Can Twilight Reefs Usher In A New Dawn For Depauperate Shallow Coral Reefs?

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CAN TWILIGHT REEFS USHER IN A NEW DAWN FOR DEPAUPERATE SHALLOW CORAL REEFS?

By
Hunter Kenneth Giles Noren

Submitted to the Faculty of
Halmos College of Natural Sciences and Oceanography
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

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and
Coastal Zone Management

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Hunter Kenneth Giles Noren

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Masters of Science:
Marine Biology and Coastal Zone Management

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ABSTRACT

As shallow reefs continue to decline, scientists are searching for the key to their persistence; as it turns out, they may just need to look deeper. Below many shallow tropical reefs, there exist healthy and more stable mesophotic coral reef communities. The ability of these reefs to act as a refuge for declining shallow populations has garnered significant interest among the scientific community; however, the reproductive and larval aspects necessary for this to occur are unknown. This study assesses the ability of deep reefs to act as a reproductive refuge for shallow counterparts by examining gametic compatibility, viability and larval settlement preferences. Gametes from *Orcicella franksi* inhabiting the shallow (14-20m) and the upper mesophotic (27-32m) were introduced in a series of inter- and intra-depth crosses and found to be compatible. Larval settlement experiments found no natal depth preference, with deep larvae significantly preferring to settle on shallow conditioned substrate. Our findings support the plausibility of healthy mesophotic reefs acting as a refuge for depauperate shallow populations by (1) providing gametes to mix with limited shallow gametes resulting in increased fertilization and (2) providing larvae that recruit and repopulate shallow reefs. This is the first study to comprehensively evaluate the Deep Reef Refugia Hypothesis from a reproductive and larval settlement standpoint. Our results suggest a close coupling between shallow and mesophotic reefs through gamete and larval export and illustrate the current and future importance of these mesophotic reefs.
CHAPTER 1 - INTRODUCTION

Importance of Coral Reefs

Coral reefs are among the most beautiful and important ecosystems on our planet. In addition to their beauty, coral reefs are among the most diverse and productive ecosystems as well (Moberg and Folke 1999). Most commonly found in shallow, oligotrophic tropical waters, coral reefs provide critical habitat and nutrition for countless fish and invertebrate organisms. Estimates of reef inhabitants worldwide vary greatly, ranging from around 1 million to greater than 9 million different species (Reaka-Kudla et al. 1997). Considering coral reefs only cover approximately <0.17% of the area of the world’s oceans, the sheer biodiversity they support is staggering (Smith 1978). Coral reefs also play integral roles in other marine ecosystems, such as mangrove forests and seagrass beds, though the cycling of nutrients, energy, and biological organisms. Many coastal species undergo habitat shifts as they mature, utilizing several types of coastal environments at different life stages. This high degree of connectivity means that the degradation of coral reefs not only impacts resident populations, but also those of nearby coastal habitats. The benefits provided by healthy coral reefs extend beyond the marine environment; reefs play a major role in coastal protection and provide support for extensive coastal populations.

In the Caribbean, over 116 million people live on or near a coastline (Burke and Maidens 2004). These large coastal populations typically rely on reefs for livelihood, protection, and sustenance. Shallow coral reefs absorb wave energy, protecting coasts from erosion and reducing storm damage (Sheppard et al. 2005). Some coral species have adapted to this high-energy environment and use the high-energy waves to reproduce asexually via fragmentation (Wallace 1985). As large storms such as hurricanes become more frequent and intense, a result of ongoing climate change (Knutson et al. 2010), these reefs prove to be instrumental in protecting vulnerable coastlines and reducing erosion. Coral reefs support local economies through fishing and tourism (Hawkins and Roberts 2004). Unfortunately, many of these populations lack regulation, resulting in the overexploitation of their local reefs through overfishing and
destructive harvesting methods. An alternative option to harvesting from coral reefs is to capitalize on the tourism generated by these natural wonders. Coral reef-associated tourism generates an estimated $9.6 billion annually (Cesar et al. 2003). Lastly, the vast biodiversity of reef houses countless potential new medicines, including painkillers and cancer-fighting compounds that benefit humanity (Bruckner 2002). Recent declines in coral reef density and diversity have already caused a reduction in the benefits that reefs are able to provide to local populations.

The devastation of shallow reefs is not a localized phenomenon; reefs worldwide are experiencing rapid and substantial decline. As coral reefs continue to deteriorate, so does the value of the services they provide, impacting all flora and fauna, not only human populations. Changing temperatures, ocean chemistry, coastal processes, as well as direct, and indirect, human interaction are several of the factors contributing to this demise. Evaluating patterns of shallow reef waning on a global scale will increase our understanding of how various aspects of corals’ biology, such as growth, distribution and location, influence these declines. This knowledge can be used to identify areas facing greatest risk as well as areas of potential refuge, both important aspects of coral reef conservation.

Global Threats

Modern scleractinian corals first appeared in the fossil record during the Triassic period, approximately 237 million years ago (Stanley and Fautin 2001). They have persisted through the end-Triassic and end-Cretaceous mass extinctions (Stanley 2003). Recently, these scleractinian coral reefs have experienced rapid and precipitous declines, mainly attributed to human activities. While it may be hard to comprehend how our actions can have consequences observable on a global scale, it is important to understand that the earth is a closed circuit system and all changes we impose within that system have consequences. Bombarded by the multitude of stressors perpetrated by humanity, reefs worldwide are dying. While every reef faces a unique set of threats based on its particular geography, biotic, and abiotic conditions, there are global threats affecting all reefs. Since the beginning of the British Industrial Revolution in the late 1700s, humans have relied on fossil fuels as the dominant source of energy. The combustion of these
fuels releases large quantities of energy used to provide heat, electricity, transportation and has played a major role in shaping our current society. Unfortunately, burning fossil fuels releases byproducts such as carbon dioxide, a major greenhouse gas, into the atmosphere (DeLuchi 1991). Since the beginning of the Industrial Revolution, carbon dioxide concentrations in the atmosphere have increased by approximately 30% (Vitousek et al. 1997).

Greenhouse gases insulate the planet, leading to global warming and increasing sea surface temperatures (SSTs) (Cox et al. 2000). Due to the shallow depth of many coral reefs, they are among the first and most severely compromised by increasing SSTs. Since many shallow corals live at their maximum thermal tolerance limit, any increase in temperature often results in massive coral bleaching, the expulsion of symbiotic algae, and, if temperatures remain high, eventual mortality (Marshall and Baird 2000). Corals undergo bleaching in response to elevated temperatures or ultraviolet radiation. When a coral bleaches it releases its pigmented Symbiodinium leaving the white calcium carbonate skeleton visible through the clear coral tissue (Brown 1997). If temperatures remain high and corals cannot re-acquire their symbiotic algae they are unable to photosynthesize. Dramatic increases in the frequency and severity of bleaching events have been reported since the early 1970s (Lough 2000). A single severe thermal event in 1998 resulted in the loss of 16% of shallow coral worldwide (Wilkinson 2004). While global bleaching events can be catastrophic, localized bleaching events are much more common and can easily result in over 60% coral mortality (Miller et al. 2009). As highest water temperatures tend to occur at the surface, shallow colonies tend to bleach more frequently and severely than their deeper counterparts (Goreau et al. 2000). Rising sea surface temperatures also contribute to more intense weather events, such as tropical storms and hurricanes. Scientists report a correlation between rising SSTs and increasing hurricane frequency and intensity (Webster et al. 2005). Intense storms can harm reefs though physical trauma, such as sand abrasion and violent surge, or more indirectly by increasing coastal runoff and reducing water quality. It can take anywhere from a couple years to centuries for a coral reef to recover from a severe hurricane. Repeated storms during this period of recovery result in high mortality (Harmelin-Vivien 1994).
The greenhouse gasses we produce are also altering the chemistry of the oceans, making them less habitable to vital organisms, including corals. In the closed circuit system of our planet, trapped greenhouse gasses have nowhere to escape. As humans continue to deforest the land, there are fewer trees to convert carbon dioxide into oxygen, leaving the ocean to absorb the majority of this acidic greenhouse gas. Estimates suggest that our oceans have absorbed approximately 48% of emissions produced by fossil fuel and cement manufacturing (Sabine et al. 2004). When carbon dioxide is absorbed by ocean water, it undergoes a chemical reaction during which released hydrogen ions react with carbonate ions. This process reduces calcium carbonate saturation in the water, making it more difficult for corals to precipitate their calcium carbonate skeletons (Cao et al. 2007). Since the start of the Industrial Revolution, carbon dioxide absorption has already reduced calcium carbonate concentrations in surface waters by over 10% (Hallock 2004). While these global threats may seem paramount because they act on such a large scale, it is important to recognize that many reefs are far more affected by local stressors.

*Local Threats*

Many reefs’ shallow coastal location means they often exist in close proximity to human settlements. Human population density in coastal zones is 112 people/km compared to a global average population density of 44 people/km (Nicholls and Small 2002). Despite the current overpopulation of many coastal regions; by 2050 the global population is expected to increase by approximately 2.6 billion individuals (Cohen 2003). Dense coastal populations often rely on nearshore habitats for sustenance and livelihood, putting additional pressure on already strained ecosystems. Even unintentionally, coastal settlements endanger reefs by polluting nearshore waters with runoff, sewage and trash. Human activities are degrading local reefs both directly and indirectly. A common activity directly impacting coral reefs is overfishing. In many less developed countries where coral reefs once thrived, there are few to no management strategies or fishing regulations (Burke et al. 2012). Many local fishermen indiscriminately fish shallow reefs without regard to size or ecological role of the species they are targeting. Herbivorous fish such as parrotfish and surgeonfish play an important role in controlling algal growth.
on coral reefs. Where key herbivores have been removed, algal growth inhibits coral recruits and outcompetes adult colonies, eventually resulting in a phase shift from coral- to algal-dominated reefs (McManus and Polsenberg 2004). Jamaican reefs are a prime example; the overharvesting of key herbivorous fish, coupled with the mass mortality of \textit{Diadema} in the 1980s contributed to a massive phase shift from coral- to algal-dominated reefs, a shift that persists to this day (Williams and Polunin 2001).

As well as overexploiting shallow reefs for food, many indigenous populations harvest the reef itself. Coral skeletons, when dried and bleached, are sold as souvenirs, used in jewelry, and even crushed to be incorporated into building materials (Moberg and Folke 1999). The looting of adult colonies generates a quick one-time economic return, but ends up severely damaging the reef by reducing rugosity, decreasing community biomass, and lowering the reproductive ability and survivorship of the reef. Due to the extremely slow growth rate of stony corals, they can take decades to recover from such systematic destruction, if they are able to recover at all. A third local activity directly affecting coral reefs is the tourism industry. While tourism can be tapped as a potential source of economic support for local populations, it is not uncommon for unscrupulous tourism operators to bring untutored visitors to reefs where they often harm coral by standing, abrading or even collecting souvenirs (Hawkins et al. 1999). Coral reefs are so delicate that even responsible eco-tourism can impair the health of the reef. Studies have shown, for example, that chemicals in sunscreen can leach into the water, causing infections which lead to bleaching and mortality at all stages of corals’ life history from larvae to adult colonies (Danovaro et al. 2008; Downs et al. 2016). Education and implementation of safe-reef diving practices offer the most hope for halting the detrimental effects of the tourism industry.

In addition to the multitude of direct stressors many reefs face, there are also indirect stressors resulting from anthropogenic activities to contend with. The consequences of human enterprise on the terrestrial environment have the potential to severely alter the marine environment as well. While indirect impacts on coral reefs may be difficult to pinpoint, there remain several indisputable facts. Terrestrial runoff comes in many forms and is a major contributor to coral reef mortality. Deforestation for
mining, logging, construction and agricultural purposes results in the destabilization and subsequent erosion of soil and sediment. These loose sediments wash into rivers where they are transported to the ocean, resulting in large sediment plumes that extend into coastal waters. The influx of suspended sediments increases turbidity and harms corals by reducing light, which interferes with photosynthesis, or by settling out over the corals and smothering them (Dodge and Vaisnys 1977). Runoff from agricultural watersheds can also contain high levels of inorganic nutrients that encourage algal overgrowth, contributing to phase shifts from coral- to algal-dominated reefs. Runoff from urban development often carries chemicals and toxins such as oil or engine coolant from our roadways that harm or kill corals. Highly populated, less developed areas often lack proper plumbing, resulting in raw sewage entering coastal waters. Even in developed areas such as the Florida Keys, this has been documented. A human fecal bacterium capable of causing white band disease in *Acropora palmata* was found in canals and nearshore waters after seeping from antiquated septic tanks (Sutherland et al. 2011).

