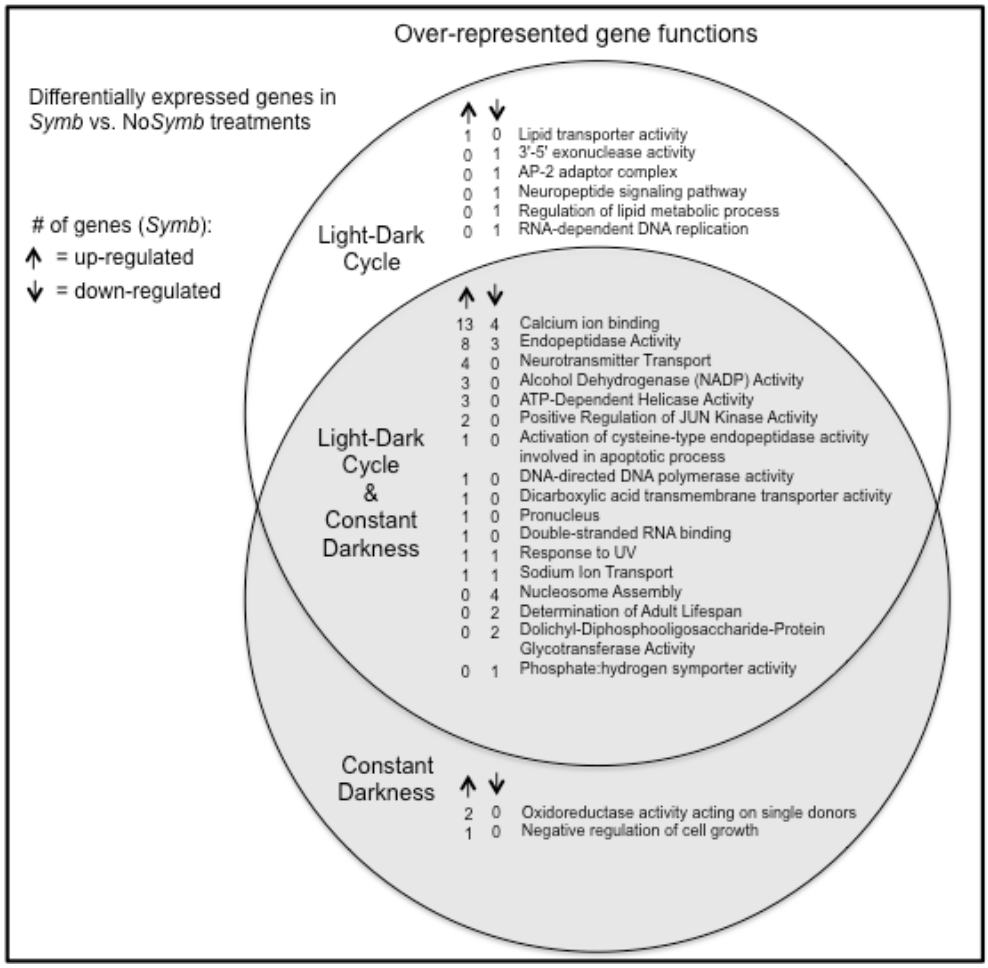


Figure 5.3: Over-represented functional groupings of genes (GO terms) that were differentially expressed between larvae infected with *Symbiodinium* ("*Symb*") and not infected ("No*Symb*") in Experiment 1. Functional groups are categorized by those that were differentially expressed between infection status regardless of light condition ("Light-Dark Cycle & Constant Darkness"), only in the diurnal light cycle treatment ("Light-Dark Cycle"), or only in constant darkness ("Constant Darkness") treatment conditions. Up arrows and corresponding numbers indicate the number of genes up-regulated in each respective category, while down arrows indicate the number of genes down-regulated.

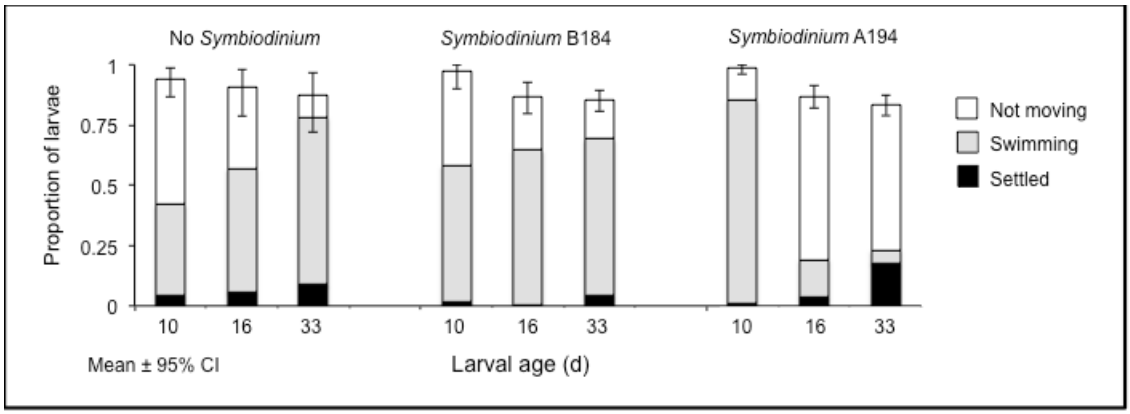


Figure 5.4: Activity, settlement and survival of infected and non-infected *O. faveolata* larvae in Experiment 2. The proportion of larvae actively swimming, not moving, settled, and alive (entire bar) 10, 16, and 33 d after spawning are relative to the initial starting population (n = 5 replicates/group; Experiment 2). The larval cohort used was the same as that in Experiment 1 (Fig. 5.2 and Fig. 5.3). Larvae were exposed to cultured *Symbiodinium* isolated from conspecific adults ("*Symbiodinium* B184"), cultured *Symbiodinium* isolated from *Cassiopea* sp. ("*Symbiodinium* A194"), or no symbionts ("No *Symbiodinium*"). Proportions of larvae in each category are relative to the initial starting population (n = 5 replicates/group). Values represent means proportions and error bars represent arcsine square root transformed 95% confidence intervals of mean proportions.

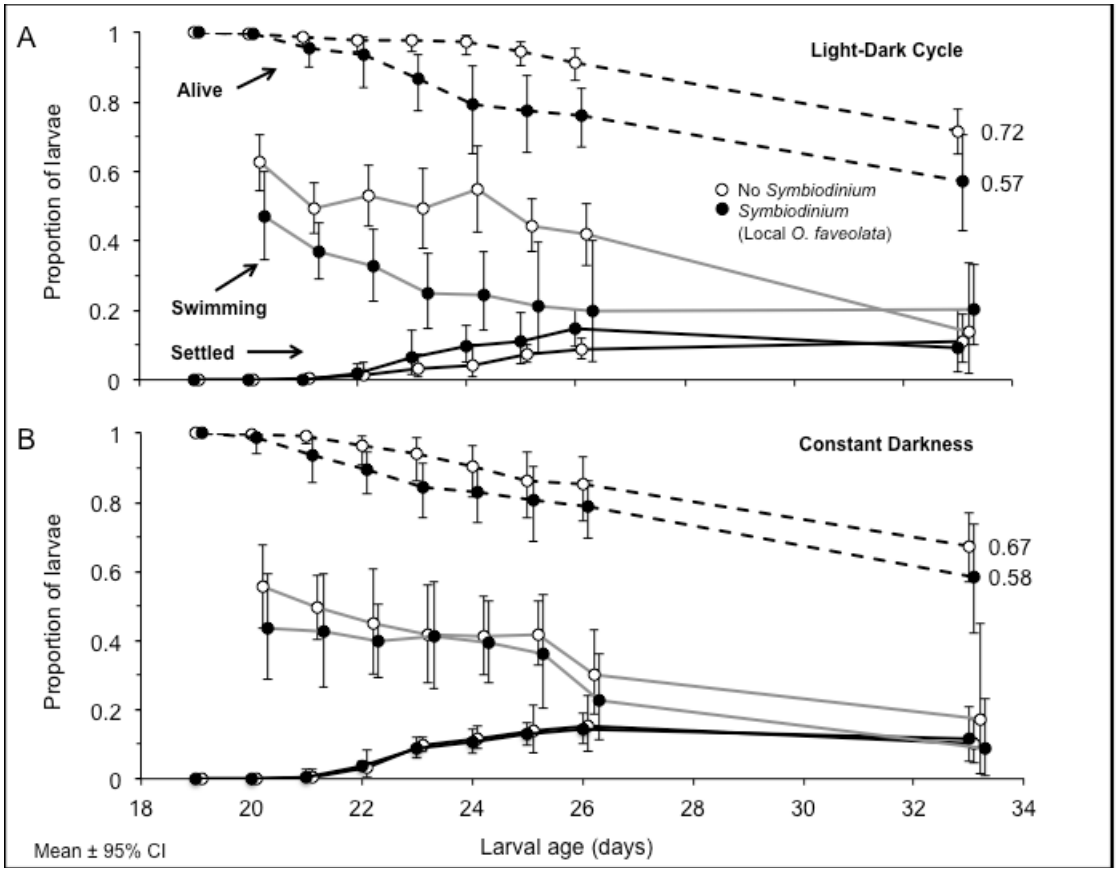


Figure 5.5: Swimming activity, settlement and survival of infected and non-infected *O. faveolata* larvae in Experiment 3. The proportion of *O. faveolata* larvae swimming (grey lines), settled (black lines), and alive (dashed lines) in (A) "Light-Dark Cycle" and (B) "Constant Darkness." Larvae were either infected with *Symbiodinium* isolated from local conspecifics (black circles) or not infected (white circles). Data were collected on seven consecutive days 19-26 days after spawning and on day 33. Arcsine square root transformed means and 95% confidence intervals are shown (n = 5 replicated/group). Proportions at the end of each time series of larvae alive represent the numerical proportion of surviving larvae in each treatment group at the end of the experiment.

