Impact of the fungal disease aspergillosis on populations of the sea fan *Gorgonia ventalina* (Octocorallia, Gorgonacea) in La Parguera, Puerto Rico

by

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Abstract

Aspergillosis is a disease caused by the fungus *Aspergillus sydowii* which affects a number of shallow-water octocorals including the common Caribbean sea fan *Gorgonia ventalina*. This work includes two studies on the current status of aspergillosis in populations of *G. ventalina* in several reefs off La Parguera, Puerto Rico. The first study evaluated disease prevalence and virulence while the second investigated the effects of the disease on the reproduction of *G. ventalina*. Aspergillosis prevalence was explored through surveys performed over a two-year period at varying depths within six reefs along an inshore-offshore gradient. The average prevalence of aspergillosis increased from 3% (95%CI: 1.8-6.0%) in March 2005 to 17% (95%CI: 14.8-19.5%) in September 2006, with values for individual reefs varying from 0-29%. Prevalence was most related to reef and depth, but season, year, reef zone and density of sea fans also had some influence. The highest prevalence was found at Turrumote, Media Luna and Enrique at depths shallower than 9.6 m in 2006. Aspergillosis virulence was monitored on tagged colonies for fifteen months in three reefs to assess the spatial and temporal dynamics of individual disease lesions, the rate of advance and tissue mortality, the seasonal variability, and the rate of recovery. Trends were consistent among all sites; the number of active lesions (May 2006: 3.08 lesions/colony, W=1-23, n=61; July 2007: 0.72 lesions/colony, W=0-4, n=61) and the area of purpling due to disease (May 2006: 3.44%, W=0.05-21.32, n=61; July 2007: 0.51%, W=0-3.35, n=61) decreased over time as the aspergillosis infection disappeared, while the area of tissue loss per colony (May 2006: 1.92%, W=0-19.53, n=61; July 2007: 13.46%, W=0-100, n=61) increased over time as a consequence of the infections. The effect of aspergillosis on reproductive output was

investigated using histological techniques to observe eggs and spermaries in tissue samples collected from both healthy colonies and colonies with signs of aspergillosis. In diseased colonies, there was a significant decrease in the proportion of reproductive polyps and in the number of eggs per polyp in the infected area (4.1% reproductive polyps, 95%CI: 2.6-6.5; 0.06 eggs/polyp, W=0-0.6, n=24) and immediately adjacent to it (12.4% reproductive polyps, 95%CI: 9.7-15.6; 0.18 eggs/polyp, W=0-2.0, n=24) compared to areas at least 10cm away from lesions in healthy-looking tissue (34.4% reproductive polyps, 95%CI: 30.2-38.7; 0.50 eggs/polyp, W=0.05-1.5, n=24). The impact seems to be systemic since even polyps in healthy-looking areas of infected colonies showed decreased egg and sperm production compared to healthy colonies (41.2% reproductive polyps, 95%CI: 38.0-44.2; 0.76 eggs/polyp, W=0.05-3.35, n=100). The long-term effect of this reduction in gamete production depends on whether it drops low enough to limit recruitment causing a decline in the population or produces a recruit population which is not reduced in size but is more resistant to disease.

Resúmen

Aspergiliosis es una enfermedad causada por el hongo *Aspergillus sydowii* que afecta varias especies de octocorales, principalmente al abanico de mar *Gorgonia ventalina*. Este trabajo incluye dos estudios sobre el estatus de aspergillosis en poblaciones de *G. ventalina* en varios arrecifes de La Parguera, Puerto Rico. El primer estudio evaluó la prevalencia y la virulencia de la enfermedad y su variación espacial y temporal y el segundo, los efectos de aspergiliosis sobre la reproducción de *G. ventalina*. La prevalencia de aspergiliosis fue estudiada mediante censos durante dos años en varias profundidades en seis arrecifes en un gradiente costero-mar afuera. La prevalencia promedio aumentó de 3% (95% CI: 1.8-6.0%) en marzo de 2005 hasta 17% (95% CI: 14.8-19.5%) en septiembre de 2006, con valores en los arrecifes individuales variando entre 0-29%. Resultados indican una mayor dependencia con la localidad (arrecife) y el hábitat (profundidad), pero la estacionalidad, el año, la cercanía a la costa y la densidad de colonias también influenciaron la prevalencia. Turrumote, Media Luna y Enrique mostraron la mayor prevalencia a profundidades menores a 9.6m en el 2006. La virulencia de aspergillosis fue monitoreada durante quince meses en colonias marcadas en tres arrecifes. La dinámica espacial y temporal de las lesiones, las tasas de avance de la enfermedad y de mortandad de tejido, la variabilidad temporal y la tasa de recuperación fue monitoreada en estas colonias. La tendencia fue consistente en todos los arrecifes; el número de lesiones activas disminuyó de 3.08 lesiones/colonia en mayo de 2006 (W=1-23, n=61) a 0.72 lesiones/colonia en julio de 2007 (W=0-4, n=61). Similarmente el área violeta-oscura causada por la enfermedad disminuyó de 3.44% en mayo de 2006 (W=0.05-21.32, n=61) a 0.51% en julio de 2007 (W=0-3.35, n=61),

posiblemente porque la infección fue contenida. El área de tejido muerto por colonia aumentó significativamente con el tiempo de 1.92% en mayo de 2006 (W=0-19.53, n=61) a 13.46% en julio de 2007 (W=0-100, n=61). El efecto de aspergillosis en el esfuerzo reproductivo fue investigado con técnicas histológicas para observar los huevos y los espermatocitos en muestras de tejido de colonias sanas y colonias con aspergillosis. En las colonias enfermas hubo una disminución significativa en la proporción de pólipos reproductivos (4.1% pólipos reproductivos, 95% CI: 2.6-6.5) y en el número de huevos por pólipo en el área infectada (0.06 huevos/pólipo, W=0-0.6, n=24), y en el área adyacente a la infección (12.4% pólipos reproductivos, 95%CI: 9.7-15.6 y 0.18 huevos/pólipo, W=0-2.0, n=24) comparado con las áreas aparentemente sanas de la colonia (34.4% pólipos reproductivos, 95%CI: 30.2-38.7 y 0.50 huevos/pólipo, W=0.05-1.5, n=24). Al parecer, el impacto es sistémico debido a que los pólipos en las áreas sanas de colonias infectadas mostraron una disminución en la fecundidad (huevos/pólipo) y número de espermatocitos comparados con las colonias control sanas (41.2% pólipos reproductivos, 95%CI: 38.0-44.2 y 0.76 huevos/pólipo, W=0.05-3.35, n=100). El efecto a largo plazo de esta reducción en la producción de gametos y mantenimiento de las poblaciones depende de si la caída en fecundidad limita el reclutamiento y causa una disminución en la población o si no reduce el tamaño de la población de reclutas pero produce una población más resistente a aspergillosis.

For my family and friends

Especially Dad, who would have appreciated this the most!

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1. Introduction

Coral reefs are highly productive and diverse ecosystems. In recent times, there has been a worldwide decline in the health of these important tropical communities, particularly in the Caribbean (Knowlton 2001a, Gardner et al. 2003). Changes in coral reefs have dramatically increased due to human impacts (Jackson 2001, Pandolfi and Jackson 2006). Human population growth has been increasingly driving coral reef decline (Birkeland 2004). Negative influences include overfishing of reef fish, pollution and sedimentation caused by growth and development in coastal areas (Jackson et al. 2001, McClanahan 2002, Wolanski et al. 2003) and more recently, increases in temperature and atmospheric carbon dioxide associated with climate change (Knowlton 2001a, Birkeland 2004). While disturbance is a natural and necessary part of every natural ecosystem, coral reefs have more difficulties recovering from the types of chronic problems often caused by anthropogenic influences than from short-term stresses such as hurricanes (Connell 1997). The greatest danger of long-term detrimental influences is in the reduction of resilience in coral reefs (Bellwood et al. 2003, Bellwood et al. 2004). Functional redundancy is one important factor in resilience; if an important organism disappears from the environment or severely declines, another organism fills its niche or ecological role in sustaining the ecosystem (Bellwood et al. 2003). The wide-spread compounding effects of multiple and chronic perturbations in reef ecosystems reduce resilience and make community collapse a possibility (Knowlton 2001a).

Coral reef diseases are one type of chronic stress on these communities. Although historical information and baseline data are incomplete, it is clear that coral reef diseases have become more prevalent in recent decades (Harvell et al. 1999, Green and Bruckner

2000, Kim et al. 2000, Porter et al. 2001, Harvell et al. 2004, Sutherland et al. 2004, Ward and Lafferty 2004, Weil 2004, Weil et al. 2006). In the last 30 years, there have been a number of disease epizootics which have severely damaged populations of many reef organisms. Some of these have caused significant shifts in the structure of the reef community (Harvell et al. 2004).

An unknown pathogen almost completely wiped out the sea urchin *Diadema antillarum* throughout the Caribbean in the early 1980s (Lessios et al. 1984). The cause of this disease remains unknown but its effects have been observed as a shift from coral-dominated to algal-dominated reefs (Hughes 1994). *D. antillarum* consume macroalgae, scraping it off hard substrate where algae and many other organisms recruit. Without the urchins to clear the substrate for other organisms, faster-growing algae have the advantage (Knowlton 2001b, Cho and Woodley 2002, McManus and Polsenberg 2004).

Also in the early 1980s, an epizootic of white band disease decimated populations of *Acropora palmata* and *A. cervicornis* (Gladfelter 1982). These two branching corals generated large amounts of calcium carbonate and habitat for reef organisms which was reduced severely by the epizootic. Only a small proportion of the habitat created by this species has been restored and there is no other species in the Caribbean with a similar growth form that was able to fill this gap (Bellwood et al. 2004).

Coral diseases are especially common and affect proportionally more species in the Caribbean which historically has much lower species diversity than the Indo-Pacific. The Caribbean is also the area where reefs have suffered the most degradation (Green and Bruckner 2000, Gardner et al. 2003, Sutherland et al. 2004, Weil 2004). Nine major diseases and a variety of other syndromes affect the majority of the reef building corals of

the Caribbean as well as several octocoral and hydrocoral species (Sutherland et al. 2004, Weil 2004). Mortalities of large coral colonies due to disease indicate that there truly has been a recent increase in the virulence and variety of disease (Weil 2004).

Increased water temperatures associated with climate change (Harvell et al. 2002) and increased nutrients (Bruno et al. 2003) associated with human development promote coral diseases. Extended periods of higher than normal temperatures are responsible for episodes of coral bleaching, a non-infectious disease which has threatened coral reefs since the late 1980s (Glynn 1993). In infectious diseases, higher temperatures tend to favor pathogens over hosts by increasing pathogen virulence, decreasing host-resistance, or a combination of the two (Alker et al. 2001, Harvell et al. 2002, Patterson et al. 2002, Richardson and Kuta 2003, Sutherland et al. 2004).

Pollution and sedimentation also promote disease by increasing nutrient availability (Kuta and Richardson 2002, Bruno et al. 2003) and transporting potential pathogens to the marine environment (Smith et al. 1996, Shinn et al. 2000, Weir-Brush et al. 2004). Pathogens make use of extra nutrients and become more virulent (Bruno et al. 2003). Coastal runoff caused by poorly-planned human development in coastal areas is a major source of sediments and nutrients, however, in some cases pristine areas actually have higher incidence of coral disease (Weil et al. 2002). Another source of sediment and nutrients is deposition from African dust clouds, a phenomenon which has been increasing in recent years because of human development and poor land management. The dust is swept up into the atmosphere in clouds which carry it around the earth allowing it to settle out in areas far from its origin in the Sahara, and impact even remote areas of the ocean (Shinn et al. 2000, Garrison et al. 2003, Weir-Brush et al. 2004).

One of the major differences between marine and terrestrial diseases is the ability of many microorganisms to survive much longer in marine environments (Harvell et al. 2004). Also, there are many more animal species and phyla in the marine environment including modular organisms, such as corals. Modular organisms tend to reproduce asexually resulting in populations with lower genetic variation (McCallum et al. 2004, Harvell et al. 2004) which allows for decreasing host resistance and increasing pathogen virulence (McCallum et al. 2004).

Baseline data and monitoring are key to understanding the effects of disease at different spatial and temporal scales (Weil et al. 2002, Weil 2004). Understanding the causes and spread of disease in the ocean can help evaluate its long-term effects on the reef environment (Harvell et al. 2004).

1.1 Aspergillosis

In 1995 an unknown disease severely damaged populations of sea fans throughout the Caribbean. The major signs were reported as lesions surrounded by tissue of either lighter or darker color than the rest of the sea fan (Nagelkerken et al. 1997a and 1997b). Initially a lighter ring appears, but as the sea fan begins to fight the infection, a darker ring appears (Smith et al. 1998). This dark ring is produced by an increased concentration of purple spicules in the area and has been proposed to be a defensive reaction to the infection (Smith et al. 1998).

Disease signs were similar to those of a disease which caused widespread mortality of sea fan colonies in the 1980s (Garzón-Ferriera and Zea 1992, Nagelkerken et al. 1997a, Kim and Harvell 2004). At that time, there was no analysis of the pathogen,

but it is possible that it was an earlier outbreak of the same disease. The major difference is that the 1980s outbreak was more severe, more often causing total mortality, while the 1995 outbreak caused only partial mortality in most cases. This could be because the pathogen involved in the 1995 outbreak was a less virulent pathogen and/or because sea fans were more resistant to the pathogen.

The disease was reported throughout the Antilles, the Bahamas, the Florida Keys and the coasts and offshore islands of the northern shore of South America (Nagelkerken et al. 1997a). The pathogen causing the disease was identified as *Aspergillus sydowii* (Smith et al. 1996, Geiser et al. 1998) and the disease was named aspergillosis. *A. sydowii* is a terrigenous fungus which has adapted to survive in the sea. It cannot sporulate in seawater; therefore, infection must be by hyphae which can survive in the water column and are consistently present with disease symptoms (Smith et al. 1996).

There are two major hypotheses proposed for the introduction of *Aspergillus sydowii* to the sea. The first is that it arrived attached to soil particles in runoff from areas experiencing erosion due to deforestation (Smith et al. 1996). This explains the entry of the pathogen, but not its large scale effects and presence in areas without deforestation and erosion problems including remote islands lacking soil, unless it is considered that the dispersion of hyphae could have happened over long periods of time from an area or areas with heavily deforested watersheds. The second major hypothesis is that it could enter the water attached to particles of African dust. The increase in marine disease in general and the episodic aspergillosis events coincide with increases in African dust transport to the Caribbean (Shinn et al. 2000). *A. sydowii* has been isolated in dust samples from these events (Shinn et al. 2000); while Geiser et al. (1998) found

that sea fans could be inoculated only with marine strands of *A. sydowii*, Weir-Brush et al. (2004) were able to inoculate healthy sea fans with fungus isolated from the dust events. Transportation of dust from Saharan Africa to the Caribbean occurs during the summer (Garrison et al. 2003). Initially, this was believed to coincide with peaks in the disease (Alker et al. 2001); however more recently it has been observed that more new infections occur in the winter and spring, and during summer they increase in size (Kim et al. 2006). If the source of *A. sydowii* can be identified, it may help us identify how to control the input of the fungus into the marine system. Harvell et al. (2004) identified this as a research priority.

In 1996, a Caribbean-wide survey determined the extent of the disease in sea fans (Nagelkerken et al. 1997a&b), followed more recently by other geographic surveys of aspergillosis prevalence (Weil et al. 2002). Aspergillosis prevalence has varied greatly with location and time: surveys throughout the Caribbean have found between 8-60% prevalence in the Florida Keys (Kim and Harvell 2004), 43-52% in Curacao (Nagelkerken et al. 1997b, Nugues and Nagelkerken 2006), 5-25% in Mexico (Mullen et al. 2006), and 6-18% in Puerto Rico (Weil et al. 2002, Toledo-Hernández et al. 2007).

Although it was originally believed to affect only *Gorgonia* spp., aspergillosis is now known to affect gorgonians of three other genera: *Pseudopterogorgia*, *Pseudoplexaura* and *Plexaurella* (Weil 2004, Smith and Weil 2004). It is most commonly reported and easiest to identify on *Gorgonia* spp.

Jolles et al. (2002) evaluated the mode of disease transmission of aspergillosis. They hypothesized three mechanisms of transmission: primary introduction to the ocean by runoff or dust particles and secondary transmission by direct contact with a diseased

organism or by waterborne hyphae released by infected colonies. They found that short distance waterborne transport takes place, because disease clusters were larger than predicted by direct contact alone. This means that diseased colonies can infect other colonies in their vicinity even if they do not have direct contact. However, prevalence seems to be more dependent on outside sources than on transmission between colonies (Kim and Harvell 2004). Viable spores can survive for long time periods and have been found in open waters and trenches (Harvell et al. 2004).

Temperature, depth, sea fan size, water motion, water quality, algal tumors and *Cyphoma* spp. grazing have all been correlated with aspergillosis. Infection is dependent on the immune system of the host and the virulence of the pathogen (Alker et al. 2001) which can be affected by many of these factors.

Sea fans produce antifungal compounds (Kim et al. 2000). Because healthy colonies contain more of these antifungal compounds at their edges while diseased colonies have similar concentrations throughout the colony it appears that these compounds play a role in resistance to the disease. While *Aspergillus sydowii* demonstrates optimal growth at 30ºC, high temperatures stress the host and may suppress its natural production of anti-fungal compounds. This combination of factors may explain why the disease peaks during the warmest water temperatures; increases in temperature shift the balance in favor of the pathogen (Alker et al. 2001).

Disease prevalence was found to increase with depth and sea fan size (Nagelkerken et al. 1997b). In shallow reefs, water motion may decrease attachment of the pathogen (Nagelkerken et al. 1997b). Smaller sea fans have lower rates of infection, probably because they produce more anti-fungal compounds (Dube et al. 2002). The

severity of aspergillosis has been positively correlated with poor water quality; while infection rates are not affected, virulence is greater (Kim and Harvell 2004). However aspergillosis has also been found in clear and clean water conditions (Harvell et al. 2004).

One study reported that sea fans suffering from aspergillosis had a decrease in gamete production in two of three months sampled (Petes et al. 2003). Recruitment was inversely related to disease prevalence in the Florida Keys (Kim and Harvell 2004). A reduction of reproduction and recruitment in sea fans affected by aspergillosis could potentially have long-term effects on the population.

1.2 Aspergillosis in La Parguera, Puerto Rico

This project was initiated in La Parguera, Puerto Rico due to the wide distribution and impact of this disease, the limited information on its distribution and prevalence and the lack of information in other aspects of the dynamics and the specific impact of the disease in the area. The status of aspergillosis in La Parguera and its impact on populations of the sea fan *Gorgonia ventalina* were evaluated. Acquiring more information on the current status and impact of aspergillosis, as well as the spatial and temporal variability, will provide important information for understanding the type of threat the disease poses and for planning management strategies and potential remedies.

The main goals of this project were (1) to assess the spatial and temporal variability in aspergillosis prevalence, (2) to assess the spatial and temporal variability in virulence (rates of advance and recovery), and (3) to assess the impact of the disease on the reproduction of *Gorgonia ventalina*.

1.3 Objectives, Questions and Null Hypotheses

Objective #1: To determine the prevalence of aspergillosis affecting *Gorgonia ventalina* and assess its temporal and spatial variability.

- What is the current status of aspergillosis affecting *G. ventalina* in La Parguera?
- Does the year or season affect the prevalence of aspergillosis?
- Does reef zone, reef, depth or sea fan density affect the prevalence of aspergillosis?

    Ho: The prevalence of aspergillosis on *G. ventalina* is not affected by year, season, reef zone, reef, depth or sea fan density.

Objective #2: To measure the virulence of aspergillosis affecting *Gorgonia ventalina* and determine whether there are temporal and spatial variations.

- How quickly does aspergillosis kill tissue in infected *G. ventalina* colonies?
- Does the rate of tissue loss vary with season or reef?

    Ho: The virulence of aspergillosis on *G. ventalina* is not affected by season or reef.

Objective #3: To determine the effects of aspergillosis on reproduction in *Gorgonia ventalina*.

- Do colonies of *G. ventalina* infected with aspergillosis produce the same number of eggs or spermaries as healthy colonies?