Humanity’s impact on our marine environment has been so extensive and comprehensive that some scientists claim we have entered a new epoch, the Anthropocene, where humans are the dominant force shaping the planet (Zalasiewicz et al. 2011). Tragically, many of our actions have negative consequences for the environment and its inhabitants. The ocean, our largest and most important ecosystem, has suffered enormous damage. Even the Great Barrier Reef, home to some of the healthiest and most protected coral reefs on Earth, continues to experience significant ecological deterioration (Pandolfi et al. 2003). Globally, one third of scleractinian corals face extinction, with Caribbean corals at greatest risk (Carpenter et al. 2008). As anthropogenically driven stressors continue to precipitate the decline of shallow coral reefs, scientists began to notice a difference in reef quality based on depth (Bak and Nieuwland, 1995). This realization that deeper reefs tended to be healthier than their shallow counterparts has generated interest among the scientific community for these previously unstudied mesophotic coral reefs.
Mesophotic Reefs

As coral cover and overall health of shallow reefs continued to deteriorate, scientists began to notice significant differences in coral cover, diversity, and overall health between shallow reefs and their deeper counterparts inhabiting the upper mesophotic zone (starting at 30m) (Hinderstein et al. 2010). Coupled with advances in SCUBA and submersible technologies, this scientific interest has led to increased exploration of deep reefs. Our understanding of the numerous stressors affecting shallow reefs, makes it apparent that mesophotic reefs are more protected by their depth and distance from shore (Smith et al. 2008). By sacrificing light availability for a more stable environment, mesophotic reefs have remained relatively healthy. This understanding has generated interest in mesophotic communities and the potential connectivity occurring between healthy mesophotic reefs and their shallow counterparts. The deep reef refugia hypothesis (DRRH) has attracted significant interest, but has yet to be definitively proven; it postulates that healthy deep reefs may serve as a thermal and reproductive refuge, providing gametes and recruits that bolster shallow populations (Bongaerts et al. 2010; Glynn 1996; Riegl and Piller 2003). It is necessary to assess our current knowledge of mesophotic coral ecosystems prior to examining their potential for interaction with shallow conspecifics. Evaluating the types of mesophotic reefs—their prevalence, as well as their community structure and adaptations to life in the mesophotic—will increase our understanding of their current and potential roles in shallow reef persistence and recovery.

Most people believe coral reefs are restricted to shallow water due to the limited light available for photosynthesis at greater depths (Anthony and Fabricius 2000). Mesophotic, or twilight, reefs defy this notion; they consist of light-dependent coral assemblages that inhabit depths beginning around 30 meters and continuing down to the bottom of the photic zone which can extend to depths greater than 120 meters (Hinderstein et al. 2010). Mesophotic reef ecosystems (MCEs) are characterized by the presence of zooxanthellate coral and algal and sponge communities inhabiting the deeper area of the photic zone in tropical and subtropical waters (Armstrong and Singh 2012; Kahng et al. 2010), typically found along continental slopes, seamounts, and island slopes.
First described by Darwin in 1982, mesophotic reefs remained relatively unstudied until recent advances in submersible and SCUBA technology enabled scientists to begin exploring these mesophotic ecosystems.

Predictive modeling within the Great Barrier Reef, one of the most studied reefs in the world, suggests that potential coral reef habitats have been greatly underestimated (Kahng et al. 2014; Harris et al. 2013). Mesophotic reefs have been reported on the Great Barrier Reef (Bridge et al. 2011; Bridge et al. 2012; van Oppen et al. 2011), in the Red Sea (Nir et al. 2014), throughout the Pacific (Rooney et al. 2010; Sinniger et al. 2013), and at many locations throughout the Caribbean (Bongaerts et al. 2010a; Holstein et al. 2015; Kahng et al. 2010; Smith et al. 2010). Despite their global distribution, until recently there was limited information concerning their community structure, depth range, habitat preferences or dominant taxa (Kahng et al. 2010). This review will focus mainly on mesophotic coral reefs in the Caribbean, which are among some of the most studied and understood.

Initially, the majority of studies on mesophotic reefs have mostly been observational, focusing on quantifying coral cover, species richness and growth patterns (Bak et al. 2005; Gleason et al. 2010; Slattery and Lesser 2012; Smith et al. 2010). With continued interest in MCEs, studies have become more focused; however, there is still much we do not understand. Observational studies find that mesophotic reefs tend to have significantly higher coral cover than their shallow counterparts. Shallow reefs in the Caribbean tend to have less than 20% live coral cover compared to 40-60% cover on mesophotic reefs (Bak et al. 2005; Menza et al. 2008). Diversity on mesophotic reefs can also be surprisingly high. An estimated 60% of scleractinian corals worldwide are able to survive at depths in excess of 20 meters (Lesser et al. 2009). For Caribbean corals that number increases to 70% (Smith et al. 2010). The majority of structural habitat on MCEs is provided by coral, sponge and several algal species (Hinderstein et al. 2010). Coral and algae are more common in the upper mesophotic (30-40 meters). As depth increases and light is decreased, sponges tend to become the dominant taxa (Slattery et al. 2011). In the Caribbean, mesophotic coral ecosystems are commonly found on submerged banks, along continental shelves and on walls (Bongaerts et al. 2010a). Caribbean
mesophotic reefs tend to begin about 30 meters and can extend down to around 80 meters, the limit of the photic zone (Kahng et al. 2010). Depth influences the community composition of mesophotic reefs; in the Caribbean, the upper mesophotic (30-45 meters) is dominated by members of the *Montastraea annularis* species complex which then transition to *Agaricia spp.* with increasing depth (Garcia-Sais 2010). To understand how depth structures coral communities, it is necessary to examine its benefits and challenges and how corals adapt to those unique conditions.

**Benefits and Limitations to Life in the Mesophotic**

Significant differences in health and coral density between shallow and mesophotic reefs suggest the benefits of inhabiting the mesophotic outweigh any limitations imposed by the environment. The depth of mesophotic corals protects them from many, but not all, of the stressors contributing to the decline of shallow reefs. Global stressors such as increasing SSTs and increased storm severity have led to the widespread bleaching and destruction of many shallow reefs. Due to the nature of these global threats, they tend to have a disproportionately greater effect closer to the surface. A study of bleaching-induced mortality among *Acropora spp.* on an Indonesian reef found 90% mortality of shallow colonies (0-2 meters), 60% mortality of colonies at 3-4 meters, and negligible mortality for colonies 6-8 meters and deeper (Bridge et al. 2014). This has also been found on mesophotic reefs in the US Virgin Islands (Smith et al. 2014). Mesophotic corals’ depth shields them from the vast majority of temperature anomalies, resulting in reduced bleaching. Violent wave action and storm surge is also greatly reduced at depth, resulting in a far more stable environment. Storm damage to coral reefs of Curacao and Bonaire from Hurricane Lenny (1999) was significantly reduced at depths of just 20 meters, well above the upper mesophotic (Bries et al. 2004; Smith et al. 2016a). Woodley et al. (1981) reported similar findings for *Diadema* on Jamaican reefs after Hurricane Allen (1980), illustrating how depth can provide refuge for other important species. Their greater depths mean that mesophotic reefs tend to be located farther from shore, reducing the influence of human coastal populations. Since mesophotic reefs begin at approximately the depth limit of recreational SCUBA, they are
far less affected by tourism and other direct human interaction. Despite all the advantages, mesophotic corals’ depth does not protect them from all stressors.

Since mesophotic corals are light dependent, they still rely on photosynthesis for a significant portion of their energetic requirements and therefore are susceptible to light attenuation with increasing depth. Eutrophication and coastal runoff can greatly increase turbidity, reducing light and inhibiting mesophotic corals’ ability to photosynthesize. Due to the predominately platy growth form of mesophotic corals, they are susceptible to smothering as suspended sediments settle out of the water column (Appeldoorn et al. 2016). Like their shallow counterparts, mesophotic reefs are vulnerable to increasing ocean acidification (Cerrano et al. 2013). The protection afforded by mesophotic corals’ depth reduces the severity and frequency of many, but not all, common stressors, allowing them to live in a relatively stable environment. In order to take advantage of the benefits of their stable environment, corals must adapt to the abiotic constraints of life in the mesophotic by altering their morphology, feeding strategy, and light-capture approach (Smith et al. 2016b).

Adaptations to Living in the Mesophotic

Light availability is one of the most important factors influencing the structure and growth of coral communities (Ziegler et al. 2015). Due to the lack of nutrients in their oligotrophic environment, many corals must rely on symbiotic dinoflagellates (zooxanthellae) to convert light into energy and nutrients which are then used for calcification (growth) or reproduction (Al-Horani et al. 2003). In the marine environment, light is attenuated with depth and can also vary greatly in response to water quality. Photosynthetically active radiation (PAR), the light available for photosynthesis, in the mesophotic is 1-10% of that found on shallow reefs (Crandall et al. 2016). In order to use the limited light available and maintain a balanced energy budget, mesophotic corals exhibit altered growth patterns, different feeding strategies, and specialized adaptations to maximize efficiency of light-capture and utilization (Brandtneris et al. 2016; Muir et al. 2015).
The most striking phenotypic difference between mesophotic colonies and their shallow counterparts is growth morphology. For depth-generalist species, such as *Montastraea cavernosa* and *Orbicella franksi*, the difference between deep and shallow morphologies is obvious. Shallow colonies tend to grow in the typical boulder form, growing vertically and horizontally at a similar pace. Mesophotic colonies exhibit a platy or thinly-branching morphology, spreading horizontally rather than growing vertically. This horizontal growth increases the surface area of the colony while simultaneously reducing self-shading to maximize light harvesting (Stambler and Dubinsky 2005). This growth adaptation to enhance light-capture would not work at shallow depths due to damage potential from high-energy waves. In order to further increase light capture efficiency, mesophotic colonies reduce self-shading on a cellular level by arranging their symbiotic cells in a single layer instead of stacks (Ziegler et al. 2015). A third adaptation to further increase the efficiency of light-capture and utilization is the acquisition of specialized *Symbiodinium* clades that are efficient at low light. Scleractinian corals are able to acquire symbionts from many different clades (Baker 2003). Scleractinian corals have been shown to acquire specialized *Symbiodinium* suited to their particular growth stage, depth or temperature. After severe bleaching events, significant shifts to more thermally tolerant *Symbiodinium* have been reported (Jones et al. 2008). Each *Symbiodinium* clade possesses a unique set of trade-offs that complements its host’s requirements. Clades that photosynthesize more efficiently but are highly susceptible to bleaching at high temperatures are often found on deeper colonies than clades that have greater thermal tolerance but may not be as photo-chemically efficient. Studies have found specialized deep water *Symbiodinium* in several species of mesophotic scleractinian corals (Bongaerts et al. 2015; Pochon et al. 2015). Caribbean corals in the *Orbicella annularis* species complex were found to host clades A and B at shallow depths and clade C at greater depths (Toller et al. 2001). Finally, in addition to increasing light-capture and efficiency, mesophotic corals exhibit higher rates of heterotrophy than their shallow water counterparts (Muscatine and Kaplan 1994). These remarkable adaptations by light dependent corals to mesophotic conditions indicates high phenotypic plasticity and specialized symbiont acquisition ability.
These adaptations illustrate how corals, once thought of as shallow water organisms, are able to adapt and thrive in mesophotic environments. Growth rates among some species of mesophotic corals were found to be comparable to those of shallow colonies (Bongaerts et al. 2015). Other species’ growth rates such as Orbicella annularis have been found to decrease with increasing depth (Bosscher and Meesters 1993). As human influence and warm SSTs continue to precipitate the decline of shallow reefs, mesophotic corals’ ability to adapt to the conditions that accompany their relatively stable environment will allow them to persist. The current and anticipated health and stability of mesophotic reefs make inter-depth interactions increasingly likely with mesophotic reefs acting as a refuge for dwindling shallow populations.

**Deep Reef Refugia Hypothesis**

The ability of healthy deep reefs to act as a refuge for declining shallow reefs was first suggested by Peter Glynn (Glynn 1996). After observing a large increase in bleaching-induced mortality among many shallow water coral reefs, Glynn (and others since) proposed that cooler habitats such as upwelling centers, higher latitudes, and moderate depths could provide refuge for shallow corals (Riegl and Piller 2003). This concept led to the formation of the deep reef refugia hypothesis (DRRH). The DRRH assumes that 1) deep reefs are healthier, stable, and protected from many disturbances affecting shallow reefs, and 2) can provide a viable reproductive source capable of repopulating shallow reefs following a disturbance (Bongaerts et al. 2010a). Mesophotic reefs must fulfill both aspects of the DRRH to be an effective refuge for declining shallow populations. The first assumption is relatively straightforward; mesophotic reefs must inhabit a stable environment that is protected from many of the sources of shallow reef mortality. Mesophotic communities can provide a stable environment and suitable substrate for shallow recruits (Glynn 1996). The second aspect is critical for maintaining and bolstering shallow coral reef populations.

After large mortality events, decreases in coral density and genetic diversity can lead to a phenomenon known as the Allee effect, a negative feedback loop brought about through population decline (Allee and Bowen 1932). Since corals are immobile, they reproduce mostly via synchronized broadcast spawning. Fertilization success for marine
broadcast spawning organisms is highly density-dependent (Levitan et al. 1992). As colonies die, population density decreases, reducing the chance of gametes encountering and fertilizing conspecifics. This leads to decreased fertilization and reduced numbers of new recruits, which further exacerbates the decline of shallow reefs (Gascoigne and Lipcius 2004). The Allee effect also interferes with population recovery after a period of stress and can result in genetic bottlenealing (Jennings 2001). By exporting gametes and larvae to bolster these depauperate communities, mesophotic reefs are theoretically capable of simultaneously reducing the Allee effect and increasing genetic diversity. Confirming the validity of the DRRH requires evaluating each of its assertions in order to identify any potential barriers that may influence mesophotic reefs’ ability to act as a refuge.

