SPAWNING DYNAMICS AND PARENTAL EFFECTS IN THE HAWAIIAN SCLERACTINIAN CORAL MONTIPORA CAPITATA

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A mi querida Pily
¿QUIÉN MUERE?

Muere lentamente quien se transforma en esclavo del hábito, repitiendo todos los días los mismos trayectos, quien no cambia de marca, no arriesga vestir un color nuevo y no le habla a quien no conoce.

Muere lentamente quien hace de la televisión su gurú.

Muere lentamente quien evita una pasión, quien prefiere el negro sobre blanco y los puntos sobre las "íes" a un remolino de emociones, justamente las que rescatan el brillo de los ojos, sonrisas de los bostezos, corazones a los tropiezos y sentimientos.

Muere lentamente quien no voltea la mesa cuando está infeliz en el trabajo, quien no arriesga lo cierto por lo incierto para ir detrás de un sueño, quien no se permite por lo menos una vez en la vida, huir de los consejos sensatos.

Muere lentamente quien no viaja, quien no lee, quien no oye música, quien no encuentra gracia en sí mismo.

Muere lentamente quien destruye su amor propio, quien no se deja ayudar.

Muere lentamente, quien pasa los días quejándose de su mala suerte o de la lluvia incesante.

Muere lentamente, quien abandona un proyecto antes de iniciararlo, no preguntando de un asunto que desconoce o no respondiendo cuando le indagan sobre algo que sabe.

Evitemos la muerte en suaves cuotas, recordando siempre que estar vivo exige un esfuerzo mucho mayor que el simple hecho de respirar. Solamente la ardiente paciencia hará que conquistemos una espléndida felicidad.

Pablo Neruda
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ABSTRACT

Coral reefs are some of the most diverse and productive ecosystems on the planet and function as important spawning, breeding and feeding areas for many organisms in the tropical seas. The persistence of these ecosystems relies on spawning events with remarkable synchronicity during limited periods each year and the ability of coral holobionts (animal host and symbiotic community) to acclimatize and/or adapt to environmental change. The goal of this dissertation was to advance our understanding about the spatial and temporal variation in coral reproductive capability and parental effects in scleractinian corals. Specifically, this work explored for the first time (1) the spawning dynamics of a major reef building coral (broadcast spawner) from the central Pacific using a systematic sampling of the gametes in the field, (2) the ultrastructure of the egg-sperm bundle and (3) how the variability in parental environmental and physiological conditions (host and Symbiodinium) relates to the phenotype of gametes in a scleractinian coral. Spawning dynamics of Montipora capitata varied among years, months and lunar days. Synchrony and proportion of spawning colonies did not reflect differences in coral colony morphology or environment between sites. Major changes in spawned material occurred in different years, suggesting that reproductive output may have been controlled by changes in the coral’s phenology and/or stress associated with larger temperature fluctuations. M. capitata is a coral with extraordinary phenotypic plasticity that can release a diverse array of gametes (depending on the parental environment) within a mass spawning event. Oocytes released by different parents were seeded with different Symbiodinium assemblages and had different biochemical traits, yielding taxonomic and phenotypic variability that may have profound implications for
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<tbody>
<tr>
<td>AMOVA</td>
<td>Analysis of molecular variance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELLSD</td>
<td>Evaporative light scattering detection</td>
</tr>
<tr>
<td>FESEM</td>
<td>Field Emission Scanning Electron Microscopy</td>
</tr>
<tr>
<td>FSW</td>
<td>Filtered seawater</td>
</tr>
<tr>
<td>GLM</td>
<td>General linear model</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly Significant Difference</td>
</tr>
<tr>
<td>ITS2</td>
<td>Internal transcribed spacer region 2</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
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<td>Polymerase chain reaction</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>Triacylglycerol</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>UV</td>
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CHAPTER 1

GENERAL INTRODUCTION

Coral reefs are one of the most productive and diverse ecosystems on Earth (Connell 1978). They provide protection and shelter for thousands of species in the tropical seas, and contribute to the economies of many island and coastal communities by supplying ecological services such as seafood, recreation, coastal protection and new biochemical compounds (Moberg and Folke 1999). Although coral reefs only cover 0.1-0.5% of the ocean floor (Smith 1978, Copper 1994, Spalding and Grenfell 1997), almost one third of the world’s marine fish species are present on coral reefs (McAllister 1991) and the catch from reef areas represents ~10% of fish consumed by humans (Smith 1978). At least tens of millions of people in more than 100 countries have coastlines with coral reefs and depend on them for their livelihoods (Salvat 1992).

Unfortunately, coral reefs are declining rapidly throughout the world due to pollution, coastal development, overexploitation (Hughes et al. 2003, Pandolfi et al. 2003, Bruno et al. 2003, Bellwood et al. 2004, Lough 2008) and effects associated to global climate change, such as rising ocean temperatures and ocean acidification (Hoegh-Guldberg et al. 2007, De’ath et al. 2009).

Rising temperatures have triggered bleaching events (loss of coral color due to the expulsion or death of symbiotic algae) and damaged corals reefs across large areas of the world’s tropical oceans. In the Indian Ocean for example, shallow areas had up to 90% of mortality after a severe bleaching episode during El Niño in 1998 (Wilkinson et al. 1999). Furthermore, the declining of pH of the upper seawater layers due to absorption of CO₂ (ocean acidification) has been linked to reduced coral calcification (De’ath et al. 2009),
which can compromise the formation of the complex three dimensional structure and primary framework of the reef which serves as an habitat of great diversity of reef-associated fauna and flora.

The persistence of healthy coral reefs depends on (a) the ability of corals to recover from environmental disturbances, (b) successful reproduction (gamete production, gamete release, fertilization) and (c) survival of the new recruits (Richmond 1997). All these processes are variable and are influenced by interactions between coral biology and spatial and temporal fluctuations in the environment (Tomascik and Sander 1987; Harrison and Wallace 1990; Richmond and Hunter 1990; Szmant and Gassman 1990; Hughes and Tanner 2000; Baird et al. 2009). Thus, understanding how coral reproductive processes can vary in space and time is critical to better predict the effects associated to climate change in these important marine ecosystems.

Reproduction in scleractinian corals

Corals reproduce both asexually and sexually. Asexual reproduction assures the continued existence of the genotype by producing genetically identical modules. Modes of asexual reproduction include fission/budding (Kramarsky-Winter and Loya 1996, Gilmour 2004), polyp bail-out (Sammarco 1982), polyp expulsion (Kramarsky-Winter and Loya 1997), fragmentation (Highsmith 1982) and development of an oocyte without fertilization (parthenogenesis) (Stoddart 1983, Ayre and Rsing 1986, Ayre and Miller 2004, Sherman et al. 2006) (Fig. 1.1). Sexual reproduction requires fertilization to occur and allows genetic recombination and the creation of new genotypes. Depending on where the fertilization occurs, corals can be classified as spawners or brooders. Spawners
release their gametes (oocytes and spermatozoa) into the water column for fertilization. Whereas brooders have their oocytes fertilized inside the polyps and larvae are released. Both spawners and brooders can be either gonochoric (male or female) or hermaphrodites (both sexes) (Harrison and Wallace 1990, Richmond and Hunter 1990). A recent review by Harrison (2011) showed that among 400 scleractinian species in which both the sexual patterns and mode of development are known, hermaphroditic broadcast spawning is the dominant form of sexual reproduction (258 species, 64%), gonochoric broadcast spawners are next in abundance (78 species, 19.5%) and the less common are hermaphroditic brooders (25 species) and gonochoric brooders (15 species). It is important to note that not all species belong strictly to one of these four main patterns and some corals (~12 species) exhibit mixed patterns of sexuality (gonochoric/hermaphroditic) or both modes of development (spawning/brooding) (Harrison 2011).

The abundance of species with distinct modes of development and sexuality differs among biogeographic regions (Baird et al. 2009). In the Atlantic, brooders are more abundant representing 50% of the corals species, compared to the Indo-Pacific in which brooders only represent 20% of the coral species (Szmant 1986, Baird et al. 2009). Hermaphroditic spawners in the Indo-Pacific are extremely abundant representing 70% of the species in this region (Baird et al. 2009).

Although the reproductive periods for many species of scleractinian corals are well documented (Harrison & Wallace 1990, Richmond & Hunter 1990, Baird et al. 2009, Harrison 2011), spatial and long-term temporal variation in coral reproductive capability has not been well studied because of the sporadic nature of reproductive
activities and the limited number of methodological approaches for exploring them (histology, dissection and periodic observations during the spawning nights); the same corals are rarely observed over multiple spawning periods and so temporal variation in spawning activities is not well understood, particularly in species that can release their gametes over consecutive nights and months (split-spawn) (Willis et al. 1985).

How climate change will affect the phenology of corals in terms of both spawning synchrony and reproductive output remains unknown. For sessile organisms, such as corals, changes in temperature could significantly impact the physiology of the colony (e.g., bleaching) and have deleterious effects on coral reproduction (Szmant & Gassman 1990, Ward et al. 2000, Mendes & Woodley 2002, McClanahan et al. 2009) and the recovery and maintenance of coral reef communities. As climate change continues, reproductive cues (e.g. temperature and light) that influence the timing of specific stages of gametogenesis and spawning could be decoupled due to an earlier arrival of spring (Hughes 2000). This could have dramatic consequences for species that require high spawning synchronicity for reproductive success and have restricted mating opportunities each year due to their length of the oogenetic cycles. Establishing a fundamental understanding of the factors controlling spawning synchrony and reproductive output is fundamental to recognizing the threats associated with climate change on the life history of the main reef builders.

**Parental effects**

Parental effects occur when the phenotype of the offspring is affected by the phenotype or environment of the parents (Mousseau and Fox 1998, Badyaev and Uller...
Although parental effects have been extensively studied in plants and insects and terrestrial vertebrates, these effects have received much less attention in the marine environment (Mousseau & Fox 1998, Marshall et al. 2008). Parental effects can significantly influence the life history (Donelson et al. 2009), competitive ability (Wulff 1986), evolutionary trajectories, speciation rates (Wade 1998) and population dynamics (Ginzburg 1998, Donohue 2009) of future generations of individuals. These effects also facilitate the perpetuation of symbiosis between generations by influencing the direct transmission of effective symbiotic microorganisms from parent to its progeny (Russell and Moran 2006).

Corals can acquire their dinoflagellate endosymbionts from their parents or the environment. Generally, offspring from brooders inherit algal symbionts (*Symbiodinium*) from their parent (vertical transmission), whereas offspring from spawners acquire their symbionts from the environment (horizontal transmission). However, there are brooders (isoporans) that can release larvae without algal symbionts and spawners from the genera *Montipora, Porites, Pocillopora* and *Anacropora* that can release oocytes with algal symbionts (Baird et al. 2009).

The cellular events associated with the uptake of *Symbiodinium* and the establishment of symbiosis have been studied in some detail in coral species that transmit *Symbiodinium* horizontally (Schwarz et al. 1999, Weis et al. 2001, 24, Little et al. 2004, Rodriguez-Lanetty et al. 2004, Marlow and Martindale 2007, Gomez-Cabrera et al. 2008, Abrego et al. 2009) and vertically (Hirose et al. 2001, Hirose and Hidaka 2006, Marlow and Martindale 2007), however, no studies have yet examined the diversity of *Symbiodinium* in the oocytes of a coral that transmits *Symbiodinium* vertically. Such
parental effects may have profound ramifications for larval behaviors, natural selection after settlement, the potential for acclimatization and ultimately the resilience of coral populations and reef communities. Corals with vertical transmission are more abundant in the Pacific, suggesting that larvae provided with algal symbionts can use energy sources provided by their photosynthetic symbionts (Rinkevich 1989) and travel for longer distances in the water column (LaJeunesse et al. 2004, Baird et al. 2009).

As climate change intensifies, parental effects may play an important role in the survival of offspring and the resilience of future generations. Offspring from parents with compromised health (e.g., corals affected by bleaching events or diseases) may have reduced physiological capacity and this could, in turn, prevent successful development and recruitment under stressful environmental conditions. For example, experimentally bleached parents of the soft coral *Lobophytum compactum* release oocytes with lower levels of protein, lipid, mycosporine-like amino acids (MAA) and carotenoid concentrations, which may jeopardize oocyte and larval viability (Michalek-Wagner and Willis 2001). That said, despite the vulnerability of most corals to drastic changes in the environment, corals from three major genera (Acropora, Pocillopora and Porites) have shown the capacity to increase their thermal tolerances following a mass bleaching event (Maynard et al. 2008). How this increase in thermal tolerance in coral adults affects the performance of their offspring remains unknown, but it is possible that parental effects may modify offspring to induce developmental variation in a particular environmental context (Sultan 2007, Badyaev and Uller 2009).

**Purpose of study**
The goal of this dissertation is to advance our understanding about (1) the spatial and temporal variation in coral reproductive capability and (2) the role of parental effects in scleractinian corals.

By exploring the *in situ* coral spawning dynamics we were able to help answer important questions such as: Do parents from different sites/morphologies spawn at the same time? Is the size of the parent important in the likelihood of spawning? Does the reproductive output differ between years, months or even days? Do parent release egg-sperm bundles with different attributes? Examining how the spawning dynamics differed through time (years, months, days) and space (sites, colony, polyp) will help to better understand the variability in reproductive behavior and obtain a necessary reproductive ecological baseline.

Parental effects were explored by determining the natural phenotypic variability of coral oocytes (host and symbiont) in order to answer questions such as: Does the diversity of symbiont assemblages in the gamete reflect the diversity of the parent? Is there a selective transmission of symbiont type to the gamete? Does the physiological status of the parent affect the biochemical status of the gamete? Is the relationship between parent and gamete influenced by differences in environments? Differences in the quality of the gamete may influence the survival and dispersal of the coral larvae and success of new recruits.

For this research we studied the reproductive biology of *Montipora capitata*, one of the most broadly distributed, morphologically plastic and important reef builders in the main Hawaiian Islands (Jokiel et al. 1983, Jokiel et al. 2004).
Specific aims addressed in this dissertation include:

1) Understand spawning dynamics of the coral *Montipora capitata* in Hawaii
   a. Temporally, how does spawning (synchrony and reproductive output) change over different years, months and lunar days.
   b. Spatially, how does spawning (synchrony and reproductive output) change over sites, colonies and polyps.

2) Explore the ultrastructure and formation of the gamete packaging (egg-sperm bundle) in the hermaphrodite broadcast spawner *Montipora capitata*.
   a. Describe the ultrastructure of the gametes and the egg-sperm bundle using electron microscopy.
   b. Propose models of egg-sperm bundle formation.

3) Examine the diversity of symbiotic algae (*Symbiodinium*) transmitted from parents to oocytes in the coral *M. capitata*.
   a. Characterize the phylogenetic diversity of *Symbiodinium* assemblages associated with different life stages of *M. capitata*.
   b. Identify how *Symbiodinium* assemblages relate to environmental and morphological characteristics of the parent.

4) Determine the parental effects of on the biochemical composition of the gamete.
   a. Characterize the biochemical composition of different life stages of *M. capitata*.
   b. Identify how the biochemical physiological status of the oocyte relates to the environmental and morphological characteristics of the parent.
Organization of dissertation

Specific aims 1-4 are addressed in chapters 2-5 respectively. Chapters 2-5 of this dissertation are written in a format for publication in peer-reviewed journals.

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CHAPTER 2

SPAWNING DYNAMICS OF A REEF BUILDING CORAL IN HAWAI’I

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ABSTRACT

The release of gametes into the water column by broadcast-spawning marine invertebrates represents an important culmination of several physiological events in the parents and the first step to the supply of new recruits. Spawning timing and synchronicity have been studied in several taxa, however there are still many gaps in our understanding of specific cues that drive the spawning events and the variability of reproductive outputs during such events under natural conditions. The persistence of important ecosystems such as coral reefs relies on spawning events with remarkable synchronicity during limited periods each year. Although spawning timing for many coral species is known, the variation in coral reproductive capabilities (e.g., coral fecundity, polyp fecundity, spawning synchronicity) has not been well studied because the limited number of methodological approaches for exploring them in situ. This study is the first to explore the spatial and temporal spawning dynamics of a major spawning broadcasting reef building coral from the central Pacific using a systematic sampling of the gametes in the field. Our results indicate that spawning dynamics of Montipora capitata vary among years, months and lunar days and that the synchrony and proportion of spawning colonies did not reflect differences in coral colony morphology and environment between sites. Major changes in reproductive output (spawned material) occurred in different years, suggesting that reproductive output may have been controlled by changes in the coral’s phenology and/or stress associated with higher temperature fluctuations. As climate change intensifies, an organism’s phenology and reproductive capabilities may be compromised by alterations in environmental cues (e.g. temperature and photoperiod). Thus it is critical to better understand spawning dynamics of important reef builders as
means to predict the effects associated with climate change in these important ecosystems.

INTRODUCTION

Spawning is the culmination of important physiological processes and is a critical event in the life history of broadcast-spawning marine invertebrates. Spawning synchrony reduces the risk of sperm dilution and enhances fertilization success (Oliver & Babcock 1992, Levitan & Petersen 1995), which can affect the reproductive success and dynamics of a population (Harrison & Wallace 1990).

The release of gametes into the environment is ubiquitous among marine invertebrates and has been studied in phyla such as echinoderms, annelids, mollusks, cnidarians and sponges (Babcock et al. 1992, Minchin 1992, Van Veghel 1993, Himmelman et al. 2008, Mercier & Hamel 2010). In terms of the timing of spawning, many species of reef building (Scleractinian) corals provide an astonishing example of spawning synchronicity (Babcock 1986). Approximately 64% of reef building (Scleractinian) coral species are hermaphroditic broadcast spawners that release their gametes to the water column for external fertilization (Richmond and Hunter 1990, Baird et al. 2009, Harrison 2011) in mass spawning events. Hermaphroditic spawners can release their gametes independently or simultaneously, but the majority package and release their gametes as positively buoyant egg-sperm bundles (Arai et al. 1993, Kinzie 1996, Padilla-Gamino et al. 2011).

The magnitude and synchronicity of the coral spawning events can vary regionally (Richmond & Hunter 1990). For example, in areas such as the Great Barrier
Reef (GBR), mass spawning events are multispecific including over 130 species. These spawning events occur during a brief period (1-2 months in the spring) and release large amounts of oocytes and larvae to the reef (Willis et al 1985, Harrison and Wallace 1990). Spawn slicks covering up to 50,000 m² in area have been observed following a mass multispecific spawning on the GBR. In contrast, in areas such as the Red Sea or the central Pacific, the spawning of ecologically dominant species occurs in different seasons, months and lunar phases (Shlesinger & Loya 1985, Richmond & Hunter 1990, Kolinski & Cox 2003).

Although the spawning periods for many species of scleractinian corals are well documented (Harrison & Wallace 1990, Richmond & Hunter 1990, Baird et al. 2009), spatial and long-term temporal variation in coral reproductive capability has not been well studied because of the sporadic nature of reproductive activities and the limited number of methodological approaches for exploring them. These methods include histology, dissection and periodic observations during the spawning nights (Harrison & Wallace 1990). Sampling limitations have been circumvented in the past by collecting colonies and observing them in the lab, but this has restricted these studies to small to medium-sized corals that can be handled and maintained in laboratory aquaria. In addition, traps used to collect gametes in the field are often small to promote ease of deployment and handling, an approach that contributes to the bias in data collected for small corals species and colonies. Lastly, the same corals are rarely observed over multiple spawning periods and so temporal variation in spawning activities is not well understood.
Furthermore, it is still unclear how different cues from the environment can interact and entrain spawning synchrony with such precision. Environmental variables that have been associated to the control of spawn timing include season, moon phase and sunset time (Harrison et al. 1984, Babcock et al. 1986, Hunter 1988, van Veghel 1993, Mendes and Woodley 2002, van Woescik et al. 2006). Seasonally, gametogenesis and spawning can be influenced by sea surface temperature (SST); many coral species spawn when the waters are warming or close to the annual maxima (Willis et al. 1985, Harrison & Wallace 1990, Richmond & Hunter 1990). However, in other coral species SST does not correlate with spawning and the rate of change of insolation can be a better predictor of spawning (van Woesik et al. 2006). Daily, spawning behavior during the reproductive season may be influenced by the moonlight and tidal changes which are associated to the lunar cycles (Willis et al. 1985). Corals possess blue-light-sensing photoreceptors (cryptochromes) that allow them to sense the moonlight (Gorbunov & Falkowsi 2002, Levy et al. 2007) and manipulative experiments have shown that shifting lunar cycles can influence the timing (lunar days) of release of gamete or larvae (Jokiel et al. 1985, Hunter 1988). Finally, the exact time of gamete release has been associated with sunset time in some species (Brady et al. 2009). Brady et al. influenced the timing of spawning (hours) of fragments from the same colony by keeping them under different light conditions during the day of the spawning; changing sunset by ~1 h caused and equal shift in spawning time.

In addition to spawning synchrony, other reproductive characteristics (e.g., oocyte size) can also be influenced by the environment (Fan & Dai 1999). However, our current understanding of this is very limited, particularly in species that can release their gametes.
over consecutive nights and months (split-spawn) (Willis et al. 1985). Moreover, corals are modular organisms composed of many interdependent polyps, each with its own birth, growth and death rates (Hughes 1989, Baird et al. 2009) and each of which is exposed to different micro-environmental conditions (Kaniewska et al. 2008). Currently there is almost no information on coral’s endogenous rhythms and how corals detect spawning signals and transduce this information to the entire colony (Atkinson & Atkinson 1992, Tarrant 1999, Twan at al. 2006, Levy et al. 2007, Brady et al. 2009).

How climate change may affect the phenology of corals in terms of both spawning synchrony and reproductive output remains unknown. For sessile organisms, such as corals, changes in temperature can significantly impact the physiology of the colony (e.g., bleaching) and have deleterious effects on coral reproduction (Szmant & Gassman 1990, Ward et al. 2000, Mendes & Woodley 2002, McClanahan et al. 2009). Reproductive cues (e.g. temperature and light) that influence the timing of specific stages of gametogenesis and spawning could be decoupled due to an earlier arrival of spring (Hughes 2000). This could have dramatic consequences for species that require high spawning synchronicity for reproductive success and that have restricted mating opportunities each year due to their length of the oogenic cycles. Thus, understanding the factors controlling spawning synchrony and reproductive output of the main reef builders is critical in predicting the responses associated with climate change on coral reefs ecosystems; which harbor an extraordinary diversity and function as important habitats for many organisms in the tropical seas (Moberg and Folke 1999, Knowlton 2001).

This work explores in situ spatial (sites, colony, polyps) and temporal (years, months, days) spawning dynamics of a broadcast spawner from the Central Pacific.
Montipora capitata is one of the most abundant and important reef building coral species in the main Hawaiian Islands (Jokiel et al. 2004), and is a hermaphrodite broadcast-spawner that releases egg-sperm bundles during the summer months (Hodgson 1985, Hunter 1988).

METHODS

Spawning collections and synchrony

Collections of gametes occurred around Moku O Lo'e Island in Kane'ohe Bay (O'ahu, Hawai'i). Gametes were collected during the spawning events of summer 2007 and 2008 from two sites: Bridge to Nowhere (BTN; 21° 25.893’ N; 157° 47.376’W) and Gilligan’s lagoon (GL; 21° 25.973’N; 157° 47.392’W) located in the western side of Moku O Lo'e Island at ~1-2 m depth. Montipora capitata colonies exhibited primarily branching morphologies at BTN and plating at GL (Fig. 2.1, Sup. Fig. S2.1). Collections of egg-sperm bundles occurred for 3 days each month (June, July & August) starting on the night of the new moon during 2007 and 2008. The only exception was in August 2007 when collections were done for only 2 days. The same colonies were followed throughout the reproductive season both years (n=21 at BTN, n=20 at GL in 2007 and n=20 for both sites in 2008); only one colony from the BTN site was excluded from the study in 2008 because it died. Gametes were collected on the reef using a novel net system specifically designed to collect spawn from shallow colonies with minimum damage to both adult coral colonies and released gametes (Chapter 5, Fig. 5.1). The nets were placed over the same adult colonies 1-2 h before spawning and were removed every night after the spawning events. To examine the reproductive synchrony of M. capitata...
colonies between sites and years, the spawning seasonality index (SI) was calculated as in Baird et al. (2009).

