Seasonal effects on the prevalence and intensity of the parasite *Bonamia* spp. in bivalves from the Indian River Lagoon

by

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SEASONAL EFFECTS ON THE PREVALENCE AND INTENSITY OF THE PARASITE *BONAMIA* SPP. IN BIVALVES FROM THE INDIAN RIVER LAGOON

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This thesis was prepared under the direction of the candidate's thesis co-advisors, Dr. Jon Moore and Dr. Susan Laramore, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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ABSTRACT

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Bonamia spp., a haplosporidian protistan parasite, was first reported in Florida in 2007 in oyster species cultured at Harbor Branch Oceanographic Institute in water from the Indian River Lagoon. Previous research (summer 2010 and 2011) evaluated prevalence and intensity of infections in IRL bivalve species. This study seeks to examine the seasonal effect on parasite prevalence and infection intensity. Bivalves from three sites in the IRL were sampled summer, fall, and winter 2012. Prevalence (general and species specific) was evaluated using PCR. Intensity of infection was evaluated using fluorescent in situ hybridization. Highest prevalence (31.9-48.9%) was seen at all three sites in the fall. Fluorescent *in situ* hybridization revealed highest intensity in the fall (2.08) and lowest in the summer (0.85). Overall prevalence and intensity of infection followed the seasonal trend observed by other researchers in more temperate regions with harsher winter seasons than Florida.

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INTRODUCTION

Bonamia spp. is a microscopic, protistan parasite, phylum Haplosporidia, that infects bivalves (Hine 1996; Carnegie and Cochennec-Laureau 2004; Lewbart 2006, Culloty and Mulcahy 2007). The parasite's placement within the phylum was initially disputed by many for several years because, unlike most haplosporidians, *Bonamia* spp. was not observed to produce spores (Carnegie *et al.*, 2000). Instead its inclusion into the phylum was based up on its possession of haplosporosomes, the persistence of microtubules during interphase, and retention of its nuclear envelope during mitosis; all of which are traits displayed by other Haplosporidians (Carnegie *et al.*, 2000; Hine *et al.*, 2001). Furthermore the plasmodia of *Bonamia* spp. closely resemble that of other *Haplosporidium* species (Hine *et al.*, 2001) and Cochennec *et al.* (2000) found that the SSU rDNA of *B. ostreae* is very similar to the Haplosporidians *Haplosporidia nelson*, *H. costale*, and *Minchinia teredinis*. Inclusion into the phylum was universally accepted following the discovery of spores in the parasite *B. perspora* (Hine *et al.*, 2001; Carnegie *et al.* 2006; Culloty and Mulcahy 2007).

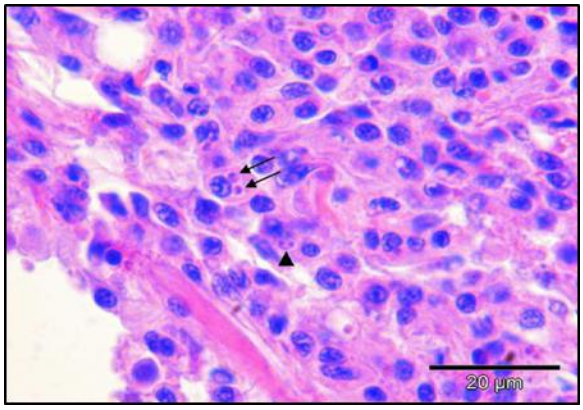

Figure 1. H&E photo of *Bonamia* spp.
Photo by Audemard *et al.* 2008

Bonamia spp. is usually observed as uninucleate microcells 2-3 μm in diameter, but some multinucleate cell types have been observed (Hine 1996; Carnegie and Cochennec-Laureau 2004; Rohde 2005; Lewbart 2012). This parasite has four developmental stages: dense, intermediate, plasmodial, and vacuolated (Hine *et al.*, 2001). The dense form has a central, circular nucleus, eccentric nucleolus, dense ribosomes, intranuclear microtubules, short strand of smooth endoplasmic reticulum, less than three ovoid bodies, and more than 20 haplosporosomes that were either oval, spherical, or pyriform (Hine *et al.*, 2001). Division of the dense form occurs through elongation of the nucleus and karyokinesis (Hine *et al.*, 2001). The dense form is believed to be the infective stage and is seen usually in the fall (Hine, 1991). The intermediate form of the parasite is larger with less densely packed ribosomes, more lipoid bodies and haplosporosomes, and an eccentric nucleus (Hine *et al.*, 2001). This form also divides by elongating the nucleus and karyokinesis (Hine *et al.*, 2001). The intermediate form is mostly seen in the fall and it will begin to outnumber the dense form at the end of fall, beginning of winter (Hine, 1991). The plasmodial form is larger than the intermediate form and contains more haplosporosomes and lipoid bodies (Hine *et al.*, 2001). The plasmodial form has an irregular nucleus, dense mitochondria that surround the nucleus, and contains endosomes with pinocytosed or phagocytosed membranous inclusions (Hine *et al.*, 2001). This form is not seen during the winter and peaks at the beginning and end of fall (Hine 1991). The final vacuolated form usually develops from cells with a poorly delineated nucleus and contains vacuous mitochondria that enlarge to become a central vacuole (Hine *et al.*, 2001). A nucleus is rarely distinguished in this

form does contain ribosomes and haplosporosomes in the cytoplasm (Hine *et al.*, 2001). Not much is known about the life cycle of *Bonamia* spp. and there are no known intermediate species or vectors (MacArdle *et al.* 1991, Cochennec *et al.* 2000).