Assessing the Health and Stability of Mesophotic Reefs

The first assertion of the DRRH stipulates that in order to be an effective refuge, mesophotic reefs must be protected from frequent and severe disturbances, resulting in a stable and healthy community. For mesophotic reefs, depth is the main factor responsible for reducing disturbances. As previously mentioned, depth is an effective buffer capable of reducing thermal stress and high-energy waves (Bongaerts et al. 2010a). Increased depth also tends to correlate with greater distance from shore, further reducing many local anthropogenic threats (Hinderstein et al. 2010). A large portion of the sediment entering the water from terrestrial runoff settles out prior to reaching the deeper reefs farther offshore (Smith et al. 2008). It is important to consider that while depth has the potential to protect against these and other stressors, every reef is unique and subject to countless other influences that determine the degree of stability. To act as an effective refuge, mesophotic reefs must exhibit high diversity, density and biomass. Accurately assessing the stability and function of a coral reef requires examining colony health, density, and diversity, as well as community aspects including fish, algal biomass, and diversity (Hodgson 1999). Unfortunately, due to the relative inaccessibility of mesophotic reefs, information is scarce and limited mostly to coral cover and diversity. Nevertheless, these observational studies provide important information on the density and diversity of mesophotic reefs and their inhabitants. Reefs located in the upper
mesophotic often exhibit higher coral cover than their shallow conspecifics (Bongaerts et al. 2010b; Menza et al. 2008; Smith et al. 2008). Fish surveys conducted along a depth gradient in Puerto Rico found highest abundance and diversity at the upper mesophotic, around 25-30 meters (Garcia-Sais 2010). They can also provide refuge for important fish and invertebrate species especially on overfished shallow reefs (Lindfield et al. 2014). While the majority of fish observed in the upper mesophotic was also found on shallow reefs, important predators such as mature mutton snapper (*Lutjanus analis*) and tiger groupers (*Mycteroperca tigris*) were only observed on mesophotic reefs (Garcia-Sais 2010).

Little is known about the long-term stability of mesophotic reefs since the vast majority of studies focus on reefs shallower than 20 meters (Bak et al. 2005). One long-term study assessing coral cover on shallow and mesophotic reefs in Curacao and Bonaire over a 30-year period found that while shallow reefs experienced significant declines, coral cover on mesophotic reefs remained constant, reaching up to 60% at some sites (Bak et al. 2005). While this study found those mesophotic reefs stable over multiple decades, every coral reef is unique and cannot be assumed to reflect these results. However, the fact that these mesophotic reefs maintained high coral cover and diversity over several decades while local shallow reefs experienced significant declines is a strong indication of the ability of mesophotic reefs to persist while shallow reefs succumb. As our knowledge of mesophotic reefs increases, we are not only discovering the potential of mesophotic reefs, but also their limitations. A recent paper by Smith et al. (2016) suggests that mesophotic reefs have become acclimated to their stable environment and even slight increases in their mean warmest temperatures, despite still being cooler than temperatures at the surface, will trigger coral bleaching. This may cast doubt on the ability of mesophotic reefs to thrive as temperatures continue to rise; however, there are ways mesophotic reefs could adapt to increasing thermal maxima.

Corals have been shown to improve their thermal tolerance simply through the incorporation of specifically adapted *Symbiodinium* (Rowan 2004). There is a possibility mesophotic corals are using light-efficient, thermally-intolerant *Symbiodinium*, and as temperatures in the mesophotic begin to rise, colonies will transition to a better-adapted
symbiont. In order to fulfill the DRRH and be considered a viable refuge, mesophotic reefs must be connected with their shallow counterparts. Without connectivity and compatibility between populations, deep reefs may simply be bystanders as shallow reefs decline.

*Reproduction, Compatibility and Recruitment of Shallow and Mesophotic Reefs*

The second aspect of the DRRH, which addresses the reproductive ability of mesophotic corals, the connectivity and compatibility between populations, as well as the viability and settlement preference of those larvae, is far more complex and challenging to confirm. Reproduction among sessile marine invertebrates, such as corals, is an intricate process subject to many external influences capable of reducing or interrupting fertilization and connectivity. Each aspect of reproduction, from spawning and fertilization, to compatibility and larval preferences, will be examined to evaluate the plausibility of the DRRH.

Mesophotic reefs inhabit a low light environment translating to less energy available for growth, metabolism and reproduction. Brown’s Principle supports this, suggesting that sub-optimal conditions at the edge of a species’ biogeographic range may result in decreased reproductive success (Brown 1984). If mesophotic corals are still energy-deficient despite their adaptations to increase light-capture, they may be investing their limited energy into growth rather than reproduction or vice-versa (Brandtneris et al. 2016; Holstein et al. 2015). Studies investigating how depth influences sexual reproduction in corals have found that corals’ response varied greatly depending on the species. The Red Sea coral *Stylophora pistillata* was found to be less fecund at depth (Rinkevich and Loya 1987). In the Caribbean, *Porites astreoides* exhibited depth-independent reproduction and *Orcicella faveolata* was found to be more fecund at mesophotic depths (Holstein et al. 2016; Holstein et al. 2015). Based on these findings, it is likely that the other closely-related corals in the *Orcicella* species complex are able to acquire sufficient energy for both growth and reproduction at mesophotic depths. With a high likelihood of gamete production, the next step to establishing connectivity is spawning.
Since corals are sessile, they must disperse their gametes to reproduce sexually and maintain genetic diversity. The most common form of sexual reproduction among scleractinian reef corals is hermaphroditic broadcast spawning (Richmond and Hunter 1990). Each colony releases its gametes into the water column where they float to the surface, interact, and fertilize conspecific gametes. Once released, gametes (notably sperm) have a limited window to locate and fertilize an egg before they lose viability. The fertilization window for *O. franksi* sperm is just two hours, illustrating the importance of synchronized spawning (Levitan et al. 2004). In order to maximize synchronization to achieve optimum fertilization, corals rely on a variety of environmental cues that become increasingly precise as spawning approaches (Levitan et al. 2011).

The first cue for corals to begin the gamete maturation process is thought to be rising SSTs throughout the spring and early summer months (van Woesik et al. 2006). As the spawning event approaches, corals become more tightly synchronized using lunar patterns (van Veghel 1994). On the night of spawning, the onset of darkness is an important cue increasing synchronization (Baird et al. 2009). The final, most precise cues prior to spawning, are not completely understood and may be a combination of several factors such as genetics and chemical cues (Levitan et al. 2011; Tarrant et al. 2004). In the marine environment there are few concrete barriers to fertilization; instead, corals rely on relatively short fertilization windows and highly synchronized gamete release. If mesophotic corals interpret these cues differently than shallow populations, this could lead to reproductive isolation between populations. Conversely, if mesophotic and shallow populations spawn simultaneously, the time it takes for mesophotic gametes to float to the surface could significantly reduce inter-depth fertilization. To achieve optimum fertilization between mesophotic and shallow gametes, mesophotic colonies would likely have to spawn earlier, providing additional time for their gametes to reach the sea surface. The earlier spawning of deeper colonies has been reported for *O. franksi* (Holstein et al. 2016; Levitan et al. 2004). This suggests that mesophotic reefs may be responding to earlier light decreases at mesophotic depths or interpreting spawning cues differently resulting in earlier spawning which gives their gametes additional time to reach the surface so they arrive with shallow conspecifics. This earlier spawning by
mesophotic colonies may serve to increase fertilization between shallow and mesophotic populations.

While connectivity between shallow and mesophotic populations is necessary, simply mixing gametes does not guarantee successful fertilization. Since many broadcast spawning invertebrates follow the same lunar and light cues, they often spawn together in one large event. On the Great Barrier Reef, 105 species of scleractinian corals from 36 genera reproduce in annual, synchronized spawning events (Babcock et al. 1986). In order to prevent cross fertilization by other species and maintain species boundaries, many broadcast spawning species rely on gametic incompatibilities which reduce or prevent fertilization by heterospecific sperm (Palumbi 1994). If such gametic incompatibilities have evolved between shallow and mesophotic populations, genetic connectivity will prove to be greatly diminished. If no such gamete incompatibilities exist, the larval and genetic pool could increase, reducing the influence of the Allee effect. In addition to reproductive isolation, random vicariance events caused by factors, such as shifting currents or horizontal distance between populations, can also cause reproductive isolation (Veron 1995). While vicariance events would completely isolate shallow and mesophotic reefs, it is important to consider if isolation occurs due to temporal isolation or gamete incompatibilities, mesophotic reefs could still aid their shallow counterparts by reproducing with mesophotic conspecifics and providing larvae to settle on and repopulate shallow reefs.

If mesophotic colonies spawn earlier in response to earlier onset of darkness in the mesophotic, this would increase inter-depth fertilization potential and the chances of inter-depth fertilization. Even after fertilization has occurred, if populations were previously reproductively isolated, there may exist barriers capable of hindering the development and viability of larvae. These post-zygotic barriers occur when populations are closely related and prezygotic-isolating barriers are weak. They can interrupt larval development, decrease viability and cause hybrid sterility (Fogarty 2012). Conversely, if deep and shallow colonies are compatible, there is also the possibility of the hybrids experiencing hybrid vigor, expressing beneficial traits from both parents and increasing their overall fitness (Willis et al. 2006).
The final aspect to consider in evaluating the ability of mesophotic reefs to serve as a reproductive source and refuge for shallow populations is larval settlement and survival. Larval behavior is complex and influenced by genetic predisposition as well as a variety of environmental cues. Since adult corals are immobile, population distribution is directly influenced by larval settlement preferences. Poor settlement choice increases the likelihood of mortality. When searching for suitable settlement substrate, coral larvae respond to rugosity, substrate orientation, irradiance, and a variety of biochemical cues given off by local biofilm and CCA (Arnold and Steneck 2011; Heyward and Negri 1999). The role of coralline algae in inducing larval settlement has been extensively studied (Harrington et al. 2004; Heyward and Negri 1999; Morse et al. 1996; Ritson-Williams et al. 2014). Studies have also found that larvae exhibit species-specific settlement responses to certain species of coralline algae (Ritson-Williams et al. 2014). Recently bacterial biofilm has also been shown to induce settlement in several Caribbean corals including *O. franksi* (Sneed et al. 2014). In addition to substrate composition, light quality and quantity have been shown to influence the settlement depth of multiple species of scleractinian coral larvae (Mundy and Babcock 1998). Larval settlement preferences are known to play an important role in influencing the population structure and distribution of adult populations. Larvae of depth-generalist corals exhibit little or no depth preference, while larvae generated from corals that exclusively inhabit shallow or deep environments significantly preferred to settle at their respective depths (Baird et al. 2003). This larval depth fidelity has only been examined among Pacific coral species and has never been tested in the Caribbean prior to this study. If larvae generated from shallow or mesophotic populations exhibit depth fidelity, then larvae generated by healthy mesophotic reefs are unlikely to recruit to and help repopulate, depauperate shallow reefs. However, since *O. franksi* is considered to be a depth generalist, larvae may settle indiscriminately on both shallow and mesophotic substrates. In order to effectively repopulate shallow reefs, larvae must not only recruit to shallow substrate but also be able to survive at that depth. Settlement and metamorphosis is a critical time for juvenile corals, with highest mortality rates occurring within the first 24 hours post settlement (Martinez and Abelson 2013). As recruits grow, their survivorship increases exponentially (Arnold and Steneck 2011). Therefore, to accurately assess refuge
potential, it is important not only to observe settlement preference but also to examine short-term survivorship of the larvae at their settled depths. If recruits generated in the upper mesophotic settle on shallow substrate and are able to persist through the critical early stages, they stand a chance at persevering and repopulating shallow reefs.

Assessing fertilization and examining the degree of connectivity between populations is a complicated undertaking. There are numerous potential barriers to gene flow between populations possibly resulting in reproductive isolation and reduced connectivity. While gamete production and synchronized spawning have been reported among mesophotic populations, gamete viability and compatibility can vary by species and location (Holstein et al. 2016). Experimental manipulation is required to accurately confirm gamete compatibility, making assessment more involved. Even if shallow and deeper colonies are not connected or their gametes are incompatible, mesophotic reefs may still be able to bolster shallow populations by reproducing with conspecifics then exporting recruits that can settle on shallow reefs. Since connectivity is subject to a variety of factors, it is difficult to determine where it is occurring. Advances in genetic fingerprinting have made it possible for scientists to examine the genetic makeup of various populations, providing an assessment of connectivity. By using species-specific microsatellite loci or polymorphic Amplified Fragment Length Polymorphism (AFLP) markers, the genetic interaction among multiple populations can be assessed. Vertical connectivity between populations is highly variable and may be influenced by several factors such as horizontal distance between populations and prevailing hydrological conditions. Studies assessing vertical connectivity among populations of *Montastraea cavernosa* at multiple sites throughout the Caribbean found evidence of connectivity at some locations but not at others (Brazeau et al. 2013; Serrano et al. 2014). This pattern of sporadic connectivity has also been reported on Australian and Japanese reefs as well (van Oppen et al. 2011; Nakajima et al. 2009). After bleaching events, connectivity has been shown to increase, with unaffected reefs providing the majority of the new recruits (Underwood et al. 2006). These studies show that spatial separation between populations does not necessarily interfere with connectivity. As shallow populations continue to decline, it is possible that gene flow will increase due the breakdown of density-dependent prezygotic barriers (Fogarty et al. 2004).
Based on the current literature, mesophotic reefs appear to be capable of fulfilling the requirements of the DRRH. They are healthier and more stable than shallow counterparts and appear to be sexually reproductive. While connectivity, gamete mixing, and larval viability aspects are more difficult to test, studies show that genetic exchange is happening at multiple locations worldwide.