**Reproductive output**

Most of the collected egg-sperm bundles were broken apart by rinsing them with 0.2 μm filtered seawater; after breakage (separation of oocyte and sperm), the volume of oocytes was measured, normalized by colony surface area and used as a proxy for spawning material released per colony (reproductive output). A small subset of the freshly collected egg-sperm bundles was used for fertilization trials between colonies from different sites and for microscopic observations to obtain the number of oocytes/bundle and the size of the oocytes. Oocyte size and number on freshly spawned material were assessed using a dissecting microscope (Olympus, SZX7); the number of oocytes/bundle was used as a proxy of polyp fecundity, since most polyps generally release only one egg-sperm bundle in a single evening (Stanton 1992) and each bundle represents the entire reproductive output of the polyp (Wallace 1999, Heyward 1986).

**Colony surface area and reproductive output**

The colonies surface area was estimated as the product of an ellipsoid surface and the complexity index. The ellipsoid surface area was calculated using the greatest aerial diameter of the colony (X) and the diameter perpendicular to it (Y). The complexity index was calculated using rugosity measurements of the colony along those diameters (X’ and Y’ respectively). The formula used to estimate the colony surface area can be expressed as: \[\pi*X*Y*(X’+Y’)] / [4*(X+Y)].
Because the coral morphology differed between sites, calix densities were examined in separate branching and plating fragments to examine if the reproductive surface area differed between morphologies. Fragments from colonies located in the same study sites were collected (4 cm away from colony tips and edges) where the polyps were reproductively active (Wallace 1985, personal observation). Coral tissue from these fragments was removed using a Waterpik Ultra; and remaining skeletons were bleached and dried. Calyx density was calculated by counting the number of calices present in a 1cm² quadrat (5 fragments per morphology, 3 quadrats per fragment).

**Fertilization experiments**

Fertilization trials were performed using a modified version of Mate et al. (1997). Two egg-sperm bundles from different colonies were placed in a 16 mL glass vial containing 5 mL of 0.2 μm filtered seawater. After 5 h, 0.5 mL of 25% Z-fix, Anatech, Ltd (diluted with seawater) was added to the vials to preserve the sample and record fertilization success at a later time using a dissecting microscope (Olympus, SZX7); oocytes that were at or beyond the two-cell stage were considered to be fertilized.

**Lipid content in the adults before and after spawning**

Lipid content was measured to gain an understanding of the energy reserves available before and after the reproductive season. Total lipids were extracted according to Rodrigues and Grottoli (2007) from colonies from the BTN (n=7) and GL (n=9) site before and after spawning during 2007 and 2008 (pre: June 9th, 2007 & May 27, 2008, post: September 30th, 2007 & September 21st, 2008. In brief, lipids were extracted from ground samples (skeleton + host tissue + symbiont cells) in a 2:1 chloroform/methanol
solution, the organic phase was washed using 0.88% KCl and the extract was dried to a constant weight (Harland et al. 1991). Lipids were normalized to total ash-free dry weight of the organic fraction of the coral holobiont (tissue and algal symbionts).

**Environmental characteristics of sites**

Long-term records (2 years) of temperature, precipitation, wind, solar and ultraviolet (UV) radiation at Moku O Lo'e Island were obtained from meteorological stations at the Hawai'i Institute of Marine Biology. Temperature was recorded using a Campbell Scientific temperature probe (model 107), precipitation was recorded using a Texas Electronics Tipping bucket rain gauge (Eppley, TE525), wind was recorded with a R.M. Young Wind Monitor (Young, 05103), sun-plus-sky radiation was measured using a silicon pyranometer with 0.2 kW m\(^{-2}\) mV\(^{-1}\) sensitivity (Campbell Scientific, L1200X-L) and UVR was measured using a total ultraviolet radiometer with 8\(\mu\)V/Wm\(^{-2}\) sensitivity (EPLAB, TUVR).

To compare environmental characteristics between the BTN and GL sites (short-term), temperature and light were examined. Temperature was measured at 10 min intervals for 1 yr in 2007 using StowAway Tidbits accurate to ±0.2°C (Onset Computer). Light was measured during two 2-wk periods in 2008 (22 September – 1 October and 25 November – 5 December, 2008) at both sites. Light measurements were taken at 10 min intervals using Odyssey Photosynthetic Irradiance Recording Systems (Odyssey).

**Statistical analysis**
A general linear model (GLM) was used to analyze the proportion of spawning colonies at each Site (BTN vs GL), year (2007 & 2008), month (June-August) and lunar day (during new moon and 1-2 days after). Proportions were arcsine transformed to achieve homogeneity of variances and normality and were verified by Kolmogorov-Smirnov and Levene’s tests. Tukey’s HSD multiple comparison tests were conducted as post-hoc tests when GLMs detected significant differences.

To analyze if the spawning probability varied with site, year, month, lunar day, and size a generalized linear model was performed using a binary logistic regression (yes/no spawning) and calculated by the maximum-likelihood method. The model was run with - size, site, year, month and lunar day - as main effects; interaction terms were chosen based on the distribution and availability of the data throughout the reproductive season (e.g. we were not able to test the effects of lunar day on different months because in August of 2008 there was no spawning at all). To determine how well the model fit the data, $\chi^2$ Pearson and Deviance goodness-of-fit tests were used.

A GLM was used to analyze the reproductive output by colonies of different sizes at each Site, year and month (June and July). August was not included in the model because most colonies did not spawn that month and if they did, they spawned very small amounts ($\leq 0.1$ mL), only colonies releasing more than 0.1 mL oocytes per night were included in this analysis. One outlier was removed from this analysis (colony ID 16, July 3, 2008). Spawn volumes were double square root transformed.

Differences in the number of oocytes per bundle and the coefficient of variation (CV) of that value were examined between sites, years, months and colony size using a GLM. The CV describes the standard deviation as a percentage of the mean and is a
useful measure of variation, independent of measurement units (Quinn & Keough 2002). Between 6 and 12 bundles per colony were used to estimate the number of oocytes per bundle. Because August spawning was very low (both years), only egg-sperm bundles collected in June and July were used in this analysis.

In addition to quantifying number of oocytes per bundle, in 2008 we also estimated the size of the oocytes in each bundle for 3 colonies per site, per month (n=2274 oocytes). The relationship between the number of oocytes per bundle and the size of the oocytes was explored using a GLM with site and months as fixed factors and number of oocytes as covariate.

Additionally, in 2008, a subset of egg-sperm bundles from the two sites were collected for fertilization trials. In June, fertilization trials were performed using 8 and 13 colonies from the BTN and GL sites respectively; in July, fertilization trials were performed with bundles of 7 and 9 colonies from the BTN and GL sites, respectively. The proportion of fertilized oocytes was arcsin transformed and analyzed using a two-way ANOVA with crosses (BTN vs BTN, GL vs GL, BTN vs GL) and month (June and July) as fixed factors.

A general linear model was used to examine the effects of year, site and pre-post spawning periods in the lipid content of the adults. Lipid data were log transformed to achieve normality and homogeneity of variances. Monthly averages and standard deviations were calculated for the environmental long-term records. To compare short-term records of light and temperature between sites, means, standard deviations and ranges (minimum–maximum) were calculated during the periods sampled. All statistical
analyses were performed using the Minitab statistical package (Minitab 15, Minitab Inc). Values of p<0.05 were considered significant.

RESULTS

Environment

Long-term environmental records

The mean temperature did not fluctuate between 2007 and 2008. However, the variability in temperature and timing of temperature shifts changed between years. In 2007 temperature ranged between 21.1°C-28.3°C, with the minimum temperatures occurring during February and the maximum values occurring over a 3 month period (June, July & August, *M. capitata* spawning season) during the summer (Fig. 2.2, Table 2.1). In contrast in 2008, temperature ranged between 20.5°C-29.3°C and the rate of change of decreasing and increasing temperatures was more abrupt, with the minimum temperatures occurring during January and the maximum temperature occurring in July. Furthermore, in 2007 monthly differences between maximum and minimum temperatures were generally lower than 4°C, except for the month of April. In 2008, 5 months had differences between temperature maximum and minimum values at or above 4°C (Fig. 2.2, Table 2.1). Overall, total solar radiation was slightly higher (3.5%) in 2008 than 2007. During the month of March however, the solar radiations was ~23% higher in 2008 than 2007 (Fig. 2.2, Table 2.2a). Precipitation was higher in 2008 than 2007 by ~17%. In 2007, rainfall was recorded in 185 days/year for a total of 74.16 cm of rain with the highest monthly average rainfall occurring in October. In 2008, precipitation was recorded in 177 days/year for a total of 86.5 cm of rain with the highest monthly average
rainfall occurring through November and December (Fig. 2.2, Table 2.2b). During the spawning season (June-August) more rainfall was recorded in 2008. For the most part, tradewinds blew from the NE and E averaging 17-18 km/hr, however the highest maximum wind velocities were observed in January in 2007 and in December in 2008 (which coincided with the rainy season for this year) (Table 2.2c). Interestingly, in 2007 there was a shift in wind direction; winds blew from the southwest during the period of January-March, which coincided with the month when maximum monthly wind velocities were observed for this year (Sup. Fig. S2.2).

**Short-term environmental records**

The monthly mean temperatures did not differ between the BTN and GL sites throughout the year. However, larger thermal variability was detected at the BTN site, with up to ~3°C fluctuations observed over a single 24-hr period (Fig. 2.3). Light levels differed among the two collection sites. The BTN site was characterized by the highest and broadest light intensities in both summer and autumn sampling times. Sunset times occurred slighter earlier (~5 minutes) at the GL site than the BTN site (Fig. 2.3).

**Colony surface area**

The estimated colony surface areas ranged between 1,385-10,002 cm² at the BTN site and between 151-9,606 cm² at the GL site. Calix density was ~14.8 ± 1.07 calices per cm² (mean, ± SE) and did not change between colonies with different morphologies (ANOVA, F=0.69, p=0.432, df=29).
**Spawning dynamics of Montipora capitata**

The release of egg-sperm bundles (spawning) occurred between 8:45-9:15 pm during the first quarter of the new moon in June, July and August of 2007 and 2008 (see Sup. Fig. S2.3 for exact dates). Spawning events in June occurred slightly earlier (3-5 min) than spawning events in the following months in both years. The spawning events in both years occurred when tidal amplitudes were at their greatest, and tide was going out. Colonies from BTN and GL sites spawned synchronously. The spawning synchrony indexes were very similar between sites and mostly differed between years. In 2007, the spawning seasonality indexes for BTN and GL were 0.371 and 0.367 respectively; in 2008 the spawning seasonality indexes for these two sites increased to 0.485 and 0.415 respectively.

There were differences in the proportion of colonies releasing gametes among years, months and lunar days, and the effect of year depended on site (Fig. 2.4a,b, Table 2.3). A higher proportion of colonies spawned in the year 2007 (Year, F=16.09, df=1, p=0.002, Table 2.3). The proportion of colonies spawning declined each month (Month, F=40.12, df=2, Tukey, p<0.005, Table 2.3). June had the greatest proportion (43.4%), then July (28.12%), and finally August (7.83%). Although we found a lunar day effect (Lunar Day, F=4.67, df=2, p=0.032, Table 2.3), the pairwise comparisons did not reveal any differences in the proportion of colonies releasing gametes between days (Tukey, p=0.065, p=0.053). In 2007, both the BTN and GL sites had similar proportions of colonies releasing gametes (35.7%, 34.38%, respectively); in 2008 the proportion of spawning colonies decreased in both sites but there were differences in the proportions
between sites (Site*Year, F=5.79, df=1, p=0.033, Table 2.3), only 15% of the colonies spawned at the BTN site, whereas 26.1% of colonies spawned at the GL site.

Out of the 41 colonies we followed in 2007, 88% of them spawned at least once. Of these colonies, 29% spawned for one month only, 39% spawned for two months and 19.5% spawned for three months. In 2008, out of the 40 colonies followed, 75% of the colonies spawned at least once; in this year most of the colonies (42.5%) spawned for one month only, 27.5% spawned for two months and only 5% spawned for three months. Most colonies spawned more than once per month, especially during the earlier months (June and July); in August, most colonies spawned only once per month (Fig. 2.5).

Throughout the 2 yr study only one colony died (BTN site).

The probability of spawning was most influenced by year and month (logistic regression, G = 125.36, df=13, p<0.0001, Table 2.4). Both Pearson and Deviance goodness-of-fit tests indicated that the model fits the data well ($\chi^2=621.1$, df=632, p=0.614, $\chi^2=635.2$, df=632, p=0.456; respectively). Coral colonies were more likely to spawn in the year 2007 (p<0.001, Table 2.4) and 6 and 3 times more likely to spawn in June and July (p<0.001, p=0.021 respectively, Table 2.4), than August. In the year 2008, colonies were 7 and 8.5 times more likely to spawn in June and July than August (p=0.023, p=0.013, respectively, Table 2.4); during this year colonies from the GL site were 2.8 times more likely to spawn than colonies from the BTN site. The likelihood of spawning did not depend on colony size (Size, p=0.111, Site*Size, p=0.079, Table 2.4).

Colonies had higher reproductive output (mL of oocytes/colony surface area) in 2007 than 2008 (Year, F=10.24, df=1, p=0.002, Fig. 2.4 c,d, Table 2.5, Sup. Fig. S2.3). In 2007, around 261mL of oocytes were released, whereas in 2008 approximately 65mL
were released. In 2007, the largest amount of spawn was released in June, whereas in 2008 the largest amount of spawn was released in July. It is important to note that 40 of the 65mL of oocytes released in 2008 were spawned by a single colony (Sup. Fig. S2.3). The lowest reproductive output was observed in August at both sites during both years. No differences in reproductive output were observed between sites or colony size (Table 2.5).

Bundles contained between 8 and 23 oocytes, with an average of 15 ± 0.3 (mean ± SE, n=483) oocytes per bundle (Fig. 2.1, 2.6a). In 2007, the number of oocytes per bundle changed between months, whereas in 2008 did not (Year*Month, F=5.02, df=1, p=0.027, Table 2.6a); averaging over both years, there were more oocytes per bundle in June than in July (Month, F=6.27, df=1, p=0.014, Table 2.6a). In June of 2007, bundles contained approximately 16.6 ± 0.53 oocytes (n=123) whereas in July of 2007 bundles contained approximately 13.8 ± 0.81 oocytes (mean ± SE, n=123). In 2008, both months had similar number of oocytes per bundle, 14.6 ± 0.69 (mean ± SE, n=123), 14 ± 0.44 (n=114), June and July respectively. Number of oocytes per bundle did not differ between sites. Colony size did not have an effect on the number of oocytes per bundle.

Differences in the Coefficient of Variation (CV) between months were found at the GL site, whereas no change in the CV was found at the BTN site (Site*Month, F=8.12, df=1, p=0.005). At the GL site, the CV increased from 18 ± 1.3 (mean ± SE, n=120) in the month of June to 28.8 ± 2.0 (mean ± SE, n=111) in the month of July. At the BTN site, the CV was very similar between months, 22.3 ± 1.5 and 22.6 ± 2.2 (mean ± SE, n=126, n=126, June and July respectively). Averaging over both sites, the CV was
The number of oocytes had an effect on the size of the oocytes (F=8.51, p=0.004, Table 7); overall oocyte size tended to decrease with higher number of oocytes per bundle. At the BTN site, oocytes size was slightly smaller in June than July (0.383 μm ± 0.003, 0.390 μm ± 0.002 respectively, means ± SE), whereas at the GL site, oocyte size was slightly larger in June than July (0.396 μm ± 0.003, 0.382 μm ± 0.004 respectively, means ± SE) (Site*Month, F=7.58, df=1, p=0.007, Table 2.7). There was no effect of site or month on the size of the oocytes.

Fertilized oocytes ranged between 55-58% per cross; and no differences were found in the fertilization success among colonies within the same site or between sites, nor between different months. Overall, lipid content in total holobiont biomass changed prior and post spawning (Prepostspawn, F=7.94, df=1, p=0.007, Fig. 2.7, Table 2.8). Interestingly, higher lipid content was found prior spawning at the BTN site, whereas no change in lipid content (prior/post spawning) was found at the GL site (Site * Prepostspawn, F=5.85, p=0.02, Fig. 2.7, Table 2.8). No differences in lipid content were found between sites or years.

DISCUSSION

Coral reefs are highly diverse and one of the most productive ecosystems on the planet. They function as important spawning, breeding and feeding areas for many organisms in the tropical seas (Moberg and Folke 1999, Knowlton 2001) and their
persistence relies on spawning events with remarkable synchronicity during limited periods each year.

This study explored (in situ) the spatial and temporal spawning dynamics of a broadcast spawner from the Central Pacific using a systematic sampling of the gametes in the field. Our results indicate that the spawning dynamics of Montipora capitata (1) changed among years, months and lunar days and (2) that the synchrony and proportion of spawning colonies did not change despite colony size and environmental differences (colony morphology) between sites.

**Temporal variability of spawning**

*Year*

Spawning dynamics changed significantly between years. In 2007, there were both more colonies participating in the split spawning events and larger reproductive output. In 2008, the reproductive output decreased dramatically, only ~25% of the volume released in 2007 was released in 2008. Although the exact cause of the decrease in reproductive output during 2008 remains unknown, we propose two hypotheses that may have contributed to the change in spawning dynamics between years.

It is possible that the environment could have caused the change in spawning dynamics between years. Spawning (gamete release) is the culmination of important physiological events that occur over an extended period of time in which the gametogenic cells multiply and gametes grow. During this entire period, generally 9-10 months for Acroporids (Wallace 1985, Shimoike et al. 1992), coral physiology can be influenced by fluctuations in the environment, which in turn may affect reproductive output. The year
2008 had higher precipitation and wind velocities during winter storm months (November-January), larger temperature fluctuations (higher maximum and lower minimums) throughout the year and slightly lower winds during the warmer season (April-August) than the year 2007. In previous years, high temperatures and low winds have been associated with coral bleaching events in Kane'ohe Bay, Hawai'i (Jokiel & Brown 2004). Furthermore, in the summer (June and July) of 2008 temperature values were near or at the bleaching threshold of 29-30 °C.

Bleaching has deleterious and long-lasting effects on the coral reproductive cycle and has been associated with reduced polyp fecundity, oocyte size, fertilization success and low overall reproductive output for at least one year after the bleaching event (Szmant & Gassman 1990, Ward et al. 2000, Michalek-Wagner & Willis 2001, Mendes & Wodley 2002, McClanahan et al. 2009). Although M. capitata colonies did not experience a severe bleaching in 2008; many colonies looked paler than in 2007 (personal observation), suggesting that they were responding to environmental stress and possibly compromising reproductive output by reabsorbing the oocytes for maintenance and recovery (Sier and Oliver 1994, Michalek-Wagner and Willis 2001).

Another reason why spawning dynamics differed between years could be a shift in the phenology (timing of spawning) of M. capitata. In 2008, temperature started rising in February, one month earlier than in 2007; and reached a higher maximum in July. These differences in the temperature patterns could have affected the rate of reproductive development causing a change in the timing of spawning. Although infrequent; observations of M. capitata spawning during the full moon have been reported (Heyward 1986, Hunter 1988), suggesting that this species can release gametes around both the new
and full moon but the major spawning occurs around the new moon (Hunter 1988). It is possible that the major spawning in 2008 occurred around the full moon or later than 1-2 days after the new moon. The decrease in lipid content after the reproductive season during both years supports this hypothesis. Lipids, which are a main component of oocytes (Arai et al. 1993, Chapter 5), were similar in 2008 than 2007, suggesting that there were enough energy reserves available for reproduction in 2008. In both years, there was a similar decrease in lipids before and after the reproductive season, and the interaction between pre and post spawning from different years was not significant, suggesting that physiologically, the coral colonies had similar changes in energy reserves prior and post spawning.

Studies of periodic coral life cycles events and their response to seasonal and interannual variations in climate are very limited. Long-term observations (over 20 years) of spawning events on the Great Barrier Reef (GBR) have revealed that corals that usually spawn in 1 month only have the ability to split spawn (over two months) every 2-3 years to realign spawning rhythms (Baird et al. 2009). Field observations (e.g. spawning slick formation, spawning odor; personal observation) of M. capitata spawning during the summers of 2006-2010, suggest that spawning events around the new moon occurred regularly, except for the unusual low spawning event in 2008. Was 2008 a year for M. capitata to realign spawning rhythms causing a shift in the timing of spawning (spawning closer to the full moon instead of the new moon)? It is unknown, and clearly longer-term data are necessary to accurately assess this hypothesis. Understanding the consequences of spawning variability across larger temporal scales is particularly
important for species with longer life spans that have the ability spawn over several decades.

**Month and Lunar Day**

*Montipora capitata* had split spawning events over a 2-3 month period (June-August); which was consistent with previous tank observations on the reproductive behavior of this species (Hodgson 1988, Hunter 1988). Spawning events in which coral populations partition the release of gametes over consecutive months are common in many coral species and have been reported in different areas around the world including the Great Barrier Reef in Australia (Harrison et al. 1984, Willis et al. 1985), Japan (Shimoike et al. 1992, Baird et al. 2009), Thailand (Kongjandtre et al. 2010), Venezuela (Bastidas et al. 2005) and the Persian Gulf (Bauman et al. 2011).

High temperatures and low precipitation characterized the reproductive season for *M. capitata* in Hawaii. Colonies were more likely to spawn during June and July in both years. However, in 2007, colonies were more likely to spawn in June whereas in 2008 colonies were more likely to spawn in July. These differences could be due to the new moon falling during the middle of June in 2007 and early in the reproductive season in 2008 (June 3rd), when the oocytes may not have been quite ready to be released (F. Cox, personal communication); in corals with vertical transmission such as *M. capitata*, symbionts are translocated to the coral oocytes between 1-12 days prior spawning (Heyward 1986, Hunter 1988, Hirose et al. 2001), thus the period before spawn is critical for the acquisition of symbionts by the next generation (Chapter 4). An early release of gametes could have detrimental consequences for the offspring and the perpetuation of symbiosis in this species.
Overall, the seasonality index (SI), which is a relative measurement of spawning synchrony between the spawning colonies in different months (Baird et al. 2009), was very close to SI values obtained from the Acropora assemblage in the GBR (Baird et al. 2009). This supports the hypothesis that spawning synchrony is higher at mid latitudes. Interestingly, the SI of *M. capitata* was higher in 2008 than 2007 suggesting that higher synchrony occurred during the year with the lowest reproductive output. The year 2007 was characterized by a very intense reproductive event in June in which most of the gametes were released in one night (June, 1st day after new moon), whereas 2008 was characterized by low reproductive output released in similar amounts during the new moon and 1-2 days after (June and July). Higher synchronicity in the year 2008 may have helped to counteract (in part) the effects of sperm dilution and low concentration of gametes during this reproductive season (Oliver & Babcock 1992, Levitan & Petersen 1995).

Thus, split-spawning events of *M. capitata* can vary at different temporal scales in terms of the amount of gametes released, the proportion of colonies participating in different months and their synchrony.

**Spatial variability of spawning**

*Site-

Spawning dynamics was explored between sites to understand how the reproductive output of colonies was influenced by different environmental characteristics. The proportion of spawning colonies did not differ between sites in 2007, suggesting that colonies from both sites exhibited similar reproductive patterns. However, in 2008 the
proportion of colonies releasing gametes at the BTN site was lower than the GL site. This suggests that colonies from an environment with higher fluctuations (e.g. temperature and light) experienced higher stress during 2008 that may have compromised their reproductive performance. However, it is important to note that even though there were differences in the proportion of individuals releasing gametes between years at different sites, the amount of spawned material (mL of oocytes) released was always higher at the BTN site. At this site, colonies had branching morphology, which increased their reproductive surface area (i.e. number of polyps) and in turn, the amounts of gametes produced and released.