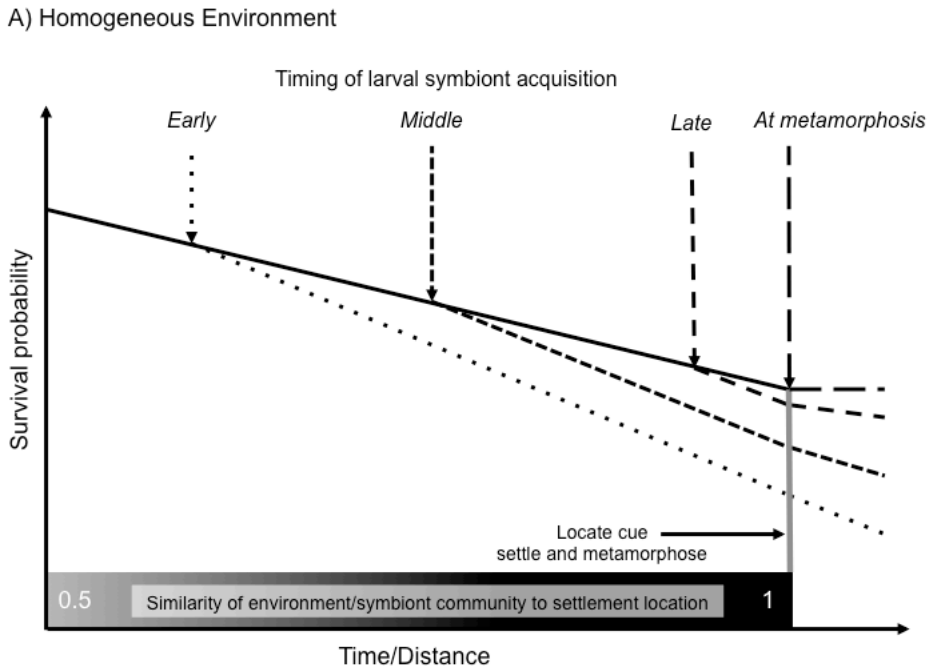


Figure 5.6: A depiction of the 'symbiont-settlement coupling' hypothesis. Shown are theoretical offspring survival probabilities through time/distance for species exhibiting horizontal symbiont transmission. Survival is dependent on the timing of symbiont acquisition relative to the timing of locating external cues for settlement and metamorphosis. Survival expectations assume a cost of symbiont acquisition prior to metamorphosis due to: (1) a small increase in mortality due to apoptotic and immune responses to symbiont uptake, (2) behavioral and molecular transitions towards preparedness to metamorphose that impinge upon "normal" larval functions (e.g., continuing to search for metamorphosis cues), (3) life-stage dependent increased risk of experiencing environmental conditions that lead to symbiotic breakdown (e.g., high irradiance at the ocean surface), and (4) heightened risk of acquiring symbionts that are inappropriate for the environment in which settlement and metamorphosis occurs. The likelihood of encountering symbiont communities or environmental conditions similar to those found on the natal reef is assumed to decline with distance from the natal reef (on a 0-1 scale). Thus, acquiring symbionts in the area in which settlement and metamorphosis occurs (1) is more beneficial to the host than acquiring symbionts earlier in dispersal (< 1). Based on this model, (A) homogenous environments (0.5-1) are expected to incur less of a cost than (B) heterogeneous environment (0-1) due to the greater similarity in environment/symbiont community between the natal reef and the settlement reef.

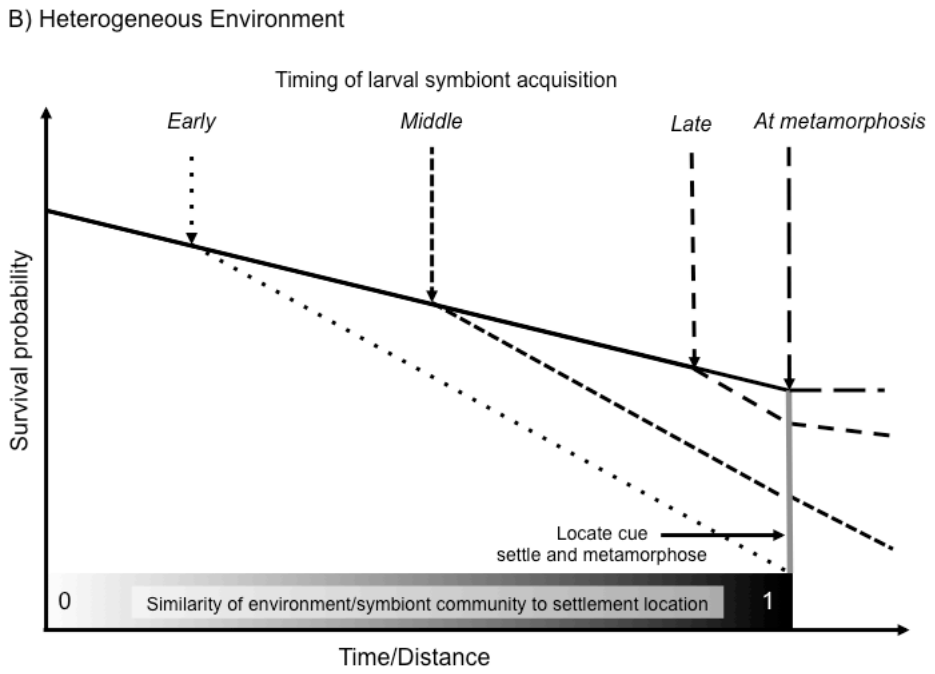


Figure 5.6 Continued

Table 5.1: Over-represented gene functions (GOEAST) and associated differentially expressed genes based on comparisons of (A) *Symb* / No*Symb*; (B) *Symb*-Light / No*Symb*-Light; (C) *Symb*-Dark / No*Symb*-Dark (D) *Symb*-Light / all else. Gene Ontology, Clone ID, common name, and Fold Change (FC) are represented. FC values > 1 represent genes up-regulated in *Symb*+ and all FC values < 0 are genes down-regulated in *Symb*. Up-regulated genes are bolded to assist in visualization.

Table 5.1A) *Symb* (Light and Dark) vs. No*Symb* (Light and Dark)

Ont.	Function/Clone ID	Common Name	FC
BP	Positive regulation of JUN kinase activity		
	CCHW8415	**Dual specificity mitogen-activated protein kinase kinase 4**	**1.67**
	CCHW11261	**Serine/threonine-protein kinase PAK 1**	**1.60**
BP	Determination of adult lifespan		
	CCHW5467	Probable glutathione S-transferase 5	-2.61
	CCHW14962	Probable glutathione S-transferase 5	-2.62
BP	Nucleosome assembly		
	CCHW5031	Histone H4	-1.82
	CCHW16596	Nucleosome assembly protein 1-like 1	-2.06
	CCHW3969	Histone H2A	-2.09
	AOSF1219	Histone H3.3	-2.13
MF	Sodium ion transport		
	CCHW8540	**Sodium-coupled monocarbox. transporter 1**	**2.08**
	CCHW16682	Sodium/potassium-transporting ATPase subunit alpha-2	-1.64
BP	Response to UV		
	CCHW4670	**Ubiquitin-conjugating enzyme E2 A**	**1.90**
	CCHW7047	Ubiquitin conjugation factor E4 B	-1.94
BP	Neurotransmitter transport		
	CCHW7729	**Protein DJ-1**	**2.07**
	CCHW7412	**Neurexin-4**	**1.85**
	CCHW15456	**Islet cell autoantigen 1**	**1.62**
	CCHW16682	**Sodium/potassium-transporting ATPase subunit alpha-2**	**1.64**
CC	Pronucleus		
	CCHW14578	**Polycomb protein EED**	**1.94**

Table 5.1A) *Symb* (Light and Dark) vs. No*Symb* (Light and Dark) Cont.

Ont.	Function/Clone ID	Common Name	FC
MF	Dicarboxylic acid transmembrane transporter activity		
	CCHW8767	**Mitochondrial dicarboxylate carrier**	**1.46**
MF	Alcohol Dehydrogenase (NADP) Activity		
	CCHW5790	**Alcohol dehydrogenase [NADP+]**	**2.63**
	CCHW1362	**Aldose reductase**	**1.72**
	CCHW6644	**Alcohol dehydrogenase [NADP+]**	**1.54**
MF	Dolichyl-diphosphooligosaccharide-protein glycotransferase activity		
	CCHW11351	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	-2.38
	CCHW10374	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	-3.39
MF	Endopeptidase activity		
	CCHW13879	**Prothrombin**	**4.20**
	CCHW10731	**Chymotrypsin-like protease CTRL-1**	**2.35**
	CCHW14207	**Proteasome subunit alpha type-1**	**2.24**
	CCHW2660	**Dipeptidyl-peptidase 1 (Fragment)**	**1.93**
	CCHW15802	**Stromelysin-1**	**1.92**
	CCHW8563	**Trypsin I-P1**	**1.89**
	CCHW7886	**Zinc metalloproteinase nas-13**	**1.72**
	CCHW17419	**Proteasome subunit alpha type-7**	**1.54**
	CCHW6865	Putative ankyrin repeat protein FPV219	-1.63
	CCHW1363	Plasma kallikrein	-1.73
	CCHW10939	Digestive cysteine proteinase 1	-1.86
MF	Neuropeptide binding		
	CCHW4493	**Neuropeptide FF receptor 1**	**1.36**
	CCHW3797	Adrenocorticotropic hormone receptor	-1.48
MF	Double-stranded RNA binding		
	CCHW4872	**Interleukin enhancer-binding factor 2**	**2.42**
MF	DNA-directed DNA polymerase activity		
	CCHW3024	**Predicted protein**	**2.27**
MF	Calcium ion binding		
	CCHW13879	**Prothrombin**	**4.20**

Table 5.1A) *Symb* (Light and Dark) vs. No*Symb* (Light and Dark) Cont.

