The Ecology and Evolution of Coral-Associated Apicomplexans

by

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Abstract

Apicomplexans are protists within the eukaryotic supergroup S.A.R. that infect a wide-range of animal species and can cause disease. Although apicomplexans are an important parasitic group, little is published regarding those associated with many invertebrates, such as reef-building scleractinian corals. There is a single described species of coral-associated apicomplexans, *Gemmocystis cylindrus*, which is hypothesized to diverge early within the coccidian lineage. This group contains many opportunistic human and livestock pathogens. To resolve the potential effect of apicomplexans on coral health, it is first necessary to further describe this enigmatic group of putative parasites and determine their prevalence among host species. This study utilized previously collected seasonal samples from four Caribbean scleractinian coral species (*Montastraea annularis*, *Montastraea faveolata, Siderastrea siderea,* and *Porites astreoides*) over a nine-year period (May 2000-2008) from two reefs in the Florida Keys as well as over five-and-a-half years (May 2001-Nov 2005) for two Bahamian reefs. Using PCR-based screening, these colonies exhibited consistent infection for the sampling duration. There was a significant effect of season and species, with apicomplexans more likely to be associated during the winter and less likely associated with *S. siderea*. High prevalence may be partially explained by life-history traits as apicomplexans were found associated with planulae larvae of *P. astreoides*, indicating vertical transmission in species that brood (i.e. undergo fertilization internally). Conversely, apicomplexans are not associated with larvae of broadcasting (i.e.

undergo external fertilization) species, implying horizontal transmission in these species. To determine the evolutionary history of these as well as coral-associated apicomplexans from an additional 16 coral hosts, small subunit (18S) ribosomal DNA (rDNA) was utilized to generate phylogenetic trees. This group of apicomplexans forms a monophyletic clade with strong bootstrap support near the coccidians. Altogether, these data provide insights into the symbiotic association between coral hosts and apicomplexans.

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List of Abbreviations

ADM	Admiral Patch Reef (Florida)
LG	Little Grecian Reef (Florida)
SP	South Perry Reef (The Bahamas)
NNP	North Norman's Pond Reef (The Bahamas)
ML	Maximum Likelihood
BS	Bootstrap Support
PP	Posterior Probability

Chapter 1. Introduction

Symbiosis, defined by Anton de Bary as the intimate “living together of two differently named organisms”, has helped shape the evolution of eukaryotic life (Douglas 1994; Margulis 1998). Such interactions between partners can range from harmful to beneficial to the host species, with mutualistic symbionts providing or facilitating nutrition (Muscatine et al. 1981; Baumann et al. 1995; Little and Currie 2007; Thornhill et al. 2008), access to resources (Pirozynski and Malloch 1975; Lesser et al. 2004), intraspecific communication (Sasaki et al. 2003), defense (Ferrari et al. 2004; Kaltenpoth et al. 2005; Kroiss et al. 2010) and camouflage (Nyholm and McFall-Ngai 2004), to name a few. In addition, symbioses can alter the physiological requirements of hosts, increasing both their range and niche (Pirozynski and Malloch 1975; Dunbar et al. 2007; Piscart et al. 2007; Kranner et al. 2008). However, not all symbionts positively affect the host and a majority of all species on earth are hypothesized to be parasitic (Poulin 1996; Windsor 1998). One such group of parasites is the Apicomplexa.

Apicomplexans are alveolates (Adl et al. 2005) and taxonomically part of the S. A. R. eukaryotic supergroup, which phylogenetically groups the stramenopiles, alveolates, and Rhizaria together (Walker et al. 2011). Well-known apicomplexans include the causative agents of human malaria, cryptosporidiosis, and toxoplasmosis as well as coccidiosis, babesiosis, and theileriosis that can result in severe illness and death in livestock (Fayer et al. 2000; Bishop et al. 2004; Kim and Weiss 2004; Snow et al. 2005; Morris and Gasser 2006; El Hussein et al. 2012; Vannier and Krause 2012). There are currently ~6000 described species of apicomplexans

(Perkins et al. 2000), but this is likely a gross underestimate of the total that exist (Levine 1973,1988). Part of the discrepancy is due to poor sampling among hosts, especially of invertebrate taxa. Determining the occurrence, transmission, and diversity of these apicomplexans can potentially identify previously unknown species (e.g. Rueckert and Leander 2008; 2009), their intermediate and definitive hosts (e.g. Mathew et al. 1998; Cooper et al. 2009), source pools for known species (e.g. Miller et al. 2008; Vilcins et al. 2009; Winiecka-Krusnell et al. 2009), elucidate cases of host-switching (e.g. Duval et al. 2007; Vilcins et al. 2009), and reveal new models for studying closely related parasites (e.g. Leander 2008). Resolving the relationships among and between the apicomplexans is crucial as many invertebrate parasites may represent intermediate "links" between groups of well-known human pathogens (Kopecna et al. 2006; Leander 2008). Therefore, understanding the association of apicomplexans and invertebrates will help clarify both ecological distributions of known parasites and evolutionary history regarding the taxa potentially leading to new areas of applied research (e.g. Laurent and Pietra 2006).

Scleractinian corals are considered the foundation of the tropical reef ecosystem, providing such services as nutrition and shelter to a wide-range of organisms including apicomplexans (Knowlton and Jackson 1994; Plaisance et al. 2011). However, little is known regarding the prevalence, host range, transmission, and diversity of these particular symbionts. A single coral-associated apicomplexan species, *Gemmocystis cylindrus*, has been described infecting six species of Caribbean corals (Upton and Peters 1986). Later, apicomplexan DNA was detected in a majority (~90%) of *Montastraea faveolata* and *Montastraea annularis* (Toller et al. 2002), two common Caribbean corals, as well as several species of gorgonian corals (Goulet and Coffroth 2003). In this light, this dissertation focuses on apicomplexan parasites

infecting both scleractinian (i.e. hard) and gorgonian (i.e. soft) corals and will further describe this enigmatic group. The objectives of my dissertation were to 1) review the current state of knowledge of marine apicomplexans in general, 2) assess the prevalence of apicomplexans within several Caribbean scleractinian coral species across distance and time, 3) examine the transmission patterns of these symbionts in Caribbean scleractinian coral species having different reproductive modes, and 4) infer the evolutionary relationships among coral-associated apicomplexans and their placement within the apicomplexan phylogeny.

While human and agricultural parasites are well known, a number of apicomplexans infect invertebrates and marine taxa in particular. Notably, many apicomplexan taxa are only found in the marine environment (Perkins et al. 2000). Describing and understanding marine apicomplexans has been influential in resolving phylogenetic relationships and the evolutionary history of the group in general (Leander 2008; Rueckert et al. 2010; Rueckert et al. 2011) as well as uncovering the first known mutualistic apicomplexan (Saffo 1988; Saffo et al. 2010). In addition, several human pathogens, such as *Toxoplasma gondii* and *Cryptosporidium parva*, have caused "reverse" zoonoses in marine species (Fayer et al. 2004; Conrad et al. 2005; Miller et al. 2008). Chapter 2 is a systematic review of marine apicomplexan taxonomy and evolution. This review encompasses apicomplexan specificity for host taxa, transmission, and pathology associated with infection. Human induced transport and introduction of novel apicomplexans also are discussed.

The remainder of this dissertation focuses on coral-associated apicomplexans in the context of their ecology and evolutionary history. Chapter 3 examined apicomplexan prevalence in four species of Caribbean scleractinian corals: *M. annularis*, *M. faveolata*, *Porites astreoides*, and *Siderastrea siderea*. Prevalence was assessed using a PCR-based screening technique for 6

marked colonies of each species on two reefs each in Florida and the Bahamas over a 9 and 5.5-yr period, respectively. These colonies were examined seasonally to test the hypothesis that prevalence would increase in summer months as is seen in other groups of apicomplexans (Sawyer et al. 1973; Tuntiwaranuruk et al. 2008; Alvarez-Pellitero et al. 2009) and scleractinian symbionts (Chen et al. 2005; Koren and Rosenberg 2006; Jones et al. 2008; Suwa et al. 2008; Cavada et al. 2011; Chen et al. 2011).

Another important aspect of this symbiosis is transmission between host individuals. Corals offer a unique perspective into symbiont transmission, because they harbor numerous symbionts and exhibit one of two different reproductive modes: brooding and broadcasting. Brooding corals undergo internal fertilization and generally release larger and well-provisioned larvae. Conversely, broadcasting species spawn gametes into the water column and the larvae tend to be small and without symbionts. Additionally, corals are able to propagate asexually via fragmentation potentially contributing to symbiont transmission. Scleractinians make an excellent study system for symbiont transmission, because they 1) harbor many different symbionts, 2) possess varying reproductive modes via either internal external or external internal fertilization, and 3) can propagate asexually. Scleractinian corals usually exhibit one of two modes of sexual reproduction depending on whether fertilization occurs internally (brooding) or externally (broadcasting) (Baird et al. 2009). Brooding species produce larvae that are generally larger and provisioned with symbionts (i.e. vertical transmission), while larvae of broadcasting species are typically smaller and exclude symbionts (i.e. horizontal transmission) (Baird et al. 2009). Chapter 4 examined the brooded larvae of *P. astreoides* taken from two reefs in Florida and from a reef in Belize to determine if apicomplexan acquisition pattern mirrors that of other symbionts. To further examine the generality of vertical transmission in brooding species,

planulae larvae were collected from an additional four brooding species in Belize. Along with this, larvae and adult tissue was sampled from five species of broadcasting species on the same reef tracts as the brooding species to determine if apicomplexans associated with the adults but not larvae, which would be indicative of horizontal transmission.

This study also attempted to determine the host range and phylogeny of the coral-associated apicomplexans. As mentioned previously, only a single species of apicomplexan has been formally described from coral species. This species was taxonomically placed as an early branching lineage within the coccidians (Levine 1988), which include numerous economically important parasites (Perkins et al. 2000). However, oocyst size and shape differences are observed among apicomplexans isolated from different hosts (Upton and Peters 1986), implying potential undiscovered diversity as these characters have been utilized to describe novel species (e.g. Duszynski 1974). Furthermore, Peters (1984) observed another clade of apicomplexans (gregarines) within the tissues of the Caribbean scleractinian *Porites porites*. Chapter 5 used a molecular approach to examine the diversity within the coral-associated clade and to determine the phylogeny. 20 coral species, encompassing both scleractinians and gorgonians, were collected from three locations in the Caribbean Sea. *P. porites*, *P. astreoides*, *M. faveolata*, *M. annularis*, and *S. siderea* were all included in the analysis. Small subunit (18S) ribosomal DNA (18S rDNA) was utilized to determine the evolutionary history of the group and search for evidence of co-evolution between the coral hosts and apicomplexan symbionts.

Finally, chapter 6 reflects on the previous chapters and outlines outstanding research questions and the future direction of this system. First and foremost is the nature of the interaction between host and symbiont. Various techniques, such as fluorescence in situ hybridization (FISH) (e.g. Ainsworth and Hoegh-Guldberg 2009), quantitative PCR (e.g.

Thurber et al. 2008) and enumeration via next-generation sequencing (e.g. Medinger et al. 2010), could be utilized to estimate differences in relative abundance between coral samples. In this context, increased parasite load often negatively correlates with fitness, which could be measured as growth or fecundity. Finally, to examine diversity within this lineage of coral-associated apicomplexans, morphological data and fine-scale molecular markers should be utilized.

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Chapter 2. The Diversity, Prevalence, and Importance of Marine Apicomplexans

Abstract: Apicomplexans are unicellular eukaryotic parasites infecting numerous metazoan hosts. The most well-known include the causative agents of human malaria, toxoplasmosis and cryptosporidiosis. Epidemics generated by these parasites significantly affect the human condition and cost billions of dollars in damages. However, these human pathogens represent a small minority of total biodiversity. Apicomplexa likely originated in the marine environment and unparalleled diversity exists within several exclusively maritime lineages. Thus a better understanding of apicomplexan evolutionary history starts by further discovering and describing these marine species.

Introduction: The evolution of life was shaped when α-proteobacteria and cyanobacteria became the mitochondrion and plastid respectively (reviewed in Margulis & Chapman 1998). Although considerable debate remains regarding the timing (Javaux et al. 2001, Katz et al. 2004, Yoon et al. 2004, Berney & Pawlowski 2006, Knoll et al. 2006, Cavalier-Smith 2009) and progenitor host (Cavalier-Smith 2006, Esser & Martin 2007, Pisani et al. 2007, Poole & Penny 2007, Rivera 2007, Saruhashi et al. 2008, Yutin et al. 2008), these symbioses almost certainly occurred in the marine environment (Raven 1997, Anbar & Knoll 2002, Mentel & Martin 2008). The success of these early symbioses has led to tremendous diversity within the eukaryote lineage. This symbiogenesis was replayed millions of years later when secondary and tertiary acquisition of plastids occurred giving rise to the chlorarachniophytes, euglenids, and alveolates

(reviewed in Archibald & Keeling 2002, Palmer 2003, Keeling 2009). Thus for billions of years, symbioses have facilitated evolution in the oceans.

Although these extreme mutualisms have lead to symbiogenesis, parasitism is not in the evolutionary best interest of the host. Therefore, parasites must evade host resistance and even temper their own virulence to optimize survival and future propagation (Frank 1993, Ebert & Herre 1996). Infection requires encounters with suitable host species and can either be vertical or horizontal, where parasites are inherited directly or acquired from the environment each generation, respectively. In the marine environment, parasite transmission tends to be more open (McCallum et al. 2004) with infection success dependent on abundance and range of suitable hosts (González & Oliva 2009), host dispersal ability (Palm & Klimpel 2007, Marcogliese 2009), environmental tolerance (Poulin & Rohde 1997, Ford & Chintala 2006), presence of competing parasites (Dobson 1985) as well as accidental hosts (Thieltges et al. 2008), and recently has been linked to climate change and other anthropogenic factors (reviewed in Harvell et al. 1999, Harvell et al. 2002, Lafferty et al. 2004). However, this has not prevented millions of parasitic symbionts from evolving numerous times within bacteria, protists, and metazoans (reviewed in Baker 1994, Poulin & Morand 2000, Moya et al. 2008) and the taxonomic diversity reflects their widespread success. In fact, it has been hypothesized that the number of parasite species surpasses all others utilizing different life history strategies (Windsor 1998). Parasitism is ubiquitous in marine environments and contributes greatly to the community composition (Dobson et al. 2008). As in terrestrial systems, parasite species encompass a wide range of metazoan phyla including Platyhelminthes, Nematoda, Annelida, Mollusca, Arthropoda, Porifera, and Chordata to name a few (reviewed in Rohde 1993, de Meeûs & Renaud 2002). In

addition, numerous groups of parasitic protists have been discovered in marine hosts (reviewed in Rohde 1993, de Meeûs & Renaud 2002).

One protistan group of interest is the Apicomplexa, within the Alveolata (Adl et al. 2005). All of the ~6000 apicomplexan species described to date are obligate endosymbiotic parasites (Levine 1988a). Although first seen in 1674 by Antony van Leeuwenhoek, they were not described until later (See below) and largely dismissed as irrelevant until *Plasmodium* species were implicated as the causative agent of malaria in the late nineteenth century (Levine 1973b, 1988a). Other notable taxonomic members include *Cryptosporidium* spp. and *Toxoplasma gondii*, which are opportunistic pathogens of humans (reviewed in Barta & Thompson 2006, Dubey 2008). Numerous other species cause epizootics in livestock (reviewed in Bishop et al. 2004, Bock et al. 2004, Morris & Gasser 2006) resulting in the loss of millions of dollars annually. In addition to terrestrial chordates, thousands of species have been described associated with strictly marine hosts and the scope of this review will focus on these parasites.

Descriptive Taxonomy of Apicomplexans:

As unicellular protists, it is not surprising that apicomplexans were discovered only after microscopes were invented. Nor is it unexpected that the first description by Cavolini in 1787 was of a gregarine, as they can reach several mm in length (Minchin 1903). However, they were erroneously interpreted as larval tapeworms and half a century passed before von Kolliker correctly identified them as protists in 1845 (Minchin 1903, Levine 1973b). Once accurately described, thousands of species have been named based on several criteria. Gross morphology and ultrastructure have been used to characterize groups within Apicomplexa (reviewed in

Clopton 2004, Leander 2008, Clopton 2009, Dubremetz & Ferguson 2009). Oocyst morphology and the number of infective sporozoites per cyst has delineated several groups (reviewed in Levine 1973b). In addition, life-cycle patterns have differentiated clades. For example, Grassé (1953) split the subclass Gregarinasina into three orders initially based on the presence or absence of schizogony, the ability to reproduce asexually by multiple fissions inside the parent -schizont- cell (but see Schrével 1971). The agammococcidians also are defined by lacking both schizogony and gametogony (the creation of gametes), reproducing solely by asexual sporogony (Levine 1979a). Host range has been implemented as an important method for differentiating taxa. Some apicomplexans, such as most gregarines and eimeriids, utilize a single host species and are termed homoxenous. Others, including the Sarcocystidae and haemosporidians, are heteroxenous requiring at least two host species: one for sexual (definitive host) and another for asexual reproduction (intermediate host) (reviewed in Vivier & Desportes 1990, Perkins et al. 2000). Often different species of Apicomplexa will inhabit distinct definitive hosts. For example, two closely-related eucoccidians are distinguished as *Toxoplasma gondii* undergoes sexual recombination in felines and *Neospora caninum* requires a canine host (Dubey et al. 2002). Cross-infection studies also have been utilized to confirm that morphologically similar *Eimeria* spp. isolated from different hosts are distinct (e.g. Upton et al. 1992). Recently, molecular markers have been applied to further explore differences between intragenic species (e.g. Yang et al. 2002, Satoh & Nakai 2007) and relatedness between and among taxa (e.g. Hnida & Duszynski 1999, Barta et al. 2001, Morrison et al. 2004). They also have been used in collaboration with morphology and life-cycle traits to elucidate phylogeny (e.g. Jakes et al. 2003, Barta & Thompson 2006, Clopton 2009). Finally, movements of feeding stages (trophozoites)

have been used to differentiate archigregarines from eugregarines (Schrével 1970, Leander et al. 2006, Rueckert & Leander 2009a).

Evolution of Apicomplexa:

Recently, the former classification system of protists and animals has been modified to reflect current phylogenetic thought regarding the evolution of eukaryotes. To this end, several putatively monophyletic “supergroups” have been proposed (Simpson & Roger 2004, Burki et al. 2008, Parfrey et al. 2010, Walker et al. 2011). The recently erected SAR supergroup unites the stramenopiles and Rhizaria with the apicomplexan-containing alveolates (Burki et al. 2007, Burki et al. 2008, Walker et al. 2011). A common secondary acquisition of red-algal plastid has been hypothesized within Alveolata and relatives (Cavalier-Smith 1999, Keeling 2009). Although considerable debate remains (Palmer 2003, Bodyl 2005, Cavalier-Smith 2009), this hypothesis has been supported by several independent lines of evidence including monophyletic grouping utilizing nuclear (Baldauf et al. 2000) and plastid genes (Bachvaroff et al. 2005, Parfrey et al. 2006), rare genomic changes (Fast et al. 2001, Harper & Keeling 2003, Patron et al. 2004), and biochemical pathways for polysaccharide storage (Coppin et al. 2005). Thus the ancestor to Apicomplexa was likely photosynthetic, possibly with a secondarily acquired red algal plastid. This is corroborated by the detection of a relict plastid in several apicomplexan groups (McFadden et al. 1996, Denny et al. 1998, Lang-Unnasch et al. 1998) and by the exciting discovery of *Chomera velia*, a photosynthetic alveolate basal to the apicomplexan group (Moore et al. 2008). This cumulative evidence implies that photosynthetic loss within the apicomplexans was widespread as they switched to heterotrophy (Obornik et al. 2009), which may have

occurred in several other groups of alveolates. Genes associated with plastids have now been discovered inside non-photosynthetic alveolates including the ciliates (Reyes-Prieto et al. 2008), dinoflagellates (Sanchez-Puerta et al. 2007), and their relatives *Perkinsus* spp. (Teles-Grilo et al. 2007, Matsuzaki et al. 2008) and *Oxyrrhis marina* (Slamovits & Keeling 2008).

Unlike the supergroups, relationships within the phylum Alveolata are much less contentious with three large groups encompassed: Ciliophora, Dinozoa, and Apicomplexa (Adl et al. 2005). The alveolates are united by possessing cortical alveoli – flattened membraneous sacs beneath the plasmalemma – , a micropore, and tubular/ampulliform cristae inside the mitochondria (Cavalier-Smith 1991, 1993, Adl et al. 2005). Within the phylum, dinoflagellates are strongly supported as a sister taxon to the apicomplexans (Escalante & Ayala 1995, Fast et al. 2002, Leander et al. 2003b, Cavalier-Smith & Chao 2004, Moore et al. 2008). Although they most likely share a photosynthetic past, apicomplexans are strictly parasitic with the exception of *Nephromyces*, a potentially mutualistic symbiont of tunicates (Saffo et al. 2010). Characteristic of a parasitic lifestyle, the apicomplexans all have an anterior apical complex during at least one life-cycle stage (Levine 1988b). Despite the secondary loss of components in some groups, the apical complex usually consists of a microtubule-associated polar ring(s) together with a closed conoid (Morrissette & Sibley 2002) and other invasion organelles such as rhopteries (Sam-Yellowe 1996, Boothroyd & Dubremetz 2008), micronemes (Tomley & Soldati 2001), and dense granules (Mercier et al. 2005). The apical complex and affiliated organelles have been attributed to active invasion, host cell attachment, feeding, movement, and creation of the intracellular parasitophorous vacuole within which many parasites reside (Tomley & Soldati 2001, Huang et al. 2004, Sibley 2004, Kats et al. 2008).

Apicomplexa is subdivided into two groups depending on the presence or absence of the conoid: Aconoidasida, containing the haemosporidians and the piroplasms, and the Conoidasida (Levine 1988b, Perkins et al. 2000). The latter comprises the gregarines and coccidians, encompassing a majority of the known species. Within the gregarines, it has been hypothesized that archigregarines are early branching among apicomplexan lineages as they share many pleisomorphic traits including using the apical complex as a myzocytotic –sucking of host cytoplasm- feeding apparatus similar to colpodellids (Simdyanov & Kuvardina 2007), extracellular attachment, homoxenous life-cycle, and exclusive residence in the digestive tract of marine invertebrates (reviewed in Vivier & Desportes 1990, Cox 1994, Leander & Keeling 2003, Leander et al. 2003b, Leander 2008, Rueckert & Leander 2009a). This hypothesis was recently examined utilizing molecular phylogenies of the 18S small subunit ribosomal DNA (18S rDNA), but lack of resolution among the basal nodes led to low bootstrap support and alternate topographies (Leander et al. 2003a, Cavalier-Smith & Chao 2004, Leander et al. 2006, Leander 2007). Despite the ambiguous phylogeny and lack of fossil record, it still seems likely that apicomplexans originated in the marine environment (Leander 2008), arose ~350-930 mya as estimated from sequence divergence (Escalante & Ayala 1995, Douzery et al. 2004), and parasitized invertebrate hosts (Kopecna et al. 2006).

Marine Apicomplexan Taxa:

Although ~6000 species have been formally named, this is likely a gross underestimate with millions more remaining to be discovered (Adl et al. 2007). Other factors clearly bias the rate of discovery of parasites including location of ecosystem and scientific researchers, size of

the organisms (Poulin 1996, Takahashi et al. 2004), relevance to human health, and economic importance of hosts. In addition, relatively less attention has been given to invertebrate hosts (Kopecna et al. 2006). It is beyond the scope of this review to provide a list of all species infecting marine hosts. Instead, it will focus on groups associated with marine organisms. For detailed species lists within these groups, the reader is referred elsewhere (Levine 1988b, c, Duszynski et al. 1998, Perkins et al. 2000). A summarized list of marine apicomplexan taxa is provided below (Table 1).

Gregarine taxa: The gregarines are unique among the Apicomplexa by exclusively parasitizing invertebrate hosts and forming equal numbers of male and female gametes in separate gamonts during gametogony (Théodoridès 1984, Adl et al. 2005). Most gregarines are homoxenous and many are extracellular parasites (Schrével & Philippe 1993). Three groups (orders) are described within Gregarinasina: Archigregarinorida, Eugregarinorida, and Neogregarinorida (Grassé 1953). The archigregarines solely infect the digestive tract of marine hosts, specifically polychaetes, sipunculids, enteropneust hemichordates, urochordates, and are strictly homoxenous (Harant 1931, Levine 1971, 1988b). The taxonomy of Archigregarinorida is still in flux and was historically split from Eugregarinorida based on the presence of schizogony observed during the life-cycle (Levine 1971, 1988b, Perkins et al. 2000). However, schizogony may simply be rare and difficult to observe in some taxa making it a poor taxonomic character. Worse, it may not accurately reflect evolutionary history (Rueckert & Leander 2009a). Recent ultrastructure and phylogenetic studies have found several pleisomorphic characters that support its original placement in the archigregarines (Kuvardina & Simdyanov 2002, Leander et al. 2003a, Leander et al. 2006, Leander 2007, Simdyanov & Kuvardina 2007, Rueckert & Leander 2009a). This modification might also include moving *Digyalum oweni*, an intestinal parasite of

Littorina gastropods tentatively placed within this family, potentially increasing the host range of this group (Dyson et al. 1993, 1994). Trophozoites of this parasite exhibit non-progressive coiling and pulsating movements characteristic of other archigregarines, although further work is needed to place this species (Koura et al. 1990). Thus they appear to be an early-branching paraphyletic stem group within the gregarines (Rueckert & Leander 2008, 2009a, b, Rueckert et al. 2010). Regardless of the current taxonomic uncertainty, archigregarines are prevalent in marine polychaetes (Ray 1930a, MacKinnon & Ray 1933, Dibb 1938, Schrével 1970, 1971, Leander 2006) and perhaps understudied due to their potential phylogenetic position basal compared to other Apicomplexa taxa (Leander 2008).

Eugregarines are speciose with ~1600 divided into ~250 genera as of the late 1990's (Perkins et al. 2000). This group is differentiated from the archigregarines by likely method of nutrient acquisition, motility, ultrastructure, location within a host, and lack of schizogony (reviewed in Leander et al. 2006). In brief, different eugregarines specialize in infecting intestines, reproductive tissues, and coelomic cavities of hosts. Therefore, the ultrastructure and movements have evolved to exploit the distinct microhabitats (Leander et al. 2006). Eugregarinorida has been traditionally split into three groups namely the blastogregarines, aseptate (acephaline) and septate (cephaline) gregarines (Levine 1988b). The Blastogregarinorina contain a single genus –*Siedleckia* - and four described species all of which infect the intestines of marine polychaetes (Chatton & Villenueve 1936a, b, Perkins et al. 2000). Due to their peculiar life-cycle in which the trophozoite feeding-form buds off gametes (reviewed in Grassé 1953), they have been grouped into Eugregarinorida simply because they abstain from schizogony (Levine 1988b, Perkins et al. 2000) or ranked as a unique gregarine order (Vivier & Desportes 1990). Unfortunately, blastogregarines have been largely ignored in studies since their initial

descriptions and require further taxonomic attention. Septate eugregarines are characterized by a septum that separates the anterior and posterior of gamont cells and aseptates have no partition (Levine 1988b). Although convenient, molecular data do not support this clean distinction as both groups are polyphyletic (Rueckert et al. 2011). Even so, Rueckert and colleagues (2011), utilizing 18S rDNA sequence data, have shown differences between "aquatic" septate and aseptate gregarines after the exclusion of all "terrestrial" clades. Thus, we retain these taxonomic terms in this review, although the phylogeny remains to be elucidated and many groups will likely be rearranged in light of new molecular evidence.

Within the Aseptatorina, ~450 species have been characterized within nine groups of classical families, three of which contain marine parasites: Monocystidae, Urosporidae and Lecudinidae (reviewed in Vivier & Desportes 1990, Perkins et al. 2000). Within Monocystidae, only two genera containing four species associate with marine annelid hosts (Bhatia & Setna 1938, Noble 1938). The Urosporidae almost exclusively contain marine species that infect a wide range of taxa including mollusks (e.g. Tuzet 1931) echinoderms (e.g. Minchin 1893, Pixell-Goodrich 1915, Coulon & Jangoux 1987), nemertean ribbon worms (reviewed in McDermott 2006), sipunculids (e.g. Pixell-Goodrich 1950), and other polychaetes (e.g. Pixell-Goodrich 1916, Porchet-Henneré & Fischer 1973, Levine 1977a, Landers 1991). Traditionally, the Lecudinidae encompass both terrestrial and marine parasites, the latter inhabiting tunicates (e.g. Harant 1931, Ciancio et al. 2001, Rueckert & Leander 2008) crustaceans (e.g. Théodoridès & Desportes 1972) and turbellarian flatworms (Cannon & Jennings 1988) in addition to the host range of the Urosporidae (MacKinnon & Ray 1931b, a, Ganapati 1946, Schrével 1963, Levine 1976, 1977b, Desportes & Théodoridès 1986, Rueckert et al. 2010). Aseptate gregarine species are likely homoxenous and can reside in the host coelomic fluid (Brownell & McCauley 1971,

De Ridder & Jangoux 1984, Landers 2002), the respiratory tree (Pixell-Goodrich 1929) or hemal system of marine echinoderms (Pixell-Goodrich 1925), and gastrointestinal tract (Kuriyama et al. 2005). Some spend different stages of their life-cycles in separate tissues (Coulon & Jangoux 1987).

The septates, sub-order Septatorina, comprise the majority of gregarines with ~1200 species partitioned into 23 families, eight of which include marine taxa (Perkins et al. 2000, Clopton 2009, Rueckert et al. 2011). Two of these families contain few thalassic genera: *Thalicola* and *Tricystis* within Actinocephalidae and *Cirrigregarina* within Gregarinidae, which infect pelagic salps, arrowworms, and barnacles, respectively (Hamon 1951, Théodoridès & Desportes 1975, Levine 1979b, 1988b, Perkins et al. 2000). The family Ganymedidae incorporates only two species infecting marine crustaceans to date (Prokopowicz et al. 2010, Rueckert et al. 2011). Another family, Cephalolobidae, consists of only five species that infect the intestines of crustaceans (Sprague & Couch 1971, Perkins et al. 2000, Chávez-Sánchez et al. 2002), including planktonic forms (Théodoridès & Desportes 1975). Most marine septate gregarines are characterized into the following three families: Porosporidae, Cephaloidophoridae, and Uradiophoridae. The Porosporidae are uniquely heteroxenous, utilizing a mollusk as an intermediate host and a crustacean as the definitive host where sexual reproduction occurs (reviewed in Prytherch 1940, Cheng 1967). Thus this family has received much attention due to the shellfish industry as several economically important species harbor these parasites (Prytherch 1940, Landau & Galtsoff 1951, Canestri-Trotti et al. 2000, Jiménez et al. 2002, Tuntiwaranuruk et al. 2008, Francisco et al. 2010). Species within Cephaloidophoridae are homoxenous and parasitize crustaceans (e.g. Ball 1959, 1963, Takahashi et al. 2004). Members of this group have been found inside the digestive tract of Antarctic krill (Takahashi et

al. 2004, Takahashi et al. 2008), pelagic amphipods (Pixell-Goodrich 1949, Théodoridès & Desportes 1975) and barnacles (Lacombe et al. 2002). The final marine septate family, Uradiophoridae, has ~20 species that mostly infect the intestines of decapods, amphipods, and barnacles (Tuzet & Ormières 1964, Théodoridès & Laird 1970, Sprague & Couch 1971, Rueckert et al. 2011).

No marine taxa exist in the final gregarine order, Neogregarinorida. However, this is a tautology as the neogregarines are partially defined by only infecting insects (Grassé 1953, Levine 1988b).

Cryptosporidium: Cryptosporidiidae has traditionally been considered a Eucoccidian family within the Eimeriorina (see below) based on morphology, life-cycle, and anisogamy, which among other characteristics define this group (reviewed in Egyed et al. 2003). However, as they are resistant to anti-coccidian drugs and have unique feeding morphology, this placement is tenuous (Huang et al. 2004, Barta & Thompson 2006). Recently, a number of molecular studies have grouped *Cryptosporidium* species outside of the coccidia and often with gregarines (Barta & Thompson 2006, Bachvaroff et al. 2011). Therefore, this group will be considered separately in this review. *Cryptosporidium* spp. have been found in the digestive tract of several marine chordates including fishes (Alvarez-Pellitero & Sitjà-Bobadilla 2002, Reid et al. 2010), turtles (Graczyk et al. 1997), seabirds (Bogomolni et al. 2008), pinnipeds (Deng et al. 2000, Santin et al. 2005) and other marine mammals (Morgan et al. 2000). In addition, oocysts have been isolated from bivalves (Miller et al. 2005, Schets et al. 2007), although it has been hypothesized that these filter-feeders have accumulated the water-resistant spores from nearby anthropogenic

runoff (Miller et al. 2005) as no signs of pathology were seen in the shellfish (Potasman et al. 2002).