Previous manipulative experiments in broadcast spawners have shown that temperature, lunar phase and day length, can influence the timing of spawning (Hunter 1988, Brady et al. 2009). Hunter (1998) found that lowering the temperature during the spawning season precluded the release of gametes in *M. capitata* (=*M. verrucosa*) in Hawaii. Furthermore, alterations in the local solar light cycle have been shown to shift the timing of the spawning in *M. capitata* (Hunter 1998) and *Montastraea franski* (Brady et al. 2009). In our study, the release of gametes of *M. capitata* occurred at the same time of day despite differences in the temperature variability, light levels, sunset times and morphology between the two sites. This suggests that both populations are responding similarly to spawning cues (e.g. hormones, light-dark rhythms) (Atkinson & Atkinson 1992, Tarrant et al. 2004, Gorbunov & Falkowski 2002, Levy et al. 2007). Similarly, Bastidas et al. (2005) found no differences in coral spawning synchrony of seven common species between an inshore and offshore reef possibly exposed to different environmental characteristics. In contrast, at the Great Barrier Reef corals from inshore
reefs spawned one month earlier than those from offshore reefs, possibly due to the rapid seasonal warming of the sallower reefs (Willis et al. 1985). Clearly further research is still required to understand how corals can regulate their remarkable synchronicity capacity and how the spawning cues can be perceived and transduced by the organism, particularly when colonies experience very different environmental conditions.

*Colony-

*M. capitata* colonies in this study had very variable sizes and morphologies (Sup. Fig. S2.1) and shared characteristics of different “morphospecies” (Forsman et al. 2010). However, our results support the hypothesis that the colonies followed in this study correspond to *M. capitata* species because they spawned simultaneously and fertilization success among colonies within the same site or between sites did not differ. Previous host phylogenetic analyses from these sites have also revealed that colonies with different morphologies shared common alleles supporting the hypothesis that the different morphotypes represent indeed the same species (Chapter 4). Tracking individuals over time throughout the reproductive seasons allowed us to discover the extraordinary variability between colonies in terms of their reproductive output and frequency of spawning (months, nights per month.). For example, some colonies released gametes only once in the reproductive season, whereas other colonies released gametes in multiple months and a number of times within each month (Sup. Fig. S2.3). Releasing gametes over several periods can be an adaptation with beneficial value because this strategy minimizes the risk associated with a catastrophic event over a single reproductive event (Richmond & Hunter 1990). This could also indicate that certain members of coral
populations have different physiological responses to factors controlling gamete development (Harrison & Wallace 1990).

*In situ* observations and collections of the gametes released also allowed us to explore the spawning dynamics of colonies with a very broad size range in their natural environment, as opposed to laboratory studies that have observed spawning of small colonies or fragments of larger colonies in tanks. Our results show that colony size did not have an effect in the likelihood of spawning and reproductive output; gamete release was very variable between colonies of all sizes. Some of the smaller colonies released similar or larger amounts of spawn than colonies that were four to seven times larger. Moreover, the largest amount of spawned material per colony per night was released by one of the smaller colonies at the BTN site.

It is important to note that colony size in corals does not always link to sexual maturity or age. Colonies can suffer fragmentation and partial mortality caused by physical and biological factors such as storm damage, bleaching, disease and anthropogenic activities (pollution, tourism, etc.); and these factors can impact reproductive output (Szmant & Gassman 1990, Guzmán & Holst 1993, Smith & Hughes 1999, Harrison & Ward 2001).

In *Goniastrea aspera* the volume of the polyps in the larger sexually mature colonies is greater than in the sexually immature corals suggesting that polyp size as well as colony size may be a crucial determinant of sexual maturity (Sakai 1998). Once a coral has reached sexual maturity, either by age or size (Kai & Sakai 2008), fecundity (or quality and quantity of gametes produced) continues to increase with colony size.

Interestingly, the largest female colonies of the soft coral *Pseudoplexaura porosa* show decreased polyp fecundity compared to their smaller female colonies (Kapela & Lasker 1999), suggesting that there is a decline in the reproductive capacity of the largest coral. The latter is consistent with the suggestion that senescence occurs in some colonial taxa (Rinkevich & Loya 1986). Also notable is the fact that not all colonies of a species will spawn in a given year (Van Veghel 1993), although the biological reasons for this variability have not yet been resolved.

While our study included colonies from several size classes and these colonies were larger than the ones used in previous laboratory studies, we still did not encompass the largest size class of the population (~2-3 m wide). Future research examining the largest colonies of the population as well as the smaller ones is necessary to explore the reproductive capacity (and possible senescence) of *M. capitata* and how size and sexual reproduction vary between colonies due to age or physiological condition.

**Polyp fecundity**

*Montipora capitata* polyps have both male and female functions and gametes are released simultaneously as egg-sperm bundles (Chapter 3). Reproductive output at the polyp level was examined using the number of oocytes per bundle as a proxy for polyp fecundity.

Despite the large difference in colony size and morphology, and environmental differences at the site level, the number of oocytes per bundle was very consistent,
suggesting that polyps were able to acquire and allocate similar energy reserves for reproduction. Similar results were observed in the polyp fecundity of Montastraea faveoloata (Villinski 2003) where no differences in the oocyte production per polyp were found between colonies from different depths (3 m vs. 18 m). Likewise Hall & Hughes (1996) found that colony size had no effect on oocyte or testes number per polyp or testes volume per polyp for any of the six reef-building corals they studied.

Only temporal differences in the number of oocytes per bundle were observed. In 2007, bundles with more oocytes were released earlier in the reproductive season. Whereas in 2008 (the year with low reproductive output) no differences were found in the number of oocytes released between the two months. Interestingly, the CV (which gives an estimate of the number of oocytes/bundle variation) changed between months (both years) at the GL site and not at the BTN site. This is contrary to what we would expect as the GL site colonies have a simpler morphology (plate) and experience lower environmental fluctuations (light and temperature) than at the BTN site; originally we had expected larger variation in the propagules of colonies with more complex morphologies (e.g. branching).

In most coral species, gametes typically develop with high synchrony among polyps (Harrison & Wallace 1990). However, in many species, marginal polyps in the periphery of colonies are “non-reproductive zones” and do not produce gametes; possibly due to the allocation of energy resources to colony defense and expansion rather than reproduction (Kojis & Quinn, 1982, Szmant-Froelich 1985, Hall & Hughes 1996, Kapela & Lasher 1999, Smith & Hughes 1999) and/ or the fact that non-reproductive zones are the sites of extra-tentacular budding that result in new polyps that are initially sexually
immature (Sakai 1998). In *Acropora* spp., differences in the timing of spawning and oocyte maturation have been observed in polyps depending on their location within the colony (Shimoike et al. 1992). Shimoike et al. (1992) found that oocytes from polyps located in the shaded part of the colony spawned at a later time and had lower volume, suggesting that different light conditions had an impact on the allocation of energy for reproduction within the same colony. It is possible that branching colonies of *M. capitata* at the BTN site may have larger intracolony variability in the allocation of energy for reproduction than plate morphologies at the GL site due to their more complex morphology.

In *M. capitata*, the number of oocytes per bundle was associated with the size of the oocytes. Generally, bundles with smaller oocytes had more in total and bundles with larger oocytes had fewer. It is still unclear what drives this variability, but it is possible that the relationship between size and number of oocytes depends on the polyp’s size, age or the position within the colony potentially affecting their ability to compile bundles prior to spawning.

Gamete size variation has not been well studied in colonial marine organisms and remains poorly understood (Marshall et al. 2008). Size of the oocyte can influence its chances of being fertilized and larger oocytes may have higher chances of sperm encounter than smaller oocytes (Levitan 1996, Marshall et al. 2000, 2002). In our study, the size of the oocytes showed different trends at the BTN and GL sites. At the BTN site, larger oocytes were found early in the season (June) and smaller oocytes late in the season (July); and the opposite were true for the GL site. However, it is not clear if these patterns are due to differences in gamete development between both sites. Future studies
should also measure sperm content per bundle and how it relates to both the oocyte size and the number of oocytes in the bundle; it is possible that a shift in size or number of oocytes may be accompanied by a shift in sperm production. Hall and Hughes (1996) found that the ratio of total oocyte volume to testes volume per polyp increased with colony size in all six reef-building corals studied.

Recent studies of gamete plasticity in colonial invertebrates in the field have revealed important insights of the parental effects on gamete size. Monro et al. (2010) studied the relationship between offspring size and performance in the colonial invertebrate Bugula neritina (bryozoan). Their work suggests that plasticity in offspring size can optimize fitness when the environment is unpredictable (bet-hedging). Crean and Marshall’s (2008) research on the broadcast spawning ascidian Styela plicata revealed that both oocyte and sperm showed phenotypic plasticity in response to changes in adult density (sperm environment). When adult density was higher, oocytes with larger ovicells (portion of the oocyte available for embryonic development) were produced and sperm were larger, more motile and remained viable for longer than the sperm collected from adults with low density.

Thus, all gametes are not created equal and when the environment of the offspring is unpredictable this variation may have a beneficial value (Crean & Marshall 2009). It is not clear how much corals can control the phenotypic plasticity of their gametes but this could have important implications for survival of coral offspring, further research is necessary in this novel and exciting field.

CONCLUSIONS
Spawning synchrony is of critical importance for broadcast-spawning marine invertebrates. However, spawning is a complex phenomenon that depends on the physiological response of organisms to several environmental cues that vary both in time and space over several scales. Despite the fact that spawning is an extremely common trait in marine invertebrates, spawning synchrony and reproductive output variability are still not well understood, mostly due to its sporadic nature and the limitation in the methodology available to explore reproductive activities in the field.

This work is one of the most comprehensive studies of spawning dynamics in the field and represents the first ecological spawning baseline for a reef-building coral in the central Pacific. Our data demonstrated that in situ spawning dynamics of the scleractinian coral *M. capitata* vary significantly between years, months and lunar days and between sites, colonies and polyps. For *M. capitata*, the environment of a “good spawning year” was characterized by warmer temperatures in the winter, cooler temperatures during the summer (<28°C) and low precipitation during the spawning season (June-August). However, longer time series would help us determine whether the differences in spawning between years were simply part of the natural variability or whether there may have been antecedent environmental/physiological differences causing the change.

Further research is necessary to understand the causes of variability at all these scales and how spawning cues may interact and affect the reproductive physiology at the population, organism and polyp (module) level. Understanding these relationships will help us to better predict the effects of climate change on the phenology and reproductive success of corals, which are critical for the resilience and persistence of coral reefs and the survival of many species in the tropical seas.
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Table 2.1 Temperature records in Moku O Lo'e Island in Kane'oehe Bay (O'ahu, Hawai'i) during 2006-2008.

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Table 2.2 Solar radiation, precipitation and wind records in Moku O Lo'e Island during 2006-2008.

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(c) Wind Average

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Table 2.3 General linear model (GLM) of the proportion of colonies releasing gametes from the BTN and GL sites during different years (2007 & 2008), months (June, July & August) and lunar days (new moon and 1-2 days after). Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.

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Table 2.4 Results of logistic regression to analyze how spawning probability varies with size, year, month, lunar day. Model comparisons: Site to BTN, Year to 2007, Month to August, Lunar day to 1, Site*Year BTN 2008, Site*Month GL August, Year*Month, 2008*August. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.

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Table 2.5 General linear model of reproductive output (mL of oocytes/colony surface area) by colonies with different sizes at each site (BTN & GL), year (2007 & 2008) and month (June & July). Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.

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Table 2.6 General linear model of number of oocytes per bundle and coefficient of variation (oocytes per bundle per colony) at each site (BTN & GL), year (2007 & 2008) and month (June & July). Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.

**a) Number of eggs per bundle**

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**b) Coefficient of variation**

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Table 2.7 GLM of size of oocytes with site and months as fixed factors and number of oocytes as covariate.

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Table 2.8 GLM of lipid content in colonies prior and post reproductive season during 2007 and 2008 at each site (BTN & GL). Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.

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<td>Error</td>
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<td>Total</td>
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Fig. 2.1 *Montipora capitata* colonies representing (a) plating and (b) branching morphologies, (c) larger branching colony spawning egg-sperm bundles, (d) oocytes post release seeded with *Symbiodinium* cells acquired from parent colonies.
**Fig. 2.2** Long-term (2yrs) environmental characteristics at Moku O Lo'e Island in Kane'ohe Bay (O'ahu, Hawai'i).
Fig. 2.3 Temperature and light data from BTN and GL sites in Moku O Lo'e Island in Kane'ohe Bay (O'ahu, Hawai'i).
Fig. 2.4 Proportion of colonies that spawned in 2007 (a) and 2008 (b). Amount of spawning material (mL of oocytes) released in 2007 (c) and 2008 (d). Reproductive output (mL/cm$^2$ $10^{-4}$) in 2007 (e) and 2008 (f). White bars correspond to the BTN site and black bars correspond to the GL site. At BTN site, n=21 (2007) and n=20 (2008), at GL site, n=20 (2007 & 2008). Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.
Fig 2.5 Proportion of colonies that spawned once, twice or three times within each month. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.
Fig. 2.6 Number of oocytes per bundle (a) and coefficients of variation (b) from colonies from the BTN and GL site during June and July (2007 & 2008). White bars correspond to the BTN site and black bars correspond to the GL site. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.
Fig. 2.7 Total lipids in coral colonies prior and post reproductive season. BTN-Bridge to Nowhere, GL-Giligan’s Lagoon. Means ± SE. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.
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Sup. Fig. S2.1- Examples of colony morphologies examined in this study.
Sup. Fig. S2.2 - Wind direction and velocity for Moku O Lo'e Island in Kane'ohe Bay (O'ahu, Hawai'i).
Sup. Fig. S2.3 - Reproductive output per colony from the BTN and GL sites. White boxes indicate that spawning occurred, number in the box is the volume (mL) of oocytes collected. Black boxes indicate no spawning.
CHAPTER 3

FORMATION AND STRUCTURAL ORGANIZATION OF THE EGG-SPERM
BUNDLE OF THE SCLERACTINIAN CORAL MONTIPORA CAPITATA

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ABSTRACT

The majority of scleractinian corals are hermaphrodites that broadcast spawn their gametes separately or packaged as egg-sperm bundles during spawning events that are timed to the lunar cycle. The egg-sperm bundle is an efficient way of transporting gametes to the ocean surface where fertilization takes place, while minimizing sperm dilution and maximizing the opportunity for gamete encounters during a spawning event. To date, there are few studies that focus on the formation and structure of egg-sperm bundle. This study explores formation, ultrastructure and longevity of the egg-sperm bundle in *Montipora capitata*, a major reef building coral in Hawai’i. Our results show that the egg-sperm bundle is formed by a mucus layer secreted by the oocytes. The sperm package is located at the center of each bundle, possibly reflecting the development of male and female gametes in different mesenteries. Once the egg-sperm bundle has reached the ocean surface it breaks open within 10-35 min, depending on the environmental conditions (i.e., wind, water turbulence). Although the bundle has an ephemeral life span, the formation of an egg-sperm bundle is a fundamental part of the reproductive process that could be strongly influenced by climate change and deterioration of water quality (due to anthropogenic effects) and thus, requires further investigation.
INTRODUCTION

Coral reefs are productive and diverse habitats that provide shelter for an extraordinary biodiversity and services that support the economies of many island and coastal communities (Connell 1978; Moberg and Folke 1999). Coral ecosystems worldwide are severely threatened by climate change, pollution and overexploitation (Hughes et al. 2003; Lough 2008). Both the persistence of healthy reefs, and the recovery of coral populations impacted by severe environmental disturbance, are dependent on gamete production, successful fertilization, development of viable offspring and survival of new recruits (Richmond 1997). All of these processes are variable and are influenced by interactions between coral biology and spatial and temporal fluctuations in the environment (Tomascik and Sander 1987; Harrison and Wallace 1990; Richmond and Hunter 1990; Szmant and Gassman 1990; Hughes and Tanner 2000; Baird et al. 2009).

Broadcast spawning is the dominant form of sexual reproduction in scleractinian corals (Harrison and Wallace 1990; Baird et al. 2009). Broadcast spawners can be either gonochoric or hermaphroditic and can release their gametes independently or simultaneously. Approximately 65% of scleractinian coral species studied thus far are hermaphroditic broadcast spawners (Richmond and Hunter 1990; Guest et al. 2008; Baird et al. 2009) and of these, the majority package and release their gametes as positively buoyant egg-sperm bundles (Arai et al. 1993; Kinzie 1996). This is in contrast to brooding corals (which can also be gonochoric or hermaphroditic), where oocytes are fertilized inside the coral polyp and well-developed larvae are released (Harrison and Wallace 1990).
Gametogenesis and gamete structure have been examined in a number of coral species representing a range of reproductive modes (Harrison and Wallace 1990; Richmond and Hunter 1990); however, relatively few studies have focused on egg-sperm bundles. Those that have, reveal each bundle to contain anywhere from 6 to 180 oocytes depending on the species (Wallace 1985; Richmond 1997). For example, in the genus *Acropora* (clade Complexa), which includes mostly hermaphroditic spawners (Baird et al. 2009), bundles contain anywhere from 6 to 13 oocytes (Wallace 1985). These are arranged peripherally around a centrally located sperm mass (Wallace 1985; Vargas-Angel et al. 2006) and the gametes develop on different, but specific mesenteries (Wallace 1985). In contrast, in bundles released by *Favites abdita* (clade Robusta), oocytes and spermatocytes are intermingled and the gametes develop on the same mesenteries (Kojis and Quinn 1982).

Egg-sperm bundles disintegrate 10-40 minutes after reaching the surface of the water, releasing the gametes and making them available for fertilization (Richmond 1997). To date, no studies have examined the ultrastructure or formation of the egg-sperm bundle. The structure and organization of the egg-sperm bundle is likely to influence the time required to break open, which has implications for fertilization success and opportunities for hybridization (Wolstenholme 2004).

Here we use electron microscopy to address this knowledge gap in *Montipora capitata* (Dana 1846; family Acroporidae), a major reef building coral in Hawai’i (Jokiel et al. 2004). This species belongs to the family Acroporidae and like most members of this family, is a simultaneous hermaphrodite that broadcast-spawns egg-sperm bundles (Wallace and Willis 1994). This family dominates coral reef assemblages throughout the
Indo-Pacific region and the Caribbean Sea and is extremely sensitive to environmental (e.g., thermal anomalies, Hoegh-Guldberg 1999) and biological disturbances (e.g., crown of thorns predation, Pratchett et al. 2009). As such, the analysis of the ultrastructure and formation of egg-sperm bundles in this family contributes to our basic understanding of reproductive processes in an ecologically important group of corals and one that is increasingly threatened by climate change.

MATERIALS AND METHODS

Collections and preliminary analysis

*Montipora capitata* releases egg-sperm bundles during the new moon from late spring through summer in Hawaiʻi (Hunter 1988). Egg-sperm bundles were collected from coral colonies on reefs adjacent to Moku O Loʻe Island in Kaneʻohe Bay, Hawaiʻi 1-2 days after the new moon during spawning events in June through August in 2007 and 2008. Coral fragments were collected and dissected every five days for one month prior to spawning in order to evaluate gamete maturity and symbiont acquisition by the oocytes. Pictures of coral fragments were taken using a dissecting microscope (Olympus, SZX7) equipped with an Olympus camera (MagnaFire SP S 99810). A subset of the collected gamete samples were observed under the dissecting microscope and compound microscope (Olympus, BX51), photographed and measured using Image J digital analysis software (NIH). The remaining collections were fixed for scanning and transmission electron microscopy as described below.

Transmission Electron Microscopy (TEM)
For transmission electron microscopy, specimens were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (with 0.1 M calcium chloride, 0.35 M sucrose, buffered to pH 7.4) for 48 h, washed in 0.1 M sodium cacodylate (with 0.4 M sucrose) for 3 times 30 min each, followed by postfixation with 1% OsO₄ in 0.1 M sodium cacodylate buffer for one hour. Tissue was dehydrated in a graded ethanol series (30%, 50%, 70%, 85%, 95%, 100%), substituted with propylene oxide, and embedded in LX112 epoxy resin. Ultrathin (60-80 nm) sections were cut with a Reichert Ultracut E ultramicrotome, double stained with uranyl acetate and lead citrate, viewed on a LEO 912 EFTEM at 100 kV, and photographed with a Proscan frame-transfer CCD.

**Field Emission Scanning Electron Microscopy (FESEM)**

For scanning electron microscopy, samples were fixed, postfixed and dehydrated in the same way as TEM samples. After ethanol dehydration, samples were critical point dried (Tousimis Samdri-795), mounted on aluminum stubs, sputter coated with gold/palladium to 5-8 nm thickness (Hummer 6.2), and viewed with Hitachi S-800 and Hitachi S-48000 field emission scanning electron microscopes.

**RESULTS**

**Development of gametes**

Observations of coral tissue before the spawning event revealed that oocytes and spermatocytes developed on separate pairs of mesenteries growing deep in the skeleton (Fig. 3.1a). Reproductive tissue was found up to 1 cm below the surface tissue intercalated in the skeleton. As the spawning time approached, oocytes and spermatids...
developed and moved towards the anterior of the polyp. Oocytes were infected by endosymbiotic dinoflagellates (referred to as *Symbiodinium* throughout) ~2-3 weeks before spawning occurred.

**Spawning**

Spawning of *Montipora capitata* occurred between 2145 hrs and 2200 hrs during the first quarter of the new moon in June, July and August in 2007 and 2008. Approximately 2 h before spawning, the polyps relaxed (Fig. 3.1b) and expanded and were observed to produce mucus. Approximately 10-15 min before spawning the egg-sperm bundles became visible beneath the oral disk. During spawning, the oral disk of each polyp became greatly extended and the tentacles contracted (Fig. 3.1c). The egg-sperm bundles were squeezed through the polyp mouth and released into the water column. The release of bundles of the coral population in the field lasted 25-30 minutes.

Positively buoyant egg-sperm bundles (brownish-pink color) floated to the water surface and broke apart releasing spermatozoa and oocytes for external fertilization (Fig. 3.2). The breakage of the bundle was signaled by the release of spermatozoa, which was observed as a white stream generally emanating from one or more small openings between oocytes (Fig. 3.2b). The oocytes subsequently separated from one another, and the egg-sperm bundle dissociated completely within 5-25 min of release (Fig. 3.2c). When initially released, the oocytes were irregularly shaped and became ovoid over a period of 25-30 min (Fig. 3.2d). At the end of the reproductive season (August 2007), some oocytes remained irregularly shaped (Fig. 3.3). Both normal and deformed oocytes were light brown in color and reflecting the presence of *Symbiodinium* (Fig. 3.3b).
Ultrastructure of the egg-sperm bundle

The egg-sperm bundles released by *M. capitata* measured approximately 1 mm and contained around 15 ± 5.1 oocytes (mean ± SD, n=214, from 26 colonies), surrounding a central mass of spermatozoa (Fig. 3.4). After rounding had occurred oocytes measured approximately 461 ± 75 μm (mean ± SD, n=214, from 26 colonies) in diameter (Fig. 3.4).

Light microscopy and SEM observations suggest that the surface of the bundles, and the spaces between the oocytes, were covered with a mucus layer (Fig. 3.5a and b). This layer was amorphous and has no structural organization and TEM observations revealed the material had a similar electron density to granules within the oocytes (Fig. 3.5c and d). The interface between the oocytes and the mass of spermatozoa within the bundle was variable; sometimes oocytes were observed in close proximity with no bounding material between them (Fig. 3.5e), and sometimes a large amount of mucus separated the oocytes and spermatozoa (Fig. 3.5f and 3.5g). Entry of spermatozoa nuclei into the oocyte or fusion of plasma membranes was never observed.