Despite their current health, some questions have been raised concerning mesophotic reefs’ ability to persist through predicted climate change scenarios. As conditions continue to deteriorate, it is possible that shallow reefs may contribute to mesophotic reef resilience through the acquisition of beneficial genes or better adapted Symbiodinium from shallow populations. In any case, mesophotic reefs currently appear to be the best option for shallow reef refugia. While studies have provided important (and sometimes contradictory) information concerning mesophotic reefs, one universal conclusion is that more research is necessary to increase our understanding of mesophotic coral ecosystems.

*Importance and Goals of this Study*

The goal of this study was to outline current knowledge of mesophotic reefs and evaluate the plausibility of the DRRH as it pertains to the Belizian Barrier reef and similar reefs with little horizontal distance between shallow coral colonies and their deeper counterparts. As the second largest barrier reef tract in the world, the barrier reef of Belize is a critically-important reef system. Recognized as a UNESCO World Heritage Site since 1996, approximately 12% of its total area is currently under some form of protection. Unfortunately, over the past decade, a significant portion of shallow Belizian reefs have experienced widespread bleaching, disease, and mortality. During the 1997-98 El Niño, live coral cover on shallow reefs in Belize declined by approximately 48% (McField 2000). Such high levels of mortality highlight the urgent need for research on connectivity between mesophotic and imperiled shallow reefs on the barrier reefs of Belize. While recent advances have been made in the detection and study of other Caribbean mesophotic reefs, the Belizian Barrier reef has been largely overlooked. Studies focusing on mesophotic reefs in the Caribbean as potential thermal refugia neglect mesophotic reefs in Belize (Bongaerts et al. 2010; Brazeau et al. 2013; Chollet...
and Mumby 2013). As the second largest reef tract in the world, it is highly likely that Belize is also home to extensive mesophotic reefs; yet they remain overlooked. In addition to documenting mesophotic reefs in Belize, this study will focus on *Orbicella franksi*, an important reef building coral that has also been understudied, especially at mesophotic depths. *Orbicella franksi* is considered to be a depth generalist with the deepest distribution of the three species that make up the *O. annularis* species complex, making them an important component of Caribbean MCEs. When mentioned in the literature, *Orbicellids* are often grouped and referred to simply as a member of the species complex. By studying *O. franksi*, we will increase our knowledge of the species, its reproductive potential, and compatibility between populations. Additionally, we will increase our understanding of its role on mesophotic and possibly, shallow reefs.

Due to the physiological constraints accompanying diving in the mesophotic and the need to achieve the highest degree of precision possible, colonies from the shallow and mesophotic will be collected prior to spawning and then spawned in a controlled laboratory environment. Conducting spawning in the lab will enable us to obtain precise setting and spawning data and also guarantee that gametes are not mixed prior to conducting specific crosses. Orchestrating inter- and intra-depth crosses using both shallow and deep eggs will reveal compatibility between depths as well as identify any specific gametic incompatibilities that may exist. To ensure no postzygotic barriers exist, larvae will be reared until they elongate and are competent to settle. Assuming that no pre- or post-zygotic barriers exist, larval settlement preferences will be examined. Once competent to settle, the larvae will be given a choice of two settlement tiles, each conditioned for six months on shallow or mesophotic reefs. By conditioning aragonite tiles at shallow and mesophotic depths, they will acquire the biofilm and CCA unique to each respective depth. Larvae will be given 48 hours in which to investigate each tile’s biofilm and settle on their preferred tile. Since larvae have been shown to investigate and settle on specific substrates, their recorded settlement selection will reveal whether larvae generated from a specific depth exhibit any preference for that depth. For settlement choice to be relevant, recruits must also be able to survive at whichever depth they chose to settle; therefore, all tiles with settled larvae will be re-deployed to the depths where they were conditioned. Early post-settlement is a critical time when coral recruits
undergo the highest levels of mortality. After approximately four weeks, the tiles will be recovered and re-examined to determine early post-settlement survival rates. Recruits that survive their first month post settlement will be considered able to persist at that depth. Finally, in order to determine the degree of connectivity between shallow and deep populations of *O. franksi*, samples will be collected from multiple shallow and upper mesophotic sites along the barrier reef and analyzed using a panel of microsatellite markers. This study will provide a comprehensive evaluation of the plausibility of the DRRH occurring between shallow and mesophotic populations of *O. franksi* along the largest reef system in the Caribbean.

Documenting the presence of mesophotic coral ecosystems in Belize and evaluating their levels of connectivity to shallow coral reefs will raise awareness of these vital, yet overlooked, coral reefs. In addition to increasing awareness of mesophotic reefs, the results of this study have the potential to better inform the decisions of policymakers regarding the implementation of new, or expansion of existing, protected marine areas. By preemptively protecting these mesophotic reefs before they begin to experience the effects of climate change, we may be able to aid shallow reefs more than previously considered. Through ecosystem-based resilience, the export of important fish and invertebrates, the provision of gametes and recruits to shallow reefs, mesophotic reefs may be more important now than in any time in human history. Protecting mesophotic reefs now may increase the survival rates of all reefs and influence future generations of humans as well as corals. Although out of sight, mesophotic reefs must not be out of mind; they must be on top of our minds and the minds of policymakers whose job it is to protect our environment and future.
CHAPTER 2 – PUBLICATION

INTRODUCTION

Coral reefs worldwide are experiencing ongoing declines in both biomass and diversity (Côté et al. 2005). In the Caribbean, coral cover on shallow reefs has decreased from approximately 50% to 10% in only three decades (Gardner et al. 2003). Anthropogenically driven climate change, increasing ocean acidity and widespread coral disease, coupled with local stressors (such as overfishing, increased coastal runoff and invasive species), are major causes of coral reef mortality (Hoegh-Guldberg et al. 2007; Riegl et al. 2009). Shallow coral reefs’ proximity to shore make them exceptionally susceptible to these stressors, resulting in significant differences in coral cover between shallow reefs and their mesophotic counterparts (Bak et al. 2005). This differential fitness has generated interest in mesophotic coral ecosystems (MCEs) and their potential to serve as a refugium for declining shallow coral populations (Bongaerts et al. 2010; Chollett and Mumby 2013).

Mesophotic coral reefs are light-dependent benthic communities that inhabit depths starting around 30 m and extending down to the end of the photic zone. The Deep Reef Refugia Hypothesis (DRRH) postulates that (1) deep reefs are more protected from many common disturbances negatively impacting shallow reefs, and (2) are able to act as a viable reproductive source for shallow reefs following a disturbance (Bongaerts et al. 2010). Depth buffers mesophotic coral reefs by reducing impact from fluctuating sea surface temperatures, human interactions, coastal pollution, and physical processes, such as waves (Harmelin-Vivien 1994; Smith et al. 2014). Studies comparing coral cover on mesophotic and shallow reefs find that MCEs have significantly higher (40-60%) coral cover than their shallow counterparts (<20%) (Bak et al. 2005; Baker et al. 2016). While many studies have validated the first assumption of the DRRH (Aronson et al. 2002; McField 2000; Mumby 1999), without confirmation of reproductive compatibility or
connectivity between deep and shallow populations, we cannot confirm plausibility of the DRRH.

While mesophotic reefs tend to have higher coral cover than their shallow counterparts, they are unlikely to act as an effective refuge if shallow and mesophotic populations are not connected. There are two ways deep corals can replenish shallow reefs: 1) supplying gametes and, 2) supplying larvae that recruit to the shallow reef. Connectivity between shallow and mesophotic reefs can be influenced by extrinsic factors, such as prevailing currents, or intrinsic factors, such as spawning time or larval settlement preferences. If there is no connectivity, over time populations may develop pre- and post-zygotic barriers that either prevent fertilization from occurring or prevent the embryo from developing (Ladner and Palumbi, 2012). Even if populations are connected, larval response to different types of settlement cues could be influenced by parental depth, resulting in more separate and structured populations (Baird et al. 2003; Harrington et al. 2004; Heyward and Negri 1999). In *Favia fragum* depth differences of as little 5m yield significant morphometric and genetic differentiation (Carlton and Budd 2002), while *Montastrea cavernosa* exhibit phenotypic but no genetic differences between shallow and mesophotic populations (Budd et al. 2012). Due to reduced light irradiance, mesophotic corals exhibit altered morphology from colony growth down to the cellular level (Kahng et al. 2010; Lesser et al. 2010).

As shallow coral reefs continue to succumb to an increasing number of natural and anthropogenic threats, mesophotic reefs will likely play an increasingly important role in the persistence of shallow coral reefs. By studying the reproductive potential between shallow and mesophotic populations of *O. franksi*, we hope to: (1) assess compatibility of gametes released from shallow and deep individuals, (2) examine the viability of larvae from shallow and mesophotic corals, (3) quantify settlement preference of larvae from shallow, mesophotic and mixed-depth crosses, (4) examine post-settlement survivorship of the settled larvae, and (5) compare genetic connectivity between shallow and mesophotic colonies. The results of this study will greatly increase our understanding of the interactions between shallow and mesophotic reefs, improve models of reef connectivity and persistence, inform management and policy decisions concerning
mesophotic coral ecosystems and confirm the importance of mesophotic reefs in the continued persistence of shallow reefs.

**MATERIALS AND METHODS**

*Study Species*

Little is known about some of the most prevalent coral species in the mesophotic zone. *Orbicella franksi* (formerly *Montastraea*) is one of the dominant scleractinian corals found on both shallow and mesophotic reefs throughout the Caribbean (Fukami et al. 2004). It is closely related to two other species in the same genus (*Orbicella annularis* and *O. faveolata*) and has limited ability to cross reproduce with *O. faveolata* (Levitan et al. 2004; Lopez et al. 1999); however, temporal isolation (Levitan et al. 2004, 2011) and conspecific sperm precedence (Fogarty et al. 2012) reduces the chance of mating with *O. annularis* as well. Whether identified to the individual species or as part of the *Orbicella annularis* species complex, its presence on mesophotic Caribbean reefs is well documented (Armstrong et al. 2006; Armstrong 2007; James and Ginsburg 1979; Vize 2006). Members of the *Orbicella* family have a wide and overlapping depth range; however, *O. franksi* (inhabiting depths 1-50m) tends to live deeper than its congeners (Szmant et al. 1997; Fukami et al. 2004). At shallow depths, *O. franksi* has a mounding morphology, yet becomes platy below 30m (Baker et al. 2016). *Orbicella faveolata* and *O. franksi* are simultaneous hermaphrodites (Szmant 1986) and reproduce via highly synchronized spawning events 5-8 days after the full moons in August and September (Szmant 1986; Knowlton et al. 1997; Levitan et al. 2011). Spawning occurs in multiple phases; during the setting phase, gamete bundles pass through the polyp’s pharynx and appear on the corals’ surface. Approximately 15 minutes after setting, the corals enter the birthing stage where gamete bundles are released into the water column (Knowlton et al. 1997). Once released, bundles float to the surface where they open, releasing gametes to mix and undergo fertilization. Deeper *O. franksi* colonies have been observed to spawn earlier so that their gametes float to the surface with their shallow counterparts. Estimates suggest that for every 10m in depth, corals spawn 18 minutes earlier (Levitan et al. 2004); however, this has yet to be confirmed at depths greater than 12m.

*Collection*
*Orbicella franksi* were collected from Raph’s Wall (N 16° 46.775’; W 88° 04.513’), located on the Belizean barrier reef. Corals were collected 1-3 days prior to spawning. In August 2013, 19 corals were collected: 9 from “shallow” depths (14-20m) and 10 from the upper mesophotic (27-32m) reefs. In September 2013, 18 colonies were collected: 7 from the shallow and 11 from the upper mesophotic reef. Coral collection was done with the same area on a vertical wall resulting in minimal horizontal separation between shallow and deep populations. Prior to collection, a small excision (1-2cm²) was made in the surface of the coral colony to examine for the presence of ripe pink eggs. For deep colonies, underwater lights were used to determine color; despite the added light, ripeness was hard to distinguish, so if eggs were visible, the colony was collected. For shallow colonies, if pink eggs were present the coral colony was considered ripe and subsequently collected and given a unique ID number. When possible, the entire colony was collected. However, due to the large size and platy growth form of mesophotic *O. franksi* colonies, in several cases only a portion of the colony was removed. The colonies were then transported in seawater to the field station where their undersides were scrubbed to remove sponge debris, tunicates and other encrusting organisms. Colonies were then placed and maintained on a raised bed under the field station dock.