Coccidian taxa:

The coccidians differ from the gregarines by creating an unequal number of male (microgametes) and female (macrogametes) gametes during gametogony; in coccidians, the male and female gamonts develop many smaller microgametes and a single larger macrogamete, respectively (Vivier & Desportes 1990). Although there are some exceptions, coccidians tend to infect vertebrates, residing intracellularly within a host and parasite derived parasitophorous vacuole membrane (PVM) (reviewed in Sibley 2004). The coccidians are a speciose group with a majority of the ~2,000 species residing within Eucoccidiorida, by far the largest of the orders (Levine 1988b).

Species within the order Eucoccidiorida exhibit sporogony, gametogony, and schizogony stages in their life-cycles (Levine 1988b). This order is further split into two groups: Adeleinorina and Eimeriorina (Perkins et al. 2000). Although most studies have focused on terrestrial systems, marine taxa exist within each. Species within Adeleinorina are unique among coccidians by pairing gamonts before gametogony in a process called syzygy (Levine 1988b). Interestingly, this is commonly seen in most gregarine taxa. Marine species are restricted to just three heteroxenous families, Haemogregarinidae, Dactylosomatidae, and Adeleidae (Perkins et al. 2000). Historically many species lumped into the haemogregarines were incompletely described often solely from the vertebrate intermediate host species that they were isolated (reviewed in Siddall 1995, Perkins et al. 2000). In an attempt to clarify the taxonomy of this

group of apicomplexans, Siddall and colleagues (1995) used morphological and life-cycle characters to determine possible evolutionary relationships. Therefore, species have been rearranged among different genera, some of which may still be in flux (e.g. Smit et al. 2003, Davies et al. 2004). All marine haemogregarines infect erythrocytes from a wide range of fish hosts (e.g. Khan 1978, Paperna 1981, Davies et al. 2004) and require leeches (Siddall & Desser 1992, Siddall & Burreson 1994) or parasitic copepods (Davies et al. 1994, Davies et al. 2004) for sexual reproduction. Within Dactylosomatidae, species also have been found inside red blood cells of marine fish (Saunders 1960). This group also requires a leech definitive host for fulfillment of its life-cycle (Barta & Desser 1989). The final family, Adeleidae, contains a single genus infecting marine bivalves and gastropods (Buchanan 1979).

The Eimeriorina encompass seven families, but the majority of marine taxa reside within just three: Aggregatidae, Eimeriidae, and Calyptosporidae (Perkins et al. 2000). The Aggregatidae are mostly heteroxenous and have many sporocysts –containing the infective sporozoites- within the oocysts, which differentiates them from the Eimeriidae and Calyptosporidae. Cephalopods and crustaceans are the definitive and intermediate hosts, respectively (Pixell-Goodrich 1914, Sardella & Martorelli 1997, Gestal et al. 2002a). These species develop intracellularly inside gastro-intestinal tissues of both hosts (Sardella & Martorelli 1997, Gestal et al. 2002a, Gestal et al. 2005). The homoxenous species infect echiurid annelids (MacKinnon & Ray 1937), ascidian urochordates (Harant 1931) and mollusks (Buchanan 1979). The Eimeridae consists of many genera that are almost completely homoxenous (Perkins et al. 2000). A vast number of marine fish parasites were historically described within the genus *Eimeria* (Dyková & Lom 1981, 1983), but differences in the oocyst ultrastructure have sub-divided some species into the genus *Goussia* (Dyková & Lom 1981,

Gestal & Azevedo 2006). Although this split has not been accepted by all (Duszynski et al. 1998, Perkins et al. 2000), it is corroborated by molecular evidence in 18S rDNA phylogenies, albeit *Goussia* appears paraphyletic (Jirku et al. 2002, Jirku et al. 2009). *Eimeria* spp. have been discovered in the testes of menhaden (Hardcastle 1944), air bladder of haddock (Morrison et al. 1993), and the digestive tract of rays (Lom & Dyková 1981, Upton et al. 1986, Upton et al. 1988), eels (Hine 1975, Lom & Dyková 1995), pipefish (Upton et al. 2000), and other marine fishes (Lom & Dyková 1981, Diouf & Toguebaye 1994, Lom & Dyková 1995). In addition, this genus has been isolated from enteropneust hemichordates (Léger & Duboscq 1917, Fernandez & Benito 1983, Fernandez et al. 1989) as well as marine mammals and birds (Upton et al. 1989, McClelland 1993, Golemansky 2011). *Goussia* spp. have also been isolated from the liver (Costa & MacKenzie 1994), swim bladder (Levine 1983) spleen (Jones 1990), kidneys (Morrison & Poynton 1989) and intestines (Alvarez-Pellitero et al. 1997) of several species of marine fishes. Other eimeriids have been discovered inside eggs of echiurid worms (MacKinnon & Ray 1929, 1937), the gut of polychaetes worms (Ray 1930b) and priapulids (McLean 1984, Levine 1985), and the kidneys of mollusks (Friedman et al. 1993, Friedman et al. 1995).

The final family, Calyptosporidae, was split from Eimeriidae after it was determined that members of this group were heteroxenous (Overstreet et al. 1984). For example, *Calyptospora funduli* infect several definitive atheriniform fish hosts (Fournie & Overstreet 1993), residing within the liver and pancreas cells (Duszynski et al. 1979). For completion of their life-cycle, oocysts need to be ingested by palemonid shrimps, where the infective sporozoites are released (Fournie & Overstreet 1983, Fournie et al. 2000). Although few species exist within this family, it has been suggested that several other species within Eimeriidae should belong as they likely

have undiscovered intermediate hosts (Overstreet 1981, Overstreet et al. 1984). However, without more substantiated results, this group remains small (Perkins et al. 2000).

Three additional groups (orders) exist within the Coccidiasina: Agamococcidiorida, Ixoheorida, and Protococcidiorida (Levine 1988b). Although most coccidians consist of a life-cycle containing three stages – gametogony, schizogony, and sporogony – these groups are defined by the lack of one or more stages (Levine 1988b, Perkins et al. 2000). However, all of these orders consist almost exclusively of marine taxa and warrant consideration. Agamococcidiorida, distinguished by the absence of gametogony and schizogony, contain two gerena and few species (Levine 1979a, Upton & Peters 1986). *Rhytidocytis* spp. infect the gastrointestinal tract of polychaetes worms (Leander & Ramey 2006, Rueckert & Leander 2009b) and *Gemmocystis cylindrus* was discovered in the digestive tissues of scleractinian corals (Peters 1984, Upton & Peters 1986). Within Ixoheorida, there is only one species, *Ixorheis psychroptae* (Levine 1984). *I. psychroptae* is missing the gametogony stage and infects blood vessels and the gastrointestinal system of a deep-sea sea cucumber (Massin et al. 1978). The Protococcidiorida is larger, composed of four families and seven genera (Perkins et al. 2000). Members of this order lack the asexual replicative phase of schizogony. These species infect the intestines and coelomic cavities of marine polychaetes (e.g. Chatton & Villenueve 1936b, Ganapati 1941, 1952, Henneré 1967, Porchet-Henneré 1967, Levine 1973a).

Aconoidasida taxa:

As the name implies, members of this group lack a conoid in the apical complex and are heteroxenous (Perkins et al. 2000, Adl et al. 2005). This clade contains the haemosporidians and

the piroplasms, infamously encompassing the causative agents of malaria and babesiosis, respectively. Although this group harbors ~1500 named species and has been intensely studied, almost all infect terrestrial species and no members of Haemosporida inhabit strictly marine hosts (Levine 1988c). However, *Plasmodium* and *Haemoproteus* spp. have been recovered from the blood of three species of seabirds (Quillfeldt et al. 2010).

Several species within the genus *Haemohormidium* have been categorized as “piroplasms” inside fish erythrocytes (So 1972, Davies 1980) with leeches hypothesized as the intermediate host (reviewed in Molnár 1995). This taxonomic position has been considered tenuous, because micropores and invasion organelles associated with an apical complex were not observed in some parasites inside host erythrocytes (Siddall et al. 1994). In addition to ultrastructure differences, Siddall and colleagues (1994) argue that the absence of schizogony and the ability of fish in captivity to remain infected without intermediate hosts should invalidate their placement and perhaps remove them from Apicomplexa altogether. Although ongoing debate remains involving this group of parasites (Davies et al. 2003), piroplasms have been discovered in an ascidian urochordate as well (Van Gaver & Stephan 1907, Ciancio et al. 2008). *Cardiosporidium cionae* was isolated from the pericardial body of the common marine tunicate, *Ciona intestinalis*. In this study, ultrastructure and molecular phlogeny of 18S rDNA group this apicomplexan within other piroplasms (Ciancio et al. 2008). The definitive host remains unknown. Finally, molecular evidence has placed the enigmatic symbiont, *Nephromyces*, within Apicomplexa sister to *C. cionae* (Saffo et al. 2010). This symbiont, which was initially described as a fungus, is an intriguing addition to the apicomplexan lineage as it appears to be a mutualist breaking down nitrogenous waste in tunicate host renal glands (Saffo 1988, Saffo et al. 2010). To our knowledge, this would be the first record of a beneficial (i.e. mutualisitic) apicomplexan.

Marine Hosts of Apicomplexans:

There are ~35 recognized animal phyla of which almost all (~33) are present in the marine environment (Ruppert et al. 2004). Apicomplexans have been found in at least 15 of these including some of the most speciose (Table 2): Cnidaria (Upton & Peters 1986), Mollusca (Azevedo & Padovan 2004), Annelida (Schrével 1971), Arthropoda (Prasadan & Janardanan 2001), Echinodermata (Jangoux 1987), and Chordata (Dyková & Lom 1981). They also have been found associated with Platyhelminthes (Cannon & Jennings 1988), crustaceans (Davies et al. 2004) and Hirudinea (Siddall & Desser 1993) as hyperparasites. Oocysts can even be taken up by ameobae and rotifers, which may be utilized as reservoir hosts (Fayer et al. 2000, Gómez-Couso et al. 2007, Winiecka-Krusnell et al. 2009).

Infection and Host Preference:

Each generation, apicomplexans must find a suitable host and have evolved to facilitate transmission. In homoxenous species, juvenile hosts have to be infected each generation. The aseptate eugregarine, *Diplauxis hatti*, has synchronized the timing of its life-cycle with its host, the polychaete worm *Perinereis cultrifera* (Prensier et al. 2008). Inside the worm, *D. hatti* remains paired in syzygy for over two years before maturing into oocysts and sticking to the gelatinous coat of host eggs, which the larval worm ingests upon hatching (Prensier et al. 2008). Other species rely on transmission via the water column. The uptake of viable oocysts can occur with frequent inhalation of seawater for respiration and sustenance (Pixell-Goodrich 1929, e.g.

Miller et al. 2005). It has been hypothesized that oral consumption of oocysts, necrotic fish, or fecal matter infects marine fish (Hardcastle 1944, Molnár 1995, Paperna 1995, Sitjà-Bobadilla et al. 2005). For example, fish fed intraspecific stomach tissue infected with *Cryptosporidium molnari* developed infection in all individuals (Sitjà-Bobadilla & Alvarez-Pellitero 2003). In captivity, oocysts of *Pseudoklossia haliotis* from abalone can be transferred to uninfected individuals via the water column as well (Friedman et al. 1993).

Other groups of Apicomplexa have utilized a second host species to ensure infection. Blood parasites are often transmitted by hematophagous intermediates, such as leeches and crustaceans (Khan 1980, Siddall & Burreson 1994, Davies et al. 2004). In addition, apicomplexans exploit prey/predator cycles. For example, oocysts of *Nematopsis* spp. (a septate eugregarine) are found in the gills, mantle, and the adductor muscles of bivalves (Landau & Galtsoff 1951, Azevedo & Cachola 1992). This has been hypothesized to weaken the definitive host making it easier to transfer infective stages to predatory crab hosts (Prytherch 1940, but see below, Pathology). Similarly, the oocysts of the eucoccidian genus *Aggregata* can accumulate in cephalopod hosts, which may make the octopus susceptible to predation and disease (Gestal et al. 2002b, Mladineo & Bocina 2007). The intermediate hosts are scavenger crustaceans and would likely ingest the necrotic tissues and fecal matter (Gestal et al. 2002a). A final example involves the eucoccidian, *Calyptospora funduli*, a parasite of killifishes (Overstreet et al. 1984). *C. funduli* can not be transferred directly from fish to fish (Solangi & Overstreet 1980), instead it requires a shrimp host to consume the oocysts (Fournie & Overstreet 1983). Only inside the intermediate host, do the oocysts rupture releasing the infective sporozoites (Fournie et al. 2000). Remarkably, the shrimp hosts harbor viable sporozoites up to 200 days post-infection increasing the probability of predation by killifish (Fournie & Overstreet 1983). Paratenic hosts, which are

not required for the full life-cycle of the apicomplexan, can also be utilized to increase the chances of infection. Sporozoites of the eimeriid *Goussia carpelli* can remain viable inside tubificid oligochaetes at least 57 days before infecting the host carp (Steinhagen & Korting 1990). Likewise, oocysts of another coccidian, *T. gondii*, remained infective to mice following experimental passage through sardine and anchovy hosts (Massie et al. 2010).

In addition, several parasites have increased infection success by increasing suitable host range. For example, the fish haemogregine, *Haemogregarina bigemina*, has been isolated in 96 fish species spanning 34 families (reviewed in Davies et al. 2004). However, the authors admit that cross-infection studies need to be conducted to ensure it is the same species. The abalone parasite, *Pseudoklossia haliotis*, was found in all six western United States *Haliotis* spp. examined (Friedman 1991) and co-infection could occur between some of the species in tank experiments (Friedman et al. 1993). Conversely, *Goussia* (formerly *Eimeria*) *gadi* was found infecting up to 58% of haddock populations off of Nova Scotia, but not in cod, pollock, or hake species (Odense & Logan 1976, but see below, Abundance). This is a bit surprising, as they were originally described in the air bladder of cod and this result may be caused by lower sample sizes of cod (n=90). Laboratory experiments have shown some hosts are not suitable. For example, cuttlefish became infected with *Aggregata eberthi* when fed carrying host shrimps *Palaemon Elegans* and *P. adpersus*, but not closely related *P. serratus* or other crustaceans (Gestal et al. 2002a). *Calyptospora funduli* could only be transferred to the definitive killifish host via palaemonid shrimp vectors, but not penaeid shrimp, amphipods, decopods, or copepods (Fournie & Overstreet 1983). Additionally, when *C. eberthi* was introduced to a non-native host, *Rivulus marmoratus*, degeneration was observed in 95% of the parasites 47 days after infection (Fournie & Overstreet 1993, see below, Pathology). Most gregarines are considered stenoxenous, in which

the parasite can only persist and grow within a few intragenic species (Perkins et al. 2000). This is true of several terrestrial and freshwater eugregarines (Clopton & Gold 1996, Wise et al. 2000, e.g. Reyes-Villanueva et al. 2003, Detwiler & Janovy 2008, Smith & Cook 2008), but cross-infection or molecular studies are needed to confirm in marine systems (Wagenbach et al. 1983).

Biogeography and Transport:

Apicomplexans have been observed in marine hosts near all seven continents (Molnár & Rohde 1988, Landers 1991, Diouf & Toguebaye 1994, Sardella et al. 2000, Ciancio et al. 2001, Tuntiwaranuruk et al. 2004) residing in a wide range of latitudes and habitats. Apicomplexans have been recovered from hosts inhabiting rocky (Dyson et al. 1993, Pomory & Lares 1998) as well as sandy (Buchanan 1979, Landers 2002) intertidal zones, estuaries (Prasadan & Janardanan 2001), and even deep-ocean seeps and vents (Takishita et al. 2007, Gestal et al. 2010). Although commonly found associated with benthic fauna, apicomplexans also infect pelagic hosts (Théodoridès & Desportes 1975, Jones 1990). Species have been isolated from polar (Takahashi et al. 2008), temperate (Costa & MacKenzie 1994), subtropical (Saunders 1960), and tropical waters (Diouf & Toguebaye 1996) worldwide. Therefore, this parasitic group has been highly successful over evolutionary timescales. Although stages of the apicomplexan life-cycle are motile (Baum et al. 2006), transport is almost certainly facilitated.

Motile hosts can increase the geographic range of apicomplexans. One of the most spectacularly successful apicomplexans is the circumglobal blood parasite, *Haemogregarina bigemina*, which has been isolated from many fish hosts (see above) in habitats including coral reefs, open ocean, coastal, and even brackish water (reviewed in Davies et al. 2004). The

eimeriid eucoccidian, *Goussia auxidis*, was found associated with pelagic tuna species across the southern Pacific Ocean (Jones 1990). Although it is likely that large pelagic fish species can transport apicomplexan parasites long distances, this is not restricted to vertebrate hosts. *Cephaloidophora pacifica*, a septate eugregarine, is associated with krill at all sampled locations across the Antarctic Ocean (Takahashi et al. 2004, Takahashi et al. 2008). These gregarines are homoxenous and have utilized the hosts' circumpolar range to expand their own. This trend was also seen in the eucoccidian *Aggregata andresi* that was isolated from the digestive tract of the circumpolar squid, *Martialia hyadesi*. This coccidian was observed in 96% (55/57) of samples collected from the Southwest Atlantic Ocean (Gestal et al. 2005).

In addition, anthropogenic activity has likely facilitated the spread of some apicomplexans. Transport of exotic host species will have unintended consequences regarding their parasite loads. For example, *Sparus aurata*, a Mediterranean fish brought to Mexico for farming recently has been found outside the enclosures (Balart et al. 2009). This fish species is a known vector for *Cryptosporidium molnari*, as well as other parasites (Alvarez-Pellitero & Sitjà-Bobadilla 2002), which may have increased their geographic range. Commercial oysters historically transplanted from the Chesapeake Bay to New York City in the United States were infected with a *Nematopsis* sp. common in the former area, but native oysters were not as of 1950 (Landau & Galtsoff 1951). Apicomplexans also can be spread to marine systems via run-off. As mentioned above, terrestrial species, *Cryptosporidium parvum*, *C. felis*, and *C. andersoni*, have been isolated from mussels on the coast of California, which may serve as reservoirs (Miller et al. 2005). Oocysts of *Cryptosporidium* were still viable and infective after being exposed to seawater for up to 12 weeks (reviewed in Fayer et al. 2004), which indicates that epizootics could occur in marine mammals from run-off. *Toxoplasma gondii* is another terrestrial

apicomplexan found in marine mussels (Miller et al. 2008) as well as marine mammals (reviewed in Fayer et al. 2004). In fact, an autopsy of over 100 California sea otters found dead between 1998 and 2001 revealed that over 60% of deaths were likely caused by *T. gondii* (Conrad et al. 2005). Infections of *T. gondii* and *Sarcocystis neurona* were implicated in another mass-mortality event along the California coast in spring of 2004 (Miller et al. 2010). Therefore, human activities have contributed to the spread of both these potential pathogens.

Abundance and Pathology:

It is not unusual for apicomplexan parasites to be prevalent, especially in filter-feeding hosts (e.g. Ray 1930b, Jones 1990, Takahashi et al. 2004). *Nematopsis* spp. can have infection rates higher than 80% in many commercially important bivalves species from coastal waters near Ecuador (Jiménez et al. 2002), Spain (Azevedo & Cachola 1992), Italy (Canestri-Trotti et al. 2000), Thailand (Tuntiwaranuruk et al. 2004), and the United States (Landau & Galtsoff 1951). High infection prevalence also was seen in the farmed fish, *Sparus aurata*, with over 80% of fish containing *Eimeria* sp. in their intestines (Alvarez-Pellitero et al. 1995). Local populations can differ in relative abundance of parasites. For example, *Goussia gadi* prevalence ranged from 4-50% in two different fishing banks off of Nova Scotia, Canada (Odense & Logan 1976). Strong seasonal and yearly differences in infection rate and severity also were observed in the same sampling area (Odense & Logan 1976, Scott 1981). In the stingray *Urolophus halleri*, prevalence of *Eimeria chollaensis* varied based on the location the host was caught: 52% (26/50) in estuaries and 20% (10/51) in open water (Upton et al. 1988). Finally, age of the host can influence parasite quantities. *Eimeria* sp. were absent in the smallest whiting fish examined (n=50), present in the

intermediate fish size (12% had sporulated oocysts: 9/55), and infected all adults examined (n=308).

It is clear that apicomplexans are prevalent; however, the pathogenicity associated with these infections is less well known. Conflicting observations have been made regarding the infection of mollusk and crustacean hosts with *Nematopsis* species. High infections were observed in the adductor muscles of bivalves, leading to paralysis and failure to close the shell (Prytherch 1940). This genus was observed to cause cell death and degeneration in gill (Tuntiwaranuruk et al. 2004) and gut (Jiménez et al. 2002) cells. *Nematopsis* spp. also were thought to cause slow growth and mortality in farmed shrimp (Fajer-Ávila et al. 2005). However, Landau and Galtsoff (1951) found intensity of infection did not correlate with mortality or visibly affect oyster health. Destruction of host tissues and irregular growth were commonly seen in histological studies of other apicomplexan species (e.g. Solangi & Overstreet 1980, Gestal & Azevedo 2006, Mladineo & Bocina 2007, Gjurcevic et al. 2008), occasionally leading to observable host malaise. The eucoccidian *Aggregata octopiana* inhabits the intestines of octopuses, which led to measurable decreases in food absorption (Gestal et al. 2002b) and muscle protein concentrations correlating with high levels of infection (Gestal et al. 2007) that increases with age (Pascual et al. 2010). Infection by *Eimeria* sp. can lead to loss of 6-10% of the host fish weight (MacKenzie 1981). Haemogregarines even cause anemia in fish hosts with a corresponding 20% reduction of erythrocytes (Kirmse 1978). There is often a host response to infections and lymphocytes and phagocytes are often seen in highly damaged areas (e.g. Costa & MacKenzie 1994, Gestal & Azevedo 2006, Tuntiwaranuruk et al. 2008). Host immune cells are particularily adept at removing non-natural species. For example, *Calyptospora funduli* can destroy up to 85% of the liver and pancreas of definitive host killifish (Solangi & Overstreet

1980, Overstreet 1981). However, in a non-natural host, there is a general inflammatory response and macrophages are associated with degeneration of 50-95% of parasite oocysts (Fournie & Overstreet 1993).

Direct fitness costs of the host are associated with apicomplexans infecting reproductive tissue. In addition, it has been hypothesized that parasitizing host germ cells will lead to optimal fitness of a horizontally acquired symbiont as this will increase host survival and thus parasite transmission (Obrebski 1975, Ebert & Herre 1996). Eimeriorina eucoccidians have been found infecting the testes of male fishes (Hardcastle 1944, Diouf & Toguebaye 1994) and this can lead to complete castration (Molnár 2005). This group of coccidians also parasitize the ovary and eggs of bivalve mollusks (Buchanan 1979) and echiurid annelids (MacKinnon & Ray 1929, 1937). Infection of the eggs leads to degredation of the ovum and a decrease in host fecundity (MacKinnon & Ray 1929, 1937). This was also seen in the aseptate eugregarine, *Gonospora goodrichae*, which infects eggs of the marine annelid, *Arenicola ecaudata* (Goodrich & Pixell-Goodrich 1920). Therefore, both coccidians and gregarines are capable of inhabiting and destroying host reproductive cells.

Conclusions:

Symbiosis has shaped the course of evolution in marine environments, and parasitism has arisen as an extremely successful life history strategy (Windsor 1998). One such group of parasites is the apicomplexans, which are commonly found in association with many different taxa of marine hosts (Levine 1988b, c). Apicomplexans are ubiquitous with prevalence approaching 100% in some hosts and localities (e.g. Fournie & Overstreet 1983, Jones 1990,

Gestal et al. 2005). As apicomplexans can infect many species harvested as food, it is important to understand the pathogenicity inside the hosts, which is often unknown and requires further attention. This effect may be exacerbated when individuals are crowded in aquaculture farms (Kirmse 1980, Alvarez-Pellitero et al. 1995, Fajer-Ávila et al. 2005, Pascual et al. 2006, Gestal et al. 2007). Furthermore, marine epizootics appear to be rising and some may be a consequence of human activities (Harvell et al. 1999, Harvell et al. 2002, Lafferty et al. 2004). Reservoirs of oocysts of human apicomplexan parasites can accumulate in filter-feeding shellfish and even infect marine mammals (Fayer et al. 2004). These newly introduced pathogens can cause significant mortality in some animals (reviewed in Conrad et al. 2005). Therefore, it is important to catalogue existing biodiversity of potential pathogens and prevent any possible new sources from occurring. Finally, it is important to study marine apicomplexans as they may be vital for understanding the evolutionary history of this sub-phylum (Kopecna et al. 2006). As the ancestral types were likely marine (reviewed in Leander & Keeling 2003, Leander 2008), including these organisms may help elucidate phylogenetic information, which has been elusive in this group to date (Morrison 2008).

Table 1: A list of apicomplexan taxonomic groups that infect marine hosts. The taxonomic rankings of Perkins and colleagues were used (2000) with modifications explained in the text.

Apicomplexan Taxon	Host Taxon	Example Citation
Gregarinasina		
Archigregarinorida	**Hemichordata:**	
	enteroptneust	Leger and Duboscq, 1917
	Urochordata:	
	Ascidians	Harant, 1931
	Annelida:	
	Sipuncula	Levine, 1971
	Flabelligera	Levine, 1971
	Terribelliformia	Ray, 1930b
	Spionids	Ganapati, 1946
	Sabellids	Levine, 1971
	Serpulids	Levine, 1976
	Amphinomidae	Bhatia and Setna, 1938
Eugregarinorida		
Blastogregarinorina	**Annelida:**	
	Spionids	Chatton and Villeneuve, 1936a; b
Aseptatorina	**Echinoidermata:**	
	Spatangoids	Pixell-Goodrich, 1915
	Holothuroideans	Pixell-Goodrich, 1925
	Arthropoda:	
	Copopoda	Theodorides and Desportes, 1972
	Decapoda	Theodorides and Desportes, 1972
	Amphipoda	Theodorides and Desportes, 1972
	Mollusca:	
	Gastropods	Tuzet, 1931
	Platyhelminthes:	Cannon and Jennings, 1988
	Urochordata:	
	Ascidians	Rueckert and Leander, 2008
	Chaetognatha:	Levine, 1976
	Nemertea:	Vinckier, 1972
	Annelida:	
	Echiura	Mackinnon and Ray, 1931a
	Sipuncula	Pixell-Goodrich, 1950
	Flabelligera	Levine, 1976
	Terribelliformia	Levine, 1977a
	Phyllodicida	Desportes and Theodorides, 1986

	Eunicida	Bhatia and Setna, 1938
	Spionids	Levine, 1971
	Capitellids	Levine, 1976
	Opheliiidae	Levine, 1977a
Septatorina	**Cnidaria:**	
	Scleractinian Corals	Peters, 1984
	Arthropoda:	
	Cirripedia	Levine, 1988c
	Decapoda	Sprague and Couch, 1971
	Amphipoda	Theodorides and Desportes, 1975
	Mollusca:	
	Gastropods	Azevedo and Padovan, 2004
	Bivalves	Azevedo and Cachola, 1992
	Urochordata:	
	Thaliaceans	Theodorides and Desportes, 1975
	Chaetognatha:	Levine, 1988c
	Annelida:	
	Eunicida	Bhatia and Setna, 1938
Cryptosporidium	**Mollusca:**	
	Bivalves	Miller et al., 2005
	Chordata:	
	Osteichthyes	Alvarez-Pellitero and Sitja-Bobadilla, 2002
	Mammalia	Morgan et al., 2000
	Reptilia	Graczyk et al., 1997
Coccidiasina		
Agamococcidiorida	**Cnidaria:**	
	Scleractinian Corals	Upton and Peters, 1986
	Annelida:	
	Opheliiidae	Levine, 1979b
Ixoreiorida	**Echinoidermata:**	
	Holothuroideans	Massin et al., 1978
Protococcidiorida	**Annelida:**	
	Phyllodicida	Porchet-Hennere, 1967
	Eunicida	Levine, 1973
	Spionids	Chatton and Villeneuve, 1936b
	Sabellids	Hennere, 1967
Eucoccodiorida		
Adeleorina	**Arthropoda:**	
	Isopoda	Davies et al., 2004
	Mollusca:	
	Gastropods	Levine, 1988c
	Bivalves	Buchanan, 1979

	Annelida:	
	Hirudinea	Siddall, 1995
	Chordata:	
	Chondrichthyes	Diouf and Toguebaye, 1994
	Osteichthyes	Davies et al., 2004
Eimeriorina	**Arthropoda:**	
	Decapoda	Sardella and Martorelli, 1997
	Mollusca:	
	Cephalopods	Pixell-Goodrich, 1914
	Gastropods	Levine, 1988c
	Bivalves	Friedman et al., 1995
	Hemichordata:	
	enteropneusts	Leger and Duboscq, 1917
	Urochordata:	
	Ascidians	Harant, 1931
	Priapulida:	McLean, 1984
	Annelida:	
	Echiura	Mackinnon and Ray, 1937
	Phyllodicida	Levine, 1983
	Spionids	Ray, 1930a
	Chordata:	
	Chondrichthyes	Dykova and Lom, 1983
	Osteichthyes	Dykova and Lom, 1983
	Mammalia	Upton et al., 1989
Piroplamorida	**Urochordata:**	
	Ascidians	Cianco et al., 2008
	Chordata:	
	Osteichthyes	Siddall et al., 1994

Table 2: Identity of apicomplexan genera infecting marine hosts. Host location, habitat, tissue preference (Location in host) and climate is annotated.