The oocytes contained numerous lipid droplets, several types of cortical and yolk granules varying in structure, shape and electron density, and *Symbiodinium* cells (Fig. 3.6). The cortical granules had, for the most part, an ovoid shape and were homogeneous and membrane-bound (Fig. 3.6a-b). Yolk granules were larger than cortical granules and had higher irregularity in their shapes (Fig. 3.6c-f). Mitochondria, lipid-like inclusions, Golgi elements, and both membrane- and non-membrane bound fibrous bodies were commonly present in the oocytes. Mitochondria were dispersed throughout the oocyte but
were most common near the *Symbiodinium* cells. Germinal vesicles were not observed in the oocytes before or after the bundle broke apart. *Symbiodinium* cells were surrounded by the symbiosome membrane complex (Fig. 3.6g) and distributed throughout the oocyte (Fig. 3.4c), with some *Symbiodinium* cells dividing (Fig. 3.6h). Our observations of the *Symbiodinium* cells in the oocytes showed a good correspondence with previously published descriptions of this symbiont in the adult coral stage (Blank 1987); the multi-lobed chloroplasts were peripherally located and their lamellae appeared as bands traversing their entire length (Fig. 3.6g-h). The nucleus contained a nucleolus and chromosomes were widely distributed in the nucleoplasm (Fig. 3.6g). The *Symbiodinium* also contained mitochondria, accumulation bodies and randomly distributed uric acid deposits (Yamashita et al. 2009).

The microvilli and cortical layer of the oocytes were different before and after the egg-sperm bundles broke (Fig. 3.7). When contained in the egg-sperm bundle, the microvilli were thick and close together (Fig. 3.7a) and the cortical granular layer contained smaller granules (approximately 860 nm) at the periphery, and larger granules towards the center of the oocyte (Fig. 3.7b). Once released from the bundle and rounded, the microvilli were thinner and more spaced out (Fig. 3.7c), and the granules at the periphery of the oocyte were larger and had a different shape and electron density (Fig. 3.7d).

The spermatozoa mass occupied approximately 20% of the total egg-sperm bundle volume and was held together in spherical shape with a mucous coating similar to the one found on the surface of the bundle and between oocytes (Fig. 3.8a-c). The
spermatozoa had no particular orientation (Fig. 3.8d-e) and a mucous web was present within the mass (Fig. 3.8f-h).

*Montipora capitata* had ovoid ect-aqua sperm (fertilize externally in contact with water, Rouse and Jamieson 1987) that comprised an approximately 1.6 μm head and 1.3 μm midpiece. The nucleus was bullet shaped and contained a zone of electron dense material at the top (Figs. 3.8 and 3.9). The anterior part of the nucleus was surrounded by small vesicles (Figs. 3.8i and 3.9a-b) and the anterior and lateral regions of the midpiece were occupied by lamellae layers stacked in parallel arrays (Fig. 3.9a-c). The midpiece also contained aggregated mitochondria and a large lipid body on one side (Fig. 3.9a-b,d). The intracentriolar ligament was found at the center of the midpiece. The cytoplasmic collar was at the base of the spermatozoa and surrounded the anterior portion of the flagellum (Fig. 3.8h and 3.9b). The flagellum exhibited the usual 9+2 arrangement of microtubules and the flagellar membrane expanded laterally (Fig. 3.9e-f). In some instances, spermatocytes (early stage sperm) were observed in bundles collected at the beginning of the reproductive season (June). The only detectable structures at this early stage were the nucleus, mitochondria and lamellate bodies (Fig. 3.9 g-h).

**DISCUSSION**

The longevity of an egg-sperm bundle is limited. It is formed a few hours before spawning (Wallace 1985) and breaks apart in less than 10-40 min after it has been released from the polyp. Although it has a short life, this bundle carries out the important function of transporting gametes to the surface, and maximizing the chances of encounter between gametes with very different buoyancies (*i.e.*, sperm is denser and sinks, whereas
oocytes are generally positively buoyant). This strategy increases sperm availability and facilitates outcrossing (Harrison and Wallace 1990; Richmond 1997). This work is the first systematic study of the ultrastructure of the egg-sperm bundles in scleractinian corals and presents important new observations to understanding bundle structure and formation. A layer of mucus is present around the oocytes and within the egg-sperm interface and this material appears to be forming and holding the egg-sperm bundle together for ejection from the polyp coral. TEM observations revealed that there is a significant discharge of granule content from the oocytes, and this material has very similar electron density to the mucous layer, suggesting that bundle formation is achieved, at least in part, by the excretion of oocyte material.

The release of cortical material from oocytes in response to seawater has been observed in other invertebrates such as the polychaete *Sabellaria vulgaris* (Waterman 1936) and the crustacean *Penaeus aztecus* (Clark et al. 1980). In *S. vulgaris* the newly-shed oocytes have a very irregular shape due to mechanical pressure in the coelomic cavities. After exposure to seawater the oocyte membrane undergoes physical alteration, and the oocytes become spherical. During this period, fertilization is limited. In *P. aztecus*, the contact of oocytes with seawater results in a dramatic and massive release of a jelly precursor material from the cortical crypts (Clark et al. 1980). The jelly precursor material is made of 25-30% carbohydrate and 70-75% protein (Lynn and Clark 1987) and is believed to be responsible for the prevention of polyspermy and establishing a microenvironment inside the oocyte suitable for embryo development (Clark et al. 1980).
From our data it appears that the mucous material in *M. capitata* is secreted by the oocytes in response to seawater contact (as they near the oral area prior to spawning). This secretion facilitates oocyte adhesion during the bundle formation. The hypothesized process of bundle formation of *M. capitata* is shown diagrammatically in figure 3.10 (I).

Male and female gametes develop along the 8 mesenteries, with two male alternating with two female mesenteries (Heyward 1986). Between 3 and 5 oocytes (Heyward 1986) are lined up vertically along the mesenteries (Fig. 3.10a, I). In *M. capitata* (a perforate coral), most of the oocyte and spermatozoa development occurs deep in the skeleton. Hours before spawning, the polyps elongate and gather the gametes near the oral disk (Heyward 1986). During this time the seawater flux inside the gastrodermal cavity may increase and induce the granule secretion of the cortical layer and facilitate oocyte adhesion (Fig. 3.10 b-d, I). As the oocytes move toward the distended oral disc, the secretion continues and the bundle forms (Fig. 3.10 e, I). Most polyps of *M. capitata* release one egg-sperm bundle per polyp in a single evening, though on rare occasions two egg-sperm bundles can be released (Babcock and Heyward 1986; Stanton 1992). Releasing two smaller bundles instead of one may be a good strategy if the gamete material is too large to fit through the polyp’s mouth (Stanton 1992). For corals in which oocytes and spermatozoa develop on the same mesentery, the process of bundle formation could result in an egg-sperm bundle in which oocytes and spermatozoa are intermingled (Figure 3.10, II), such as the coral *Favites abdita* (Kojis and Quinn 1982).

With the mucus being secreted by the oocytes, the energy investment from the coral polyp is minimized. More complex structures such as membranes (composed of phospholipids and proteins) would be both more difficult to produce in a short period of
time and more energetically costly (Vance 2002; Voelker 2002). SEM observations of the mucous layer in the bundle (Figs. 3.5, 3.8) resemble SEM observations of the mucous floc and web material produced by the coral *Porites astreoides* (Ducklow and Mitchell 1979). Coral mucus is mostly composed of carbohydrates (Coffroth 1990), and to a lesser degree glycoproteins (Krupp 1985; Vacelet and Thomassin 1991) and lipids (Benson and Muscatine 1974; Crossland et al. 1980). Further studies of the structure and macromolecular composition in the bundle mucus layer are necessary.

TEM observations of the egg-sperm interface showed areas where mucus separated the oocytes and spermatozoa and other areas where this layer was minimal or otherwise not visualized. Regardless of the presence of mucus, no fusion of gametes was observed within the bundles. This is consistent with previous research that has found that self-fertilization is very uncommon in *M. capitata* (Hodgson 1988; Maté et al. 1997). It is also possible that, while inside the egg-sperm bundle, the oocytes possess biochemical blocks to self- or total fertilization or that cortical rearrangement and acquisition of spherical shape of the oocyte must occur before fertilization can take place (by which time spermatozoa have been released and dispersed through water currents to other colonies). Hodgson (1988) found evidence that oocytes of *M. capitata* do not self- or cross-fertilize for at least 1 min after the bundle has broken open and self-fertilization blocks have been reported to last 3 h or more in *Favia pallida, Platygyra pini* and *P. ryukyensis* (Heyward and Babcock 1986).

Most egg-sperm bundles broke open within 30 min after release, which is approximately the time that the spawning events lasted. Agitation of water or exposure to higher temperatures accelerated the bundle breakage. However, faster breakage does not
necessarily mean more or faster fertilization. On the contrary, when the egg-sperm bundles broke quickly (i.e., by higher wave turbulence), the spermatozoa mass separated from the oocytes and sank before the spermatozoa were released. Sinking of the sperm mass thus likely reduced the likelihood of these sperm encountering oocytes because oocytes are positively buoyant and remain on the surface while the spermatozoa sink in the water column and became diluted at depth. Thus, environmental conditions during spawning events have the potential to significantly influence egg-sperm bundle breakage and fertilization rates and may diminish or promote local reproductive success.

The release of deformed oocytes towards the end of the spawning season (August) did not coincide with unusual environmental conditions, and colonies releasing deformed oocytes were found adjacent to colonies that produced regular egg-sperm bundles. These observations raise a number of interesting questions regarding the abiotic and biotic conditions leading to the production of deformed oocytes, whether or not the deformed oocytes are viable, and the selective value of releasing deformed oocytes rather than reabsorbing them. Reabsorption of unspawned oocytes has been described for brooding (Rinkevich and Loya 1979) and spawning (Neves and Pires 2002) corals, and could be an important means of conserving nutrients. If the deformed oocytes result from stress, releasing deformed oocytes could be either a response with beneficial value (i.e., release of a toxin via the gametes), or an indication of impaired reproductive capacity. Failure of bundle formation and release of prematurely aborted oocytes were observed in *Leptoria phrygia* in response to stress (Kojis and Quinn 1982). Elucidating the factors leading to the failure of bundle formation and production of deformed oocytes and their occurrence
in nature could prove to be an important component of understanding the dynamics of coral populations.

The formation of an egg-sperm bundle and synchronicity of spawning are critical aspects for the reproductive success of broadcast spawners, which represent the majority of coral species (Fadlallah 1983; Harrison and Wallace 1990; Baird 2009). These two aspects increase the abundance of female and male gametes in seawater and increase the chances of outcrossing. The selective advantage of the egg-sperm bundle in corals is unequivocal and is exemplified by its occurrence in many species of corals from both the Robust and Complex clades (Kerr 2005). However, it is unclear if the egg-sperm bundle has been acquired or lost several times throughout evolution. Bundles from the two coral clades show differences in structure, with bundles of Acroporids (clade complexa) having the oocytes around the sperm package, whereas bundles of *Favites* sp. (clade robusta) have the oocytes embedded within the sperm cluster (Kojis and Quinn 1982; Wallace 1985; Harrison and Wallace 1990; Richmond 1997). Interestingly, in both clades the oocytes released in the bundle do not have a visible germinal vesicle (Kojis and Quinn 1982, this study) and it is unknown if this observation is associated with the process of bundle formation and/or the release of cortical material in response to seawater. Future studies on bundle structure of both clades may provide insights into different mechanisms of bundle formation and evolution of reproductive strategies in corals.

Little is published on the egg-sperm bundle due to its ephemeral lifespan. However, the failure of bundle formation could have detrimental effects on the reproduction and success of coral populations. As climate change and water quality deterioration (due to anthropogenic effects) continue, environmental cues for spawning
may change and impact the different steps of the reproductive cycle. Bundle formation and breakage are critical components that can be strongly influenced by the environment and thus require further investigation.

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Figure 3.1 *Montipora capitata* prior to spawning, (a) Oocytes and spermatozoa developing along the mesenteries deep in the skeleton. (b) Polyp starting to relax. (c) Polyps ready to release the bundle ~1 min before spawning, with bundle visible beneath the oral disc.
**Figure 3.2** Egg-sperm bundle after release, (a) Egg-sperm bundle. (b) Sudden release of spermatozoa. (c) Bundle breaking apart, oocytes with irregular shape and spermatozoa still present in high concentrations. (d) 30 min later, oocytes spherically shaped and, spermatozoa diluted and no longer visible.
Figure 3.3 Deformed oocytes released at the end of the spawning season (arrows pointing to the deformed oocytes).
Figure 3.4 Micro-structure in *M. capitata* egg-sperm bundles, (a) Scanning electron micrograph of egg-sperm bundle, (b) SEM of broken egg-sperm bundle, “sperm cluster” visible at the center (c) Light micrograph of cross section of egg-sperm bundle, darker dots inside the oocytes are *Symbiodinium* cells.
Figure 3.5 Bundle material in the egg-sperm bundle, (a) Light micrograph of bundle material (bm) holding together the oocytes in the egg-sperm bundle (b) SEM of bm on the oocytes of the egg-sperm bundle (c-d) TEM of bm possibly secreted by the oocytes to form the egg-sperm bundle. TEM of egg-sperm interface (e) no mucus between gametes (f) large amount of mucus separating oocytes and spermatozoa (g) SEM of egg-sperm interface separated by bundle material.
Figure 3.6 TEM of components present inside the oocytes. (a-b) cortical granules with ovoid shape, membrane-bound and homogeneous, (c-f) several types of cortical granules varying in structure shape and electron density, membrane- and non-membrane bound fibrous bodies were regularly present (g) *Symbiodinium* cell in the oocyte, a=accumulation bodies, chl=chloroplast, chr=chromosomes, m=mitochondria n=nucleolus, arrow pointing at the uric acids (h) Some *Symbiodinium* cells were dividing.
Figure 3.7 Microvilli and cortical layer of the oocytes before and after egg-sperm bundle breakage. (a) SEM of microvilli and (b) TEM of cortical layer before bundle breakage when oocytes still have an irregular shape and some bundle material (bm) is still present. (c) SEM of microvilli and (d) TEM cortical layer after oocytes have been released from the bundle and became rounded.
Figure 3.8 SEM of sperm bundle (spermatozoa mass). (a) Sperm clump isolated from an egg-sperm bundle, (b) “glue material” surrounding the sperm clump, (c) hole in the sperm clump, spermatozoa are visible, (d-e) spermatozoa with no particular orientation, (f) “sperm net” material possibly holding together the spermatozoa inside the sperm clump, (g-i) spermatozoa close up.
Figure 3.9 TEM of *M. capitata* spermatozoa structure (a,b): a, anterior less dense nuclear zone, c, collar, dc, distal centriole, l, lipid body, lg, intercentriolar ligament, lm, lamellae, m, mitochondria, n, nucleus, pc, proximal centriole, vn, vesicle apically of nucleus. (c) Spermatozoa cross section, frontal plane (d) spermatozoa cross section, caudal plane, (e) spermatozoa tails with membrane oblique section, (f) Axonemes (9+2 arrangement of microtubules), and (g-h) spermatocytes (early stage sperm) released in the egg-sperm bundle during the beginning of spawning season.
Figure 3.10 Proposed models of egg-sperm bundle formation. I. Model if oocytes and sperm packages develop in different mesenteries. II. Model if oocytes and sperm packages develop intermingled in the same mesentery. Top views show the gametes on the mesenteries prior to initiation of bundle formation.
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CHAPTER 4
FROM PARENT TO THE GAMETE: VERTICAL TRANSMISSION OF
SYMBIODINIUM (DINOPHYCEAE) IN THE SCLERACTINIAN CORAL
MONTIPORA CAPITATA

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ABSTRACT

Parental effects are ubiquitous in nature and play a particularly critical role in the transfer of symbionts across generations in many organisms, however, their influence and relative importance in the marine environment has rarely been considered. Coral reefs are biologically diverse and productive marine ecosystems, whose success is framed by symbiosis between scleractinian (reef-building) corals and the unicellular dinoflagellates in the genus *Symbiodinium*. Many corals produce asymbiotic larvae that are infected by *Symbiodinium* from the environment (horizontal transmission), which creates the opportunity to acquire new endosymbionts each generation. In the remaining species, *Symbiodinium* are transmitted directly from parent to offspring via oocytes (vertical transmission), a mechanism that perpetuates the relationship between some or all of the *Symbiodinium* diversity found in the parent through multiple generations. Here we examine vertical transmission in the Hawaiian coral *Montipora capitata* by comparing the *Symbiodinium* ITS2 sequence assemblages in parent colonies and the oocytes they produce. Parental effects on *Symbiodinium* assemblages in oocytes are explored in the context of the genotype, colony morphology, and the environment of parent colonies. Our results indicate that *Symbiodinium* ITS2 sequence assemblages in oocytes are generally similar to their parents, and patterns in parental *Symbiodinium* assemblages are different, and reflect environmental conditions, but not colony morphology or coral genotype. We conclude that oocytes released by parent colonies during mass spawning events are seeded with different *Symbiodinium* assemblages, taxonomic variability that may have profound implications for the development, settlement and survival of coral offspring.
INTRODUCTION

Parental effects are fundamentally important in biological systems. They occur when the phenotype of the offspring is affected by the phenotype or environment of the parents (Mousseau & Fox 1998, Badyaev & Uller 2009). These effects significantly influence the life history (Donelson et al. 2009), competitive ability (Wulff 1986), evolutionary trajectories, speciation rates (Wade 1998) and population dynamics (Ginzburg 1998, Donohue 2009) of future generations of individuals. Parental effects also facilitate the perpetuation of symbiosis between generations by influencing the direct transmission of symbiotic microorganisms from parent to progeny (Russell & Moran 2006). Although parental effects have been extensively studied in plants and insects and terrestrial vertebrates, these effects have received much less attention in the marine environment (Mousseau & Fox 1998, Marshall et al. 2008).

In the ocean, a variety of algal and cyanobacterial symbionts live in association with protists and animal hosts in habitats ranging from the coastal sediments to the deep-sea hydrothermal vents (Smith & Douglas 1987). Coral reefs are ecosystems whose ecological success is framed by endosymbiotic associations between scleractinian corals and unicellular dinoflagellates in the genus *Symbiodinium* (Muscatine et al. 1981). *Symbiodinium* photosynthesis contributes to the productivity of the ecosystem and newly fixed carbon translocated to coral hosts powers respiration and enhances the deposition of calcium carbonate skeletons that create habitat for the extraordinary biodiversity that characterizes coral reef ecosystems (Muscatine et al. 1984, Allemand et al. 2004).

*Symbiodinium* is a taxonomically diverse genus comprising nine major lineages called clades A through I (Pochon & Gates 2010), that each contain from 2 - >100
subclade types (Baker 2003, LaJeunesse 2005, Pochon et al. 2006). Some coral species form highly specific associations with one or two closely related *Symbiodinium* types from one clade (e.g. Poritids, LaJeunesse 2005, Van Oppen & Gates 2006), while others form relationships with multiple *Symbiodinium* types that span the taxonomic breadth of the genus *Symbiodinium* (e.g. Pocilloporids, Van Oppen & Gates 2006). The taxonomic composition of *Symbiodinium* assemblages found at different locations on the same coral colony, within a single coral species sampled from different reefs, depths or different times of the year may also vary (Baker 2003, Coffroth & Santos 2005, Stat et al. 2009), illustrating a high degree of spatio-temporal variation in these endosymbiotic associations (Rowan & Knowlton 1995, Baker et al. 2004, Iglesias-Prieto et al. 2004). The broad taxonomic divisions in the genus *Symbiodinium* reflect in functional diversity with distinct clades having different physiological optima (Little et al. 2004, Abrego et al. 2008, Cantin et al. 2009, Rowan 2004). Importantly, these differences in performance influence physiological characteristics of the coral host, rendering them more or less susceptible to environmental disturbances and disease (e.g. Baker 2003, Baker et al. 2004, Coffroth & Santos 2005, Stat et al. 2008b).

Given the fundamental role that *Symbiodinium* plays in the basic biology of corals, the perpetuation of this symbiosis is pivotal to the persistence of corals through time. Most coral species release asymbiotic gametes (*Symbiodinium* free) that are fertilized in the water column and then develop into asymbiotic larvae that must acquire *Symbiodinium* from environmental pools (horizontal transmission). In other species, however, *Symbiodinium* are passed directly from the parent to the developing oocytes. These oocytes are released during mass spawning events, are fertilized in the water
column, and then develop into larvae pre-seeded with *Symbiodinium* (vertical transmission). Approximately 25% of coral species that spawn gametes transmit *Symbiodinium* vertically through their oocytes and 90% of corals that brood larvae release larvae containing *Symbiodinium* (Baird et al. 2009). In general, vertically transmitting species exhibit higher specificity in their endosymbiotic unions than horizontal transmitters (Barneah et al. 2004, Thornhill et al. 2006, Stat et al. 2008a). *Symbiodinium* assemblages in coral offspring can be comprised of a single *Symbiodinium* type or a range of types, and as in adults, the taxonomic composition of these assemblages affects the growth and physiological tolerance of the juvenile coral (Little et al. 2004, Abrego et al. 2008, Abrego et al. 2009).

No studies to date have examined the diversity of *Symbiodinium* in the oocytes of a coral that transmits *Symbiodinium* vertically. For coral species that host diverse *Symbiodinium* assemblages, the transmission of *Symbiodinium* directly from parent to the oocyte potentially provides an opportunity for the parent to select the type(s) of *Symbiodinium* transmitted to the gamete, and thus influence the physiological range, survival and recruitment success of their offspring. Further, differences in the endosymbiotic assemblages among adult colonies that reflect environmental conditions, life-history stage, health state or morphology may result in oocytes seeded with very different *Symbiodinium* assemblages. Such parental effects have never been examined in corals but likely have implications for larval behaviors, natural selection after settlement, the potential for acclimatization and ultimately the resilience of coral populations and reef communities.
The timing of spawning in many corals is temporally constrained and predictable, making the collection and comparison of assemblages of *Symbiodinium* in newly spawned oocytes tractable. We used this approach to examine the diversity of *Symbiodinium* transmitted from parents to oocytes in the coral *Montipora capitata*, a simultaneous hermaphrodite that releases egg-sperm bundles during the new moon from late spring through summer in Hawai‘i (Hunter 1988). This coral is one of the most broadly distributed, morphologically plastic and important reef building corals in the main Hawaiian Islands (Jokiel et al. 1983, Jokiel et al. 2004). This combination of traits makes this species an excellent model for examining *Symbiodinium* transmission in the context of environment and morphology. Specifically, this study tests the hypotheses that (i) *Symbiodinium* assemblages between parent and oocytes do not differ and that (ii) differences in the *Symbiodinium* assemblages in the oocytes do not reflect differences in environmental conditions or morphology of the coral adult.

Studying vertical transmission of *Symbiodinium* is especially relevant in Hawai‘i because the reefs of the Hawaiian Archipelago are dominated by coral species that transmit *Symbiodinium* vertically (Jokiel et al. 2004, LaJeunesse et al. 2004) and they represent some of the most isolated reefs in the world. Thus, parental effects may play a particularly important role in shaping the reefs in this region.

**METHODS**

*Study sites and sample collections*

Parent colonies and gametes were sampled at three sites around Moku O Lo‘e Island in Kane‘ohe Bay, Hawai‘i: Bridge to Nowhere (BTN), Gilligan’s Lagoon (GL), and
Point Reef (PR) (Fig. 4.1) during the summers of 2007 and 2008. These sites are located at N 21 25.893’ and W 157 47.376’, N 21 25.973’ and W 157 47.392’, N 21 25.988’ and W 157 47.192’, respectively. Montipora capitata colonies exhibited primarily branching morphologies at BTN, plating at GL, and both branching and plating at PR (Fig. 4.2). The PR site was only sampled in 2008 and was included to examine whether Symbiodinium ITS2 sequence assemblages differed in corals sampled at a site where both branching and plating morphologies co-occur.

Egg-sperm bundles were released (spawned) between 8:45 and 9:15 pm during the first quarter of the new moon in June, July and August of 2007 and 2008. Samples of parent colonies at depths of 1-2 m were collected 5 d before spawning nights by removing small tissue fragments 4 cm away from tips and edges of the colony where the polyps were reproductively active. Egg-sperm bundles released by corals at the BTN and GL sites were collected in the field using cylindrical nets placed over the adult colonies. Due to rough conditions at PR in 2008, ten fragments of adult colonies were moved from PR into tanks (~ 2 weeks before spawning) exposed to ambient light levels and gametes were collected as in Cox (2007). After collection, all coral fragments and gametes were immediately frozen and stored at -80°C until processed.