This parasitic genus has three species: *B. ostreae*, *B. perspora*, and *B. exitiosa*. *B. ostreae* was first described in 1979 from Brittany, France (Comps *et al.* 1980). Hine *et al.* (2001) suggested that a new species of *Bonamia* be named *B. exitiosa* after thorough comparison of the new species with *B. ostreae*. The final species *B. perspora* was discovered while researchers were searching for a reservoir species in the oyster *Crassostrea ariakensis* in North Carolina (Canegie *et al.* 2006).

All three *Bonamia* spp. infect the hemocytes of several different genera of oysters, causing the disease Bonamiosis (MacArdle *et al.* 1991; Montes *et al.* 1994; Hervio *et al.*, 1995; Cochennec *et al.*, 2000; Cochennec-Laureau *et al.*, 2003; Engelsma *et al.* 2010). There are three types of hemocytes that have been observed within bivalve tissues: granulocytes, large agranular cells, and basophilic agranular cells. It has been found that oysters that are infected with *Bonamia* spp. have significantly less granulocytes and significantly more of the large agranular cells; this indicates a preference large agranular cells as only a very low number of parasites have been observed in the granulocytes and basophilic agranular cells (Cigarria and Elston, 1997). It has also been found that resistant oysters tend to have lower levels of large agranular cells than susceptible oysters (Cigarria and Elston, 1997; Naciri-Graven *et al.* 1998).

The hemocytes of bivalves serve several functions such as wound repair, shell repair, nutrient digestion, transport, and excretion, but most importantly they act as one of the bivalve's first internal defenses (Cigarria and Elston, 1997). As hemocytes defend against pathogens by phagocytosis or encapsulation, in order for *Bonamia* spp. to be able to infect the hemocytes it must have some defense against the host's immune system (Cigarria and Elston, 1997). There are two ways in which a parasite can enter a hemocyte: diacytosis and endocytosis. In diacytosis the parasite creates a hole in the host's cell membrane through which it enters, in endocytosis the parasite does nothing but instead allows the host to enclose it in a membrane (Chagot *et al.* 1992). *Bonamia* spp. enters the bivalve hemocyte through phagocytosis which is an endocytotic process in which the hemocytes create pseudopods that surround the parasite and pull it into the cytoplasm and surround it by a parasitophorous vacuole (Chagot *et al.* 1992). It is unclear how the parasite is able to survive phagolysosomal fusion because it does not escape nor does it inhibit fusion (Chagot *et al.* 1992).

Once *Bonamia* spp. has entered an hemocyte it multiplies by binary fission until the cell ruptures; ten or more parasites have been observed in one hemocyte (Culloty and Mulcahy 2007). Once the host cell has ruptured the parasites infect other hemocytes and repeat the process (Culloty and Mulcahy 2007). Bivalves that are infected with *Bonamia* spp. display few clinical symptoms but may gape (MacArdle *et al.* 1991; Culloty and Mulcahy 2007). Infected bivalves may also have lesions in the connective tissue and gill erosion, with severe infections resulting in death (MacArdle *et al.* 1991; Culloty and Mulcahy 2007).

The earliest report of *Bonamia* spp. was found in California in *Ostrea edulis*, where it has been prevalent since the 1960's (Hine, 1991; Cigarria and Elston, 1997). From there it spread to France's *Ostrea edulis* stocks most likely through the importation of infected seed in 1979 (Cigarria and Elston, 1997; Engelsma *et al.* 2010). By 1998, oyster production in France was 10% of what it was before the *Bonamia* spp. outbreak (Naciri-Graven *et al.* 1998). Spain and Denmark also experienced severe outbreaks of Bonamiosis *O. edulis*, seeds were imported from California (Friedman and Perkins 1994; Cigarria and Elston, 1997; Culloty and Mulcahy 2007). By 1980 the flat oyster industry in Spain was nonexistent (Cigarria and Elston, 1997). *Bonamia ostreae* was first reported in the Netherlands in 1980 and, despite strict hygiene, continued spread caused mortalities of up to 80% by 1988 (Engelsma *et al.* 2010). In the mid-1980's Bonamiosis was found in Washington, USA; it is believed spread was due to exposure oysters from California (Friedman and Perkins 1994). The parasite was found in England in 1982 (Culloty and Mulcahy 2007) and in 1985 New Zealand began to experience large scale mortalities in its *Triostrea chilensis* stocks due to Bonamiosis (Hine *et al.* 2001). By 1992 the New Zealand stocks had been reduced by 91% and the fishery was officially closed in 1993 (Hine *et al.* 2001). The first outbreak of *Bonamia* spp. in Ireland was reported in 1987, but it is believed that the parasite was present as early as 1984 (MacArdle *et al.* 1991; Culloty and Mulcahy 1996; Culloty *et al.* 2001). It is believed that *Bonamia* spp. was introduced to Ireland through illegal seed importations from France (MacArdle *et al.* 1991). In 1991 the parasite was discovered that Bonamiosis had spread to Maine (Freidman and Perkins 1994; Cochennec *et al.* 2000). The parasite was also reported in British Columbia, Canada in 2004, in Morroco in 2005, and in Scotland

and Wales in 2006 (Culloty and Mulcahy 2007). In 2003 North Carolina experienced severe mortalities in its *Crassostrea ariakensis* populations that were later attributed to *Bonamia* spp. (Carnegie *et al.* 2006; Audemard *et al.* 2008). The parasite was reported in Florida waters for the first time in *Crassostrea virginica* and *C. ariakensis* in the Indian River Lagoon after it was discovered in oyster mesocosm experiments (Dungan *et al.* 2012).