Host Taxon	Apicomplexa Taxon	Species	Geographic Location	Host Habitat	Location in Host	Citation
Invertebrates						
Cnidaria:						
Scleractinian Corals	Agamococcidian	*Gemmocystis cylindrus*	Caribbean Sea	Tropical	Gastroderm	Upton and Peters, 1986
	Septate Eugregarine	*Nematopsis sp.*	Caribbean Sea	Tropical	Calicodermis	Peters, 1984
Echinoidermata:						
Spatangoids	Aseptate Eugregarine	*Lithocystis spp.*	North Sea/ English Channel/ NE Pacific	Temperate	Coelomic/ Intrahemal	Pixell-Goodrich, 1915; Theodorides and Laird, 1970;Coulon and Jangoux, 1987
	Aseptate Eugregarine	*Urospora spp.*	North Sea/ Mediterranean Sea/ English Channel	Temperate	Coelomic/ Intrahemal	Coulon and Jangoux, 1987; Pixell-Goodrich, 1915
Holothuroideans	Aseptate Eugregarine	*Urospora* spp.	NW Atlantic/ Mediterranean Sea/ Sea of Japan	Temperate/ Subtropical	Blood/ Coelom/ Gastrointestinal	Pixell-Goodrich, 1925; Levine, 1977a
	Aseptate Eugregarine	*Gonospora* spp.	English Channel/ Barents Sea	Arctic/ Temperate	Blood Vessels/ Gonads/ Coelom	Minchin, 1893; Levine, 1977a
	Aseptate Eugregarine	*Lithocystis spp.*	NW Atlantic, English Channel	Temperate	Coelomic/ Gastrointestinal/ Respiratory Tree	Pixell-Goodrich, 1925; Levine, 1977a
	Ixoreiorida coccidian	*Ixorheis psychropotae*	NE Atlantic	Temperate (4000 m)	Blood Vessels/ Coelom	Massin et al., 1978
Arthropoda:						
Crustecea:						
Cirripedia	Septate Eugregarine	*Cirrigregarina* spp.	NE Pacific/ Sea of Japan	Temperate/ Subtropical	Gastrointestinal	Levine 1979a
	Septate Eugregarine	*Cephaloidophora* spp.	NE Pacific	Temperate	Gastrointestinal	Levine, 1988c
	Septate Eugregarine	*Pyxiniodes* spp.	NW Atlantic/ NE Pacific/	Temperate/ Subtropical	Gastrointestinal	Theodorides and Laird, 1970;

			Sea of Japan			Levine 1988c
	Septate Eugregarine	*Nematoides fusiformis*	Mediterranean Sea	Temperate	Gastrointestinal	Levine, 1988c
	Septate Eugregarine	*Bifilida rara*	Europe	Temperate	Gastrointestinal	Tuzet and Ormieres, 1964
Copopoda	Aseptate Eugregarine	*Paraophioidina* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1972; Levine, 1977b
	Aseptate Eugregarine	*Cephaloidophora petiti*	Mediterranean Sea	Temperate	Gastrointestinal	Levine, 1988c
Malacostraca						
Decapoda	Aseptate Eugregarine	*Paraophioidina* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1972; Levine, 1977b
	Septate Eugregarine	*Cephalolobus penaeus*	Caribbean Sea/ Mediterranean Sea	Temperate/ Tropical	Gastrointestinal	Sprague and Couch, 1971; Chavez-Sanchez, 2002
	Septate Eugregarine	*Nematopsis* spp.	Caribbean Sea/ Sea of Japan/ Mediterranean Sea/ NE Pacific/ NW Atlantic	Temperate/ Subtropical/ Tropical	Gastrointestinal	Chavez-Sanchez, 2002
	Septate Eugregarine	*Porospora* spp.	English Channel/ NW Atlantic	Temperate	Gastrointestinal	Sprague and Couch, 1971; Theodorides and Laird, 1970
	Septate Eugregarine	*Pachyporospora* spp.	Mediterranean Sea/ Sea of Japan	Temperate/ Subtropical	Gastrointestinal	Sprague and Couch, 1971
	Septate Eugregarine	*Cephaloidophora* spp.	Mediterranean Sea/ Arabian Sea/ Southern Ocean	Antarctic/ Temperate/ Tropical	Gastrointestinal	Ball, 1959; Theodorides and Desportes, 1975; Takahashi et al., 2008
	Septate	*Caridohabitans*	Arabian	Tropical	Gastrointesti	Ball, 1959;

	Eugregarine	spp.	Sea		nal	Levine, 1988c
	Eimeriorina	*Selenococcidium intermedium*	Atlantic Ocean	Temperate	Gastrointestinal	Perkins et al., 2000
	Eimeriorina	*Aggregata spp.*	SW Atlantic/ Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1975; Sardella and Martorelli, 1997
Amphipoda	Aseptate Eugregarine	*Lateroprotomeritus conicus*	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1976
	Aseptate Eugregarine	*Paraophioidina* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1972; Levine, 1977b
	Septate Eugregarine	*Callyntrochlamys phronimae*	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1975
	Septate Eugregarine	*Cephalolobus* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1975
	Septate Eugregarine	*Uradiophora* spp.	NW Atlantic	Temperate	Gastrointestinal	Theodorides and Laird, 1970
	Septate Eugregarine	*Nematopsis* spp.			Gastrointestinal	Prasadan and Janardanan, 2001
	Septate Eugregarine	*Cephaloidophora* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1975; Levine, 1988c
	Septate Eugregarine	*Rotundula* spp.	English Channel	Temperate	Gastrointestinal	Pixell-Goodrich, 1949; Levine 1988c
	Septate Eugregarine					
Isopoda	Adeleid Eucoccidian	*Haemogregarina* spp.	Global	Global	Blood Cells	Davies et al., 2004
Mollusca:						
Cephalopods	Eimeriorina	*Aggregata* spp.	SW Atlantic/ NE	Temperate	Gastrointestinal/ Gills	Pixell-Goodrich, 1914;

			Atlantic/ English Channel/ Mediterranean Sea			Sardella et al., 2000; Pascual et al., 2006
Gastropods	Aseptate Eugregarine	*Digyalum owenii*	North Sea/NW Atlantic	Temperate	Gastrointestinal	Dyson et al., 1993
	Aseptate Eugregarine	*Gonospora* spp.	Mediterranean Sea	Temperate	Testes	Tuzet, 1931
	Septate Eugregarine	*Nematopsis* spp.	SW Atlantic	Tropical	Phagocytes in Mantle	Azevedo and Padovan, 2004
	Adeleid Eucoccidian	*Klossia* spp.	NE Atlantic	Tropical	Renal Tissue	Levine, 1988c
	Eimeriorina	*Merocystis kathae*	Europe	Temperate	Kidney	Levine, 1988c
Bivalves	Septate Eugregarine	*Nematopsis* spp.	Gulf of Thailand/ Mediterranean Sea/ NE Atlantic	Temperate/ Tropical	Gills	Azevedo and Cachola, 1992; Tuntiwaranuruk et al., 2004
	Adeleid Eucoccidian	*Klossia* spp.	NE Atlantic/ North Sea	Temperate	Renal Tissue	Buchanan, 1979
	Cryptosporidium	*Cryptosporidium* sp.	NE Pacific	Temperate	Haemolymph in adductor muscle	Miller et al., 2005
	Eimeriorina	*Pseudoklossia* spp.	NE Pacific/ NE Atlantic/ North Sea	Temperate	Ovary/ Kidney	Buchanan, 1979; Levine, 1988c; Friedman et al., 1995
Platyhelminthes:	Aseptate Eugregarine	*Monocystella* spp.	Mediterranean Sea/ Coral Sea	Temperate/ Tropical	Gastrointestinal	Levine, 1977b; Cannon and Jennings, 1988
Hemichordata:						
enteropneusts	Archigregarine	*Selenidium metchnikovi*	Mediterranean Sea	Temperate	Gastrointestinal	Leger and Duboscq, 1917
	Eimeriorina	*Eimeria* spp.	NE Atlantic Ocean/ Mediterranean Sea	Temperate	Hepatic caeca/ Epithelial cells	Leger and Duboscq, 1917; Fernandez et al., 1989
Urochordata:						

Ascidians	Archigregarine	*Selenidium giganteum*	Mediterranean Sea	Temperate	Gastrointestinal	Harant, 1931
	Archigregarine	*Merogregarina amaroucii*	English Channel/ Tasmanian Sea	Temperate/ Subtropical	Gastrointestinal	Porter, 1908; Harant, 1931
	Aseptate Eugregarine	*Lankesteria spp.*	English Channel/ Mediterranean Sea/ Sea of Japan/ NE Pacific	Temperate/ Subtropical	Gastrointestinal	Levine, 1977b; Cianco et al., 2001; Rueckert and Leander, 2008
	Eimeriorina	*Selysina* spp.	English Channel/ Mediterranean Sea	Temperate	Gastrointestinal/ Epidermis	Harant, 1931
	Piroplasm	*Cardiosporidium cionae*	Mediterranean Sea	Temperate	Haemocytes	Cianco et al., 2008
Thaliaceans	Septate Eugregarine	*Thalicola* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Theodorides and Desportes, 1975
Chaetognatha:	Aseptate Eugregarine	*Lecudina leuckartii*	Mediterranean Sea	Temperate	Gastrointestinal	Levine, 1976
	Septate Eugregarine	*Tricystis* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Hamon, 1951; Levine, 1988c
Nemertea:	Aseptate Eugregarine	*Lecudina linei*	North Sea	Temperate	Gastrointestinal	Vinckier, 1972
	Aseptate Eugregarine	*Difficilina* spp.	NE Pacific	Temperate	Gastrointestinal	Rueckert et al., 2010
	Aseptate Eugregarine	*Urospora nemertis*	Mediterranean Sea/ English Channel	Temperate	Gastrointestinal	Levine, 1977a
Annelida:						
Echiura	Aseptate Eugregarine	*Hyperidion thalassemae*	English Channel	Temperate	Gastrointestinal	Mackinnon and Ray, 1931a
	Aseptate Eugregarine	*Hentschelia thalassemae*	English Channel	Temperate	Gastrointestinal	Mackinnon and Ray, 1931b
	Aseptate Eugregarine	*Lecudina fluctus*	Sea of Japan	Subtropical	Gastrointestinal	Levine, 1976
	Aseptate Eugregarine	*Lecythion thalassemae*	English Channel	Temperate	Gastrointestinal	Mackinnon and Ray, 1931b
	Aseptate Eugregarine	*Zygosoma* spp.	NE Pacific Ocean/ Meriterranean Sea	Temperate	Gastrointestinal	Noble, 1938; Levine, 1977b
	Aseptate	*Ophiodina*	Mediterran	Temperate	Gastrointesti	Levine,

	Eugregarine	*bonelliae*	ean Sea		nal	1977b
	Aseptate Eugregarine	*Echiurocystis spp.*	NE Pacific Ocean	Temperate	Gastrointestinal	Noble, 1938
	Eimeriorina	*Ovivora thalassemae*	English Channel	Temperate	Gonads	Mackinnon and Ray, 1937
Sipuncula	Archigregarine	*Selenidium* spp.	NE Atlantic/ Sea of Japan/ NE Pacific	Temperate	Gastrointestinal	Levine, 1971; Leander, 2006
	Aseptate Eugregarine	*Lithocystis lankesteri*	Mediterranean Sea	Temperate	Coelom	Pixell-Goodrich, 1950
	Aseptate Eugregarine	*Urospora* spp.	Mediterranean Sea	Temperate	Coelom	Pixell-Goodrich, 1950
	Aseptate Eugregarine	*Filipodium* spp.	Caribbean Sea/ Sea of Japan/ Mediterranean Sea	Subtropical/ Tropical/ Temperate	Gastrointestinal	Levine, 1977b
	Aseptate Eugregarine	*Extremocystis dendrostomi*	Indian Ocean	Tropical	Coelom	Levine, 1977b
Flabelligera	Archigregarine	*Selenidium* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Levine, 1971
	Aseptate Eugregarine	*Lecudina zimmeri*	NE Pacific Ocean	Temperate	Gastrointestinal	Levine, 1976
Terribelliformia	Archigregarine	*Selenidium* spp.	English Channel	Temperate	Gastrointestinal	Ray, 1930b
	Aseptate Eugregarine	*Gonospora varia*	English Channel	Temperate	Coelom	Levine, 1977a
	Aseptate Eugregarine	*Paragonospora typica*	North Sea	Temperate	Coelom	Levine, 1977a
Phyllodicida	Aseptate Eugregarine	*Cygnicollum lankesteri*	Mediterranean Sea/ Iles Crozet	Temperate	Gastrointestinal	Desportes and Theodorides, 1986
	Aseptate Eugregarine	*Lecudina* spp.	Sea of Japan/ English Channel/ Indian Ocean/ NW Atlantic/ Mediterranean Sea	Temperate/ Subtropical/ Tropical	Gastrointestinal	Theodorides and Laird, 1970; Levine, 1976
	Aseptate Eugregarine	*Ulivina* spp.	SW Atlantic Ocean	Tropical	Gastrointestinal	Levine, 1977b
	Aseptate Eugregarine	*Diplauxis* spp.	Mediterranean Sea	Temperate	Coelom	Porchet-Hennere and Fischer, 1973;

						Levine, 1977b
	Aseptate Eugregarine	*Gonospora* spp.	Mediterranean Sea/ Sea of Japan	Temperate/ Subtropical	Coelom	Pixell-Goodrich, 1916; Levine, 1977a
	Aseptate Eugregarine	*Ceratospora mirabilis*	France	Temperate	Coelom	Levine, 1977a
	Protococcidian	*Coelotropha* spp.	France	Temperate	Coelom	Porchet-Hennere, 1967
	Agamococcidian	*Rhytidocystis sthenelais*	NE Atlantic/ English Channel	Temperate	Coelom	Levine, 1979b
	Eimeriorina	*Defretinella eulaliae*	English Channel	Temperate	Epidermis	Levine, 1983
Eunicida	Aseptate Eugregarine	*Lecudina* spp.	Sea of Japan/ Indian Ocean/ Mediterranean Sea/ English Channel, North Sea	Temperate/ Subtropical/ Tropical	Gastrointestinal	Bhatia and Setna, 1938; Schrevel, 1963; Levine, 1976
	Aseptate Eugregarine	*Ulivina eunicae*	Andaman Sea	Tropical	Gastrointestinal	Bhatia and Setna, 1938; Levine, 1977b
	Aseptate Eugregarine	*Bhatiella* spp.	Andaman Sea/ Sea of Japan	Tropical	Gastrointestinal	Levine, 1977b
	Aseptate Eugregarine	*Viviera marphysae*	English Channel	Temperate	Gastrointestinal	Levine, 1988c
	Aseptate Eugregarine	*Cochleomeritus* spp.	NE Pacific Ocean/ Sea of Japan	Temperate/ Subtropical	Gastrointestinal	Levine, 1977b
	Aseptate Eugregarine	*Contortiocorpa prashadi*	Andaman Sea	Tropical	Gastrointestinal	Levine, 1977b
	Aseptate Eugregarine	*Stomatophora primitiva*	Andaman Sea	Tropical	Gastrointestinal	Bhatia and Setna, 1938
	Septate Eugregarine	*Deuteromera cleava*	Andaman Sea	Tropical	Gastrointestinal	Bhatia and Setna, 1938
	Protococcidian	Grellia ophryotrochae	Mediterranean Sea	Temperate	Coelom	Levine, 1973
Spionids	Archigregarine	*Selenidium* spp.	Bay of Bengal	Tropical	Gastrointestinal	Ganapati, 1946; Levine, 1971
	Blastogregarine	*Siedleckia* spp.	English Channel/ Bay of Bengal	Temperate/ Tropical	Gastrointestinal	Chatton and Villeneuve, 1936a; b

	Aseptate Eugregarine	*Lecudina bogolepova*	Sea of Japan	Subtropical	Gastrointestinal	Levine, 1976
	Aseptate Eugregarine	*Ulivina* spp.	Mediterranean Sea	Temperate	Gastrointestinal	Levine, 1977b
	Aseptate Eugregarine	*Selenocystis foliata*	English Channel	Temperate	Gastrointestinal	Dibb, 1938
	Aseptate Eugregarine	*Polyrhabdina* spp.	English Channel/ Indian Ocean	Temperate/ Tropical	Gastrointestinal	Mackinnon and Ray, 1931b; Ganapati, 1946
	Aseptate Eugregarine	*Sycia* spp.	Sea of Japan/ Mediterranean Sea/ Bay of Bengal	Subtropical/Temperate	Gastrointestinal	Ganapti, 1946; Levine, 1977b
	Aseptate Eugregarine	*Ditrypanocystis* spp.	English Channel/ North Sea	Temperate	Gastrointestinal	Levine, 1971
	Aseptate Eugregarine	*Urospora* spp.	Mediterranean Sea	Temperate	Unknown	Levine, 1977a
	Aseptate Eugregarine	*Gonospora varia*	English Channel	Temperate	Coelom	Levine, 1977a
	Protococcidian	*Eleutheroschizon dubosqui*	English Channel	Temperate	Gastrointestinal	Chatton and Villeneuve, 1936b
	Protococcidian	*Myriospora* spp.	Bay of Bengal	Tropical	Coelom/ Gastrointestinal	Ganapati, 1941; Ganapati, 1952
	Eimeriorina	*Dorisiella scolelepidis*	English Channel	Temperate	Gastrointestinal	Ray, 1930a
Sabellids	Archigregarine	*Selenidium* spp.	NE Atlantic/ NE Pacific	Temperate	Gastrointestinal	Levine, 1971; Ray, 1930b; Leander, 2007
	Protococcidian	*Myriosporides amphiglenae*	English Channel/ Mediterranean Sea	Temperate	Coelom	Hennere, 1967
Serpulids	Archigregarine	*Selenidium* spp.	English Channel/ Mediterranean Sea	Temperate	Gastrointestinal	Levine, 1971
Capitellids	Aseptate Eugregarine	*Lecudina capitellae*	SW Atlantic Ocean	Tropical	Gastrointestinal	Levine, 1976
	Aseptate Eugregarine	*Ancora* spp.	SW Atlantic Ocean/ Mediterranean Sea/ Bay of	Tropical/ Temperate	Gastrointestinal	Ganapati, 1946; Levine, 1977b

			Bengal			
	Aseptate Eugregarine	*Gonospora goodrichae*	English Channel	Temperate	Coelom/ eggs	Goodrich and Pixell-Goodrich, 1920
	Aseptate Eugregarine	*Pterospora* spp.	English Channel/ NW Atlantic/ Barents Sea/ Caribbean Sea/ NE Pacific	Arctic/ Temperate	Coelom	Theodorides and Laird, 1970; Levine, 1977a; Landers, 2002; Leander et al., 2006
Opheliiidae	Aseptate Eugregarine	*Urospora travisiae*	North Sea	Temperate	Coelom	Levine, 1977a
	Agamococci dian	*Rhytidocystis* spp.	NE Atlantic/ NE Pacific/ English Channel	Temperate	Coelom	Levine, 1979b; Rueckert and Leander, 2009b
Amphinomida e	Archigregari ne	*Selenidium amphinomi*	Andaman Sea	Tropical	Coelom	Bhatia and Setna, 1938
Hirudinea	Adeleid Eucoccidian	*Desseria* spp.			Gastrointesti nal	Siddall, 1995
	Adeleid Eucoccidian	*Haemogregari na* spp.	Bering Sea	Arctic	Gastrointesti nal	Siddall and Burreson, 1994
unknown?	Protococcidia n	*Grellia* spp.	Mediterran ean Sea	Temperate	Coelom	Levine, 1973b
	Agamococci dian	*Rhytidocystis polygordiae*	NE Pacific Ocean	Temperate	Gastrointesti nal	Leander and Ramey, 2006
Priapulida:	Eimeriorina	*Pfeifferinella macrocoronata*	NE Pacific Ocean/ North Sea/ White Sea	Temperate	Gastrointesti nal	McLean, 1984; Levine, 1985; Duszynski et al., 1998
Chordata:						
Vertebrates						
Chondrichthy es	Eimeriorina	*Eimeria spp.*	Caribbean Sea/ NE Atlantic/ Indian Ocean/ English Channel/ Mediterran ean Sea	Subtropical/ Tropical	Gastrointesti nal	Lom and Dykova, 1983; Upton et al., 1986; Upton et al., 1988; Diouf and Toguebaye, 1994
	Eimeriorina	*Goussia lucida*	Atlantic Ocean/ Mediterran ean Sea	Temperate/ Subtropical	Gastrointesti nal	Dykova and Lom, 1983

	Adeleid Eucoccidian	*Haemogregarina* spp.	NW Atlantic Ocean	Temperate	Blood Cells	So, 1972
Osteichthyes	Adeleid Eucoccidian	*Haemogregarina* spp.	Global	Temperate/ Subtropical/ Tropical	Blood Cells	Davies et al., 2004
	Adeleid Eucoccidian	*Cyrilia uncinata*	NW Atlantic Ocean	Temperate	Blood Cells	Khan, 1978
	Adeleid Eucoccidian	*Desseria* spp.	SE Atlantic	Subtropical	Blood Cells	Siddall, 1995; Smit and Davies, 2006
	Cryptosporidium	*Cryptosporidium molnari*	Mediterranean Sea/ NE Atlantic	Temperate	Gastrointestinal	Alvarez-Pellitero and Sitja-Bobadilla, 2002
	Adeleid Eucoccidian	*Dactylosoma* spp.	Red Sea/ Indian Ocean	Subtropical	Blood Cells	Saunders, 1960; Levine, 1988c
	Adeleid Eucoccidian	*Babesiosoma* spp.	NW Atlantic Ocean/ Red Sea	Temperate/ Subtropical	Blood Cells	Saunders, 1960; Paperna, 1981
	Eimeriorina	*Eimeria* spp.	Global	Temperate/ Subtropical/ Tropical	Gastrointestinal/ Testes/ Liver/ Kidney	Dykova and Lom, 1983; Lom and Dykova, 1995; Diouf and Toguebaye, 1994
	Eimeriorina	*Goussia* spp.	Sea of Japan/ N Atlantic Ocean/ Mediterranean Sea/ Caribbean Sea/ Tasman Sea/ Baltic Sea	Subtropical/ Tropical	Gastrointestinal/ Liver/ Swim bladder	Levine, 1983; Upton et al., 1989; Lom and Dykova, 1995; Gestal and Azevedo, 2006
	Eimeriorina	*Calyptospora* spp.	Gulf of Mexico, SW Atlantic	Subtropical	Liver/ Hepatocytes	Overstreet et al., 1984
	Piroplasm	*Haemohormidium* spp.	NW and NE Atlantic Ocean/ Southern Ocean	Temperate/ Subtropical	Blood Cells	Khan, 1980; Siddall et al., 1994; Davies et al., 2003

Mammalia	Sarcocystidae	*Toxoplasma gondii*	North-Eastern Pacific	Temperate	Gastrointestinal	Millet et al., 2008
	Cryptosporidium	*Cryptosporidium* sp.	South-Western Pacific	Subtropical	Gastrointestinal	Morgan et al., 2000
	Eimeriorina	*Eimeria* spp.	NW Atantic Ocean/ Caribbean Sea	Temperate/ Subtropical	Gastrointestinal	Upton et al., 1989, McClelland, 1993
Reptilia	Cryptosporidium	*Cryptosporidium* sp.	Hawaii	Tropical	Gastrointestinal	Graczyk et al., 1997

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Chapter 3. Persistent Associations Between Apicomplexan Symbionts and Caribbean Reef Corals

Abstract Although apicomplexans are a widely recognized and important parasitic group, little is known about those associated with invertebrates, such as reef-building scleractinian corals. To resolve the potential impact of apicomplexans on coral health, it is first necessary to further describe this group of putative parasites and determine their prevalence among host species. Here, it was hypothesized that apicomplexan prevalence would increase in summer and decrease in winter, similar to what occurs in other marine apicomplexans as well as some coral symbionts. To test this, samples were examined from the Caribbean scleractinian species *Porites astreoides*, *Montastraea annularis*, *M. faveolata*, and *Siderastrea siderea* that had been collected seasonally from two reefs each in the Florida Keys and the Bahamas over nine and five and a half year periods, respectively. Utilizing a PCR-based screening assay, apicomplexan DNA was detected from most Floridian (80.1%: n = 555/693) and Bahamian (90.7%: n = 311/343) coral tissue samples collected over these multi-year periods. Furthermore, apicomplexan DNA was detected from nearly all (98.7%: n = 78/79) single-polyps sampled at multiple locations within six *M. faveolata* colonies, indicating little to no intracolonial variation in the screening assay. Mixed-model logistic regression was utilized to determine the effects of season, host species, and reef on apicomplexan prevalence. The model identified a significant seasonal effect, with the highest apicomplexan prevalence occurring during winter, contrary to the hypothesis. There also was a large effect of host species, with apicomplexan prevalence significantly lower among *S. siderea* colonies relative to the other species. While reef did not

have a significant effect in the full model, there was a significant difference in apicomplexan prevalence between Floridian and Bahamian reefs for *S. siderea*, implying regional differences in this host species. Despite seasonal and species-specific differences in prevalence, apicomplexans are ubiquitous constituents of these particular scleractinian coral species from Florida and the Bahamas.

Introduction

Tropical coral reefs are a highly productive ecosystem occurring in oligotrophic waters. The physiology and metabolism of scleractinian corals, the foundation of this ecosystem, is largely dependent on symbioses with dinoflagellates in the genus *Symbiodinium* (reviewed in Yellowlees et al. 2008). While this symbiosis is well known, many other organisms reside on, or within, coral tissues and over the last 15 years, histological and molecular techniques have detected a wide range of eukaryotic, as well as prokaryotic symbionts (Humes 1985; Rohwer et al. 2002; Toller et al. 2002; Knowlton and Rohwer 2003; Lesser et al. 2004; Wegley et al. 2004; Wilson et al. 2005; Cróquer et al. 2006; Ritchie 2006; Bourne et al. 2008; Qiu et al. 2010). These members of the “holobiont”, which include the coral colony itself and the assemblage of other organisms on and within it (i.e., Archaea, Eubacteria, Protista, etc.), are hypothesized to contribute significantly to host physiology and health (Reshef et al. 2006; Rosenberg et al. 2007). Despite advances in identifying the lesser-known constituents of the holobiont, many remain undescribed and understudied and their interactions with the host are only beginning to be hypothesized. One such group of coral symbionts is the apicomplexans.

Apicomplexans are classified within the eukaryotic supergroup S.A.R., which unites the stramenopiles, alveolates, and rhizaria (Parfrey et al. 2010; Walker et al. 2011), and are the sister group to the dinoflagellates (Cavalier-Smith 1999; Adl et al. 2007; Bachvaroff et al. 2011). Notable apicomplexans include *Plasmodium falciparum*, a causative agent of human malaria (Snow et al. 2005), as well as the opportunistic pathogens *Toxoplasma gondii* and *Cryptosporidium parvum* that can cause severe illness or death in immuno-compromised humans (Kim and Weiss 2004; Wanyiri and Ward 2006). Epidemics caused by apicomplexans affect millions of people and cost billions of dollars in damages (Sachs and Malaney 2002; Abrahamsen et al. 2004; Snow et al. 2005; Alonso 2006; Jones et al. 2007; Khan et al. 2007; Jones et al. 2008b; Snow et al. 2008). Although apicomplexans are an important parasitic group, considerably less is known about their interactions with invertebrates, particularly scleractinian corals. Apicomplexans were first observed in apparently healthy Caribbean corals using light microscopy (Peters 1984; Upton and Peters 1986). Later, it was determined that ~ 90% (n = 52) of examined *Montastraea annularis* colonies harbored apicomplexans according to DNA-based molecular markers (Toller et al. 2002). Therefore, it is plausible that apicomplexans are widespread in a variety of corals (e.g. Toller et al. 2002; Goulet and Coffroth 2004) and infection may not be associated with visible symptoms of disease.

One important aspect of elucidating the nature of coral-apicomplexan interactions is to establish patterns of prevalence (i.e., the percentage of infected host individuals within a sample) and seasonality (i.e., differences in prevalence among seasons) across scleractinians since these generally vary according to the particular host and/or symbiont taxa being examined. For example, seasonal stability in prevalence has been identified for several groups of hosts and their apicomplexans (Smallridge and Bull 2000; Amo et al. 2005; Prokopowicz et al. 2010; Godfrey et

al. 2011). In contrast, seasonal and interannual variation in apicomplexan prevalence has been reported for a number of terrestrial vertebrates and invertebrates (Weatherhead and Bennett 1992; Leinwand et al. 2005; Vezzani and Wisnivesky 2006; Albicócco and Vezzani 2009; Locklin and Vodopich 2010; Schultz et al. 2011; Forbes et al. 2012; Morsy et al. 2012). In marine systems, *Goussia gadi*, an apicomplexan associated with fish, exhibits large differences in prevalence both seasonally and geographically in Canada (Odense and Logan 1976; Scott 1981). Other examples of seasonality include the marine apicomplexans *Cephaloidophora alii* and *Aggregata* sp. that associate with shrimp in the Mediterranean Sea (Théodoridès and Desportes 1975) as well as in an unknown gregarine sp. infecting the American oyster, *Crassostrea virginica*, in the Chesapeake Bay (Sawyer et al. 1973). In this latter example, seasonal prevalence is apparently driven by rapid springtime growth followed by clearing of the symbiont via the host immune response (Sawyer et al. 1973). Other instances of seasonality, which may correlate with temperature, have been observed in aquaculture stocks of mussels and fish (Tuntiwaranuruk et al. 2008; Alvarez-Pellitero et al. 2009). Thus, variation in prevalence due to seasonality, geography and/or host species appears to be characteristic of symbioses involving apicomplexans.

Here, patterns of prevalence and seasonality in scleractinian apicomplexan symbionts were assessed via examination of repeatedly-collected temporal samples of colonies belonging to four Caribbean coral species. These colonies were previously utilized in quantifying seasonal variation in tissue biomass as well as density and genetic identity of their *Symbiodinium* (i.e., mutualistic dinoflagellate) populations (Thornhill et al. 2006a; Thornhill et al. 2006b; Thornhill et al. 2011). Thus, estimates of apicomplexan prevalence can be put into the larger context of tissue biomass, which is indicative of host energy reserves (Fitt et al. 2000; Thornhill et al.

2011), in addition to *Symbiodinium* complement and density. Based on the seasonality patterns observed in other apicomplexan associations, it was hypothesized that apicomplexan prevalence would correlate with seasonal changes in temperature, specifically being higher in summer and lower in winter. Such changes in prevalence have been observed in other members of the coral holobiont, including particular *Symbiodinium*-host combinations (Chen et al. 2005; Jones et al. 2008a; Suwa et al. 2008), microalgal assemblages (Cavada et al. 2011) as well as mutualistic and pathogenic bacteria (Koren and Rosenberg 2006; Chen et al. 2011). Furthermore, previously-collected samples from two reefs each in the Florida Keys and Bahamas were examined towards determining whether general aspects of apicomplexan prevalence among scleractinians were attributable to 1) host species and/or 2) geography.

Materials and Methods

Sample collection

This study took advantage of previously collected samples from individual colonies of four species of Caribbean scleractinian corals (Thornhill et al. 2006a; Thornhill et al. 2006b). All samples utilized in this study were previously collected to determine the "*Symbiodinium*" type present and were preserved in 95% ethanol. The sibling species *Montastraea faveolata* and *M. annularis* were previously sampled from two reefs in the Florida Keys (Little Grecian Reef [LG; 3-4 m depth] and Admiral Patch Reef, [ADM; 1-2 m]) and from two reefs in the Bahamas (South Perry Reef, [SP; 12-15 m] and North Norman's Patch Reef, [NNP; 2-4 m]) (see Fig 1, Thornhill et al. 2009). A third species, *Porites astreoides*, was only collected from ADM and NNP in

Florida and the Bahamas, respectively (Thornhill et al. 2006a). The final species, *Siderastrea siderea*, was collected from both Floridian reefs and at NNP in the Bahamas (Thornhill et al. 2006b). Six tagged colonies of each species were sampled at four different time periods annually (Winter: Jan-Mar, Spring: Apr-Jun, Summer: Jul-Sep, and Fall: Oct-Dec) for a majority of the multi-year study periods in Florida and the Bahamas (Table 1). Due to circumstances, such as inclement weather, precluding complete sampling on an annual basis, the number of seasonal collections ranged from two to four per year. A total of 1,157 samples were examined across all species, reefs, seasons and years.

Along with the above, additional samples of a single scleractinian coral species were examined in order to estimate prevalence among individuals on a reef. Specifically, the sample size of *M. annularis* was increased from 6 to 18 individuals in March (Winter) and 12 individuals during May (Spring) and August (Summer) of 2003 (i.e., timepoints representing midway through the overall study period) at ADM in Florida.

To determine if location within a colony influenced the detectability of apicomplexans, previously-collected samples utilizing the microsampling technique of Kemp et al. (2008) to survey multiple polyps from a single colony at a given time point were screened. For this, two *M. faveolata* colonies from NNP collected in Sep 2005, one from SP in Nov 2005, and three colonies from LG in Aug 2006 were selected. From these collections, 79 were haphazardly selected from the top, middle and bottom of these six colonies to include the widest breadth of single-polyp samples across these colonies.

Testing specificity of PCR primers for apicomplexans

For this study, the presence or absence of apicomplexans in any given sample was ascertained via PCR using primers 18N-F2 and 18N-R1, which specifically target an 860 bp fragment of the small subunit (18S) ribosomal DNA (18S rDNA) (Toller et al. 2002). Prior to being utilized on field samples, primers were first compared to 18S rDNA sequences from closely related protists that associate with scleractinian corals to determine their specificity *in silico*. These comparisons identified seven and ten mismatches between the photosynthetic alveolate, *Chromera velia* (Moore et al. 2008) and the forward and reverse primers, respectively. Additionally, the primers were designed to anneal to the 18S rDNA in apicomplexan-specific insertion-deletion (indel) regions that should preclude amplification of *Symbiodinium* DNA (Toller et al. 2002). Nevertheless, to ensure that 18N-F2 and 18N-R1 did not amplify *Symbiodinium* DNA, the primers were screened against 47 cultures, representing 5 of the major sub-generic clades in the genus (Table 2). These included 30 cultures isolated from Caribbean hosts as well as 13 specifically derived from *Symbiodinium* populations of *M. faveolata*. Nucleic acids from *Symbiodinium* cultures were extracted using a 2xCTAB protocol (Coffroth et al. 1992) and reactions were conducted in 10 µL volumes with 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 0.3 µM of each primer, 1 U of *Taq* DNA polymerase, and ~5 ng of template DNA. A touchdown PCR was employed, with a 5 min initial denaturation step at 94°C, 45 sec at 94°C, 45 sec at 60°C, decreasing 1°C/cycle until 50°C was reached, and a 1 min extension at 72°C. This was followed by 30 additional cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min, and a 5 min final extension at 72°C. PCR products were run in a 1% w/v agarose gel, visualized with ethidium bromide and scored for presence or absence of amplicons (see Results).

Presence/absence screening for apicomplexans in scleractinian corals

Extraction of coral holobiont DNA utilized the Wizard® (Promega) DNA preparation protocol and was conducted previously at the University of Georgia (Thornhill et al. 2006b; Kemp et al. 2008). All 1,157 samples, and the additional 24 *M. annularis* for estimating prevalence, were shipped to Auburn University in Fall 2008 and screened via PCR for apicomplexan DNA as described above. Reactions were repeated (at least) once for cases were a sample did not initially amplify with primers 18N-F2 and 18N-R1. If a sample consistently failed to amplify with these apicomplexan-specific primers, it was subsequently screened with the alveolate-specific primers SS5 and SS3Z, which targets both apicomplexan and *Symbiodinium* DNA (Toller et al. 2002; Goulet and Coffroth 2004), using previously described PCR protocols (Rowan and Powers 1991). Screening of such samples with the SS5 and SS3Z primer pair was done to confirm the presence of suitable template and to eliminate the possibility that DNA degradation was the underlying cause of amplification attempts scored as apicomplexan "absent".