    Ho: Colonies of *G. ventalina* infected with aspergillosis produce the same number of eggs and spermaries per polyp as healthy colonies.

- Do colonies of *G. ventalina* affected with aspergillosis produce eggs and spermaries at the same time as healthy colonies?

Ho: Colonies of *G. ventalina* affected with aspergillosis produce eggs and spermaries at the same time as healthy colonies.

- Is there a systemic effect of aspergillosis in infected colonies?

  Ho: There is no systemic effect of aspergillosis in infected colonies.

2. Prevalence, distribution and virulence of aspergillosis affecting *Gorgonia ventalina* in La Parguera, Puerto Rico

2.1 Abstract

This study assessed the spatial and temporal variability in prevalence and virulence of the infectious fungal disease, aspergillosis, in populations of the common sea fan *Gorgonia ventalina* in several reefs off La Parguera, Puerto Rico. Prevalence was assessed over a two-year period through seasonal surveys of 20m² band transects in several different depth intervals, ranging from 0 to 23 m, in six reefs along an inshore-offshore gradient. The average prevalence of aspergillosis increased from 3.3% (95%CI: 1.8-6.0%) in March 2005 to 17.0% (95%CI: 14.8-19.5%) in September 2006, with values for individual reefs varying from 0-29%. Differences in prevalence between years, seasons, reef zones, reefs, depths and density of sea fans were explored using G-tests and a classification tree. All factors were found to significantly affect the prevalence of aspergillosis; reef site and depth had the strongest influence on disease prevalence followed by the year. The highest prevalence was found at Turrumote, Media Luna and Enrique at depths shallower than 9.6 m in 2006. Spatial and temporal variability in aspergillosis virulence was monitored for fifteen months in different reefs to assess the spatial and temporal dynamics of individual disease lesions, the rate of advance and tissue mortality, the seasonal variability, and the rate of recovery. Friedman Repeated-Measures ANOVAs on Ranks were used to analyze differences in (1) the number of active lesions, (2) areas of purpling and (3) areas of tissue loss. Trends were consistent among all sites; the number of active lesions (May 2006: 3.08 lesions/colony, W=1-23, n=61; July 2007: 0.72 lesions/colony, W=0-4, n=61) and the area of purpling due to

disease (May 2006: 3.44%, W=0.05-21.32, n=61; July 2007: 0.51%, W=0-3.35, n=61) decreased over time as the aspergillosis infection disappeared while the area of tissue loss per colony (May 2006: 1.92%, W=0-19.53, n=61; July 2007: 13.46%, W=0-100, n=61) increased over time as a consequence of the infections. Seasonality had little effect on virulence in tagged colonies; observed trends appeared to be due to the disease running its course in individual colonies. There was high temporal and spatial variability of aspergillosis prevalence and virulence. Abnormally high sea temperatures associated with the 2005 bleaching event may have influenced the increase in prevalence over the course of this study, especially in shallow areas. Smaller scale factors, however, must have affected prevalence at individual sites which is where the highest variability was observed.

2.2 Introduction

Marine diseases have been on the rise in recent decades (Harvell et al. 1999, Knowlton et al. 2001, Harvell et al. 2002, Weil et al. 2002, Harvell et al. 2004, Ward and Lafferty 2004, Weil 2004). While most of the research on marine diseases has been done at one locality and either at one point in time or at widely separated time intervals (Weil et al. 2006) during initial disease outbreaks (Santavy et al. 2001), continuous follow up over time of disease dynamics is lacking. There is also a gap in the understanding of colony recovery and resistance for many diseases (Kim and Harvell 2004). Understanding the spatial and temporal variability of disease dynamics at the colony and population levels over local and geographic scales and the dynamics of disease

progression and/or host recovery will provide information important for understanding the etiology and epidemiology of these syndromes in coral reef communities.

Although a few coral diseases were observed in the 1960s and 1970s, many more have been reported and described since the mid-1990s (Sutherland et al. 2004). The study of coral diseases began with information collected during field observations, and more recently the etiology, epizootiology and physiology of several diseases have been explored (Dark spots disease: Gil-Agudelo and Garzón-Ferreira 2001; Black band disease: Kuta and Richardson 2002; White pox: Patterson et al. 2002). Aspergillosis is one disease which had been the focus of much research; it is caused by the fungus *Aspergillus sydowii* (Smith et al. 1996, Geiser et al. 1998) and affects a number of shallow-water gorgonian species (Weil et al. 2002, Smith and Weil 2004). The major sign of the disease, apparent necrosis surrounded by dark purple tissue caused by a concentration of spicules, is most obvious on the three Caribbean sea fans of the genus *Gorgonia* (*G. ventalina*, *G. flabellum* and *G. mariae*). The net-like structure of sea fan colonies is usually oriented perpendicular to water motion, which is ideal for catching particles from the water that flows through them, including *A. sydowii* spores or hyphae. Surveys at the population level in Caribbean localities indicate that *G. ventalina* is more susceptible to infection with aspergillosis than *G. flabellum* and *G. mariae* (Smith and Weil 2004, Mullen et al. 2006). *G. ventalina* is the most common and abundant of the three species, with a wider geographic and depth distribution.

Aspergillosis prevalence, like that of many coral diseases, is extremely variable in space and time. In 1996, Caribbean-wide surveys were done to determine the extent of the outbreak of aspergillosis affecting sea fan populations (Nagelkerken et al. 1997a&b).

Subsequent studies throughout the Caribbean have consistently found the disease to be present, but have shown high variability in prevalence (Weil et al. 2002, Kim and Harvell 2004, Nugues and Nagelkerken 2006). Although studies from different areas may use different techniques for determining local aspergillosis prevalence, values can be variable even on a temporal basis for a single locality. For example, Kim and Harvell (2004) reported that prevalence of aspergillosis affecting sea fans on reefs of the Florida Keys decreased from 31% in August 1997 to 5.9% in August 2003. In Curacao, the trend has been different; surveys found higher disease prevalence than in the Florida Keys with no significant difference between 1995 (52%) and 2005 (43%) (Nagelkerken et al. 1997b, Nugues and Nagelkerken 2006). In Puerto Rico the percentage of diseased sea fans in 1996 was much lower than in Curacao (10-12%, Nagelkerken et al. 1997b), but it also remained relatively constant in 1999 (11%, Weil et al. 2002) and 2002 (6-18%, Toledo-Hernández et al. 2007).

Depth has been found to be an important factor affecting the prevalence of aspergillosis. For all sites throughout the Caribbean surveyed in 1996, a positive correlation between prevalence and depth up to 12 m was found (Nagelkerken et al. 1997b). A more recent study in the Yucatan reported higher prevalence at sites between 9 and 13.5 m compared to both deeper and shallower sites (Mullen et al. 2006). Size of the host is another factor which may play an important role in the epizootiology of aspergillosis. While some studies have found that larger colonies were more likely to be infected than smaller colonies (Nagelkerken et al. 1997b, Dube et al. 2002, Mullen et al. 2006), one study found colony size not to be an important factor in determining infection rates (Toledo-Hernández et al. 2007).

Virulence of aspergillosis has been positively correlated with depth (Nagelkerken et al. 1997b), sea fan size (Mullen et al. 2006), temperature (Alker et al. 2001) and nutrients (Bruno et al. 2003). In a two-year study in the Florida Keys, 80% of sea fans had less than 25% diseased tissue and 15% of colonies recovered, but only temporarily (Kim et al. 2006). Only 5% of sea fans suffered complete mortality per year, but outbreaks in the area had substantially higher mortalities, between 8 and 95% per year (Kim et al. 2006). Other surveys in Curacao reported that tissue loss related to aspergillosis varied between 3 - 45% on individual colonies (Nugues and Nagelkerken 2006) with no significant differences between 1995 (8.8%) and 2005 (9.6%) (Nugues and Nagelkerken 2006). Understanding disease dynamics in different locations at different times could eventually indicate sources of the pathogen, effects of disease on population structure and long-term survivorship of affected populations. It will also help to evaluate the short- and long-term impacts of disease at the colony, population and community levels in the reef environment (Harvell et al. 2004, Weil et al. 2006).

This study assessed the current status of aspergillosis in populations of the common sea fan *Gorgonia ventalina* in several reefs off La Parguera, on the southwest coast of Puerto Rico. The first major goal was the assessment of the temporal and spatial variability in the prevalence of aspergillosis. Disease surveys were performed over a two-year period at varying depths within several reefs along an inshore-offshore gradient. We expected to see variations in disease prevalence with (a) seasons, because of changes in water temperature, (b) depth, because of differences in water motion and (c) shelf location, because of distance from the shore and related anthropogenic influences such as nutrient and sediment influx. The second major goal of this study was to evaluate the

virulence of aspergillosis in terms of the number of lesions, area of tissue with purpling due to infection and area of tissue loss per colony. The variability in virulence was monitored for fifteen months in different reefs to assess the spatial and temporal dynamics of individual disease lesions, the rate of advance and tissue mortality, the seasonal variability, and the rate of recovery, if any. We expected to see variability in the virulence of the disease over time in response to seasonal changes in water temperature and disease progression within a colony.

Results of this study will provide insight into the current prevalence and virulence of aspergillosis in the area. Combined with similar studies in this area and others, it will provide information on the status and variability of the disease at local, geographic and temporal scales. Knowing the current status of aspergillosis is important for understanding what kind of threat the disease poses to Caribbean sea fan populations and for planning potential management and remedial measures.

2.3 Methods

2.3.1 Study area

This study was carried out in the Natural Reserve of La Parguera, on the southwest coast of Puerto Rico (Figure 2.1). Six reef sites in an inshore-offshore gradient were selected and surveyed to assess the spatial and temporal variability in prevalence of aspergillosis in the area. These reefs were chosen as a good representation of the common reef morphologies in La Parguera. Enrique and Pelotas are both inner-shelf fringing reefs. They are the reefs closest to shore and are the most protected from wave energy, but also the most subject to local human influences. Media Luna and Turrumote

are mid-shelf reefs, another line of fringing reefs farther from shore and more exposed to wave energy. All of these fringing reefs have steep slopes with depths ranging from 1 to 16 m. The two reefs farthest from shore, Weinberg and El Hoyo, are in the gradually sloping area at the edge of the insular shelf with depths ranging from 18 to 23 m. Weinberg is a spur and groove, deep bank reef and El Hoyo is a hard bottom community with sparse coral colonies (Table 2.1, Fig 2.1).

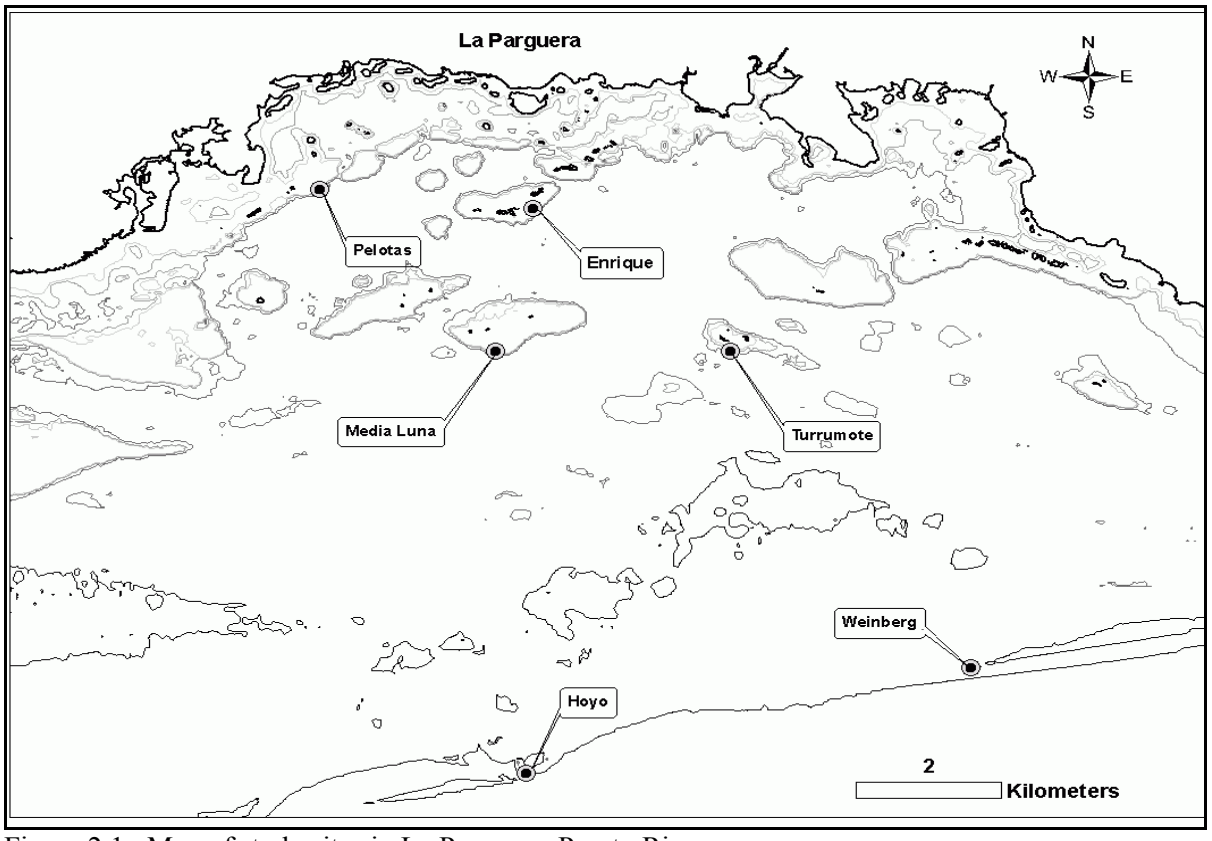

Figure 2.1: Map of study sites in La Parguera, Puerto Rico.

Table 2.1: Characteristics of the six reef sites off La Parguera where the different aspects of this study were conducted. The first four are fringing reefs bordering coral/mangrove keys. Weinberg is a spur and groove, deep bank reef at the edge of the insular platform, and El Hoyo is a hard bottom community with sparse coral colonies. Prevalence surveys were conducted at all six reef sites. Colonies were tagged for the virulence study at the three reefs marked with asterisks (*).

Site	Zone	Coordinates (N)	Coordinates (W)	Distance from shore (km)	Depth range (m)	Slope	Coral cover	Octocoral density
Enrique*	Inner-shelf	17°56.658	67°02.213	1.5	1-14	steep	moderate	moderate
Pelotas	Inner-shelf	17°57.442	67°04.176	1	1-12	steep	low	low
Media Luna*	Mid-shelf	17°56.093	67°02.931	2	4-16	steep	moderate	high (dominate in shallow areas)
Turrumote	Mid-shelf	17°56.097	67°01.130	2	2-15	steep	low	low
Weinberg*	Shelf edge	17°53.429	66°59.320	6	18-23	gradual	high	moderate
El Hoyo	Shelf edge	17°52.559	67° 02.619	8	19-22	gradual	very low	very low

Prevalence surveys were performed at all six of the reefs described and colonies were tagged to monitor the virulence of aspergillosis in three of the reefs (Table 2.1). From each of the three zones, the reef with the highest disease prevalence and sea fan density were chosen for the virulence study. Weinberg and Enrique were chosen because they had both higher disease prevalence and higher sea fan density than El Hoyo and Pelotas. Media Luna was chosen for its high sea fan density and high disease prevalence, even though in some sampling periods prevalence was higher at Turrumote.

2.3.2 Disease signs and definitions

Aspergillosis can be difficult to identify in the field as the presence of hyphae in the tissues is difficult to determine with the naked eye. For this study, aspergillosis was identified by lesions surrounded by hard, dark purple tissue, the common sign used for

field identification (Nagelkerken et al. 1997a&b, Kim and Harvell 2004). This purpling of tissue is indicative of a high concentration of spicules, a general sign of stress which could also be displayed by a colony affected by abrasion, predation or overgrowth. Therefore, in the prevalence surveys, any colony experiencing one of these processes was not considered diseased but was categorized separately and included with healthy colonies for statistical analyses. In the virulence study, no colonies with any sign of abrasion, predation or severe overgrowth were tagged.

Prevalence was defined as the proportion of diseased colonies in the *Gorgonia ventalina* population in a given area (i.e. all colonies in a reef, all colonies at a certain depth) at a given time. Virulence was defined as the change over time in the amount of damage to a colony caused by disease.

2.3.3 Temporal and spatial variability of prevalence

In order to assess the temporal and spatial variability of aspergillosis infections in *Gorgonia ventalina*, sixteen 20m² permanent band transects were surveyed four times over a two year period at each of the six reef areas described above (Fig. 2.1, Table 2.1). At each of the inner and mid-shelf reefs, 8 transects were shallow (4 transects <4m and 4 transects 4-8m) and 8 were intermediate (4 transects 8-12m and 4 transects 12-16m), while at the shelf-edge, all transects were deep (≥16m). Based on the number of sea fan colonies in transects surveyed at each site, site densities were categorized as high (>1.25 colonies/m^2 - Media Luna), medium (0.75-1.25 colonies/m^2 - Enrique, Weinberg) and low (<0.75 colonies/m^2 - Pelotas, Turrumote, El Hoyo). In 2005-2006, each transect was surveyed during March of each year when sea temperatures were near their coolest and

during September of each year when temperatures were near their warmest. At each site, HOBO temperature loggers were deployed from mid-2004 through 2007. A graph of the average temperature for all sites was produced. During each survey, all *G. ventalina* colonies within each band transect were checked for disease signs and counted.

The numbers of healthy and diseased colonies were used for calculating prevalence values (i.e. percent of all colonies affected by disease at a given time in a given reef or at a given depth, etc.) and for statistics. Differences between years and between seasons were explored using G-tests. Within each of the four sampling periods, G-tests were performed to seek differences between zones, sites, depths and sea fan densities. For each of these factors, G-tests were also used to examine differences between sampling periods. For prevalence surveys, the same colonies were surveyed repeatedly in different sampling periods; therefore, data were not independent and significance levels of G-test results should be viewed with caution.

Additionally, in order to explore interactions among factors, a classification tree was produced with the statistical software Statistica 7.1, using all factors and based on presence or absence of disease in each colony. Classification trees are ideal for evaluating ecological data because they can demonstrate interactions between multiple factors even with non-parametric data (De'ath and Fabricus 2000). Splits ($p \leq 0.05$) in the classification tree were made to best predict whether an individual colony has above or below average chance of being diseased.

2.3.4 Temporal and spatial variability of virulence

Sixty-one diseased *Gorgonia ventalina* colonies were tagged at three of the six reefs surveyed above to evaluate the temporal and spatial variability in aspergillosis virulence. Fifteen colonies were tagged at Weinberg (shelf-edge reef), 31 were tagged at Media Luna (mid-shelf fringing reef) and 15 were tagged at Enrique (inner-shelf fringing reef) (Fig. 2.1, Table 2.1). Only colonies exhibiting the common sign of active disease, dead areas surrounded by dark purple tissue, were tagged and mapped within each reef.

All tagged colonies and their affected areas were photographed with a scale on seven occasions: May, July, September and November of 2006 and February, May and July of 2007. Photographs were analyzed using SigmaScan image analysis software. From the initial photographs the total area of each colony was measured. For each sampling period, three measurements of lesions were made for each colony: (1) the number of active lesions, (2) the area of purpling due to active disease and (3) the total area of tissue loss (including both bare and missing skeleton). After the initial sampling period, lesions were considered disease-free if they no longer showed purpling; in these cases only the measurement for tissue loss was taken. For each colony, comparisons between these measurements were made between sampling periods. Data was summarized for each colony, site and sampling period in order to assess the dynamics of the infections (i.e. advancing or arresting) and the rates of tissue mortality and/or regeneration.

Because data did not fulfill the requirements for parametric tests and could not be normalized using standard transformations, Friedman Repeated-Measures ANOVAs on Ranks were used to analyze differences in (1) the number of active lesions, (2) areas of

purpling and (3) areas of tissue loss between sampling periods with pooled sites. For each individual site, Friedman Repeated-Measures ANOVAs were used to evaluate the same three factors. For colonies with a change in the area of tissue loss, rates of tissue loss and regeneration were analyzed separately for differences between sampling periods and sites using Kruskal-Wallis ANOVAs.