**Spawning**

Beginning on the 5th night after the full moon, approximately 30 minutes before sunset, corals were transferred from under the dock to individual buckets containing seawater and transported to the open-air laboratory. All artificial lights were extinguished to mimic natural light conditions and to reduce light cue disturbances. Beginning 75 minutes after sunset (19:30 local time), the corals were monitored every 10 minutes for evidence of setting. Once setting was observed, monitoring increased to every 5 minutes. The proportion of the colony that set was recorded as an indication of spawn potential. In August, a total of 6 shallow and 4 deep corals spawned. In September, 5 shallow and 7 deep corals spawned. As soon as spawning occurred, the buoyant gamete bundles were carefully pipetted into labeled specimen cups. The cups were gently swirled to mimic wave action and assist in breaking up any intact gamete bundles. The released gametes were then poured through a 100µm Nitex mesh that
allowed sperm to pass but retained eggs. The sperm that passed through the mesh were
collected in a new labeled cup for sperm stock. To remove any remaining sperm, the egg
stocks then underwent a four stage rinse in seawater (collected prior to spawning to avoid
potential sperm contamination from nearby reefs), and then transferred into a new labeled
specimen cup and used as egg stock. A previous study by Levitan et al. (2004) noted
decreased fertilization potential in the first 30 minutes after spawning; therefore, the
gametes were allowed to activate for approximately half an hour prior to fertilization.
Extremely concentrated sperm stocks were diluted with filtered seawater to reduce the
risk of polyspermy. Prior to fertilization, a 1ml sperm sample was collected from each
stock and fixed with formaldehyde for later quantification. Counts were done by taking
the average of 8 replications using a hemocytometer.

Gamete Compatibility

Multiple fertilization crosses were conducted to assess the compatibility of
gametes between and within deep and shallow populations of O. franksi. The gamete
compatibility experiment consisted of 64 scintillation vials in a matrix, which crossed
each individual with itself and others from its own and opposite depth. Due to the limited
window (6 hours) in which developing embryos could be scored before they became
indistinguishable from unfertilized eggs, the maximum number of corals that could be
tested using this matrix were 4 deep and 4 shallow (D 1-4 and Sh 1-4). If more than four
deep or shallow colonies spawned in a single night, the four that released the most egg-
sperm bundles were used in the matrix. If scoring was not complete by the time embryos
had begun to resemble unfertilized eggs, the sample was fixed with Z-fix and scored at a
later date. Each scintillation vial was labeled with its specific cross and filled with 8ml
0.2µm filtered seawater (FSW). Eggs (1ml) and sperm (1ml) from the appropriate stock
solutions were gently pipetted into the scintillation vials and gently mixed. To eliminate
the possibility of sperm contamination, controls were conducted by only adding eggs
(1ml) to 9ml of filtered seawater. All vials were allowed to fertilize for 3 hours, then
scored for fertilization with a dissecting microscope.
**Larval Rearing**

All stock gamete solutions remaining after fertilization experiments had concluded were fertilized in batches using intra-depth gametes (ShxSh and DxD). These batch larvae were then transferred into multiple 1.5 liter plastic bowls filled with FSW. Low-density batches were maintained to reduce bacterial buildup and resulting larval mortality. Apart from occasional gentle agitation to reduce clumping, larvae were reared in stagnant FSW with water changes occurring approximately every 8-12 hours. Water changes were done by pipetting larvae into clean bowls containing fresh FSW. Used bowls were emptied, rinsed with a freshwater hose and wiped to remove the lipid layer adhering to the edges (a result of the breakdown of decaying larvae). Larvae were maintained in this manner until they reached settlement competency 4 days after fertilization.

**Larval Viability**

To determine larval viability, 100 embryos were placed in a Petri dish containing 50ml FSW. Experimental methodology was altered between the first and second month. In August, embryos were introduced to the petri dish and left undisturbed for 5 days until they were counted on day 6 after spawning. In September, 100 embryos were again transferred to a Petri dish containing 50ml FSW. These were then scored daily for the next 5 days. After each daily count in September, survivors were transferred to a new petri dish containing fresh FSW.

**Settlement Preference and Post-Settlement Survivorship**

Larval batch cultures were raised for 4 days until they reached competency to settle. To examine if the larvae from the deep and shallow *O. franksi* exhibited any settlement preference (or depth fidelity); settlement preference experiments were conducted in 34 square 750 ml plastic containers. Two small daubs of hot glue were placed 2.5 cm from opposite corners of each of the containers. After the glue hardened, containers were then soaked in seawater for 24 hours to allow for leaching of potential chemicals. The corners with glue were randomly labeled as shallow or deep. One deep and one shallow conditioned aragonite settlement disk were then placed in the
corresponding location in each container containing 750ml FSW. The disks were previously conditioned for 3 months at either 18m (shallow) or 39 m (deep). The disks were placed at opposite edges and propped up between the glue dot and the wall of the container (to maximize larval settlement area) at approximately 60° angles. The container was then randomly assigned a sample of 50 deep or shallow larvae. After 48 hours, the tiles were scored for settlement using a dissecting microscope. All tiles with settlers were then secured to a plastic framework using a cable tie around the perimeter with approximately 1cm between tiles and redeployed to the depth at which they were conditioned. After 4 weeks, the tiles were collected and reexamined under a dissecting scope to determine the number of survivors. Due to their cryptic nature, it is possible that some spat could be overlooked while scoring initial settlement preference. If while rescoring, there are tiles where post settlement survival ratios exceeded 1.0, analysis will be run twice; firstly those tiles will be adjusted down to 1.0 (assuming full survival) and analyzed, then those tiles will be excluded from the analysis and the results will be compared.

Genetic Population Analysis

Dive teams collected tissue samples from a total of 33 shallow colonies (collected from between 15.4m and 18.9m) and 37 deep colonies (collected between 33m and 37.8m). Divers descended to around 35m and collected 4 to 7 colonies, then ascended to about 16m and collected approximately the same number of colonies. To reduce the risk of sampling the same clone, *O. franksi* colonies were sampled at least 3 m apart. Due to low rates of asexual propagation among *O. franksi*, this distance was considered sufficient (Levitan et al. 2011). Tissue samples were preserved in Chaos (4 M guanidine thiocyanate, 0.1% sodium N-lauroyl sarcosine, 10 mM Tris-HCl pH8, 0.1 M 2-mercaptoethanol) (Fukami et al. 2004) and frozen for transport to Nova Southeastern University for analysis. Eight polymorphic microsatellite markers were used to genotype the corals (taken from Severance et al. 2004; Davies et al. 2013). Seven of these primers (*M_fav5^A* (CGA)17, *M_fav7^A* (CAT)24, *M_fav3^A* (ATG)25, *M_fav6^B* (CA)33, maMS 2-4, maMS 2-8, *M_fav30^C* (TTTTG)8) were run on a Promega PCR cocktail (Promega, Madison, WI). The PCR recipe consisted of 2.4μl 5X PCR buffer, 1.2μl 1mM
dNTPs, 0.15μl GoTaq, 1.0μl 10μM BSA (bovine serum albumin), 1.5-3mM MgCl₂ (depending on the primer), 0.3μl of forward primer, 0.6μl of reverse primer, 0.6μl fluorescent tag (FAM, NED or HEX depending on the primer), 2.0μl of DNA (5ng/μl), and PCR-grade water to bring the total volume to 12μl. The last primer (M_fav9^B (CAAT)21) was run using Quiagen® reagents in a PCR cocktail which consisted of 1.2μl 10X PCR buffer, 1.2μl 1mM dNTPs, 1.44mM MgCl₂, 2.4μl 5X Q-solution, 0.06μl HotStarTaq®Plus DNA Polymerase, 0.25μl forward primer, 0.5μl reverse primer, 0.5μl fluorescent tag, and PCR-grade water to bring the total volume to 12μl. The PCR amplification process for the Promega primers was: 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 57°C (primers M_fav5^A (CGA)17, M_fav7^A (CAT)24, M_fav3^A (ATG)25, M_fav6^B (CA)8, maMS 2-4, M_fav30^C (TTTG)8) or 55°C (primer maMS 2-8) for 45 sec, 72°C for 45 seconds, followed by 8 cycles of 94°C for 30 sec, 53°C for 45 sec, 72°C for 45 sec then a final extension time of 10 min at 72°C. The primer run with Quiagen® was run at 95° for 5 min, then 34 cycles of 94°C for 1 min, 57°C for 1 min, 72° for 1 min followed by a final extension time of 10 min at 72°C. PCR products were multiplexed with HiDi Foramide (1:12) and 0.5μl Genescan 400 ROX (Applied Biosystems, Foster City, CA) and sent for analysis at FSU. Samples that did not amplify were re-run a maximum of two additional times. Finally, all samples that amplified were binned using GeneMapper 5 and then run through MicroChecker 2.2.3 in order to identify potential null alleles, large allele dropout, and stutter peaks. The data were then run in STRUCTURE 2.3.4 (Pritchard et al. 2000), to determine the degree of connectivity between shallow and deep O. franksi colonies. Finally, the STRUCTURE results were analyzed with Structure Harvester (Earl and vonHoldt 2012) to determine the number of distinct genetic populations.

**Statistical Analysis**

The statistical software program JMP 12© was used to elucidate any statistical significances within the data. Datasets that were recorded as a proportion were arcsine transformed prior to analysis. Before any statistics were conducted, the data was tested to ensure it met the assumptions of normality and equal variances. Normality was determined using the Shapiro-Wilk test and Levene’s test was used to determine
homogeneity of variances. If data was found to be normal and variances were equal, a one-way or two-way ANOVA or ANCOVA was applied. If statistical significance (p<0.05) was found, a Tukey’s Post-Hoc analysis was subsequently performed. If the dataset was unable to meet the assumption of normality or equal variances, a non-parametric test such as Wilcoxon (2 samples) or Kruskal-Wallis (>2 samples) was performed.

RESULTS

Spawn Times and Observations

There were significant differences in spawning times by spawning month and colony depth, but not by day after the full moon [ANOVA, F(1,20)=1.3982, p ≥0.05]. Spawning times in August were significantly later (180 minutes after sunset on average) than those recorded in September (120.5 minutes after sunset on average Fig.1) [ANOVA, F(1,20)=26.33, p= <0.0001]. Later spawning in August was observed for both deep [ANOVA, F(1,8)=13.04, p=0.0069] and shallow [Wilcoxon, χ²=8.456, p=0.0036] colonies. Deep colonies also spawned earlier (122 minutes after sunset on average) than their shallow counterparts (164 minutes after sunset on average) [ANOVA, F(1,20)=8.38, p=0.0089], a trend consistent across both months (Fig.2). Of the 37 colonies collected, 22 released gametes; 42.9% of deep colonies and 50% of shallow colonies spawned. Several individuals (3 shallow and 2 deep) released gametes on consecutive days. Deep
colonies were significantly larger than shallow colonies [Wilcoxon, \( \chi^2=7.531, p=0.0061 \)], but no correlation between colony size and spawning likelihood, or proportion of the colony that set, was found.

\[ \text{Figure 2. Spawn time by colony depth and month. Bars represent standard error. No significant differences exist between any of the treatments} \]

**Fertilization Success and Sperm Concentrations**

Fertilization success between inter- and intra-depth crosses were compared to reveal any potential incompatibilities between the parental genotypes obtained from the shallow reef and deeper mesophotic reef. Successful fertilization was recorded for all crosses. The average proportion of eggs fertilized across all crosses was 0.61. Controls had less than 1.2% fertilization. Comparing fertilization for all crosses (D♂xD♀, D♂xSh♀, Sh♂xD♀, and Sh♂xSh♀) using a Wilcoxon test found no significant differences in fertilization.
success for any crosses (Fig. 3) \[\chi^2=5.97, \ p \geq 0.05\]. When grouped by depth of egg (ignoring sperm donor), there were still no significant differences in fertilization success \[\text{Wilcoxon, } \chi^2=0.3033, \ p \geq 0.05\]. Significant differences in fertilization success were observed between months (Fig.4), with both shallow and deep gametes exhibiting greater fertilization in August.

![Figure 4](image)

*Figure 4. Average fertilization rates for all crosses by spawning month. Fertilization was significantly higher in August. Bars indicate standard error.*

than in September \[\text{Wilcoxon, } \chi^2=6.68, \ p =<0.0001\]. When comparing fertilization by month and day, a Tukey’s test reported no difference in fertilization during August spawning but significant differences between the two months and between both spawning days in September \[\text{Wilcoxon, } \chi^2=53.673, \ p =<0.0001, \text{ Fig.5}\]. A non-parametric Wilcoxon test was used to evaluate whether spawn day influenced fertilization success.

![Figure 5](image)

*Figure 5. Fertilization success by month and day after full moon. Columns connected by the same letter are not significantly different as determined by a Tukey’s Post Hoc Analysis. Error bars represent standard error.*
Combining both months yielded no significant difference in fertilization between individuals that spawned on day 5 and day 6 ($\chi^2=0.1468, p \geq 0.05$).