To verify that primers 18N-F2 and 18N-R1 were in fact amplifying apicomplexan DNA from the scleractinian corals being screened, 100 apicomplexan "present" reactions were selected using a random number generator as a representative sub-sample from across all host species, reefs, seasons and years for sequencing using 18N-F2. Electropherograms were trimmed using Sequencher v5.0.1 and IUPAC ambiguity codes were substituted for nucleotide positions possessing multiple peaks. Identical sequences were collapsed into a unique haplotype, each of which were submitted to the NCBI GenBank database using blastn (Altschul et al. 1990) to identify their most similar matches. Unique haplotype sequences were deposited to GenBank under accession numbers JX943865- JX943883.

Prevalence comparisons among groups and statistical modeling

Apicomplexan prevalence among *M. annularis* individuals at ADM in Florida was calculated with corresponding 95% confidence intervals using Sterne's exact method, as corrected by Reiczigel (2003), in qp3.0 (Rózsa et al. 2000). Due to the low number of samples where apicomplexan DNA was not detected (see Results), estimates of prevalence between the three months were compared using Fisher's exact test (Rózsa et al. 2000). Likewise, this test also was applied to compare apicomplexan prevalence of the single-polyp samples among the six different *M. faveolata* colonies.

To test the hypothesis of seasonality, and to examine affects of host species and reef on prevalence, apicomplexan presence was modeled as a binary response variable in a mixed-model logistic regression (Jaeger 2008). Season, host species, and reef were considered categorical explanatory fixed variables. Individual colony hierarchically nested within species, followed by within reef, location, and year were the random factors included in the statistical mixed model. The lme4 package (Bates et al. 2011) was used to create the models in the R statistical environment (ver. 2.13.2; R Development Core Team, 2011). Generalized variance inflation factors (GVIF) were calculated to examine any collinearity between the variables using the car package in R (Fox and Weisberg 2011).

Season, host species, and reef also were coded as dummy variables to examine differences in apicomplexan prevalence according to these three variables. Pairwise differences among seasons, host species and reef were evaluated with partial likelihood ratio tests (lrt) comparing the full model (i.e., all seasons separately) to those combining two seasons (e.g., Summer + Spring). This was then repeated for all possible pairwise combinations of seasons

(e.g., Summer + Winter, etc.). The comparison was structured in this manner to enable the difference between the paired models to be fully attributed to the differences between the two combined seasons. Similarly, all pairwise combinations of reefs and host species were compared to the full model using this approach. All possible pairwise comparisons were performed and α was adjusted by Bonferroni correction (Legendre and Legendre 1998) for each set of fixed variables (i.e., season, host species, or reef) as they represent separate hypotheses (Ruxton and Beauchamp 2008).

Logistic regression cannot be used when all the binary response variables in one group are identical. For example, when apicomplexan prevalence is 0 or 100% within a group, it cannot be included in the model. Although this does not affect the previously described model, interactions between reef, season, and host species could not be determined due to numerous instances of ~100% prevalence when categories are broken down further (see Results). Thus, to examine the interaction between reef and host species, it was necessary to use Fisher's exact test instead. To control for multiple tests, α was adjusted to (P = 6.25×10^{-3}) by the Bonferroni correction.

Results

Primer specificity and DNA quality

The apicomplexan-specific primer pair 18N-F2 and 18N-R1 amplified four (4) of 47 (8.5%) *Symbiodinium* cultures (Table 2), producing amplicons that could not be distinguished via size from reactions scored as apicomplexan "present". Two of these cultures were members of

Symbiodinium clade F, a lineage typically harbored by foraminifera and uncommonly associated with scleractinian corals (Pochon et al. 2006). The other two were cultures belonging to *Symbiodinium* clade A. Notably, an additional eleven *Symbiodinium* clade A cultures did not amplify, including four isolated from Florida *Montastraea faveolata*.

As an additional test of primer specificity for apicomplexans, a random sub-set of 100 samples scored as apicomplexan "present" were sequenced. Nineteen unique haplotypes of 514-690 bp were identified, with 16 being recovered individually from 2-13 samples and the remaining three being singletons (Table 3). The blastn search identified all 19 haplotypes as being most similar (97-100% identical) in sequence to apicomplexans previously associated with scleractinian corals (Table 3). Given this, as well as the low false positive rate from *Symbiodinium* (see above), PCRs yielding an amplicon of ~860 bp were considered "positive" for apicomplexan template DNA in the scleractinian coral samples.

Estimates of apicomplexan prevalence

A total of 1,157 field-collected coral tissue samples were examined for the presence of apicomplexans, with 719 and 438 coming from Florida and the Bahamas, respectively. Of these, 10.6% (n = 123) of all samples failed to amplify with either the apicomplexan-specific or the alveolate-general (i.e., SS5 and SS3Z) primer sets. Specifically, the failure rate among Florida samples was 3.6% (n = 26/719) and 22.1% (n = 97/438) for the Bahamas, especially during a six-month period from June 2002 to Jan 2003 (69.8%: n = 67/96). Excluding these two years, the failure rate for the Bahamas samples was 8.7% (n = 30/342).

The majority of scleractinian coral samples from both Florida (80.1%: *n* = 555/693) and the Bahamas (90.7%: *n* = 311/343) tested positive for apicomplexan DNA (Fig. 2, 3). Apicomplexan prevalence ranged from 83.3-100% of *M. annularis* colonies sampled from ADM in Florida during March (Winter), May (Spring), and August (Summer) of 2003 (Table 4). There was no significant difference in prevalence between sampling months (*P* = 0.153).

No statistical difference in prevalence was detected among polyps taken from transects along multiple colonies of *M. faveolata*. At LG reef in Florida, 96% (*n* = 24/25) of single polyps sampled from one *M. faveolata* colony had detectable apicomplexan DNA, as did all polyps from two other *M. faveolata* colonies (*n* = 7 and 12, respectively). In the Bahamas, apicomplexan DNA was detected in all single-polyps (*n* = 18 and 5, respectively) from two colonies at NNP, although two samples from the first colony failed to amplify with both primer sets. Similarly, apicomplexan DNA was detected in all single-polyps (*n* = 7) sampled from a *M. faveolata* colony at SP. In this latter case, seven samples failed with either primer set (50% failure rate). Overall, there was no significant difference in pairwise comparisons of apicomplexan prevalence between all *M. faveolata* colonies from which single polyps were screened (*P* = 1.00 for all intercolonial comparisons).

Statistical modeling of apicomplexan prevalence by season, host species, and reef

Calculated generalized variance inflation factors (GVIF) ranged from 1.31-1.61, indicating low collinearity between season, reef, and host species. A significant difference in apicomplexan prevalence was identified for winter relative to fall, spring, and summer (Table 5). Specifically, apicomplexan DNA was 4.38 times as likely (1.72-11.12, 95% Confidence Limit [C. L.]) to be

detected during winter compared to summer. Likewise, apicomplexan DNA was detected 5.28 (1.97-14.12, 95% C. L.) and 9.21 (3.72-22.84, 95% C. L.) times less frequently during spring and fall, respectively, relative to winter.

A significant difference in apicomplexan prevalence also was found between *S. siderea* and the other examined host species (Table 5). Relative to *S. siderea*, apicomplexan DNA was 58 (10.83-310.57, 95% C. L.), 602.09 (86.49-4191.42, 95% C. L.), and 605.95 (42.39-8662.86, 95% C. L.) times more likely to be detected in *M. annularis*, *M. faveolata* and *P. astreoides*, respectively. Over all reefs, there was no significant difference in apicomplexan prevalence detected between *P. astreoides* and either *M. annularis* (lrt: $\chi^2 = 3.73$, d.f. = 1, $P = 0.053$, Table 5) or *M. faveolata* (lrt: $\chi^2 = 0.00$, d.f.=1, $P = 0.996$, Table 5). Likewise, there was no significant difference between *M. annularis* and *M. faveolata* following Bonferroni correction (lrt: $\chi^2 = 6.87$, d.f. = 1, $P = 0.0087$, Table 5).

There was no statistical difference between total apicomplexan prevalence at any of the reefs (Table 5). Total prevalence was lowest at ADM in Florida (79.2%: 294/371) and colonies were 2.40 (0.47-12.26; 95% C. L.), 2.01 (0.22-18.70; 95% C. L.), and 3.52 (0.59-21.13; 95% C.L.) times as likely to harbor apicomplexans at LG, SP, and NNP, respectively.

On a reef-by-reef basis, there was a significant difference in apicomplexan prevalence between the two *Montastraea* species at ADM in Florida ($P = 4.67^{-10}$, Fig. 4) while no difference was recovered at any other reef (LG, $P = 0.314$; SP, $P = 0.0960$; NNP, $P = 0.702$) in Florida or the Bahamas. There also was a significant difference between apicomplexan prevalence of *S. siderea* between Floridian and Bahamian reefs (ADM vs. NNP: $P = 1.20\text{x}10^{-4}$, LG vs. NNP: $P = 1.27\text{x}10^{-8}$), but not between colonies at the two Florida Reefs after Bonferroni correction (ADM

vs. LG: *P* = 0.0478). Finally, there was no significant difference in apicomplexan prevalence between Floridian (ADM) and Bahamian (NNP) *P. astreoides* (*P* = 0.0943).

Discussion

The data presented here imply that members of the Apicomplexa associate with multiple scleractinian coral species in the Western Atlantic, Florida and the Caribbean Sea. Furthermore, these protists appear to be persistent members of the holobiont since their DNA was detected in multiple samplings of the same colonies from Florida and the Bahamas over time periods of 5.5-9 years. In the case of *M. annularis*, estimated prevalence ranged from 83.3-100% of colonies on the same reef. This corroborates a previous report where 90% of apparently-healthy *M. annularis* and *M. faveolata* colonies in Panama harbored apicomplexans according to a similar PCR assay (Toller et al. 2002). Furthermore, apicomplexan oocysts were reported in histological samples of eight scleractinian coral species from Jamaica and Puerto Rico, encompassing 65.6% (*n* = 21/32) of examined colonies (Peters 1984; Upton and Peters 1986). To date, apicomplexans have been documented from eleven Caribbean scleractinian, three Caribbean gorgonian, and two temperate Pacific scleractinians (Upton and Peters 1986; Toller et al. 2002; Goulet and Coffroth 2004, this study) species, signifying a widespread and persistent cnidarian-apicomplexan symbiosis.

Here, screening was conducted using a PCR-based assay with both alveolate (Rowan and Powers 1991) and apicomplexan specific primers (Toller et al. 2002). Such methodology can rapidly and accurately estimate apicomplexan prevalence in other systems (Morris and Gasser 2006; Schall and Smith 2006; Hide et al. 2009; Vilcins et al. 2009; Miller et al. 2010). In this context, it is important to note that a negative PCR result does not necessarily demonstrate that

apicomplexan DNA was absent from a sample since it could be below the template threshold for successful amplification. Furthermore, it is also possible that some apicomplexan symbionts of scleractinian corals do not amplify with the primer set 18N-F2 and 18N-R1. Therefore, these data should be interpreted as minimum estimates of apicomplexan prevalence in the scleractinian species examined here.

Consistent detection of apicomplexans within corals in general is indicative of a stable symbiosis, a conclusion that is congruent with the histological evidence and the infection process. Apicomplexans have been observed intracellularly in the gastrodermis (Peters 1984) Thus, these endosymbionts are likely actively entering and inhabiting tissues. In other hosts, apicomplexans actively invade host cells and persist within a parasitophorous vacuole that prevents lysosomal fusion and subsequent degradation (Sibley 2004). They can further manipulate host cellular machinery and gene expression to facilitate their growth and reproduction (Entzeroth et al. 1998; Gilbert et al. 2007; Saeij et al. 2007; Boothroyd and Dubremetz 2008; Plattner and Soldati-Favre 2008). Therefore, apicomplexans associating with scleractinian corals likely use similar means of active entry and proliferation within a colony.

Seasonal prevalence of apicomplexans in scleractinian corals

Although most individual coral colonies were consistently infected throughout multiyear timespans, exceptions are evident. For instance, a statistically significant seasonal effect was identified, with apicomplexan detection more likely in winter compared to other seasons. Notably, higher winter prevalence was contrary to the initial hypothesis (see Introduction), wherein it was predicted that these symbionts would be more prevalent in summer when coral

colonies are generally more susceptible to opportunistic pathogens (Edmunds 1991; Lafferty et al. 2004; Willis et al. 2004; Lesser et al. 2007; Sato et al. 2009). This hypothesis is based on the assumptions that (1) decreased host resistance in the summer would be coupled with increased parasite growth and (2) apicomplexans associating with corals are parasites, analogous to the role these protists play in other organisms (see below).

Several possible explanations may account for the increased winter prevalence of apicomplexans across scleractinian coral species. Firstly, the presence and prevalence of organisms in the holobiont have been shown to be influenced by host- and symbiont-derived antimicrobial compounds (Kim et al. 2000; Ritchie 2006; Shnit-Orland and Kushmaro 2009; Mydlarz et al. 2010; Rypien et al. 2010; Gantar et al. 2011), with season and temperature altering the number, abundance, and efficacy of such compounds (Alker et al. 2001; Gochfeld et al. 2006; Ward et al. 2007; Rypien et al. 2010). Furthermore, some of these antimicrobial metabolites, as well as wandering amoebocytes which are involved in induction of the immune response (Mydlarz et al. 2008), are upregulated during high temperatures (Ward et al. 2007; Mydlarz et al. 2009; Mydlarz et al. 2010). Taken together, such "defenses" could alter the symbiont assemblage of a given holobiont, leading to lower summer and higher winter patterns of prevalence in scleractinian coral-associated apicomplexans.

Secondly, increases in host resource availability during winter could influence seasonality in apicomplexan prevalence. For instance, seasonal variability in host-derived nutrients is a hypothesized driver for the presence or absence of particular microeukaryotes within the mucous layer of scleractinian corals (Cavada et al. 2011). Furthermore, previous work on the coral colonies examined here demonstrated significant seasonal fluctuations in *Symbiodinium* density and maximum quantum yield of charge separation of the photosystem II

as well as host tissue biomass, with all three of these parameters being highest during winter months (Fitt et al. 2000; Warner et al. 2002; Thornhill et al. 2011). Thus, the correlation between a peak in host tissue biomass and increased prevalence may suggest apicomplexans are exploiting the higher levels of host energy reserves during this time of the year. It also may simply be a function of greater biomass leading to increased habitat availability for symbiont colonization and/or reproduction (and hence, detection) of these apicomplexans. Interestingly, this pattern was not observed in *S. siderea* (see below), whose biomass and *Symbiodinium* densities are not correlated (Thornhill et al. 2011).

Thirdly, it is possible that sampling position within a colony may explain summer decrease in apicomplexan detection. All samples in this study, except those collected from single-polyps, were collected from the unshaded colony tops (Thornhill et al. 2006b), potentially a more variable location (e.g., in terms of light exposure and oxidative stress) for detecting the presence of apicomplexans. Although apicomplexans are not photosynthetic (McFadden et al. 1996), they may be sensitive to differences in microenvironment throughout the colony, such as those generated by *Symbiodinium* (Lesser 2006; Ulstrup et al. 2008). The release of reactive oxygen species by *Symbiodinium* spp. may cause apicomplexans to vacate the high-light environment of the top and take refuge in the shaded periphery of the colonies. Higher apicomplexan mortality within sun-exposed surfaces of the colony also would explain this pattern. Upton and Peters (1986) observed decreased apicomplexan prevalence in tissues collected from the middle of *P. astreoides* colonies in the spring and summer, but not in samples collected from the periphery of the colonies, supporting this hypothesis. However, it is important to note that nearly all the single-polyp samples (98.7%; n = 73/74) from multiple locations on single *M. faveolata* colonies indicated the presence of apicomplexan DNA, which were

specifically chosen to include locations from unshaded top and peripheral polyps. Thus, the consistent detection of apicomplexan DNA from these positions indicates that summer sampling location within the colony would not have affected this PCR-based assay while demonstrating that apicomplexan DNA can be detected from single scleractinian polyps in non-bleaching years.

Differences in apicomplexan prevalence among scleractinian coral species and reefs

Significant differences in apicomplexan prevalence were also detected among the four scleractinian coral species examined in this study. For example, apicomplexans were 48 to 582 times less likely to be detected in *S. siderea* compared to the other three species examined. As noted above, biomass does not positively correlate with increased densities of *Symbiodinium* in *S. siderea* colonies (Thornhill et al. 2011). Thus, for this host species, tissue biomass does not correlate with habitat availability for symbionts in general and other factors likely influence the prevalence patterns of this particular coral-apicomplexan association.

Each host species combines taxon-specific differences in metabolism, physiology, immune response and symbiotic assemblage, which can all influence the habitat for a given symbiont through both facilitation and inhibition (Engel et al. 2002). For example, extracts of *S. siderea* significantly increased the survivorship of the human gut-bacteria *Serratia marcescens* when compared to extracts of *M. faveolata* or *Acropora palmata* (Looney et al. 2010). This indicates that metabolites isolated from *S. siderea* facilitate growth of this eubacteria, a causative agent of white pox disease in *Acropora* spp. (Sutherland et al. 2004; Sutherland et al. 2011). Conversely, coral extracts from many different species have varied inhibitory activity against other marine bacteria (Koh 1997; Shnit-Orland and Kushmaro 2009). Furthermore, different

coral species harbor different symbiont assemblages (Frias-Lopez et al. 2002; LaJeunesse 2002; Rohwer et al. 2002; van Oppen et al. 2005; Brück et al. 2007; Cavada et al. 2011; Gutner-Hoch and Fine 2011) and cultured bacteria isolated from coral colonies can potentially inhibit growth of other bacterial constituents of the holobiont (Nissimov et al. 2009; Rypien et al. 2010; Gantar et al. 2011; Kvennefors et al. 2012). It is possible that the symbiotic assemblages of *P. astreoides* and the two *Montastraea* spp. are more amenable to apicomplexans than *S. siderea*, contributing to observed differences in apicomplexan prevalence. In addition, apicomplexan prevalence in *S. siderea* was significantly lower in Florida (ADM and LG) than the Bahamas (NNP). Differences in the dominant *Symbiodinium* type were observed in these same individuals with C3 consistently associated with all colonies at NNP, and with B5a, C3, or a combination of the two, were found associated with colonies at LG and ADM (Thornhill et al. 2006b). Likewise, site-specific differences in the bacterial symbiont assemblage are observed in Australian *Acropora* spp. and *Stylophora pistillata* (Littman et al. 2009; Kvennefors et al. 2010). Thus, site-specific difference in the holobiont or host genetic background may facilitate or inhibit apicomplexans in their association with *S. siderea*.

Although there was not an overall significant effect of reef location in the full model, a significant difference in apicomplexan prevalence between the two *Montastraea* species at ADM was identified. Specifically, apicomplexan DNA was not detected in any *M. annularis* colonies prior to the winter of 2002 and subsequent detection starting in the following spring may represent a colonization event. Interestingly, for colonies of the congeneric species, *M. faveolata*, apicomplexans were detected throughout this same time period on the same reef. This may imply genetic differences between the apicomplexan symbionts harbored by these species. Previous work on *M. annularis* and *M. faveolata*, as well as their associated *Symbiodinium* populations, at

ADM demonstrated that both are genetically distinct (Thornhill et al. 2009). Although there are examples of host specificity in many groups of apicomplexans (Upton et al. 1992; Garcia et al. 1994; Clopton and Gold 1996; Jäkel et al. 1996; Molnár et al. 2005; Kvicerova et al. 2007; Smith and Cook 2008), the 18S rDNA haplotypes recovered in this study were virtually identical across host species (Table 3). This is not surprising given that 18S rDNA is generally conserved due to its relatively slow rate of evolution driven by purifying selection and concerted evolution (Hillis and Dixon 1991; Gagnon et al. 1996; Barta 1997). Molecular markers under less selective constraint, such as the internal transcribed spacer regions of the ribosomal region (i.e., ITS1 and/or ITS2), have be used to distinguish between closely-related apicomplexan species and sub-species (Olivier et al. 2001; Morales et al. 2005; Boullianne et al. 2007; Motriuk-Smith et al. 2009) as well as other constituents of the coral holobiont (van Oppen et al. 2001; LaJeunesse 2002). Thus, markers with higher substitution rates are likely needed to potentially resolve specificity within this group of apicomplexans.

Potential interaction of apicomplexans in the holobiont

Although the exact nature of the association between apicomplexans and scleractinian corals is unknown, the interaction between this group of symbionts and other hosts spans the range of interactions, from mutualism to parasitism. For example, gregarines can be benign compared to other apicomplexans and have been described as commensal (Kamm 1922). Furthermore, while apicomplexan mutualisms are rare, there is a single documented case between the apicomplexan genus *Nephromyces* and its tunicate host (Saffo et al. 2010). In this association, the apicomplexans reside within the tunicate's renal sac breaking-down and recycling nitrogenous

wastes (Saffo 1982). However, barring this unusual and interesting example, the vast majority of apicomplexan interactions are considered parasitisms. Thus, the likeliest scenario is that apicomplexan-coral associations are also parasitic in nature. In general, apicomplexan parasites destroy host cells and tissues (Solangi and Overstreet 1980; Jiménez et al. 2002), decrease food absorption (Gestal et al. 2002), retard somatic and reproductive growth (MacKinnon and Ray 1937; MacKenzie 1981; Field and Michiels 2005), decrease muscle protein content (Gestal et al. 2007), and/or cause host malaise and even death. Furthermore, increased parasite load negatively affects host health for many groups of apicomplexans (Dyková and Lom 1981; MacKenzie 1981; Collantes-Fernández et al. 2004; Gestal et al. 2007; Tuntiwaranuruk et al. 2008). This includes gregarines in the genus *Monocystis* sp., which infect the earthworm *Lumbricus terrestris*, and have previously been described as "commensal", "benign", and "completely non-pathogenic" (Field and Michiels 2005). As a result, the fitness costs of harboring apicomplexan parasites could potentially be significant for scleractinian and other coral species. Previous work has shown that three Caribbean coral species (*Montastraea cavernosa*, *Agaricia agaricites*, and *Meandrina meandrites*) exhibited signs of partial bleaching and tissue necrosis when associated with apicomplexans; however, it is uncertain if apicomplexans were causative, as three additional host species (*Porites porites*, *P. astreoides*, and *Dendrogyra cylindrus*) showed none of these same signs (Upton and Peters 1986). Although not measured in this study, fitness costs of harboring apicomplexans have been assessed in other systems by correlating parasite load to growth, body condition, survivorship, and/or fecundity (Landau and Galtsoff 1951; Siva-Jothy and Plaistow 1999; Collantes-Fernández et al. 2004; Amo et al. 2005; Field and Michiels 2005; Leinwand et al. 2005). Corals, especially small brooding species like *P. astreoides*, are amenable to measurements of both growth and fecundity as they predictably release brooded larvae on the

new moon in May and June (McGuire 1998; Edmunds et al. 2001). Correlating infection intensity to larvae produced and/or adult colony growth rates would greatly improve our understanding of this symbiosis.

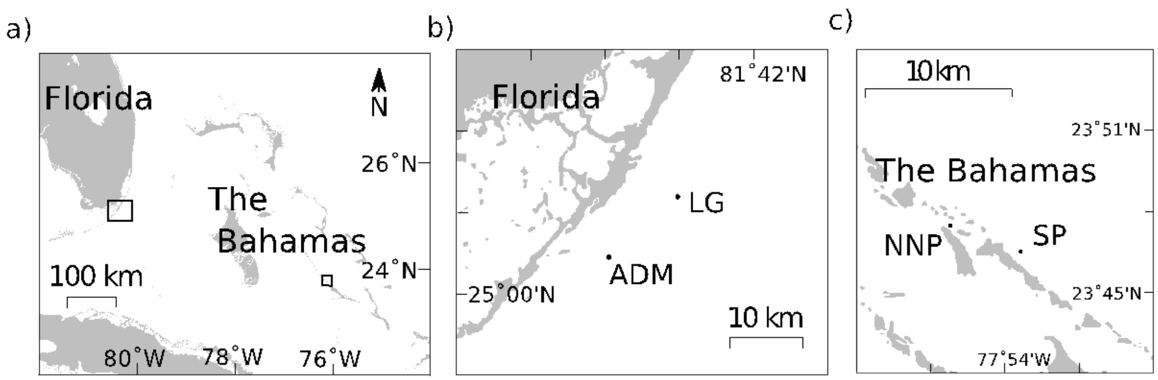

Fig. 1 a) Locations of the reefs sampled in this study with enlargements of b) Florida and c) the Bahamas. Sampled reefs in Florida: Admiral Patch Reef (ADM) and Little Grecian Reef (LG). Sampled reefs in the Bahamas: North Norman's Pond (NNP) and South Perry Reef (SP).

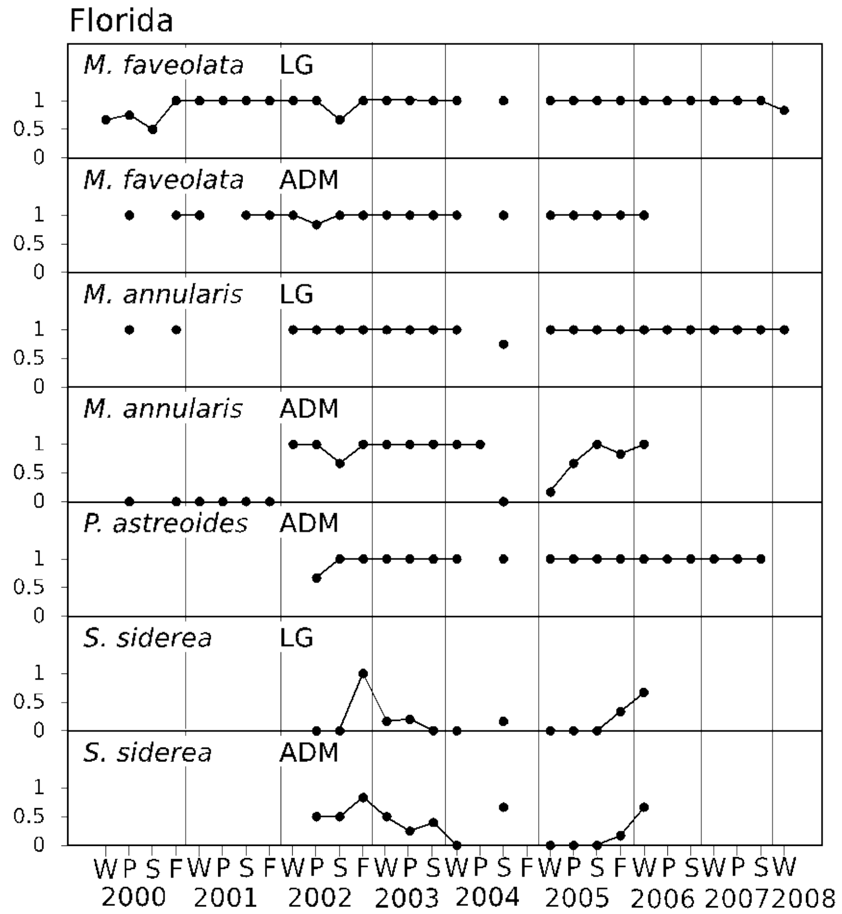

Fig. 2 Apicomplexan prevalence by sampling date, species for Admiral Patch Reef (ADM) and Little Grecian Reef (LG) in Florida. Seasons are abbreviated: Winter=W, Spring=P, Summer=S, Fall=F.

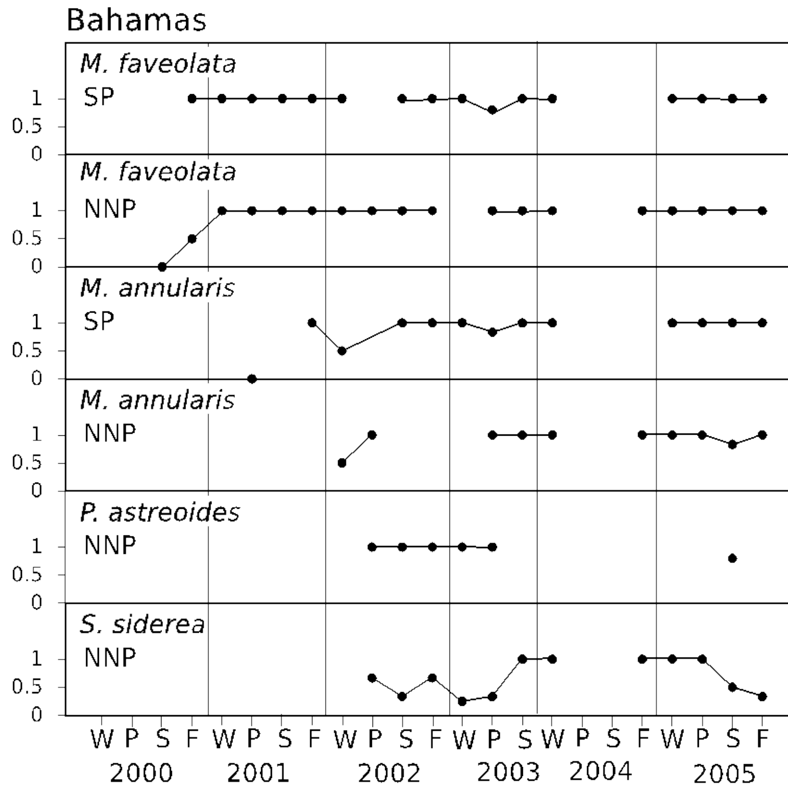

Fig. 3 Apicomplexan prevalence by sampling date, species for North Norman's Pond (NNP) and South Perry Reef (SP) in the Bahamas. Seasons are abbreviated: Winter=W, Spring=P, Summer=S, Fall=F.

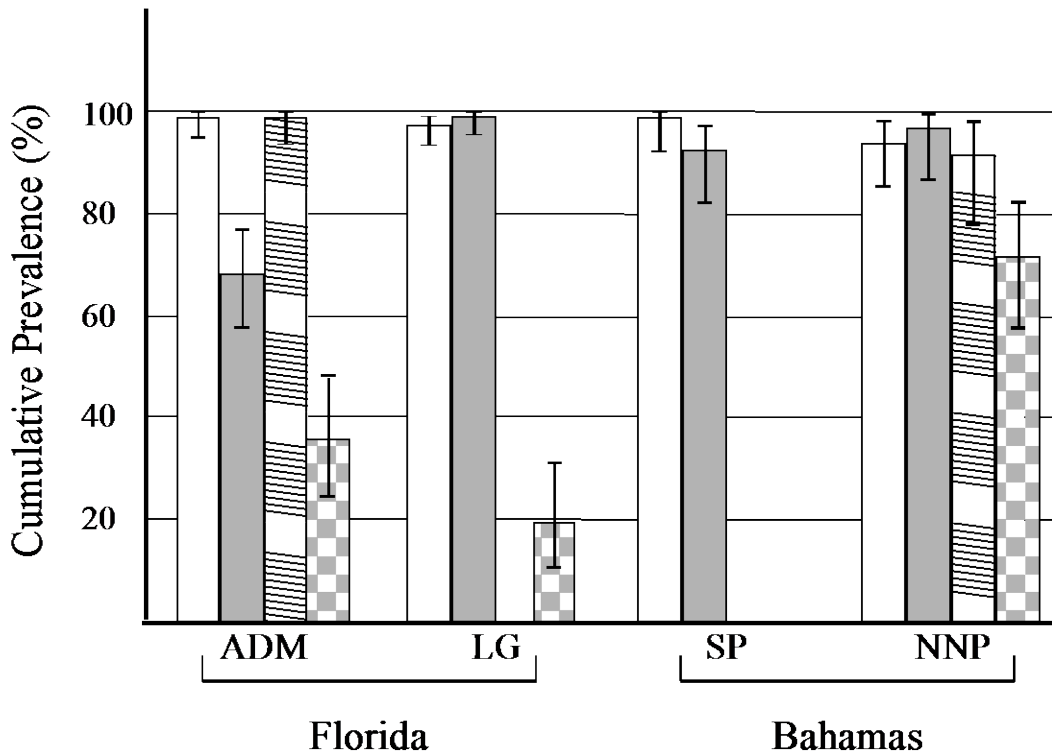

Fig. 4 Cumulative apicomplexan prevalence with corresponding 95% Confidence intervals of all samples of a given species collected at Admiral Patch Reef (ADM) and Little Grecian Reef (LG) in Florida, as well as South Perry Reef (SP) and North Norman's Pond (NNP) in the Bahamas. *M. faveolata*=white, *M. annularis*=grey, *P. astreoides*=stippled, *S. siderea*=checkered.