2.4 Results

2.4.1 Temporal and spatial variability in prevalence

The average prevalence of aspergillosis affecting *Gorgonia ventalina* colonies in La Parguera increased from 3.3% (95%CI: 1.8%-6.0%) in March 2005 to 17.0% (95%CI: 14.8%-19.5%) in September 2006, with values for individual reefs varying from 0-29% (Table 2.2).

Table 2.2: Aspergillosis prevalence values (proportion of sea fans with signs of disease) and sample size (in parenthesis) for all levels of each factor tested (zone, reef, depth and density) for each sampling period.

		Aspergillosis prevalence (%)			
Factor	Level	March 2005	September 2005	March 2006	September 2006
Zone	Inner	0.7 (291)	10.4 (471)	9.7 (493)	12.4 (493)
	Mid-shelf	6.5 (275)	8.0 (549)	12.7 (655)	25.0 (655)
	Shelf-edge	2.3 (132)	6.7 (299)	4.7 (344)	8.4 (344)
Reef	Enrique	1.6 (122)	15 (227)	17.5 (274)	19.3 (274)
	Pelotas	0 (169)	6.1 (244)	0 (219)	3.7 (219)
	Media Luna	3.9 (179)	6.3 (394)	11.7 (472)	23.5 (472)
	Turrumote	11.5 (96)	12.3 (155)	15.3 (183)	29.0 (183)
	El Hoyo	4.7 (64)	5.1 (79)	0 (72)	11.1 (72)
	Weinberg	0 (68)	7.3 (220)	5.9 (272)	7.7 (272)
Depth	Shallow	1.1 (374)	11.0 (668)	15.0 (776)	30.6 (776)
	Intermediate	8.8 (192)	7.8 (352)	8.0 (372)	13.1 (372)
	Deep	3.0 (132)	7.8 (299)	5.7 (344)	9.2 (344)
Density	Low	4.3 (329)	7.9 (478)	5.9 (474)	14.6 (474)
	Medium	1.1 (190)	11.2 (447)	11.7 (546)	13.6 (546)
	High	3.9 (179)	6.3 (394)	11.7 (472)	23.5 (472)
Total		3.3 (698)	8.6 (1319)	9.9 (1492)	17.0 (1492)

The number of diseased and healthy *Gorgonia ventalina* colonies was dependent on both year within each season (G-tests: $G_{(March)} = 32.878$, $G_{(September)} = 45.355$, df=1, $p \leq 0.05$) and season within each year (G-tests: $G_{(2005)} = 22.536$, $G_{(2006)} = 33.334$, df=1, $p \leq 0.05$). For each season (March, September) there was a significant increase in the proportion of diseased colonies from 2005 to 2006 (Unplanned test for goodness of fit: df=1, $p \leq 0.05$). For each year, there was a significant increase in diseased colonies from March to September (Unplanned test for goodness of fit: df=1, $p \leq 0.05$) (Fig. 2.2, Table 2.2).

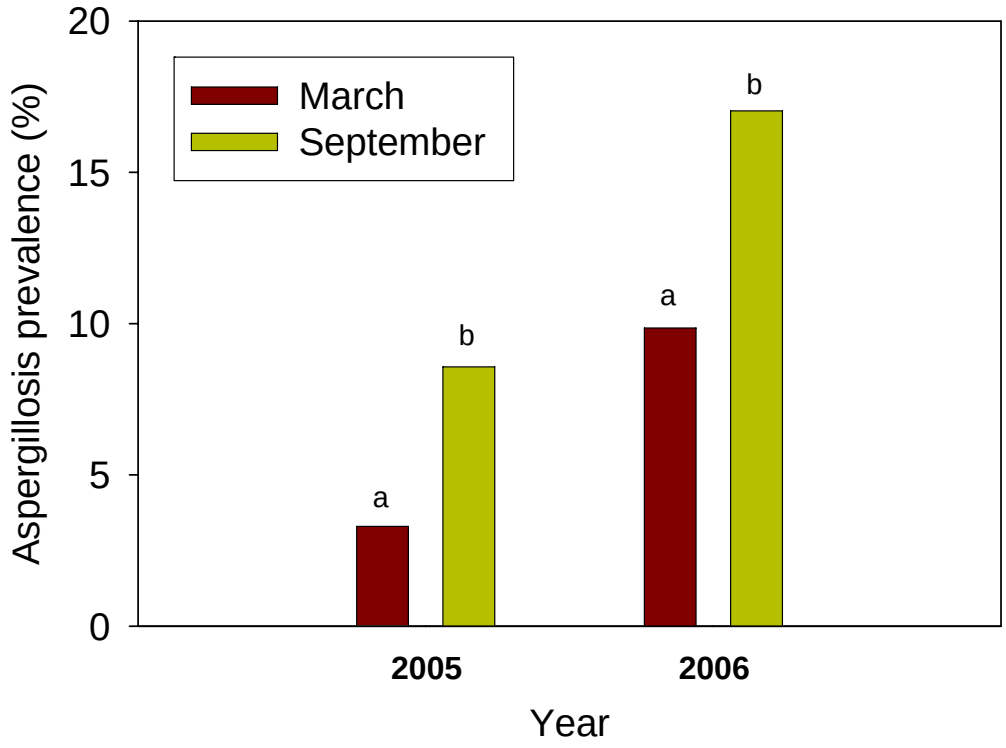

Figure 2.2: Grouped bar graph of the increase in aspergillosis prevalence from March 2005 to September 2006. ($n_{(March\ 2005)}$=698, $n_{(September\ 2005)}$=1319, $n_{(March\ 2006)}$=1492, $n_{(September\ 2006)}$=1492) Letters denote significant differences within groups (months within years) (G-tests with Unplanned tests for goodness of fit: df=1, $p \leq 0.05$).

Temperatures in the summer of 2005 were warmer than in 2006 and 2007 while temperatures in the winter of 2005-6 did not drop as low as in 2004-5 (Fig. 2.3).

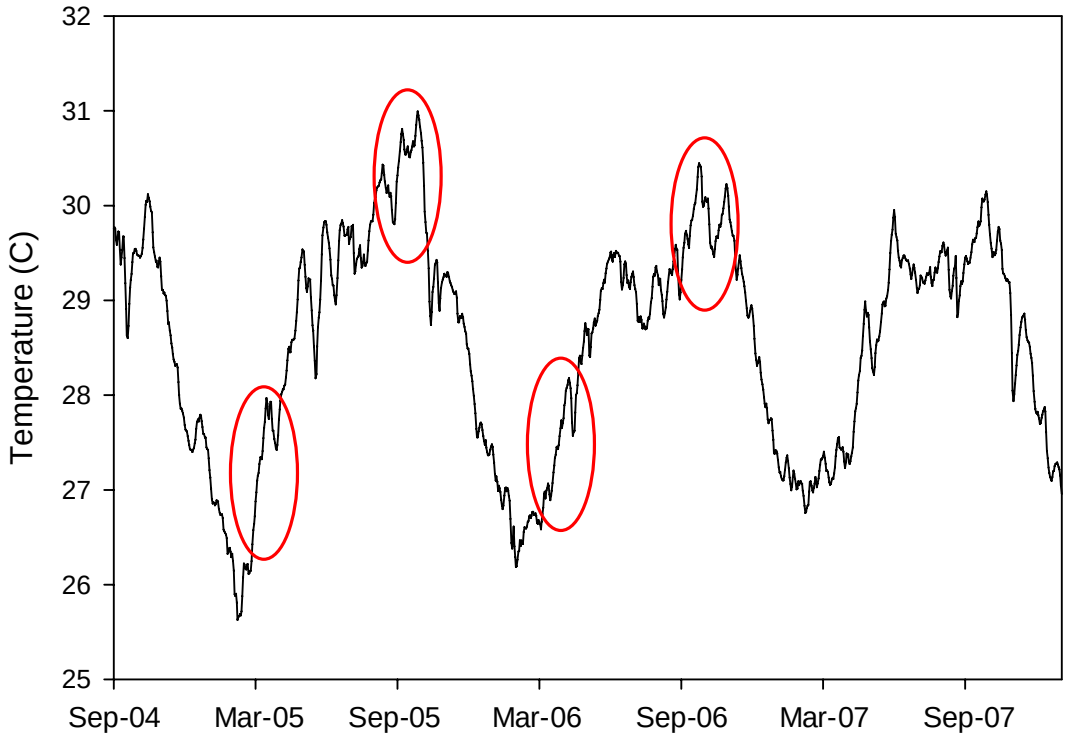

Figure 2.3: Line graph of average water temperature from September 2004 to December 2007 at the six sites surveyed in La Parguera, constructed using data collected by HOBO temperature loggers at those sites. Red ovals indicate sampling periods.

The mid-shelf reefs had significantly higher disease prevalence than the inner reefs in March 2005 and significantly higher disease prevalence than both the inner reefs and the shelf-edge reefs in both March and September of 2006 (G-tests: $G_{(March\ 2005)}$ = 16.730, $G_{(March\ 2006)}$ = 18.212, $G_{(September\ 2006)}$ = 56.304, df=2, $p \leq 0.05$; Unplanned tests for goodness of fit: df=2, $p \leq 0.05$). The most significant increases were for inner reefs between March and September 2005 and for mid-shelf reefs between March and

September 2006 (G-tests: $G_{(inner)} = 47.787$, $G_{(mid\text{-}shelf)} = 90.595$, df=2, $p \leq 0.05$; Unplanned tests for goodness of fit: df=2, $p \leq 0.05$) (Fig. 2.4a, Table 2.2).

In all sampling periods, there were significant differences in the prevalence of aspergillosis between sites (G-tests: $G_{(March\ 2005)} = 10.654$, $G_{(September\ 2005)} = 18.975$, $G_{(March\ 2006)} = 20.391$, $G_{(September\ 2006)} = 90.387$,df=5, $p \leq 0.05$). Turrumote, Media Luna and Enrique had significantly higher prevalence than the other three sites in September 2006 (Unplanned tests for goodness of fit: df=5, $p \leq 0.05$) and each had among the highest prevalence in two of the three earlier sampling periods. Within each of these three sites, there were significant increases in disease prevalence over the course of the study (G-tests: $G_{(Turrumote)} = 20.967$, $G_{(Media\ Luna)} = 75.251$, $G_{(Enrique)} = 31.208$, df=3, $p \leq 0.05$; Unplanned tests for goodness of fit: df=3, $p \leq 0.05$), while the other three sites (Pelotas, El Hoyo and Weinberg) had no significant differences between sampling periods (Fig. 2.4b, Table 2.2).

The depth of a colony also affected whether it was infected with aspergillosis (G-tests: $G_{(March\ 2005)} = 17.460$, $G_{(March\ 2006)} = 19.914$, $G_{(September\ 2006)} = 50.806$, df=2, $p \leq 0.05$). In March 2005 colonies at intermediate depths had significantly higher disease prevalence than shallower colonies, however in both March and September 2006, shallow colonies had significantly higher disease prevalence than intermediate and deep colonies (Unplanned tests for goodness of fit: df=2, $p \leq 0.05$). Over time, there were significant increases in prevalence among colonies in shallow habitats (G-test: $G_{(shallow)} = 143.184$, df=3, $p \leq 0.05$; Unplanned test for goodness of fit: df=3, $p \leq 0.05$), but not in intermediate and deep habitats (Fig. 2.4c, Table 2.2).

Three of the four sampling periods had significant differences in disease prevalence at sites with different sea fan densities (G-tests: $G_{(September\ 2005)} = 6.545$, $G_{(March\ 2006)} = 13.235$, $G_{(September\ 2006)} = 19.963$, df=2, $p \leq 0.05$). In September 2005, medium density sites had significantly higher disease prevalence than high density sites, but in March 2006, colonies in reefs with high and medium densities of *G. ventalina* were more likely to be diseased than colonies at low-density sites, and in September 2006 high density sites had more disease than medium density sites (Unplanned test for goodness of fit: df=2, $p \leq 0.05$). Within all levels of sea fan density, there were significant increases in aspergillosis prevalence over time (G-tests: $G_{(low)} = 32.700$, $G_{(medium)} = 34.166$, $G_{(high)} = 75.251$, df=3, $p \leq 0.05$; Unplanned tests for goodness of fit: df=3, $p \leq 0.05$), but the most dramatic differences were in the high-density sites (Fig. 2.4d, Table 2.2).

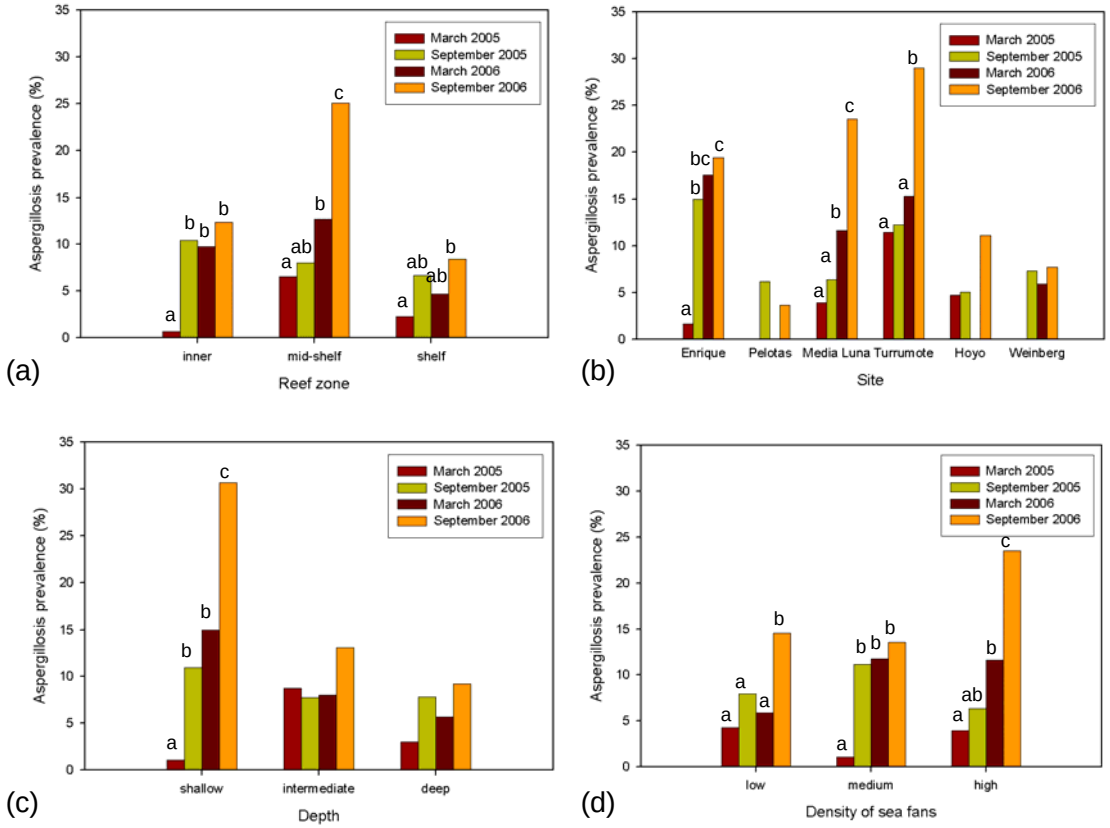

Figure 2.4: Grouped bar charts of aspergillosis prevalence among *Gorgonia ventalina* colonies in (a) different reef zones (b) different reefs (c) different depths and (d) sites with different sea fan densities. (For n values see Table 2.2.) Letters denote significant differences within groups (sampling periods within site, depth, reef zone and sea fan density) (G-tests with Unplanned tests for goodness of fit: df=2 (depths, reef zones, densities), df=5 (sites), $p \leq 0.05$). Groups with no letters had no significant differences.

All factors (year, season, zone, site, depth and the density of sea fans at the site) were found to significantly affect the prevalence of aspergillosis in the G-tests above and were therefore used to build the classification tree (Fig. 2.5). The first split in the classification tree was based on sites. The three reef sites that split to the left (Weinberg, Pelotas and Hoyo) had a disease prevalence of 4.8% which is much lower than the average for all sites (10.7%). There were no more splits from this group indicating that no other factor had a strong influence on the prevalence of disease at these low-

prevalence sites. The three reef sites that split to the right (Media Luna, Turrumote and Enrique) had a disease prevalence of 15.4% which is higher than the average prevalence of aspergillosis for all the sites. Depth was the next most important factor influencing prevalence at these high-prevalence sites; depth was not an important factor determining prevalence at the other sites indicating an interaction between site and depth. Deeper than 9.7 meters at the high-prevalence sites, prevalence was significantly lower than average (8.1%); this group split again based on reef site. The average disease prevalence in colonies deeper than 9.7 m at Media Luna and Enrique was 6.1%, while at Turrumote, it was 16.8%. Among colonies shallower than 9.7 meters at the high-prevalence sites, prevalence was significantly higher than average (16.1%), and the year had the next strongest effect on prevalence. In 2005 only 8.9% of colonies were diseased while in 2006, disease prevalence was 20.4%.

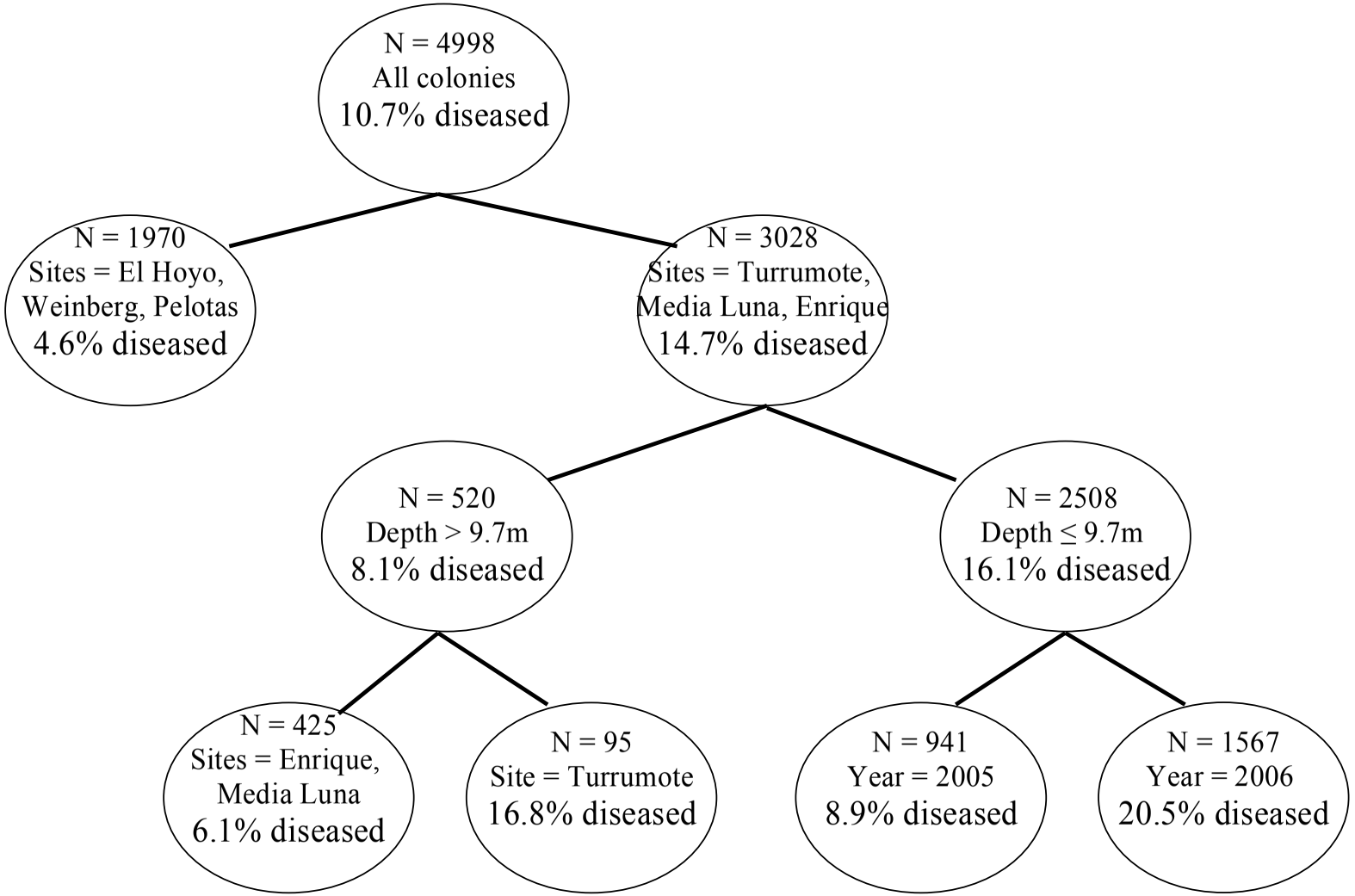

Figure 2.5: Classification tree created using six factors (year, season, zone, site, depth and sea fan density). Circles display the levels of the factor on which the split was based along with the number of colonies included in the group and the percentage of those colonies which were diseased. Splits were made at the p=0.05 level.