To ensure fertilization rates were not influenced by variation among the various sperm stocks, the potential interaction between sperm concentration and cross was examined. Sperm concentration was used as a covariate and the cross as the main effect. Fertilization as a function of sperm concentration was examined separately for shallow and deep crosses. An effects test showed significant interaction for shallow eggs and between sperm and eggs. For deep eggs there appeared to be interactions of sperm and eggs x log sperm (Table 1). Shallow eggs fertilized with shallow sperm had significantly higher fertilization compared to shallow eggs fertilized with sperm from deep colonies (Fig.6A). While mesophotic eggs were more promiscuous, appearing to be equally susceptible to both shallow and deep sperm, sperm concentration did have a significant

![Figure 6](image-url)

*Figure 6. Fertilization ANCOVA, conspecific crosses are expressed as a solid line, heterospecific crosses are expressed as dotted line. (A) Fertilization of shallow *O. franksi* eggs across a sperm concentration continuum. (B) Fertilization with deep *O. franksi* eggs across a sperm continuum.*
influence on fertilization success. Unlike the shallow, deep eggs did not appear to experience decreased fertilization at elevated sperm concentrations (Fig. 6B).

### Larval Viability

In August, larval survivors were quantified after 5 days when the larvae were competent. There were a total of 11 replicates (Sh♂xSh♀ n=3; Sh♂xD♀ n=4; D♂xSh♀ n=2; D♂xD♀ n=2). The average survival across all treatments was 50.4%. The highest survival was recorded for Sh♂xSh♀ (64.3%) and lowest survival was reported among Sh♂xD♀ (41.7%). Due to small sample size and that larval survival is likely more influenced by maternal provisioning (Gagliano and McCormick 2007), replicates were grouped by egg donor, but no significant difference in survival after day five was found [ANOVA, F(1,9)=0.0354, p ≥0.05].

In September, larvae were scored and transferred to new FSW every day for 5 days. The September trial consisted of 12 replicates (Sh♂xSh♀ n=3; Sh♂xD♀ n=2; D♂xSh♀ n=4; D♂xD♀ n=3); the highest mortality occurred during the initial day post-fertilization. As time progressed, mortality rates declined among all crosses. In both the

| Table 1. Effects test indicating significant interactions for shallow and deep eggs. * denotes significance |
|----------------------------------|-------|----------------|---|-----|
| Source                     | DF    | Sum of Squares | F-Ratio | P   |
| Shallow Eggs               |       |                |         |     |
| Egg                        | 1     | 0.7335         | 6.427   | 0.0149* |
| Egg x Log Sperm           | 1     | 0.6925         | 6.068   | 0.0177* |
| Deep Eggs                  |       |                |         |     |
| Egg                        | 1     | 0.0585         | 0.3523  | 0.5558 |
| Log Sperm                  | 1     | 1.0226         | 6.1554  | 0.0169* |
| Egg x Log Sperm           | 1     | 1.2393         | 7.46    | 0.009* |

**Figure 7. Larval survivorship in September by cross. Day 0 is fertilization. Bars represent standard error (Egg x Sperm)**
D♂xSh♀ and D♂xD♀ treatments, no mortality occurred after the fourth day. Average survival across all treatments was 24.7\% (Fig. 7). Both highest and lowest survival was observed among inter-depth crosses where eggs were fertilized by sperm of opposite depth. While survival varied by individual cross, when grouped by egg donor, larvae originating from shallow larvae had significantly elevated survival rates [Wilcoxon, $\chi^2=93.896$, $p<0.0001$].

When combining survivorship values for both months, no significance was observed among any of the four crosses [ANOVA, $F(3,19)=0.4322$, $p \geq 0.05$] or between egg donor [ANOVA, $F(1,21)=0.9489$, $p \geq 0.05$]. To investigate survivorship between months, average survival rates for all crosses were pooled by month. This showed that August crosses resulted in significantly higher survivorship than those from September (Fig. 8) [ANOVA, $F(1,21)=6.73$, $p=0.017$].

![Figure 8. Larval survivorship by month. Bars represent standard error.](image)

**Larval Settlement Preference**

Larvae had elongated and reached settlement competency four days after fertilization, at which time they were presented with a choice of settlement substrate conditioned for three months on the shallow (18m) or mesophotic reef (40m). Larvae were given 48 hours in which to investigate the biofilm and CCA on each tile, choose their preferred substrate and undergo settlement before settlement preference was scored. The average proportion of settlement across all treatments was 0.41. There was no
significant difference in the proportion of deep (D♀+D♂) or shallow (Sh♀+Sh♂) larvae that settled [ANOVA, F(1,30)=2.56, p≥0.05]. Larvae did however, exhibit significant depth settlement preferences [ANOVA, F(3,60)=3.62, p=0.0180]. While shallow larvae exhibit no preference for substrate depth, deep larvae significantly prefer to settle on shallow rather than on deep conditioned substrate [ANOVA, F(1,30)=8.28, p=0.0073, Fig.9].

![Figure 9. Larval depth settlement preference. Light blue columns represent the number of settlers on shallow conditioned tile, dark blue represents tile conditioned in the mesophotic. Columns connected by the same letter are not significantly different. Bars represent standard error.](image)

No significant difference was reported in the number of deep and shallow larvae still swimming after the 48 hour settlement window, suggesting similar competency and survival of shallow and deep larvae [ANOVA, F(1,30)=0.882, p ≥0.05]. There was no significant difference in the total number of larvae that settled on the top or bottom of each tile [ANOVA, F(1,62)=1.811, p≥0.05]. When broken down by larval depth there was still no significant preference for settlement location [ANOVA, F(3,60)=1.25, p≥0.05].

**Post-Settlement Survival**

Post-settlement survival was determined by re-deploying all tiles to the depths at which they were originally conditioned. After four weeks, the tiles were recovered and, again, scored for settled larvae. The average proportion of survival across all treatments
was 0.40. A comparison of survival rates showed no significant differences among any of the four treatments (Fig.10) [Wilcoxon, $X^2(3)=1.7229, p=0.05$]. On four tiles (one of each tile type), post settlement survival ratios exceeded 1.0. Regardless if these tiles were included at 100% survival or excluded from the analysis, the results did not change. Therefore, these tiles were removed from the analysis.

![Figure 10. Post settlement survival after four weeks. Light blue columns represent survival of recruits deployed to the shallow reef. Dark blue columns represent survival of recruits after being deployed to the mesophotic for 4 weeks. Bars indicate standard error](image)

Population Genetics

The results of the genetic analysis indicate a single population of *O. franksi* with no genetic differentiation between depths or sites. This supports our gametic compatibility results, confirming that deep and shallow populations are compatible and that gene flow is occurring between depths. The characteristics of the microsatellites loci are summarized in Table 2. Two samples were eliminated from analysis due to issues at 2 loci, where the samples either did not amplify or amplified as a polyploid. An additional 2 samples flagged by the microsatellite toolkit for Excel as being potential clones were also excluded from the analysis so as not to bias the population estimates. When analyzing data with Micro-Checker, analyses were performed using both 100% and Bonferroni confidence intervals. When run at 100% confidence, Micro-Checker identified one primer (M_fav5^A (CGA)17) as containing potential null alleles. When utilizing the Bonferroni confidence intervals, an additional primer was flagged as containing potential null alleles (M_fav9^B (CAAT)21). When all primers were
included in the Structure analysis, it was difficult to determine if there were one or two distinct populations. When the primer identified as containing potential null alleles by both confidence intervals was excluded, it became apparent that one population was the most likely. When both potential problem primers were excluded, it became conclusive that our samples all belong to a single population (Fig.11).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Size (Published)</th>
<th>Size (Observed)</th>
<th>Nr of Alleles</th>
<th>Nr of Triploids</th>
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<tr>
<td>M_fav5^A (CGA) 17</td>
<td>340-394</td>
<td>338-452</td>
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<td>M_fav7^A (CAT) 24</td>
<td>465-519</td>
<td>451-541</td>
<td>22</td>
<td>1</td>
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<td>M_fav3^A (ATG) 25</td>
<td>161-218</td>
<td>164-224</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>M_fav6^B (CA) 33</td>
<td>401-445</td>
<td>406-457</td>
<td>5</td>
<td>2</td>
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<tr>
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<td>262-318</td>
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<td>0</td>
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<td>192-237</td>
<td>12</td>
<td>28</td>
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<tr>
<td>maMS2-4</td>
<td>275-357</td>
<td>312-348</td>
<td>13</td>
<td>2</td>
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<tr>
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<td>227-247</td>
<td>238-243</td>
<td>3</td>
<td>0</td>
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</tbody>
</table>

Table 2. Characteristics of microsatellites loci used in population analysis. For each primer the published and observed sizes, number of alleles and the number of polyploids (triploids) are reported.

**Figure 11.** Structure Harvester graph indicating the most probable number of populations based on six microsatellite loci. Bars represent standard error. K=1 has highest probability and least error.
**DISCUSSION**

The demonstration of mesophotic corals’ ability to reproduce among themselves and with shallow conspecifics confirm the plausibility of the Deep Reef Refugia Hypothesis along the Belize barrier reef. Upper mesophotic *O. franksi* colonies exhibited early spawning times that facilitated gamete encounters with shallow corals, interdepth gamete compatibility, viable larvae, settlement preference to shallow reefs, and equivalent post-settlement survival in the shallows compared to natal depth. These findings imply the potential for ongoing connectivity between healthy deep and declining shallow populations. As anthropogenically driven climate change continues to negatively impact shallow reefs, deeper reefs will play an increasingly important role in maintaining genetic diversity and larval recruitment to shallow reefs (Holstein et al. 2015).

Spawning synchrony is a key component of fertilization success for sessile broadcast spawners; differences in spawning times as little as 10 minutes can lead to reproductive isolation (Levitan et al. 2011). While our spawn times for all deep and shallow *O. franksi* were similar to published spawn times (Knowlton et al. 1997; Levitan et al. 2004), the deep colonies were found to spawn approximately 40 minutes earlier than their shallow counterparts. It is possible that deep populations may be spawning earlier in response to earlier light onset of darkness in the mesophotic; low levels of light that penetrate the shallows at dusk are unlikely to penetrate down to the upper mesophotic. This suggests earlier spawning by mesophotic colonies is due to environment. However, it is important to note that shallow and mesophotic colonies were held under identical light regimes in the days prior to spawning and mesophotic corals still spawned significantly earlier. This may indicate the presence of a genetic factor influencing spawn times among shallow and mesophotic populations or spawning times are determined in advance and altering the light regime briefly (1-3 days) prior to spawning does not influence spawning times. The most important fact is; rather than reducing connectivity, earlier spawn by deeper colonies increases the potential for inter-depth gametic mixing. Spawn times were significantly later in August than in September; this held true for both shallow and deep colonies. Similar trends between months indicates that both deep and shallow populations are responding to similar cues.
such as water temperatures and changing light regimes. While slightly fewer deep corals spawned in both August and September, this could be due to our difficulty in differentiating between ripe and unripe eggs at depth; it is possible some colonies with immature eggs were collected. Despite deep colonies having significantly larger surface area, there was no difference in the size of colonies that spawned or in the setting area of those corals, suggesting that size was not a factor influencing spawning potential. However, it is important to keep in mind that for several individuals, only a portion of the colony was collected so our sample is not indicative of actual colony size.

Fertilization rates recorded for all crosses indicate a lack of prezygotic and postzygotic barriers between deep and shallow *O. franksi* corals. It appears that mesophotic colonies are able to produce viable gametes capable of fertilizing shallow conspecifics. We recorded an average fertilization success of 61% across all crosses with no single cross exhibiting less than 55% fertilization. These fertilization rates are quite high considering that previously reported fertilization rates for *O. franksi* range from approximately 0 to 40% (Levitan et al. 2004). For shallow eggs, sperm concentration did not have a significant effect of fertilization; this is likely due to relatively uniform sperm concentrations. Significantly greater fertilization was observed among shallow eggs fertilized by conspecific sperm. Fertilization among deep eggs was significantly influenced by sperm concentration; this was likely driven by polyspermy in several crosses at the highest sperm concentration that exhibited no fertilization. A lack of significant differences in larval survival rates across all treatments also suggests a lack of post-zygotic barriers and points to the presence of a single, connected population rather than two distinct depth-mediated populations.

Our genetic analysis also indicated the presence of a single population of *O. franksi*. As potential problem primers were excluded, the results became increasingly obvious. Comparing populations by depth within each site and the populations between each sampling site found no genetic differences which indicate a single population. The presence of a single population spanning substantial vertical and horizontal distances indicates that significant gene flow is occurring not only between depths but also along the reef. This corroborates our previous findings that mesophotic colonies are
reproducing with shallow counterparts, confirming the plausibility of the DRRH occurring along the Belize Barrier Reef. While this is the first study examining vertical connectivity among *O. franksi*, other studies have examined vertical and horizontal connectivity of other Caribbean corals with mixed findings. Genetic connectivity within populations of *Montastraea cavernosa* in the Florida Keys, Bermuda and USVI were examined; high horizontal connectivity at shallow depths was observed at all locations; however, vertical connectivity was only observed among populations in the USVI and Bermuda (Serrano et al. 2014). A similar study examining connectivity among and between populations of *Porites astreoides* (a brooding coral) in Bermuda, USVI and the Florida Keys found uniformly high vertical and horizontal connectivity within all locations and even some connectivity between study locations (Serrano et al. 2016). In light of the significant vertical and horizontal connectivity occurring among *M. cavernosa* and *P. astreoides* (Serrano et al 2014; Serrano et al. 2016), it is not surprising that we found genetic flow between shallow and mesophotic populations of *O. franksi* with slight vertical separation. Our findings, supported by previous literature, suggest that there is significant potential for genetic exchange between shallow and mesophotic reefs and provides further validation for the DRRH.