Figure 2. Worldwide distribution of *Bonamia* spp.

The disease has been found in both subtidal and intertidal areas and has been reported mostly in oysters (Naciri-Graven *et al.*, 1998). *Bonamia* spp. has been found to infect oysters in the genus *Ostrea* (*O. edulis*, *O. concaphila*, *O, puelchana, O. angasi*, and *O. chilensis*), *Triostrea chilensis*, *Crassostrea rivularis*, *Crassostrea virginica*, *Crassostrea ariakensis*, *Saccostrea glomerata*, and *Osteola equestris* (Carnegie *et al.*

2006; Lewbart 2006; Culloty and Mulcahy 2007). The only oyster species known to be resistant to *Bonamia* spp. is *Crassostrea gigas* (Engelsma *et al.* 2010).

Research has shown that *Bonamia* spp. prefers warm high salinity waters, 31-35 psu (Audemard *et al.* 2008). The parasite is transmitted directly through the water and thrives in systems such as troughs and enclosed estuaries or bays in which the water is recirculated and there are high densities of bivalves (Friedman and Perkins 1994; Culloty and Mulcahy 2007). The parasite is believed to enter the tissues during respiration and filtration of sea water (Culloty and Mulcahy 2007). It may take several weeks to a few months before the parasite can be detected within the bivalve (Culloty and Mulcahy 2007). The first signs of disease are usually seen around two years of age and infection increases with age (MacArdle *et al.* 1991; Audemard *et al.* 2008; Culloty and Mulcahy 1996). *Bonamia* appears to follow a seasonal pattern, it generally has low prevalence and intensity levels in the spring and the parasites seen are small, dense basophilic forms in the basement membrane of the gut (Hine *et al.* 1991; Carnegie *et al.* 2008). *Bonamia* spp. proliferates in the middle/end of summer (mid/end July) to the beginning of fall (September) and the parasites seen are usually dense, eosinophilic forms; highest intensities of infection are seen in the fall (September to early November) (Hine 1991; Culloty and Mulcahy 2007; Carnegie *et al.* 2008). The parasite's prevalence as well as intensity usually decreases in winter (Hine 1991; Carnegie *et al.* 2008). It is hypothesized that the reason the parasite is able to proliferate during the fall is due to summer stress on the hemocytes (Hine *et al.* 1991). Mortality due to Bonamiosis is most often seen during late fall or early winter not long after spawning occurs (Hine *et al.*

1991). It is believed that spawning leaves the oysters too weak to combat the parasite so it proliferates quickly through the tissues, eventually killing the bivalve (Hine *et al.* 1991).

The standard diagnosis method for Bonamiosis has changed over the years. In the late 1980's and early 1990's, researchers examined gill, digestive gland, and heart tissue smears using light microscopy as a diagnosis tool for *Bonamia* spp., a quick and relatively inexpensive method (Boulo *et al.* 1989). In 1989, Boulo *et al.* found that testing with monoclonal anti-bodies provided a quicker means of diagnosis since it allowed examination of the smears at lower magnification. By 2000 fixing and staining of heart and hemolymph smears, was commonly used as a cheap and quick detection method but the low sensitivity of this method soon became problematic (Carnegie *et al.* 2000). Histological methods allow researchers to observe the foci of infection but this method is very slow, furthermore the parasite can resemble routine intrahemocytic inclusions and misidentification of the parasite could lead to false positives (Carnegie *et al.* 2000; Culloty and Mulcahy 2007). A specific immunohistological method that was adequately sensitive was developed in Europe, but it had limited use for other countries since the antibodies reacted weakly to parasites outside of the European populations (Carnegie *et al.* 2000). One popular immunohistological method is Fluorescent *in situ* hybridization, in which a fluorescent probe is added to a samples and hybridizes only to parts of the sample that contain the complementary sequence (Bartlett 2004). When examined under a fluorescent microscope the portion of the sample which the probe has hybridized with will appear to be a different color than the rest of the tissue (Bartlett

2004). In 2000 polymerase chain reaction (PCR) assays were developed to detect the presence of the parasite (Carnegie *et al.* 2000). PCR utilizes DNA polymerase's ability to create a new strand of complementary DNA when offered a template strand, a researcher can use a primer to indicate what portion of the template strand that should be amplified and the DNA polymerase will create billions of copies of this region of DNA (Schochetman *et al.* 1988). Probes were developed to detect *B. ostreae* in *Ostrea edulis* based on an 18S ribosomal DNA sequence present in both the USA and European populations as well as to detect *B. ostreae* and other *Bonamia* spp (Carnegie *et al.* 2000; Cochennec *et al.* 2000). It is currently recommended that both PCR assays and histology be used together in order to diagnose *Bonamia* spp. infections (Carnegie *et al.* 2000; Cochennec *et al.* 2000).