2.4.2 Temporal and spatial variability in virulence

The time-series photographs of individual tagged colonies indicated a high variability in disease dynamics at the colony level over time. Although 61% (95%CI: 47.0-73.9) of colonies had a net increase in tissue loss during the 15-month study (Fig. 2.6a) and 43% (95%CI: 29.9-57.0) suffered from new lesions (Fig. 2.6b), 94% (95%CI: 83.3-98.3) of colonies showed some recovery from purpling due to infections (Figure 2.6c) and 21% (95%CI: 11.5-34.9) had a net regeneration of tissue during the study (Fig. 2.6d).

Figure 2.6a: Photographic time series of a colony at Enrique with significant tissue loss (partial mortality). (i) May 2006, (ii) November 2006 and (iii) February 2007.

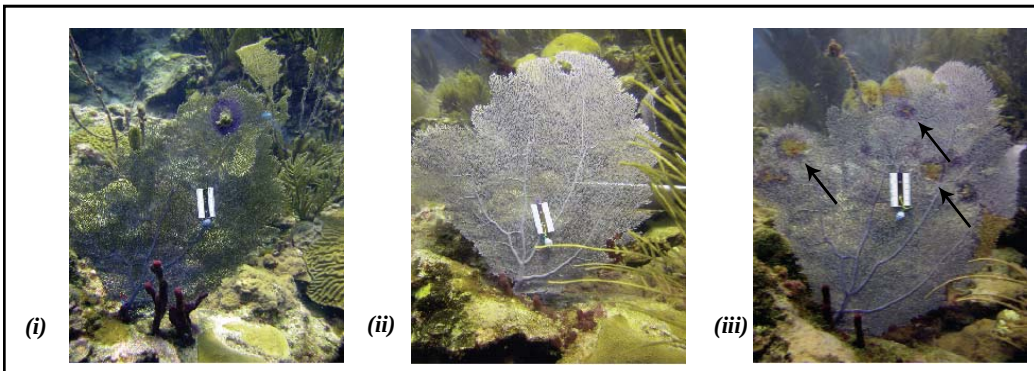

Figure 2.6b: Photographic time series of a colony at Media Luna which developed new lesions (arrows) during the course of the study. (i) May 2006, (ii) September 2006 and (iii) February 2007.

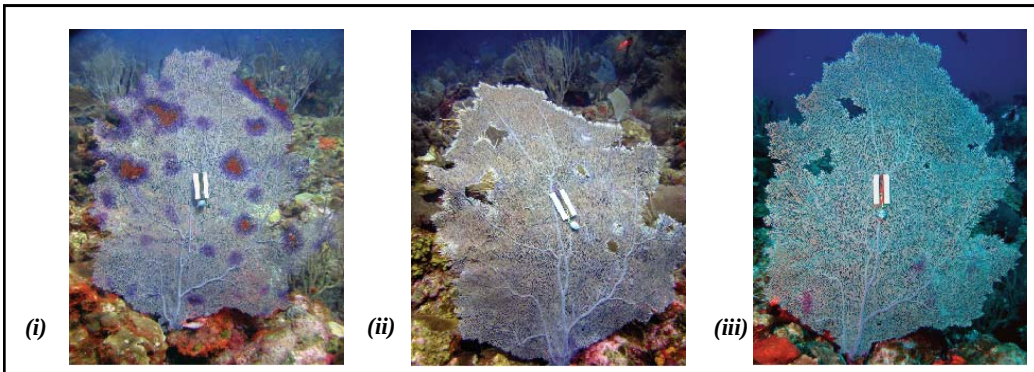

Figure 2.6c: Photographic time series of a colony at Weinberg which recovered from a large number of lesions. (i) May 2006, (ii) September 2006 and (iii) February 2007.

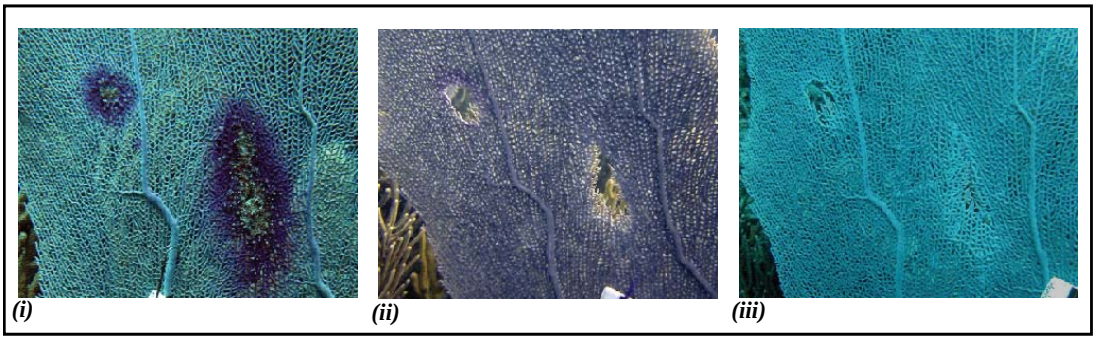

Figure 2.6d: Photographic time series of a lesion showing tissue regeneration. (i) May 2006, (ii) September 2006 and (iii) November 2007.

2.4.2.1 Number of Active Lesions

There was a significant decrease in the average number of lesions per colony between sampling periods from 3.08 lesions per colony (W(range)=1-23, n=61) in May 2006 to 0.72 (W=0-4, n=61) in July 2007 when all sites were pooled (Friedman Repeated-measures ANOVA on ranks: $X^2 = 163.684$, df=6, $p<0.0001$) (Fig. 2.7a). All sites showed a decrease in the average number of active lesions per colony by the end of the study (Fig. 2.7b); only 3% (95%CI: 0.4-12.2) of colonies (one at Media Luna and one at Enrique) showed an increase in the number of lesions per colony.

Weinberg showed the strongest significant decrease in the average number of active lesions per colony (Friedman Repeated-measures ANOVA on ranks: $X^2 = 64.262$, df=6, $p<0.0001$). This decrease was clear even in July 2006 despite the appearance of 13 new lesions on five of the 15 colonies. In November 2006, there were no active lesions on the tagged colonies at Weinberg and during the last three sampling periods, only three colonies had a recurrence of active lesions. Enrique had a decrease in the number of active lesions per colony in each sampling period (Friedman Repeated-measures ANOVA on ranks: $X^2 = 44.014$, df=6, $p<0.0001$). Media Luna also had an overall decline in the average number of active lesions per colony with time (Friedman Repeated-measures ANOVA on ranks: $X^2 = 65.547$, df=6, $p<0.0001$); however, there was an increase in the average number of active lesions per colony in February 2007 when nine of the 31 colonies at Media Luna had a total of 26 new lesions (Figs. 2.6b, 2.7b). By May 2007, 81% of those new (95%CI: 67.1-92.1) lesions had disappeared.

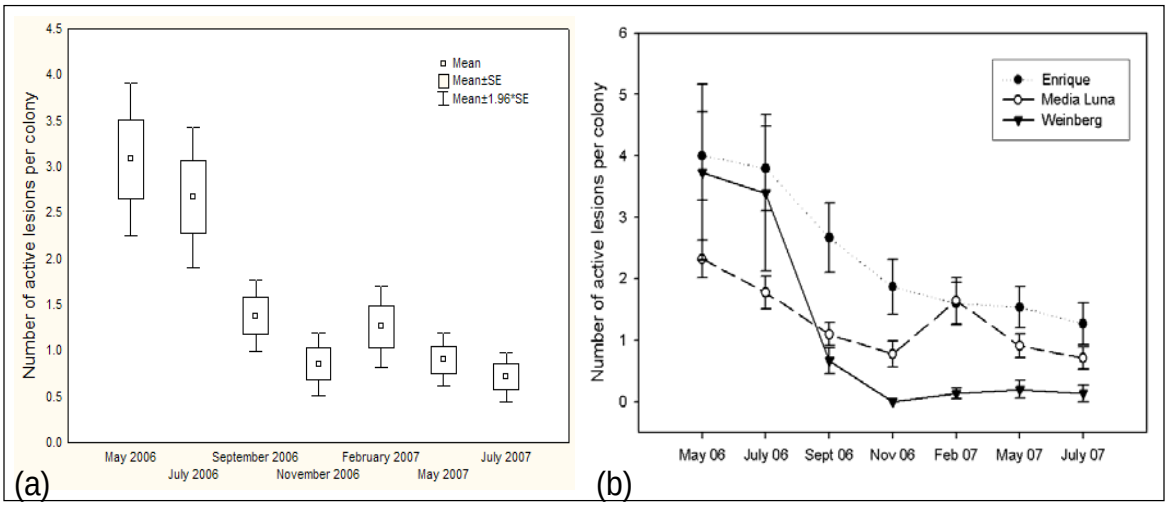

Figure 2.7: Decreasing trend in the average number of active lesions per colony. (a) Boxplot for all sites pooled. (b) Line graph with standard error bars for each individual site. ($n_{(total)}$=61, $n_{(Enrique)}$=15, $n_{(Media\ Luna)}$=31, $n_{(Weinberg)}$=15)

2.4.2.2 Area of purpling due to disease and area of tissue loss

When all sites were pooled, there were significant differences between sampling periods for both the average proportion of a colony with purpling due to disease (Friedman Repeated-measures ANOVA on ranks: X^2 = 161.407, df=6, p<0.0001) (Fig. 2.8a) and the average proportion of a colony with tissue loss (Friedman Repeated-measures ANOVA on ranks: X^2 = 58.573, df=6, p<0.0001) (Fig. 2.9a). The average proportion of a colony with purpling decreased from 3.44% (W=0.05-21.32%, n=61) at the beginning of the study in May 2006 to 0.51% (W=0-3.35%, n=61) by the end of the study in July 2007. The decrease in purpling was due to a combination of recovery from infection and tissue death. The average proportion of a colony with tissue loss increased from 1.92% (W=0-19.53%, n=61) at the beginning of the study to 13.46% (W=0-100%, n=61) at the end. When the proportion of purpling and the proportion of tissue loss were

combined, the overall effect of the disease increased over time from 5.44% (W=0.05-30.62, n=61) in May 2006 to 13.84% (W=0-100, n=61) in July 2007.

Trends were consistent among all sites; the area of purpling due to disease decreased over time (Fig. 2.8b) as the aspergillosis infection disappeared, while the area of tissue loss per colony increased over time (Fig. 2.9b) as a consequence of the infections, resulting in an overall increase in the total effect of the disease. Weinberg, at the shelf edge, showed the most significant decline in the average proportion of a colony with purpling (Friedman Repeated-measures ANOVAs on ranks: Weinberg - X^2=65.529, df=6, $p<0.0001$; Media Luna - X^2=69.622, df=6, $p<0.0001$; Enrique - X^2=38.146, df = 6, $p<0.0001$) and the most significant increase in the average proportion of a colony with tissue loss (Friedman Repeated-measures ANOVAs on ranks: Weinberg - X^2=20.746, df=6, p=0.00204; Media Luna - X^2=21.337, df=6, p=0.00160; Enrique - X^2=22.035, df = 6, p=0.00119)(Figs. 2.8b, 2.9b). Over the course of the study only one colony, which was located at Weinberg, suffered complete mortality, a mortality rate of 1.4% for the duration of this study.

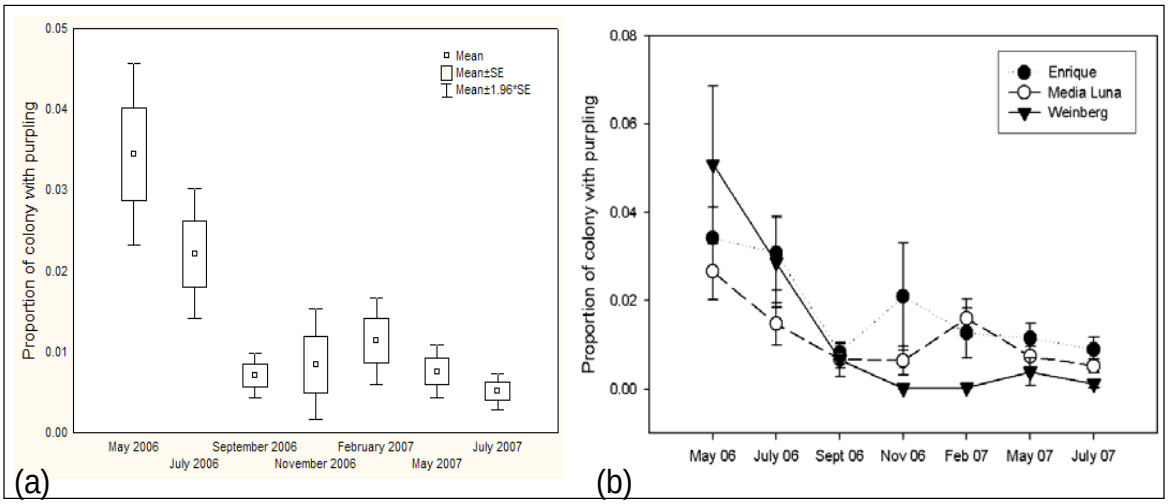

Figure 2.8: Decreasing trend in the average proportion of each colony with purpling due to disease. (a) Boxplot for all sites pooled. (b) Line graph with standard error bars for each individual site. ($n_{(total)}$=61, $n_{(Enrique)}$=15, $n_{(Media\ Luna)}$=31, $n_{(Weinberg)}$=15)

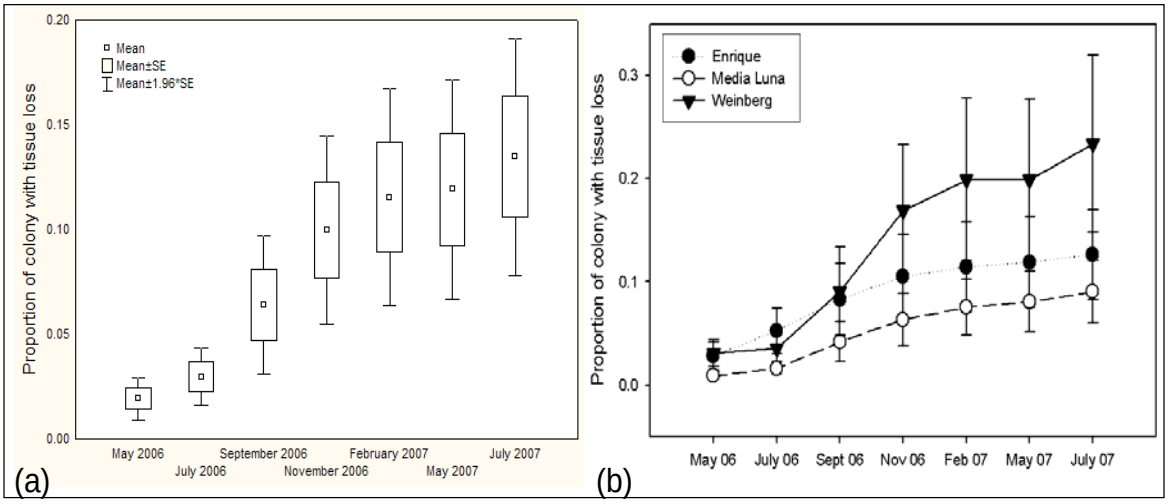

Figure 2.9: Increasing trend in the average proportion of each colony with tissue loss. (a) Boxplot for all sites pooled. (b) Line graph with standard error bars for each individual site. ($n_{(total)}$=61, $n_{(Enrique)}$=15, $n_{(Media\ Luna)}$=31, $n_{(Weinberg)}$=15)

2.4.2.3 Rate of tissue loss

There was no significant difference in the rate of tissue loss between sites (Kruskal Wallis ANOVA: X^2=0.553, df=2, p=0.7585) or sampling periods (Kruskal Wallis ANOVA: X^2=5.381, df=2, p=0.3711). The rate of tissue regeneration was significantly faster (Kruskal-Wallis ANOVA: X^2=9.403, df=2, p=0.0091) at Weinberg (0.257 cm^2/day, W=0.002-1.476, n=18) than at Media Luna (0.112 cm^2/day, W=0.001-1.300, n=30) (Multiple comparisons: $p \leq 0.05$). This could be partly because colonies at Weinberg suffered more damage and, therefore, there was more potential for recovery. There was no significant difference in the rate of regeneration between sampling periods (Kruskal- Wallis ANOVA: X^2=4.548, df=6, p=0.4735). Tissue was killed by disease much faster than it was regenerated in recovered colonies. Also, more colonies in each sampling period experienced tissue loss than tissue regeneration, resulting in a net increase in average tissue loss per colony (Table 2.3, Fig. 2.8) at an overall average rate of 0.372 cm^2/day (W = -1.476-13.105, n=364).

The colony in Figure 2.5a had a rate of tissue loss of 1.80 cm^2/day which is approximately 54 cm^2/month. The larger lesion in Figure 2.5d recovered tissue at a rate of 0.065 cm^2/day or approximately 2 cm^2/month.

Table 2.3: Number of colonies experiencing tissue loss, tissue regeneration, advance of disease, retreat of disease or no change during different sampling periods. Data is pooled across sites.

	May-July 2006	July-Sept 2006	Sept-Nov 2006	Nov 2006-Feb 2007	Feb-May 2007	May-July 2007
# of colonies with increase in purple area	12	6	11	19	11	8
# of colonies with decrease in purple area	48	49	29	11	20	21
# of colonies with tissue loss	26	32	23	24	17	15
# of colonies with tissue regeneration	10	9	10	9	14	10
# of colonies with no change	1	4	14	18	19	23

2.4.2.4 Mystery tissue loss

Throughout this study, tissue loss was associated with purpling either in the same photograph in which tissue loss was observed or in photographs of the colony from the previous sampling period. However, two colonies experienced large amounts of rapid tissue loss in areas where purpling was not observed in photographs (Fig. 2.10). Both of the colonies were located at Weinberg and had shown complete recovery of original aspergillosis lesions by July 2006; then in September 2006 each displayed a small amount of tissue loss (~30 cm^2) followed by significant tissue loss (~800 cm^2) in November at a rate of about 12 cm^2/day which was by far the fastest tissue loss observed in the study. There was no purpling of the edges which might indicate that whatever was killing the tissue was too fast to allow the concentration of spicules in the affected areas. One of these colonies had regenerated some tissue (~15 cm^2) by February and continued to regenerate tissue through July 2007 (~100 cm^2); between May and July 2007, tissue was regenerated at a rate of 1.5 cm^2/day, the fastest that any colony regenerated tissue during this study. No photographs of these colonies showed the source of damage. Even

in the first photographs showing small amounts of tissue loss, there were signs of tissue regeneration (tissue at edges of lesions displayed pointed tips similar to healthy colony edges where growth occurs), indicating that the colony was already recovering from damage before the major damage occurred. The damage could be due to disease or to some other source such as predation, but the rates of tissue loss and regeneration were both the fastest observed in this study indicating that the source of damage may have been different and that tissue regeneration was not slowed by recovery from disease. The results of statistical tests were not affected when these two colonies were excluded from analyses.