Ont.	Function/Clone ID	Common Name	FC
	CCHW2642	**Neuronal pentraxin 2**	**2.13**
	CCHW15802	**Stromelysin-1**	**1.92**
	CCHW2215	**Myosin regulatory light chain 2, smooth muscle isoform**	**2.40**
	CCHW5259	**Crumbs homolog 2**	**1.92**
	CCHW4504	**EF-hand calcium-binding domain-containing protein 7**	**1.59**
	CCHW8563	**Trypsin I-P1**	**1.89**
	CCHW1047	**Follistatin-related protein 1**	**1.74**
	CCHW8252	**Caltractin (Fragment)**	**1.45**
	CCHW16592	**Myosin regulatory light chain 2, smooth muscle isoform**	**1.79**
	CCHW15643	**Hemicentin-1**	**1.69**
	CCHW11274	**Calmodulin**	**1.66**
	CCHW9567	**Neuronal pentraxin receptor**	**1.58**
	AOSF1123	Predicted protein	-2.49
	AOSC457	Predicted protein	-5.69
	CCHW4518	FK506-binding protein 14	-3.49
	CAGI3048	Putative uncharacterized protein	-4.74
MF	ATP-dependent helicase activity		
	CCHW3508	**Superkiller viralicidic activity 2-like 2**	**2.21**
	CCHW15971	**Probable ATP-dependent RNA helicase DDX17**	**2.13**
	CCHW8252	**Caltractin (Fragment)**	**1.45**
MF	Phosphate:hydrogen symporter activity		
	CCHW13747	Phosphate carrier protein, mitochondrial	-2.10
	Not Associated		
	CCHW16034	**Cat eye syndrome critical region protein 2**	**3.27**
	CCHW2211	**Beta,beta-carotene 9,10-oxygenase**	**2.93**
	CCHW8641	**Bystin**	**2.72**
	CCHW17177	**Stromal membrane-associated protein 1**	**2.69**
	AOSF1022	**Vitellogenin-2**	**2.61**
	CCHW16396	**UPF0384 protein CGI-117 homolog**	**2.56**
	CCHW9116	**Beta,beta-carotene 15,15-monooxygenase**	**2.37**
	CCHW9832	**Vacuolar protein sorting-associated protein 52 homolog**	**2.13**
	CCHW9677	**Peptidyl-prolyl cis-trans isomerase B**	**2.12**
	CCHW4165	**Tetratricopeptide repeat protein 25**	**2.11**

Table 5.1A) *Symb* (Light and Dark) vs. No*Symb* (Light and Dark) Cont.

Ont.	Function/Clone ID	Common Name	FC
	CCHW2309	Uncharacterized protein C3orf34	2.08
	CCHW15795	Muscle-specific protein 20	2.07
	CCHW13662	Gamma-glutamyl hydrolase	2.06
	CCHW13965	Cytoplasmic aconitate hydratase	2.06
	CCHW7407	Rhamnose-binding lectin	2.02
	CCHW4862	60S ribosomal protein L37a	2.01
	CCHW9422	Coiled-coil domain-containing protein 25	2.00
	CCHW4485	Oxidative stress-induced growth inhibitor 1	1.98
	CCHW11069	Elongation factor 1-delta	1.97
	CCHW12092	Alpha-aminoadipic semialdehyde dehydrogenase	1.97
	CCHW9300	Mitochondrial intermembrane space import and assembly protein 40	1.96
	CCHW14559	T-complex protein 1 subunit zeta	1.95
	CCHW8452	BTB/POZ domain-containing protein 3	1.94
	CCHW10984	Protein tyrosine phosphatase-like protein PTPLAD1	1.94
	CCHW11592	4-hydroxyphenylpyruvate dioxygenase	1.93
	CCHW5818	COMM domain-containing protein 5	1.93
	CCHW10047	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	1.92
	CCHW10998	Cytochrome b-c1 complex subunit 9	1.90
	CCHW4554	Serine hydrolase-like protein 2	1.88
	CCHW4608	Poly [ADP-ribose] polymerase 15	1.87
	AOSF1356	40S ribosomal protein S15a	1.86
	CCHW8282	Single-stranded DNA-binding protein, mitochondrial	1.86
	CAON1649	60S ribosomal protein L35a	1.85
	CCHW13787	SUMO-conjugating enzyme UBC9-A	1.85
	CCHW10506	Ubiquitin carboxyl-terminal hydrolase isozyme L5	1.84
	CCHW9968	Gamma-secretase subunit PEN-2	1.83
	CCHW11353	UDP-N-acetylglucosamine transferase subunit ALG13 homolog	1.80
	CCHW15696	26S proteasome non-ATPase regulatory subunit 8	1.80
	CCHW10856	Spartin	1.79
	CCHW3747	Translocon-associated protein subunit delta	1.78
	CCHW11459	Cyclopropane-fatty-acyl-phospholipid synthase	1.78
	CCHW9660	Actin-2	1.75
	CCHW3063	Selenoprotein K	1.74
	CCHW12779	Transgelin	1.73

Table 5.1A) *Symb* (Light and Dark) vs. No*Symb* (Light and Dark) Cont.

Ont.	Function/Clone ID	Common Name	FC
	CCHW2764	**Thiosulfate sulfurtransferase**	**1.72**
	CCHW7676	**ETS domain-containing protein Elk-4**	**1.70**
	CCHW5756	**GDP-fucose transporter 1**	**1.69**
	CCHW6692	**Protein AF-9**	**1.69**
	CCHW9495	**3-hydroxyisobutyryl-CoA hydrolase, mitochondrial**	**1.69**
	CCHW3510	**Signal recognition particle receptor subunit beta**	**1.68**
	CCHW7185	**Pappalysin-2**	**1.68**
	CCHW7690	**28 kDa heat- and acid-stable phosphoprotein**	**1.66**
	CCHW5787	**PHD finger protein 21A**	**1.65**
	CCHW17630	**Voltage-dependent anion-selective channel protein 2**	**1.64**
	CCHW2357	**HIT-type zinc finger-containing protein C1orf181 homolog**	**1.63**
	CCHW14024	**Coronin-6**	**1.62**
	CCHW8628	**Protein FAM18B**	**1.59**
	CCHW10816	**Probable U3 small nucleolar RNA-associated protein 11**	**1.56**
	CCHW3475	**OV-16 antigen**	**1.56**
	CCHW2074	**Trichocyst matrix protein T2-B**	**1.55**
	CCHW8058	**Phosphoglycerate kinase 1**	**1.55**
	CCHW10801	**Sorting nexin-32**	**1.55**
	CCHW8814	**Zinc finger HIT domain-containing protein 3**	**1.54**
	CCHW4982	**Pancreatic secretory granule membrane major glycoprotein GP2**	**1.54**
	CCHW13590	**Nuclear protein localization protein 4 homolog**	**1.53**
	CCHW9449	**SH3 domain-binding glutamic acid-rich-like protein 3**	**1.53**
	CCHW4661	**Coiled-coil domain-containing protein 49**	**1.52**
	CCHW11523	**Trpc4-associated protein**	**1.52**
	CCHW14015	Uncharacterized protein LOC129881	-1.60
	CCHW1048	Tektin-1	-1.70
	CCHW9236	Putative uncharacterized protein yghX	-1.74
	CCHW16404	Uncharacterized protein C1orf192 homolog	-1.83
	CCHW13000	Extended synaptotagmin-2	-1.96
	CCHW11806	Endothelial differentiation-related factor 1	-1.96
	CCHW7262	Serine/threonine-protein kinase DCLK2	-1.98
	CCHW4422	ATP synthase subunit beta, mitochondrial	-1.99
	CCHW9335	Eukaryotic translation initiation factor 4 gamma 3	-1.99
	CCHW5752	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	-2.36

Table 5.1A) *Symb* (Light and Dark) vs. No*Symb* (Light and Dark) Cont.

Ont.	Function/Clone ID	Common Name	FC
	AOSB638	Intron-binding protein aquarius	-2.37
	CCHW3590	Transmembrane protein 147	-2.41
	AOSF591	40S ribosomal protein S29	-2.44
	CCHW11703	Selenoprotein T2	-2.59
	CAGI2850	Transposon TX1 uncharacterized 149 kDa protein	-2.64
	CCHW8912	Lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog	-2.65
	CCHW14697	Multifunctional protein ADE2	-3.17
	AOSB392	Recombination repair protein 1	-3.32
	CCHW9084	DnaJ homolog subfamily B member 14	-3.82
	CCHW11487	Cryptochrome-1	-3.85
	CCHW9811	60S ribosomal protein L13	-3.97
	AOSB445	Glyoxylate reductase	-4.24
	CCHW14458	Vitamin K-dependent gamma-carboxylase	-4.28
	CAGI2744	Uncharacterized protein U88	-5.04

Table 5.1B) *Symb*-Light vs. No*Symb*-Light

Ont.	Function/Clone ID	Common Name	FC
BP	Regulation of lipid metabolic process		
	CCHW11806	Endothelial differentiation-related factor 1	-2.10
BP	Activation of cysteine-type endopeptidase activity involved in apoptotic process		
	CCHW13879	**Prothrombin**	**3.55**
BP	Determination of adult lifespan		
	CCHW14962	Probable glutathione S-transferase 5	-3.22
	CCHW5467	Probable glutathione S-transferase 5	-2.95
BP	RNA-dependent DNA replication		
	CAGI2850	Transposon TX1 uncharacterized 149 kDa protein	-3.36
BP	Neuropeptide signaling pathway		
	CAGI3048	Putative uncharacterized protein	-4.74
MF	Lipid transporter activity		
	AOSF1022	**Vitellogenin-2**	**2.97**

Table 5.1B) *Symb*-Light vs. No*Symb*-Light Cont.