Attempts to eradicate *Bonamia* spp. from oyster populations have not been successful; therefore containment and prevention, such as improved husbandry techniques that include reduced handling and decreased stock densities, are used to control the spread of the parasite (Culloty and Mulcahy 2007). Furthermore, establishing future oyster cultures in areas of lower salinity, less than 30 psu, may reduce the risk of a *Bonamia* spp. outbreak (Audemard *et al.* 2008). For most areas rapid and accurate diagnosis will also help to control the spread of Bonamiosis (Carnegie *et al.* 2000; Cochennec *et al.* 2000). Some researchers have attempted to breed Bonamiosis resistant oyster species, something that has not been observed in the wild due to the fact that susceptible oysters are often able to breed before the parasite kills them (Naciri-Graven *et al.* 1998). Naciri-Graven *et al.* 1998 reported that two strains of resistant oysters (*O.*

edulis), have been developed by using the selection pressure of purified parasites. Culloty *et al.* (2001) also attempted to develop *Bonamia* spp. *O. edulis* resistant oysters by breeding the survivors of a Bonamiosis outbreak. The most important tool in preventing the spread of Bonamiosis is regulation, ensuring that infected animals and seed are not imported to uninfected areas and emphasis on permitting and regulations have been put in place by most countries that have been affected by the disease (Culloty and Mulcahy 2007).

Previous summer Harbor Branch Oceanographic summer interns confirmed the presence of *Bonamia* spp. in Florida's Indian River (IRL) and Lake Worth (LWL) Lagoons by PCR through the use of general *Bonamia* and specific *Bonamia* primers (*B. exitiosa* and *B. perspora*). Those projects determined which IRL sites where the parasite was present, parasitized bivalve species, and the specific *Bonamia* species. They found *Bonamia* sp. in seven different bivalve species, four oysters and three mussels: *C. virginica*, *O. equestris*, *Isognomon bicolor*, *I. alatus*, *Iscahdium recurvum*, *Branchiodontes exustus*, and *Geukensia granosissima* in fifteen sites ranging from as far north as the Sebastian River in the IRL and as far South as Bird Island in the LWL (Unpublished data, Lave 2010; Miller 2011). *B. exitiosa* was found in six species (*C. virginica*, *O. equestris*, *I. alatus*, *B. exustus*, *I. recurvum*, and *G. granosissima*) and eight sites (Spoil Island 12, Wild Cat Cove, Port Sewall, Fort Pierce Inlet, Taylor Creek, and Roosevelt Bridge in the IRL and at Peanut Island and Bird Island in the LWL) and *B. perspora* was found in six species (*C. virginica*, *I. alatus*, *I. bicolor*, *B. exustus*, *I. recurvum*, and *G. granosissima*) at nine sites (Sebastian River, Oslo, Wild Cat Cove,

Taylor Creek, St. Lucie Estuary, North Causeway, and Port Sewall in the IRL and at Peanut Island and Bird Island in the LWL) (Unpublished data, Miller 2011; Lave 2010). Five sites in the IRL (Wabasso, Bear Point, Jack's Island, Oyster Cut Island, and Spoil Island 13) were positive for general *Bonamia* but negative for *B. exitiosa* and *B. perspora*, indicating that another *Bonamia* species may be present in the IRL, it is possible that this species may be *B. ostreae* but this species has yet to be tested for in Florida waters (Unpublished data, Miller 2011; Lave 2010). Low to moderate prevalence levels was seen at all sites, the prevalence observed in Florida has been much lower than areas where *Bonamia* spp. outbreaks have occurred. As a Link summer intern in 2012 I examined the PCR positive summer 2010 samples using fluorescent *in situ* hybridization in order to determine the intensity of infection in those bivalves. The majority of the samples exhibited very low infection intensities.

Based on past research and my own research in 2012, it occurred to me that perhaps the low prevalence and intensity levels that were seen may have been due to the time of year (summer) in which the sampling occurred. Previous published research documented a decline in parasite prevalence in early summer; however most of the regions have very distinct seasons compared to Florida's subtropical environment. This project was focused on determining if we would observe higher prevalence and intensity levels in the fall and winter as has been documented in more temperate regions. To that end three positive previously high-salinity sites (Wild Cat Cove (WCC), North Causeway (NC), and Wabasso Bridge (WB)) were examined for which summer prevalence (PCR) data existed so that some comparisons could be made with previously reported results.

Three seasons were compared: summer, fall, and winter (time precluded the evaluation of a spring sampling period). To evaluate parasite prevalence three PCR primers were used: general *Bonamia*, *B. exitiosa*, and *B. perspora*. To examine infection intensity levels fluorescent *in situ* hybridization was used to examine samples that were found to be positive for the parasite by PCR.

MATERIALS AND METHODS

Site Selection

The three sites for this project were chosen from seventeen previously visited sites studied in the Indian River Lagoon (unpublished data, Lave 2010; Miller 2011). The criteria for selecting the sites for this study were based upon having previously been positive for the presence of *Bonamia*, similarities in salinities, and ease of access. Two sites had been sampled previously: Wabasso bridge (fall 2010 and summer 2011) and Wild Cat Cove (summer 2010 and 2011): while North Causeway had only been sampled once previously (summer 2010). Wabasso Bridge is the northernmost site and North Causeway is the southernmost site. Most of the samples were collected from the intertidal zone and were associated with mangroves. Samples were collected at low tide and the majority was exposed when collected.