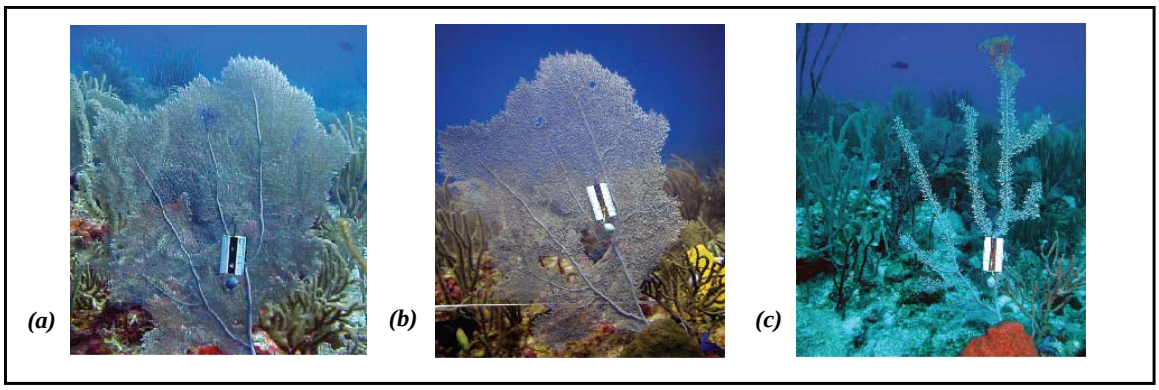

Figure 2.10: Photographic time series of a colony at Weinberg which had tissue loss with no sign of disease. (a) July 2006, (b) September 2006 and (c) February 2007.

2.5 Discussion

2.5.1 Prevalence and Distribution

Overall, aspergillosis prevalence in La Parguera increased between March 2005 (3.3%) and September 2006 (17.0%). There was high variability in disease prevalence in both space and time (0%-29%). This high variability has been found in other surveys of

aspergillosis prevalence in Puerto Rico and around the Caribbean. Average aspergillosis prevalence values for individual reefs were similar to values from surveys in Puerto Rico since the 1995 outbreak (Puerto Rico: 6-18%, 2002 (Toledo-Hernández et al. 2007), La Parguera, Puerto Rico: 4-22%, 2003-2004 (Weil et al. unpublished data)), but were lower than many values from around the Caribbean ((Florida Keys: 8-60%, 1997-2003 (Kim and Harvell 2004); Mexico: 5-75%, 2000-2001 (Mullen et al. 2006); Curacao: 10-80%, 1995, 2005 (Nugues and Nagelkerken 2006)).

The overall increase over time indicated a regional factor affecting aspergillosis prevalence, while the significant increases over time in shallow but not intermediate or deep areas and in only some reefs indicate that small-scale factors may have a stronger influence than, or act synergistically with, large-scale factors.

Two major large-scale factors could be affecting aspergillosis prevalence: increases in fungal spores carried to Caribbean reefs by African dust events and increases in water temperature. The African dust season was more prolonged in 2006 than in 2005 (Roy Armstrong pers. comm.) providing a potential source for *Aspergillus sydowii* (Shinn et al. 2000, Garrison et al. 2003, Weir-Brush et al. 2004); however, the dust only settles during rain events which were less frequent in 2006 (CARICOMP data collected by E. Weil). While African dust clouds may be an important source of *A. sydowii* and may have contributed to the increase in aspergillosis prevalence within each year, they do not appear to be driving all trends observed in this study. Temperature appears to be a more important factor.

While causality is extremely difficult to prove, there is evidence that high temperatures may increase prevalence of diseases in general and coral diseases in

particular (Harvell et al. 2002). Prevalence of white syndrome in the Pacific has been linked to high water temperatures (Bruno et al. 2007). Both the aspergillosis epizootic in 1995 and an earlier epizootic of an unidentified disease affecting sea fans in the early 1980s coincided with rapid temperature increases (Kim and Harvell 2004). While each year there was a significant increase in prevalence from March to September which correlated with seasonal increases in water temperature, there was no decrease in prevalence between September 2005 and March 2006 as expected. This may be the result of the higher-than-normal winter sea surface water temperatures associated with the bleaching event of 2005. Water temperatures were higher than normal during the late summer of 2005 and remained higher than normal throughout the fall and winter. Higher temperatures have been reported to benefit the fungus over the host because the growth rate of *Aspergillus sydowii* increases with increasing temperature up to 30ºC while the anti-fungal extracts produced by *Gorgonia ventalina* are less effective at such high temperatures (Alker et al. 2001); this may explain the lack of a decrease in prevalence over the winter of 2005-2006.

Higher than normal water temperatures could also explain the higher aspergillosis prevalence in shallow areas during 2006. Shallow areas accounted for most of the increase in aspergillosis prevalence during the course of this study, and the increases in prevalence in shallow areas followed the same trend as the overall increases. In this study, colonies in areas shallower than 9.7 m had higher prevalence than those in deeper areas; contradicting earlier surveys which found that aspergillosis prevalence increased with depth (Nagelkerken et al. 1997b) or peaked at intermediate depths (Mullen et al. 2006). Part of this discrepancy could be due to other factors influencing disease

prevalence that vary with depth. Water motion, nutrient influx, turbidity and temperature are factors that vary with depth and may affect the dynamics of aspergillosis, but may not be consistent over time and in different areas. Increased water motion may decrease the chance of aspergillosis infection (Nagelkerken et al. 1997b), but if shallower areas are warmer and more prone to nutrients and soil particles remaining suspended in the water, this may have a more important effect on disease prevalence.

Prevalence varied greatly across reef sites which might be related to the location of the reef. Weinberg and El Hoyo are deep localities at the shelf edge of southwestern Puerto Rico and both had lower than average prevalence in all sampling periods. The shelf-edge sites are far from point-source coastal anthropogenic influences which may reduce the susceptibility of local sea fans or limit the input of the spores or hyphae which infect colonies. Media Luna and Turrumote, the two mid-shelf reefs, had high disease prevalence; while Pelotas grouped with the low-prevalence shelf-edge reefs, Enrique had high disease prevalence along with the two mid-shelf reefs. This indicates that although there were some statistical differences between reef zones, differences between individual reefs had more influence over aspergillosis prevalence. More reefs in each zone would have to be sampled to generalize about trends within reef zones.

Media Luna had a high density of sea fans (~1.5 colonies/m^2) and other gorgonians. Density is one of the most important aspects in the epidemiology of a disease which is contagious at closer range (contact or water borne transmission). The closer the colonies are to one another, the higher the probability of infection (Jolles et al. 2002). In 2006, disease prevalence increased with the density of sea fan colonies at the site in contradiction to other studies, which found disease to be density independent (Kim

and Harvell 2004, Toledo-Hernández et al. 2007). However, this trend was not strong; population densities were not an important enough factor to produce any splits in the classification tree and the significant results of the G-tests for sea fan densities could be a reflection of the differences across sites since Media Luna was the only high density site.

Disease prevalence at Turrumote was among the highest of all reefs in all sampling periods, peaking close to 30% in the summer of 2006. It is also a reef which has suffered greatly from other diseases such as yellow band disease in *Montastraea* spp. (Weil unpublished data). Distance from shore and density of sea fans may have accounted for some of the differences in aspergillosis prevalence between sites, but the data show an interaction between sites and sampling periods indicating that there are variable factors affecting prevalence. This patchiness with time and location has been found in other aspergillosis surveys (Kim and Harvell 2004, Nugues and Nagelkerken 2006, Mullen et al. 2006). Studies of the local conditions at Turrumote could indicate what factors make the local organisms more susceptible to disease.

2.5.2 Virulence

Virulence showed variability between colonies and between reefs. There were strong trends in virulence over time which did not appear to be attributable to the seasonality of aspergillosis. The decrease in purpling was relatively steady, most likely representing a combination of death of infected tissue and the progressive decline of the disease as the host sequesters the pathogen and increases its defenses. Outbreaks were localized, similar to results in the Florida Keys (Kim and Harvell 2004). Peaks of new infections were observed in July 2006 at Weinberg and in February 2007 at Media Luna.

The July 2006 event at Weinberg coincides with the season when African dust clouds pass over the Caribbean, which is a potential source of *Aspergillus sydowii* spores (Shinn et al. 2000, Garrison et al. 2003, Weir-Brush et al. 2004); however, there were no new lesions at Media Luna or Pelotas at this time indicating that the source of the outbreak was localized rather than wide-spread as would be expected with African dust. The sudden increase in incidence at Media Luna in February 2007 seemed to follow the predicted trend of new lesions in the winter and spring developing into larger lesions during the warmer months (Alker et al. 2001, Harvell et al. 2002, Kim and Harvell 2004, Kim et al. 2006), but most of these new infections arrested by the next visit in May 2007, and the overall rate of retreat of the disease was faster during the warmer months. Based on how quickly the disease virulence changed, the prevalence surveys may have missed some of the variability in aspergillosis prevalence.

Overall, there was a decrease in the proportion of diseased tissue and an increase in the proportion of tissue loss during the study. The decrease in diseased tissue may reflect the trend only in the tagged colonies and not necessarily the entire sea fan population; since only diseased colonies were tagged, there was no measure of infections in previously healthy colonies. Twenty-one percent (21%) of colonies lost large proportions of tissue (>20% of original colony area) to aspergillosis during the study while 25% had little (<5%) and 20% had no tissue loss. Sixty-one percent (61%) of colonies suffered tissue loss due to the disease while only 20% showed tissue regeneration and the rate of tissue regeneration was slower than the rate of tissue loss resulting in a net increase in average tissue loss per colony over time. Colonies can recover from tissue purpling due to aspergillosis faster than they can recover from tissue

loss partly because fouling of bare skeleton prevents tissue regeneration (Kim et al. 2006). It was also observed that when dead skeleton (and the fouling organisms associated with it) falls away, it clears the way for tissue recovery; however, this process is much slower than recovery from purpling because the colony must also generate new skeleton.

The long-term survival of each individual colony depends on how much tissue destruction the disease produces, whether the colony returns to a disease-free state and how much time the colony has to recover from tissue loss while disease free.

2.6 Conclusions

Aspergillosis prevalence varied significantly with both year and season. The seasonal increase in prevalence was expected due to the annual cycle of temperature increases, however, the general increase between 2005 and 2006 was not expected. This can most likely be attributed to higher than normal temperatures associated with the 2005 bleaching event.

Disease prevalence varied with each of the spatial factors tested in at least three of the four sampling periods. Results of G-tests combined with the classification tree indicated that reef and depth were the most important factors affecting aspergillosis prevalence in *Gorgonia ventalina*.

Prevalence was inversely related to depth contrary to expectations. High temperatures may be the cause of some of the increase; however, not all sites had increases in shallow areas in 2006 indicating that other factors also contributed.

Reef zone was expected to have a strong effect on prevalence, but this was overshadowed by the effect of individual reefs. More in depth studies are needed to identify small scale factors that influence prevalence at individual reefs.

Virulence varied greatly between individual colonies and over time. Overall, tissue loss increased over the course of the study while purpling due to disease decreased. This appeared to reflect the disease running its course and the host developing resistance rather than any seasonal influence. Infections were generally, short-lived, and colonies were capable of regenerating tissue.

Temporal and spatial variability in aspergillosis prevalence and virulence and how this variability affects the balance between tissue loss and regeneration, could affect the future of local sea fan populations. Episodes of abnormally high temperatures such as that which led to the 2005 bleaching event had a strong effect on the prevalence of the disease and the predicted increase in the occurrence of these events due to global warming could significantly increase the prevalence, incidence and virulence of aspergillosis and reduce the recovery time available to damaged colonies, effectively changing the balance in favor of the pathogen and against the host.

With relatively low disease prevalence and the potential for rapid recovery, aspergillosis is not likely to have devastating effects on the *Gorgonia ventalina* population unless changing conditions shift the balance in favor of the pathogen.

3. Impact of aspergillosis on sexual reproduction of the octocoral *Gorgonia ventalina*

3.1 Abstract

Coral and octocoral diseases have obvious negative effects on their hosts in the form of tissue and colony mortality. Other effects, such as decreases in physiological function and reproductive output, while not as obvious, may have long-term ecological and evolutionary consequences for populations affected by disease. Tissue samples from both healthy colonies and colonies with signs of the fungal disease aspergillosis were collected monthly from randomly selected *Gorgonia ventalina* colonies between November 2005 and October 2006 in La Parguera, Puerto Rico to assess the impact of aspergillosis on the reproductive biology of this sea fan. Using histological samples, the reproductive cycle of *G. ventalina* and the effects of aspergillosis infection on the reproduction of the species were examined. *G. ventalina* is a gonochoric spawner, and both the proportion of reproductive polyps and the number of eggs per polyp in healthy female colonies peak at the same time indicating that spawning may have taken place in the winter of 2005-2006; however, considering the high proportion of reproductive polyps for males and the high proportion of mature eggs, it is likely that spawning continued through the spring. Results showed that aspergillosis had a negative effect on the reproduction of *G. ventalina.* In diseased colonies, there was a significant decrease in the proportion of reproductive polyps and in the number of eggs per polyp in the infected area (4.1% reproductive polyps, 95%CI: 2.6-6.5; 0.06 eggs/polyp, W=0-0.6, n=24) and immediately adjacent to it (12.4% reproductive polyps, 95%CI: 9.7-15.6; 0.18 eggs/polyp, W=0-2.0, n=24) compared to areas at least 10cm away from lesions in

healthy-looking tissue (34.4% reproductive polyps, 95%CI: 30.2-38.7; 0.50 eggs/polyp, W=0.05-1.5, n=24). The impact seems to be systemic since even polyps in healthy-looking areas of infected colonies showed decreased egg and sperm production compared to healthy colonies (41.1% reproductive polyps, 95%CI: 38.0-44.2; 0.76 eggs/polyp, W=0.05-3.35, n=100). The long-term consequences of this reduction in gamete production depends on whether it drops low enough to limit recruitment causing a decline in local population densities or produces a recruit population which is not reduced in size but is more resistant to disease.

3.2 Introduction

Coral and octocoral diseases have increased dramatically in recent years and have produced significant ecological changes at local and geographical scales over relatively short periods of time; however, the long-term effects are still unknown (Harvell et al. 1999, Knowlton 2001a, Harvell et al. 2002, Weil et al. 2002, Harvell et al. 2004, Ward and Lafferty 2004, Weil 2004). Diseases affecting colonial organisms have obvious negative effects on their hosts in the form of tissue and colony mortality. Less obvious effects, such as a reduction in reproductive output, may have long-term ecological and evolutionary consequences for populations affected by disease (Weil 2004, Weil et al. 2006).

Beyond the simple loss of tissue for gamete production, three aspects of coral and octocoral reproduction may amplify the effects of partial mortality. First, the reproductive effort of a colony is typically lower at its edges compared to its central areas, as more energy is invested in asexual growth than in sexual reproduction (Brazeau

and Lasker 1990, Harrison and Wallace 1990); partial mortality due to disease often creates new edge areas in the center of a colony. Second, corals and octocorals do not reproduce sexually below a minimum reproductive size because energy is instead being devoted to growth (asexual reproduction) (Coma et al. 1995b, Szmant-Froelich et al. 1985, Beiring and Lasker 2000); colonies which lose large areas of live tissue to disease may fall below the minimum size threshold for sexual reproduction. Third, larger colonies of some octocoral species have a disproportionate effect on the gene pool (Brazeau and Lasker 1989, Brazeau and Lasker 1990, Beiring and Lasker 2000, Coma et al. 1995b). For example, large colonies of *Plexaura flexuosa* produced six times as many eggs per polyp as smaller colonies (Beiring and Lasker 2000). In this case, a size reduction due to disease would drastically reduce the egg production of a colony. Large sea fan colonies have been found to have lower resistance to infection than smaller colonies and, therefore, much higher prevalence of the disease aspergillosis than smaller colonies (Nagelkerken et al. 1997b, Dube et al. 2002, Kim and Harvell 2004), further compounding the effects of the disease on the reproductive effort of a population.

Severe negative effects of disease on sexual reproduction may be due not just to tissue mortality but also to the allocation of energy and resources to fighting and recovering from disease. Distribution of energy and resources is important to the survival of an organism. Egg and sperm production require energy which may not be available if that energy is being used to fight disease. In many species of octocoral, eggs develop over a much longer time period than spermaries (Coma et al. 1995a) and require more energy and resource input. Therefore egg production, which is the limiting factor of reproductive success (Beiring and Lasker 2000), may be more affected by disease than

sperm production. Compared to healthy colonies, disease-affected colonies may display a disruption of the gametogenic cycle and/or a delay in the timing of reproduction and therefore, a decrease in fecundity (eggs/polyp) and reproductive output.

The few studies that have focused on the effects of coral and octocoral diseases on the reproductive fitness of their hosts, have found negative disease effects. Disease-affected areas of the coral, *Montastraea faveolata,* had significant reductions in fecundity compared to healthy colonies during an outbreak of yellow band disease in La Parguera, Puerto Rico in 2006 (Weil et al. unpublished data). *Gorgonia ventalina* colonies suffering from aspergillosis had decreased egg production in two of the three months sampled in the Florida Keys (Petes et al. 2003).

Aspergillosis is a disease caused by the terrestrial fungus *Aspergillus sydowii* (Smith et al. 1996, Geiser et al. 1998) which affects octocorals of the genus *Gorgonia* (*G. ventalina*, *G. flabellum* and *G. mariae*), as well as other species of octocoral (Smith and Weil 2004). The pathogen was first identified after an outbreak of the disease in the mid-1990s which caused widespread mortality of sea fans throughout the Caribbean (Nagelkerken et al. 1997a,b). Since then, aspergillosis has been identified and reported throughout the Caribbean in low to epizootic levels (Weil et al. 2002, Kim and Harvell 2004, Mullen et al. 2006, Nugues and Nagelkerken 2006, Toledo-Hernández et al. 2007).

The main goals of this study were to first characterize the normal reproductive cycle of *Gorgonia ventalina* in La Parguera, on the southwest coast of Puerto Rico, and then to determine how the fungal infection aspergillosis affects this cycle. Since large eggs have been found in this species mostly during spring and summer months (Fitzsimmons-Sosa et al. 2004), we expected to find similar trends indicating summer

spawning which is common in gorgonians (Brazeau and Lasker 1989, Coma et al. 1995a, Beiring and Lasker 2000). Secondary goals included determining the sex ratio of the species, whether reproductive output varied across different reef zones in the area, whether colony depth or colony size had any relationship to the number of eggs produced per polyp, and whether the impact of aspergillosis was localized or systemic within the colony.

3.3 Materials and Methods

Histological techniques were used to characterize the reproductive cycle of *Gorgonia ventalina* colonies and to investigate the effects of aspergillosis infection on the reproduction of the species.

3.3.1 Study area and sample collection

This study was carried out at three reefs off La Parguera, on the southwest coast of Puerto Rico (Fig. 3.1): Pelotas (17º57.442N, 67º04.176W), an inner fringing reef near the coast line; Media Luna (17º56.093N, 67º02.931W), a mid-shelf fringing reef; and the Buoy (17º53.300N, 66º59.879W), a deep shelf-edge reef.

Figure 3.1: Aerial photograph of La Parguera, Puerto Rico showing location of reef sites where sampling was carried out (Red dots).

Tissue samples were collected from randomly selected *Gorgonia ventalina* colonies during the first week of each month between November 2005 and October 2006. At least five healthy colonies were sampled from each of the three sites for the entire year. For the entire year, three diseased colonies from Pelotas and four from Media Luna

were randomly sampled. Beginning in June 2006, three diseased colonies were also randomly sampled at the Buoy.

Since the species is reproductive year round (Fitzsimmons-Sosa et al. 2004), we also explored the possibility of a lunar or monthly reproductive cycle, as found in the scleractinian brooder *Favia fragum* (Szmant-Froelich et al. 1985). Ten healthy colonies and five diseased colonies were tagged at Media Luna immediately following the year-long sampling, and were sampled every three to four days between November 5th and December 4th, 2006 based on the results of the year-long study of healthy sea fans: Media Luna had the highest proportion of reproductive polyps of the three sites, and November had the highest number of eggs per polyp of all the months surveyed.