Ont.	Function/Clone ID	Common Name	FC
MF	3'-5' exonuclease activity		
	AOSB392	Recombination repair protein 1	-3.86
CC	AP-2 adaptor complex		
	AOSB1075	AP-2 complex subunit alpha-2	-2.08
	Not Associated		
	CCHW4165	**Tetratricopeptide repeat protein 25**	**2.78**
	CCHW1362	**Aldose reductase**	**1.89**
	CCHW9236	Putative uncharacterized protein yghX	-2.00
	CCHW7262	Serine/threonine-protein kinase DCLK2	-2.21
	AOSF1219	Histone H3.3	-2.48
	CCHW11703	Selenoprotein T2	-2.70
	CAON1234	Coiled-coil domain-containing protein 94	-2.81
	CCHW8912	Lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog	-3.16
	CCHW9811	60S ribosomal protein L13	-3.69
	AOSB445	Glyoxylate reductase	-5.07
	CAGI2744	Uncharacterized protein U88	-6.01

Table 5.1C) *Symb*-Dark vs. No*Symb*-Dark

Ont.	Function/Clone ID	Common Name	FC
BP	Proteolysis		
	CCHW10731	**Chymotrypsin-like protease CTRL-1**	**3.14**
	CCHW13879	**Prothrombin**	**5.44**
	CCHW15802	**Stromelysin-1**	**2.09**
	CCHW7886	**Zinc metalloproteinase nas-13**	**1.67**
BP	Negative regulation of cell growth		
	CCHW4485	**Oxidative stress-induced growth inhibitor 1**	**2.26**
BP	Activation of cysteine-type endopeptidase activity involved in apoptotic process		
	CCHW13879	**Prothrombin**	**5.44**
MF	Calcium ion binding		
	CCHW2642	**Neuronal pentraxin2**	**2.15**

Table 5.1C) *Symb*-Dark vs. No*Symb*-Dark Cont.

Ont.	Function/Clone ID	Common Name	FC
	CCHW2215	**Beta,beta-carotene 9,10-oxygenase**	**3.84**
	CCHW5259	**Crumbs homolog 2**	**2.12**
	CCHW13879	**Prothrombin**	**5.44**
	CCHW15802	**Stromelysin-1**	**2.09**
MF	Oxidoreductase activity, single donors with incorporation of molecular oxygen		
	CCHW9116	**Beta,beta-carotene 15,15-monooxygenase**	**2.91**
	CCHW2211	**Beta,beta-carotene 9,10-oxygenase**	**3.84**
MF	Alcohol dehydrogenase (NADP+) activity		
	CCHW5790	**Alcohol dehydrogenase [NADP+]**	**2.69**
MF	DNA-Directed DNA Polymerase Activity		
	CCHW3024	**Predicted Protein**	**2.29**
	Not Associated		
	CCHW8641	**Bystin**	**2.75**
	CCHW2215	**Myosin regulatory light chain 2, smooth muscle isoform**	**2.19**
	CCHW10856	**Spartin**	**2.08**
	CCHW4554	**Serine hydrolase-like protein 2**	**1.92**
	CCHW6692	**Protein AF-9**	**1.87**
	CCHW2971	**PERQ amino acid-rich with GYF domain-containing protein 2**	**1.57**

Table 5.1D) *Symb*-Light vs. all else

Ont.	Function/Clone ID	Common Name	FC
BP	Carbohydrate metabolic process		
	CCHW1362	**Aldose reductase**	**1.72**
BP	Negative regulation of cell growth		
	CCHW4485	**Oxidative stress-induced growth inhibitor 1**	**1.92**

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Chapter 6.

EVOLUTION OF SYMBIONT TRANSMISSION STRATEGIES AMONG REEF CORALS

Aaron C Hartmann[1] & Danwei Huang[2,3]

[1]Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, USA

[2]Department of Earth and Environmental Sciences, University of Iowa, Iowa City, IA 52242, USA

[3]Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore

Abstract. The role of symbiont transmission strategies in the evolution of species with complex life histories is poorly understood, despite their critical role in maintaining symbioses across generations. By reconstructing the evolution of life history traits on the phylogeny of reef corals, we find that symbiont transmission strategy (vertical or horizontal) and reproductive mode (brooding or spawning) are correlated over evolutionary timescales. Transition rates suggested selection favored horizontal transmission in species with long pre-adult periods (spawning) and vertical transmission in species with short pre-adult periods (brooding). Our results also showed strong phylogenetic constraints, as vertical transmission could only be gained or lost among brooding species. The lack of transitions among spawners highlights the importance of horizontal brooding, an apparently unstable intermediary, in transitions between the two states dominating modern coral communities. The evolution of horizontal from vertical transmission represents the interruption of symbiont transfer across generations, likely changing the host-symbiont relationship on a fundamental level. We found that symbiont transmission strategy also correlated with the susceptibility of adults to bleaching, and evolutionary transitions favored the loss of vertical transmission in reducing bleaching susceptibility. Thus, symbiont transmission strategies may play a previously unknown role in bleaching risk among corals, and the loss of transgenerational symbiosis could be critical for lowering bleaching susceptibility by allowing larvae to obtain symbionts that have adapted to novel environmental conditions.

INTRODUCTION

Symbioses represent one of the most fundamental interactions in nature. They likely facilitated the rise of complex organisms and persist today as vital survival strategies in numerous taxa. By forming symbiotic associations, host species obtain necessary resources not sufficiently available from their environment, such as fixed nitrogen in plant roots or organic carbon in marine invertebrates (e.g., Muscatine 1967, Smith and Read 1997). Despite their essential nature, hosts do not always pass symbionts across generations. Symbionts can be directly transmitted to offspring via eggs (vertical transmission) or must be reacquired from the external environment with each successive generation (horizontal transmission; Trench 1987). Once acquired in a lineage, vertical transmission is theoretically stable from an evolutionary perspective, so there must be mechanisms maintaining horizontal transfer despite the need to re-establish obligate symbiosis in each generation (Wilkinson and Sherrat 2001).

Examples of vertical and horizontal symbiont transfer strategies are widespread among species with complex life histories, such as those that progress from larval to adult stages. Among them, there is remarkable consistency in the association between vertical transmission and short larval periods or horizontal transmission and long larval periods (Fitt and Trench 1981, Won et al. 2003, Nussbaumer et al. 2006, Baird et al. 2009, Ramos and Banaszak 2013). This functional redundancy may have evolved as a result of environmental specificity exhibited by different symbiont types—offspring of hosts that remain in the natal environment may favor the same symbiont community as their parent, while widely-dispersing offspring likely prefer acquisition of symbionts adapted to the

environmental conditions where they assume adulthood (Schwarz et al. 1999, Knowlton and Rohwer 2003, LaJeunesse et al. 2004, Nussbaumer et al. 2006).

Stony corals of the order Scleractinia show a high degree of association between broadcast spawning gametes and horizontally transmitting symbionts (dinoflagellates of the genus *Symbiodinium*) or internally brooding larvae and vertically transmitting symbionts (Baird et al. 2009). Offspring of spawning species have an obligate dispersal period and tend to have longer larval periods than brooding species (Sammarco and Andrews 1989, Graham et al. 2008, Underwood et al. 2009). Thus, the strong association between symbiont transmission strategy and reproductive mode in corals may provide a mechanism for ensuring larvae acquire locally adapted symbionts via patterns outlined above, which we term the "local symbiont" hypothesis.

Symbiodinium types show surprisingly high diversity along a spectrum of preferred environmental regimes and efficiencies as a symbiotic partner (e.g., Warner et al. 1996, LaJeunesse 2002, Ulstrup and van Oppen 2003, see also Baker 2003). There is also a remarkable degree of differentiation between symbiont type that associate with vertically or horizontally-transmitting coral species, potentially creating marked differences in the host-symbiont relationship as a result of how *Symbiodinium* are acquired (Fabina et al. 2013).

The divergence of *Symbiodinium* types between vertical and horizontal transmitters may have arisen in response to (or as a result of) co-evolution of host and symbionts, which is expected to only occur with vertical transmission because of the uninterrupted symbiosis across generations (LaJeunnesse et al. 2004, van Oppen 2004). While the extent of co-evolution is largely unknown, behavioral and metabolic evidence

suggests offspring from vertical and horizontal transmitters have fundamentally different interactions with *Symbiodinium* during the larval stage (Alamaru et al. 2009, Yakovleva et al. 2009, Harii et al. 2010, Hartmann et al. 2013, Chapter 5 this dissertation). In the presence of a single or specialized set of symbionts across generations, selection may optimize the relationship for both host and symbiont, and particularly so in stable environmental conditions (McMullin et al. 2003, Wade 2007). Yet, adapting to specific symbionts may carry the risk that host-specific symbiont communities lack the plasticity to tolerate new environmental regimes. When environmental shifts are large enough, the metabolic flexibility presumably required to maintain a horizontally transmitting lifestyle may become particularly advantageous as a means to adapt to environmental change.

Coral diversity is declining precipitously worldwide in large part due to increases in global ocean temperatures (Glynn 1993, Hoegh-Guldberg 1999, Knowlton 2001, Bruno et al. 2007, Carpenter et al. 2008). Much of this temperature-induced mortality arises after corals bleach, or lose their *Symbiodinium*, in response to the breakdown of photosynthesis (Lesser 1997, Brown 1997). Despite evidence that vertical and horizontally-transmitting species associate with disparate *Symbiodinium* communities, little attention has been paid to how the evolution of symbiont transmission strategies may influence bleaching susceptibility among species.

We tested the local symbiont hypothesis (Fig. 6.1A) by first quantifying the degree of phylogenetic conservatism associated with symbiont transmission strategy and reproductive mode, based on the most comprehensive phylogeny of scleractinian corals to date (Huang 2012, Huang and Roy 2013). We then evaluated the relationship between these traits by estimating evolutionary transition rates among transmission strategies and

reproductive modes. These computations were also used to investigate the role of the symbiont transmission strategies in mediating the susceptibility of coral species to bleaching. Given the flexibility in symbiont partners afforded to horizontal transmitters, we predicted that evolution would show directional selection favoring horizontal transmission and low susceptibility to bleaching.

Better understanding of the evolution of symbiont transmission strategies may reveal how unique trajectories of symbiont acquisition across generations influence adaptation potential among species. Through this approach, we highlight the affects of an often-overlooked life history strategy on species' extinction risk in a changing climate.