Table 1 Sampling locations and corresponding start and end dates for all species collected at a site.

	Reef	Species	Start	End
Florida	Admiral Patch Reef (ADM)	*Porites astreoides*	May 2002	Aug 2007
	(25.04˚ N, 80.39˚ W)	*Montastraea annularis*	May 2000	Mar 2006
		Montastraea faveolata	May 2000	Mar 2006
		Siderastrea siderea	May 2002	Mar 2006
	Little Grecian Reef (LG)	*M. annularis*	May 2000	Mar 2008
	(25.12˚ N, 80.30˚ W)	*M. faveolata*	Mar 2000	Mar 2008
		S. siderea	May 2002	Mar 2006
Bahamas	North Norman's Patch (NNP)	*P. astreoides*	Jun 2002	Sep 2005
	(23.79˚ N, 76.14˚ W)	*M. annularis*	Mar 2002	Nov 2005
		M. faveolata	Aug 2000	Nov 2005
		S. siderea	Jun 2002	Nov 2005
	South Perry Reef (SP)	*M. annularis*	May 2001	Nov 2005
	(23.78˚ N, 76.09˚ W)	*M. faveolata*	Nov 2000	Nov 2005

Table 2 Amplification results (amp) of 47 *Symbiodinium* cultures tested with the primers 18N-F2 and 18N-R1. Provided are culture ID, host species (Host), location and Ocean/Sea the culture was isolated from as well as the *Symbiodinium* clade (CLA).

	culture	Host	Location	Ocean/Sea	CLA	amp
1	**CassELI**	*Cassiopea* sp.	Hawaii	C. Pacific	**A**	no
2	**CasskB8**	*Cassiopea* sp.	Hawaii	C. Pacific	**A**	no
3	**Cx**	*Cassiopea xamachana*	Jamacia	Caribbean	**A**	**YES**
4	**Flap1 10ab**	*Aiptasia pallida*	Florida	Caribbean	**A**	no
5	**Mf 13.14**	*Montastrea faveolata*	Florida	Caribbean	**A**	no
6	**Mf10.1**	*Montastrea faveolata*	Florida	Caribbean	**A**	no
7	**Mf11.4**	*Montastrea faveolata*	Florida	Caribbean	**A**	no
8	**Zs**	*Zoanthus sociatus*	Jamacia	Caribbean	**A**	**YES**
9	**9**	*Plexaura kuna*	Florida	Caribbean	**A**	no
10	**11**	*Plexaura kuna*	Florida	Caribbean	**A**	no
11	**Mf10.2a**	*Montastrea faveolata*	Florida	Caribbean	**A**	no
12	**Fla Cass**	*Cassiopea xamachana*	Florida	Caribbean	**A**	no
13	**Y109**	Unknown host	Okinawa	W. Pacific	**A**	no
14	**Ap**	*Aiptasia pulchella*	Hawaii	C. Pacific	**B**	no
15	**Cane**	Unknown anemone	Hawaii	C. Pacific	**B**	no
16	**FLAp3**	*Aiptasia pallida*	Florida	Caribbean	**B**	no
17	**FLCass**	*Cassiopea xamachana*	Florida	Caribbean	**B**	no
18	**Gv5.6a**	Gorgonia ventalina	Florida	Caribbean	**B**	no
19	**Mf1.5b**	*Montastrea faveolata*	Florida	Caribbean	**B**	no
20	**Mf11.5b1**	*Montastrea faveolata*	Florida	Caribbean	**B**	no
21	**P P flex**	*Plexaura flexuosa*	Florida	Caribbean	**B**	no
22	**PtBr**	*Briareum* sp.	Florida	Caribbean	**B**	no
23	**Schypus**	Unknown host	Hawaii	C. Pacific	**B**	no
24	**FLAp3 10ab**	*Aiptasia pallida*	Florida	Caribbean	**B+**	no
25	**FLAp4**	*Aiptasia pallida*	Florida	Caribbean	**B+**	no
26	**Mf 6.1T**	*Montastrea faveolata*	Florida	Caribbean	**C**	no
27	**Mf 7.5T**	*Montastrea faveolata*	Florida	Caribbean	**C**	no
28	**Mf 8.5Tb**	*Montastrea faveolata*	Florida	Caribbean	**C**	no
29	**Mf 8.5TB.2**	*Montastrea faveolata*	Florida	Caribbean	**C**	no
30	**Mf8.3 TC4**	*Montastrea faveolata*	Florida	Caribbean	**C**	no
31	**Mp**	*Mastigia paupa*	Palau	W. Pacific	**C**	no
32	**Pa44b**	*Porites divaricata*	Florida	Caribbean	**C**	no
33	**Pa45a**	*Porites divaricata*	Florida	Caribbean	**C**	no
34	**A001**	*Acropora* sp-0	Okinawa	W. Pacific	**D**	no
35	**A006**	*Acropora* sp-3	Okinawa	W. Pacific	**D**	no
36	**A013**	*Porites annae*	Okinawa	W. Pacific	**D**	no

37	**A014**	*Porites australiensis*	Okinawa	W. Pacific	**D**	no
38	**Ap32**	Unknown anemone	Okinawa	W. Pacific	**D**	no
39	**Ap37**	Unknown anemone	Okinawa	W. Pacific	**D**	no
40	**Mf10.8a**	*Montastrea faveolata*	Florida	Caribbean	**D**	no
41	**CCMP 421**	From CCMP	New Zealand	W. Pacific	**E**	no
42	**Mv**	*Montipora verrucosa*	Hawaii	C. Pacific	**F**	**YES**
43	**Sin**	*Sinularia* sp.	Guam	W. Pacific	**F**	**YES**
44	**711**	*Plexaura kuna*	Panama	Caribbean	**Het**	no
45	**JN120.1**	*Plexaura kuna*	Panama	Caribbean	**Het**	no
46	**Mf13.3b**	*Montastrea faveolata*	Florida	Caribbean	**Het**	no
47	**Pdam3 N**	*Pocillopora damnicornis*	Hawaii	C. Pacific	**Het**	no

Table 3 Blast report for the 19 haplotypes (Hap) found among the 100 sequenced samples. The number of times a haplotype was recovered (# Hap), the length (in basepairs), description and accession number (Hit-Name) are provided. The type species (sp), Reef, Season and year are provided as are the sp, reef, season and year of all (Total) samples sharing identical haplotypes. Abbreviations for reef and season are provided in Figure 2. Mf=*M. faveolata*, Ma=*M. annularis*, Pa=*P. astreoides*, and Ss=*S. siderea*. Numbers in parentheses are the total number sharing that characteristic. The most common species, reef, season or year sharing the haplotype are in bold.

Hap	# Hap	Length (bp)	Hit-Name	Description	Type Sp	Type Reef	Type Sea	Type Yr	Total Sp	Total Reef	Total Sea	Total Loc	Total yrs
1	13	636	Af238265	Unidentified symbiont Type N clone N:0-2 18S ribosomal RNA gene, partial sequence	Mf	SP	P	2005	**Ma (6), Mf (6)**, Ss	NNP (6), **SP (7)**	**W (7)**, P, S, F (4)	**B (13)**	**2001 (4)**, 2003 (2), 2004 (3), **2005 (4)**
2	12	636	Af238265	Unidentified symbiont Type N clone N:0-2 18S ribosomal RNA gene, partial sequence	Ma	LG	S	2005	Ma (4), **Mf (8)**	ADM (4), **LG (6)**, NNP (2)	W (2), P, S (3), **F (6)**	**F (10)**, B (2)	2000 (2), 2002 (4), **2005 (5)**, 2006
3	10	636	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Mf	LG	S	2003	**Ma (5), Mf (5)**	ADM (3), **LG (6)**, NNP	W, P, **S (5)**, F (2)	**F (9)**, B	2001, **2002 (4)**, 2003 (2), 2004 (2), 2005
4	9	593	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Ma	LG	W	2005	Ma (2), Mf (2), **Pa (3)**, Ss (2)	**ADM (7)**, LG (2)	**W (6)**, P (2), F	**F (9)**	**2002 (2)**, 2003, 2004, **2005 (2), 2006 (2)**, 2007
5	8	668	Af238265	Unidentified symbiont Type N clone N:0-2 18S	Pa	ADM	P	2003	Mf, **Pa (7)**	**ADM (7)**, LG	W (3), **P (4)**, F	**F (8)**	2002, **2003 (4)**, 2005

				ribosomal RNA gene, partial sequence									, 2006 (2)
6	6	593	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Mf	LG	P	2002	Ma, **Mf (5)**	**LG (5)**, SP (1)	**W (4)**, P, S	**F (5), B**	2002, **2004 (3)**, 2005, 2008
7	6	688	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Ma	LG	W	2006	Ma (2), **Mf (4)**	**ADM (5)**, LG	**W (4)**, S (2)	**F (6)**	**2004 (3)**, 2005, 2006 (2)
8	5	688	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Ma	ADM	S	2005	Ma (2), **Mf (3)**	ADM (2), **LG (3)**	**W (2)**, P, **S (2)**	**F (5)**	2003, 2005, 2006, 2007, 2008
9	5	637	Af238265	Unidentified symbiont Type N clone N:0-2 18S ribosomal RNA gene, partial sequence	Ss	ADM	W	2006	**Ss (5)**	**ADM (5)**	W, **S (2), F (2)**	**F (5)**	**2002 (2)**, 2004, 2005, 2006
10	5	648	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Ma	ADM	S	2003	**Ma (4)**, Mf	**ADM (3)**, SP (2)	**W (4)**, S	**F (3)**, B (2)	2003, 2004, 2005, 2006
11	4	690	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene,	Pa	NNP	S	2002	Mf, **Pa (3)**	LG, **NNP (3)**	P, **S (3)**	F, **B (3)**	**2002 (4)**

				partial sequence									
12	4	636	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Mf	ADM	F	2000	Ma, Mf, **Ss (2)**	ADM, LG, **NNP (2)**	W (3), F	**F (2), B (2)**	2000, **2004 (3)**
13	3	636	Af238265	Unidentified symbiont Type N clone N:0-2 18S ribosomal RNA gene, partial sequence	Mf	NNP	F	2004	Ma, **Mf (2)**	**NNP (3)**	**P (2)**, S	**B (3)**	2003, 2004, 2005
14	3	652	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Ss	NNP	W	2005	**Ss (3)**	**NNP (3)**	W, P, S	**B (3)**	**2002 (2)**, 2005
15	2	517	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Mf	ADM	S	2002	Mf, Pa	**ADM (2)**	W, S	**F (2)**	2002, 2007
16	2	636	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Pa	ADM	S	2006	PA (2)	**ADM (2)**	S, F	**F (2)**	2005, 2006
17	1	514	AF238264	Unidentified symbiont Type N clone N:0-1 18S ribosomal RNA gene, partial sequence	Ma	NNP	P	2005					

18	1	593	AF23826 4	Unidentifie d symbiont Type N clone N:0- 1 18S ribosomal RNA gene, partial sequence	Ma	SP	F	200 5					
19	1	593	AF23826 4	Unidentifie d symbiont Type N clone N:0- 1 18S ribosomal RNA gene, partial sequence	Pa	NNP	S	200 5					

Table 4 Estimates of apicomplexan prevalence on a reef with corresponding 95% confidence intervals (95% C I) for the *Montastraea annularis* colonies on Admiral Reef for 3 months during 2003. The sample size and number of colonies infected (No. Inf) are provided.

Date	Sample Size	No. Inf	Prevalence	95% C I
Mar 2003	18	18	100%	83.73-100%
May 2003	12	12	100%	79.17-100%
Aug 2003	12	10	83.3%	58.32-95.47%

Table 5 Partial likelihood ratio tests (lrt) comparing the full model to those combining two species at a time (top), those combining two months at a time (middle) and those combining two reefs at a time (bottom). See text for more explanation. These values model a χ^2 distribution with 1 degree of freedom. Bold values are significant at $\alpha = 0.05$ after Bonferroni correction for sequential comparisons.

	M. annularis	*M. faveolata*	*P. astreoides*	*S. siderea*	
Spring	XX	6.8734	3.7301	**14.99**	*M. annularis*
Summer	0.2006	XX	0.0517	**30.248**	*M. faveolata*
Fall	2.3557	3.7803	XX	**23.538**	*P. astreoides*
Winter	**12.019**	**10.659**	**27.827**	XX	*S. siderea*
	Spring	Summer	Fall	Winter	

FLORIDA		**BAHAMAS**		
ADM	LG	SP	NNP	
XXX	1.1673	0.4049	1.9225	ADM
	XXX	0.0245	0.1618	LG
		XXX	0.2159	SP
			XXX	NNP

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Chapter 4. Tracking Transmission of Apicomplexan Symbionts in Diverse Caribbean Corals

Abstract

In each generation, symbionts must reassociate with their hosts to ensure continuity of the relationship. Scleractinian corals make an excellent study system for understanding patterns of symbiont transmission because they harbor many diverse symbionts, possess varying reproductive modes via either internal (brooding) or external (broadcast spawning) fertilization, and can propagate clonally, potentially passing symbionts to new individuals in the process. Here, it was hypothesized that apicomplexans (Chromalvaeolata: Alveolata), a widely recognized and important group of parasites, would be vertically and horizontally transmitted in brooding and broadcasting species, respectively, which are patterns consistent with many other coral symbionts. Utilizing a PCR-based screening assay, apicomplexan DNA was detected from all larvae produced by the majority (93.9%; *n* = 62/66) of *Porites astreoides* colonies, a brooding species examined in Florida and Belize. Apicomplexan DNA also was detected in planula larvae from all colonies of four additional brooding species, *Agaricia agaricites*, *Agaricia tenuifolia*, *Favia fragum* and *Mycetophyllia ferox*, collected in Belize. In contrast, no apicomplexan DNA was detected from planulae larvae of three (*Acropora palmata*, *Diploria strigosa*, and *Montastraea faveolata*) and four (*Acropora cervicornis*, *A. palmata*, *D. strigosa*, and *M. faveolata*) broadcast spawning species collected in the Florida Keys and Belize, respectively, and rarely from gametes of these same species. Notably, tissue samples from nearly all (92.0%; *n* = 81/88) colonies of *M. faveolata*, *A. cervicornis*, and *A. palmata* that either provided gametes for

planulae crosses, or were from wild populations, harbored apicomplexan DNA. Lastly, almost all individuals and clones of *A. cervicornis* (100%; n = 7/7) and *A. palmata* (84.2%; n = 16/19) were associated with apicomplexans. Taken together, these data suggest brooding species transmit their apicomplexans vertically while broadcast spawning species acquire these symbionts horizontally, patterns that are consistent with other scleractinian coral symbionts.

Introduction

Symbioses have helped shape the evolution of eukaryotic life (Douglas 1994; Margulis 1998) and their ubiquity (Poulin 1996; Windsor 1998) and antiquity (Simon et al. 1993; Lake 2009) demonstrates the widespread success of such relationships in general. Of importance to any symbiosis is the continuity of the relationship, particularly across each generation. In this context, symbionts may be passed vertically from parents to offspring or acquired horizontally via a vector or from the local environment. For the symbiont, there is an obvious benefit from vertical transmission, as a new host individual is guaranteed. However, the fate of the symbiont is often tied to the extinction or local extirpation of their host species in strictly vertical systems (Stork and Lyal 1993; Koh et al. 2004; Poulin and Keeney 2008). On the other hand, horizontal transmission requires the symbiont encounter a suitable host each generation. It is hypothesized that vertical transmission will lead to more benevolent symbionts (Ewald 1987; Herre et al. 1999) and this has been demonstrated in manipulation studies (Bull et al. 1991; Stewart et al. 2005; Sachs and Wilcox 2006; Magalon et al. 2010). While exceptions to these generalizations exist, it is in the evolutionary interest of the symbiont to occasionally transfer horizontally (if

possible) in order to maximize fitness while preventing the accumulation of deleterious mutations due to inbreeding (Hamilton and May 1977; Lipsitch et al. 1995; Frank 1996b).

Serving as the foundation of the tropical reef ecosystem, scleractinian corals, within the phylum Cnidaria, provide services such as nutrition and shelter to a wide-range of other organisms (Knowlton and Jackson 1994; Plaisance et al. 2011). Furthermore, scleractinian corals are an ideal system to study symbiont acquisition as they harbor numerous, diverse symbionts. As hosts, corals form symbioses with members from all three domains of life: Eubacteria, Archaea, and Eukaryota (Knowlton and Rohwer 2003). Their most well-known relationship involves dinoflagellates in the genus *Symbiodinium*, which translocate up to 90% of the photosynthetically fixed carbon they produce to the host (Muscatine and Porter 1977; Muscatine et al. 1981). Along with *Symbiodinium*, other mutualists as well as parasites of scleractinian corals influence host health and physiology in both positive and negative ways (Aeby 1991; Lesser et al. 2004; Reshef et al. 2006; Ritchie 2006; Nissimov et al. 2009; Rosenberg and Zilber-Rosenberg 2011).

Symbiont transmission in scleractinian corals is often related to the reproductive mode of the particular host species (reviewed in Stat et al. 2006; Baird et al. 2009), with different modes dependent upon whether syngamy occurs internally or externally (Fadlallah 1983; Szmant 1986; Richmond and Hunter 1990; Baird et al. 2009). For example, species possessing internal fertilization produce planula larvae that develop within the maternal colony prior to release, termed “brooding”. These host species tend to provision symbionts like *Symbiodinium* vertically (Baird et al. 2009). Conversely, species releasing gametes into the water column, in a process called “broadcasting”, have external fertilization and the resulting planulae tend to obtain symbionts, such as *Symbiodinium*, horizontally upon settling and metamorphosis (Babcock et al.

1986; Little et al. 2004; Baird et al. 2009). While the transmission patterns of other coral symbionts are beginning to be described, this general trend appears to apply to Eubacteria (Apprill et al. 2009; Sharp et al. 2010; Sharp et al. 2012) and eukaryotic stramenopiles as well (Siboni et al. 2010). Finally, corals are modular organisms capable of clonal growth and propagation. Over time, they add additional polyps to create single colonies defined as ramets (Begon et al. 1996; Lasker and Coffroth 1999) and individual ramets derived from a single zygote represent a genet (Begon et al. 1996). Colony fragmentation and subsequent growth can lead to clonal individuals becoming abundant on some reefs (Highsmith 1982; Jackson and Hughes 1985; Ayre and Willis 1988; Ayre and Hughes 2000). This is exemplified by Caribbean corals like *Acropora cervicornis* and *A. palmata*, both of which grow rapidly (Gladfelter et al. 1978) and readily form clonal aggregates due to frequent fragmentation (Gilmore and Hall 1976; Bak and Engel 1979; Tunnicliffe 1981; Highsmith 1982; Lirman 2000). Such a situation can increase habitat availability for symbionts while potentially transporting them across a reef. This can also lead to increases in the source pools of symbionts that are horizontally transmitted.

This study focuses on elucidating the transmission patterns of another group of coral symbionts, the eukaryotic apicomplexans. Evolutionarily, this large clade (~6,000 described species) of protists is sister to the dinoflagellates and almost exclusively comprised of parasites, including the causative agents of malaria and toxoplasmosis (Snow et al. 2005; Dubey 2008). Apicomplexans were first documented in coral hosts and described as a single species, *Gemmocystis cylindrus*, based on morphology and life-cycle (Peters 1984; Upton and Peters 1986) and have been subsequently detected in Caribbean scleractinian corals and gorgonians using molecular markers (Toller et al. 2002; Goulet and Coffroth 2004). However, the impacts these symbionts have on their scleractinian coral hosts, such as fitness costs, remain unknown.

Furthermore, their route of transmission among host individuals, which could be vertical, horizontal or both, is unresolved. Given the transmission patterns of other symbionts like *Symbiodinium* (see above), we hypothesized that 1) planulae from brooding species of scleractinian corals would harbor apicomplexans; 2) gametes and/or planulae of broadcasting scleractinian corals would lack apicomplexans, and; 3) asexual propagation would lead to high prevalence of apicomplexans among individual ramets belonging to the same genet. To test these hypotheses, gametes, planulae and/or adults were examined from multiple species of brooding and broadcasting scleractinian corals in both the Florida Keys and Belize.

Methods

Ethics Statement

Collection of all scleractinian coral larvae and adult tissue samples was permitted through appropriate regulatory bodies and in accordance to the permits and laws of the issuing body. Specifically, Florida samples were collected in accordance to the following permits from the Florida Keys National Marine Sanctuary: 2010 *Porites astreoides* adult and larvae colonies: (FKNMS-2010-039); 2011 *P. astreoides* larvae: (FKNMS-2010-023): broadcasting gametes and larvae (FKNMS-2009-081-A and FKNMS-2010-055). In Belize, all colonies and larvae were collected (by permit of Belize Fisheries) and imported according to CITES permits (131, 385, 1817, 1818).

Collection of Planulae and Adults from the Brooding Species *Porites astreoides* in Florida

In May 2010, 30 colonies of the brooding scleractinian coral *Porites astreoides* were collected at 4-6m depth using a hammer and chisel from an artificial patch reef established in 1986 from Long Key viaduct rubble in the Middle Keys (Bureau of Marine Fisheries Management (1999); Rubble Piles [RP]: N 24.7428°, W 80.8147°, Fig. 1). Individual colonies were at least 7.7 cm in diameter ($\bar{x}$=13.1±2.2) in order to maximize the probability of being reproductive (Chornesky and Peters 1987) and collections occurred three days prior to the new moon (13 May) when *P. astreoides* was predicted to spawn (Chornesky and Peters 1987; McGuire 1998). Colonies were brought to the Keys Marine Laboratory and kept outside in shaded raceways with constant seawater flow. Daily and prior to dusk, colonies were individually placed into planulae collection buckets (Jokiel 1985; Jokiel et al. 1985) and flow was decreased and diverted into each container. Released brooded planulae were collected the following mornings over the subsequent three days and preserved in 95% ethanol for downstream molecular analyses. Additionally, two 1 cm^2 tissue samples were removed from the edge of all maternal colonies and preserved as above to test whether adults harbored apicomplexan symbionts.

As few colonies spawned in 2010 (see Results), the experiment was repeated in 2011 in the lower Florida Keys (Wonderland Reef [WR]: N 24.5603°, W 81.5013°, Fig. 1). Fifty-one *P. astreoides* colonies were collected from a patch reef in April 2011, five days prior to the new moon, and transported to MOTE Marine Laboratory in the Lower Keys (Fig. 1). Here colonies were kept in shaded raceways until dusk when they were placed in collection buckets as before. Planulae were collected in the morning of first release and preserved as described above. Furthermore, to determine whether apicomplexans were present in all planulae or just in those

that were released on the first day of spawning, collections were made from five maternal colonies over three consecutive days, which was the duration of the April 2011 spawning period.

Collection of Planulae from Other Brooding Species in Belize

To determine if apicomplexans were present in planulae of other Caribbean brooding corals, 15-40 colonies from four additional species as well as *P. astreoides* (Table 1) were collected from reefs surrounding Carrie Bow Cay (CBC: N 16.8025°, W 88.0819°, Fig. 1) in the Belizean Barrier Reef and transported to the water adjacent to the dock at Carrie Bow Cay Marine Laboratory. Nightly, colonies were placed in individual buckets on the dock and larvae were collected the following morning and batch preserved in 95% ethanol. Specifically, colonies of *P. astreoides* and *Agaricia tenuifolia* were collected on 1 June 2011 (the night of the full moon when both were predicted to spawn) from the reef flat directly adjacent and northeast of CBC and from a small patch reef ~200 m north of CBC, respectively. The following day (2 June 2011), colonies of *Mycetophyllia ferox* and *Agaricia agaricites* were collected from the fore-reef ~200 m east of CBC. Twenty colonies of *Favia fragum* were collected on 8 June and again on 10 August from the same patch reef as *A. tenuifolia*. This species was collected later in the lunar cycle when individuals have a higher likelihood of releasing brooded planulae (Szmant-Froelich et al. 1985). All planulae were collected the morning following release and preserved as described above.

Collection of Gametes, Planulae and Adults from Broadcasting Species in Florida and Belize

Gametes and planulae were collected from three and five Caribbean broadcasting coral species in the Florida Keys and Belize, respectively (Table 2). In August 2011, gametes were

collected in the Florida Keys 2-4 hours after sunset by tenting individual colonies prior to release. Gamete bundles were returned the laboratory, allowed to break apart and then mixed with bundles from different colonies to increase fertilization success (Ritson-Williams et al. 2010). Planulae were reared in filtered seawater for between 18 hrs and 9 days and then preserved in 95% ethanol prior to metamorphic competence. All Floridian *Acropora palmata* planulae came from a single parental cross-fertilization involving a single genet (i.e. clone) from Horseshoe Reef and a single colony from Elbow Reef (Fig 1, Table 2). Additionally, eggs were separated from sperm prior to fertilization in this species and both preserved separately in 95% ethanol. Similarly, all Floridian *Diploria strigosa* planulae came from a single bi-parental cross of individuals from Horseshoe Reef. Finally, three separate batches of *Montastraea faveolata* larvae were generated from reef-specific, multi-parental crosses using gametes collected from three reefs in the Florida Keys: Looe Key (10-15 adults), Horseshoe Reef (3-5 adults), and Alligator Reef (10-15 adults) (Fig 1, Table 2).

The hypothesis that apicomplexans are transmitted horizontally in broadcasting coral species assumes that adult colonies in the population (including those not contributing to the gametic pool) would be found associating with these symbionts. Thus, it was necessary to sample adults in the population. In Florida, however, it was not possible to sample the exact colonies providing gametes for planula generation due to logistic difficulties. Instead, single polyps from the top, middle and bottom of 30 Floridian *M. faveolata* colonies were sampled at Alligator Reef (one of the sampling sites where gametes were collected) a few days after the spawning event using the syringe technique of Correa et al. (2009) and preserved in 95% ethanol.

In Belize, all colonies of the four broadcasting species were collected from the reef 50-100 m south of CBC (Table 2). The only exception was the deeper (~20 m) water colonies of *Montastraea franksi*, which were from a reef ~1.5 km south of CBC (N 16.7797°, W 88.0753°). Once detached from the reef, colonies were transported to the lab and placed in individual 5-gallon containers similar to the brooding species (see above). Planulae were generated for all species in August and September 2011 from multi-parental crosses of gametes similar to the Florida samples, except eggs were separated from sperm prior to crossing using 100 μM Nitex mesh (Table 2). Since gametes from all adults were mixed for the fertilization attempts, it was impossible to track the exact parental colonies of the planulae. Therefore, gametes were also preserved for comparison to that of adult colonies for all species except *D. strigosa*, where the few bundles obtained were utilized solely for generating planulae. Lastly, gametes were harvested from *M. franksi* by hand, separated, and preserved as previously described. However, no viable planulae were obtained from this species.

At CBC in Belize, tissue from the six *A. cervicornis* and three *A. palmata* colonies that provided gametes for crosses were sampled and preserved in CHAOS buffer (Fukami et al. 2004). Additionally, all *A. cervicornis* and *A. palmata* individuals from the same reef flat as the colonies providing gametes for crosses were sampled prior to spawning towards determining 1) prevalence of apicomplexans among reproductive colonies and 2) levels of host clonality on the reef. From these, 30 colonies of each *Acropora* species were selected by a random number generator to include those of reproductive age using a size cutoff metric of colony length x width; the minimum threshold sizes for *A. palmata* and *A. cervicornis* colonies included in this study were 2,500 cm^2 and 600 cm^2, respectively. If the colonies providing gametes were not selected as part of this random subset, they were also included into the sampling scheme. This

increased the number of individuals examined (N) to 31 and 33 for *A. palmata* and *A. cervicornis*, respectively.

DNA extraction and Presence/Absence Screening for Apicomplexans

Preliminary experiments determined that 3-5 brooding and 20 broadcasting planulae consistently provided sufficient template DNA to produce a strong amplicon via PCR (data not shown). Therefore, DNA was extracted from single batches of either 5 planulae for each adult sampled of all brooding species or 20 planulae for each conducted cross of broadcasting gametes. Additionally, the presence of apicomplexans in gametes prior to syngamy was assessed in Belize by combining all sperm collected from an individual or ~100 eggs for downstream molecular analyses. DNA was extracted from these egg and sperm samples for all colonies providing gametes in Belize, except *D. strigosa* (Table 2, see above). The majority of DNA extractions were done solely using 2X CTAB buffer, with tissue homogenization by pestles and bead-beat prior to standard phenol:chloroform extraction (Coffroth et al. 1992). However, due to co-precipitation of inhibitors using the 2X CTAB protocol, all *P. astreoides* samples taken from adult colonies also were gel purified using Spin-X filters (Corning Costar®). For the *Acropora* colonies from Belize, DNA was isolated using the protocol described in Levitan et al. (2011). To control for potential contamination during DNA isolation, no-larvae controls were included during all extractions; these controls utilized all the same buffers, plastic consumables, and protocol steps as the other samples, except planulae were not included.

To confirm the presence of amplifiable DNA in all samples, the small subunit ribosomal DNA (18S rDNA) "universal" primer SS5 was coupled with two reverse primers, the "universal" SS3 and the alveolate-specific primer SS3z, using previously described PCR conditions and

cycling (Rowan and Powers 1991). This was followed by screening for apicomplexans via a presence/absence PCR based assay utilizing the apicomplexan-specific 18S rDNA primers 18N-F2 and 18N-R1 (Toller et al. 2002). To ensure that these latter amplicons were derived from apicomplexan DNA, twenty samples from the brooded planulae dataset were randomly selected as described above and sequenced in the forward direction with the primer 18N-F2 (Toller et al. 2002). This set of random samples was representative of all examined brooding species: Florida and Belize *P. astreoides* as well as *A. agaricites*, *A. tenuifolia*, *F. fragum*, and *M. ferox* from Belize. Lastly, twelve gamete or planulae samples also were randomly selected from the Florida and Belize broadcasting species and an ~710 bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene amplified utilizing the universal primers of Folmer et al. (1994) and protocol of Craft et al. (2008). These twelve samples were sequenced using the forward primer LCO1490 (Folmer et al. 1994). All sequences were trimmed in Sequencher v5.0.1 prior to being submitted to GenBank's non-redundant (nr) database using blastn (Altschul et al. 1990) to identify their most similar matches. Sequences were submitted to GenBank under accession numbers (###-###).

Assessing Clonality of Parental *Acropora* sp. in Belize

Both *A. cervicornis* and *A. palmata* commonly undergo clonal propagation by fragmentation (Tunnicliffe 1981; Lirman 2000). To assess the clonality among adult colonies included here, samples were genotyped for three polymorphic microsatellite loci: 181, 182, and 187 (Baums et al. 2005a). All samples were amplified using the protocol of Fogarty et al. (2012) and separated on an ABI 3100 xl (Applied Biosystems®) capillary sequencer. Allele sizes were determined using Genescan v3.1 and Genotyper v3.7 software (Applied Biosystems). The

probability of identity (P.I.), which represents the potential that identical and shared genotypes are by chance and not common descent (i.e. ramets of the same genet), was calculated for each of the three loci in Gimlet v1.3.3 (Valiére 2002). The combined probability of identifying such clonemates utilizing three loci was calculated by multiplying the three individual locus probabilities (Baums et al. 2005b). The number of genets (N_g) and ratio of genets to ramets (N; i.e., the sample size examined) also were calculated.

Light and Transmission Electron Microscopy

To visualize potential apicomplexans in the planulae of brooding scleractinian corals, twenty Florida *P. astreoides* planulae collected on 1 Jun 2011 were fixed in 2.5% gluteraldehyde for 24 hrs prior to being transferred to Millonig's phosphate buffer. These were then post-fixed in 2% osmium tetraoxide for 2 hrs and embedded in Epon/Araldite resin (kit, EMS). Sectioning at 1 µm was done on a Reichert Ultracut S ultramicrotome followed by staining with toluidine blue and examination via light microscopy. Samples with suspected apicomplexans were cut into ultra-thin sections, stained with uranyl acetate and viewed on a Philips 301 transmission electron microscope.

Statistical Analyses

Prevalence (i.e., the number of times apicomplexan DNA was detected divided by the number of samples examined, expressed as a percentage) was calculated along with their 95% Confidence Intervals (C.I.) using Sterne's exact method in qp v3.0 (Rózsa et al. 2000). Prevalence was compared among planulae of the brooding species in Belize using Fisher's exact test as there were few samples were apicomplexans were not detected (see Results) and

significance was adjusted by the Bonferroni correction (Legendre and Legendre 1998). Likewise, prevalence was compared between all pairs of broadcasting species in Belize for sperm and egg samples. Fisher's exact test also was utilized to determine whether apicomplexan prevalence between brooding and broadcasting species were significantly different. Specifically, comparisons were made between all brooding planulae from Belize (n = 25) and sperm (n = 23), eggs (n = 23) and all batches of planulae (n = 4) from the broadcasting species in Belize.