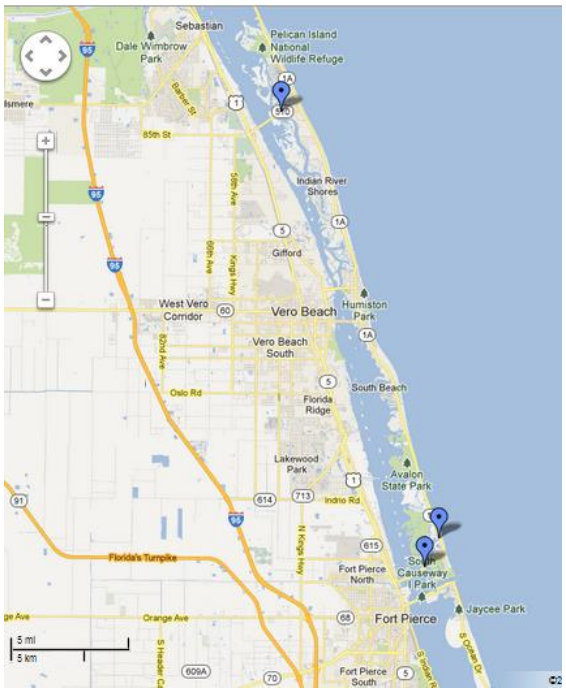

Figure 3. Map of Collection Sites

Sampling and Tissue Processing

Wild Cat Cove (WCC) was sampled summer, fall, and winter while Wabasso Bridge (WB) and North Causeway (NC) were only sampled fall and winter. Initial the project scope involved the seasonal examination of only one site (WCC), but was later expanded (fall 2012) to include three sites. Although there was no sample collection for

Wabasso bridge and North Causeway during the summer of 2012, there is PCR data available for both of these sites from previous summers that can used to compare the seasonal prevalence of the parasite at those sites.

The sites were sampled at low tide and we attempted to collect approximately twenty samples of any bivalve species we could find. Collected samples were placed in a cooler filled with water from the site and transported back to Harbor Branch Oceanographic Institute (HBOI). In the laboratory the samples were identified taxonomically, cleaned, weighed, and measured. The samples were then processed for PCR and histology. For PCR a section of gill and mantle was placed in a microcentrifuge tube containing 95% ethanol. For histology a cross section containing gills, mantle, and digestive tissue was taken and placed in Davidson's fixative for approximately 48 hours, after which the samples were washed and transferred to 70% ethanol.

PCR Analysis

DNA was extracted from the preserved gill and mantle tissues using a Wizard Genomic DNA purification kit (Promega Corp.). A nanodrop fluorospectrometer was used to determine the DNA concentration of the extracted samples after we observed extraneous bands in the electrophoresis gels, the DNA concentration was found to be approximately 10 times greater (200-500 mm) than the maximum recommendation for PCR. The extracted DNA was then diluted 1:10 by placing 5 µl of extracted solution in 45 µl of nuclease free water; this reduced the number of extraneous bands seen in the initial gels. Extracted samples were amplified in a 25 µl sample volume containing 12 µl

of Go Taq Master Mix, 1µl of DNA, 10 µl of dH_2O, and 1 µl each of the general *Bonamia* primer, BoF/BoaR, in a BioRad "icycler" thermocycler. After the PCR reaction, 10 µl of the solution was loaded onto a 1% TAE gel and run for one hour at 80v. Pictures of the gel were taken and the results noted. Previous results (Lave 2010; Miller 2011) had shown that gel bands around 700 and 380 molecular weights were indicative of a positive result for *Bonamia* spp. Any sample that gave positive results with the *Bonamia* general primers were then tested against species specific primers for *B. exitiosa* and *B. perspora*. The primer used for *B. exitiosa* was CaBon 461R/CaBon 146F. The primer used for *B. perspora* was OEBonN 472R/OEBoN 154F. All primers were created by Sigma Genosys (The Woodlands, TX).

Fluorescent *in situ* hybridization

Only samples that gave PCR positive results for *Bonamia* spp. were examined histologically. Preserved positive samples were processed in a Thermo Scientific Excelsior ES. Processed tissues were embedded in paraffin using a Thermo Scientific Histostar. Once the paraffin solidified 8 µm thick serial sections were cut using a rotary microtome and placed on Fischer colorfrost slides. A modified fluorescent in situ hybridization protocol, developed by the Carnegie laboratory at the Virginia Institute of Marine Science, was used on the sectioned slides. The slides were first placed in a Leica autostainer to de-paraffinize the tissue using xylene and then were rehydrated using ethanol. The slides were then placed in a pre-warmed (37ºC) 50 µg/mL proteinase K solution (14 minutes), which allows proteolysis, a process in which the probe penetrates the tissues. Slides were transferred to a phosphate buffered saline (PBS) solution with

0.2% glycine to stop proteolysis (five minutes). The slides are then placed into an acetic anhydride solution (12 minutes) to reduce the probe from electrostatically bonding to positive charges in the tissue to reduce background. This was followed by a wash in PBS (5 minutes) and 5X SET (5 minutes). A Bonamia Fluorescent Alexa 488 oligo probe, created by Invitrogen, was placed in a pre-prepared prehybridization buffer and 100 µl of the solution was placed on each slide. The slides are then placed in a humid chamber overnight. The next day the slides were washed in 0.2X SET (3 × 1 minute), cover slipped, and mounted with Fluoromount-G (SouthernBiotech).

Slides were examined using an Olympus fluorescent microscope with a universal blue green filter. When the probe binds with *Bonamia* spp. it gives off a specific fluorescent pattern: small, green rings (Carnegie et al. 2003). Other spurious fluorescence, usually the result of granules in the epithelium, was seen in the tissues but was disregarded (Carnegie et al. 2003). Intensity of infection was evaluated on a 0-5 scale that was based on the one used by Audemard *et al.* (2008). The samples were scored a 0 if no parasites were seen; a 0.5 rating for a rare infection (Figure 4a), less than 10 parasites observed in the entirety of the tissue; a 1 for light infection intensity (Figure 4b), parasites focused in two or three areas of tissue with two to three parasites per field of view; a 3 for moderate infection intensities (Figure 4c), >50% of the tissue contained parasites with three to four parasites per field of view (40X); a 5 for heavy infection intensities (Figure 4d), almost every field of view contained parasites, usually in high density, five to six parasites per field of view.