MATERIALS AND METHODS

Source data for reproductive mode and symbiont transmission strategy were previously tabulated by Baird et al. (2009). Susceptibility to bleaching scores were generated by Carpenter et al. (2008) in which species were classified as “Moderately or Highly” susceptible to bleaching, which were deemed as “High Susceptibility” in the present study. Species without this designation were deemed as “Low Susceptibility.” For the purposes of this analysis, we coded bleaching susceptibility as a binary trait based on the Carpenter et al. (2008) classification, though acknowledging that it likely acts on a continuum among species. Traits were mapped onto the most recent completely-sampled phylogenetic tree of reef Scleractinia, which was inferred based on seven molecular markers, 13 morphological trees and taxonomic information (Huang 2012). The tree topology was time calibrated (based on Simpson et al. 2011, Stolarski et al. 2011) by fitting the molecular data using BEAST 1.6.2 (Drummond and Rambaut 2007), and then

randomly resolved for nodes not supported by DNA sequence data with PolytomyResolver (Kuhn et al. 2011). The result was 1000 Bayesian posterior trees that incorporate phylogenetic (i.e., topological and branch length) uncertainties (Huang and Roy 2013).

Trees were pruned to subsets of species based on the availability of various trait data, following the approach in Kerr et al. (2011). This yielded two sets of trees with taxa totaling 357 (reproductive mode) and 252 (symbiont transmission strategy). The degree of clustering of each binary trait on the tree was calculated with the *D* statistic (Fritz and Purvis 2010), which provides a measure of phylogenetic signal on each trait ($D = 0$, clumped under Brownian motion; $D = 1$, random).

The two life history strategies, reproductive mode and symbiont transmission strategy, comprise four possible states when examined in combination—horizontal brooding, horizontal spawning, vertical spawning, and vertical brooding. We estimated transition rates on the tree between these states using BayesTraits version 2 (Pagel 1994, Pagel 2013). The algorithm assumes that the evolution of both traits simultaneously is infinitesimally small, therefore only single trait transitions were considered (i.e., transitions across the internal space in Fig. 6.1 were impossible). For complete overlap of taxon sampling between both traits, the 252-species phylogeny was used. Transition rates for a given set of parameters were modeled for each of the 1000 posterior trees with 100 maximum likelihood replicates per tree (1000 for the dependent full model). Each tree generated a maximum likelihood score for the particular model examined, the mean of which was compared between models using a likelihood ratio test based on the Akaike Information Criterion (AIC). We first fit a full dependent (8-parameter) model, in which

the rate of change in one trait was dependent on the state of the other, and an independent (4-parameter) model, which assumed that the traits evolved independently. If the full dependent model was a significantly better fit than the independent model, complexity was reduced in a stepwise fashion by equating transition rates that were close in value or to zero, one at a time. The fewer-parameter models were subsequently compared against the dependent model with the AIC. For the best-fit model, rate uncertainties were estimated using a Markov chain Monte Carlo (MCMC) approach, analyzing the model five times for 11 million generations each. The first million iterations were discarded, and the remaining posterior samples were concatenated, from which the median transition rate and 95% highest posterior density (HPD) were summarized for each transition.

We repeated the correlation analysis to examine the relationship between symbiont transmission strategy and susceptibility to bleaching. Transitions between the states horizontal with low susceptibility, horizontal with high susceptibility, vertical with high susceptibility, and vertical with low susceptibility, were modeled using BayesTraits version 2 with the same procedure above. The 252-species phylogeny was further trimmed to 232 tips for complete taxon overlap between the two traits examined here.

RESULTS

Negative values of the *D* statistic revealed strong phylogenetic signal for both reproductive mode (mean -4.70 ± S.D. 2.80) and symbiont transmission strategy (mean -4.70 ± 2.81). The full dependent model of symbiont transmission strategy and reproductive mode was a significantly better fit to the tree than the independent model ($p < 0.0001$) indicating that these two characters are correlated with each other. Model

complexity reductions settled on a best-fit model comprised of six unique transition rates (Fig. 6.2).

Symbiont transmission strategy evolved at eight times the rate of reproductive mode (*q13*+*q31*+*q24*+*q42*/*q12*+*q21*+*q34*+*q43*). Strikingly, transitions between symbiont transmission strategies among spawners were equal to zero in both directions (*q42* and *q24*) and quite high in brooders. This suggests vertical transmission was never gained or lost within spawning species, but arose only through transitions from vertical brooders (*q34*).

In brooding species, vertical brooding evolved from horizontal brooding (*q13*) at ten times the rate of the opposite change (*q31*). Brooders were also eight times more likely to evolve spawning (*q12*+*q34*/*q21*+*q43*), and this transition was thirty times greater among horizontal transmitters (*q12*) relative to vertical transmitters (*q34*). When transitions from spawning to brooding occurred, the rate was two and a half times greater among vertical transmitters (*q43*/*q21*).

In sum, our calculated rates of evolution suggested three noteworthy conclusions: 1) symbiont transmission strategies evolved only among brooders, 2) transitions between the common states of horizontal spawning and vertical brooding evolved through a horizontal brooding intermediary, and 3) directional selection was greatest when transitions occurred between reproductive strategies (rather than symbiont transmission strategy).

Susceptibility to bleaching showed strong clumping on the tree (mean $D = -1.2 \pm 0.11$). The independent model was a significantly worse fit to the tree than the full dependent model ($p < 0.01$), which was further reduced to four parameters (Fig. 6.3).

Evolutionary rates toward low susceptibility were one and a half times higher than the reverse and depended on symbiont transmission strategy. Highly susceptible vertically-transmitting species did not evolve low susceptibility without first losing vertical symbiont transmission ($q43 = 0$ and $q42 > 0$), though they did evolve high susceptibility to bleaching ($q34$). Horizontally-transmitting species were more than twice as likely to evolve low susceptibility than high ($q21/q12$), though there was a relatively high rate of evolution from low to high susceptibility among horizontal transmitters ($q12$) relative to all other rates. With respect to bleaching susceptibility, highly susceptible species only evolved horizontal ($q42$) but not vertical ($q24$) transmission.

DISCUSSION

The high level of phylogenetic clumping associated with symbiont transmission strategy and reproductive mode demonstrates there is tendency for closely related species to have similar life history characteristics (Derrickson and Ricklefs 1988). Biologically meaningful analyses of the trait associations examined here thus require the phylogenetic comparative method to account for variations resulting from common descent (Ashton 2000, Blomberg et al. 2002, 2003, Fritz and Purvis 2010). The highly conserved nature of coral sexuality (gonochorism vs. hermaphroditism) has been highlighted recently by Baird et al. (2009). Here we show quantitatively that reproductive mode, which the previous study notes is relatively plastic, and transmission strategy are also conserved on the coral tree of life.

Based on the best-fit model of trait correlation between symbiont transmission strategy and reproductive mode (Fig. 6.2), our rate estimates reveal that transmission

strategy is characterized by rapid transitions among reproductive life histories: it evolved eight times faster than reproductive mode, which has been found to evolve four times faster than sexuality (Kerr et al. 2011). This demonstrates that symbiont transmission strategy is highly labile over evolutionary timescales among corals, although this flexibility is constrained to brooding species. While symbiont transmission strategies show high rates of transitions, directional selection (rate of one transition relative to the reverse) is stronger between reproductive modes. As a result, we infer greater selection for specific reproductive modes than specific symbiont transmission strategies, consistent with the highly labile nature of the latter.

The BayesTraits model assumes that extinction is minimal and independent of the character traits being assessed. Yet, because character trait reconstructions rely on the traits exhibited by extant species, calculated transition rates may also reflect differential extinction (Paradis 2005; Maddison 2006), in this case due to reproductive mode or symbiont transmission strategy. Furthermore, the model assumes speciation rates do not differ among trait states. In nightshade plants for example, species that are self-incompatible tend to speciate faster than species capable of self fertilizing (Goldberg et al. 2008). Brooding coral species are more likely to be self-compatible than spawning species (Carlon 1999), possibly leading to currently unknown differences in speciation rates between brooders and spawners. Therefore, additional analyses that compare fixed versus differential rates of speciation between traits can add further insight into the findings presented here (e.g., Binary State Speciation and Extinction, or BiSSE; Maddison et al. 2007; FitzJohn 2010, 2012).

Spawning species show negligible flexibility in symbiont transmission strategy

The strong statistical support for correlated evolution of symbiont transmission strategy and reproductive mode shows that the probability of transitioning between vertical and horizontal symbiont transmission depends on whether a species spawns or broods. Evolutionary transition rates are most consistent with the "local symbiont" hypothesis (Fig. 6.1A), with one exception. Unexpectedly, transitions between vertical and horizontal symbiont transmission among spawners were equal to zero in both directions. This demonstrates that vertical symbiont transmission was only gained or lost among brooding species, suggesting the genetic factors responsible for vertical transmission evolved only among brooders.

Vertical transmitters were twenty-five times more likely to evolve brooding, consistent with associated costs of bearing symbionts during dispersal in spawning species (Yakovleva et al. 2009, Nesa et al. 2012, Chapter 5 this dissertation). Interestingly, horizontal transmitters were almost thirty times more likely to evolve spawning, suggesting a high degree of selection *for* horizontal spawning rather than an arrival at this state due to selection *against* vertical spawning. This raises the question of why horizontal brooders would evolve horizontal spawning, in particular when short-dispersing, symbiont-free larvae (horizontal brooding) are presumably capable of acquiring locally-adapted symbionts.

Horizontal transmission and larval dispersal patterns

Behavioral evidence suggests harboring symbionts enhances survival in brooding coral species (Harii et al. 2002, Harii et al. 2009) and consistent with this inference, our

model found that brooders are ten times more likely to evolve vertical transmission. Thus, lacking the apparent benefit of harboring *Symbiodinium* may put larvae of horizontal brooders at a competitive disadvantage. On the other hand, evolving horizontal spawning from horizontal brooding may be advantageous by providing a means to disperse to locations of lower community density. Indeed, spawning can facilitate greater dispersal than brooding (Sammarco and Andrews 1989, Graham et al. 2008, Underwood et al. 2009) and prolonged dispersals can reduce competition, parent-offspring conflict, and allowing individuals to locate high quality habitats (Grosberg 1981, Kamel et al. 2010).