Larval settlement preference is an important factor of the DRRH; if deep larvae exhibit depth fidelity, it decreases their potential to repopulate shallow reefs. Overall, our results showed no significant differences in larval settlement between deep- and shallow-generated larvae. Interestingly, while shallow larvae settle equally on shallow and deep substrate, deep larvae significantly prefer to settle on shallow-conditioned substrate. Since settlement occurred under identical light and temperature conditions, the only difference is the tile and its associated biofilm. This suggests that deep larvae may be attracted to shallow-water biofilms or CCA species. While this does not tell us exactly what the larvae are responding to, overall settlement preference strongly supports mesophotic corals’ potential to assist in shallow reef recovery. While settlement rates may appear to be low, averaging 41% across all treatments, they are higher than settlement rates of 15-40% reported for the closely related *O. faveolata* (Ritson-Williams et al. 2014).
While larval settlement preference supports the DRRH, coral recruits are sensitive and can experience high mortality if the substrate or environmental conditions are not suitable. After four weeks of tiles deployed to their original depths of 18 or 39 m, post-settlement survival was not significantly different between larval origin or deployment depth. Based on these results, it appears that both shallow and mesophotic recruits can survive at depths other than where they were generated. Mesophotic reefs’ ability to successfully export larvae to shallow depths increases their efficacy as a refuge and provides further support for the DRRH.

*Orbicella franksi* is just one species present on Caribbean coral reefs, and while this study confirms that mesophotic populations of *O. franksi* are capable of acting as a refuge for shallow populations, it is important to assess whether the DRRH applies to other dominant reef-building coral species such as *Orbicella faveolata* and *Montastraea cavernosa*. While this study focuses on how vertical separation influences connectivity; future studies must examine connectivity between deep and shallow populations over large horizontal distances. By increasing our understanding of the connectivity between mesophotic and shallow populations on both vertical and horizontal spatial scales, we will be better able to predict future patterns of resilience and recovery. In addition to expanding our knowledge of connectivity between reefs, it would be beneficial to conduct long-term monitoring of shallow and deep reefs to evaluate their responses to and recovery from various stressors, as well as the role mesophotic reefs play in that recovery.

Mesophotic reefs appear to be acting as a refuge for shallow populations at some locations; as shallow reefs continue to decline the role of mesophotic reefs will likely increase. Mesophotic reefs current and future role as a spatial and reproductive refuge underscores their significance and highlights the need for their conservation. While increasing our knowledge of mesophotic reefs, we must lobby for their protection and preservation. It is imperative that we consider mesophotic reef habitats when implementing marine parks and protected areas. These findings may also be used to increase the resilience and efficacy of artificial reefs. Since coral reefs in the upper mesophotic are not only healthier than their shallow counterparts but also able to act as a
refuge for declining shallow populations; constructing artificial reefs or conducting restoration efforts at greater depths may increase their health and stability resulting in greater benefit to adjacent populations. Finally, since mesophotic reefs are susceptible to light limitation, we must focus on projects aimed at increasing water quality, reducing coastal runoff, eutrophication, and sedimentation. The protection of mesophotic coral ecosystems will increase the resilience and recovery of shallow coral assemblages, allowing them to persist in the face of increasingly hostile conditions.
CHAPTER 3 – DISCUSSION

The goal of this research project was to increase our knowledge of mesophotic reefs, investigate their potential role in shallow reef recovery and evaluate the plausibility of the Deep Reef Refugia Hypothesis occurring along the second largest barrier reef in the world. By confirming the presence of extensive, healthy mesophotic reefs and demonstrating their ability to reproduce among themselves and with shallow conspecifics, our results affirm the applicability of the DRRH to the Belize Barrier Reef. In addition to spawning earlier improving inter-depth fertilization potential, *Orbicella franksi* colonies from upper mesophotic reef exhibited high levels of, gamete compatibility, larval viability, settlement and post settlement survival. The settlement trends of mesophotic larvae that we observed, while unexpected, bode well for shallow reef recovery. Our findings, coupled with a lack of any obvious pre- or post-zygotic isolating barriers, suggest potential for connectivity between healthy deep and declining shallow populations. As anthropogenic climate change continues to have negative impacts on shallow reefs, these deeper reefs will play an increasingly large role in maintaining the genetic diversity and recruitment to shallow reefs. The topography along the Belize Barrier Reef is such that populations are separated by vertical rather than horizontal distance. This minimal horizontal separation made the Belize Barrier Reef an ideal candidate to evaluate the plausibility of mesophotic reefs acting as a refuge to bolster and support shallow reefs.

The Belizean Barrier Reef system is host to large, dense, and diverse mesophotic coral ecosystems consisting of healthy assemblages of scleractinian corals, sponges and reef fish (James and Ginsburg 1979). Mesophotic coral cover is variable with increased colony densities observed on sloping portions of the wall and reduced densities on steeper or underhanging surfaces. The growth patterns are influenced by a combination of light availability and antecedent topography and are consistent with the structure of mesophotic populations described by Bridge et al. (2011). While our study area was on the scale of a few kilometers, the presence of healthy mesophotic reefs at every site visited confirmed the presence of extensive mesophotic ecosystems on this section of the
Belizean barrier reef. This supports the first aspect of the DRRH stating that deep reefs are stable, protected, and healthy. This also suggests that despite a lack of acknowledgement in the literature, the barrier reefs of Belize are home to extensive and important mesophotic reef ecosystems. *Orcicella franksi* were observed at all sites and at all depths, highlighting their important role on both shallow and mesophotic reefs. The continuous distribution and dense populations of *O. franksi* colonies from relatively shallow reef crest of the wall (>15 m) to the upper mesophotic (at least 45m) and beyond (the max depth of *O. franksi* on the Belizean Barrier Reef has not been established), also supports the likelihood of interconnected populations rather than distinct populations separated by depth. Finally, the wall itself may increase gamete mixing and fertilization as the barrier created by the wall may funnel both currents and gamete bundles as they rise to the surface, increasing mixing and potential inter-depth fertilization.

All colonies that spawned did so within previously reported *O. franksi* spawning times. Both mesophotic and shallow colonies spawned on the 5th and 6th days after the full moons in August and September within the reported spawning window of four to eight days after the full moon (Levitan et al. 2004; Levitan et al. 2011). Our spawn times for all deep and shallow *O. franksi* were similar to published spawn times (Knowlton et al. 1997; Levitan et al. 2004), and consistent with the literature, the deep colonies we observed spawned an average of 40 minutes earlier than their shallow counterparts. Earlier spawning by deep colonies is interesting since both deep and shallow colonies were exposed to identical light cues in the days prior to gamete release. Earlier spawning by mesophotic colonies is possible evidence of genetic differences between depths. Spawning synchrony is a key component of fertilization success for sessile broadcast spawners; a seemingly insignificant ten-minute difference in spawning times can lead to reproductive isolation (Levitan et al. 2011). Rather than reducing connectivity, earlier spawning by deeper colonies is shown to increase the potential for inter-depth gametic mixing and connectivity between populations. Spawn times were significantly later in August than in September, a pattern observed for both shallow and deep colonies. This earlier spawning in September may be due to increased water temperatures exerting thermal stress on the colonies, this may also explain reduced fertilization observed among September’s gametes. Reproductive investment and spawning by mesophotic colonies
illustrates their ability to not only survive but also thrive in mesophotic conditions. In addition, the consistently offset spawning to increase inter-depth gametic mixing across months suggests that these populations are relatively tightly coupled and likely connected.

While slightly fewer deep colonies spawned, the difference was not significant and could be attributed to our difficulties in determining egg ripeness at depth. Artificial lights tend to wash out color, which could have resulted in the collection of some unripe colonies. Surface area of the colony did not influence that colony’s likelihood of spawning, nor did it correlate to the proportion of the colony that set or spawned. This suggests that size was not a factor influencing spawning potential. It is important to keep in mind that for several individuals, only a portion of the colony was collected so sampled size is not always indicative of actual colony size.

Successful fertilization was recorded between and within depths in both August and September, indicative of fully reproductive populations lacking apparent prezygotic barriers between deep and shallow *O. franksi*. When grouping fertilization rates by month, there were significant differences in fertilization success, with greater fertilization rates reported in August than in September. This pattern of a main spawn followed by a smaller secondary spawning event the following month has previously been reported for *Orbicellids* (van Veghel 1994), possibly signifying that August was the main spawning event and September was more of a bet hedging strategy to spread the risk of reproductive failure if only relying on one month. Overall, fertilization rates recorded in this study were relatively high. Average fertilization success in this study was 61% across all crosses with no single cross exhibiting less than 55% fertilization. This was much higher than previously reported fertilization rates of 0 to 40% for *O. franksi*; however, they were targeting a broader range in sperm concentration (Levitan et al. 2004). The consistent and successful fertilization rates reported among all crosses suggest that mesophotic and shallow populations are fully fertile and capable of successful fertilization in no-choice (non-competitive) crosses.

To examine egg-sperm preference, a parametric ANCOVA was used. Despite the data lacking a normal distribution, the parametric test was performed. According to
Vickers (2005), unless data exhibits an extreme skew, the parametric ANCOVA is a more powerful and accurate test than comparable non-parametric options (such as the Mann-Whitney). The results of the ANCOVA indicate that shallow eggs had greater fertilization rates when exposed to shallow sperm than when presented with comparable concentrations of deep sperm. Interestingly, while overall fertilization was less than shallow sperm, deep sperm achieved their optimum fertilization at lower sperm concentrations, which is important especially as populations dwindle and gametes become more dilute. Deep eggs were more promiscuous, compatible with both shallow and deep sperm. Deep eggs, when crossed with deep sperm, showed consistent fertilization across a wide range of concentrations. Shallow sperm, on the other hand, required slightly higher concentrations to fertilize deep eggs, but at those elevated concentrations, they were able to achieve extremely high fertilization. Overall, patterns of gamete susceptibility suggest a lack of prezygotic isolating mechanisms or gamete incompatibilities between shallow and mesophotic colonies. While slight gametic preferences were observed between shallow and deep gametes, fertilization among inter-depth crosses exceeded 58%. High fertilization between depths, coupled with successful fertilization along a spectrum of gamete densities, suggests that as shallow populations continue to decline, shallow gametes will likely be fertilized with gametes generated by mesophotic populations.

While uniformly high fertilization rates across all trials suggest that shallow and mesophotic populations are compatible, it is important to monitor early development for any evidence of post-zygotic inviability. Uniformly high mortality among a specific cross could indicate genetic incompatibilities, ultimately resulting in developmental failure (Palumbi 1994). The high fertilization and survival rates across all treatments in this study suggest the presence of a single connected population rather than two isolated depth-mediated populations. While no early developmental issues were detected in any particular cross, larval survivorship overall was significantly higher in August than in September, a finding that is consistent with previously reported fertilization success between August and September. Reduced fertilization and survival among larvae generated in September, regardless of depth or cross and despite daily water changes, is further indication that August was the main spawning event and September was a
secondary event. However, there are other possible explanations for this pattern of increased survivorship recorded among August crosses. One possible reason for reduced survival among larvae generated in September could be due to the method used to rear the larvae. In August, the larvae were left undisturbed in a Petri dish for five days until the survivors were counted. In September, the larvae were counted and transferred to a clean Petri dish with new filtered seawater every day for the same five-day period. It is possible that larvae could have been injured or damaged by the pipette during the daily transfers in September, ultimately resulting in increased mortality. Increased thermal stress is another possible explanation that would account for both decreased fertilization and survivorship among September larvae. Temperature loggers located adjacent to where the colonies were held recorded an average temperature increase of 0.71°C in September (Smithsonian Institute, CCRE 2016). This slight increase in ambient temperature could have stressed the colonies resulting in decreased gamete quality.