Results

Apicomplexan Screening of *Porites astreoides* from Florida

In May 2010, 14 of 30 *Porites astreoides* colonies released brooding planulae over a six-day period. The number of planulae released ranged from 2-105 per colony ($\bar{x}$ = 27.7±26.6) and apicomplexan DNA was detected via PCR in planulae collected from all of these colonies (n = 14/14; 76.2-100%, 95% C.I.). Furthermore, 96.2% of the adult colonies (n = 25/26; 81.2-99.8%, 95% C.I.), including all (n = 14) that released planulae, tested positive for the presence of apicomplexan DNA. Template DNA from four (4) of the 30 colonies failed to amplify with any of the three primer sets (i.e., universal, alveolate-specific, and apicomplexan-specific) and were thus excluded from the prevalence calculation, as they were unsuitable for PCR. In 2011, 50 of 51 (98%) *P. astreoides* colonies from the lower Florida Keys population produced planulae. In this case, planulae from 92.0% of these colonies (n = 46; 81.21-97.22%, 95% C.I.) screened positive for apicomplexans. Overall, there was no significant difference in apicomplexan prevalence of *P. astreoides* planulae between the two years (P = 0.568). Two and nine amplicons from these apicomplexan-specific PCR reactions were sequenced for the 2010 and 2011 larvae,

respectively, (Supplementary Table 1) and all 11 sequences (142-750 bp in length; $\bar{x}$ = 559 bp) were most similar (97-100%) to that of a coral-associated apicomplexan (GenBank Accession #AF23826) from Toller et al. (2002). Apicomplexan DNA also was detected in batches of 5 planulae collected from five colonies on three consecutive mornings from 1 June-3 June 2011. Thus, apicomplexans are apparently associated with brooded planulae over the course of a spawning event. Lastly, no amplicons were produced from either the planulae-free extractions or negative (i.e., no template added) PCR controls with any of the three primer sets, implying that contamination was not a contributing factor to the above results.

Putative apicomplexans were observed inside the ectoderm and the endoderm of *P. astreoides* planulae collected in 2011 (Fig. 2). These symbionts were located within membrane-bound vacuoles inside host cells. The TEM micrographs of one of the putative apicomplexans have numerous electron-lucent bodies, resembling the amylopectin granules found in some stages of members of the group (Wang et al. 1975; Asai and Tomavo 2007; Breshears et al. 2009).

Apicomplexan Screening of *P. astreoides* and Other Brooding Species from Belize

In June 2011, larvae were collected from 6 of 22 Belizean *P. astreoides* colonies throughout a six-day period, starting two days after the new moon (1 June). Apicomplexan DNA was detected in planulae of all releasing colonies (n = 6/6; 58.9-100%, 95% C.I.). Batches of planulae also were collected from five colonies of *A. agaricite*s, two colonies of *A. tenuifolia*, and eleven colonies of *F. fragum* and in all cases, apicomplexan DNA was detected via the PCR assay from planula of these four brooding species. Here, nine sequences generated from a random selection of these samples with the 18S rDNA apicomplexan-specific primers were most

similar (97-100%) to the same GenBank accession (Table 3) from Toller et al. (2002). Again, planulae-free extraction and negative PCR controls for these samples were amplicon-free using all three primer sets.

Apicomplexan Screening of Broadcasting Species from Florida and Belize

In contrast to the brooding scleractinian species, apicomplexan DNA was not detected via the PCR assay from any planulae in the three broadcasting species, *A. palmata*, *M. faveolata* and *D. strigosa*, of the Florida Keys (Table 4a). However, amplicons could be generated using the 18S rDNA universal primers (i.e., SS5 and SS3), implying the presence of suitable template and an absence (or levels incapable of generating a PCR product) of apicomplexan DNA. Furthermore, eggs from one of the *A. palmata* colonies used in the cross-fertilization also produced a negative result for the presence of apicomplexans. Notably, *M. faveolata* planulae from the cross between individuals collected at Alligator and Horseshoe Reefs were the only instance where the alveolate-specific primers (i.e., SS5 and SS3z) produced an amplicon, possibly indicating the presence of *Symbiodinium* in these planulae.

All colonies of *M. faveolata* at Alligator Reef were PCR-positive for apicomplexan DNA (n = 24/24, 100%; 86.1-100, 95% C.I.) from at least one of the three polyps sampled. Specifically, apicomplexan DNA was detected in 21, 22, and 22 of each of 30 samples taken from the bottom, middle and top, respectively, and all but one colony (n = 23/24, 95.8%; 78.9-99.9, 95% C.I.) was positive for the presence of apicomplexans in at least two of the three polyps taken from a colony.

Similarly, apicomplexan DNA was not detected via the PCR assay in any planulae of the four broadcasting species from Belize while all of these same samples produced amplicons using

the universal 18S rDNA primers, again implying the existence of amplifiable DNA. Unexpectedly, apicomplexan DNA was detected in gametes collected from some of the Belize colonies. Specifically, these included sperm from an *A. cervicornis* colony (*n* = 1/6; 16.7%, 0-58.86% C.I.), eggs from an *A. palmata* colony (*n* = 1/3; 33.3%, 0-86.46% C.I.), sperm from two *M. faveolata* colonies (*n* = 2/7; 28.6%, 0-70.96% C.I.), and eggs from one of these same colonies (*n* = 1/7; 14.3%, 0-55.42% C.I.) (Table 4b).

As an additional test of DNA template integrity, twelve samples were randomly selected from the complete set of broadcasting planulae and gamete samples for PCR of a partial region of the mitochondrial COI gene (see Materials and Methods). Subsequent sequencing and BLAST searches of these amplicons found all to be 100% identical to GenBank accessions for corals of their respective genera (Table 5).

Apicomplexan Prevalence and Clonality of *Acropora* sp. in Belize

Apicomplexan DNA was detected in 87.9% (*n* = 29/33; 72.4-95.9, 95% C.I.) of the examined *A. cervicornis* colonies from Belize. This includes five of six colonies that contributed gametes for planula generation. Furthermore, the PCR assay was positive for apicomplexan DNA in 90.3% (*n* = 28/31; 74.5-97.3, 95% C.I.) of the *A. palmata* colonies on the same reef, including all three from which gametes were collected. There was no significant difference between apicomplexan prevalence in adults of the two species (*P* = 0.786).

Utilizing three microsatellite loci, the biased and unbiased P.I. between colonies of *A. cervicornis* were 1.36×10^{-2} and 3.65×10^{-2}, respectively, while the same estimates for *A. palmata* were 3.37×10^{-4} and 1.37×10^{-4}, respectively. Thus, these microsatellite loci have sufficient power to discriminate between ramets belonging to different genets of both species present on the same

reef. From the 33 *A. cervicornis* and 31 *A. palmata* individuals (N) sampled, seven (genet to ramet [N_g/N] ratio= 0.212) and 19 ($N_g/N = 0.623$) unique genets (N_g) were identified for *A. cervicornis* and *A. palmata*, respectively. For *A. cervicornis*, 66.7% (n = 22/33) of the examined individuals were ramets belonging to a single genet (Table 6). Overall, all (n = 7/7) *A. cervicornis* and 84.2% of the *A. palmata* (n = 16/19) genets tested positive for apicomplexan DNA. A single ramet represented the genets (n = 3/19) of *A. palmata* where apicomplexan DNA was not detected in this data set. However, there was no statistical difference between the apicomplexan prevalence of genets represented once and those comprised of multiple ramets (P = 0.530).

Statistical Comparison of Apicomplexan Prevalence in Brooding and Broadcasting Species in Belize

Statistical pairwise comparisons found no significant differences in apicomplexan prevalence between planulae of the brooding coral species examined from Belize before, or after, sequential Bonferroni correction (Table 7). Likewise, no significant difference was identified between all species of broadcasting species when comparing among egg or sperm samples (Table 8). Thus, samples from each category were combined so as to compare apicomplexan prevalence between planulae and/or gametes from all brooding and broadcasting species (i.e., brooding planulae [total prevalence = 100%; n = 25/25], broadcasting eggs [8.7%; n = 2/23], broadcasting sperm [13.0%; n = 3/23], and broadcasting planulae [0%, n = 0/4]). For this overall comparison, significant differences in apicomplexan prevalence were apparent between brooded planulae relative to eggs ($P < 0.001$), sperm ($P < 0.001$), and planulae ($P < 0.001$) of the broadcasting coral species.

Discussion

This study detected the presence of apicomplexan DNA in nearly all brooded planulae of *P. astreoides* from the Florida Keys and Belize. Similar results were obtained from planulae of four other scleractinian species with the same reproductive mode. Additionally, sequences derived from a randomly selected subset of these samples were most similar to a previously identified apicomplexan harbored by Caribbean corals (Toller et al. 2002), implying that primers 18N-F2 and 18N-R1 are specific to these symbionts. For coral species that brood their planulae, vertical transmission is the most parsimonious explanation for this pattern, particularly since apicomplexan DNA also was detected among sampled adult colonies. Conversely, apicomplexan DNA was not detected in any planulae, and at a low frequency in gametes, from five broadcasting scleractinian species despite being present in a majority of adult colonies (Peters 1984; Upton and Peters 1986; Toller et al. 2002; Kirk et al. In Prep). Given this, apicomplexans associated with broadcasting species must be horizontally acquired via another means, such as from the sediment, water-column, or a paratenic (i.e. transport) and/or intermediate host.

Vertical Transmission of Apicomplexan in Brooding Corals

Apicomplexans (by virtue of their DNA) were found to be associated with planulae from a majority of *P. astreoides* colonies (n = 62/66, 93.9%) sampled in two different years on Floridian and Belizean reefs as well as from planulae of four additional brooding species in Belize. To our knowledge, this represents the first evidence of apicomplexans associating with this particular life stage of scleractinian corals. In addition, apicomplexans were detected from

96% of adult *P. astreoides* at the Middle Keys site as well as a majority of individuals of this species sampled elsewhere in the Florida Keys along with the Bahamas, Jamaica and Puerto Rico (Kirk et al., in prep, Peters 1984; Upton and Peters 1986). Thus, this symbiosis appears to be widespread in the Caribbean Sea, with prevalence reaching 100% of host individuals on certain reefs. Apicomplexans were associated with *P. astreoides* larvae collected up to three days after first release, which may represent a form of bet-hedging (sensu Begon et al. 1996) by these symbionts. Constant association with planulae, which represent a highly variable environment (i.e., they exhibit high mortality), would assure symbiont perpetuation via successful host recruits. Interestingly, *P. astreoides* has been increasing in relative abundance throughout the Caribbean Sea (Green et al. 2008), with relatively high numbers of recruits and juveniles seen on many reefs (Bak and Engel 1979; Smith 1992; Vermeij et al. 2011). Taken together, vertical symbiont transmission coupled with high rates of successful recruitment may offer one explanation for the apicomplexan prevalence reported here among colonies of *P. astreoides*.

In general, the planulae of brooding scleractinian species are larger and provisioned with mutualistic dinoflagellates (Baird et al. 2009) as evident by the fact that *Symbiodinium* has been reported in planulae of the five brooding species examined in this study (Szmant-Froelich et al. 1985; Gleason and Wellington 1995; Edmunds et al. 2005; Gleason et al. 2009). Furthermore, vertical transmission of bacterial symbionts also has been documented for *P. astreoides* (Sharp et al. 2012). Thus, transmission of apicomplexans in a vertical fashion for these brooding scleractinian species is parsimonious with both the inheritance pattern of their other symbionts as well as with other host-apicomplexan systems (Bergeron et al. 2000; Hide et al. 2009). Specifically, apicomplexans are directly passed in zygotes and larvae of other aquatic species (Prensier et al. 2008; Fellous and Koella 2009) and in one case, the eugregarine *Diplauxis hatti* is

capable of arresting development to couple its reproduction with that of its host (Prensier et al. 2008). It remains to be determined, however, whether brooding scleractinian species have the ability to acquire these symbionts horizontally as well.

One intriguing aspect of vertical transmission is how coral larvae cope with a potential parasitic infection as larval and juvenile mortality is high (Vermeij and Sandin 2008). In this light, there are several potential explanations. Firstly, infection intensity is positively correlated to virulence in many groups of parasites (Field and Michiels 2005; Hill and Su 2012). Apicomplexan DNA was not detected regularly (~1/20) within single brooded planulae of *P. astreoides*, but they were detected in almost all samples (n = 66/70, 94.3%) after pooling 3-5 larvae. Low abundance within planulae is consistent with this observation and may explain the future survival of the planulae. Secondly, apicomplexans are capable of forming resting stages (e.g. tissue cysts and oocysts) that remain viable in the environment, paratenic and, in some cases, aberrant hosts (Fayer et al. 1998; Lindsay et al. 2003; Fayer et al. 2004; Winiecka-Krusnell et al. 2009; Massie et al. 2010). For example, bradyzoites (i.e. cells within tissue cysts) of the apicomplexan *Toxoplasma gondii* decrease growth and enter a quiescent stage, which is likely caused by slowing the cell cycle (Weiss and Kim 2007). Furthermore, bradyzoites accumulate and utilize amylopectin granules, which serve as a source of energy for survival, sporulation, invasion, and initial development inside the host (Guérardel et al. 2005; Weiss and Kim 2007). Oocysts have been observed in coral tissues (Peters 1984; Upton and Peters 1986) and the putative apicomplexans observed in this study appear to have these amylopectin granules. Thus the coral-associated apicomplexans may not utilize the larval energy stores in favor of their own. Finally, it is hypothesized that vertical transmission will lead to more benevolent symbionts (Ewald 1987; Bull et al. 1991; Mangin et al. 1995; Herre et al. 1999;

Sachs and Wilcox 2006; Magalon et al. 2010). Virulence must be diminished within species that are strictly transmitted vertically or there would be a severe fitness cost for infected individuals, leading to strong selection for those that are unparasitized (Lipsitch et al. 1995; Mangin et al. 1995). Low abundance, forming resting stages (i.e. slowing metabolism), and an evolutionary decrease in pathogenicity are not mutually exclusive hypotheses, and a combination of these scenarios (or others) may explain the predominance of apicomplexans harbored by brooded planulae.

Horizontal Transmission of Apicomplexans in Broadcasting Spawners

In contrast to the brooding species, apicomplexans were not detected in the planulae of any broadcasting scleractinian species examined in Florida or Belize. As most of the adult colonies in this and others studies (Upton and Peters 1986; Toller et al. 2002) harbor apicomplexans, they must be acquired horizontally. This mirrors the transmission pattern of other symbionts by most broadcasting scleractinian species (Baird et al. 2009; Sharp et al. 2010), including *Symbiodinium* and bacterial symbionts for the five hosts examined here (Bassim and Sammarco 2003; Wellington and Fitt 2003; Schwarz et al. 2008; Sharp et al. 2010; Mason and Cohen 2012).

Horizontal transmission involves the encounter of a suitable host and a symbiont, either in the environment and/or via paratenic (i.e., intermediate) hosts. Since apicomplexan DNA was detected in a few gamete samples from three of the broadcasting species in Belize (n = 4/54, 7.4%), this may represent one mechanism of horizontal transmission. Specifically, the five broadcasting species examined here are hermaphroditic, releasing positively buoyant gametes in bundles that break apart and cross-fertilize in surface waters (Szmant 1986). These bundles are

bound in a mucous covering, allowing negatively-buoyant sperm to float (Padilla-Gamiño et al. 2011). Given that apicomplexans were not detected in larvae generated from the gametes in these bundles, it is unlikely they are inside the oocytes or spermatozoa themselves. Instead, they are most likely associated with the mucous covering of the gamete bundles prior to fertilization. Corallivorous fish may also play a role in horizontal transmission by acting as paratenic hosts. For example, fish predation of broadcasting gamete bundles is significant (Babcock et al. 1986; Pratchett et al. 2001) and ingestion of the oocysts may occur at this stage. Fish defecation of viable cells upon the surface of coral colonies may help initiate this symbiosis. Such a route of transmission has been hypothesized for these apicomplexans (Upton and Peters 1986) and other coral symbionts like *Symbiodinium* (Muller-Parker 1984; Porto et al. 2008). It may also be possible that apicomplexans are freely transported in the water column as viable oocysts from terrestrial host species are detected in filter-feeding mussels near sources of runoff (e.g. Miller et al., 2005). Lastly, established colonies and benthic sediments may act as horizontal sources of apicomplexans for broadcasting planulae recruiting into a population. For the latter, *Symbiodinium* capable of forming symbioses (Carlos et al. 1999; Coffroth et al. 2006) as well as the alveolate parasites of bivalves, *Perkinsus* spp. (Bushek et al. 2002; Audemard et al. 2004; Park et al. 2010), have been recovered from this habitat. Thus, apicomplexans, either as physiologically active cells or in resting stages (i.e. oocysts), may reside in the benthos until being horizontally acquired by newly settled planulae of broadcasting scleractinian species.

Although there are drawbacks to horizontal transmission (i.e. finding a suitable host), there are advantages. Namely, horizontally acquired symbionts tend to be more virulent than those transmitted vertically with virulence balancing a tradeoff among parasite growth, transmission, and host mortality (Anderson and May 1979; Frank 1996a; Lipsitch et al. 1996).

Thus, growth and consequent virulence of the symbiont is able to increase, which can be a selective advantage. In addition, avoiding the higher mortality stages of broadcast spawning corals might increase survivorship of these apicomplexans. Szmant (1986) hypothesized that brooder planulae would have higher survival than broadcasted planulae as they are larger and reach competency sooner, thus avoiding long planktonic duration that can lead to high mortality. A combination of increased virulence and decreased planulae size, which decreases survivorship (Isomura and Nishihira 2001), may make vertical transmission an unstable strategy in planulae of broadcasting spawners.

Clonality and Host as a Symbionts' Habitat

Estimates of clonality reported here for the two *Acropora* species are consistent with those from reefs in the Caribbean Sea (Baums et al. 2006; Vollmer and Palumbi 2007). In fact, the high prevalence of clonal growth in these species can lead to >20 individuals on some reefs being ramets derived from a single genet (Neigel and Avise 1983; Baums et al. 2005b; Baums et al. 2006). In this context, nearly all ramets of a given *Acropora* spp. genet harbored apicomplexans. Transmission of symbionts among individuals due to clonal reproduction of a host also has been documented in other systems. For example, bacterial symbionts of aphids and flatworms are transmitted to clones during asexual reproduction (Sandström et al. 2001; Dirks et al. 2012). In addition, myxozoan parasites are propagated in freshwater bryozoans by fragmentation of the host (Morris and Adams 2006), leading to high prevalence (Hill and Okamura 2007). Thus, clonal propagation increases symbiont transmission and can contribute

towards the high apicomplexan prevalence seen among ramets of the same scleractinian coral genet.

It has been demonstrated here that scleractinian corals can acquire apicomplexan symbionts via three routes of transmission (i.e., vertically, horizontally, and clonal propagation) over two different life stages (i.e., larval and adult). While this information furthers our knowledge regarding this widespread, but under-recognized symbiosis, numerous questions remained to be addressed. For example, are there persistent populations of scleractinian-associated apicomplexans present in the environment? Are intermediate/paratenic hosts involved in these relationships and, if so, do they potentially provide transportation to symbionts dispersing between and among reefs? Do apicomplexans infect broadcast spawning corals as recruits, as *Symbiodinium* does, or adults? Lastly, do coral-associated apicomplexans exhibit host specificity or are they "generalists", being shared between both brooding and broadcasting species? Given that these apicomplexans have been found in coral species throughout the Caribbean Sea (Peters 1984; Upton and Peters 1986; Goulet and Coffroth 2003) and the Eastern Pacific Ocean (Toller et al. 2002), transmission appears to be efficient. Therefore, mechanisms of long distance transport for apicomplexans associated with broadcasting spawner species remains to be elucidated.

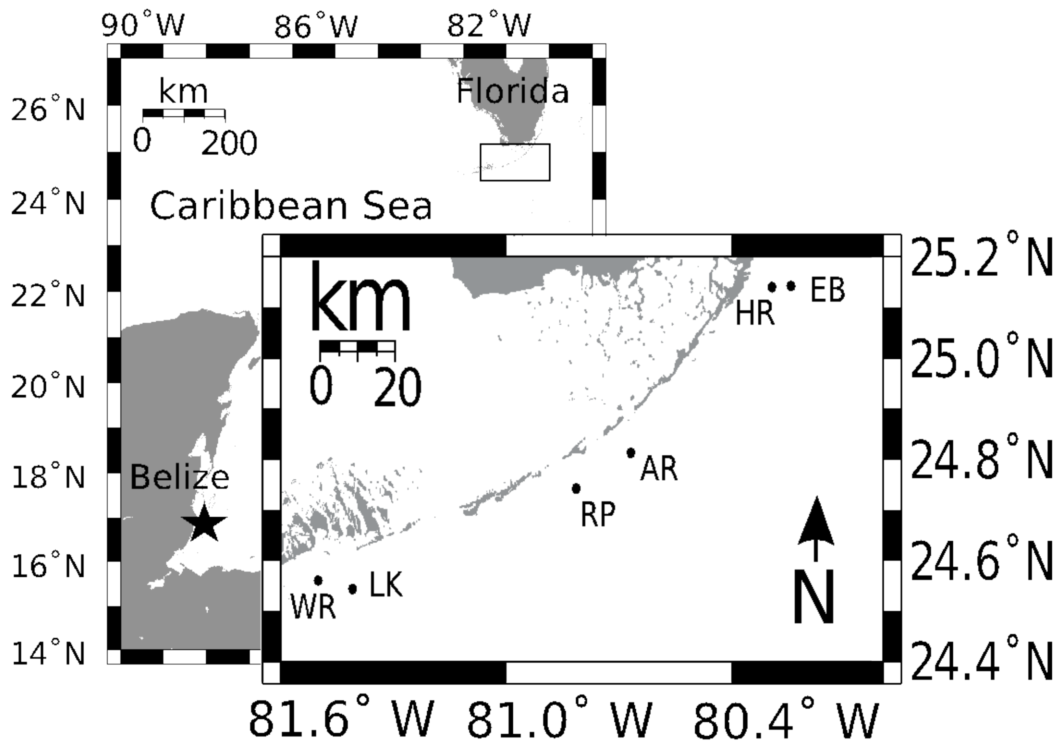

Fig 1. Map of sampling locations with inset of Florida reefs. Elbow Reef=EB, Horseshow Reef=HR, Alligator Reef=AR, Rubble Piles=RP, Looe Key=LK, Wonderland Reef=WR.

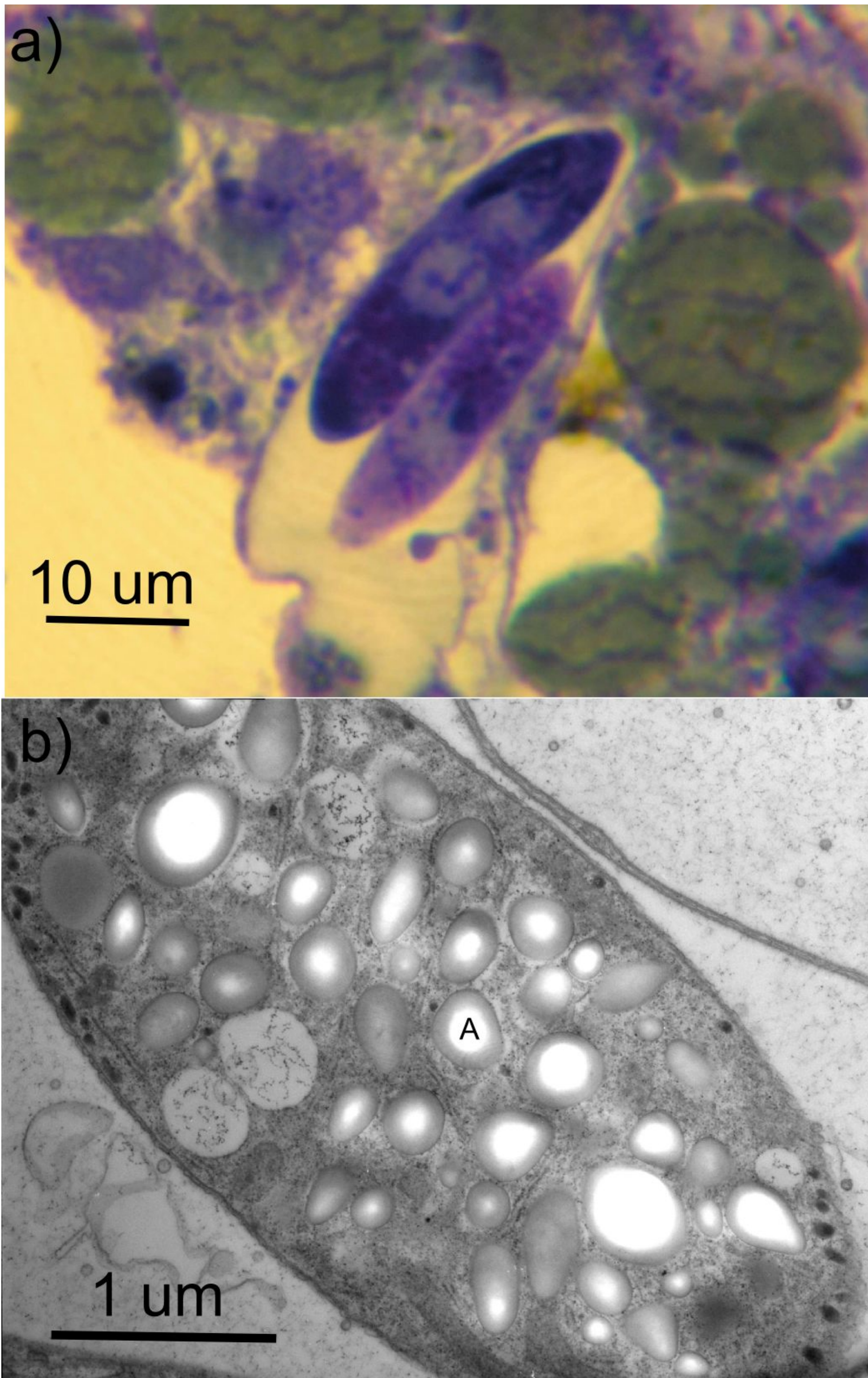

Fig 2. a) Light microscopy image and b) transmission electron micrographs of putative apicomplexans associated with *Porites astreoides* larvae. A= potential amylopectin granule.

Table 1 Species of brooding species collected in Belize. Depth is the collection depth, Collect Ad is the date the adults were collected, Collect Spn is the period of release of brooded larvae, No. Adults and No. Spawn are the number of colonies collected total and the number that released brooded larvae.

Species	Depth	Collect Ad	Collect Spn	No. Adults	No. Spawn
P. astreoides	1 m	2 June	3-8 June	22	6
A. agaricites	10 m	2 June	3-6 June	~14	5
A. tenuifolia	1-3 m	1 June	3 June	~12	2
M. ferox	20 m	2 June	5 June	~15	1
F. fragum	1-3 m	9 June	11-14 June	21	6
F. fragum	1-3 m	10 August	12 August	20	5

Table 2 Broadcast spawning species collected in Florida (FLA) and Belize (BEL). Date of gamete collection and fertilization (Fert) is noted as it whether Eggs (E), sperm (S), both (E/S), or none (0) were collected. The number of parents utilized in the gamete cross and the Parental Reef with corresponding GPS coordinates are also given.

Loc	Species	Fert	E/S	# Par	Parental Reef	GPS
FLA	*D. Strigosa*	19-Aug	0	2	Horseshoe Reef	N 25.1399°, W 80.2944°
	M. faveolata	20-Aug	0	3-5	Horseshoe Reef	
	M. faveolata	19-Aug	0	10-15	Looe Key	N 24.5449°, W 81.4094°
	M. faveolata	19-Aug	0	10-15	Alligator Reef	N 24.8129°, W 80.6695°
	A. palmata	16-Aug	E	1	Elbow Reef	N 25.1435°, W 80.2574°
			E	1	Horseshoe Reef	
	A. palmata	17-Aug	0	1	Molasses Reef	N 25.0102°, W 80.3733°
			0	1	Horseshoe Reef	
BEL	*D. Strigosa*	19-Sep	0	3	CBC Reef	N 16.8025°, W 88.0819°
	A. palmata	16-Aug	E/S	5	CBC Reef	
	A. cervicornis	17-Aug	E/S	7	CBC Reef	
	M. faveolata	19-Sep	E/S	7	CBC Reef	
	M. franksi	19-Aug	E/S	5	CBC Wall	N 16.7797°, W 88.0753°

Table 3 The blastn report for the larvae of brooding species samples from Florida (Fla) and Belize (Bel) amplified with the 18S rDNA apicomplexan-specific primers. Blast scores (Score), percent identity (% Id) and E-values are provided as well as the NCBI GenBank accession numbers (Acc #) and species and molecule (Top Hit) of the top blastn hit.

Fla/Bel	Query Species	E-Values	% Id	Score	Acc #	Top Hit
Bel	*A. agaricites*	0	100%	583	AF238264	Coral symbiont (18S rDNA)
Bel	*A. tenuifolia*	0	99%	704	AF238264	Coral symbiont (18S rDNA)
Bel	*F. fragum*	0	100%	672	AF238264	Coral symbiont (18S rDNA)
Bel	*F. fragum*	0	100%	622	AF238264	Coral symbiont (18S rDNA)
Bel	*F. fragum*	0	100%	728	AF238264	Coral symbiont (18S rDNA)
Bel	*F. fragum*	2.0×10^{-104}	97%	210	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	0	99%	704	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	7.0×10^{-54}	99%	119	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	0	99%	557	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	5.0×10^{-105}	99%	211	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	0	98%	599	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	1.0×10^{-56}	99%	124	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	0	97%	588	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	0	97%	586	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	0	99%	723	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	4.0×10^{-56}	99%	123	AF238264	Coral symbiont (18S rDNA)
Fla	*P. astreoides*	3.0×10^{-57}	99%	125	AF238264	Coral symbiont (18S rDNA)
Bel	*M. ferox*	0	99%	737	AF238264	Coral symbiont (18S rDNA)
Bel	*P. astreoides*	0	99%	539	AF238264	Coral symbiont (18S rDNA)
Bel	*P. astreoides*	6.0×10^{-65}	99%	139	AF238264	Coral symbiont (18S

rDNA)

Table 4 The number of times apicomplexan DNA was detected (Present) in larvae a) or gametes b) from broadcasting spawner species in Florida or the Bahamas. The parental reef (Reef) and time after fertilization (Fert Time) is noted for the larvae. The larvae were generated from pooled gametes from all corals, so the sample size is always 1. The total number of colonies providing gametes (Total) also are reported. The 95% confidence interval (C.I.) for prevalence were calculated using Sterne's exact method for all colonies that were PCR positive for apicomplexan DNA in at least one sample. **Florida Reefs:** HR=Horseshoe Reef, AR=Alligator Reef, LK=Looe Key, EB=Elbow Reef. **Belize:** CBC=Carrie Bow Cay.

		Species	Reef	Fert Time	Present
a)	**Florida**	*D. Strigosa*	HR	40 hr	0
				4 d	0
		M. faveolata	AR	6 d	0
			HR	18 hr	0
				40 hr	0
			LK	4 d	0
				7 d	0
		A. palmata	HR x EB	18 hrs	0
				5 d	0
				9 d	0

		Species	Reef	Gamete	Present	Total	95% C. I.
b)	**Florida**	*A. palmata*	HR	Eggs	0	2	
	Belize	*A. palmata*	CBC	Eggs	1	3	0-86.46%
			CBC	Sperm	0	3	
		A. cervicornis	CBC	Eggs	0	6	
			CBC	Sperm	1	6	0-58.86%
		M. faveolata	CBC	Eggs	1	7	0-55.42%
			CBC	Sperm	2	7	0-70.96%
		M. franksi	CBC	Eggs	0	5	
		(August)	CBC	Sperm	0	5	
		M. franksi	CBC	Eggs	0	5	
		(September)	CBC	Sperm	0	5	

Table 5 The blastn report for the larvae (L), eggs (E) and sperm (S) broadcasting spawner samples from Florida (Fla) and Belize (Bel) amplified with the COI primers. Blast Scores, percent identity (% Id) and E-Values are provided as well as the NCBI GenBank accession numbers (Acc #) and species and molecule (Top Hit) of the top blastn hit.