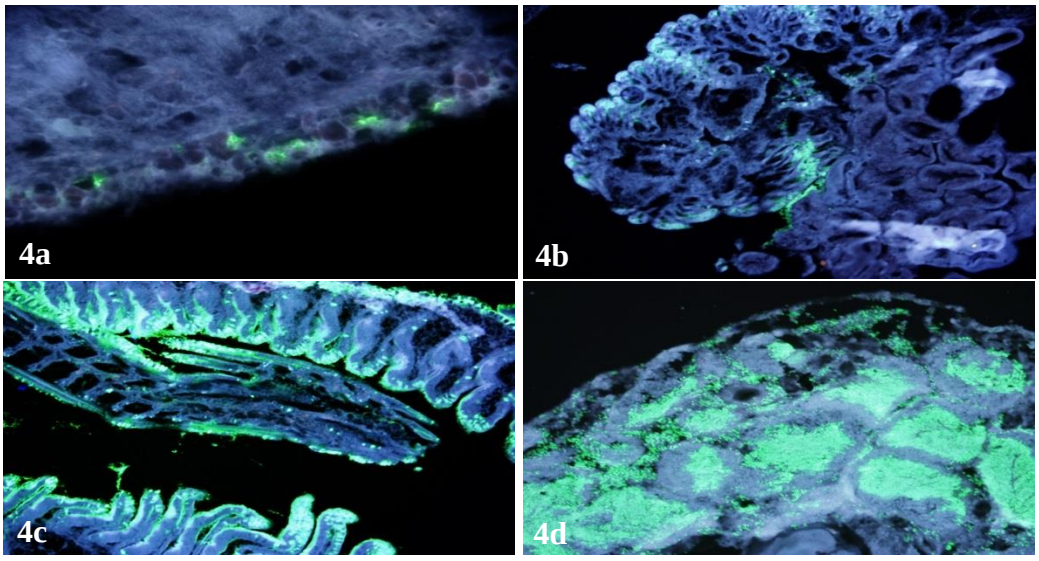

Figure 4. Examples of Infection Intensity Ratings; 4a. 0.5 or rare intensity, 4b. 1 or light intensity, 4c. 3 or moderate intensity, 4d. 5 or heavy intensity

Statistical Analysis

Prevalence (number of infected samples/number of total samples) was calculated for each of the three sites at every season. The percent of *Bonamia* spp. positive samples that were infected by *B. exitiosa* and *B. perspora* was then calculated. Prevalence was calculated similarly for each bivalve species in each season for the three sites.

Infection intensity calculated at Wild Cat Cove for every season. This was calculated by adding up the intensity scores for all PCR positive samples from that site at that season and dividing the sum by the number of samples. The infection intensity was also calculated for each of the bivalve species at each season.

RESULTS

PCR Results

Of 555 bivalve samples examined for *Bonamia* by PCR 314 (56.58%) were positive for general *Bonamia* spp., none were positive for *B. exitiosa*, and 29 (5.25%) were positive for *B. perspora* (Table 1).

Wild Cat Cove summer (56 samples) had a prevalence of 3.57% for *Bonamia* spp. (Figure 5, Table 1). None of the samples were positive for either *B. exitiosa* or *B. perspora* (Figure 5, Table 1). Only one species (*Crassostrea virginica*) of four collected species was positive for the parasite (8.33%, n=24) (Table 2). At the time of collection (June) the water temperature was 29.5ºC, salinity was 29.5 psu, and dissolved oxygen was 4.60 mg/L.

Wild Cat Cove fall (72 samples) had a prevalence of 47.73% for *Bonamia* spp. (Figure 5, Table 1). None of the samples were positive for either *B. exitiosa* or *B. perspora* (Figure 5, Table 1). Six of seven species collected were positive for *Bonmia*: *C. virginica* (30%, n=30), *O. equestris* (33.3%, n=3), *Geukensia granosissima* (42.1%, n=19), *Ischadium recurvum* (40%, n=5), *Isognomon alatus* (83.33%, n=24), and *I. bicolor* (50%, n=4) (Table 2). At the time of collection (September) the water temperature was 29.5ºC, salinity was 24 psu, and dissolved oxygen was 4.78 mg/L.

Wild Cat Cove winter (100 samples) had a prevalence of 24% for *Bonamia* spp. (Figure 5, Table 1). None of the samples were positive for either *B. exitiosa* or *B. perspora* (Figure 5, Table 1). *C. virginica* (70%, n=20), *O. equestris* (100%, n=1), and *I.*

alatus (45%, n=20) were all positive for *Bonamia* spp (Table 2). At the time of collection (December) the water temperature was 24.7ºC, salinity was 35 psu, and dissolved oxygen was 4.60 mg/L.

North Causeway fall (96 samples) had a prevalence of 47.92% for *Bonamia* spp. and 63% of those positive samples tested positive for *B. perspora* (Figure 6, Table 1). None of the samples were positive for *B. exitiosa* (Figure 6, Table 1). Six of seven collected species were positive for *Bonamia*: C. *virginica* (73.33%, n=30), *O. equestris* (10%, n=10), *G. granosissima* (66.67%, n=3), *B. exustus* (22.72%, n=22), *I. alatus* (36.84%, n=19), and *I. bicolor* (75%, n=12) (Table 3). The same species were also positive for *B. perspora*. The percentage of *Bonamia* spp. positive samples that were also positive for *B. perspora* are: 77.27% *C. virginica*, 100% *O. equestris*, 100% *G. granosissima*, 20% *B. exustus*, 42.86% *I. alatus*, and 55.56% *I. bicolor* (Table 3). At the time of collection (September) the water temperature was 27.7ºC, salinity was 27 psu, and dissolved oxygen was 5.69 mg/L.