3.3.2 Sampling method

All tissue samples were collected from the central area of the colony, at least 10 cm from the edge. Samples of approximately 1 cm^2 were cut from the colony using different scissors for healthy and diseased colonies to prevent contagion. They were placed in labeled plastic bags and brought to the lab. One tissue sample was collected from each healthy colony and three tissue samples were collected from each diseased colony: one sample from the dark-purple, actively diseased area (ADA), one sample from the transition area immediately adjacent to the ADA (TA) and one sample from the healthy-looking area of the diseased colony (HA) at a distance >10 cm from the ADA (Fig. 3.2).

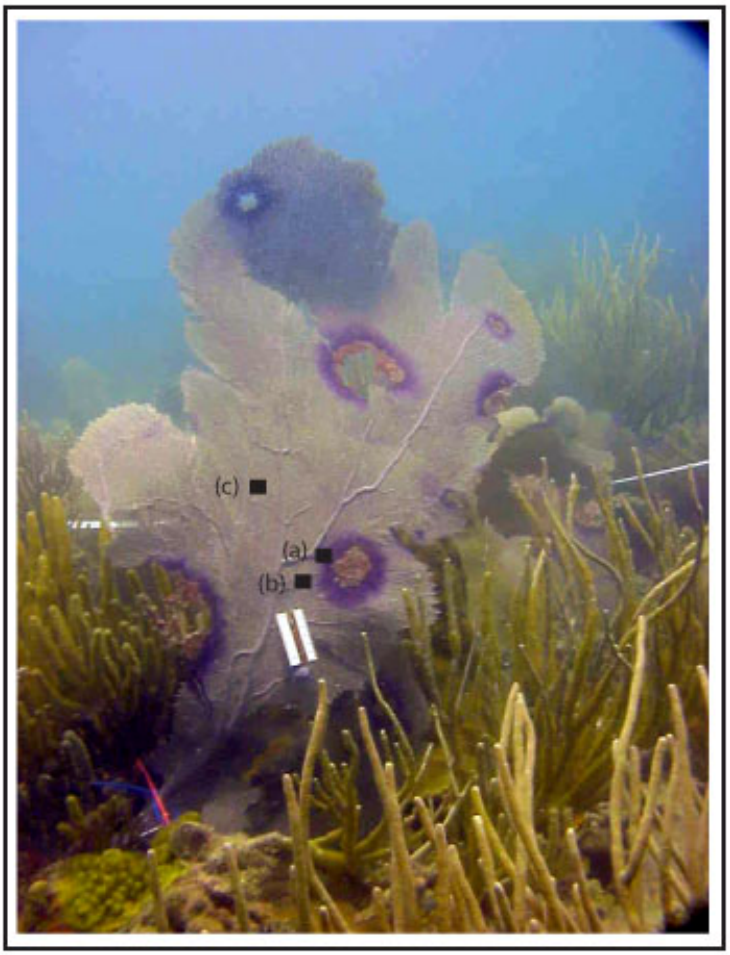

Figure 3.2: Sampling method for diseased *Gorgonia ventalina* colonies. Three 1 cm^2 samples were collected: (a) one from the dark-purple diseased area (ADA), (b) one from the transitional area immediately adjacent to the diseased area (TA) and (c) one from the apparently healthy tissue at least 10 cm from the lesion (HA).

3.3.3 Sample preparation

All tissue samples were brought back to the lab and immediately preserved in Zenker formalin solution for 12-24 hours. Tissues were then rinsed with running tap water for 24 hours, decalcified with 10% HCl for 3-4 days and preserved in 70% ETOH. Tissue samples were embedded in paraffin and 6μm sections were cut with a microtome

and mounted on slides. Tissues were stained with Heidenhain's Aniline Blue Method (Coolidge and Howard 1979) to enhance the color of the eggs and spermaries.

3.3.4 Slide reading

Slides were observed at 10x and 40x under a compound microscope. All slides were viewed to check for gametes in order to determine whether or not each sample was reproductive. A colony was considered reproductive if one or more eggs or spermaries were observed in the prepared slides. For each reproductive sample, the sex was recorded and 20 polyps were randomly selected for observation. Of these 20 polyps, the number of reproductive polyps was counted. A polyp was considered reproductive if one or more eggs or spermaries were observed in the polyp. For each reproductive polyp in female colonies, the total number of eggs per polyp was counted (Fig. 3.3).

Also for each female colony, all eggs in five reproductive polyps were classified according to three stages of development based on Szmant-Froelich et al. (1980)(Fig. 3.3). Stage II eggs were the earliest stage observed and were characterized by a nucleus in the center of a small amount of cytoplasm. Stage III eggs were larger with more cytoplasm, and the nucleus was no longer in the center. Stage IV eggs were larger with a more dense, granular cytoplasm that stained darker than stage III eggs with Heidenhain's Aniline Blue stain. These were considered mature eggs. Szmant-Froelich et al. (1980) characterized Stage I as an accumulation of interstitial cells which were not recorded in this study. To normalize the number of eggs of each stage per polyp, the proportion of eggs of each stage (in the five polyps in which egg stage was recorded for each colony)

was multiplied by the total number of eggs per polyp (in the 20 polyps in which eggs were counted for that colony).

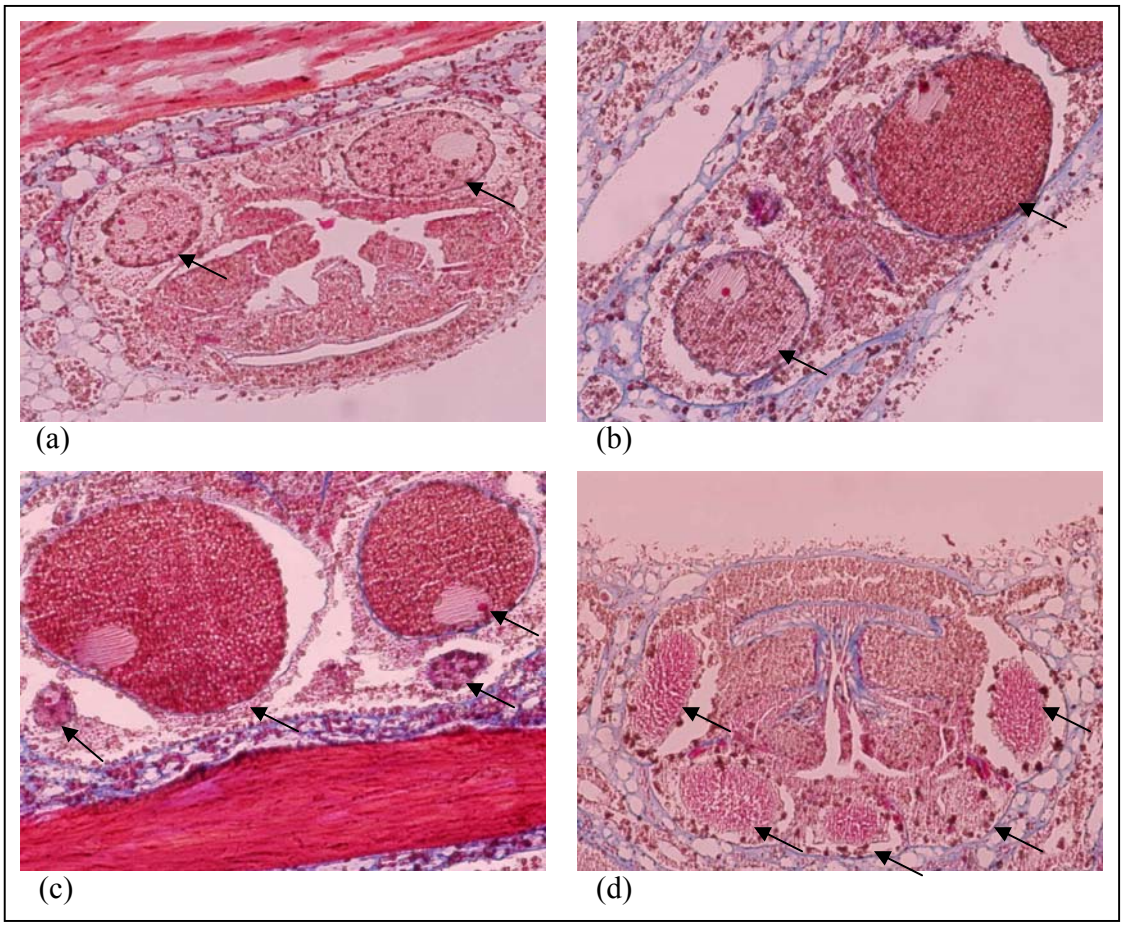

Figure 3.3: *Gorgonia ventalina* polyps with arrows indicating (a) two stage III eggs, (b) one stage III (left) and one stage IV (right) egg, (c) two stage II (bottom) and two stage IV eggs (top), and (d) spermaries.

3.3.5 Statistics

In order to explore whether healthy *Gorgonia ventalina* colonies were more likely to be reproductive in some months than others, G-tests were run to determine whether the number of reproductive and non-reproductive polyps were dependent on the month for all colonies and for female and male colonies individually. A Kruskal-Wallis ANOVA was used for healthy samples to determine whether female colonies had more eggs per polyp in some months than in others. Differences between months in the number of stage IV eggs per polyp were investigated using a Kruskal-Wallis ANOVA.

Repeated-Measures ANOVAs were used to determine whether healthy tagged female colonies that were sampled repeatedly through November and early December 2006 had a higher proportion of reproductive polyps or more eggs per polyp in some sampling periods than in others.

A G-test was used to determine whether the number of reproductive and non-reproductive polyps were dependent on the reef. Spearman Rank Order Correlations were performed to explore the potential effects of colony size and colony depth on the number of eggs per polyp in reproductive female colonies.

A G-test was used to compare the number of reproductive and non-reproductive polyps in samples of different conditions: healthy colonies, and HA, TA and ADA samples from diseased colonies. Kruskal-Wallis ANOVAs were used to compare the number of eggs per polyp between female samples of each condition.

In order to determine whether HA samples from diseased colonies were more likely to be reproductive in some months than others, a G-test was run to determine whether the number of reproductive and non-reproductive polyps were dependent on the

month. A Kruskal-Wallis ANOVA was used for HA samples to determine whether female colonies had more eggs per polyp in some months than in others.

3.3.6 Between vs. within colony variability

Only one tissue sample was collected from each healthy colony and from each condition of diseased colonies. To determine whether some of the between colony variability found could actually be attributed to within colony variability, a one-time sampling was performed. From each of eleven healthy colonies, five individual samples were collected from within the normal sampling area (>10 cm from edge). As in the main study, twenty polyps were randomly selected and the proportion of reproductive polyps and the number of eggs per polyp were compared between samples using Kruskal-Wallis ANOVAs.

While all healthy and HA samples were collected from central areas of colonies, TA and ADA samples from diseased colonies were collected from an edge formed by tissue mortality due to disease. In order to determine whether sampling from this edge accounted for some of the decreased reproductive activity found in TA and ADA samples, one sample was collected from the edge of each of the eleven colonies sampled above. Again, for twenty randomly selected polyps, the proportion of reproductive polyps and the number of eggs per polyp were compared between edge and central samples from each colony using Kruskal-Wallis ANOVAs.

3.4 Results

3.4.1 Reproductive cycle of healthy *Gorgonia ventalina* colonies

Results from the histological samples of 148 healthy reproductive colonies confirmed that *Gorgonia ventalina* is a gonochoric species, with a female:male ratio of 2:1 for healthy colonies sampled throughout this study. The species appears to spawn gametes as no evidence of planulae was observed in polyps.

For healthy colonies, there were significant differences between months in the number of reproductive versus non-reproductive polyps for all colonies and for female and male colonies individually (G-tests: $G_{(all)}$=170.544, $G_{(female)}$=168.051 , $G_{(male)}$=147.688, df=11; $p \leq 0.05$). Overall, more polyps in healthy samples were reproductive from October to April than from May to August (Unplanned test for goodness of fit: df=11, $p \leq 0.05$) (Table 3.1).

Table 3.1: The total number of reproductive and non-reproductive polyps and proportion of reproductive polyps in healthy *Gorgonia ventalina* colonies collected monthly from November 2005 to October 2006 from all three sites. Letters indicate significant differences between months for each condition (G-test with unplanned test for goodness of fit: $p \leq 0.05$).

Month	Reproductive polyps	Non-reproductive polyps	Proportion reproductive polyps	Significant differences
Nov-05	175	145	0.55 (0.48-0.62)	a
Dec-05	155	185	0.46 (0.38-0.53)	a
Jan-06	156	164	0.49 (0.42-0.56)	a
Feb-06	149	171	0.47 (0.40-0.54)	a
Mar-06	144	176	0.45 (0.38-0.52)	a
Apr-06	149	171	0.47 (0.40-0.54)	a
May-06	95	205	0.32 (0.25-0.39)	b
Jun-06	83	217	0.28 (0.22-0.35)	b
Jul-06	61	219	0.22 (0.17-0.28)	b
Aug-06	79	221	0.26 (0.20-0.33)	b
Sep-06	135	165	0.45 (0.38-0.52)	ab
Oct-06	156	144	0.52 (0.45-0.60)	a

Healthy female colonies contributed more to the proportion of reproductive polyps in late summer and fall (Unplanned test for goodness of fit: df=11, $p \leq 0.05$) while males accounted for more of the reproductive polyps in the winter and early spring (Unplanned test for goodness of fit: df=11, $p \leq 0.05$) (Fig. 3.4).

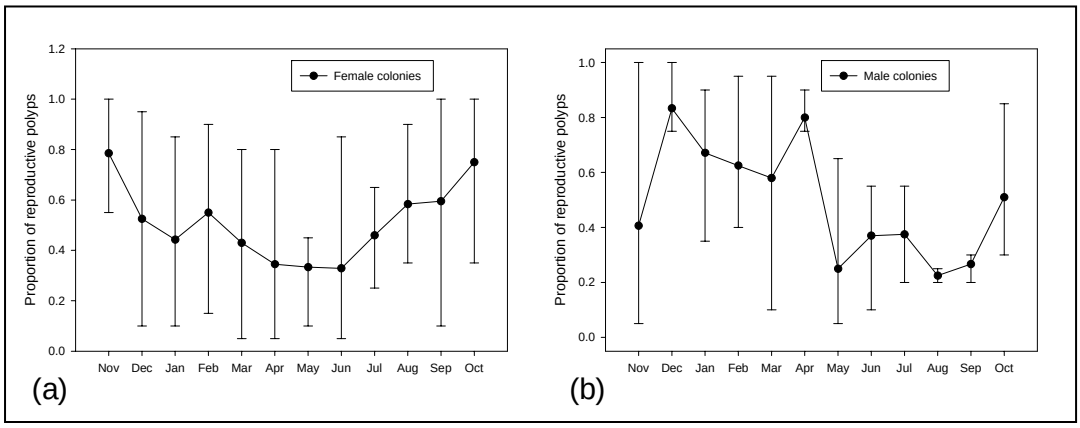

Figure 3.4: Line graphs of the average proportion of reproductive polyps in healthy (a) female and (b) male *Gorgonia ventalina* colonies collected monthly from November 2005 to October 2006 from all three reefs. Error bars show the range of values.

For healthy reproductive female colonies, the number of eggs per polyp varied significantly across months (Kruskal-Wallis ANOVA: $X^2=32.871$, df=11, n=100, p=0.0006). Fecundity values peaked in November (1.7 eggs per polyp, W(range)=0.8-3.4, n=7), which had a significantly higher number of eggs per polyp than April through June 2006 (April: 0.4 eggs/polyp, W=0.05-1.4, n=10; May: 0.4 eggs/polyp, W=0.1-0.6, n=12; June: 0.4 eggs/polyp, W=0.05-1.5, n=7) (Multiple comparisons: df=11, $p \leq 0.05$) (Fig. 3.5).

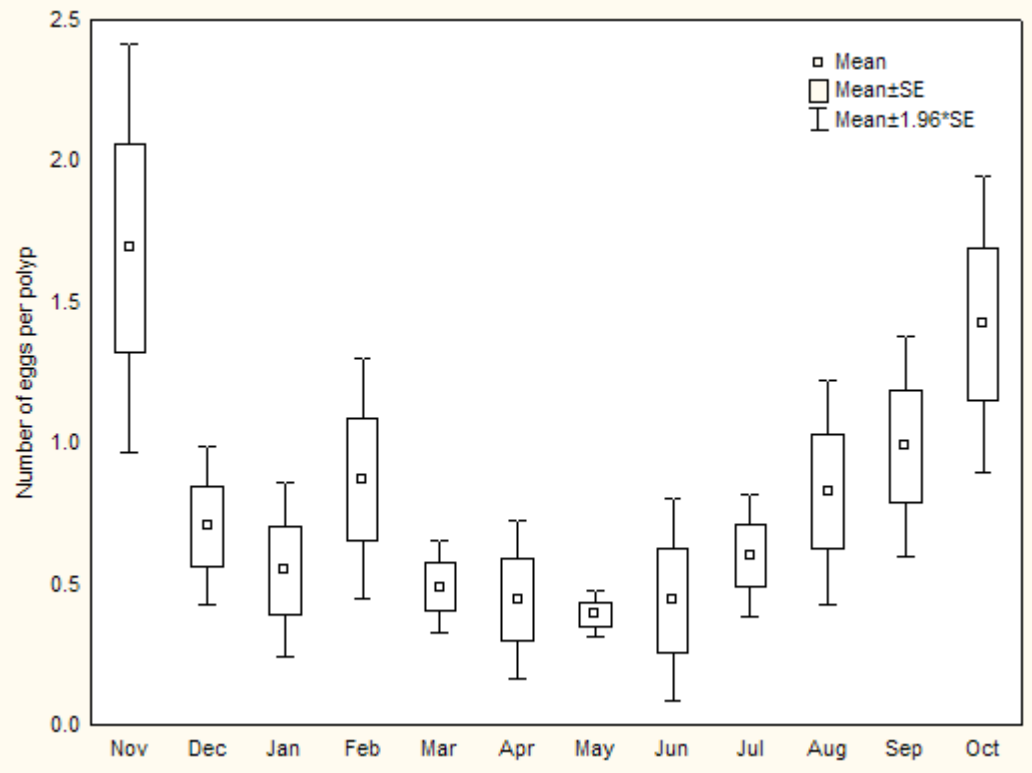

Figure 3.5: Boxplot of the average number of eggs per polyp in healthy *Gorgonia ventalina* colonies collected monthly from November 2005 to October 2006 from all three reefs. ($n_{(November)}$=7, $n_{(December)}$=10, $n_{(January)}$=7, $n_{(February)}$=9, $n_{(March)}$=10, $n_{(April)}$=10, $n_{(May)}$=12, $n_{(June)}$=7, $n_{(July)}$=5, $n_{(August)}$=6, $n_{(September)}$=10, $n_{(October)}$=7)

The number of stage IV eggs per polyp did not differ significantly between months indicating that the species might have multiple spawning events during the year (Kruskal-Wallis ANOVA: X^2=18.512, df=11, n=100, p=0.07); February (0.37 stage IV eggs/polyp, W=0-1.1, n=9,) had the highest number of stage IV eggs per polyp followed by November (0.23 stage IV eggs/polyp, W=0-0.66, n=7) and October (0.22 stage IV eggs/polyp, W=0-0.75, n=7). July was the only month in which no stage IV eggs were observed (Fig. 3.6).