Fla/Bel	**Query Species**	**E/S/L**	**E-Values**	**% Id**	**Score**	**Acc #**	**Top Hit**
Bel	*A. cervicornis*	S	$1.0x10^{-143}$	100%	280	AY451340	*Acropora cervicornis* (COI)
Bel	*A. cervicornis*	E	0	100%	553	AY451340	*Acropora cervicornis* (COI)
Bel	*A. cervicornis*	E	0	100%	549	AY451340	*Acropora cervicornis* (COI)
Bel	*A. palmata*	E	0	100%	613	AY451341	*Acropora palmata* (COI)
Bel	*D. strigosa*	L	0	100%	578	AY451349	*Diploria strigosa* (COI)
Fla	*M. faveolata*	L	0	100%	374	HQ203282	*Montastraea annularis* (COI)
Bel	*M. faveolata*	E	0	100%	522	HQ203283	*Montastraea annularis* (COI)
Bel	*M. faveolata*	L	0	100%	549	HQ203284	*Montastraea annularis* (COI)
Bel	*M. faveolata*	E	0	100%	454	HQ203285	*Montastraea annularis* (COI)
Fla	*M. faveolata*	L	0	100%	506	HQ203286	*Montastraea annularis* (COI)
Fla	*M. faveolata*	L	0	100%	554	HQ203287	*Montastraea annularis* (COI)
Bel	*M. franksi*	S	0	100%	426	HQ203288	*Montastraea annularis* (COI)

Table 6 The apicomplexan prevalence in clones of A. palmata and A. cervicornis. For each clone the number of ramets (i.e. individuals per clone; N ramet) and the number infected (N infect) is given as is the 95% confidence interval (C.I.).

Acropora palmata **(n=31)**

Clone	N ramet	N infect	prevalence	95% C.I.
1	8	8	1	.6354-1
2	3	3	1	.3685-1
3	2	2	1	.2237-1
4	2	2	1	.2237-1
5	2	2	1	.2237-1
6	1	1	1	0.05-1
7	1	1	1	0.05-1
8	1	1	1	0.05-1
9	1	1	1	0.05-1
10	1	1	1	0.05-1
11	1	1	1	0.05-1
12	1	1	1	0.05-1
13	1	1	1	0.05-1
14	1	0	0	0-.9499
15	1	1	1	0.05-1
16	1	0	0	0-.9499
17	1	0	0	0-.9499
18	1	1	1	0.05-1
19	1	1	1	0.05-1

Acropora cervicornis **(n=33)**

Clone	N ramet	N infect	prevalence	95% C.I.
1	22	18	0.818	.6111-.9354
2	3	3	1	.3685-1
3	3	3	1	.3685-1
4	2	2	1	.2237-1
5	1	1	1	0.05-1
6	1	1	1	0.05-1
7	1	1	1	0.05-1

Table 7 Pairwise comparison of prevalence between larvae of brooding species in Belize. P values from Fisher's exact test are presented. None were significant before or after Bonferroni correction.

P. astreoides	*M. ferox*	*F. fragum*	*A. agaricites*	*A. tenuifolia*	
XXX	1.000	1.000	1.000	1.000	*P. astreoides*
	XXX	1.000	1.000	1.000	*M. ferox*
		XXX	1.000	1.000	*F. fragum*
			XXX	1.000	*A. agaricites*
				XXX	*tenuifolia*

Table 8 Pairwise comparison of prevalence between eggs (top) and sperm (bottom) of broadcasting spawning species in Belize. P values from Fisher's exact test are presented. None were significant before or after Bonferroni correction.

M. faveolata	*M. franksi*	*A. cervicornis*	*A. palmata*	
XXX	1.000	1.000	1.000	*M. faveolata*
0.205	XXX	1.000	0.177	*M. franksi*
1.000	0.483	XXX	0.258	*A. cervicornis*
1.000	1.000	1.000	XXX	*A. palmata*

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Chapter 5. Evolutionary History of the Coral-Associated Apicomplexans

ABSTRACT. Apicomplexans (Alveolata: Apicomplexa) are an important parasitic group, but phylogenetic relationships among the major lineages are only now being elucidated in part due to poor taxon sampling. One of the most overlooked sources of novel, as well as recognized, apicomplexan lineages are invertebrates, and corals in particular. A single coral-associated species has been formally described and based on life history, taxonomically united within the apicomplexan order Agamococcidiorida with the rhytidocystids, which are symbionts of polychaete worms. To test this phylogenetic hypothesis and to determine the evolutionary relationships among coral-associated symbionts, 18S rDNA sequences were generated from a broad sampling of 16 scleractinian coral and 3 gorgonian host species. Maximum likelihood and Bayesian phylogenetic trees recovered a monophyletic clade containing all coral apicomplexan symbionts with strong support. This coral-associated clade was closely related to the coccidian families Sarcocystidae and Eimeriidae, which both contain economically important taxa. However, the coral-associated clade was phylogenetically distinct from the rhytidocystids and a constraint tree forcing such a grouping was significantly worse than the best tree. Within the clade there was little evidence of specificity and constraint trees enforcing monophyly of apicomplexans isolated from five of the seven same coral families also were significantly worse than the best tree. Likewise, a constraint tree binning coral-associated apicomplexans by the two major scleractinian coral clades (complex and robust) also was rejected. Thus, there was little

support for Agamococcidiorida or specificity within the clade, which may be indicative of host-switching and/or incomplete lineage sorting.

INTRODUCTION

Molecular sequence data have considerably improved our understanding of eukaryotic evolutionary relationships over the last 25 years (Simpson and Roger 2004; Adl et al. 2005; Keeling et al. 2005; Adl et al. 2007). Six putatively monophyletic "supergroups", based on ultrastructural, phylogenetic and genomic data, have been erected to reflect this current classification system (Adl et al. 2005). However, the relationships between and among supergroups remain in flux (Parfrey et al. 2006; Burki et al. 2007; Burki et al. 2008; Roger and Simpson 2009; Parfrey et al. 2010; Walker et al. 2011). A prime example is the former supergroup Chromalveolata, first hypothesized by Cavalier-Smith (1999) to involve a secondary endosymbiotic event between a photosynthetic red-alga lineage and the precursor to several eukaryotic groups. Today, both phylogenomics and other genetic evidence suggest Chromalveolata and another supergroup, Rhizaria (Burki et al. 2007; Hackett et al. 2007; Burki et al. 2008; Elias et al. 2009; Burki et al. 2010; Cavalier-Smith 2010) belong to a single, new supergroup uniting the **s**tramenopiles, **a**lveolates and **r**hizaria (SAR) (Burki et al. 2007). This has important implications in understanding the evolutionary history of the encompassed taxa as many have lost their photosynthetic capacity and evolved into obligate parasites. One group is the apicomplexans (Alveolata: Apicomplexa), who retain a vestigial plastid (McFadden et al. 1996; Wilson and Williamson 1997). However, as significant as it is to understand the relationships between eukaryotic supergroups, it is equally important to determine the phylogeny

of member clades as well. Due to their parasitic nature and impact in areas such as the quality of human life, elucidating the evolutionary history of the apicomplexans can be considered of utmost importance.

Historically, Apicomplexa has been split into four major lineages, the haemosporidians, piroplasms, coccidians, and gregarines, based on motility, life-cycle, host range, tissue localization, and morphology (Perkins et al. 2000). Along with this, small subunit (18S) ribosomal DNA (rDNA) sequence data have supported some clades while highlighting morphological synapomorphies that unite particular groups. Conversely, these data also have reassigned taxonomic placements. For example, *Cryptosporidium parvum*, an opportunistic human pathogen, was moved from the coccidians to the gregarines (Barta and Thompson 2006; Saffo et al. 2010; Rueckert et al. 2011b), based on analyses of 18S rDNA data. However, evolutionary relationships among many of the major apicomplexan groups remain elusive in part due to poor taxon sampling (Leander 2008; Morrison 2008,2009). Historically, most phylogenetic studies focused on taxa infecting hosts with human economic importance and little attention has been paid to others, such as invertebrates (Leander et al. 2003; Kopecna et al. 2006; Leander 2008; Clopton 2009; Rueckert et al. 2010; Rueckert et al. 2011a). This is unfortunate since many apicomplexans from invertebrates represent previously unknown species (Rueckert and Leander 2008; Dubey et al. 2009; Rueckert and Leander 2009b). Thus, describing the diversity of apicomplexans in invertebrates will clarify both ecological distributions of known parasites and the evolutionary history of the involved (and other) taxa.

One understudied group of host invertebrates is the scleractinian and gorgonian corals (Cnidaria: Anthozoa). Scleractinians (Hexacorallia: Scleractinia) are the foundation of the coral reef ecosystem and phylogenetically group into two clades: complex and robust (Romano and

Palumbi 1996). Within these clades, multiple phylogenetic studies have clarified our understanding of coral evolutionary history (Kerr 2005; Fukami et al. 2008; Kitahara et al. 2010). Likewise, gorgonians (Octocorallia: Gorgonacea) are numerically dominant on shallow Caribbean reefs and their phylogenetic relationships also have been well-elucidated (Sanchez et al. 2003; McFadden et al. 2006). Scleractinians and gorgonians harbor numerous bacterial and protist symbionts, including apicomplexans (Upton and Peters 1986; Toller et al. 2002; Knowlton and Rohwer 2003; Goulet and Coffroth 2004). A single apicomplexan species, *Gemmocystis cylindrus*, was formally described and due to its unusual life-cycle (i.e. no merogony or sporogony), it was placed within the coccidian order Agamococcidiorida (Upton and Peters 1986), which included a single other genus, *Rhytodocystis* (Levine 1979). Although no 18S rDNA data exist for *G. cylindrus*, *Rhytodocystis* spp. have recently been described with corresponding sequence data and found to phylogenetically group with a symbiont isolated from a giant clam in a clade called the "rhytidocystids" (Leander and Ramey 2006; Rueckert and Leander 2009a). Therefore, these taxa can be utilized towards determining whether coral-associated apicomplexans and *Rhytodocystis* spp. share a most recent common ancestor, as hypothesized by their mutual inclusion in Agamococcidiorida.

To test this hypothesis, 18S rDNA sequences were utilized to resolve phylogenetic relationships 1) among apicomplexans associated with scleractinian and gorgonian hosts in the Caribbean Sea and 2) determine their evolutionary placement within the Apicomplexa. Sixteen coral species, including both host clades (i.e. complex and robust) as well as three gorgonian species, were chosen to provide wide-breadth of diversity and to test for potential specificity between hosts and their symbionts. The host species *Dendrogyra cylindrus* also was specifically targeted since it is the host of the type species, *G. cylindrus*. Lastly, apicomplexan 18S rDNA

sequences isolated from other hosts of the same ecosystem were included to determine if symbionts from corals and other coral reef taxa belong to a common lineage. These samples encompassed two coccidians from coral reef fishes and an apicomplexan from a giant clam, which has previously grouped with the rhytidocystids.

MATERIALS AND METHODS

Coral samples utilized. Samples were collected from 16 Caribbean scleractinian coral species representing four families in the robust and complex clades, respectively (Table 1). Two or three species were selected from each of the families to test the hypothesis of specificity, which assumes monophyly within the families. The one exception was Siderastreidae, which contains only two Caribbean species. Apicomplexan DNA was not detected in any colonies of *Siderastrea radians* examined (n = 15) and this scleractinian family was confined to symbionts exclusively from *Siderastrea siderea*. As several host families are paraphyletic, host species were chosen to include members that exhibited common ancestry in a recent host phylogeny (Fukami et al. 2008). Two colonies of *Porites astreoides* were included to determine if symbionts from the same host were identical and/or most closely related to each other. Samples also were collected from three gorgonian species commonly found in the Caribbean Sea (Kinzie 1973; Jordán-Dahlgren 2002). These host species were selected to include sister genera, *Gorgonia* and *Pseudopterogorgia* (Family Gorgoniidae) as well as a more distant related genus, *Muriceopsis* (Family Plexauridae) (Sanchez et al. 2003).

DNA isolation, PCR amplification, and cloning. Scleractinian tissue was removed from the skeleton using a water-pik (LaJeunesse 2002) and DNA isolated using a modified Promega

Wizard protocol as described in LaJeunesse et al. (2003). DNA was extracted from gorgonian samples using the 2xCTAB method (Coffroth et al. 1992).

To specifically target apicomplexan DNA while excluding other coral symbionts, an ~860 bp fragment of the 18S small subunit ribosomal DNA (18S rDNA) molecule was amplified using the apicomplexan-specific primers 18N-F2 and 18N-R1 under previously published conditions (Toller et al. 2002) and a touchdown PCR protocol with a 5 min initial denaturation step at 94°C, followed by 45 sec at 94°C, 45 sec at 60°C, decreasing 1°C/cycle until 50°C was reached, and a 1 min extension at 72°C. This was immediately followed by 30 additional cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min, and a 30 min final extension at 72°C. Amplicons from all 20 samples were sequenced using the forward primer (18N-F2) to determine the dominant (i.e. most abundant) 18S rDNA copy from each coral host.

To acquire nearly full-length (~1,800 bp) 18S rDNA sequences for the coral hosts, two overlapping segments of the molecule were individually amplified. Specifically, samples were amplified using the universal eukaryotic primers 18S-82F and 18S-1498R (Slapeta et al. 2005) coupled with 18N-R1 and 18N-F2 (Toller et al. 2002), respectively. The first half of the molecule (18Sa) was amplified using a 5 min denaturation step, and 35 cycles of 15 sec at 94° C, 15 sec at 55° C, 30 sec at 72° C, followed by a 30 min extension at 72° C. The second half of the molecule (18Sb) was amplified utilizing the touchdown PCR protocol detailed above. PCR products from 18Sa and 18Sb were immediately cloned using the pCR® 2.1 TOPO vector and TOP10 chemically competent cells (Invitrogen, Life Technologies, USA). Twenty bacterial colonies were haphazardly picked and PCR screened for insert size. From these, five clones per coral host sample were chosen by a random number generator. Clones were grown overnight in

LB broth, plasmids were purified by alkaline lysis extraction (Birnboim 1983), and then sequenced bi-directionally by HtSeq (Seattle, Washington).

Assembly and alignment of 18S rDNA sequences. Vector sequence was removed and bi-direction reads assembled for 18Sa and 18Sb separately using Geneious (Drummond et al. 2011). To identify potential PCR chimeras within the dataset, sequences of 18Sa and 18Sb were assessed using a 300 bp sliding window and compared to all other sequences on both sides of the breakpoint with a partial tree-building approach based on distance (i.e. sequence similarity) in Bellerophon (Huber et al. 2004). Conflicting topologies derived from the sequence to the left or right of the breakpoint are indicative of chimeras and were removed from the dataset. The 18Sa and 18Sb sequences were then aligned to the dominant apicomplexan-copy from the same coral sample if they contained 100% sequence identity and no gaps to generate the almost full-length 18S rDNA. These consensus sequences were rechecked using Bellerophon to ensure no chimeric sequences were artificially generated in this process.

The following Apicomplexa 18S rDNA sequences were downloaded from GenBank to place those associating with corals into an evolutionary context with other taxa from the group: *Adelina bambarooniae* (AF494058), *Adelina dimidiata* (DQ096835), *Adelina grylli* (DQ096836), *Aggregata eberthi* (DQ096838), *Ascogregarina taiwanensis* (JX131297), *Babesia rodhaini* (M87565), *Besnoitia besnoiti* (AF109678), *Caryospora bigenetica* (AF060976), *Choleoeimeria* sp. (AY043207), *Cryptosporidium parvum* (DQ898159), *Cryptosporidium serpentis* (AF093502), *Cystoisospora felis* (L76471), *Dactylosoma ranarum* (HQ224957), *Eimeria leucisci* (GU479649), *Eimeria intestinalis* (EF694012), *Eimeria maxima* (DQ538348), *Frenkelia glareoli* (AF009245), *Goussia janae* (AY043206), *Gregarina chortiocetes* (L31841), *Gregarina niphandrodes* (AF129882), *Hepatozoon ayorgbor* (EF157822), *Hepatozoon canis*

(EU289222), *Hepatozoon felis* (AY628681), *Isospora orlovi* (AY365026), *Isospora suis* (U97523), *Klossia helicina* (HQ224955), *Lankesterella minima* (AF080611), *Lankesteria abbotti* (DQ093796), *Lecudina polymorpha* (AY196706), *Lecudina tuzetae* (AF457128), *Leidyana migrator* (AF457130), Lithocystis sp. (DQ093795), *Mattesia geminata* (AY334568), *Monocystis agilis* (AF457127), *Neospora caninum* (U03069), *Pterospora floridiensis* (DQ093794), *Pterospora schizosoma* (DQ093793), *Rhytidocystis cyamus* (GQ149767), *Rhytidocystis polygordiae* (DQ273988), *Sarcocystis canis* (DQ146148), *Sarcocystis hominis* (AF176945), *Theileria annulata* (DQ287944), *Theileria parva* (HQ684067), and *Toxoplasma gondii* (U03070). Additionally, sequences from unidentified apicomplexan species from aquatic environments (AF372780, GQ330636, AY664971, AY426932, GU825688), the coral reef environment (two isolated from fishes, *Lutjanus kasmira* and *Mulloidichthys pfluegeri*, (HM117907 and HM117908) and one from a giant clam, *Tridacna crocea* (AB000912), and three apicomplexan 18S rDNA clones (AF238264-6), originally isolated in Panama from the coral *Montastraea* sp., were included.

The final data matrix encompassed 76 18S rDNA sequences including *Chromera velia* (Genbank accession number JN986789), a photosynthetic alveolate branching near the base of the apicomplexans (Moore et al. 2008; Janouskovec et al. 2011), which served as the outgroup in phylogenetic analyses. Sequences were aligned using the L-INS-in algorithm with 1000 iterative refinements in MAFFT v6.861b (Katoh and Toh 2008). The alignment was inspected by eye and further refined in Mesquite v2.75 (Maddison and Maddison 2011), as recommended for improved accuracy (Edgar and Batzoglou 2006). To increase the likelihood of comparing homologous characters in the alignment, ambiguously aligned regions were removed using Gblocks v0.91b (Castresana 2000). “Relaxed” parameters were used to trim the alignments as

they performed best on simulated datasets of similar length (Talavera and Castresana 2007). Under these parameters, gaps were allowed in half of the sequences, which permitted inclusion of shorter environmental sequences (e.g. marine water column sample: GU825688, 1095 bp) without truncating the final alignment to that length. This also allowed the inclusion of characters within insertion/deletion events (indels) that might provide additional phylogenetic information.

Phylogenetic analyses. The most appropriate model of evolution for the alignment was selected using hierarchical likelihood ratio tests (hLRT) and Akaike Information Criterion corrected for sample size (i.e. number of sites in the alignment: AICc (Hurvich and Tsai 1989)) in jModelTest v0.1.1 (Posada 2008). Both methods selected the general time reversible model with rate variation across sites as modeled by the gamma distribution (GTR+γ) (Sullivan and Joyce 2005) and this model of evolution was utilized in all phylogenetic analyses. Maximum likelihood (ML) trees were inferred in RAxML v.7.0.4 (Stamatakis 2006), with nodal confidence assessed using 1,000 bootstrap replicates. Trees also were generated using Bayesian inference (Huelsenbeck et al. 2001) in MrBayes v3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). All Bayesian phylogenies comprised of the best tree from 2 runs (with 4 chains per run) of $10x10^6$ generations, sampling every $10x10^3$ steps after removing the first 25% (i.e. $2.5x10^5$) as burnin. The runs were terminated after $10x10^6$ generations as the average standard deviation between them was less than 0.02, suggesting both converged on a similar topology despite starting in different, randomly-selected, areas of tree space (Ronquist et al. 2009).

Hypothesis testing of phylogenetic trees. To test the hypothesis that the coral-associated apicomplexans are the sister taxa to the rhytidocystids, which also included the clam symbiont,

the topology of the best ML tree was compared to a constraint tree (i.e. coral symbionts and rhytidocystids forced to be sister taxa *a priori*). Likewise, a constraint tree uniting the coral-associated apicomplexans and coccidians infecting reef fishes was created to test whether they belong to a single lineage. Additionally, the monophyly of apicomplexans collected from the coral families belonging to the complex clade (i.e., Acroporidae, Agariciidae, Poritidae, Siderastreidae) and to the robust clade (i.e., Faviidae, Meandrinidae, Pocilloporidae, Mussidae) separately to determine if specificity was apparent in this group. Separate trees constraining apicomplexans specifically to each of the seven (no *Siderastreidae* test was possible, as only one species was examined) coral families also were tested. Lastly, three previously sequenced apicomplexans from *Montastraea* sp. (Toller et al. 2002) were included within the Faviidae to test monophyly of this group. ML constraint trees were generated for all ten hypotheses (Table 2) using the GTR+γ model and compared to the most likely ML tree generated above using SH tests (Shimodaira and Hasegawa 1999) in RAxML v.7.0.4.

RESULTS

Final data matrix. Twenty coral-associated apicomplexan 18S rDNA consensus sequences generated in this study were used in all analyses, ranging in size from 1705-1712 bp. The alignment, which included the other apicomplexans and *C. velia*, was 2014 bp including gaps. This included 1031 parsimony-informative, 340 uninformative, and 643 constant (i.e. invariable) characters. Following removal of ambiguously aligned regions, the final data matrix contained 1436 total characters with included gaps, with 727 parsimony-informative, 237 uninformative, and 472 constant characters.

Phylogenetic analysis. Strong support (99% bootstrap [BS] support) was found for a single clade containing all coral-associated apicomplexans, as well as a single environmental sample from the Southern Caribbean Sea, in the ML tree (Fig. 1). This clade grouped with the Sarcocystidae and the Eimeriidae, two coccidian families, with moderate (71% BS) support. Three distinct groups, based on >85% BS support, were identified within the coral-associated apicomplexans. Firstly, apicomplexans associated with gorgonians represented Group I with strong (92% BS) support. Notably, a symbiont of *P. astreoides* was basal to this group with moderate (77% BS) support. The second group united symbionts from *Madracis decactis*, *Agaricia grahamae*, *Scolymia* sp. and a *Mycetophyllia* sp. (90% BS, Group II). The third group (Group III) encompassed apicomplexans from *Dendrogyra cylindrus*, *Madracis mirabilis*, *Acropora palmata*, *Mussa* sp., *Porites porites*, and the other symbiont from *P. astreoides* with strong (86% BS) support. None of the symbionts grouped with others isolated from con-familial corals (Fig. 1).

The phylogeny based on Bayesian inference also recovered a monophyletic clade of coral-associated apicomplexans and the same environmental sample (Fig. 2). Once again, the coral-associated apicomplexan clade was united with the Eimeriidae and the Sarcocystidae with a posterior probability (PP) of 0.84 (Fig. 2). Likewise, the three clades of coral-associated apicomplexans were recovered with strong support (PPs of 1.00, 0.98, and 1.00, respectively). Thus, while the Bayesian tree possessed minor differences in its basal topology relative to the ML tree (e.g., placement of the coccidian *Aggregata eberthi*; see Discussion), the inferred position of the coral-associated clade, and the groups within it, were identical across methodologies (Fig. 2).

Hypothesis testing. There were significant differences between the best ML tree and 8 of the 10 hypothesis-testing constraint trees. A clade uniting the coral-associated apicomplexans with the rhytidocystids significantly decreased the likelihood of the phylogeny (Table 3). Similarly, the SH test rejected a clade containing the coral symbionts and apicomplexans isolated from coral-reef fish. Within the coral-associated clade, trees grouping apicomplexans based on whether their host came from the complex or robust clade were significantly worse. There also were significant differences between the best ML tree and constraint trees when grouping symbionts from five of the seven coral families tested (Table 3). The two exceptions were the coral families Acroporidae and Faviidae, where no significant difference was found between the most likely topology and those constraining these families (Table 3).

DISCUSSION

Here, coral-associated apicomplexans were found to form a strongly supported monophyletic clade. Furthermore, trees inferred via both ML and Bayesian approaches were largely congruent with previous studies in regards to the phylogenetic placement of other apicomplexan taxa. For example, the Bayesian phylogeny of the adeleid coccidians mirrored that of a targeted evolutionary study for this group (Barta et al. 2012). While this relationship was not found in the ML tree, with *A. eberthi* being within the clade, this alternative topology (*A. eberthi* + the adeleid clade) has also been identified in another phylogenetic study (Kopecna et al. 2006). Branching within the Sarcocystidae and Eimeriidae also agreed with published phylogenies containing these groups (Jirku et al. 2002; Morrison et al. 2004; Jirku et al. 2009; Barta et al. 2012). Finally, the "marine eugregarine" clade from Rueckert et al. (2010) and the "terrestrial

gregarine" clades I and II from Rueckert et al. (2011b) were also recovered in the ML and Bayesian trees presented here. Taken together, the high congruence among multiply phylogenies provides strong support for the inferred phylogenetic placement of the coral-associated clade presented here.

Evolutionary placement of the coral-associated clade. There was moderate support for coral-associated apicomplexans as being a sister group to the eucoccidian families Sarcocystidae and Eimeriidae. The Sarcocystidae are mostly heteroxenous, requiring an intermediate host in their life-cycle and infecting mammals, birds and occasionally reptiles (Smith 1981; Olias et al. 2011). Although some marine taxa host sarcocystid apicomplexans, all documented cases involve mammals (Miller et al. 2008; Miller et al. 2010; Colegrove et al. 2011; Carlson-Bremer et al. 2012) and they likely originate from terrestrial sources (Fayer et al. 2004). On the other hand, Eimeriidae are generally homoxenous, and can infect a wider range of marine animals including mammals (Hsu et al. 1974), fishes (Dyková and Lom 1981), hemichordates (Fernandez et al. 1989), polychaetes (Ray 1930), and mollusks (Friedman et al. 1995). Therefore, while coral-associated apicomplexans are likely coccidians, they do not belong within either the Sarcocystidae or Eimeriidae.

In the phylogenies presented here, the symbiont found within the hemolymph of the giant clam *Tridacna crocea* grouped with polychaete symbionts of the genus *Rhytidocystis*. This relationship was first described by Leander and Ramey (2006) and has been recovered in subsequent phylogenies (Rueckert and Leander 2009a; Saffo et al. 2010). As the genus *Rhytidocystis* was placed taxonomically within the coccidian order Agamococcidiorida (Levine 1979), which contains the coral-associated genus *Gemmocystis* (Perkins et al. 2000), this study

tested this hypothesized evolutionary relationship. While Leander and Ramey (2006) hypothesized that the *Tridacna crocea* symbiont may be closer related to those from corals, the coral-associated clade of apicomplexans are distinct from the rhytidocystids with strong (100% bootstrap, 1.00 posterior probability) support. Furthermore, forcing the rhytidocystids to group with the coral apicomplexans significantly decreased the likelihood of the tree (Table 3). Although it is uncertain whether the morphological description and host tissue localization of *G. cylindrus* is representative of all members of the coral-associated clade in this study, it is clear these symbionts, harbored by a diverse scleractinian coral species throughout the Caribbean Sea, including several gorgonians, 1) do not share a recent common ancestor with *Rhytodocystis* spp. and 2) should potentially be removed from within the Agamococcidiorida.

The coral-associated clade is also phylogenetically distinct from apicomplexans of other host species in the coral reef environment as well. For example, two coccidians infecting the kidneys and spleen of Hawaiian reef fishes (Work et al. 2003) formed a monophyletic clade with two additional fish symbionts, *Eimeria leucisci* (Molnár 1996) and *Goussia janae* (Lukes and Dyková 1990), basal to the Sarcocystidae/Eimeriidae split. This position of *G. janae* has been recovered in other published phylogenies (Jirku et al. 2002; Morrison et al. 2004; Jirku et al. 2009), so it appears that this group of fish parasites are a distinct lineage relative to the Sarcocystidae and Eimeriidae. Again, similar to the *Tridacna* symbiont, these symbionts are phylogenetically discrete from the coral symbionts with strong (100% bootstrap; 1.00 posterior probability) support and constraint trees grouping the fish parasites with the coral symbionts were significantly longer than the best ML tree (Table 3).

Phylogenetic relationships among the coral-associated apicomplexans. There was little to no support for specificity between members of the coral-associated apicomplexan clade and their hosts. Specifically, a constraint tree parsing apicomplexans associated with complex corals and those from robust corals was significantly worse than the best ML topology (Table 3). The complex/robust division among host taxa has been strongly supported using different molecular markers and thus likely represents the evolutionary history for the group (Romano and Palumbi 1996; Kerr 2005; Medina et al. 2006; Fukami et al. 2008; Kitahara et al. 2010). Furthermore, symbionts did not branch following host familial lineages and most constraint trees forcing monophyly of symbionts from coral families also were significantly worse than the best ML tree. The two exceptions were the Faviidae and Acroporidae. However, it seems unlikely that this represents specificity within the family, as apicomplexans associating with *Montastraea* spp. 1) were scattered throughout the clade and 2) shared identical sequences with those hosted by *S. siderea* and *P. astreoides* (See Chapter 3). This potentially suggests rapid host switching and/or incomplete sorting (sensu Page and Charleston 1998) within the coral-associated clade (Johnson and Clayton 2004; Ricklefs et al. 2004).

Three groups of coral-associated apicomplexans were identified and consistently recovered in both the ML and Bayesian trees. One (Group I) united all sequences recovered from gorgonians and shared a most recent common ancestor with symbionts from *P. astreoides*. This group was nested within the coral-associated clade with strong support, despite encompassing distantly-related hosts (Kitahara et al. 2010). One potential explanation for this pattern is again host-switching within the symbiotic lineage. Within the gorgonian-associated apicomplexans, there was equivocal support for the groupings. The Gorgoniidae (*Pseudopterogorgia americana* + *Gorgonia ventalina*) was recovered in the Bayesian, but not the ML tree. Once again this is a

strongly supported host division (Sanchez et al. 2003). Although two additional clades were detected (Clades II and III), host phylogeny does not fully explain the groups. This could indicate the coral-associated clade are “generalist” symbionts or that 18S rDNA lacks the appropriate resolution to identify potential host specificity. This has been seen in other groups of apicomplexans, where markers, such as the internal transcriber spacer (ITS) regions of the ribosomal array, have resolved host specificity whereas the 18S rDNA could not (Hnida and Duszynski 1999; Motriuk-Smith et al. 2009). Likewise, while 18S rDNA data groups the coral dinoflagellate symbiont *Symbiodinium* into eight distinct clades (Pochon and Gates 2010) that appear to be “generalists” (Rowan and Powers 1992), ITS2 data have uncovers many subgroup “types” displaying host-specificity (LaJeunesse 2002; Coffroth and Santos 2005). Thus, its is suggested that future studies employ genetic markers with higher resolution to address questions of specificity in this coral-associated clade of apicomplexans.

Finally, a single environmental sample from the Caribbean Sea grouped with the coral-associated apicomplexan clade with strong support in all trees. The specific 18S rDNA sequence came from the “anoxic water column” of the Cariaco Basin, which is in the Southern Caribbean near Venezuela (Edgcomb et al. 2011) and contained three indels (5 bp, 5 bp, and 9 bp in length) distinguishing it from all other sequences. Notably, these rare genomic changes (sensu Rokas and Holland 2000) were also unique relative to all other coccidian sequences included in this study. Therefore, they are not phylogenetically informative as characters other than to differentiate this environmental sequence from those of the coral-associated apicomplexans. However, given that apicomplexans associate with corals throughout the Caribbean Sea, they likely are transported via currents and/or intermediate/paratenic hosts (see Chapter 4). Thus, further sampling of environmental samples could facilitate understanding of apicomplexan

transport and potentially recover novel 18S rDNA sequences that would further improve the phylogenies presented here.