Despite the apparent benefits of spawning for dispersal, Strathmann et al. (2002) find little convincing evidence that dispersal is selected for in species with planktonic larval stages, but argue it is instead a byproduct of the migration into and out of the water column. These authors suggest larvae should remain in the plankton so long as survival is higher than on the benthos. In this context, we suggest that a divergence of pressures arises among corals whereby symbiont-free larvae benefit from staying in the water column (avoiding competitive dominants) while symbiont-bearing larvae favor rapid settlement (competitive dominance, remaining on the natal reef, avoiding symbiont-induced stress in the plankton; Hartmann et al. 2013).

This divergence would explain evidence for strong selection away from horizontal brooding in two directions, and in particular why selection for horizontal spawning over horizontal brooding is relatively strong. Consistent with this, spawned eggs tend to be positively buoyant, a character that is beneficial for life in the plankton (Chapter 2 this dissertation). Brooded larvae, in contrast, tend to become neutrally or negatively buoyant

soon after release and are often larger in size, likely facilitating high competitive ability on the benthos (Carlon and Olson 1993).

Evolution of symbiont transmission strategy and reproductive mode in response to environmental change

Short dispersal and high recruitment rates associated with brooding likely explains the proportional increase in Atlantic brooding species after a mass extinction during the Oligocene-Miocene transition, thought to result from decreased water temperature and increased turbidity (Edinger and Risk 1994, Edinger and Risk 1995). A recent ancestral state reconstruction of coral sexuality and reproductive mode found that evolutionary transitions to brooding could also explain the skew towards brooding species in the Atlantic during the Oligocene-Miocene transition (Kerr et al. 2011). The authors found that brooders were one hundred times more likely to evolve hermaphroditism (from gonochorism) than were spawners. Relative to spawning species, brooders have a higher degree of parental care and are better able to produce viable offspring via self-fertilization, which is only possible among hermaphrodites (Benzie et al. 1995, Brazeau et al. 1998, Carlon 1999). Thus, the combination of brooding and hermaphroditism may increase local and absolute recruitment in brooders, which is likely advantageous during times of decreased adult survival and increased risk of allee effects.

While brooding enhances success during periods of environmental change (Edinger and Risk 1995, Knowlton 2001), shifts in environmental conditions are not likely to favor vertical symbiont transmission (Yakovleva et al. 2009, Hartmann et al. 2013). Our model found appreciable rates of evolution from vertical to horizontal

brooding, a transition that represents the interruption of transgenerational symbiosis between a host and symbiont that have presumably co-evolved (LaJeunesse et al. 2004, van Oppen 2004). Such a scenario could be expected when the type of symbiont(s) harbored by parents become unsuitable for offspring, regardless of dispersal distance, as would be expecting during rapid environmental change. The loss of vertical transmission could allow offspring the flexibility necessary to take up symbiont types better suited for novel environmental regimes, analogous to the scenario experienced by long-dispersing, horizontally-transmitting species with each generation (Knowlton and Rohwer 2003, Nussbaumer et al. 2006).

Symbiont transfer strategies mediate species-specific bleaching susceptibility

In order to better understand associations between adult mortality and reproductive life histories, we examine how the degree of adult bleaching susceptibility relates to symbiont transmission strategy. Bleaching is a primary cause of mass coral mortality in response to increasing seawater temperatures, thus we regard it as a proxy for mortality risk and species responsiveness to environmental fluctuations. The correlated nature of our model demonstrates that bleaching susceptibility depended on symbiont transfer strategy, with consistent evidence that horizontal symbiont transfer might lower susceptibility to bleaching.

The strength and direction of transitions were consistent with a possible selective force for the loss of vertical transfer, which allows offspring to acquire locally appropriate symbionts. Specifically, vertically-transmitting species could only evolve low susceptibility after first evolving horizontal transmission, and horizontally-transmitting

species were more than twice as likely to evolve low susceptibility than high. Highly susceptible species only evolved horizontal transmission, while species with low susceptibility moved between symbiont transmission strategies, evolving horizontal transmission at a rate of more than two to one. Applied to periods of environmental change, this highlights how species may lose vertical transfer in order to reduce their bleaching risk.

The correlation between symbiont transfer strategy and susceptibility of adults to bleaching further suggests that strategies for transferring symbionts across generations play an important role in mediating species' risk of symbiotic failure. Our results also imply that despite apparently higher tolerance of marginal conditions and environmental change, brooding corals are vulnerable to reduced reproductive success and higher adult bleaching as global temperatures continue to rise due to their strong association with vertical symbiont transmission.

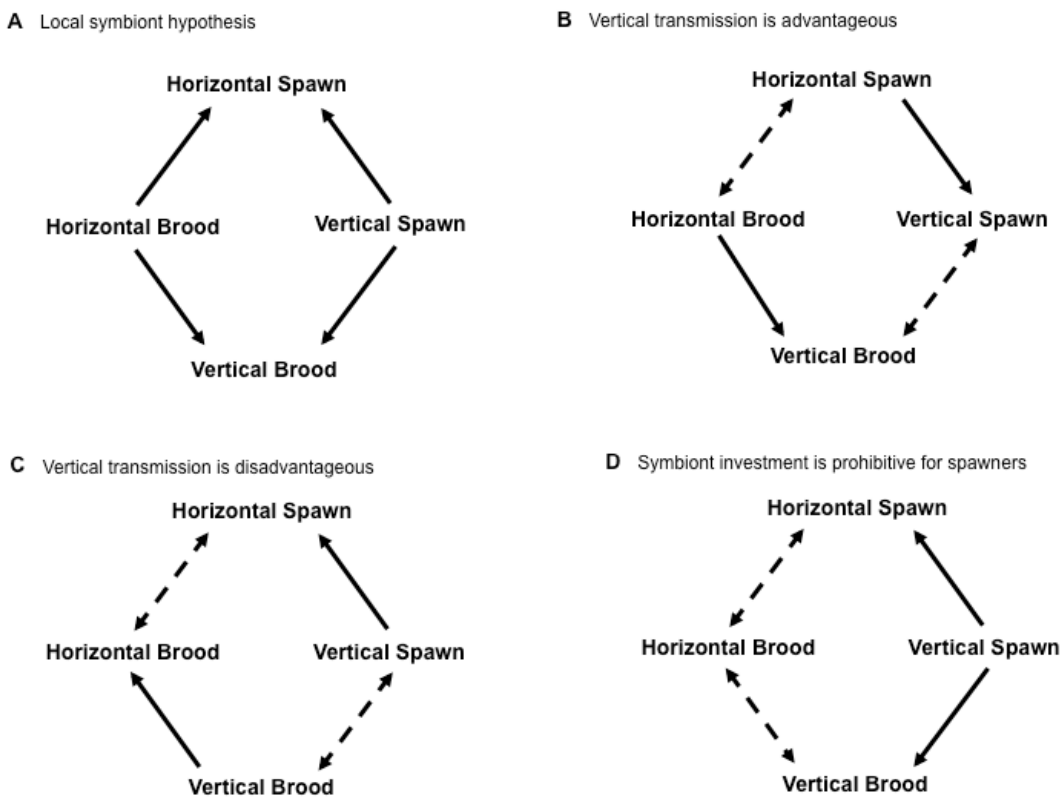


Figure 6.1: Hypothesized differences in evolutionary rates between horizontal brooding, horizontal spawning, vertical spawning, and vertical brooding strategies based on potential selection pressures. It is assumed that transitions between both states cannot occur simultaneously. A) The "local symbiont" hypothesis, which predicts that brooding species were more likely to gain vertical transmission than to lose it and spawning species were more likely to lose vertical transmission than to gain it. B) Vertical transmission provides energy to offspring, which predicts that species exhibiting either reproductive modes are more likely to gain than to lose vertical symbiont transmission. C) Horizontal transmission protects larvae from stress-induced mortality, which predicts that species exhibiting either reproductive modes are more likely to lose than to gain vertical symbiont transmission. D) The investment of symbionts is prohibitively costly in spawning species due to the stochastic investment of algal cells in eggs, which predicts that spawning species more likely to lose than to gain vertical symbiont transmission.

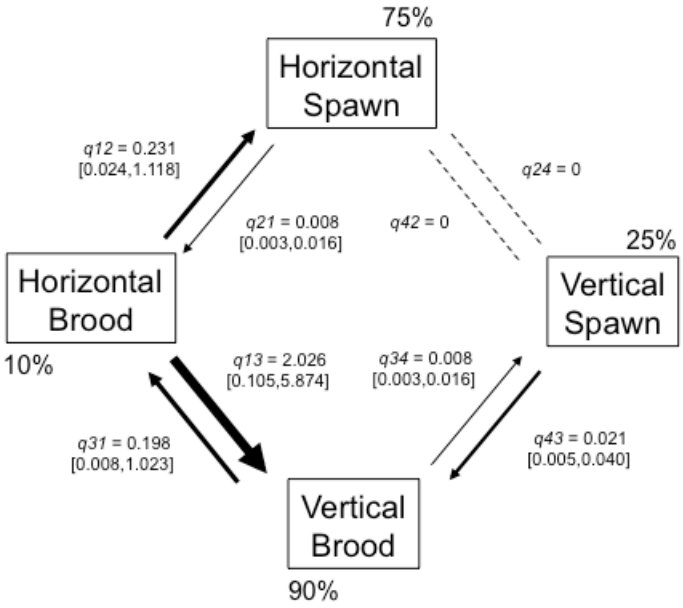


Figure 6.2: A flow diagram of derived transition rates between horizontal brooding (0,0), horizontal spawning (0,1), vertical spawning (1,1) and vertical brooding (1,0) based on the best-fit, six-parameter model. Transition rates (*qij*) represent the instantaneous rate of transition from trait *i* to *j*, as defined in Pagel (1994). The ranges in parentheses represent the 95% HPD. Arrow thicknesses reflect order of magnitude differences in transition rates and dashed lines represent transition rates equal to zero. Percentage values associated with each of the four states represent the percentage of extant species demonstrating that state with respect to reproductive mode (i.e., among brooders or among spawners).