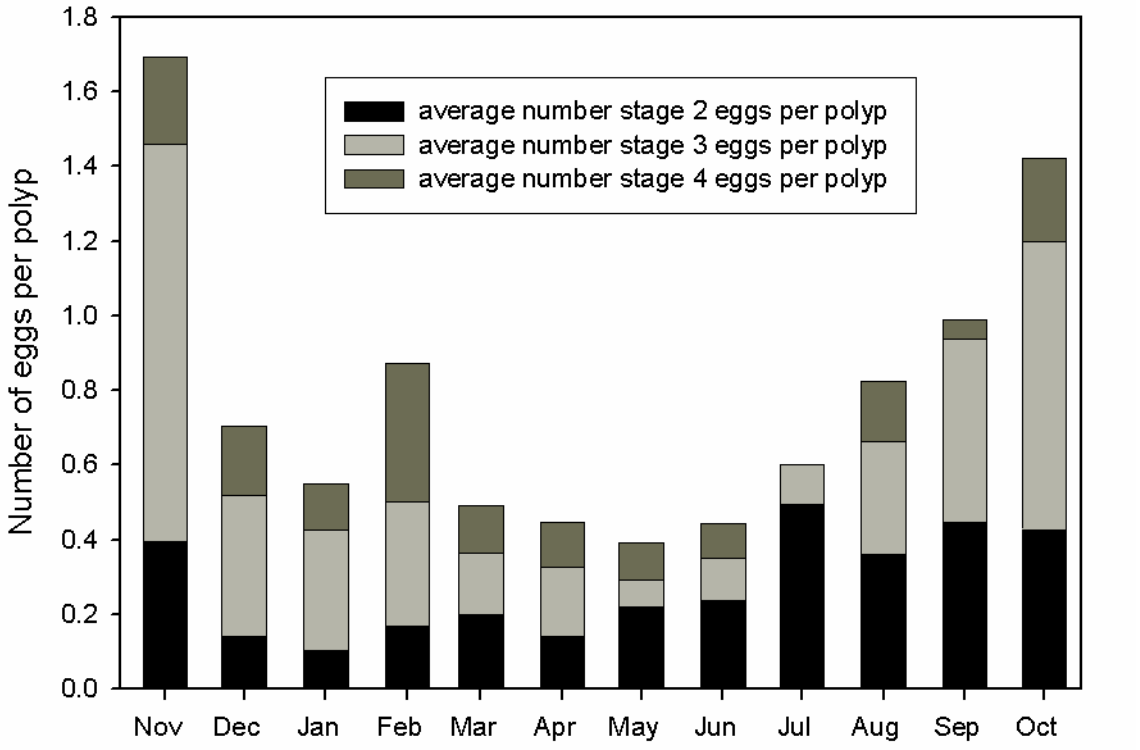

Figure 3.6: Stacked bar chart of the normalized value for the number of eggs of each stage per polyp in healthy *Gorgonia ventalina* colonies sampled monthly from November 2005 to October 2006 from all three reefs. ($n_{(November)}$=7, $n_{(December)}$=10, $n_{(January)}$=7, $n_{(February)}$=9, $n_{(March)}$=10, $n_{(April)}$=10, $n_{(May)}$=12, $n_{(June)}$=7, $n_{(July)}$=5, $n_{(August)}$=6, $n_{(September)}$=10, $n_{(October)}$=7)

No evidence of a lunar reproductive cycle was found. There was no difference in the number of reproductive polyps (Repeated-Measures ANOVA: df=8, p=0.96) or the fecundity (eggs/polyp) (Repeated-Measures ANOVA: df=8, p=0.83) between the nine samplings performed during November and early December 2006.

3.4.2 Reef Sites

Reproductive colonies were found year round in all three reef sites. However, the proportion of reproductive versus non-reproductive polyps varied and was dependent on site (G-test: G=57.599, df=2, $p \leq 0.05$). Samples collected from healthy colonies at Media Luna (49.4% reproductive polyps, 95% CI: 46.3-52.5), the exposed mid-shelf fringing reef, had a significantly higher ratio of reproductive polyps to non-reproductive polyps than samples from Pelotas (35.2% reproductive polyps, 95% CI: 32.3-38.3), the more protected inner reef, and the Buoy (38.5% reproductive polyps, 95% CI: 35.5-41.6), the offshore shelf-edge reef (Unplanned test for goodness of fit: df=2, $p \leq 0.05$) (Fig. 3.7).

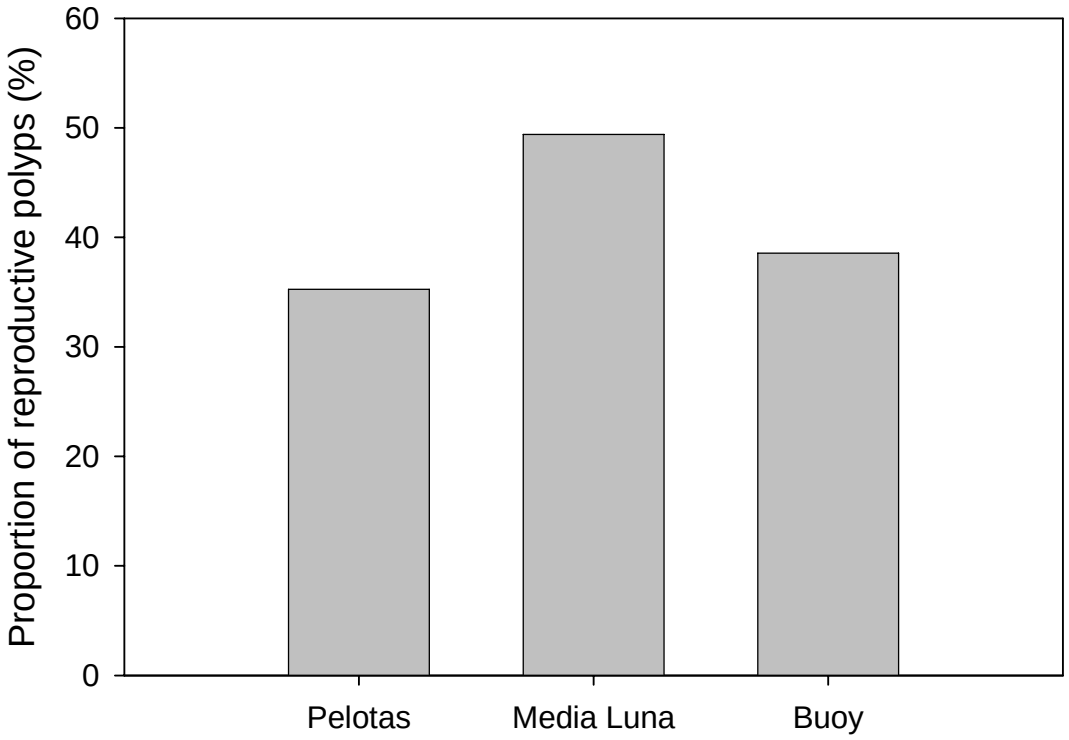

Figure 3.7: Bar chart showing the variability between sites in the proportion of reproductive polyps for all healthy *Gorgonia ventalina* colonies sampled throughout the study. The proportion of reproductive polyps was higher at Media Luna than at the Buoy or Pelotas (G-test with Unplanned test for goodness of fit: $p<0.05$).

3.4.3 Polyp fecundity vs. colony size and depth

For healthy female colonies, there was a weak but significant positive correlation between fecundity and size of colony. (Spearman Rank Order Correlation: r=0.3046; p=0.0024) (Fig. 3.8). Larger colonies produced more eggs per polyp than smaller colonies. No correlation between fecundity and depth (habitat) was found.

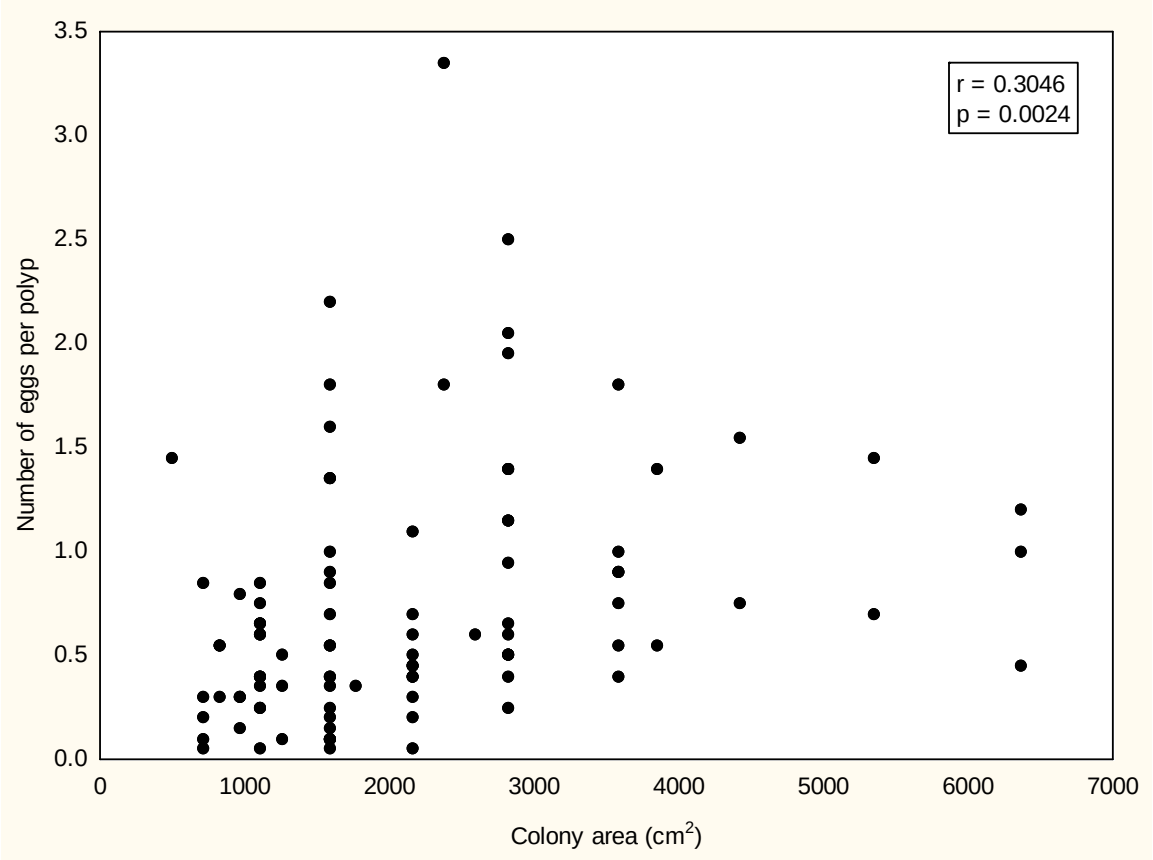

Figure 3.8: Scatterplot showing the significant positive correlation between the number of eggs per polyp and colony area for all healthy *Gorgonia ventalina* colonies sampled throughout the study. (Spearman Rank Order Correlation: r=0.3046; p=0.0024)

3.4.4 Impact of aspergillosis on the reproductive cycle of *Gorgonia ventalina*

For the 41 reproductive diseased colonies, the female:male ratio was 1.7:1. Compared to healthy colonies, this is a slight decrease in the proportion of females to males.

When all samples collected throughout the study were combined and analyzed, results indicated that the number of reproductive versus non-reproductive polyps was dependent on the condition of the sample (the health of the colony and the distance from infection)(G-test: $G=683.316$, $df=3$; $p<0.01$). The number of reproductive polyps decreased significantly from healthy colonies to the healthy-looking areas of diseased colonies (HA), to transition areas (TA) to the actively diseased areas (ADA) of diseased colonies (Unplanned test for goodness of fit: $df=3$, $p\leq0.01$) (Table 3.2).

Table 3.2: The number of reproductive and non-reproductive polyps, the proportion of reproductive polyps with 95% confidence interval (in parenthesis) and letters indicating significant differences (Unplanned test for goodness of fit: $p\leq0.05$) for *Gorgonia ventalina* colonies of different conditions: healthy, HA (healthy-looking areas of diseased colonies), TA (transitional areas of diseased colonies) and ADA (purple, actively-diseased areas of diseased colonies).

Condition	Number reproductive polyps	Number non-reproductive polyps	Proportion reproductive polyps	Significant Differences
Healthy	1537	2203	0.411 (0.380-0.442)	a
HA	269	511	0.344 (0.302-0.387)	b
TA	97	683	0.124 (0.097-0.156)	c
ADA	32	748	0.041 (0.026-0.065)	d

There were also differences in the number of eggs per polyp in female colonies of the different conditions (Kruskal-Wallis ANOVA: $X^2=73.124$, $df=3$, $n=172$, $p<0.0001$). Healthy colonies (0.76 eggs/polyp, W=0.05-3.35, n=100) and HA areas of diseased

colonies (0.50 eggs/polyp, W=0.05-1.5, n=24) had significantly higher numbers of eggs per polyp than TA (0.18 eggs/polyp, W=0-2.0, n=24) and ADA (0.06 eggs/polyp, W=0-0.6, n=24) areas of diseased colonies (Multiple comparisons: df=3, $p \leq 0.05$) (Fig. 3.9).

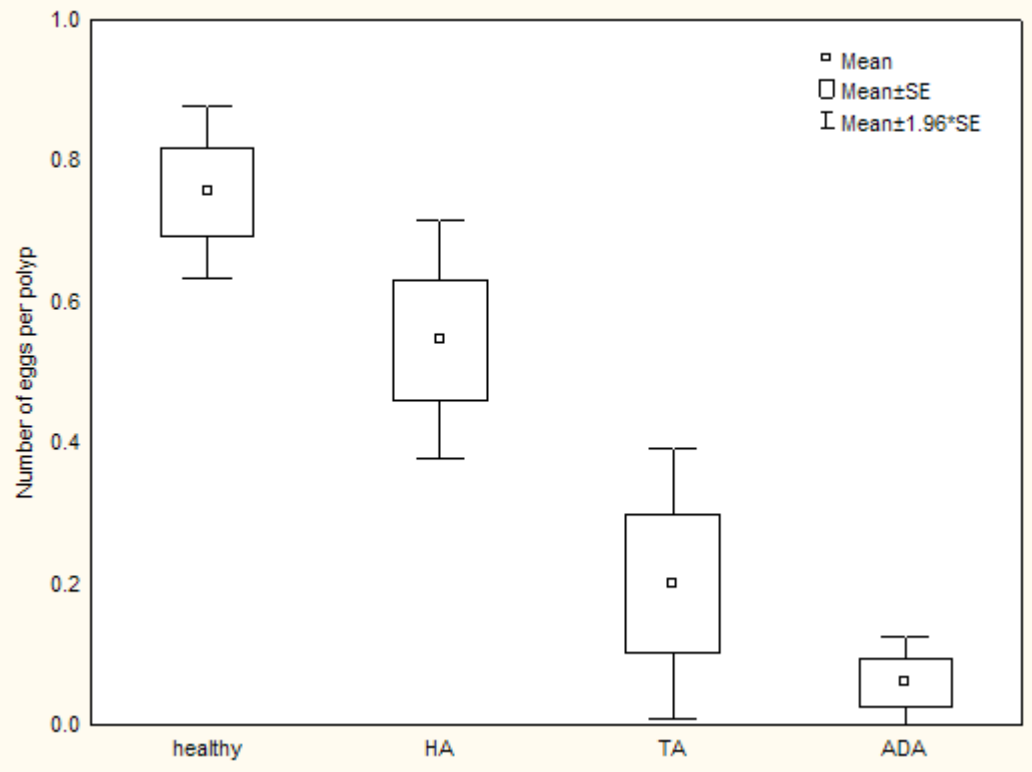

Figure 3.9: Boxplot showing the significant decrease in the average number of eggs per polyp from healthy (n=100) *Gorgonia ventalina* colonies and healthy-looking areas of diseased colonies (HA, n=24) to transition areas (TA, n=24) and actively diseased areas (ADA, n=24) of diseased colonies.

There appears to be a systemic impact of aspergillosis since the number of reproductive polyps in the healthy-looking areas of diseased colonies (HA) was significantly lower than in healthy-looking colonies without any disease signs (Table

3.2). While this difference was not significant for the number of eggs per polyp, this could be attributed to the small sample size for diseased female colonies.

The average proportion of reproductive polyps each month was negatively affected by the disease. Fecundity in all areas of diseased colonies was consistently lower than healthy colonies except in the summer; HA samples had a higher proportion of reproductive polyps than healthy colonies in July and August during the time when healthy samples had their lowest values (Fig. 3.10).

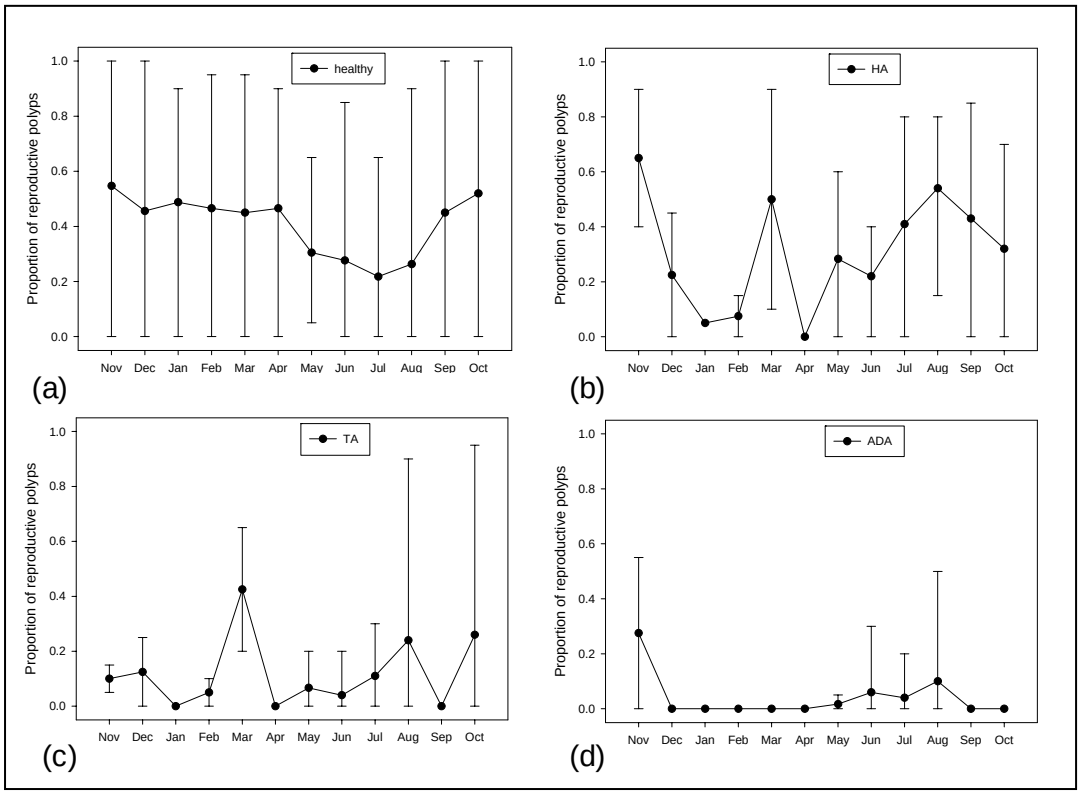

Figure 3.10: Line graphs of the average proportion of reproductive polyps in (a) healthy, (b) HA, (c) TA and (d) ADA *Gorgonia ventalina* samples collected monthly from November 2005 to October 2006 from all three reefs. Error bars show the range of values.

For HA samples from diseased colonies, there were significant differences between months in the number of reproductive versus non-reproductive polyps (G-tests: G=88.381, df=10, $p \leq 0.05$). HA samples had more reproductive polyps in November, March, and July through October than in December through February and June (Unplanned test for goodness of fit: df=10, $p \leq 0.05$) (Table 3.3). For HA samples, male and female colonies did not display individual trends probably because sample sizes were too small.

Table 3.3: The number of reproductive and non-reproductive polyps, the proportion of reproductive polyps with 95% confidence interval (in parenthesis) and letters indicating significant differences (Unplanned test for goodness of fit: $p \leq 0.05$) in HA (healthy-looking) samples from diseased *Gorgonia ventalina* colonies collected monthly from November 2005 to October 2006. *April 2006 could not be tested because there were no reproductive polyps.

Month	Reproductive Polyps	Non-reproductive polyps	Proportion reproductive polyps	Significant differences
Nov-05	26	14	0.65 (0.46-0.80)	a
Dec-05	9	31	0.23 (0.10-0.42)	b
Jan-06	2	38	0.05 (0.006-0.20)	b
Feb-06	3	37	0.08 (0.01-0.25)	b
Mar-06	20	20	0.50 (0.32-0.68)	a
Apr-06	0	20	0	*
May-06	17	43	0.28 (0.16-0.42)	ab
Jun-06	22	78	0.22 (0.14-0.32)	b
Jul-06	41	59	0.41(0.31-0.51)	a
Aug-06	54	46	0.54 (0.44-0.63)	a
Sep-06	43	57	0.43 (0.33-0.53)	a
Oct-06	32	68	0.32 (0.23-0.42)	a

The average number of eggs per polyp in HA samples was consistently lower than in healthy colonies. There was no significant variability in the number of eggs per polyp in different months for HA samples (Kruskal-Wallis ANOVA: $X^2 = 10.201$, df=11,

p=0.5124); however, values were higher in the summer and fall which is similar to the trend in healthy colonies (Fig. 3.11).