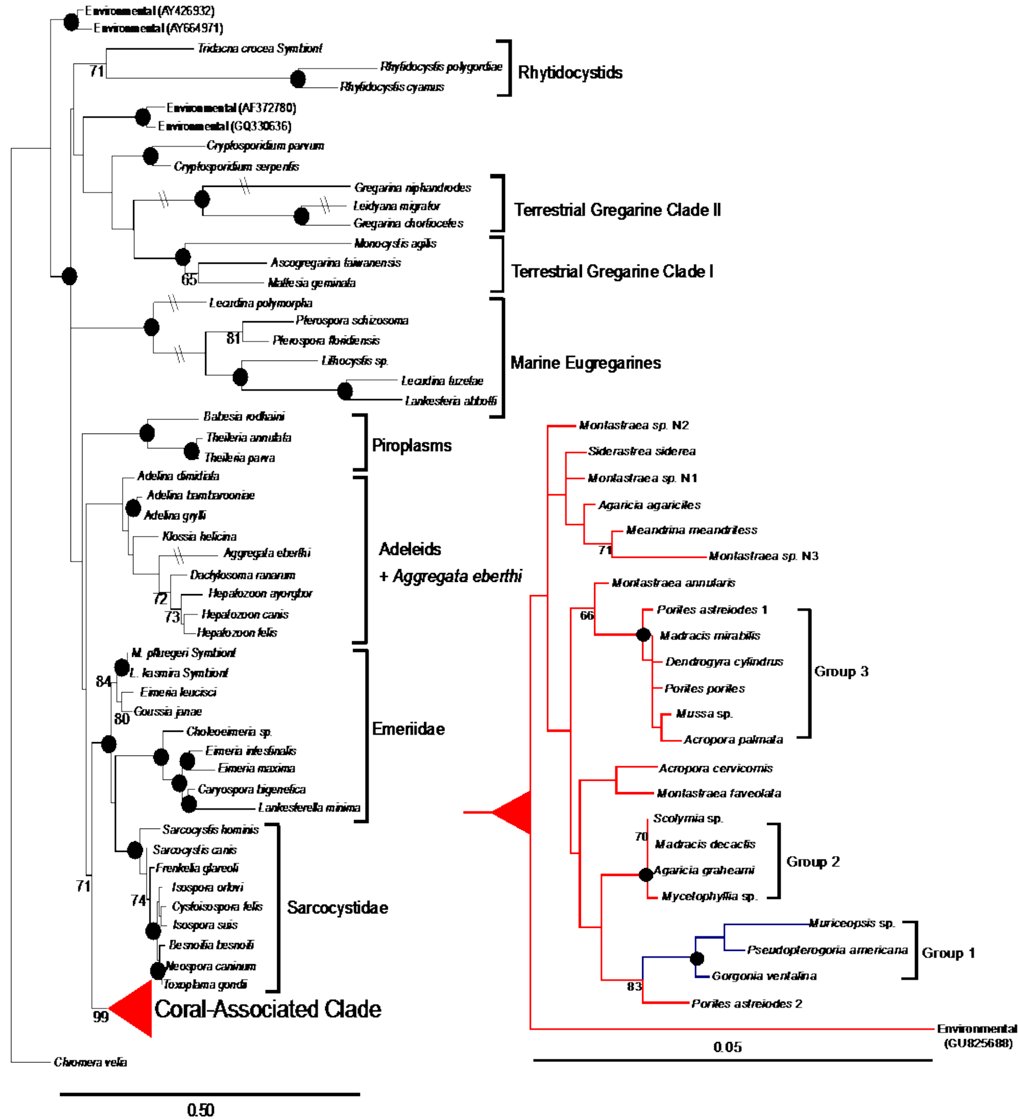

Figure 1. Maximum likelihood (ML) tree constructed from nearly-complete 18S rDNA sequences of Apicomplexa (-ln L= -19823.78 and shape parameter (α)=0.35). Inset is a blow-up of the coral-associated apicomplexan clade and connects to the main tree at the red triangle. Coral-associated apicomplexans are denoted by their host species. Nodes with >85% bootstrap support (BS) are denoted with a black dot and BS support <65% is not provided. Other values on nodes are specifically mentioned in text. Parallel lines indicate a break in the branch length (i.e. the mean number of substitutions per site) to aid in visualization. Substitution rates were higher for transitions than transversions and ranged from 0.72 (C $\Leftrightarrow$ G) to 5.97 (C $\Leftrightarrow$ T) and base frequencies were estimated (π_A=0.277; π_C=0.188; π_G=0.254; π_T=0.281).

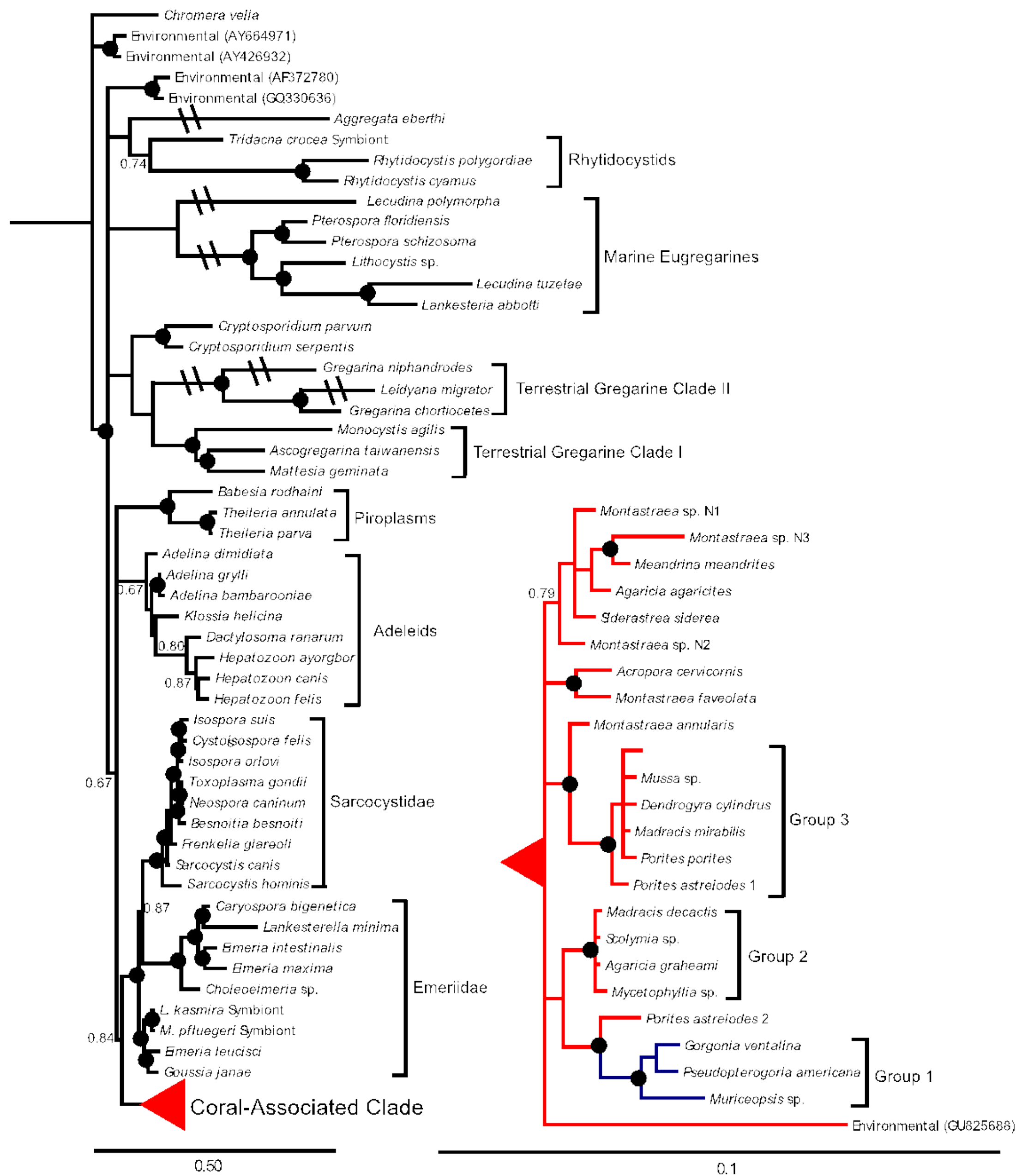

Figure 2. Bayesian phylogeny constructed from nearly-complete 18S rDNA sequences of Apicomplexa based on 50% consensus. Inset is a close-up of the coral-associated clade and connects back the main tree at the red triangle. Coral-associated apicomplexans are denoted by their host species. Nodes >0.90 posterior probability (PP) are denoted with a black dot and values <0.65 are not provided. Parallel lines indicate a break in the branch length (i.e. the mean number of substitutions per site) to aid in visualization.

Table 1. Samples utilized in the study. Given are the host species, Host family (Fam), Clade of the coral host, and sample collection location. FLA=Florida, BAH=Bahamas, BAR=Barbados, CUR=Curaçao.

		Host Fam	Host Species	Clade	Loc
Scleractinia	1	Poritidae	*Porites porites*	Complex	CUR
	2	Poritidae	Porites astreoides 1	Complex	FLA
	3	Poritidae	*Porites astreoides* 2	Complex	FLA
	4	Acroporidae	*Acropora palmata*	Complex	BAH
	5	Acroporidae	*Acropora cervicornis*	Complex	BAH
	6	Agaricidae	*Agaricia agaricites*	Complex	BAH
	7	Agaricidae	*Agaricia graheami*	Complex	CUR
	8	Siderastrea	*Siderastrea siderea*	Complex	BAH
	9	Pocilloporidae	*Madracis mirabilis*	Robust	BAH
	10	Pocilloporidae	*Madracis decactis*	Robust	CUR
	11	Meandrinidae	*Dendrogyra cylindrus*	Robust	BAR
	12	Meandrinidae	*Meandrina meandrites*	Robust	BAR
	13	Mussidae	*Mussa* sp.	Robust	BAR
	14	Mussidae	Mycetophyllia sp.	Robust	CUR
	15	Mussidae	Scolymia sp.	Robust	BAR
	16	Faviidae	*Montastraea annularis*	Robust	BAH
	17	Faviidae	*Montastraea faveolata*	Robust	FLA
Gorgonacea	18	Gorgoniidae	*Gorgonia ventalina*		FLA
	19	Gorgoniidae	*Pseudopterogoria americana*		FLA
	20	Plexauridae	*Muriceopsis* sp.		FLA

Table 2. Hypotheses for the constraint trees generated.

	Hypothesis	**Constraint**
1	Coral symbionts + Rhytidocystids are monophyletic	(All coral symbionts + *Tridacna* symbiont + *Rhytidocystis* spp.)
2	Coral symbionts + fish coccidians are monophyletic	(All coral symbionts + 2 fish coccidians)
3	Monophyly of symbionts from Acroporidae	(*A. palmata* + *A. cervicornis*)
4	Monophyly of symbionts from Agariciidae	(*A. agaricites* + *A. graheami*)
5	Monophyly of symbionts from Faviidae	(*M. faveolata* + *M. annularis* + *Montastraea* sp. 1, 2, and 3)
6	Monophyly of symbionts from Meandrinidae	(*M. meandrites* + *D. cylindrus*)
7	Monophyly of symbionts from Mussidae	(*Mussa* sp. + M*ycetophyllia* sp. + *Scolymia* sp.)
8	Monophyly of symbionts from Pocilloporidae	(*M. decactis* + *M. mirablis*)
9	Monophyly of symbionts from Poritidae	(*P. astreoides* I + II + *P. porites*)
10	Dichotomy of complex vs. robust corals	Complex and robust corals separate Complex: (*P. astreoides* I, II + *P. porites* + *A. palmata* +*A. cervicornis* + *A. agaricites* + *A. graheami* + *S. siderea*) Robust: (*M. mirabilis* + *M. decactis* + *D. cylindrus* + *M. meandrites* + *Mussa* sp. + *Mycetophyllia* sp. + *Scolymia* sp. + *Montastraea* sp.)

Table 3. Results of the SH test comparing constraint trees (Model) and their log likelihood (Ln likelihood) to that of the best ML tree (-19823.78). The difference, standard deviation (Std Dev) and significance is provided. The hypotheses (H #) are detailed in Table 2.

H #	Model	Ln likelihood	Difference	Std Dev	Significantly worse
1	**Coral + Rhytidocystids**	-19873.03286	-49.252119	19.36463	Yes
2	**Coral + Fish**	-19883.88225	-60.101505	22.072439	Yes
3	**Acroporidae**	-19857.84557	-34.064821	19.565327	No
4	**Agaricidae**	-19873.8407	-50.059958	16.902246	Yes
5	**Faviidae**	-19865.47634	-41.695593	22.315588	No
6	**Meandrinidae**	-19884.54512	-60.764374	23.579406	Yes
7	**Mussidae**	-19876.42379	-52.643041	20.453795	Yes
8	**Pocilloporidae**	-19888.5194	-64.738659	21.972723	Yes
9	**Poritidae**	-19881.92729	-58.146546	18.81824	Yes
10	**(Complex) + (Robust)**	-19941.18074	-117.399991	30.446318	Yes

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Chapter 6. Conclusions

This dissertation demonstrates that apicomplexans are intimately associated with scleractinian (and to a lesser extent, gorgonian) corals throughout the Caribbean Sea. This association is stable through time, although there is a significant decrease in prevalence during the summer (Chapter 3). High prevalence can be partially explained by vertical transmission in brooding hosts and by the clonal propagation exhibited by some species (Chapter 4). In this largely unexplored system, there are a number of outstanding research directions. First and foremost is the nature of this symbiosis and the interactions between these potential pathogens and their host species. Second, it is important to determine the drivers of variation in prevalence among hosts. Third, the functional diversity of the coral-associated apicomplexan clade warrants further description. Although there is little evidence of coevolution (Chapter 5), host-symbiont relationships, such as specificity, may still exist. Finally, as anthropogenic climate change occurs, the corresponding increase in host stressors will likely alter the interaction, potentially for the worse.

Interaction between coral and apicomplexan symbionts

Although the nature of the coral/apicomplexan symbiosis has yet to be determined, interactions between corals and their other symbionts have been elucidated. In general, alveolates form symbioses ranging from mutualism to parasitism. Ciliates can be food (Ferrier-Pagès et al.

1998), predators to recruits (Cooper et al. 2007), commensals (Qiu et al. 2010), and opportunistic pathogens (Willis et al. 2004) in scleractinian corals. For example, the recently described brown band syndrome involves a ciliate engulfing *Symbiodinium* adjacent to denuded skeleton (Willis et al. 2004; Bourne et al. 2008). Additionally, the folliculinid ciliate, *Halofolliculina corallasia*, has been implicated in skeletal eroding band (SEB) disease, which can spread quickly (up to 2 cm/day) and lead to necrosis and erosion of the skeleton (Winkler et al. 2004; Page and Willis 2008). This ciliate can rapidly colonize injured tissue, but has limited capacity to invade intact coral tissues (Page and Willis 2008). This indicates it is likely opportunistic and may require additional organisms, stressors, or insults to become pathogenic. In fact, there is an increase in number and change in species composition of ascomycetes fungi assemblages associated with both BrB and SEB (Yarden et al. 2007). It should be noted that this is only correlative as the etiology of these fungi groups and potential synergistic effects with ciliates remain unknown. Dinoflagellates are also symbionts with corals and can be parasites, commensals and mutualists. The most intimate and well characterized of coral mutualisms involves species within the genus *Symbiodinium*, which can provide their hosts with up to 90% of its photosynthetically fixed carbon (Muscatine et al. 1981). This symbiosis allows corals to survive in the oligotrophic waters of tropical reefs. However, even these symbionts have been hypothesized to become parasitic in response to either changing environmental conditions or host acquisition method (Sachs and Wilcox 2006; Thornhill et al. 2008).

One potential way to assess the impact of the interaction would be to correlate growth or fecundity to parasite load as is done in other apicomplexan systems (e.g. Field and Michiels 2005). Thus, it is important to determine the abundance (sensu Rózsa et al. 2000) of apicomplexans within a given host colony. Fluorescence *in situ* hybridization (FISH) and

quantitative PCR have both been utilized to detect and enumerate symbionts in corals (Ainsworth et al. 2006; Loram et al. 2007; Thurber et al. 2008; Ainsworth and Hoegh-Guldberg 2009). Another potential way to enumerate symbionts within a host involves "next-generation" technologies such as Illumina sequencing where hyper-variable domains of the 18S rDNA (e.g. V4 or V9; Dunthorn et al. 2012) are enumerated, detecting millions of copies of this gene within a sample (Gloor et al. 2010). With this technique, seasonal changes in protist relative abundance in an aquatic environment have already been identified (Nolte et al. 2010); however, it is important to note that the 18S rDNA is a multicopy gene and correlation to the number of individuals present is difficult. Therefore, single copy genes have been recommended for this approach (Medinger et al. 2010) and published genomes of other coccidians, such as *Toxoplasma gondii* (Kissinger et al. 2003), may help identify such single-copy markers. Symbioses represent a unique challenge, as the host and other symbionts will also amplify with universal primers. To avoid this problem, Troedsson et al. (2008) added a "blocking probe" that was specific to their particular host species to the PCR reaction. This probe specifically annealed to the host DNA and prevented amplification, resulting in PCR products only from the symbiont. Each of these techniques, separately or in combination, could be used to estimate abundance of coral-associated apicomplexans.

To determine the effects of apicomplexan association and transmission patterns in the broadcast spawners, it would be helpful to remove these protists from their coral hosts. It may be possible to use anticoccidial drugs to clear apicomplexan infections in scleractinian corals. A number of anticoccidial drugs designed to treat avian and mammalian coccidiosis have been effective in clearing apicomplexan parasites in freshwater and marine fishes (Solangi and Overstreet, 1980; Molnár and Ostoros 2007). If infection intensity (i.e. parasitic load) can be

manipulated, growth rates and fecundity (e.g. % reproductive mesentaries, number of oocytes/cm^2 of tissue, number of brooded planulae released, etc.) can be measured to assess differences between the infection treatments. These metrics were used to show a negative effect of white plague and yellow band diseases on the fecundity of *Montastraea annularis* colonies (Weil et al. 2009; Borger and Colley 2010). In addition, planula larvae from broadcast spawning species could be a source of uninfected individuals. Infection rates can be estimated by tracking recruits placed into the field as has been done for *Symbiodinium* (Little et al. 2004; Coffroth et al. 2006) and other apicomplexan systems (Mladineo et al. 2009). Additionally, placing aposymbiotic larvae in tanks with apicomplexan-associated adults will potentially address whether these symbionts can be passed through the water-column. Waterborne transmission has been demonstrated in other apicomplexan symbioses using this approach (Steinhagen and Körting 1988; Friedman et al. 1993).

Immune response of corals and corresponding differences in prevalence and abundance

Apicomplexans are found in a variety of Caribbean corals, but their prevalence also is host dependent (Chapter 3). Furthermore, apicomplexans did not appear to correlate with visible signs of disease in gross morphology nor at the tissue level in half of the species examined (Upton and Peters 1986). Thus, there is a species-specific host response to apicomplexans that warrants future study. Apicomplexans, as well as other symbionts, must enter host cells though a defensive coral mucous layer (Reed et al. 2010), avoid digestion, grow, and reproduce (van Woesik and Jordán-Garza 2011; Davy et al. 2012). This is not a trivial task as corals have an innate immunological response capable of differentiating self from non-self (Theodor 1970;

Hildemann et al. 1977; Salter-Cid and Bigger 1991; Jokiel and Bigger 1994). Moreover, corals have some degree of immune memory, where they are able to react quicker to subsequent challenges (Hildemann et al. 1977; Hildemann et al. 1980; Salter-Cid and Bigger 1991; Reed et al. 2010). In addition, some infectious agents that have previously caused disease are no longer able to infect hosts on the same reefs (Reshef et al. 2006; Rosenberg et al. 2007) as there is intercolonial variation in disease resistance (Vollmer and Kline 2008). This is potentially influenced and modulated by other microorganisms present in the coral holobiont (Reshef et al. 2006; Rosenberg et al. 2007; Rosenberg and Zilber-Rosenberg 2011). Thus, understanding differences in prevalence and pathology associated with different host/apicomplexan interactions could provide insight in the innate immune response or scleractinian corals in general.

In the context of the immune response, clonality could explain high prevalence of apicomplexans in some corals (Chapter 4). Abundant clonal individuals are more likely to be infected with parasites following a time-lag (Dybdahl and Lively 1995,1998; Jokela et al. 2009). Consistent apicomplexan infection indicates these symbionts are well adapted to live inside their coral hosts. Thus if apicomplexans are well adapted to avoid digestion and persist within host cells in one colony, they would likely be able to infect other ramets (i.e. individuals of the same clone). The host would be potentially naïve to infection while the symbiont was conditioned in another ramet of the same genet (i.e. clone). This hypothesis could be tested with the species *Acropora cervicornis* and *Acropora palmata*, as their population structure varies throughout the Caribbean in terms of clonality (Baums et al. 2006; Vollmer and Palumbi 2007). Correlating apicomplexan prevalence with clonality could further our understanding disease dynamics on reefs.

Diversity within the coral-associated clade

Apicomplexans have traditionally been classified based on morphology, ultrastructure, life-cycle and host range (Chapter 2). To compare the species description of *G. cylindrus* to the 18S-rDNA sequences generated in this dissertation**,** morphological data will have to be collected. Utilizing adult samples for histology is relatively difficult due to the presence of a calcium carbonate skeleton. However, coral larvae of brooding species can be used since PCR assays have detected apicomplexans in this life stage in Florida and Belize (Chapter 4). In addition, apicomplexans should be easier to detect in larval histological sections as they represent a smaller surface area to search. A combination of FISH and light microscopy would enable visualization for enumeration and determining location within the larvae. Currently, apicomplexans have been in observed within the gastrodermis and rarely the calicodermis of adult colonies (Peters 1984; Upton and Peters 1986). However, their location within larvae in unpublished and likely unknown. Light microscopy of coral-associated apicomplexans is essential for taxonomy as most coccidian species are described in part due to oocyst morphology (Perkins et al. 2000). Therefore, larval samples are ideally suited for comparing morphological and molecular data necessary for species descriptions.

While complete 18S-rDNA sequence data was able to infer the likely phylogenetic position and relationships among coral-associated apicomplexans, further differentiation within this clade will require molecular genetic markers with the appropriate resolution, such as the internal transcribed spacer (ITS) regions of the ribosomal array. These regions are transcribed, but are excised and degraded, during rRNA maturation. Thus, they are under less selective constraint at the nucleotide level than the 18S-rDNA and typically more variable (LaJeunesse

2001; Coleman 2003). These markers have distinguished closely related species in the genus *Eimeria* corresponding to different species of host rodents (Hnida and Duszynski 1999; Kvicerova et al. 2008; Motriuk-Smith et al. 2009). Additionally, this spacer region was capable of resolving differences between species and subspecies of other apicomplexans (Barta et al. 1998; Zahler et al. 1998; Boullianne et al. 2007). Developing more variable molecular genetic markers is important for addressing ecological concepts such as host-specificity, population genetics and connectivity.

Impending climate change and its affect on this symbiosis

A worldwide decline in coral reefs has been documented and is predicted to worsen. An increase in marine diseases over the last 30 years, and in corals in particular (Porter et al. 2001; Ward and Lafferty 2004; Bourne et al. 2009), has been noted with increasing water temperatures associated with global climate change implicated as a possible cause (Harvell et al. 1999; Harvell et al. 2002). A specific example is the unprecedented decline of Caribbean *A. cervicornis* and *A. palmata* over this time period, partially caused by outbreaks of white-band disease (Aronson and Precht 2001; Sutherland et al. 2004). Historically, no record of a decline on this scale can be found in the entire fossil record since the Holocene (Aronson and Precht 2001). These two species exhibit low recruitment (Bak and Engel 1979; Williams et al. 2008), but have come to dominate reefs via fragmentation and clonal propagation (Tunnicliffe 1981; Lirman 2000,2003). Apicomplexans were associated with ~85% of adult colonies of *A. cervicornis* and *A. palmata* in Belize. Although the interaction between corals and apciomplexans likely predates the Caribbean-wide decrease in *Acropora* abundances, environmental change can change the nature

of this symbiosis. For example, the marine fungal pathogen *Aspergillus sydowii* increases growth rates with increasing temperatures. This is accompanied by a decrease in the efficacy of the host anti-fungal activity, which would lead to an increase in pathogenicity of this coral symbiont (Alker et al. 2001).

Visible signs of disease are variable in corals associated with apicomplexans, with several species displaying no effects, even in cases of high parasite load (Upton and Peters 1986; Toller et al. 2002). That suggests that these symbionts do not appear to be causative agents of disease or mortality in corals (i.e. they may be commensal). Nevertheless, changes in host physiology or environment can lead to proliferation of opportunistic pathogens (Le Campion-Alsumard et al. 1995; Alverdy et al. 2005; Marçais and Bréda 2006; Lesser et al. 2007) and/or perturb the nature of symbiotic interactions (Sachs and Simms 2006). Thus, altering environmental conditions, as is predicted in many global climate change scenarios (Donner et al. 2005; Donner et al. 2007; Hoegh-Guldberg et al. 2007), may also affect the interaction between apicomplexans and their coral hosts, potentially for the worse. In order to explore such possibilities and determine the nature of this association, it was first necessary to establish that apicomplexans are common, persistent constituents of coral holobionts as has been demonstrated here.

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Appendix 1

Ecological modeling allows complex processes to be reduced to a few chosen variables. To address differences in apicomplexan prevalence (i.e., the percentage of infected host individuals within a sample), a logistic regression model was employed with four independent variables (See Chapter 3 for more detail):

Location: (Bahamas [BAH] or Florida [FLA])

Reef: (FLA: Little Grecian Reef [LG], Admiral Reef [ADM]; BAH: South Perry Reef [SP], North Norman's Pond [NNP])

Season: (Winter [win], Spring [spg], Summer [sum], Fall)

Host Species (sp): (*Montastraea annularis* [Ma], *Montastraea faveolata* [MF], *Porites astreoides* [Pa], *Siderastrea siderea* [Ss]).

Thus, all were categorical and coded as dummy variables to explore the effects of combining them 2 at a time in the model. For example, each season was entered separately and a sample from winter would be coded as Win=1, SPG=0, SUM=0, FAL=0. In this way, each of these seasons also could serve as the reference in the model. The R statistical environment was utilized to build all models (ver. 2.13.2; R Development Core Team, 2011).

As a first step, all variables were included in a linear model to determine if there was collinearity among them using the car package (Fox and Weisberg 2011). In brief, if collinearity exists between variables, then they are correlated with each other and can either be confounding (i.e. negatively correlated) or redundant (i.e. positively correlated). This increases the variance associated with the variable, making it more difficult to identify significance. The easiest way to

determine the effects of collinearity is to calculate the variance inflation factors (VIF), which is equal to $1/(1-R^2)$ (Rogerson 2001). The square root of this is equal to the factor the variance is multiplied by due to the effects of collinearity (Fox and Weisberg 2011). The code for this in R is:

```
lm1=lm(presence~sp+season+year+reef, data=mydata)
vif(lm1)
```

where presence (0 or 1) is prevalence for a given colony at a given time. Thus, the VIF and square root (Sq. Rt.) for the variables are presented in Table 1 (below).

Table 1. Variance inflation factors (VIF) for the three variables noted above and year. DF=degrees of freedom, Sq. Rt. is the square root of the VIF. See text for detail.

	VIF	DF	Sq. Rt.
sp	1.470073	3	1.066326
Season	1.311846	3	1.046278
Reef	1.719698	8	1.034465
Year	1.509232	3	1.071008

Thus, collinearity was not a confounding factor in this dataset.

Next, a mixed model was generated to account for the blocking of the variables. Individuals (ind) were nested with species, which were nested within reefs, locations, and years collected (i.e. year/location/reef/sp/ind). Adding the nesting structure eliminates pseudoreplication from the dataset, which would artificially increase the sample size (Hurlbert 1984). Thus, the full model is:

```
results=glmer(presence ~ Ma+Mf+Pa+sum+fall+spg+reef+(1|year/location/reef/sp/ind),
        data=mydata, family="binomial", na.omit=TRUE)
```

where glmer is a generalized model and the “binomial” distribution makes it a logistic regression with a link function of ln(y/(1-y) (Fox and Weisberg 2011). The benefit of the link function is

that it restricts the range of y from 0-1, as is appropriate for presence/absence data, and does not restrict the coefficients of the variables in the model (Rogerson 2001).

To determine if there were significant differences among the different host species, season, and reef, these variables had to be combined. For example, the full model takes into account each variable separately (Table 2).

Table 2. Estimates of the coefficients (Coeff) associated with the full model along with the standard errors (S.E.), z scores (Z), p values (P) and whether it was significant (**) or not (NS).

	Coeff	S.E	Z value	P	Sig
(Intercept)	-0.1297	1.0192	-0.127	0.898712	NS
Ma	4.0604	0.839	4.84	1.30E-06	**
Mf	6.4004	0.9702	6.597	4.20E-11	**
Pa	6.4068	1.33	4.817	1.46E-06	**
sum	-1.4762	0.4661	-3.167	0.001539	**
fall	-2.2207	0.454	-4.891	1.00E-06	**
spg	-1.6638	0.4918	-3.383	0.000716	**
reefLG	0.8774	0.8143	1.077	0.281272	NS
reefNNP	1.2581	0.8962	1.404	0.160343	NS
reefSP	0.6994	1.1146	0.628	0.530321	NS

To examine the differences between prevalence in *M. annularis* vs. *M. faveolata*, the model was specified to combine Ma + Mf as a single variable (Table 3).

```
results=glmer(presence ~ I(Ma+Mf)+Pa+sum+fall+spg+reef+ (1|year/location/reef/sp/ind),
data=mydata, family="binomial", na.omit=TRUE)
```

Table 3. Estimates of the coefficients (Coeff) associated with the model combining Ma and Mf along with the standard errors (S.E.), z scores (Z), p values (P) and whether it was significant (**) or not (NS).

	Coeff	S.E	Z value	P	Sig
(Intercept)	0.2933	1.016	0.289	0.77279	NS
(Ma+Mf)	4.8944	0.9658	5.068	4.02E-07	***
Pa	6.9078	1.7349	3.982	6.84E-05	***
sum	-1.4878	0.4782	-3.112	0.00186	**
fall	-2.1377	0.4601	-4.646	3.39E-06	***
spg	-1.5443	0.5011	-3.082	0.00206	**
reefLG	1.2754	1.0564	1.207	0.22732	NS
reefNNP	1.211	1.1345	1.067	0.28576	NS

reefSP	0.728	1.3994	0.52	0.60291	NS

At this point the models could be compared using a likelihood ratio test and all differences between the models could be attributed to the differences between Ma and Mf (Table 4).

Table 4. Likelihood ratio test, which examines the log likelihoods (LogLik) of the full model and a model combining *M. annularis* and *M. faveolata* (Ma + Mf). There is one degree of freedom (Df) and the test statistic models a chi-square distribution.

	logLik	Chisq	Df	P value
(Ma + Mf)	-240.99			
Full model	-237.56	6.8734	1	0.008749

After the Bonferroni correction, α=0.008. Therefore, there was no significant difference between prevalence in *M. faveolata* and *M. annularis* (See Chapter 3 for more detail). This test was done for all possible combinations of species as well as reef and season.

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Appendix 2

Two different protocols were followed to determine the best way to fix coral larval samples for transmission electron microscopy (TEM). The first (1) fixative consisted of 2.5% glutaraldehyde, 0.14 M $NaCl_2$ and 0.2 M Millonig's (monobasic) phosphate and the second (2) was 2% glutaraldehyde, 0.14 M $NaCl_2$, 0.05 sodium cacodylate, and 0.2% tannic acid, which is a protocol developed for preserving marine oocysts (Landers 2011). Larvae were preserved separately in both fixatives for 24 hours and transferred to Millonig's phosphate buffer (0.2% with 0.3 M $NaCl_2$) and (0.05 M Na cacodylate and 0.3 M 0.3 M $NaCl_2$) for 1 and 2, respectively. Samples were kept at 4° C for 1 week prior to osmication and embedding.

Samples were washed in their respective wash buffers (see above) and post-fixed in OsO_4 for 24 hrs (there was no difference between those fixed for 2hrs). The samples from the first preservative were fixed in 2% OsO_4 and 0.2 M Millonig's phosphate and the second were fixed in 0.5% OsO_4, 0.05 M Na cacodylate, and 1% potassium ferrocyanide followed by 2x washes in their respective buffers for 15 min. Following this step, both sets of samples were treated identically. Samples were transferred to 0.2 μm filtered water for 15 min and sequentially transferred to 30, 50, 70, 80 and 95% ethanol for 10 min at each step. They were then dehydrated in 2 rinses of absolute (100%) ethanol for 15 min each and rinsed twice with propylene oxide (PO) for 15 min.

Samples were infiltrated and embedded in Araldite 502/epon (Embed-812) (EMS) resin. For infiltration, 1:1 (PO : resin) solution was added to the sample and incubated at room temperature for 1 hr in a rotator. This was followed by another 1 hr incubation in a 1:3 (PO :

resin) solution. Finally, samples was incubated at room temperature in 100% resin for 6 hrs, transferred to molds containing new resin and hardened at 60° C for 24 hrs.

Samples were trimmed, 1.5 μm sections cut on a Reichert Ultracut S ultramicrotome and dried prior to staining in Toluidine Blue (2% toluidine blue, 2% Sodium borate). Ultrathin (95 nm) sections were stained in uranyl acetate prior to visualization with a Philips 301 transmission electron microscope.

Overall, the samples fixed in phosphate buffer had the best fixation and figures are shown within (Chapter 4, Fig. 2). Samples fixed in the cacodylate buffer appeared brittle and sections contained numerous cracks originating in the ectoderm and spreading through to the endoderm. Therefore, this fixation protocol is not recommended for coral larvae.

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