To compare the thermal environments at the collection sites, temperature was measured at 10 min intervals for 1 yr in 2007 using StowAway Tidbits accurate to ±0.2°C (Onset Computer) at BTN and GL. Light and temperature levels were also measured at all sites during two 2-wk periods in 2008 (22 September – 1 October and 25 November – 5 December, 2008). Light measurements were taken at 10 min intervals using Odyssey Photosynthetic Irradiance Recording Systems (Odyssey). These measurements were
made to compare the light levels among sites and the time of these analyses constrained by the availability of instrumentation. Temperatures were also recorded at PR during the same two 2-wk periods in 2008 (22 September – 1 October and 25 November – 5 December, 2008) that light levels were recorded.

**Symbiodinium DNA isolation, PCR, cloning, sequencing and phylogenetic analyses**

Genomic DNAs from coral fragments and gametes were extracted using guanidinium as described in Stat et al. (2009). The *Symbiodinium* ITS2 rDNA cistron was amplified (primers ‘ITS-DINO’ and ‘ITS2rev2’), cloned and sequenced following procedures detailed in Pochon and Gates (2010). DNA sequences were inspected and assembled using Sequencher v4.7 (Gene Codes Corporation, USA), identified via the Basic Local Alignment Search Tool (BLAST) in GenBank, and manually aligned with the BioEdit v5.0.9 sequence alignment software (Stat et al. 2011).

Sequences included in the downstream analyses met the following criteria: (1) they had either been published previously and the sequences retrieved and verified in multiple independent studies, or (2) were recovered in this study three or more times from clone libraries representing three or more independent coral samples. The remaining clone doubletons and singletons were excluded following Stat et al. (2009). Finally, ITS2 secondary structure folding was analyzed to identify potential pseudogenes as described in Stat et al. (2011). Two ITS2 alignments were then created for phylogenetic analyses, one for *Symbiodinium* clade C sequences and the other one for *Symbiodinium* clade D. Statistical parsimony networks were constructed using the software TCS version 1.21 (Clement et al. 2000) with a 95% connection limit and gaps were treated as a 5th state.
The *Symbiodinium* ITS2 sequence assemblage refers to the total diversity of ITS2 sequences recovered from each sample analyzed. *Symbiodinium* ITS2 DNA sequence assemblages were obtained from 26 colonies of *Montipora capitata* and gametes produced by these colonies (n=52). A subset of six of these adult colonies and their gametes (Colony ID: 8, 10, 19, 23, 29 and 37) were analyzed over two reproductive seasons (summers 2007 and 2008) to examine interannual variability in the *Symbiodinium* ITS2 community sequence profiles (n=12).

**Host DNA isolation, PCR and sequencing**

A second subsample of the frozen adult corals was used to extract DNA from the host described in Concepcion et al. (2006). The ATP synthetase subunit β intron (*atpsβ*) was amplified and sequenced as detailed in Stat et al. (2011). The resulting 300 bp sequences were edited and aligned using SEQUENCHER4.8 (Gene codes, Ann Arbor, MI). Gametic phases were determined computationally with PHASE (Stephens & Donnelly 2003).

**Statistical analyses**

*Symbiodinium ITS2 sequences* - Data from sites BTN and GL (2007 and 2008) and site PR (2008) were analyzed separately because colony morphologies did not co-occur at all sites. Environmental and temporal effects on *Symbiodinium* ITS2 sequence assemblages in both parent and oocytes were analyzed using data from BTN and GL sites. Morphological effects were analyzed using data from PR where both morphologies co-occurred. BTW and GL data were analyzed according to a four-factor experimental
design (site, life-stage, year and colony). PR data was analyzed using a three factor experimental design (morphology, life-stage and colony). Analyses of molecular variance (AMOVA, Excoffier et al. 1992) were used to test whether the composition of *Symbiodinium* assemblages differed between factors (environments, years, life stages and morphology) using the genetic distance between the sequences as described in Stat et al. (2011). Matrices of simple pairwise genetic distances were generated in ARLEQUIN 3.1, the square root of each distance was taken, and the matrices were imported to PERMANOVA+ 1.0.2 software add on for PRIMER 6 (Clarke & Warwick 2001). Φ statistics between different life stages were calculated for each colony according to Excoffier et al. (1992). Φ ranges between 0 to 1, where zero values of Φ indicate identical genetic composition between samples, and Φ values of one indicate alternate fixation of alleles. Shannon and Simpson diversity indices were calculated at the clade level for each sample (parents and gametes); zero values of Shannon and Simpson diversity indices correspond to no diversity. A Mann-Whitney test was used to evaluate differences in the diversity indices between life stages.

**Host genetics**-AMOVAs performed in ARLEQUIN ver 3.11 (Excoffier et al. 2005) were used to test whether host genetic variation was partitioned by morphology or collection site. Global exact tests of non-differentiation (Raymond & Rousset 1995) were then performed (α = 0.05, Markov chain steps = 10,000) to verify the results from the AMOVA.

**Environment** - Means, standard deviations and ranges (minimum–maximum) were calculated for each site during the periods sampled. A Kruskal-Wallis test was used to evaluate the differences in temperature and light between sites.
RESULTS

*Symbiodinium* ITS2 sequence diversity in *Montipora capitata*

A total of 659 sequences were recovered from the 64 samples (32 adults and their respective oocytes), representing 7-13 *Symbiodinium* ITS2 sequences per sample (10 ± 1.87, average ± SE; Table 4.1, Fig. 4.3, GenBank accessions JF683321-JF683339). Our initial screen of sequences resolved 29 ITS2 sequences that have either been published before, or were retrieved from multiple samples here. 24 of these sequences types belonged to *Symbiodinium* clades C, and 5 to clade D (GenBank accession numbers in Supplementary Electronic Table 1). Nine of the sequences matched previously published sequences (C3, C17, C21, C21.6, C21.11, C31, C31.1, D1, and D1a). The remaining 20 sequences were novel and were assigned names indicating the clade, the number of the most closely related published sequence type, and a decimal and a number to distinguish them from published types and one another (Stat et al. 2009).

The 29 ITS2 sequence types collapsed into seven structural folds, 5 representing clade C sequences and 2 clade D (Fig. 4.3, Fig. 4.4, Supplemental Electronic Fig. 4.1). Less abundant sequences generally exhibited identical folding structures to the most closely related dominant sequence. Two sequences, C32.1 and C32.2, exhibited secondary structures that diverged significantly from the fold of their closest relative C21.11 (Fig. 4.3). Based on these structural abnormalities, these sequences did not meet all our criteria for inclusion in the downstream statistical analyses and were excluded.

Patterns in *Symbiodinium* ITS2 sequence assemblages

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Individual coral and gamete samples harboured from 2 to 7 co-occurring ITS2 sequences (Table 4.1) and Shannon and Simpson diversity indices (clade level) were generally greater in the oocytes than adults ($W=902.5$, $p=0.0312$, Table 4.1). The ITS2 sequences C31, C21, D1a, D1, C17.2, and C21.11 were common in parents and oocytes being recovered 159, 119, 69, 52, 44, and 31 times respectively. The less abundant sequences generally differed from a dominant sequence by one to three base pairs. All ITS2 sequences were identified at least once in both life stages except C21.1, which was only detected in adults (Table 4.1, Fig. 4.3, Fig. 4.4). Similarly, all ITS2 sequences were detected at least once in both morphologies, except C31.3, which was only found in adults and gametes of plating morphologies from GL site (Table 4.1, Fig 4.4). Fourteen of the 29 ITS2 sequences types were found at all three sites (Table 4.1), however ITS2 sequence types were not distributed evenly among sites with some being more abundant at one or two sites than the others (Table 4.1, Fig. 4.4).

We examined how *Symbiodinium* ITS2 sequence diversity and assemblages partitioned among colonies, life stages, sites, and time (Table 4.1). *Symbiodinium* ITS2 sequence diversity differed between BTN and GL sites ($p=0.0199$) and between the adult colonies and their oocytes ($p=0.0202$, Table 4.2). There was also a significant interaction among all factors investigated ($p=0.0001$, Table 4.2a), suggesting that *Symbiodinium* assemblages can differ at the colony level (and within life stages) depending on the year and site. $\Phi$ statistics were higher in colonies from BTN ($p=0.005$) than both other sites, reflecting larger differences in the ITS2 sequence assemblages between life stages at this site.
Pairwise comparisons of sites, grouping ITS2 sequences by life stage indicated a significant difference in the *Symbiodinium* sequence assemblages in adult corals from BTN and GL (p=0.0093), but not in their oocytes (p=0.2566). Although adult corals from BTN had a higher abundance of clade D sequences (ITS2 sequences D1, D1a, D1a.1) than those from GL, ITS2 sequence C31 was the most abundant in oocytes from both sites (Table 4.1, Fig. 4.4a,b). In addition, pairwise comparisons of life stage within sites indicated a significant difference in *Symbiodinium* sequence assemblages between adult corals and their oocytes from the BTN (p=0.0408), but not at GL (p=0.4186), with BTN adults having a greater proportion of clade D sequences than their oocytes (Table 4.2, Fig. 4.4a). Despite the fact that oocytes did not differ in *Symbiodinium* sequence composition between the two sites, we found significant differences in ITS2 sequence assemblages in oocytes released by different colonies within each site. This indicates that there are major differences in the *Symbiodinium* sequence assemblages transmitted to oocytes that are simultaneously released by adjacent coral colonies during a mass spawning event. Pairwise comparisons of life stage by colony in 2007 revealed that adult and oocytes had different ITS2 sequence assemblages in 4 of the 7 colonies sampled at BTN (#8,10,12,18, p=0.0005, p=0.0057, p=0.0003, p=0.0001 respectively), and in 1 out of 7 colonies at GL (#37, p=0.0004). No significant differences in the *Symbiodinium* composition between adults and oocytes were found when comparing samples from the same colonies taken in 2007 and 2008 (p=0.4783, Table 4.2a). Generally, differences in the sequence assemblages between life stages were observed in parental colonies harboring predominantly clade D; their oocytes contained higher abundances of clade C (Table 4.1).
Montipora capitata colonies at the BTN were predominantly branching, while those at GL were plating. To test whether the site differences in Symbiodinium sequence assemblages between BTN and GL were due to morphology, we analyzed corals sampled in 2008 from a third site (PR) where the two morphologies co-occur. This analysis revealed no significant difference in the Symbiodinium ITS2 assemblages between plating and branching morphologies at this site (p=0.1944, Table 4.2b), nor between life stages (p=0.2185, Table 4.2b). The only differences in ITS2 sequence assemblages were between life stages and morphology within individual colonies, p=0.0001 (Table 4.2b), a pattern that is consistent with the interpretation that individual colonies at this site had different assemblages of Symbiodinium ITS2.

The six ITS2 secondary structure folds were found in corals from all three sites. There were however, differences in the relative abundance of ITS2 folds at the three sites, which reflected the differences in the ITS2 sequence diversity detailed above. For example, folding clusters D1 and D1a were most abundant at BTN site, whereas folding cluster C21 and cluster C31 were more abundant at the PR and GL sites, respectively (Fig. 4.4).

**Host phylogenetic analysis**

A 280 bp fragment of atpsβ was amplified from 47 adult colonies (20 from BTN, 15 from GL, and 13 from PR). The alignment identified 7 polymorphic sites and 17 distinct atpsβ alleles among the 47 individuals. Using the Akaike information criterion (AIC) with a likelihood approach in MODELTEST v3.06 (Posada & Crandall 1998), the
best fit model of sequence evolution was HKY with base frequencies A=0.3171, C=0.1400, G=0.1333, T=0.4097 and a transition/transversion (Ti/Tv) value of 1.4826.

Branching and plating colonies of *Montipora capitata* shared common alleles supporting the hypothesis that the two morphologies are the same species. AMOVA results indicate that the majority of variance could be explained at the among individual level for groupings based on both morphology and collection site (Pvar = 49.09%; p < 0.001 and Pvar = 52.80% p < 0.001 respectively, Table 4.3). Variance at the highest hierarchical level was low and non-significant in both tests (p > 0.05). Global exact tests of overall non-differentiation are significant indicating no partitioning based on differences between morphologies (p<0.0001) or collection site (p<0.0001). These results suggest a lack of genetic structuring due to either morphology or collection site at the scale examined in this study (Table 4.3).

**Environmental Characteristics of Sites**

Temperature differed between the three sites in both late summer and late autumn (H=972.5, df=2, p<0.0001, H=69.2, df=2, p<0.0001 respectively, Supplementary electronic Table 4.2). Temperature was higher and more variable at the BTN site, with up to ~3°C fluctuations observed over a single 24-hr period (data not shown). Light levels differed significantly among the three collection sites (H=57.9, df=2, p<0.0001, H=59.7, df=2, p<0.0001, summer and autumn respectively). The BTN site had the highest and broadest range of light levels in both summer and autumn sampling times (Supplementary electronic Table 4.2), and exhibited a recorded summer maximum of 1540 μmol quanta/m²s.
The *Montipora capitata* colonies at this site were predominantly branching morphologies. The GL site had the lowest light levels where *M. capitata* were predominantly plating in morphology. Medium light levels were observed at the PR site where plating and branching morphologies of *M. capitata* co-occurred (Supplementary electronic Table 4.2). Overall, PR and GL sites had around 45% and 23% the light levels of BTN.

DISCUSSION

Parental effects in corals with vertical transmission of *Symbiodinium* have the potential to play a significant role in the phenotype of propagules, perpetuation of symbiosis and ultimately the interaction of larvae with the environment. Vertical transmission ensures that offspring are seeded with *Symbiodinium* optimized to interact with the host. This strategy thus reduces the risk of forming unsuccessful symbiotic unions that might occur when acquiring *Symbiodinium* from the environment and that could reduce the growth and fitness of the coral (Weis et al. 2001). This study is the first to explore the taxonomic composition of *Symbiodinium* assemblages vertically transmitted from parent to oocytes in corals. Our results indicate that *Symbiodinium* ITS2 sequence assemblages in the oocytes of *Montipora capitata* are strongly influenced by the taxonomic composition of the endosymbionts of the parent colony, and that the *Symbiodinium* assemblages in the parent colonies differ and reflect characteristics of their physical environment.

A variety of *Symbiodinium* sequence types were identified in the *M. capitata* adults and oocytes, representing clades C and D, *Symbiodinium* lineages known to have
different physiological characteristics and environmental thresholds. In this study, adults and oocytes associated with clade D *Symbiodinium* were hosted by corals in more challenging environments. For example, clade D *Symbiodinium* were found in branching colonies located in areas with high light and variable thermal regimes. This distribution is consistent with previous studies that document broader environmental thresholds for corals that associate with *Symbiodinium* clade D (Baker et al. 2004, Berkelmans et al. 2006, Garren et al. 2006, Oliver & Palumbi 2009).

Previous work on *Symbiodinium* diversity of *M. capitata* in O'ahu (Hawai'i) described a highly specific symbiosis between *M. capitata* brown morph and *Symbiodinium* ITS2 C31, and between the shallow orange morph and *Symbiodinium* ITS2 D1a (LaJeunesse et al. 2004). In many cases the distinction between brown and orange morphs is ambiguous so we used shallow colonies that mostly resembled the “orange” morphotype. Our results show higher diversity than previously reported in the “orange” morph and suggest that the presence and abundance of *Symbiodinium* ITS2 types are not specific to colony color or morphology. Despite the phenotypic plasticity of *M. capitata*, no host genetic differentiation was detected between sites or morphologies. This illustrates the important role that environment plays on structuring *Symbiodinium* ITS2 assemblages. For example, branching morphologies in a high light environment (BTN site) had higher abundances of ITS2 sequence types D1 and D1a, whereas branching morphologies in a lower light environment (GL) had higher abundances of C31 and C21. Likewise, plate morphologies had C21 and C31 as dominant ITS2 sequences at PR and GL sites, respectively. *M. capitata* therefore appears to combine two strategies for
acclimatizing to environmental change via differences in the composition of their 

*Symbiodinium* assemblages and through its extraordinary morphological plasticity.

The taxonomic composition of *Symbiodinium* assemblages isolated from oocytes was generally similar to their respective parent colony, encompassing anywhere from 2 – 7 *Symbiodinium* sequence types. There was, however, a general trend for *Symbiodinium* assemblages in oocytes to have slightly greater diversity (clade level) than their parent colonies. This pattern may reflect adult host control or competition within the *Symbiodinium* assemblages and perhaps a race to occupy the less populated tissues found in the oocytes (Hirose et al. 2001). To date, interactions between vertically transmitted symbionts remains underexplored and perhaps underestimated (Vautrin & Vavre 2009).

For example, it is unknown if a *Symbiodinium* type present in low abundance in an oocyte can proliferate and become dominant in the adult colony under the right environmental conditions, or if there is a threshold in abundance required for a *Symbiodinium* type to be viable in adult colonies.

The ITS2 sequence assemblages in oocytes from parents dominated by C clade *Symbiodinium* were very similar in taxonomic composition to their parents. However, differences were detected in oocytes originating from 4 of 7 parent colonies sampled at the BTN site, an environment where the corals exhibited branching morphologies. These 4 parent colonies all harbored clade D *Symbiodinium*; however, the oocytes they produced all contained clade C ITS2 sequence assemblages. This result suggests that parent colony may preferentially transfer clade C *Symbiodinium* to their oocytes rather than clade D. *Symbiodinium* clade D are often described as opportunistic and although their presence positively influences environmental thresholds in corals, there are known
fitness tradeoffs, and corals hosting clade D do not grow as well as con-specifics that host clade C (Little et al. 2004, Abrego et al. 2009, reviewed in Stat & Gates 2011). The idea that corals can detect these differences in physiology and preferentially select those that will provide the greatest benefit to their offspring is provocative and worthy of further investigation. An alternative explanation for these results is that the differences reflect sampling bias. The oocytes examined were released from multiple polyps located across the colony; in contrast, adult samples were taken from a single location on the colony. *M. capitata* colonies are extremely plastic in their colony morphology, and this structural complexity creates microenvironments with very different light regimes, micro-spatial variations that could influence the distribution of *Symbiodinium* within colony (Kaniewska et al. 2008). Indeed, spatial patterning of *Symbiodinium* clades as a result of differences in irradiance has been reported in *Montastraea* sp. (Rowan & Knowlton 1995) and *Acropora* sp. (Van Oppen et al. 2001, Ulstrup & Van Oppen 2003). It is also noteworthy that *M. capitata* has tissues that penetrate deeply into a porous skeleton. *M. capitata* oocytes develop deep into the skeleton (Padilla-Gamino et al. 2011) and as such, acquire *Symbiodinium* from adult tissues within the skeleton that represent different microenvironments to surface tissues (Santos et al. 2009). These differences may drive micro-zonation of *Symbiodinium* within coral polyps. Although such micro-spatial patterns of *Symbiodinium* assemblages are not well understood, they may have important ramifications for the performance of these corals and are worthy of further investigation.

It is not known whether the assemblages of *Symbiodinium* in larvae or newly metamorphosed juveniles change during development, settlement and metamorphosis. Settling in environments similar to the parent may be more advantageous for the
offspring if the early-stage acclimatization capabilities are limited (i.e. inability to change
Symbiodinium assemblages and/or acquire new Symbiodinium from the environment, “switching/shuffling” (Baker 2003), inability to change host morphology). Furthermore, and as mentioned previously, different Symbiodinium assemblages in offspring can confer different physiological attributes to larvae and/or juvenile corals. For example, Little et al. (2004) found that juvenile Acropora (same family as M. capitata) grow faster when infected with clade C than with clade D, regardless of whether clade C was the homologous or heterologous subclade type. Furthermore, Abrego et al. (2008) showed that Acropora juveniles infected with Symbiodinium type C1 had enhanced physiological tolerance (measured by photosynthesis, respiration and fluorescence) over juveniles infected with clade D. Juveniles with Symbiodinium type C1 also had higher 14C photosynthate incorporation and increased carbon delivery to the host (2009).

Our data demonstrate that M. capitata colonies simultaneously release oocytes that contain very different assemblages of Symbiodinium during a mass spawning event. Once released, the oocytes rise to the surface, mix, get fertilized and develop into larvae, with gastrulae and actively swimming larvae forming 11 h and ~2.5 days after spawning, respectively (Mate et al. 1998). Although settlement can occur as soon as embryogenesis is complete (3 d), M. capitata larvae in the field can remain planktonic for ~6 weeks, and in the lab, have been reported to delay the onset of settlement competency or maintain it for 7 months or longer (Kolinski 2004). These extended larval competency periods likely reflect immediate energetic inputs from their parentally transmitted Symbiodinium assemblages (Harii et al. 2010), an exchange that is also likely to satisfy the energetic demands for these larvae to travel long distances (LaJeunesse et al. 2004). The
production of a pool of oocytes containing individuals with *Symbiodinium* assemblages that exhibit different physiological optima could potentially allow larvae to exploit a variety of habitats and survive a range of environmental conditions both in the water column and after settlement. As such, this characteristic may serve as an adaptive strategy to maximize reproductive success when the environments that offspring face vary unpredictably. The variable composition of the larval *Symbiodinium* assemblages coupled with extended larval competencies potentially facilitated by the early infection of *M. capitata* larvae with *Symbiodinium* as a result of vertical transmission highlights the fundamental importance of this endosymbiosis in this coral species and perhaps explains the ecological success and broad spatial distribution of *M. capitata* across the Hawaiian archipelago.

This study is the first to demonstrate that the environment plays a significant role in the parental effects of a coral with vertical transmission. These effects have the potential to significantly influence the biology and ecology of host and the *Symbiodinium* (Rowan & Knowlton 1995, Herre et al. 1999), the evolutionary processes (i.e. speciation rates) and the perpetuation and evolution of coral holobiont mutualisms (Thompson 1994). *Montipora capitata* is a coral with high morphological plasticity that is able to host multiple *Symbiodinium* genotypes, and these genotypes differ in abundance depending on the environment and the colony. *Symbiodinium* diversity in the gametes of this coral is a dynamic trait under parental influence. By releasing gametes with different *Symbiodinium* compositions, *M. capitata* populations maximize the chances of the early-stage holobionts to recruit and grow in microenvironments with very different environmental conditions and possibly reduce competition between the recruits. The
diverse array of early-stage holobionts can enhance the resilience of future generations of
*M. capitata* and may possibly increase the potential for adaptive responses to rapid
environmental change.

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contribution number XXXX, SOEST contribution number XXXX and 2007 Pauley
Summer Program Contribution number X.
Table 4.1 *Symbiodinium* ITS2 sequences types in adults and oocytes of *Montipora capitata*, and ATP synthetase subunit β genotypes for the adult corals sampled at three sites on Moku O Lo‘e Island, Kaneohe Bay, Hawai‘i. Site abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon, PR – Point Reef. Column Headings: *S* - number of clades/sample, *H’* - Shannon diversity index, *I-*\(\lambda\) - Simpson diversity index, \(\phi\) - genetic composition, G – adult genotype. Numerals superscripts indicate the number of times a specific *Symbiodinium* ITS2 sequence was retrieved.

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<th><em>Symbiodinium ITS2</em> sequences/s ADULTS</th>
<th><em>Symbiodinium ITS2</em> sequences/s OOCYTE</th>
<th><em>S</em></th>
<th><em>H’</em></th>
<th><em>I-</em>(\lambda)</th>
<th>(\phi)</th>
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Table 4.2 Results of AMOVA testing for differences in *Symbiodinium* ITS2 sequence assemblages among sites, years and life stage (BTN, GL sites) (A) and between morphology and life stage (PR site) (B). Significance was determined by permutation test (10,000 permutations) of the pseudo-F statistic. Significant values (p < 0.05) are indicated with bold font. Factor abbreviations: *Si* - site, *Li* – life stage, *Ye* - year, *Co* - colony, *Mo* - morphology.

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Table 4.3 Results of AMOVA showing how genetic variance is partitioned for *M. capitata* when grouped according to morphology (A) and collection site (B) for the diploid nuclear locus atpsβ.