To compare overall survival, larvae were grouped by the depth of the egg donor. This was done both to increase sample size and thus power, and because larval survival is more dependent on egg quality. Lacking mouths or zooxanthellae, young *O. franksi* larvae are unable to feed or photosynthesize until they settle and undergo metamorphosis (Wellington and Fitt 2003). During the larval stage, they rely mainly on lipid energy stores provided by the maternal egg; if that egg is deficient chances of survival are greatly diminished (Harii et al. 2010). Sperm selection occurs during the process of fertilization. Deficient sperm are unlikely to outcompete healthy, motile sperm and are therefore less likely to fertilize an egg. Survival rates of deep and shallow larvae in August were not significantly different; both depths exhibited strong survival. Larvae generated during the September spawning event did experience significant differences in survival, with shallow eggs surviving at a significantly higher rate than larvae generated from deep eggs. When both larval survival rates recorded in August and September are grouped, there was no significant difference in survival between depths. Increased mortality observed among deep larvae produced during the September spawning event could have been due to the elevated temperatures recorded before and during spawning. Due to their more stable environment, some studies suggest that mesophotic colonies are more susceptible to thermal stress than their shallow counterparts (Smith et al. 2016b). A
variety of stressors including sedimentation and thermal stress have been shown to negatively influence reproductive capacity through lipid and, in extreme cases, gamete reabsorption of several scleractinian corals (Cox et al. 1998). It stands to reason that these mesophotic colonies, unaccustomed to temperature increases, would experience greater stress than their shallow counterparts, ultimately resulting in decreased survival.

Overall, while larvae generated from shallow eggs in September exhibited significantly higher pre-competency survival, those differences were not significant when grouped with survival rates in August, indicating similar survival among both deep and shallow larvae. While there is a lack of published data on early post-settlement larval survival for *O. franksi*, larval survival rates for congener *O. faveolata* three days after fertilization are similar to survival rates we recorded after day three in September (Vermeij et al. 2006). Larval survival rates in August were even higher; survival rates after seven days were comparable to survival rates reported on day three for *O. faveolata*. Successful survival recorded among all crosses suggests a lack of early post-zygotic isolating mechanisms.

Since corals are motile only during their larval stage, their settlement choice is critical, influencing survival and growth rates and ultimately determining parental distribution and population structure (Graham et al. 2008; Baird et al. 2003). Larval settlement choice is influenced by a variety of factors including irradiance levels, substrate orientation and chemical cues from local biofilm (Baird et al. 2003; Heyward and Negri 1999; Mundy and Babcock 1998). Our settlement tiles were deployed at shallow (18 m) and mesophotic depths (39 m) for three months prior to settlement in order to cultivate the local biofilm and crustose coralline algae present at each depth. This conditioning period was chosen because it is within the ideal coral settlement window where settlement facilitators, such as biofilm and crustose coralline algae (CCA) are present but settlement inhibitors such as invertebrate crusts or turf algae have not established (Arnold and Steneck 2011). We recorded total settlement for larvae at 41% when all treatments were grouped. While this may seem low, previous studies have reported settlement rates between 15-40% for the closely related *O. faveolata* (Ritson-Williams et al. 2014). Our settlement values were in the upper range of previously
published parameters, possibly due to the health of the larvae, presence of preferred substrate or a combination of the two. It is possible that given additional time to settle, a greater number of larvae may have identified suitable substrate and settled. However, since settlement preference was the objective of this experiment, we limited the settlement window to 48hr; long enough for larvae to choose and settle on their preferred substrate but not long enough for larvae to become desperate to settle that they do so without regard to substrate cues. Since larval settlement preference directly influences adult populations, it is an important component of the DRRH. If larvae exhibit depth fidelity, the potential for mesophotic populations to repopulate shallow reefs is greatly diminished.

Our settlement data did not indicate any evidence of depth fidelity. Shallow larvae did not exhibit any preference for substrate, settling equally on both shallow- and deep-conditioned tiles. Deep larvae displayed a promising trend, significantly preferring to settle on the tile conditioned in the shallows. This settlement trend provides significant support for the plausibility of the DRRH occurring along these reefs. It suggests that these healthy mesophotic corals along the Belize Barrier Reef are not only reproductive, but have the potential to settle and repopulate shallow coral reefs. This pattern of settlement observed among deep larvae portends their likelihood to recruit to shallow substrates as they become available after shallow reef mortality events. Without these mesophotic reefs providing gametes and recruits, shallow reefs may be more threatened. As with many scientific pursuits, answering one question leads to more questions, and our study is no different. To further investigate this critical component of the DRRH, it would be interesting to evaluate larval settlement preferences with multiple tiles conditioned at a range of depths rather than just shallow and mesophotic in order to understand fine-scale larval preferences. Also, it is important to evaluate the larval settlement preferences of larvae generated from a cross between shallow and mesophotic parents. As populations become more depauperate, cross-fertilization between depths is increasingly likely. Our ability to predict reef resilience and recovery would be greatly enhanced by understanding the settlement trends of mixed-depth larvae. Unfortunately, due to the amount of effort required to rear *O. franksi* larvae, this experiment was unable
to rear enough larvae to test settlement preferences of mixed-depth larvae in addition to those generated from deep and shallow parents.

All components of this project have yielded evidence supporting the DRRH, suggesting that mesophotic reefs are, in fact, interacting with shallow conspecifics. The final area that we addressed to determine the efficacy of the DRRH focused on the early post-settlement survival of the recruits. Young coral recruits are extremely sensitive and experience high rates of mortality if their substrate or environmental conditions are not suitable. By scoring for recruits, deploying the settlement tiles to their original conditioned depths (18 and 39 m) for four weeks, and then re-scoring survivors, we were able to determine early post-settlement success. Our larval survival was highly variable and yielded no significant differences regardless of larval origin or deployment depth. This lack of significance indicates that both shallow and mesophotic recruits have comparable survival rates and can settle and survive the critical, initial post-settlement period at depths other than where they were generated. Due to a lack of available data on early post-settlement survival for *O. franksi*, our results cannot be directly compared. However, settlement experiments with *O. faveolata* in the Florida Keys found <5% survival after 4 weeks (Fogarty pers. comm.). Although the methods of this unpublished study differed in that tiles were secured parallel to the reef rather than cable tied to a framework and secured so tiles were perpendicular to the reef slope, it demonstrates the vulnerability of *Orbicella* settlers during the early life history stages. Other studies focused on recruit survival tracked established recruits rather than tracking from settlement (Bak and Engel 1979). Wilson and Harrison (2005) settled larvae from multiple Pacific coral species on to artificial substrate then deployed them on the reef and followed survivorship. They reported greatest recruit mortality within the first few months after settlement and a survivorship of less than 15% during the first four weeks for the stony coral *Acanthastrea lordhowensis*. In comparison, during the first four weeks post-settlement, total survivorship among our recruits, regardless of depth, was 39.6%. The high survival in this study may be attributed to the way in which the tiles were secured to the reef prior to deployment; each tile was secured to a panel of plastic egg-crate, resulting in limited space between each tile. The tight arrangement of settlement tiles may have offered some protection from grazing herbivores that have been
shown to significantly influence recruit mortality (Penin et al. 2010). This tile-rack setup may have contributed, however, to the low survival rates recorded among shallow larvae deployed to the deep reef. Once *O. franksi* larvae settle they acquire symbiotic zooxanthellae from the water or substrate. These *Symbiodinium* then photosynthesize, providing energy to supplement dwindling maternal lipid stores. It is possible that the tightly spaced tiles increased shading, further reducing the limited light available at mesophotic depths therefore reducing the amount of energy available to the new recruits. While scoring survivors on the tiles after the four-week re-deployment, several were found to have more recruits than originally recorded. This is most likely due to the cryptic nature of young recruits settling in holes and being overlooked during the initial scoring. When first settled, *O. franksi* larvae are translucent and difficult to discern; after four weeks, they have grown and acquired pigmented *Symbiodinium*, making the recruits more easily distinguishable. For the four tiles where all original settlers survived and a previously overlooked recruit was found, survival percentages were adjusted down to 100% to assume full survival. One tile from each of the four treatments was found to have survivorship in excess of 100%; eliminating those tiles from statistical analysis had no effect on significance. This study only examined short-term survival; for more complete survival information, the recruits should be recovered and re-scored after one year. By surviving an entire year, the recruits prove their ability to persist through every season and its accompanying stressors. Also, for future studies, when deploying settlement tiles with settlers, it would be better to deploy the tiles in less dense arrays so that the tiles receive the full ambient light and are exposed to grazing by local fish and invertebrates in order to better simulate natural conditions.

Our genetic population analysis supports our other findings, indicating the presence of a single connected population of *O. franksi*. No genetic differences were detected by depths or among sampling locations. This lack of genetic differentiation implies that deep and shallow populations are well connected; this finding was supported by the lack of pre- or post-zygotic isolating barriers between deep and shallow larvae. When all eight primers were included in the analysis, it was difficult to discern whether the samples came from a single or two distinct populations. However, after removing primer M_fav5^A (CGA)17 that had been flagged by Micro-Checker as containing
potential null alleles, it became obvious that our samples formed a single population. To further ensure that our samples were truly from a single population, the second primer M_fav9^B (CAAT)21 (identified by Micro-Checker as harboring possible null alleles when run at Bonferroni confidence) was also eliminated and the samples re-run. The removal of both problem primers resulted in even stronger indication of a single population. Our results appear to be more similar to *O. faveolata*, which has been shown to exhibit relatively well mixed populations, rather than *O. annularis* which tends to exhibit stronger population structure (Baums et al. 2010; Davies et al. 2013; Severance and Karl 2006). It is important to consider the influence of topography on the genetic population structure; since our sampling sites all occurred on a wall, it is likely that prevailing currents travelling along the wall funneled gametes as they float to the surface helping to increase mixing and genetic exchange, resulting in a single, well-mixed population of *O. franksi*.

This study comprehensively evaluates and confirms the plausibility of mesophotic populations acting as a reproductive refuge by breaking down the assumptions of the Deep Reef Refugia Hypothesis and testing each component to evaluate the applicability of the hypothesis occurring on Belizean reefs. Although overlooked in the literature, the Belize Barrier Reef is home to extensive and healthy mesophotic coral reef communities. Our genetic population analysis indicated that both deep and shallow *O. franksi* occurring along a section of the barrier reef comprise a single population rather than multiple populations separated by depth or horizontal distance. These genetic findings were corroborated by offset spawning times and reproductive success between depths. Furthermore, larval settlement preferences indicate that larvae are able to settle and survive at depths other than where they were generated. Mesophotic larvae exhibited an especially intriguing trend; we documented their preference to settle on substrate conditioned in the shallows. All the evidence suggests that mesophotic *O. franksi* are supporting shallow populations on the Belizean barrier reef.

The results of this study corroborate many previously published mesophotic findings and furnish several novel insights into the reproductive and larval ecology of mesophotic reefs and their shallow counterparts. Similar to mesophotic reefs in USVI,
we found healthy coral assemblages with high numbers of *Orbicella* (Smith et al. 2010). In concordance with Holstein et al.’s work on mesophotic coral reproduction in *Porites astreoides* and *O. faveolata*, we also found mesophotic *O. franksi* to be fully reproductive (Holstein et al. 2016; Holstein et al. 2015). Our genetic results, which indicate a single mixed population rather than multiple distinct populations, concur with previous findings by Baums et al. (2010) and Severance and Karl (2006). In addition to confirming several previously reported aspects of mesophotic coral ecology, this study is the first to study mesophotic *O. franksi* and the first mesophotic ecology conducted on the Belizean barrier reef. By confirming the compatibility, settlement preferences and survival of mesophotic larvae, we are filling in the gaps in our knowledge of mesophotic ecosystems. Answering some questions invariably leads to further queries and this study is no different. To further increase our understanding of mesophotic reefs and glean greater insight into the processes that govern them further studies are needed. Firstly, this study should be repeated with different species that inhabit shallow and mesophotic reefs. Also, in the natural environment gametes from shallow and deep colonies are likely to cross fertilize; therefore, it is important to test the settlement preferences of these mixed-depth larvae to see if maternal or paternal inheritance has any influence on larval depth preference.

While the first month post-settlement is a critical time for coral recruits, larval survival should also be monitored over a longer period to ensure that larvae that settle are able to persist though all conditions rather than just their first month. Finally, it is imperative that connectivity and compatibility between shallow reefs and their mesophotic counterparts be tested at multiple different locations and at multiple spatial scales.

Our results have greatly increased our knowledge of the reproduction and connectivity of mesophotic corals. They comprise a compelling first step in understanding the relationship between shallow and mesophotic coral assemblages. While further research is needed on different species and at different locations, our study confirms that mesophotic populations can act as a reproductive refuge for depauperate shallow communities. Scientists are too often regarded as prophets of doom; however, the evidence of this study ushers in a welcome breath of hope for the preservation of coral populations in the face of adverse climate conditions. Confirmation of mesophotic reefs’ role as a refuge has many important ramifications, not the least of which is our
improved ability to safeguard present and future corals. Our findings can also be used to expand the efficacy of marine protected areas. By including mesophotic coral ecosystems in the planning of new and expansion of existing protected areas, we will maximize our ability to maintain refuge for shallow conspecifics. The optimistic conclusions of this study augur well for our life-sustaining coral reefs. As stewards of our seas, we need to act on the good news of our findings. Legislation designed to protect our already-compromised fragile reef systems must include deep reef ecosystems. Since mesophotic reefs appear to play a crucial role in shallow reef recovery and persistence, it is necessary to enact regulations aimed at improving water quality to ensure the continued health of mesophotic ecosystems before anthropogenic stressors compromise their ability to support both themselves and the shallow corals they are clearly bolstering. As our oceans become increasingly inhospitable, mesophotic reefs may be the key to coral reef persistence; failing to act on the evidence of this study would be tantamount to throwing away that key.
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