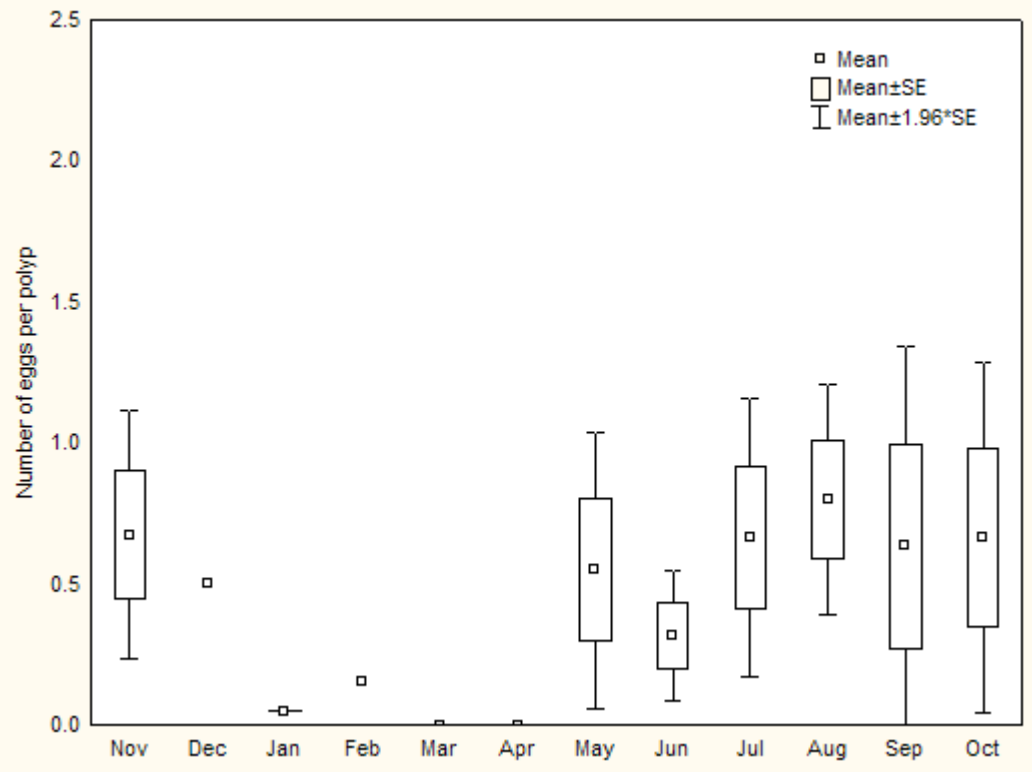

Figure 3.11: Boxplot of the average number of eggs per polyp in HA (healthy-looking) samples from diseased *Gorgonia ventalina* colonies collected monthly from November 2005 to October 2006 from all three reefs. ($n_{(November)}$=2, $n_{(December)}$=1, $n_{(January)}$=2, $n_{(February)}$=1, $n_{(March)}$=0, $n_{(April)}$=0, $n_{(May)}$=2, $n_{(June)}$=3, $n_{(July)}$=3, $n_{(August)}$=3, $n_{(September)}$=3, $n_{(October)}$=4)

The number of stage IV eggs per polyp in HA samples from diseased colonies did not differ significantly between months (Kruskal-Wallis ANOVA: p=0.1721). Stage IV eggs were present in HA samples in November-January, May, July and October with the highest amount present in May and December (Fig. 3.12). Very few stage IV eggs were present in TA and ADA samples.

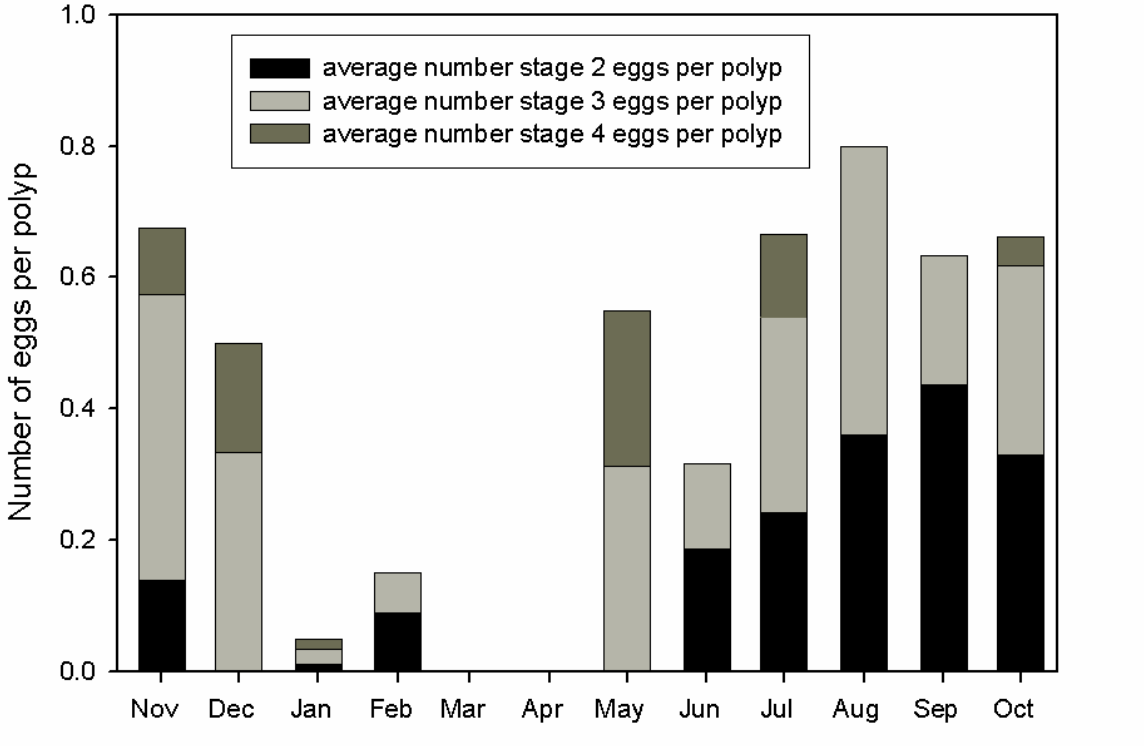

Figure 3.12: Stacked bar chart of the normalized value for the number of eggs of each stage per polyp in HA samples from diseased *Gorgonia ventalina* colonies sampled monthly from November 2005 to October 2006 from all three reefs. ($n_{(November)}$=2, $n_{(December)}$=1, $n_{(January)}$=2, $n_{(February)}$=1, $n_{(March)}$=0, $n_{(April)}$=0, $n_{(May)}$=2, $n_{(June)}$=3, $n_{(July)}$=3, $n_{(August)}$=3, $n_{(September)}$=3, $n_{(October)}$=4)

3.4.5 Between vs. within colony variability

Of the eleven colonies sampled to assess within colony variability, two colonies, one female and one male, had significant differences between the proportion of reproductive polyps in samples collected from different parts of the colony (Kruskal-Wallis ANOVAs: X^2=11.647, X^2=32.598, df =4; $p \leq 0.05$). Of the six females, two had significant differences in the number of eggs per polyp between different areas of the colony (Kruskal-Wallis ANOVAs: X^2=15.078, X^2=31.143, df=4; $p \leq 0.05$). Of the eleven

colonies, three had a significantly lower proportion of reproductive polyps in the edge sample compared to all central samples (Kruskal-Wallis ANOVAs: X^2=20.517, X^2=34.378, X^2=16.878, df=5, $p \leq 0.05$), while two had a significantly lower proportion of reproductive polyps at the edge compared to only some of the central samples (Kruskal-Wallis ANOVAs: X^2=44.210, X^2=29.522, df=5, $p \leq 0.05$). The remaining six colonies showed no difference in the proportion of reproductive polyps between edge and central samples. Only two of the six female colonies had a significant decrease in the number of eggs per polyp for the edge sample compared to all central samples (Kruskal-Wallis ANOVAs: X^2=29.945, X^2=22.175, df=5, $p \leq 0.05$). One had a decrease in the number of eggs per polyp in the edge sample compared to only some of the central samples (Kruskal-Wallis ANOVA: X^2=38.562, df=5, $p \leq 0.05$). Three had no differences in the number of eggs per polyp between the edge and central samples.

3.5 Discussion

3.5.1 Reproduction of healthy *Gorgonia ventalina* colonies

Gorgonia ventalina is a gonochoric broadcast-spawning gorgonian. Gonochorism is the dominant trait for gorgonians (Brazeau and Lasker 1989, Brazeau and Lasker 1990, Coma et al. 1995b, Lasker et al. 1996, Beiring and Lasker 2000, Fitzsimmons-Sosa et al. 2004). Both spawning of gametes (Brazeau and Lasker 1989, Coma et al. 1995b, Lasker et al. 1996, Beiring and Lasker 2000, Fitzsimmons-Sosa et al. 2004) and brooding of larvae are common modes of development in octocorals (Brazeau and Lasker 1989, 1990).

Results of this year-long study showed that the life cycle of *Gorgonia ventalina* is not as clearly defined as for other octocorals and most scleractinian corals. Both eggs and spermaries were produced in all months of the year. Even in the summer when gamete production was the lowest, approximately 25% of all polyps were reproductive, compared to a maximum of 55% in November. Mature, stage IV, eggs were found in all months except July. Similarly, Fitzsimmons-Sosa et al. (2004) reported year-round sexual reproductive activity in this species in Florida, however they found mature eggs only in March through July and November.

As egg production has higher energy costs than sperm production and usually takes place over a longer period of time, female colonies are more likely to be a better indication of the reproductive cycle of the species. It is interesting that among healthy colonies, the peak reproduction for females preceded that for males. In female colonies, both the proportion of reproductive polyps and the average number of eggs per polyp increased in the fall and dropped between November and December 2005. While it was not significant, more mature eggs were found during the winter, especially in February 2006. Males had a steep increase in the proportion of reproductive polyps between November and December and dropped most dramatically between April and May 2006. Although the peak in the proportion of reproductive polyps for males lagged behind the females, there were mature eggs in female colonies throughout this time. Female colonies indicate that spawning may have taken place in the winter of 2005-2006, however, considering the high proportion of reproductive polyps for males and the high proportion of mature eggs, it is likely that spawning continued through the spring. This

contrasts with Fitzsimmons-Sosa et al. (2004) who found a peak in the number of large eggs in the summer months indicating summer spawning.

3.5.2 Effects of aspergillosis on reproduction of *Gorgonia ventalina* colonies

Aspergillosis had a significant negative effect on the reproduction of infected *Gorgonia ventalina* colonies. Polyps closer to infected areas (ADA and TA) had significantly lower fecundity than those farther away (HA). Furthermore, the impact seems to be systemic since polyps in healthy-looking areas of infected colonies (HA) showed significant decreases in egg and sperm production compared to healthy colonies with no signs of infection. Similar results were recently found for yellow band disease (YBD) affecting the scleractinian coral *Montastraea faveolata* in the same area of La Parguera (Weil et al. unpublished data). This systemic effect has also been found in *Favia favus*, where tissue regeneration of large lesions reduced fecundity in polyps up to 15 cm from lesions (Oren et al. 2001) indicating that energy and resources were preferentially used for growth and repair as opposed to sexual reproduction (Harrison and Wallace 1990).

This systemic effect of aspergillosis affecting total colony reproductive output and possibly other physiological functions of infected colonies, combined with the reduction in fecundity produced by the loss of tissue and increase in edge areas, might hamper population maintenance and/or recovery for this species and may have some long-term evolutionary effects in areas where epizootic events have produced high mortalities.

Based on the number of reproductive polyps, the number of eggs per polyp and the presence of spermaries and mature eggs, the reproductive peak for healthy colonies of this species appears to be in the winter and spring. Aspergillosis prevalence is lower in these months than in the warmer summer months (Flynn and Weil unpublished data, see Chapter 2), therefore, the impact of the disease on the population may be less than if the species had a summer spawning. However, since the production of eggs takes an extended period of time, healthy colonies may start oogenesis in the summer when disease is more prevalent. Therefore the impact of the disease would be increased in infected colonies by means of delayed oogenesis, decreased egg production or both.

Results indicate that this species has several spawning events throughout the year which might reduce the impact of aspergillosis on the overall reproductive effort. The proportion of reproductive polyps was higher in HA than healthy samples during the summer low for healthy colonies, which may represent a delay in the timing of reproduction; however very few eggs were mature at this time. Samples from healthy areas of diseased colonies also had peaks in the number of reproductive polyps in November and March during the reproductive peak for healthy colonies. The number of eggs per polyp increased in the summer and fall, following the same pattern in healthy and HA samples. The number of mature eggs was relatively high for both healthy and HA samples in November and December, however, while the proportion of mature eggs in healthy colonies remained high for several months, HA samples did not have mature eggs again until May. If there are multiple periods of spawning for this species, diseased colonies would be less likely to be completely excluded from reproduction if they quickly recover reproductive capacity.

The sex ratio of females to males was lower in diseased colonies than in healthy colonies. As only reproductive colonies are included in calculating the sex ratio, this may indicate that suppression of reproduction due to disease occurs disproportionately in females colonies. However this could be attributed to the smaller sample size of reproductive diseased colonies than healthy colonies.

3.5.3 Colony size

In this study, large reproductive female colonies were found to produce more eggs per polyp than smaller colonies indicating that a higher proportion of energy is devoted to egg production as opposed to growth. The trend was weak and the increase in egg production with increased size was not exponential. The number of eggs per polyp approximately doubled from one half to one as the colony area increased five-fold from 1000 to 5000 cm^2. This means that the size reduction associated with partial mortality would not have the drastic negative affect on gamete production found in some other species (Beiring and Lasker 2000).

Minimum reproductive diameters for scleractinian corals range from centimeters to tens of centimeters for different species (Harrison and Wallace 1990). Both the Mediterranean octocoral *Paramuricea clavata* (Coma et al. 1995b) and the Caribbean octocoral *Plexaura kuna* (Brazeau and Lasker 1989, referred to as *Plexaura* A and named *P. kuna* by Lasker et al. 1996) are mostly non-reproductive below 10cm in height. While minimum reproductive size was not determined during this study, it did not likely affect results for healthy colonies because the smallest healthy colony sampled was 500cm^2 (approximately 25 cm in height) and was reproductive with an average of 1.5 eggs per

polyp, which is well above the overall average. It is, however, possible that the live tissue area of some diseased colonies dropped below minimum reproductive size due to partial mortality.

3.5.4 Between vs. within colony variability

There was significant variability in the proportion of reproductive polyps and the number of eggs per polyp between colonies in all months of the study. Some of this variability is probably due to sampling methods. Since only one sample was collected per colony, within colony variability might be a confounding factor preventing characterization of clear trends in the reproductive cycle of *Gorgonia ventalina*. The investigation of within colony variability found that only 18% of colonies had significant variability in the proportion of reproductive polyps in different samples while 33% varied in the number of eggs per polyp. This clearly affected the results, but cannot account for all of the between colony variability observed.

The study of the edge effect found that 55% of healthy colonies had no significant difference between the edge sample and any of the central samples in the proportion of reproductive polyps and 50% had no difference in the number of eggs per polyp. This is another example of the high variability in sexual reproduction between colonies. The net impact of aspergillosis on reproduction is then probably a combination of the infection itself altering polyp physiology and the generation of new edge areas through tissue mortality where sexual reproduction may be reduced.

3.5.5 The 2005 bleaching event

Part of this sampling overlapped with the intensive coral bleaching event of 2005. It is possible that this event had some effect on the reproduction of the species. Bleaching has been shown to negatively affect oogenesis in corals (Szmant and Gassman 1990) and octocorals (Michalek-Wagner and Willis 2001) both by delaying the cycle of oogenesis and by decreasing egg production for up to two years after the end of the bleaching event. *Montastraea faveolata* colonies did not spawn during the summer of 2006, following the 2005 bleaching event in La Parguera. This intense event lasted for several months with colonies totally bleached for up to six months, effectively disrupting the normal reproductive cycle in this species (McClanahan et al. 2008; Weil, pers. observations).

3.5.6 Population-level impact of aspergillosis

Besides the mortality it produces, aspergillosis could have further consequences for *Gorgonia ventalina* populations in La Parguera and other areas. Decreased egg production in diseased colonies could result in (1) a decrease in recruitment rates which could eventually produce a reduction in population densities and (2) an increase in disease resistance due to the higher reproductive output of resistant colonies compared to diseased ones. Recruitment is inversely related to disease prevalence in the Florida Keys (Kim and Harvell 2004). This indicates that decreased reproductive output in diseased sea fans could be a major factor affecting recruitment and/or recruit survivorship. If decreased recruitment results from the observed decrease in reproductive output, then aspergillosis could have severe negative long-term effects on the population, far beyond

the mortality caused directly by disease. Dube et al. (2002) found some evidence of natural selection in favor of *G. ventalina* colonies that produce higher quantities of anti-fungal compounds.

The impact of the decrease in gamete production due to disease on future *Gorgonia ventalina* populations depends on how gamete production affects the dynamics of the population. In La Parguera, variability in recruitment has been found to strongly affect fluctuations in the size of gorgonian populations with post-settlement factors having more influence over recruitment than pre-settlement factors, including gamete production (Yoshioka 1996, 1998). In this case, decreased reproductive output by diseased colonies which are more susceptible to aspergillosis may not negatively affect recruitment, instead resulting in a recruit population which is more resistant to disease. However, during that study, large colony survivorship was consistently high (Yoshioka 1998) which may not be the case in a population affected by disease. Complete and partial mortality of large colonies due to aspergillosis and the reduced reproductive output found in this study even in tissues that appear to be unaffected by the disease, could potentially decrease gamete production enough to significantly reduce larvae production and recruitment. Further studies of population dynamics and mortality due to disease, and longer term studies on reproductive dynamics of diseased colonies (return to normal reproductive output following recovery from infection) could indicate which of these scenarios is a more likely outcome.

3.6 Conclusions

Healthy *Gorgonia ventalina* colonies in La Parguera, Puerto Rico produced eggs and spermaries year round from November 2005 to October 2006. Spawning most likely occurred over several months as mature eggs were present in all months except July. Aspergillosis had a negative effect on gamete production. Polyps closer to infected areas (ADA and TA) had significantly lower fecundity than those farther away (HA). The impact seems to be systemic since polyps in healthy-looking areas of infected colonies (HA) showed significant decreases in egg and sperm production compared to healthy colonies. The long-term effect of this reduction depends on whether gamete production (1) drops low enough to limit recruitment causing a decline in the population or (2) produces a recruit population which is not reduced in size but is more resistant to disease.

4. Conclusions and recommendations for future work

- Aspergillosis prevalence was highly variable in both space and time. Site and depth were the most important factors affecting aspergillosis prevalence. Prevalence increased with decreasing depth in September 2006 contrary to previously observed trends. This could be due to the high temperatures associated with the 2005 bleaching event which appeared to drive the overall increase in prevalence between 2005 and 2006. Continuing prevalence surveys would help evaluate the temporal variability of the disease. This baseline data would help clarify whether disease prevalence can be tied to high water temperatures. Also, studies comparing reefs within a locality with high and low prevalence could indicate what factors drive the spatial distribution of aspergillosis.
- Aspergillosis virulence varied significantly between colonies, and rates of tissue regeneration were much slower than rates of tissue loss. The future of the population depends on the balance between tissue loss and regeneration. Long-term follow-up of colonies which have recovered from disease could help determine whether colonies remain disease-free long enough to regenerate the tissue lost to disease.
- Aspergillosis had a significant negative effect on the reproduction of infected *Gorgonia ventalina* colonies. Further examination of reproductive development could determine how the size and duration of the infection affects the reduction in gamete production, and how long gamete production remains low in colonies after they recover from disease. Life history studies of *G. ventalina* would determine

what life stages affect the long-term growth and survival of the population. Combined, this information could show whether the reduced reproductive output of diseased colonies will have long-term detrimental effects on the population.

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