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<td><em>φ</em>&lt;sub&gt;AI(AS(AM))&lt;/sub&gt;</td>
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*P < 0.05; **P < 0.005; ***P << 0.001; statistical probabilities derived from 1023 permutations*
Figure 4.1 Location of study sites: Parent colonies and gametes were sampled at three sites around Moku O Lo’e Island: Bridge to Nowhere (BTN), Gilligan’s Lagoon (GL), and Point Reef (PR). Moku O Lo’e Island is located in Kaneohe Bay on the windward coast of the island of O'ahu, Hawai‘i, USA. Montipora capitata colonies exhibited primarily branching morphologies at BTN, plating at GL, and both branching and plating at PR.
Figure 4.2 *Montipora capitata* colonies representing (a) plating and (b) branching morphologies, and (c) oocytes post release seeded with *Symbiodinium* cells acquired from parent colonies.
Figure 4.3 Statistical parsimony networks of *Symbiodinium* ITS-2 sequence diversity (N=659 sequences) identified from 64 *Montipora capitata* coral samples (see Table 1), showing the relationships among the 24 distinct ITS-2 sequence types retrieved in *Symbiodinium* clade C, and 5 in *Symbiodinium* clade D. The pie charts correspond to individual *Symbiodinium* ITS-2 sequences, with the diameter of the pie charts proportional to the number of sequences retrieved corresponding to the circular inset scale (exact numbers given in brackets). Grey and black areas correspond to sequences obtained from adult coral colonies and coral oocytes, respectively. Panels labeled a-g indicate the seven ITS2 secondary structures obtained. Two potential pseudogene sequences (C32.1 and C32.2) resulted in abnormal folds in the stems of helix IIIb (fold ‘e’; see red arrow), and were removed from further statistical analyses. Details on secondary structure folds are shown in Supplementary Electronic Figure S4.1.
Figure 4.4 Symbiodinium ITS2 sequence assemblages found in Montipora capitata colonies sampled from reefs at three sites on Moku O Loʻe Island, Hawaiʻi. The frequency of Symbiodinium ITS2 sequences per site (adult and gamete) is displayed as bar graphs. The pie charts represent the frequency of Symbiodinium based on six of the seven ITS2 secondary structures (folds a, b, c, d, f and g; see Fig 1); note that fold type e did not meet our criteria for inclusion and was omitted from the analysis.
REFERENCES


Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene


Donelson JM, Munday PL, McCormick MI (2009) Parental effects on offspring life histories: when are they important? Biology Letters 5:262-265


Edwin W Pauley summer program in marine biology. Hawaii Institute of Marine Biology, Kane'ohe 27-40


Van Oppen MJH, Palstra FP, Piquet AMT, Miller DJ (2001) Patterns of coral-dinoflagellate associations in *Acropora*: significance of local availability and physiology of *Symbiodinium* strains and host-symbiont selectivity. Proceedings of


Supplementary Table S4.1 GenBank Accession Numbers for the *Symbiodinium* ITS2 sequences identified in the present study.

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Supplementary Table 4.2 Temperature (°C) and light (μmol quanta/m²/s) data from the three study sites in Moku O Loʻe Island, Kaneohe Bay Hawaiʻi. Asterisk (*) indicates that the sampling period was restricted to a short time interval (~2 weeks). Abbreviations: max – maximum, min - minimum, std - standard deviation, cv - coefficient of variation.

### BRIDGE TO NOWHERE

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Supplementary Figure S4.1 *Symbiodinium* ITS2 secondary structures representing the 29 ITS2 sequence haplotypes shown in Fig. 1 (schematized here on the upper left corner).
Seven distinct fold clusters (a-g) were characterized based on criteria described in Stat et al. (2010). The seven secondary folding structures shown here correspond to the most dominant ITS2 sequence found in each cluster (i.e., C3, C31, C17.2, C21, C32.2, D1a, and D1). The location of mutations (insertions, deletions, or hemi-CBC changes) for each ITS2 sequence variant found in each cluster are indicated with a green arrow and corresponding variant number. Four sequence variants are not indicated here, because the observed mutations are found within the 5.8S rDNA (i.e., outside of ITS2 secondary structure). Furthermore, 1 out of 2 and 1 out of 3 observed mutations were also found within the 5.8S rDNA for sequences C21.4 and C21.1, respectively.
CHAPTER 5

FROM PARENT TO THE GAMETE: PARENTAL EFFECTS ON OOCYTE BIOCHEMICAL PROPERTIES IN THE SCLERACTINIAN CORAL MONTIPORA CAPITATA

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ABSTRACT-

Coral reefs are extremely diverse and productive ecosystems that are threatened by a variety of environmental stressors associated with climate change and other anthropogenic perturbations. The persistence of coral reefs depends largely on the ability of coral holobionts (animal host and their symbiotic community) to acclimatize and/or adapt to environmental change. Corals that are able to adapt/acclimatize may influence the physiological capabilities of future offspring via parental effects. Parental effects occur when the phenotype of an individual is affected by the phenotype of the parent or the environment that the parent is experiencing. To date, parental effects are largely unexplored in corals but may play a significant role in dictating the phenotypic range of traits in coral offspring and influence their ability to interact with future environmental challenges. This study explored parental effects in the Hawaiian reef building coral *Montipora capitata* by examining the biochemical composition of mature colonies and their oocytes sampled from different environments. Our results indicate that the biochemical composition of oocytes from different environments was most influenced by differences in *Symbiodinium* characteristics of the adults. Oocytes released from coral colonies located in the high light environment (branching morphology) had higher densities of *Symbiodinium* and lower pigment concentrations per algal cell than the oocytes released from coral colonies in the low light environment (plating morphology). Oocytes and adults differed significantly biochemically, with oocytes exhibiting higher concentration of lipids (mostly wax esters), ubiquinated proteins (which may indicate high turnover rate of proteins) and antioxidants (e.g. manganese superoxide dismutase). α-carotene, a green-algal characteristic pigment and powerful antioxidant, was also
present in higher concentrations in the oocytes, however it remains unclear whether this pigment is acquired by *M. capitata* from its diet or from the endolithic green algae living in the coral skeleton. *M. capitata* is a coral with extraordinary phenotypic plasticity (host and symbiont) that can release gametes with a diverse array of traits (depending on the parental environment). Such a strategy may maximize the ability of the offspring to colonize different habitats and increase the potential for environmental change adaptation of this species.

**INTRODUCTION**

The persistence of coral reefs depends on the ability of the coral holobiont (host and symbionts) to respond to changes in their environment either by acclimatization or adaptation (Buddemeier and Fautin 1993, Fitt et al. 2000, Coles and Brown 2003, Hughes 2003, Sotka and Thacker 2005, Maynard et al. 2008, Csaszar et al. 2010).

Acclimatization strategies in corals may have important consequences for the survival of new recruits and influence the recovery and resilience of existing populations. Currently, coral reefs are experiencing unprecedented pressure and extinction risk due to climate change (e.g. global warming, ocean acidification) and other local impacts associated with anthropogenic disturbances, including pollution, dredging, and overexploitation (Hughes et al. 2003, Hoegh-Guldberg et al. 2007, Carpenter et al. 2008, Lough 2008). How these environmental changes will impact the physiological status of adult corals and in turn influence their offspring’s phenotype is largely unexplored.

Parental effects occur when the phenotype of the offspring is affected by the phenotype or environment of the parents (Mousseau and Fox 1998, Badyaev and Uller
These effects can significantly influence the life history (Donelson et al. 2009), competitive ability (Wulff 1986), evolutionary trajectories, speciation rates (Wade 1998) and population dynamics (Ginzburg 1998, Donohue 2009) of future generations of individuals. Parental effects may occur prior to or following fertilization (Wade 1998). Prezygotic influences affect the gamete properties that are potentially utilized during development (e.g., oocyte size, symbiont transmission), whereas postzygotic influences control parent-offspring interactions over the period of gestation (e.g., larvae of brooding corals).

Parental effects have been extensively studied in plants, insects and terrestrial vertebrates, but have received much less attention in the marine environment (Mousseau & Fox 1998, Marshall et al. 2008). For example, only three studies have focused on the phenotypic relationship between parents and offspring in corals (Michalek-Wagner and Willis 2001, Wellington & Fitt 2003, Alamaru et al. 2009). These studies have revealed pre- and post-zygotic parental effects reflecting differences in the reproductive strategies of the corals studied. Oocytes and larvae of the broadcast-spawning reef corals *Acropora palmata*, *Montastraea annularis* and *M. franksi* have exhibited different levels of photoprotective compounds and survival capabilities depending on the depth of the parent colonies (Wellington and Fitt 2003). Similarly, a strong relationship between the isotopic signatures of parental tissues and that of their planulae has been found in the brooder *Stylophora pistillata* sampled across different depths (Alamaru et al. 2009).

While oocytes and embryos develop in the water column they experience environmental conditions that are very different from the parental colonies and that may exceed their physiological limits such as high levels of UV radiation, temperature,
pathogens, free radicals and toxins (Epel et al. 1999, Polato et al. 2010, Yakovleva 2009, Marquis et al. 2005, Markey et al. 2007, Hamdoun & Epel 2007). Thus, transfer of protective mechanisms from the coral parent to the gamete/larvae may be critical in provisioning the offspring to cope with environmental challenges and develop successfully (Hamdoun & Epel 2007, O’Connor 2007).

As climate change intensifies, parental effects may play an important role in the survival of offspring and the resilience of future generations. Offspring from parents with compromised health (e.g., corals affected by bleaching events or diseases, partial predation or breakage) may have reduced physiological capacity and this could, in turn, prevent successful development and recruitment under stressful environmental conditions. For example, compared to healthy colonies, experimentally bleached parents of the soft coral Lobophytum compactum release oocytes with lower levels of protein, lipid, mycosporine-like amino acids (MAA) and carotenoid concentrations, which may jeopardize oocyte and larval viability (Michalek-Wagner and Willis 2001). That said, despite the vulnerability of most corals to drastic changes in the environment, corals from three major genera (Acropora, Pocillopora and Porites) have shown the capacity to increase their thermal tolerances following a mass bleaching event (Maynard et al. 2008). How this increase in thermal tolerance in coral adults affects the performance of their offspring remains unknown, but it is possible that parental effects may modify offspring to induce developmental variation in a particular environmental condition (Sultan 2007, Badyaev and Uller 2010).

In this study, we compared biological traits in adult colonies of Montipora capitata (Family Acroporidae) and their oocytes (Table 1) to better understand the
relationship between the phenotype of parent colonies and their oocytes, and determine how this relationship is influenced by different environments. Parental effects were explored in the context of their energetic (e.g. lipid reservoirs, symbiotic algae, isotopic signatures) and protective (e.g. antioxidants) functions, which can influence the ability of the embryo/larvae to disperse and cope with oxidative stress due to higher levels of both PAR, UV and temperature compared to the adults (Gleason & Wellington 1995, Gleason et al. 2006, Wick et al. 1996).

*Montipora capitata* is one of the most abundant and important reef building coral species in the main Hawaiian Islands (Jokiel et al. 2004), is a broadcast-spawner that acquires its endosymbiotic dinoflagellates from the parent (i.e., vertically) and exhibits a high degree of phenotypic plasticity (Maragos 1972). This work helps to better understand what determines the natural phenotypic variability of *M. capitata* oocytes (host and symbiont), which may influence the success of new recruits, and contribute to the resilience of coral reef ecosystems in Hawaii.

**METHODS**

*Study sites and sample collections*

Samples of parent colonies and their gametes were collected during the spawning events in summer 2007 from two sites located in the western side of Moku O Lo'e Island in Kane'ohe Bay (O'ahu, Hawai'i). Coral colonies at ~1-2 m depth were identified and photographed at Bridge to Nowhere (BTN; 21° 25.893’ N; 157° 47.376’W, n=21) and Gilligan’s Lagoon (GL; 21° 25.973’N; 157° 47.392’W, n=20).
To compare temperature and light conditions at the two collection sites, temperature was measured at 10 min intervals for ~1 year (July 2007-August 2008) using StowAway Tidbits (Onset Computer) accurate to ±0.2°C. Light and temperature levels were also measured at each site during two 2-week periods in 2008 (22 September – 1 October and 25 November – 5 December, 2008). Light measurements were taken at 10 min intervals using Odyssey Photosynthetic Irradiance Recording Systems (Odyssey).

Gametes were collected from each adult coral using a net system designed to isolate individual colonies for gamete collection, while minimizing damage to the colonies or their released gametes and avoiding mixing of gametes from neighbor colonies. The cylindrical nets consisted of three rings connected to each other with a cylinder of fabric with a mesh size of ~200 μm (Fig. 5.1). The negatively buoyant ring anchored the net on the substrate; the positively buoyant ring floated on the surface, extending the net through the water column and allowing it to encompass the entire colony, and the slightly negatively buoyant sinking ring automatically self-adjusted the height of the net to accommodate tidal changes in the height of the water column (Fig. 5.1).

_Montipora capitata_ released (spawned) egg-sperm bundles (Fig. 5.2a) between 8:45 and 9:15 p.m. during the first quarter of the new moon in June of 2007. The nets were placed over the corals 1-2 h before spawning and removed each night after spawning. The positively buoyant egg-sperm bundles were collected using scoop nets, transferred to plastic beakers and broken apart by rinsing with 0.2 μm filtered seawater (Fig. 5.2b). A subset of the freshly collected oocytes were observed and photographed immediately upon collection using dissecting and compound microscopes (Olympus
SZX7 and BX51, respectively) (Fig. 2a-d). Most oocytes were stored at -80°C until further analysis. Samples from adult coral colonies were collected 5 days prior to spawning by breaking small fragments (~25 cm²) from a region of the colony at least 4 cm away from the tips and edges where polyps were anticipated to be reproductively active (Wallace 1985). These samples were immediately placed in dry ice and stored at -80°C for further analysis.

**Laboratory analysis**

All measurements were conducted on coral samples that included the host tissue, the endosymbiotic *Symbiodinium* community, and the skeleton (for adults). Samples were ground (if skeleton was present), homogenized and normalized to total ash-free dry tissue biomass of the organic fraction (host tissue and algal symbionts) according to Grottoli et al. (2004).

**Total lipids and lipid classes**

Total lipids were extracted according to Rodrigues and Grottoli (2007). In brief, lipids were extracted from ground samples (skeleton + host tissue + symbiont cells) in a 2:1 chloroform/methanol solution, the organic phase was washed using 0.88% KCl and the extract dried to a constant weight (Harland et al. 1991). Triacylglycerol (TAG) and wax ester (WE) concentrations in total lipid extracts were determined by high-performance liquid chromatography/evaporative light scattering detection (HPLC/ELSD) using triolein (Sigma-Aldrich, #1787-1AMP) and oleyl oleate (Sigma-Aldrich, # O3380) as reference standards (Silversand and Haux, 1997). The percentage of the “other lipids”
(OLs; polar lipids, free fatty acids, sterols, diacylglycerols, and monoacylglycerols) was estimated as the difference between total lipids and the sum of TAGs and WEs.

**Stable isotopes and element ratios**

Glass Fiber Filters (0.7 μm pore size, GF/F; Whatman) containing the homogenized coral samples were dried prior to isotopic and phosphorus analyses. Samples were analyzed on a Costech Elemental Analyzer coupled to a Finnigan Delta IV Plus stable isotope ratio mass spectrophotometer under continuous flow using a CONFLO III interface in the Stable Isotope Biogeochemistry Laboratory at the Ohio State University. Approximately 10% of all samples were run in duplicate. Stable carbon ($\delta^{13}C = \%o$ deviation of the ratio of $^{13}C:^{12}C$ relative to the Vienna Peedee Belmenite Limestone standard) and stable nitrogen ($\delta^{15}N = \%o$ deviation of $^{15}N:^{14}N$ relative to air) measurements were made where the average standard deviation of repeated measurements of the USGS24 and IAEA-N1 standards were 0.06‰ for $\delta^{13}C$ and 0.12‰ for $\delta^{15}N$. Total phosphorus was obtained using the modified high-temperature ashing/hydrolysis method (Monaghan and Ruttenberg, 1999). For each coral tissue homogenate (1-0.5ml), 40 μL of MgNO$_3$ were added; then, the solution was dried and burned at 550°C for 2 hours. After combustion, the residual material was hydrolyzed in 1mL of 1M HCl and analyzed via the molybdenum blue method (Grasshoff 1972).

**Protein expression**

The expression of manganese superoxide dismutase (MnSOD) and ubiquitin-conjugated proteins was studied via western blot according to Barshis et al. (2010).
MnSOD is a mitochondrial enzyme that plays an important role during oxidative stress (Halliwell & Gutteridge 1999). Ubiquitin is a cellular protein that tags protein for degradation in the proteosomes. Increased levels of ubiquitin conjugates indicate high levels of protein degradation (Welchman 2005). Standard curve dilutions of 0, 2, 6, 10, 14 and 18 μg total protein for ubiquitin-conjugate assays and 0, 1, 3, 5, 7 and 9 μg total protein for all other assays of a single, standard coral sample were added to all gels for reference. Note that an increased range of standard was needed for ubiquitin-conjugate assays to accurately capture the wider range of the experimental samples. For each experimental sample, 2 μg total protein was added to 4 μL of E-PAGE Sample Loading Buffer (Invitrogen, Carlsbad, CA), and water to a final volume of 18 μL. All samples and standards were run in triplicate, boiled at 95˚C for 5 min before loading onto E-PAGE 96-well 6% gels (Invitrogen) and run for 14 min with the provided E-PAGE power supply according to manufacturer’s instructions.

Gels were removed, incubated for 30 minutes in a western transfer buffer consisting of 25 mM Tris, 192 mM Glycine, 20% v/v Methanol, 0.025% w/v Sodium Doecyl Sulfate (SDS) pH 8.3 before transfer using a Criterion blotting system (Bio-Rad) according to manufacturer’s instructions with PVDF (Millipore, Bedford, MA) membrane. Transfers were conducted for 30-40 min at 100 V. Membranes were blocked overnight in 50 mL 1x TBST (50 mM Tris, 150 mM NaCl pH to 7.4, 0.1% (v/v) Tween-20) and 1% (w/v) Bovine Serum Albumin (BSA) at 4˚C. After blocking, membranes were incubated with primary antibody in TBST/BSA blocking buffer for 2 h at room temperature, washed three times for 10 min each with fresh changes of TBST, incubated with secondary antibody in blocking buffer for 1 h, washed three times for 5 min each in
1x TBS with 0.3% Tween-20 (v/v), and three times for 5 min each in 1x TBST before detection with SuperSignal West Dura Extended Duration Substrate (Pierce). Antibodies against Ubiquitin (Cat, #SPA-200), Manganese Superoxide Dismutase (MnSOD; Cat.#SOD-110), and anti-rabbit- and anti-mouse-conjugated (Cat. #SAB-300, SAB-100) horseradish peroxidase were obtained from Stressgen Biotechnologies (Victoria, British Columbia, Canada). Primary antibody dilutions were 1:5,000 for MnSOD, and 1:2,000 for Ubiquitin, secondary antibody dilutions were all 1:10,000. All images were recorded using a ChemiDoc XRS molecular imager (Bio-Rad) and analyzed with National Institutes of Health Image J software.

Fluorescent properties of oocytes and sperm were explored using epifluorescence microscopy. Emission characteristics of coral fluorescent proteins (FP) in oocytes and adults were examined using a fluorescence spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, California).

**Algal pigments**

Glass Fiber Filters (0.7 μm pore size, GF/F; Whatman) containing the coral and algal tissue homogenate were extracted in 3 mL of HPLC-grade acetone in culture tubes along with 50 μL of an internal standard (canthaxanthin) at 4°C (in the dark) for 24 h. The extracts were then brought to room temperature, vortexed, and centrifuged for 5 min to remove cellular and filter debris. A mixture containing 1 mL of the sample extract (with internal standard) plus 0.3 mL HPLC grade water was prepared in opaque autosampler vials. Then, 200 μL of this mixture was injected onto a Varian 9012 HPLC system equipped with a Varian 9300 autosampler, a Timberline column heater (26°C),
and a Waters Spherisorb® 5 µm ODS-2 analytical (4.6 x 250 mm) column and corresponding guard cartridge (7.5 x 4.6 mm). Pigments were detected with a ThermoSeparation Products UV2000 detector ($\lambda_1 = 436$, $\lambda_2 = 450$). A ternary solvent system was used for pigment analysis: Eluent A (methanol:0.5 M ammonium acetate, 80:20, v/v), Eluent B (acetonitrile:water, 87.5:12.5, v/v), and Eluent C (100% ethyl acetate). Solvents A and B contained an additional 0.01% 2,6-di-ter-butyl-

$p$-cresol (0.01% BHT, w/v; Sigma-Aldrich) to prevent the conversion of chlorophyll $a$ into chlorophyll $a$ allomers. The linear gradient used for pigment separation was a modified version of the Wright et al. (1991) method: 0.0’ (90% A, 10% B), 1.00’ (100% B), 11.00’ (78% B, 22% C), 27.50’ (10% B, 90% C), 29.00’ (100% B), 30.00’ (100% B), 31.00’ (95% A, 5% B), 37.00’ (95% A, 5% B), and 38.00’ (90% A, 10% B) (Bidigare et al., 2005). Eluent flow rate was held constant at 1 mL min$^{-1}$. Peak identity was determined by comparing retention times of pure standards with those of extracts prepared from algal cultures of known pigment composition. A dichromatic equation was used to resolve mixtures of monovinyl and divinyl chlorophyll $a$ spectrally (Bidigare and Trees, 2000). Pigment concentrations and ratios at the colony and algal cellular level were computed by normalizing to sample dry tissue biomass ($\mu$g pigment/g dw, symbiont density (pg pigment/cell) and chlorophyll $a$ concentrations (pigment/Chl $a$), respectively (please see Table 1 to review the differences between pigment normalizations).

**Symbiodinium densities**

*Symbiodinium* cells were separated from the host tissue (adult and oocytes) by homogenizing the ground coral samples, centrifuging and performing multiple washing
steps using 0.2μm filtered seawater (FSW). After separation, the *Symbiodinium* pellet was resuspended in FSW, homogenized and three subsamples counted manually with a hemocytometer and a light microscope (Olympus BX-51).

**Statistical analyses**

Prior to analysis, data were normalized as necessary using logarithmic or inverse transformations to achieve homogeneity of variances and normality. The xanthophylls DDX and DTX had similar patterns throughout and were combined for the statistical analysis. All variables were analyzed using a general linear model, with developmental stage and site as fixed factors and colony modeled as a random factor nested within site to account for the repeated measurements between adults and oocytes. Site is the between subjects factor and stage is the within subject factor. Parental effects were identified when both site and stage factors were significant and differences between the oocytes reflected the differences between the adults. A linear regression was used to estimate the number of bundles (or oocytes) per ash-free dry tissue mass of the oocyte sample, in order to get an estimate of the amount of symbiont cells per oocyte. Means, standard deviations and ranges (minimum–maximum) of temperature and light were calculated for each site during the periods sampled. Temperature and light measurements were compared between sites using a Mann Whitney test. All statistical analyses were performed using Minitab statistical software (version 15). Values of p<0.05 were considered significant.

**RESULTS**
**Study site environment** –

Temperature was higher and more variable at the BTN site throughout the year (W=45411522436, p<0.0001, Fig. 5.3), with up to ~3°C fluctuations observed over a single 24-hr period (data not shown). The BTN site was also characterized by the highest and broadest light intensities in both late summer and late autumn sampling times compared to the GL site (W=1773932, p<0.0001, W=1413959, p<0.0001, September and November respectively, Fig. 5.3). The GL site had around 23% light levels of BTN. *Montipora capitata* colonies at the BTN site were generally branching in morphology (Fig. 5.2e). The lowest light intensities were found in the GL site where *M. capitata* colonies were predominantly plating in morphology (Fig. 5.2f).