North Causeway winter (93 samples) had a prevalence of 20.43% for *Bonamia* spp (Figure 6, Table 1). None of the samples were positive for either *B. exitiosa* or *B. perspora* (Figure 6, Table 1). Five of nine collected species were positive for *Bonamia*: *C. virginica* (30%, n=20), *B. exustus* (14.29%, n=7), *M. citrinus* (16.67%, n=6), *I. alatus* (33.33%, n=24), and *I. bicolor* (42.86%, n=7) (Table 3). At the time of collection (December) the water temperature was 24.1ºC, salinity was 38 psu, and dissolved oxygen was 6.79 mg/L.

Wabasso Bridge fall (72 samples) had a prevalence of 33.33% for *Bonamia* spp. (Figure 7, Table 1). None of the samples were positive for either *B. exitiosa* or *B. perspora* (Figure 7, Table 1). Four of five collected species were positive for *Bonamia*: *C. virginica* (62.07%, n=29), *O. equestris* (20%, n=15), *G. granosissima* (100%, n=1), and *B. exustus* (5.56%, n=8) (Table 4). At the time of collection (September) the water temperature was 28.3ºC, salinity was 22 psu, and dissolved oxygen was 5.06 mg/L.

None of the Wabasso Bridge winter (50) samples were positive for *Bonamia* spp. (Figure 7, Table 1). At the time of collection the water temperature was 21ºC, salinity was 35 psu, and dissolved oxygen was 7.45 mg/L.

FISH Results

Only the PCR positive samples from Wild Cat Cove fall and winter were examined using Fluorescent *in situ* hybridization (FISH). Only two *C. virginica* samples were PCR positive samples from the summer sampling, therefore we decided to examine all *C. virginica* samples from this site. Overall 97 samples were examined using FISH and 77.32% were histologically positive. The overall intensity of infection was 1.58, which is just slightly above a light infection intensity ranking.

A total of 24 *C. virginica* samples were examined using FISH for Wild Cat Cove summer and 70.83% were histologically positive for *Bonamia* spp. (Table 5). Only 8.33% of these samples had been PCR positive. The intensity of infection for summer was 0.85, between a rare and light ranking (Figure 9, Table 5).

For Wild Cat Cove fall samples 76% of PCR positive samples were positive by FISH, histologically (Figure 8, Table 5). The intensity of infection for this season was 2.08, midway between a light and moderate ranking (Figure 9, Table 5). The species that were FISH positive for *Bonamia* spp. were *C. virginica* (73.33%, 15 samples), *G. granosissima* (69.23%, 13 samples), *I. alatus* (84.21%, 19 samples), and *I. bicolor* (100%, 2 samples) (Table 6). The intensity of infection for each species was 1.13 for *C. virginica*, 1.92 for *G. granosissima*, 3.05 for *I. alatus*, and 2 for *I. bicolor* (Table 6).

For Wild Cat Cove winter samples 87% of PCR positive samples were histologically positive (Figure 8, Table 5). The overall intensity of infection was 1.24, slightly above a light infection rating (Figure 9, Table 5). The species that were FISH positive for *Bonamia* spp. were *C. virginica* (85.71%, 14 samples), *O. equestris* (100%, 1 sample), and *I. alatus* (87.5%, 8 samples) (Table 6). The intensity of infection for each species was 1 for *C. virginica*, 0.5 for *O. equestris*, and 1.75 for *I. alatus* (Table 6).

DISCUSSION

PCR

Bonamia spp.

In this study I found that at all three sites the highest *Bonamia* spp. prevalence was seen during the fall (September) sampling. For Wild Cat Cove (WCC) the lowest prevalence was observed in the summer. No samples were collected during the 2012 summer season for either North Causeway (NC) or Wabasso Bridge (WB) but there is data available from past summer collections for both of these sites (unpublished data, Lave 2010; Miller 2011). When the past data was added for comparison it was found that the 2012 winter sampling had the lowest prevalence seen yet for both North Causeway and Wabasso Bridge; *Bonamia* spp. prevalence for NC in the summer 2010 was 39%, for WB it was 23% in summer 2011 and 5% in fall 2010. The results seen from the three sites follow the pattern that is described by both Carnegie *et al.* 2008 and Hine 1991, which is highest prevalence in fall and lower prevalence in summer and winter.

The prevalence observed in WCC 2012 summer is the lowest summer prevalence reported so far for this site (3.57% versus 42% in 2010 and 10% in 2011), however this low prevalence cannot be explained by salinity and temperature differences. The salinity for the site in 2012 was slightly lower (29 psu versus 33.78 psu in 2010 and 37.27 psu in 2011), but this cannot be the causative factor since the salinity was even lower (24 psu) during the fall sampling period and the prevalence seemed unaffected. The water temperature was the same for both the summer and fall 2012 collections so temperature was most likely not the cause either.

With the exception of the WCC summer collection the lowest prevalence at all sites was in winter. No infected samples were found at Wabasso Bridge during the winter sampling. This collection period was expected to have a lower prevalence than the previous fall sampling but it was surprising that none of the samples contained parasites since both of the other two sites still contained *Bonamia* spp. infected samples during the winter. The lack of parasite presence at Wabasso Bridge in the winter may be due to a drop in water temperature. During the fall sampling at WB the water temperature was 28.3ºC and in the winter the water temperature dropped to 21ºC, which was lower than the temperature at the other two sites (24.7ºC and 24.1ºC).