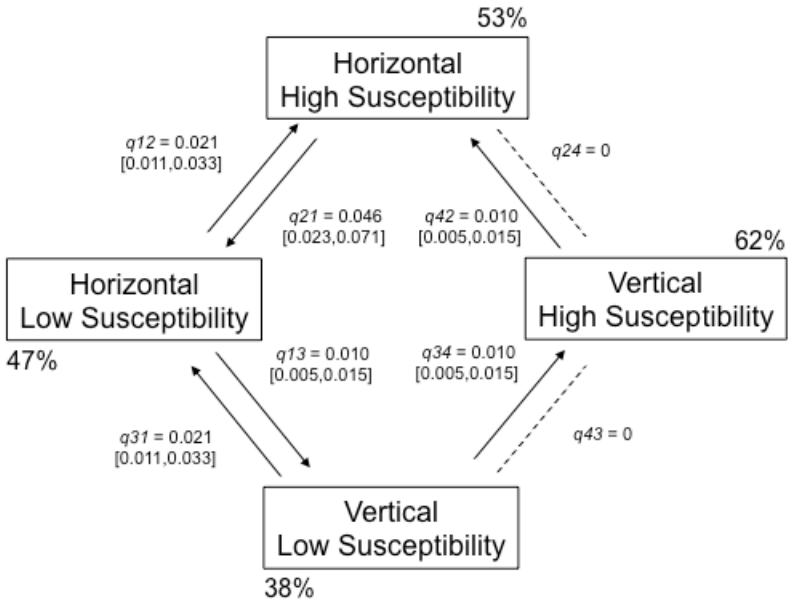


Figure 6.3: A flow diagram representation of derived transition rates between symbiont transmission strategy and susceptibility to bleaching. Transition rates (*qij*) represent the instantaneous rate of transition from trait *i* to *j*, as defined in Pagel (1994). The ranges in parentheses represent the 95% HPD. Arrow thicknesses reflect order of magnitude differences in transition rates and dashed lines represent transition rates equal to zero. Percentage values associated with each of the four states represent the percentage of extant species demonstrating that state with respect to reproductive mode (i.e., among brooders or among spawners).

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Chapter 6, in full, is currently being prepared for submission for publication of the material. Hartmann AC, D Huang. The dissertation author was the primary investigator and author of this paper.

Chapter 7.

CONCLUSIONS

The study of the coral-algal symbiosis dates back decades. Early work primarily focused on the transfer of energy from symbionts to host (e.g., Muscatine and Hand 1958, Muscatine and Porter 1977), while more recent studies have examined the breakdown of symbiosis in response to environmental change (e.g., Lesser 1996, Lesser 1997). This shift in research directions largely arose in response to the global crisis facing coral reefs, brought on by precipitous declines in corals as a result of symbiotic breakdown caused by rising global temperatures (Brown 1997, Hoegh-Guldberg et al. 2007).

In recent decades these two lines of questioning—energy transfer and the initiation or failure of symbiosis—have spilled over into the study of larval corals (e.g., Richmond 1987, Schwarz et al. 1999, Voolstra et al. 2009, Dunn and Weis 2009). In corals, larvae of some species are born with symbionts (vertical transmission) while others must acquire them from the environment (horizontal transmission). Thus, larvae of certain species can be without symbionts yet remain fully functional, in contrast to the stress experienced by adults. Thus, larvae are particularly interesting and useful for the study of energetic exchange and the initiation of symbiosis in corals.

Investigations into these two topics have occurred primarily in isolation, in part because energetic studies have focused on vertically-transmitting species while symbiotic initiation studies have been understandably confined to horizontal transmitters. Among all studies, there have been few experimental tests of the ecological implications of symbiosis in larvae or the ways in which associated life history characters influence species-specific tolerance of environmental change. In this dissertation I sought to bring

these two lines of inquiry together by examining the potential energetic role of symbionts in horizontally-transmitting species, comparing how vertical and horizontal transmitters respond to harsh environmental conditions, and determining how each of these strategies influences species' susceptibility to bleaching.

Evidence from both directly tracking of fixed carbon and inferring from the reduced use of other energy sources suggests that symbionts are a source of fixed carbon for coral larvae in vertically-transmitting species (Richmond 1987, Harii et al. 2002, Alamaru et al. 2009, Harii et al. 2010, Gaither and Rowan 2010). In one species more energetic lipid stores were consumed in larvae placed in the dark and these individuals experienced lower survivorship, suggesting that energy derived from symbionts increases fitness (Harii et al. 2010). Evidence for energetic benefits of symbionts in vertical transmitters have led many to conclude that this strategy allows larvae increase survival and prolong competency (Richmond 1987, Raimondi and Morse 2000, Baird et al. 2009, Harii et al. 2010). Despite this, vertical transmission is associated with brooding reproduction and short dispersal periods (Graham et al. 2008, Underwood et al. 2009, Baird et al. 2009) rather than the expected long larval periods based on the presence of a renewable energy source.

To address this discrepancy I measured energy use as lipids in a horizontally-transmitting species with a long larval period, which was readily infected with symbionts in the laboratory (Chapter 5). I found that while symbionts were actively photosynthesizing within larvae, symbiont photosynthesis did not offset lipid use, suggesting symbiont-derived energy is not useful in this species despite a long larval period. Instead, symbiont uptake led to disadvantageous behavioral changes, such as

small reductions in survival as well as reduced swimming activity, which may reduce an individual's likelihood of settling. This implies that energetic exchange is fundamentally different in larvae that gain symbionts via horizontal or vertical transmission. The lack of energetic gains in the horizontally-transmitting species may have occurred because of the large changes in metabolism necessary to accommodate the symbiont, evidence for which I found in gene expression of symbiont-infected larvae (Chapter 5).

I identified gene expression patterns associated with the initiation of metamorphosis upon infection, consistent with past studies of symbiont acquisition in larvae (Reyes Bermudez et al. 2009, Grasso et al. 2011, O'Rourke 2011). This result implies that the uptake of symbionts does not prolong the larval period in horizontal transmitters. Based on the lack of energetic gains, as well as decreased survival and patterns of apoptosis and immune responses upon infection, I concluded that acquiring symbionts is likely an important and beneficial precursor to metamorphosis in horizontally-transmitting species, but is not beneficial during the larval period. Thus, in species that require cues for settlement, the early uptake of symbionts can be disadvantageous.

The apparent energetic and survival benefits afforded to larvae of vertical transmitters, coupled with evidence that horizontal transmitters experience behavioral dysfunction and small levels of mortality upon infection, begs the question of why horizontal transmission exists at all. I began to explore this question by comparing the tolerance of larvae from horizontally- and vertically-transmitting species to harsh environmental conditions (Chapter 4). Furthermore, I examined how larval size plays a role in determining survival within and between species. I found that the vertical

transmitter was less tolerant of harsh environmental conditions, even months after larvae had experienced them. In contrast, the horizontal transmitter was unaffected by harsh conditions. The sensitivity of larvae of vertically-transmitting species suggests they lack the physiological flexibility to tolerate different environments, likely the result of stress induced by the presence of larvae, much like the bleaching in adults (Yakovleva et al. 2009, Nesa et al. 2012).

My experiment also found that relatively large larvae in the vertical transmitter had higher settlement and post-settlement survivals compared to smaller conspecifics. The benefits of large birth size persisted for nearly half a year and across the metamorphic boundary, which involves a massive remodeling of tissues. The advantage of being large may have been the result of proportionally higher stores of slowly-metabolized wax ester lipids in large larvae (Chapter 3) that may beneficial despite evidence that mass-specific metabolic demand is similar between large and small invertebrate larvae (Moran and Allen 2007). Indeed, the process of metamorphosis can be more energetically taxing for small invertebrate larvae when compared to larger conspecifics (Wendt 2000). This evidence suggests that the benefits of being large may have been derived from differences in the relative amount of energy required for metamorphosis relative to smaller individuals.

The above findings suggested a paradigm whereby larvae of vertically-transmitting species (and associations with brooding and short larval periods) are energy-driven early in life, gaining nutrition from symbionts and an advantage from being relatively large at birth. These factors may allow these species to be strong competitors and may be common among species with similar life histories (Vermeij and Sandin

2008). Yet, competitive advantages appear to come at the expense of environmental tolerance, largely due to their affinity for harboring symbionts. In contrast, larvae of horizontally-transmitting species tend to be smaller and likely competitive inferiors, which may explain their long searching behaviors (Vermeij et al. 2006), high degree of specificity for positive metamorphosis cues (Grasso et al. 2011, Tebben et al. 2011, Siboni et al. 2012), and microbe-mediated aversion for settling near conspecific adults (Marhaver et al. 2013).

Horizontal transmitters gain an advantage by lacking symbionts, as this trait contributes to higher tolerance of harsh environments and allows larvae to take up symbionts that are adapted to the location in which they settle and metamorphose, a paradigm I have called the 'symbiont-settlement coupling' hypothesis. This flexibility may be particularly beneficial during times of environmental change because of the stress that may arise in vertical transmitters due to their association with symbionts that become maladaptive to the novel environmental regime. As a result, vertical transmitters may benefit from local recruitment and be vulnerable during times of environmental change.

I found evidence for differential sensitivity using a character trait reconstruction that suggested species with vertical transmission favored transitions to low bleaching susceptibility, likely as an evolved strategy to reduce bleaching risk (Chapter 6). By putting these strategies into an evolutionary context, I gained further evidence of their adaptive potential. The connection I identified between the presence of symbionts in larvae and adult susceptibility to bleaching implies that variability in environmental conditions (the primary cause of bleaching) is an important force maintaining horizontal and vertical symbiont transmission, which is consistent with my experiments.