**Total lipids and lipid composition**

Total lipid concentrations were higher in oocytes than in adults (p<0.0001, Fig. 5.4a, Table 5.2) representing 80% and 25% of the total dry tissue weight, in oocytes and adults, respectively. Although not statistically significant, adult colonies from the BTN site exhibited slightly higher lipid contents than those from the GL site, however the lipid contents of oocytes from both sites were very similar (Fig. 5.4a). Lipid composition differed between life stages, with wax esters representing approximately 56% of the total lipid weight in the oocytes while only 9-17% of the total lipid weight in adults (Fig. 5.4b, d, Table 5.2). Oocytes from the GL site contained higher levels of WE than oocytes from the BTN site, even though parents from the BTN site had higher levels of WE (p<0.0001, Fig. 5.4b, d, Table 5.2). Triacylglycerols on the other hand, were found in higher concentrations in the adults than the oocytes (p=0.04, Fig. 5.4c-d, Table 5.2). The “other
lipids” category ranged between 36-92% (mean 74.9%) in adults and 25-57% (mean 45.5%) in oocytes (Fig. 5.4d).

$\delta^{13}C$, $\delta^{15}N$, % carbon % nitrogen, % phosphorous, and elemental ratios (C:N, C:P and N:P).

Adult colonies were enriched in $\delta^{13}C$ compared to oocytes (p=0.009, Fig. 5.5a, Table 5.2). Average $\delta^{15}N$ values for adults and oocytes from both sites ranged between 3-5.7 ‰ and were not different between life stages or sites (data not shown, Table 5.2). Carbon content was higher in oocytes than adult corals (p<0.0001, Fig 5.5.b, Table 5.2). Oocytes from the BTN site contained higher levels of carbon than oocytes from the GL site, even though parents from the GL site had higher levels of carbon (p=0.042, Fig. 5.5b, Table 5.2). Nitrogen and total phosphorus contents were higher in the adult coral tissue than in the oocytes (Fig. 5.5c-d, Table 5.2). C:N ratios differed between both life stages and sites (p<0.0001, p=0.028 respectively). C:N ratios in oocytes were higher than the adult colonies and different between the two sites with oocytes collected from BTN (high light) having higher C:N ratios compared with oocytes collected from GL (low light) (Fig. 5.5e, Table 5.2). C:P ratios were higher in the oocytes than in adults (p<0.0001, Fig. 5.5f, Table 5.2) but similar across sites. N:P ratios did not differ among life stages or sites (data not shown, Table 5.2)

Protein expressions

Relative concentrations of MnSOD were higher in the oocytes than in the adults (p<0.0001, Fig. 5.6a, Table 5.2). Relative concentrations of ubiquitin-conjugates were
greater in oocytes than in parents, and greater at the GL than the BTN site (p<0.0001, p=0.024 respectively, Fig. 5.6b, Table 5.2).

Fluorescent proteins were detected in both oocytes and adult tissues. Both emitted cyan-green light, characteristic of fluorescent proteins (λ=491nm, 3nm resolution) and far-red light from chlorophyll (λ=682nm, 3nm resolution) when excited by blue light (450nm) (Fig 5.2 c-d). Sperm were not fluorescent.

**Algal pigments and Symbiodinium densities**

The dinoflagellate pigments Chl $a$, Chl $c_2$, peridinin, β-carotene, dinoxanthin, diadinoxanthin (DDX) and diatoxathin (DTX) were detected in samples from all colonies for both adults (n=12) and oocytes (n=12). Dinoflagellate pigment concentrations per g ash free dry tissue were always higher in the adults compared to the oocytes (p<0.001, Fig. 5.7a-f, Table 5.3). No differences were found between sites within each life stage (Fig. 5.7, Table 5.3).

The pigment alpha-carotene (α-carotene), characteristic of green algae, was also detected in all colonies and both life stages. In contrast to dinoflagellate (brown algae) pigments (Fig. 5.7a-f), α-carotene was found in higher concentrations in the oocytes compared to the adults (p<0.001, Fig. 5.7g, Table 5.3).

In addition, *Symbiodinium* densities (cell number normalized by tissue dry mass) were higher in adult corals than in oocytes (p<0.0001, Fig. 5.7h, Table 5.3). Higher *Symbiodinium* densities were also found in adults and oocytes from the BTN site as compared to counterparts from GL (p=0.022 respectively, Fig. 5.8b, Table 5.3).
*Symbiodinium* densities in the oocytes ranged between 2.3 and 4.2 x $10^3$ symbiont cells per oocyte.

Pigment concentrations normalized per *Symbiodinium* cell, showed differences between stages and sites (Fig 5.8, Table 5.3). The concentrations of Chl *a*/cell, β-carotene/cell, dinoxanthin/cell, DDX+DTX/cell were greater in adults than in oocytes (p=0.001, p=0.008, p=0.004, p=0.007 respectively; Fig. 5.8a, d, e-f, Table 5.3). Peridinin/cell, β-carotene/cell, dinoxanthin/cell and DDX+DTX/cell (p=0.036, p=0.012, p=0.043, p=0.029 respectively) were also higher at the GL site as compared to BTN (Fig. 5.8c-f).

Pigment concentrations normalized by Chl *a* concentrations revealed differences between adults and oocytes as well (p≤0.001, Fig. 5.9a-e, Table 5.3). Chl *c*<sub>2</sub>/Chl *a*, peridinin/ Chl *a*, β-carotene/Chl *a*, dinoxanthin/ Chl *a* and DDX+DTX/ Chl *a* were higher in the oocytes compared to the adults (Fig. 5.9a-e). Moreover, β-carotene/Chl *a* ratios were higher in colonies (both adults and oocytes) from the GL compared to the BTN site (Fig. 5.9b, Table 5.3).

**DISCUSSION-**

The perpetuation of coral reefs relies on the capacity of the corals to respond to changes in the environment at different spatial and temporal scales (Csazar et al. 2010). Parental effects have great importance in the life-history of corals because they influence the ability and flexibility of future generations to interact with environmental change. This study is the first to explore how the variability in parental physiological conditions (host and *Symbiodinium*) relates to the physiology of gametes in a scleractinian coral. Our
results indicated that the biochemical phenotype of oocytes released by colonies from different environments was influenced by the physiological state of the parental colony and that there are large differences in the biochemical composition between oocytes and adult corals (Table 5.4).

**Variability in the biochemical phenotype of oocyte traits due to parental effects**

Parental effects (variations in the physiological characteristics of the oocyte due to differences in the adult corals) in *M. capitata* were evident in the densities and pigment levels of *Symbiodinium* (i.e., pigments per algal cell) transferred to the oocytes and ubiquitin conjugated protein levels (Table 5.4a).

Adults and oocytes from the BTN site had lower β-carotene/Chl a ratios, and lower concentrations of accessory pigments (peridinin, dinoxanthin, diadinoxanthin, and diatoxanthin) per *Symbiodinium* cell compared to those from the GL site (Table 5.4a). This indicates that the *Symbiodinium* acclimatized differently to the environments at BTN and GL. By reducing the levels of accessory pigments at the BTN site, *Symbiodinium* (from adults and oocytes) could be minimizing photodamage by capturing less light energy, and increasing the dissipation of excess energy capture and quenching of reactive oxygen species (ROS). Thus, our results suggest that offspring released by corals acclimatized/adapted to different environments are provisioned differently in terms of light utilization mechanisms and photodamage protection. This is consistent with the findings of Wellington and Fitt (2003) who found that larvae released by shallow water corals had higher concentrations of UVR-protective compounds (e.g. mycosporine-like
amino acids) and survived better than larvae from deeper environments when exposed to ambient surface levels of ultraviolet radiation (UVR).

Interestingly, ubiquitinated proteins were found in higher concentrations in parents and oocytes from the low light environment (GL site) (Table 5.4a). This finding was unexpected, since increased levels of ubiquitin-conjugated proteins are generally thought to signify greater levels of stress-induced damage (e.g. Brown et al. 2002). However, a recent study in the coral *Porites lobata* found elevated levels of ubiquitin-conjugated proteins to be associated with increased growth and survival (Barshis et al. 2010), suggesting a beneficial aspect to increased proteasome activity. Thus, the higher ubiquitin concentration in the low light corals in our study may also be beneficial and could indicate more repair systems are available to promote growth or to prevent photodamage in the low-light acclimatized parent and oocyte. Corals in the low light environment have a plating morphology which may make them more vulnerable to light stress than corals at the BTN site, which have a branching morphology and thus more self shading.

Adult tissues from the BTN site (increased light) had lower carbon content than adults from the GL site. This finding was surprising since adults from the BTN site had higher *Symbiodinium* densities and possibly higher rates of photosynthetic carbon fixation. However, it is possible that symbionts from the GL site had higher photosynthetic rates due to the increased availability of carbon dioxide associated with their lower symbiont density (Fitt & Cook 2001). Another alternative is that corals from the GL site may have acquired more carbon heterotrophically (Falkowski et al. 1984). In *M. capitata*, photosynthetic carbon is primarily respired to meet daily metabolic demand.
and used for calcification while heterotrophic carbon is used for tissue building and storage (Hughes et al. 2010). Interestingly, the carbon content of the oocytes showed the opposite pattern to the adults. Oocytes with higher Symbiodinium density had higher carbon content than oocytes with lower Symbiodinium density, which is consistent with the idea that photosynthetically-derived carbon can be incorporated into the propagule (Rinkevich 1989, Gaither & Rowan 2010). However, carbon in the oocytes was not significantly different (between sites) in the form of total lipid suggesting that similar carbon sources (heterotrophy) between sites were used for oocyte formation. Thus, both heterotrophy (by adults) and photoautotrophy (by symbionts in the oocyte) may contribute to the differences in carbon content in the oocytes between sites, further research is necessary to investigate both qualitatively and quantitatively how the main sources of gamete formation can change by differences in parental environment and/or health state (e.g. bleached vs. healthy).

**Differences in traits between life stages**

For sedentary organisms, like adult corals, the environmental conditions the mobile offspring experience may differ significantly from those of their parents. Once released, the oocytes rise to the surface, mix, get fertilized and develop into larvae. The coral larvae swim near the surface (Storlazzi 2006) and are exposed to higher levels of both PAR and UV (Gleason & Wellington 1995, Gleason et al. 2006) and broader temperature fluctuations (Wick et al. 1996) compared to the adults, which can cause oxidative stress in the early embryo/larvae. Moreover, oocytes of *M. capitata* can harbor thousands of Symbiodinium cells, in contrast to parental cells which generally harbor only
one *Symbiodinium* cell that occupies most of the host cell cytoplasm (Muscatine et al. 1998). Greater densities of *Symbiodinium* per host cell can also be a potential source of oxidative stress in the oocytes, which may affect the physiology of the offspring (early embryo/larvae) and its ability to interact with the environment. Nevertheless, oocytes of *M. capitata* seemed to be preconditioned to cope with oxidative stress by possessing elevated antioxidant levels and increased turnover rate of proteins when compared to the adults (Table 5.4b).

Fluorescent proteins in the oocytes may serve as antioxidants by scavenging ROS such as hydrogen peroxide (H$_2$O$_2$) (Palmer et al. 2009), which is able to move easily through biological membranes and cause lipid peroxidation (Halliwell & Gutteridge 1999). The higher concentrations of MnSOD in the oocytes (two fold higher than adults, Table 5.4b) also suggest that MnSOD participates to prevent the accumulation of the ROS superoxide in the early life stages of the coral holobiont and reduce oxidative damage. Higher expression of ubiquitin-conjugated proteins in the oocytes, suggests that the oocytes are undergoing higher rates of protein turnover (Table 5.4b). In sea urchin embryos, ubiquitin protein synthesis has been associated with selective protein degradation during embryogenesis where total ubiquitin content of the embryo increased almost 10-fold between fertilization and the pluteus larva stage (Pickart et al. 1991).

The photobiology of *Symbiodinium* between life stages differed considerably. The dinoflagellate pigment concentrations and population densities were generally higher in adult tissues than in oocytes. The greater accessory pigments/Chl $a$ ratios and lower pigments/symbiont cell in oocytes suggests that *Symbiodinium* cells photoacclimatize to lower light levels inside the oocytes than inside adult tissues (Table 5.4b). Although
never documented, it is likely that the internal light environment experienced by
*Symbiodinium* in coral oocytes or larvae is low because oocyte cytoplasm is very dense,
composed mainly of yolk granules and lipid droplets (Arai et al. 1993, Padilla-Gamino et
al. 2011), and the oocytes and larvae lack the diffuse reflective surface provided by the

Chl c$_2$ and peridinin (per cell) were the only two pigments that were similar in
concentration among life stages. These two pigments are efficient light harvesting
accessory pigments in dinoflagellates that transfer excitation energy to Chl a (Govindjee
et al. 1979) and are part of the Chl-protein complexes (peridinin-Chl a-protein and Chl a-
Chl c$_2$-peridinin-protein complexes, PCP and acpPC respectively) (Jeffrey 1976).
Iglesias-Prieto and Trench (1994) found that dinoflagellates (*Symbiodinium kawagutii*)
isolated from the coral *Montipora verrucosa* (=*M. capitata*) had higher Chl c$_2$/Chl a and
peridinin/ Chl a ratios when grown under low light (40 $\mu$m mol quanta m$^{-2}$s$^{-1}$) than under
high light (250 $\mu$m mol quanta m$^{-2}$s$^{-1}$). Similar characteristics were found in the oocytes
sampled in the current study where Chl c$_2$/Chl a and peridinin/ Chl a were significantly
higher in the oocytes than their corresponding parent colonies (Table 5.4b). These results
suggest that the *Symbiodinium* acclimatizes differently between life-stages by increasing
the ratios of accessory pigments (both light harvesting and photoprotective) to Chl a
when present in the oocytes. How changes in pigment concentrations affect the
photochemistry of *Symbiodinium* and photosynthate translocation rates to the
oocyte/larvae is not well understood and is worthy of further investigation.

Interestingly, $\alpha$-carotene, a pigment characteristic of green-algae, was also
detected in both coral adults and oocytes. In contrast to all other dinoflagellate pigments
examined here in which pigment concentrations range from 2.5 to 8 fold greater in parent tissue (Fig. 5.7), α-carotene concentration was approximately six fold greater in the oocytes (Fig. 5.7). α-carotene is a fat-soluble pigment (unsaturated hydrocarbon) and a powerful antioxidant that can stop free radicals from causing cells to break down in algae (Niyogi et al. 1997) and may prevent oxidative stress inside the coral oocytes. α-carotene is also a major carotene pigment in endolithic green algae (Ostreobium) (Jeffrey 1968) that commonly resides in the skeletons of the coral genus Montipora (Fine 2005, Magnusson et al. 2007), a location that is adjacent to where the oocytes develop (Padilla-Gamino et al. 2011). Although it is unclear whether this green-algal pigment is acquired by the oocytes via the endolithic algae living in the coral skeleton (Schlichter et al. 1995, Fine and Loya 2002) or the parent’s diet, the fact that it is present in greater concentrations in the oocytes (from both sites) suggests that this pigment may be of functional value to the oocyte. This pigment could be actively translocated by the host (from the gastrodermis or tissue within the skeleton) or acquired by the oocytes due to similarities in solubility. If this pigment is transferred by endolithic algae to the oocyte, this could be the first evidence of endolithic algae contributing to coral reproduction and/or vertical transmission of endolithic algal pigments/metabolites to the coral oocyte.

Oocytes had almost 4 times the amount of lipids concentrations than the adults (Table 5.4b). Most lipids in the oocytes were in the form of wax esters (WE) and represented approximately 56% of the total lipid weight. High WE levels have also been reported in oocytes (Arai et al. 1993 and Harii et al. 2010) and larvae (Harii et al. 2007) of other Acroporid corals, and are the main component of lipid droplets inside coral oocytes (Arai et al. 1993). Wax esters are responsible for the positive buoyancy of the
oocytes, which is critical for successful fertilization, and represent a long-term energy store that is consumed throughout development to provide energy for larval dispersal, settlement and metamorphosis (Arai et al. 1993, Lee et al. 2006, Harii et al. 2010).

In contrast to wax esters, triacylglycerols (TAGs) and the “other lipids” fraction (which corresponds mainly to polar lipids and in minor proportions to free fatty acids, sterols, diacylglycerols, and monoacylglycerols) were higher in adult tissues than in oocytes. Triacylglycerols are the most common storage lipid in animals, and in marine invertebrates serve as a short-term energy source (Villinski et al. 2002, Lee et al. 2006). In *M. capitata*, TAGs were immediately reduced after an experimental bleaching event (Rodrigues et al. 2008), which suggest that these storage lipids can be quickly assimilated by the coral adult during stressful conditions or when both photosynthesis and heterotrophy are not feasible (Rodrigues et al. 2008). Although heterotrophic carbon is the main source for tissue and energy building in *M. capitata* (Hughes 2010), TAGs can also be synthesized by *Symbiodinium* in coral adults (Crossland 1980). Higher TAGs in the adults may reflect heterotrophic sources acquired by the host and/or higher densities of *Symbiodinium* cells in adult tissues as compared to oocytes. Polar lipids are key structural components of cell membranes (Lee et al 2006) and the higher levels of polar lipids in the adults may reflect the greater proportion of cell membrane material (host and symbiont) present in the adult tissue as compared to oocytes.

Differences in δ¹³C values were also observed between life stages of the coral *M. capitata*. Overall, oocytes had lighter δ¹³C values than adults, most likely due to the high concentrations of lipids (Bodin et al. 2007, Alamaru et al. 2009), which are typically δ¹³C depleted (DeNiro and Epstein 1977). Alamaru et al. (2009) showed that following lipid
extraction, both lipid-free planulae and parental tissue of the coral *Stylophora pistillata* had similar $\delta^{13}C$ values and C:N ratios. This indicates that differences observed prior to the lipid extraction were due to large differences in lipid content between stages rather than isotopic fractionations during embryological development. In our study, nitrogen (%) and phosphorus (%) concentrations were significantly greater in adult tissue than oocytes, but the N:P ratios were the same for both life stages. Thus, the major difference in tissue composition between life stages is due to the higher levels of carbon in the oocytes compared to the adults.

Nitrogen sources were examined using stable nitrogen isotopes. Isotopic nitrogen signatures ($\delta^{15}N$) of *M. capitata* ranged between 4 and 5 ‰ and were similar across sites and between life stages. Similar $\delta^{15}N$ values were reported for this species at another site in Moku O'Lo'e Island (Rodrigues and Grottoli 2006), suggesting that adults from all these sites were feeding at similar trophic levels and that similar nitrogen sources are available to make the oocytes.

Overall, we found different physiological characteristics in the host tissue and *Symbiodinium* between the parents and the oocytes of the coral *M. capitata*. Oocytes were characterized by higher levels of lipids (mainly wax esters), an elevated amount of proteolytic activity (ubiquitin-conjugation) and possessed protective mechanisms such as increased levels of the antioxidant enzyme MnSOD, and even a green algal pigment possibly acquired through coral nutrition or from an endolithic algal source. Oocytes had lower densities of *Symbiodinium* (per dry tissue), lower concentrations of Chl $a$, $\beta$-carotene, dinoxanthin and diadinoxanthin per cell and greater pigment/Chl $a$ ratios than adults. Thus, oocytes appear preconditioned with physiological mechanisms to prevent
oxidative stress (Fig. 11b), which in turn may help the future larvae to reduce the
damaging effects of ultraviolet radiation (UVR) and successfully disperse until a habitat
suitable for settlement is found.

*Montipora capitata* is a coral with high morphological plasticity that can inhabit a
large number of environments and release gametes with different physiological
characteristics depending on the parental environmental history and acclimatization
capabilities. This strategy is particularly advantageous when the future environment of
the offspring is unpredictable. By releasing a diverse array of gametes *M. capitata*
populations can maximize their reproductive success during a single reproductive event
by producing offspring that can cope with different environmental conditions and
colonize new habitats, an important characteristic in an era of rapid climate change.

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Foundation. This is HIMB contribution number XXXX, SOEST contribution number XXXX and 2007 Pauley Summer Program Contribution number X.
Table 5.1 Traits investigated in adults and oocytes of the coral *Montipora capitata* and their biological function.

<table>
<thead>
<tr>
<th>HOLOBIONT TRAIT</th>
<th>BIOLOGICAL FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipids</strong></td>
<td>Long-term energy reserves, storage of excess fixed carbon in host tissue. Triacylglycerols and wax esters are primary energy sources utilized during development and metamorphosis</td>
</tr>
<tr>
<td><strong>Isotopic signatures</strong></td>
<td>$\delta^{13}C$ is a marker of carbon sources, $\delta^{13}C$ decreases as photosynthesis decrease and as heterotrophy increases. $\delta^{15}N$ is a marker of trophic level</td>
</tr>
<tr>
<td><strong>C:N:P ratios</strong></td>
<td>Carbon, nitrogen and phosphorous are essential bioelements of living organisms</td>
</tr>
<tr>
<td><strong>Ubiquitin</strong></td>
<td>Cellular protein that tags proteins for degradation in the proteosomes. Increased levels indicate higher levels of protein degradation</td>
</tr>
<tr>
<td><strong>MnSOD</strong></td>
<td>Manganese superoxidase dismutase, a mitochondrial antioxidant enzyme, plays a role in oxidative stress</td>
</tr>
<tr>
<td><strong>Fluorescent proteins</strong></td>
<td>Fluorescent proteins serve as antioxidants by scavenging ROS such as hydrogen peroxide (H$_2$O$_2$)</td>
</tr>
<tr>
<td><strong>Photosynthetic pigment profiles</strong></td>
<td>Light harvesting compounds: Chl $\alpha$ and accessory pigments Chl $c_2$ and peridinin Photoprotective compounds (carotenoids): non-chlorophyll accessory pigments that serve as antioxidants, carotenes ($\alpha$-carotene, $\beta$-carotene) and xanthophylls (dinoxanthin, diadinoxanthin, and diatoxanthin) a) Pigments/g dw- pigment concentration per holobiont dry mass b) Pigment/cell- pigment concentration in each dinoflagellate cell c) Pigment/Chl $\alpha$- ratio between accessory pigments that absorb or transfer light and reaction centers available</td>
</tr>
<tr>
<td><strong>Dinoflagellate density</strong></td>
<td><em>Symbiodinium</em> dinoflagellate cells provide the host with energy in the form of translocated reduced carbon compounds (glucose, glycerol and aminoacids), which are products of photosynthesis</td>
</tr>
</tbody>
</table>
Table 5.2 Results of the general linear model testing the effects of life stage and site on the traits investigated. Significant values at 95% confidence (p<0.05) are in bold, df-degrees of freedom.

<table>
<thead>
<tr>
<th>Physiological parameter</th>
<th>Effect of Stage</th>
<th>Effect of Site</th>
<th>Effect of Colony (Site)</th>
<th>Effect of Site x Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>Lipid (df=31)</td>
<td>1006.77</td>
<td>&lt;0.0001</td>
<td>2.93</td>
<td>0.109</td>
</tr>
<tr>
<td>Wax esters (df=25)</td>
<td>189.65</td>
<td>&lt;0.0001</td>
<td>3.33</td>
<td>0.093</td>
</tr>
<tr>
<td>Triacylglycerols (df=25)</td>
<td>5.4</td>
<td>0.04</td>
<td>1.59</td>
<td>0.234</td>
</tr>
<tr>
<td>δ¹³C (df=23)</td>
<td>10.61</td>
<td>0.009</td>
<td>1.74</td>
<td>0.216</td>
</tr>
<tr>
<td>δ¹⁵N (df=23)</td>
<td>0.52</td>
<td>0.489</td>
<td>3.98</td>
<td>0.074</td>
</tr>
<tr>
<td>C (%) (df=23)</td>
<td>35.19</td>
<td>&lt;0.0001</td>
<td>0.09</td>
<td>0.773</td>
</tr>
<tr>
<td>N (%) (df=23)</td>
<td>22.88</td>
<td>0.001</td>
<td>1.37</td>
<td>0.269</td>
</tr>
<tr>
<td>Total P (df=23)</td>
<td>22.45</td>
<td>0.001</td>
<td>0.84</td>
<td>0.382</td>
</tr>
<tr>
<td>C:N (df=23)</td>
<td>233.55</td>
<td>&lt;0.0001</td>
<td>6.63</td>
<td>0.028</td>
</tr>
<tr>
<td>C:P (df=21)</td>
<td>64.24</td>
<td>&lt;0.0001</td>
<td>0.01</td>
<td>0.935</td>
</tr>
<tr>
<td>N:P (df=21)</td>
<td>0.18</td>
<td>0.680</td>
<td>1.15</td>
<td>0.312</td>
</tr>
<tr>
<td>MnSOD (df=35)</td>
<td>63.57</td>
<td>&lt;0.0001</td>
<td>1.11</td>
<td>0.308</td>
</tr>
<tr>
<td>Ubiquitin-conjugates (df=34)</td>
<td>34.84</td>
<td>&lt;0.0001</td>
<td>6.26</td>
<td>0.024</td>
</tr>
<tr>
<td>Symbiont no. of cells (df=21)</td>
<td>42.31</td>
<td>&lt;0.0001</td>
<td>7.68</td>
<td>0.022</td>
</tr>
</tbody>
</table>
Table 5.3 Results of the general linear model testing the effects of life stage and site on the photosynthetic pigments of *Montipora capitata*. Pigment concentrations were normalized to dry mass (dm), chlorophyll a (Chl a) and *Symbiodinium* cell density. Significant values at 95% confidence (p<0.05) are in bold. Abbreviations: Chl a-chlorophyll a; Chl c₂-chlorophyll c₂; DDX-diadinoxanthin; DTX-diatoxanthin; and, df-degrees of freedom.*alpha-carotene is a green algal pigment.

<table>
<thead>
<tr>
<th>PIGMENT</th>
<th>Effect of Stage</th>
<th>Effect of Site</th>
<th>Effect of Colony(Site)</th>
<th>Effect of Site x Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Pigment/tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl a/mg dm</td>
<td>22</td>
<td>220.99</td>
<td>&lt;0.0001</td>
<td>0.53</td>
</tr>
<tr>
<td>Chl c₂/mg dm</td>
<td>22</td>
<td>112.65</td>
<td>&lt;0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td>Peridinin/mg dm</td>
<td>23</td>
<td>129.66</td>
<td>&lt;0.0001</td>
<td>0.00</td>
</tr>
<tr>
<td>β-carotene/mg dm</td>
<td>23</td>
<td>113.23</td>
<td>&lt;0.0001</td>
<td>0.07</td>
</tr>
<tr>
<td>Dinoxanthin/mg dm</td>
<td>23</td>
<td>283.93</td>
<td>&lt;0.0001</td>
<td>0.00</td>
</tr>
<tr>
<td>DDX+DTX/mg dm</td>
<td>23</td>
<td>346.24</td>
<td>&lt;0.0001</td>
<td>0.01</td>
</tr>
<tr>
<td>α-carotene/mg dm *</td>
<td>23</td>
<td>85.13</td>
<td>&lt;0.0001</td>
<td>2.21</td>
</tr>
<tr>
<td>Pigment/# cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl a/cell</td>
<td>22</td>
<td>25.72</td>
<td><strong>0.001</strong></td>
<td>3.75</td>
</tr>
<tr>
<td>Chl c₂/cell</td>
<td>22</td>
<td>2.31</td>
<td>0.163</td>
<td>2.07</td>
</tr>
<tr>
<td>Peridinin/cell</td>
<td>22</td>
<td>0.01</td>
<td>0.934</td>
<td>5.75</td>
</tr>
<tr>
<td>β-carotene/cell</td>
<td>22</td>
<td>11.4</td>
<td><strong>0.008</strong></td>
<td>9.17</td>
</tr>
<tr>
<td>Dinoxanthin/cell</td>
<td>22</td>
<td>14.83</td>
<td><strong>0.004</strong></td>
<td>5.26</td>
</tr>
<tr>
<td>DDX+DTX/cell</td>
<td>22</td>
<td>12.07</td>
<td><strong>0.007</strong></td>
<td>6.35</td>
</tr>
<tr>
<td>[Pigment]/[Chl a]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl c₂/Chl a</td>
<td>21</td>
<td>38.85</td>
<td>&lt;0.0001</td>
<td>0.04</td>
</tr>
<tr>
<td>Peridinin/Chl a</td>
<td>22</td>
<td>396.35</td>
<td>&lt;0.0001</td>
<td>4.65</td>
</tr>
<tr>
<td>β-carotene/Chl a</td>
<td>22</td>
<td>26.71</td>
<td><strong>0.001</strong></td>
<td>5.65</td>
</tr>
<tr>
<td>Dinoxanthin/Chl a</td>
<td>22</td>
<td>41.24</td>
<td>&lt;0.0001</td>
<td>2.07</td>
</tr>
<tr>
<td>DDX+DTX/Chl a</td>
<td>22</td>
<td>37.35</td>
<td>&lt;0.0001</td>
<td>3.44</td>
</tr>
</tbody>
</table>
Table 5.4 Summary of trends observed as parental effects (effects of stage and site were both significant) and differences in traits between adults and oocytes of *Montipora capitata*.

<table>
<thead>
<tr>
<th>a. PARENTAL EFFECTS</th>
<th>b. DIFFERENCES BETWEEN STAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High light environment/branching morphology</strong></td>
<td><strong>Higher in adults</strong></td>
</tr>
<tr>
<td>↑ # symbiont cells/tissue</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td></td>
<td>δ¹³C (heavier)</td>
</tr>
<tr>
<td></td>
<td>Total phosphorous</td>
</tr>
<tr>
<td></td>
<td>Symbiont cells/mg dw</td>
</tr>
<tr>
<td></td>
<td>Chla/mg dw</td>
</tr>
<tr>
<td></td>
<td>Chlc/mg dw</td>
</tr>
<tr>
<td></td>
<td>β-carotene/mg dw</td>
</tr>
<tr>
<td></td>
<td>Peridinin/mg dw</td>
</tr>
<tr>
<td></td>
<td>Dinoxanthin/mg dw</td>
</tr>
<tr>
<td></td>
<td>Chla/# symb. cells</td>
</tr>
<tr>
<td></td>
<td>Chlc/# symb cells</td>
</tr>
<tr>
<td></td>
<td>β-carotene/# symb. cells</td>
</tr>
<tr>
<td></td>
<td>Peridinin/# symb. cells</td>
</tr>
<tr>
<td></td>
<td>Dinoxanthin/# symb. cells</td>
</tr>
<tr>
<td></td>
<td>DDX+DTX/# symb. cells</td>
</tr>
<tr>
<td><strong>Low light environment/plating morphology</strong></td>
<td></td>
</tr>
<tr>
<td>↑ ubiquitin</td>
<td></td>
</tr>
<tr>
<td>↑ peridinin/symb cell</td>
<td></td>
</tr>
<tr>
<td>↑ β-carotene/symb. cell</td>
<td></td>
</tr>
<tr>
<td>↑ dinoxanthin/symb cell</td>
<td></td>
</tr>
<tr>
<td>↑ DDX+DTX/symb cell</td>
<td></td>
</tr>
<tr>
<td>↑ β-carotene/Chla</td>
<td></td>
</tr>
</tbody>
</table>

**Diagram:**

- Parent: **Parent**
- Oocytes: **Oocytes**
**Floating ring →**
(hula hoop)

**Sinking ring →**
(allow net to self-adjust during tide changes)

**Anchoring ring →**
(heavy ring)

*Figure 5.1* Photograph and schematic of a field device used to collect buoyant egg-sperm bundles from individual colonies of the shallow water *Montipora capitata.*
**Figure 5.2** *Montipora capitata* is a simultaneous hermaphrodite that releases egg-sperm bundles (a). Bundles contain approximately 14 oocytes and a sperm mass. (b) Oocytes contain *Symbiodinium* cells transferred from the parent. *Montipora capitata* oocyte under (c) white light and (d) blue light. In (d), the green fluorescence is from fluorescent proteins and the red fluorescence is from the chlorophyll in the symbiotic dinoflagellates. (e) Plating and (f) branching morphologies of *Montipora capitata*. 
Figure 5.3 (a) Temperature (°C) and (b-c) light (μmol quanta/m²s) data from the two study sites in Moku O Lo'e Island, Kane‘ohe Bay Hawaii, (b) 22 September – 1 October and (c) 25 November – 5 December, 2008. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon.
Figure 5.4 (a) Total lipids, (b) Wax Esters (WEs), (c) Triacylglycerols (TAGs), and (d) percentage of lipid classes in parents and oocytes of *Montipora capitata*. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon. Means ± SE.
Figure 5.5 $\delta^{13}$C (a), % Carbon (b), % Nitrogen (c), % Phosphorus (d), C:N (e) and C:P (f) ratios in parents and oocytes of *Montipora capitata*. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon. Means ± SE.
Figure 5.6 MnSOD (a) and Ubiquitin conjugate (b) levels and in parents and oocytes of *Montipora capitata*. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon. Means ± SE.
Figure 5.7 Photosynthetic pigment concentrations and *Symbiodinium* densities of parents and oocytes of *Montipora capitata* normalized by tissue ash free dry weight. (a) Chl *a*-chlorophyll *a*, (b) Chl *c*₂-chlorophyll *c*₂, (c) peridinin,(d) β –carotene, (e) dinoxanthin, (f) DDX+DDT- diadinoxanthin + diatoxanthin, (g) α-carotene, (h) *Symbiodinium* density.

Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon. Means ± SE.
Figure 5.8 Photosynthetic pigment concentrations of parents and oocytes of *Montipora capitata* normalized by density of *Symbiodinium* cells. (a) Chl *a*-chlorophyll *a*, (b) Chl *c*₂-chlorophyll *c*₂, (c) peridinin,(d) β –carotene, (e) dinoxanthin, (f) DDX+DDT-diadinoxanthin + diatoxanthin. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon. Means ± SE.
Figure 5.9 Photosynthetic pigment concentrations of parents and oocytes of Montipora capitata normalized by concentrations of Chl a. (a) Chl a-chlorophyll a, (b) Chl c₂-chlorophyll c₂, (c) peridinin,(d) β-carotene, (e) dinoxanthin, (f) DDX+DDT-diadinoxanthin + diatoxanthin. Abbreviations: BTN – Bridge to Nowhere, GL – Gilligan’s Lagoon. Means ± SE.
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CHAPTER 6

SUMMARY AND CONCLUSIONS

The purpose of this dissertation was to advance our understanding about (1) the spatial and temporal variation in coral reproductive capability and (2) parental effects in the scleractinian coral *Montipora capitata*. These topics were addressed by examining the *in situ* spawning dynamics of a major reef building coral in Hawai‘i during the summers of 2007 and 2008 (Chapter 2), the formation and ultrastructure of the egg-sperm bundle (Chapter 3) and diversity of *Symbiodinium* assemblages and biochemical characteristics in both parents and oocytes (Chapters 4 and 5). In this chapter I summarize and discuss the main findings of my dissertation work and highlight interesting questions for future research to advance the field of coral reproduction.

SUMMARY OF FINDINGS

The major findings of this dissertation include:

1. Spawning dynamics of *Montipora capitata* vary among years, months and lunar days. Synchrony and proportion of spawning colonies did not reflect differences in coral colony morphology and environment between sites.

2. Major changes in reproductive output (spawned material) occurred in different years, suggesting that reproductive output (and possibly timing of spawning) may have been controlled by changes in the coral’s phenology and/or stress associated with temperature fluctuations.

3. Colony size did not have an effect in the likelihood of spawning and reproductive output; spawn release was very variable between colonies from all sizes.
4. The egg-sperm bundle is formed by a mucus layer secreted by the oocytes. The sperm package is located at the center of each bundle, possibly reflecting the development of male and female gametes in different mesenteries.

5. In *M. capitata*, the number of oocytes per bundle (polyp fecundity) did not change between colonies with different sizes, morphologies or coming from different sites. However, the number of oocytes per bundle was associated to the size of the oocytes. Generally, bundles with smaller oocytes had more oocytes and bundles with larger oocytes had fewer oocytes.

6. *Symbiodinium* ITS2 sequence assemblages in oocytes are generally similar to their parents; however, the *Symbiodinium* assemblages in parent colonies and their oocytes, vary on a spatial scales of 1 – 100 m.

7. Patterns in parental *Symbiodinium* assemblages reflect environmental conditions, but not morphology or coral genotype.

8. The biochemical composition of oocytes from different environments is strongly influenced by the physiological state of their parent. Oocytes from parents located in the high light environment had higher C:N ratios and densities of *Symbiodinium* and lower pigment concentrations per cell than the oocytes released from parents from the low light environment.

9. Oocytes and adults differed significantly biochemically, with oocytes exhibiting higher concentration of lipids (mostly wax esters), ubiquinated proteins (which may indicate high metabolism and turnover rate of proteins) and antioxidant levels.
10. $\alpha$-carotene, a green-algal characteristic pigment and powerful antioxidant, was also present in higher concentrations in the oocytes, however it remains unclear whether this pigment is acquired by $M$. capitata from its diet or from the endolithic algae living in the coral skeleton.

11. $M$. capitata is a coral with extraordinary phenotypic plasticity that can release gametes with a diverse array of traits (depending on the parental environment) within a single reproductive event.

SIGNIFICANCE AND IMPLICATIONS

**Spawning dynamics in the field and ultrastructure of the egg-sperm bundle**

Spawning is of critical importance to the persistence of many marine invertebrates and is a complex phenomenon that can depend on the physiological response of organisms to several environmental cues that vary both in time and space. Chapter 2 presents one of the most comprehensive studies of spawning dynamics in the field and represents the first ecological spawning baseline for a reef-building coral in the central Pacific. This species belongs to the family *Acroporidae* and like most members of this family, is a simultaneous hermaphrodite that broadcast-spawns egg-sperm bundles (Wallace and Willis 1994). This family dominates coral reef assemblages throughout the Indo-Pacific region and the Caribbean Sea and is extremely sensitive to environmental (e.g., thermal anomalies, Hoegh-Guldberg 1999) and biological disturbances (e.g., crown of thorns predation, Pratchett et al. 2009). As such, the analysis of *in situ* spawning dynamics in this family contributes to our basic understanding of reproductive processes.
in an ecologically important group of corals and one that is increasingly threatened by climate change. Our data demonstrated that split spawning events of *M. capitata* can vary at different temporal scales in terms of the amount of gametes released, the proportion of colonies participating in different months and their synchrony. Spawning dynamics changed significantly between years. In 2007, there were both more colonies participating in the split spawning events and larger reproductive output. In 2008, the reproductive output decreased dramatically, only ~25% of the volume released in 2007 was released in 2008. Although the exact cause of the decrease in reproductive output during 2008 remains unknown, we feel two issues may have contributed to the change in spawning dynamics between years. (1) It is possible that the environment (larger temperature fluctuations) could have caused the change in spawning dynamics between years. Although *M. capitata* colonies did not experience a severe bleaching in 2008; many colonies looked paler than in 2007, suggesting that they were responding to environmental stress and possibly compromising reproductive output by reabsorbing the oocytes for maintenance and recovery (Sier and Oliver 1994, Michalek-Wagner and Willis 2001). (2) Another reason why spawning dynamics differed between years could be a shift in the phenology (timing of spawning) of *M. capitata*. In 2008, temperature started rising in February, one month earlier than in the year 2007; and reached a higher maximum in July. These differences in the temperature patterns could have affected the rate of reproductive development causing a change in the timing of spawning. Longer term data are necessary to accurately assess these hypotheses and understand the causes of variability in the detection of spawning cues, which may interact and affect the reproductive physiology at the population, organism and polyp (module) level.
Colony size in *Montipora capitata* did not have an effect in the likelihood of spawning or reproductive output. This finding is very important because it highlights the fact that larger colonies are not necessarily the most reproductively active individuals in a population, which is particularly informative when designing and implementing strategies for reef protection and management.

To date, no studies have examined the ultrastructure or formation of the egg-sperm bundle. The structure and organization of the egg-sperm bundle is likely to influence the time required to break open, which has implications for fertilization success and opportunities for hybridization (Wolstenholme 2004). Chapter 3 presents the first systematic study of the ultrastructure of the egg-sperm bundles in *M. capitata* and provides important new observations to understanding bundle structure and formation. Our results show that the egg-sperm bundle is held together by a mucus layer secreted by the oocytes. Although the bundle has an ephemeral life span, the formation of an egg-sperm bundle is a fundamental part of the reproductive process that could be strongly influenced by climate change and deterioration of water quality (due to anthropogenic effects). This is particularly relevant in Hawaii, where changes in land-use practices and pollution associated with increasing human population sizes are reflecting in reduced water quality and contamination of near shore coastal environments.

**Parental effects**

As climate change intensifies, parental effects may play an important role in the survival of offspring and the resilience of future generations. Offspring from parents with compromised health (e.g., corals affected by bleaching events or diseases) may have
reduced physiological capacity and this could, in turn, prevent successful development and recruitment under stressful environmental conditions. Chapter 4 and 5 explored for the first time how the variability in parental environmental and physiological conditions (host and Symbiodinium) relates to the phenotype (Symbiodinium assemblages and biochemical characteristics) of oocytes in a scleractinian coral. Our findings indicate that the environment plays a very important role in the parental effects of M. capitata.

Oocytes released by different parents during mass spawning events are seeded with different Symbiodinium assemblages and biochemical and photobiological traits, yielding taxonomic and phenotypic variability that may have profound implications for the early development, settlement and survival of coral offspring. How parental effects will influence the phenotype of future coral generations is not well understood. Corals affected by bleaching events may change their Symbiodinium assemblages in response to temperature stress (Jones et al. 2008, Sampayo et al. 2008). Selective acquisition and allocation of “resistant” Symbiodinium by the parents for specific environmental conditions (Jones et al. 2008) could be a powerful mechanism to enhance survival of the future offspring of corals with vertical transmission.

By observing the natural variability in parental characteristics and their relationship to the gametes, we can gain an understanding of the importance of parental effects in the resilience of marine communities. The findings on this dissertation can help guide the design and implementation of management strategies aimed at protecting reproductively active individuals and maximizing the genotypic and phenotypic variability of future coral generations, which are important attributes in an era of rapid climate change.
UNANSWERED QUESTIONS AND FUTURE RESEARCH

Throughout my research work and writing of this dissertation, I identified interesting questions that have never been explored and I believe might advance the field of reproduction and parental effects in corals.

**How the initiation of gamete formation and further development are affected at different temporal and spatial scales?**

We know very little about what drives the spatial and temporal variability of reproductive behavior in colonies from different environments or physiological condition. Examining the gamete formation and development of corals from different environments and over several time scales will help us to understand the enormous variability in spawning behavior observed in many coral species.

Over a long time scale, monitoring the development of gametes and the environment over several reproductive cycles could give us an idea of whether the differences in reproductive output between colonies are due to environment or physiological condition of the parent or both. Histological methods would allow identifying key stages during development that may be compromised by drastic changes of environmental conditions (e.g. heavy rain, sedimentation, temperatures above the bleaching threshold) and compromise the health state of the colony.

Over a short time scale, further research is necessary to explore gamete plasticity in corals. In this study we observed that egg-sperm bundles contained higher number of oocytes per bundle early in the spawning season. Furthermore, the number of oocytes (per bundle) was associated to oocyte size; larger oocytes were found in bundles
containing less oocytes and vice versa. But, what about the spermatozoa? Was the shift in size/number of oocytes accompanied by a shift in sperm characteristics (e.g. size, mobility) as well? A recent study in a broadcasting spawning ascidian (Crean & Marshall 2008) revealed that both, oocytes and spermatozoa showed phenotypic plasticity in response to changes in adult density (sperm environment). When adult density was higher, ascidians produced oocytes with larger ovicells (portion of the oocyte available for embryonic development) and spermatozoa were larger, more motile and remained viable for longer than spermatozoa collected from adults with low density. It is unknown how much corals can control the phenotypic plasticity of their gametes and/or if it is related to the synchronicity and frequency of the spawning events (spawning vs. split spawning), but this could have important implications for survival of coral offspring; further research is necessary in this novel and exciting field.

**What microorganisms can infect the coral oocytes and how do they do it?**

Parental transmission of dinoflagellate endosymbionts to the oocytes is a critical step for the perpetuation of symbiosis in many species of corals. Approximately 25% of spawning species and 90% of brooding species transmit their dinoflagellate endosymbionts to the oocyte/larvae before release (Baird et al. 2009). But how are the symbionts transferred to the oocyte and what physiological cue(s) trigger the transmission?

To date, only one study has focused on exploring the process of entry of dinoflagellate endosymbionts into the oocytes of reef building corals. Hirose et al. (2001) found that dinoflagellate symbionts seem to enter the mature oocytes by passing through
follicle cells surrounding the oocytes in gaps temporarily formed in the mesoglea. However, it is still unknown what activates the conformational changes of the oocyte membrane and the cellular processes that occur in the oocytes during symbiont transmission.

Moreover, oocytes of *M. capitata* can harbor thousands of *Symbiodinium* cells in contrast to parental cells which generally harbor only one *Symbiodinium* cell that occupies most of the host cell cytoplasm (Muscatine et al. 1998). How do *Symbiodinium* cells function and interact when present inside the oocyte? Could the interactions among symbiont cells affect the biochemistry of the oocyte? Can oocytes be dominated at the same speed by all *Symbiodinium* types or are some types preferentially transferred to the oocyte? How can coral oocytes balance their internal osmolarity and maintain a compatible osmotic environment for their endosymbionts? Future research should focus on the diversity and the interactions that can occur between different *Symbiodinium* types when present in the oocyte in order to understand the role of heritable symbionts in the acclimatization and adaptation of corals with vertical transmission.

Likewise, it would be very interesting to know if other microorganisms (e.g. bacteria, archaea, viruses, endolithic algae) can be transferred during the period when *Symbiodinium* infection occurs. In my research I detected alpha-carotene in the oocytes of *M. capitata*, this pigment is characteristic of green algae and absent in dinoflagellates. Endolithic green algae (*Ostreobium*) living in the skeletons of *Montipora* spp. possess this pigment (Jeffrey 1968) and may be responsible for the detection of this pigment in the oocytes. Alpha-carotene is a powerful antioxidant that can stop free radicals from causing cells to break down in algae (Niyogi et al. 1997) and could help to prevent
oxidative stress inside the oocytes and contribute to larval development. This pigment could be actively translocated by the host (from the gatrodermis or tissue within the skeleton) or acquired by the oocytes due to similarities in solubility. If this pigment is transferred by endolithic algae to the oocyte, this could be the first evidence of endolithic algae contributing to coral reproduction and/or vertical transmission of endolithic algae to the coral oocyte.

What about microbial communities (bacteria, archaea) associated with corals, could they also be transferred to the oocytes? Not much is known about the onset of microbial associations during the earlier stages of development in corals. Interestingly, Apprill et al. (2009) found that in *Pocillopora meandrina*, bacterial cells were localized in the bundle covering the oocytes rather than within individual oocytes or younger embryos. Although *P. meandrina* seeded its oocytes with dinoflagellate endosymbionts, it did not seeded the oocytes with bacterial cells. Protecting the oocyte from bacterial (and perhaps viral) infections may be an adaptation with beneficial value during oocyte development.

But, how can the host select/limit microbial transmission to the oocyte? The antimicrobial activity of extracts from coral oocytes was studied in 11 species of the Great Barrier Reef in Australia (Marquis et al. 2005). Interestingly, of all coral species examined, only extracts of *Montipora digitata* (coral with parental transmission) showed evidence of antimicrobial activity (Marquis et al. 2005). The authors suggest a possible role of the symbionts in the production of antimicrobial compounds but further experimentation is required to evaluate the potential role of density and diversity of
symbionts in the physiological capacity of the oocyte and synthesis of antimicrobial compounds.

The application of several techniques such as: next generation sequencing and electron and fluorescent microscopy could be useful in future studies examining the transmission of microorganisms to the oocyte. Specifically, live and fixed imaging via scanning confocal microscopy could be a powerful approach to identify the stages and processes of microbial infection in both coral adults and oocytes.

Understanding the interactions among dinoflagellate endosymbionts and other microbial organisms present in the coral host holobiont during symbiont transmission to the oocyte will help us to better understand the role of heritable symbionts and the adaptive significance of symbiosis in corals, which are critical for the resilience and persistence of coral reefs and the survival of many species in the tropical seas.

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