By combining disparate lines of scientific reasoning related to energy transfer and environmental tolerance in coral-algal associations during the larval stage, I have provided mechanistic insights that may explain the apparent paradox that long-dispersing species must find symbionts from their environment while short dispersers are born with a renewable energy source. My questions were guided by the life histories of extant corals and took into account fundamental knowledge of their biology, built over decades of careful study of this important symbiosis in the sea.

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LETTERS

edited by Jennifer Sills

Conservation Concerns in the Deep

IN THEIR PERSPECTIVE "A DIVE TO CHALLENGER DEEP" (20 APRIL, P. 301), R. A. Lutz and P. G. Falkowski highlight the exciting advancements in engineering that allowed director James Cameron to dive to Challenger Deep, the deepest point on Earth. As Lutz and Falkowski point out, ever-improving submarine technologies are broadening scientific research capabilities in the planet's largest and least-explored ecosystem—the deep sea. Cameron's historic dive, and the international attention it garnered, has reinvigorated humanity's fascination with this otherworldly ecosystem. Missing from most reports, however, is that although reaching the deepest points on Earth requires great feats of engineering, the deep sea is not beyond the touch of human activities.

At depths close to that of Challenger Deep, bovine DNA was found in the guts of scavenging amphipods, presumably from ship galley discards (*1*). Moreover, large-scale disposal of refuse and extraction of resources such as fishes, hydrocarbons, and soon minerals currently threaten this ecosystem, and changes in ocean temperature, oxygen availability, and pH are expected to have increasing impacts in coming decades (*2*). Thus, when considering future research objectives, it is important to consider not only the scientific and natural history gains to be made, but also the conservation concerns that are both notable and accelerating in the farthest reaches of our planet. Further exploration of the type championed by James Cameron will lead to greater understanding of species interactions and geochemical cycling in the deep sea. With this ever-growing knowledge, we must protect and manage deep-sea resources in a sustainable manner, and in doing so avoid destruction borne from ignorance like that on the imaginary planet Pandora in Cameron's other recent success—the film *Avatar*.



***Hirondella gigas*.** Collected in the Challenger Deep.

AARON C. HARTMANN* AND LISA A. LEVIN

Center for Marine Biodiversity and Conservation, Scripps Institution of Oceanography, La Jolla, CA 92043, USA.

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Presumed Guilt in the Anthrax Case

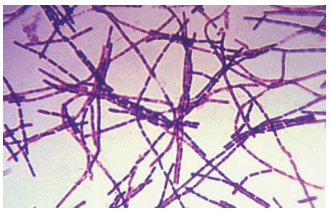
IN HIS REVIEW OF MY BOOK *AMERICAN Anthrax* on the 2001 anthrax letter attacks ("Have we 'met the enemy?,'" 3 February, p. 540), D. A. Relman accuses me of imposing a "presumption of guilt" on the FBI's prime suspect, U.S. Army microbiologist Bruce Ivins. In fact, I described many ambiguities in the case against Ivins and took no personal position on his guilt or innocence. I relied on the report of the National Research Council committee that evaluated the FBI science, and, regarding a possible foreign source for the letter spores, I accepted its conclusion that "We consider these data to be inconclusive regarding the possible presence of *B. anthracis* Ames at this undisclosed overseas site" (*1*). Relman served as vice chair of the committee that reached this conclusion.

JEANNE GUILLEMIN

Security Studies Program, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. E-mail: jeg@mit.edu

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1. National Research Council, "Review of the scientific approaches used during the FBI's investigation of the 2001 anthrax letters" (National Academies Press, Washington, DC, 2011); www.nap.edu/catalog.php?record_id=13098.



Bacillus anthracis

CREDIT: CENTER FOR DISEASE CONTROL AND PREVENTION

Response

IN MY REVIEW OF *AMERICAN ANTHRAX*, I SOUGHT to highlight the valuable contributions of this book to the public understanding of this complex and controversial case, as well as the book's shortcomings. It is true that Guillemin makes no explicit statement about her position regarding the guilt or innocence of Ivins. However, statements such as, "the FBI had solid scientific proof that the spores in the anthrax letters matched those in a flask, labeled RMR (Reference Material Receipt)-1029, that was in Ivins' keeping at the Army's medical institute at Detrick" (p. xxii), and "a criminal—either Ivins or someone else—had used the institute's Ames anthrax spores to commit murder" (p. 251), misrepresent the strength of the scientific evidence that points to this flask as the source of the spores in the letters (and

Twenty-four years ago, the US government was also trying to attract young people into STEM (T. Packard *Eos* **70,** 709; 1989). Then, 66% of the ocean-science community was living hand-to-mouth on short-term government grants. A university professor was expected to do 40 hours of teaching and administration a week, and 40 hours of research. Researchers who did not receive funding from their universities dared not spend time away from their work, lest their publication record should drop.

It was clear to me at the time that if research centres, universities, governments and societies wanted more people to work in science and technology, then salaries, job stability and job security would have to improve. They still have not.

Young people continue to shun research and instead opt to use their mathematical skills in accounting, their analytical skills in investment banking and their love of science in medicine. Macilwain blames business for the woeful range of scientific opportunities available to graduates. Whether the fault lies with business, government or universities, the educational pipeline in science and engineering does not work because graduates are scared off by what they see as a meat grinder at the other end.

Theodore T. Packard *University of Las Palmas de Gran Canaria, Spain.*
theodore.packard@ulpgc.es

Education: science literacy benefits all

Colin Macilwain wields too wide a brush in painting US federal funding of STEM education (for promoting 'science, technology, engineering and mathematics') as having the sole purpose of bolstering the workforce (*Nature* **497,** 289; 2013). This funding also achieves general science literacy, particularly when it is directed towards children in primary and secondary education or undergraduate students.

No matter how far they are pushed, most teens and young adults will not become scientists. Fortunately, many STEM programmes familiarize students with the scientific process and with the natural world. Learning fundamental concepts also teaches them how to interpret and handle scientific information.

Science literacy subsequently benefits individuals throughout their lives, from forming opinions about proposed government policies to making health-care decisions. A well-informed citizenry, in turn, pays dividends to society as a whole.

Aaron C. Hartmann *University of California, San Diego, USA.*
achartma@ucsd.edu

Climate and war: a call for more research

The possibility that climate change could be responsible for violent conflict (A. Solow *Nature* **497,** 179–180; 2013) is starting to influence how governments frame and react to climate change. However, a real problem in this area is a paucity of theory to explain the associations (if any) between climate change and the outbreak of violence.

One overlooked factor is that populations caught up in conflicts or living in post-conflict societies are often more vulnerable to climate change. For example, the presence of landmines makes productive land inaccessible.

Climate policies can themselves be a source of conflict (see go.nature.com/zutmox). Measures that manage carbon sources and sinks or treat them as commodities — such as land-use changes, hydropower development or initiatives to reduce emissions from deforestation — can stimulate civil unrest if implemented without adequate checks.

Poverty, a history of fighting, and weak governance are well-established risk factors for conflict. The likelihood of violent conflict is reduced by democracy, social protection, effective justice systems and the protection of property rights. The influence of climate change on these factors warrants further investigation to guide policy-makers in promoting peace and prosperity in a changing climate.

Neil Adger *University of Exeter, UK.*
n.adger@exeter.ac.uk
Jon Barnett *University of Melbourne, Victoria, Australia.*
Geoff Dabelko *Ohio University, Athens, USA.*

Climate and war: no clear-cut schism

We are sceptical about the effectiveness of Andrew Solow's proposals for cooling the debate over a possible link between wars and climate change (*Nature* **497,** 179–180; 2013). We think that the division between the two sides ('quants' versus 'quals') is not as clear-cut as he implies.

Solow argues that this dividing line distinguishes between those who search for connections between violence and natural phenomena, including climate-related factors (quants), and those who prefer to explain conflicts as social processes (quals). But both approaches are studied by quants as well as quals. Quants may study climate-related effects on conflicts by analysing single events in detail or by considering many wars on aggregate using statistics.

There are also strong disagreements among those on each side of Solow's dividing line. For example, quants as well as quals include both proponents and sceptics of the connections between climate change and violent conflict.

In our view, the true divide is not so much about substance as about perspective.

Michael Brzoska, Jürgen Scheffran *University of Hamburg, Germany.*
brzoska@ifsh.de

Gender equality in Australian academies

Women are not under-represented across all learned academies in Australia (see *Nature* **497,** 7 and *Nature* **497,** 439; 2013). For example, the Australian Academy of Technological Sciences and Engineering (ATSE, of which I am president) has taken steps to ensure that women are appropriately recognized and included in all its activities.

Gender imbalance can adversely affect all stages of a scientific career, from tertiary education to employer recruitment, retention and promotion, with implications for a country's productivity and prosperity. Over the past three years, ATSE has led the way in identifying and promoting female talent across the science and technology sector in Australia, and within the academy itself.

One key element of ATSE's gender-equality policy is to identify women candidates for fellowship nomination through active search and mentoring processes. Last year, 10 of 37 elected fellows were female, and women now comprise 40% of ATSE's governing board.

Alan Finkel *ATSE, Toorak, Australia.*
alan@finkel.net

European concerns over GM salmon

As investigators for the European Food Safety Authority into the environmental risks posed by genetically modified (GM) fish, we are concerned about the US Food and Drug Administration's imminent approval of GM salmon (*Nature* **497,** 17–18; 2013).

This is a huge step that could encourage aquaculture of other GM fish in other countries, and not necessarily under strictly biosecure conditions.

There is still considerable uncertainty surrounding the environmental and physiological effects of escaped, fast-growing GM fish on aquatic systems. This reflects a poor understanding of how different species might be affected as the modified gene is expressed in the wild.

Europe's regulatory guidelines for aquaculture of GM fish and other alien species in Europe will therefore be underpinned by rigorous risk assessment (see go.nature.com/p6x2qb).

J. Robert Britton *Bournemouth University, Poole, UK.*
Rodolphe E. Gozlan *Bournemouth University, Poole, UK; and Institut de Recherche pour le Développement (UMR 207), Paris, France.*
rudy.gozlan@ird.fr

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