B. exitiosa

B. exitiosa was not found in any of the three sites that were sampled, which is surprising since this species has previously been documented at Wild Cat Cove, although not at the other two sites. It is possible that *B. exitiosa* was found only at WCC because of the landscape differences between the sites; WCC is a protected cove with little water flow, while the other two sites have much more water flow. In the summer of 2010 and 2011 the *B. exitoisa* prevalence was ~20-25%. It is uncertain why this species was not found at WCC this year. One possible cause may be salinity, during this year's sampling the salinity was 29.5 psu, in 2010 it was 33.8 psu and in 2011 it was 37.3 psu. Perhaps this species is more sensitive to changes in salinity and therefore was not able to persist this year due to the lower salinity.

B. perspora

B. perspora was only found at NC during the fall sampling. We expected to find *B. perspora* during the winter sampling as well since it had been observed previously during the summer in 2010 (~31%, Lave 2010). It was also surprising to not find this species at Wild Cat Cove since it had been previously documented at this site during the summers of 2010 (~7%) and 2011 (~60%) (Lave 2010, Miller 2011). As with *B. exitiosa*, the reason *B. perspora* may not have been found at WCC is that the site had a lower salinity than when this species was found in the past. It is unclear as to why *B. perspora* was not able to persist into the winter months at North Causeway since the salinity was quite high, about 38 ppt, and temperature was not much lower than seen in the summer.

Bivalve Species

Consistent with site seasonality data, almost all the bivalve species sampled had their highest *Bonamia* spp. prevalence in the fall sampling period. *Mercenaria mercenaria* was the only species that had 0% prevalence at every site and season. *M. mercenaria* also was observed to not contain any parasites during the summer 2010 sampling. This leads me to believe that *M. mercenaria* may be resistant to *Bonamia* spp.

The crested oyster, *Ostrea equestris* had relatively low prevalence in both the fall and winter sampling, which is similar to what was found in the summer of 2010. In contrast, in the summer of 2011 it had a much higher prevalence (50%). Low prevalence levels in *O. equestris* is unusual; Carnegie *et al.* 2000 have shown that it is a reservoir for *Bonamia perspora*, so if the parasite is present it is expected to exhibit moderate to high

prevalence levels. I thought that the low prevalence may be caused by lowered salinities, but a comparison of salinities seen in 2010 and 2011 revealed that only Wild Cat Cove showed a noticeable difference in salinities. Therefore, the lower prevalence for *O. equestris* at Wild Cat Cove may have been caused by a decreased salinity, but that would not explain the results at the other two sites. It is just as possible that the high prevalence reported in the summer 2010 samples were artificially inflated due to small sample size.

The scorched mussel, *Brachiodontes exustus,* also had relatively low prevalence during the fall and winter sampling periods. This agrees with what has been observed during previous collections. During the summer of 2010 and 2011 this species had a prevalence of ~13-15% (Lave 2010, Miller 2011). Since *B. exustus* only seems to display low prevalence levels I believe that this species may be somewhat resistant to *Bonamia* spp.

At Wild Cat Cove it was found that the eastern oyster, *Crassostrea virginica,* had a higher prevalence during the winter than in the fall; at the other two sites the *C. virginica* prevalence was higher in the fall than in the winter. This may be partially caused by the fact that fewer samples were taken during the winter collection, but I believe that this indicates that the parasite is well established in this host species and is able to persist longer within this species. The moderate to high prevalence in the two flat oyster species, *Isognomon alatus* and *I. bicolor*, during the fall sampling period may indicate that the parasite is also well established within these two species. *O. equestris* also exhibited a higher prevalence in the winter than in the fall but I believe this is due to small sample (n=1) examined during the winter. *B. exustus* and *M. mercenaria* appear not

to be affected by *Bonamia* spp. at this site since both had 0% prevalence; supporting my earlier statement that they may possibly be resistant to the parasite.

At North Causeway the fall sampling revealed moderate to high *Bonamia* spp. prevalence in *C. virginica, G. granosissima, I. alatus*, and *I. bicolor*. This indicates that *Bonamia* spp. may be well established in these species at this site. *O. equestris*, *B. exustus*, and *M. mercenaria* had low *Bonamia* spp. prevalence levels, which was also seen at WCC. As mentioned before, it is unusual for *O. equestris* to have low prevalence (Carnegie *et al.* 2000) and may indicate that *B. exustus* and *M. mercenaria* are resistant to *Bonamia* spp. This was the only site that was PCR positive for *B. perspora*, however not all samples that were positive for *Bonamia* spp. were positive for *B. perspora*, indicating that another species is present at North Causeway. The other species does not appear to be *B. exitiosa*, which is surprising since it has been found at this site previously (summer 2010).

The high *Bonamia* spp. prevalence in *C. virginica* at Wabasso Bridge indicates that the parasite is well established in this species at this site as well. It appears that there is a high prevalence level in *G. granosissima* as well, but this is likely due to the small sample size (n=1 in the fall, n=10 in the winter). Once again *O. equestris*, *B. exustus*, and *M. mercenaria* displayed low prevalence levels, upholding the hypothesis that *B. exustus*, and *M. mercenaria* may be resistant to *Bonamia* spp. Neither *B. exitiosa* nor *B. perspora* were detected at this site, however this was expected since neither species has ever been found at this site previously.

None of the PCR positive samples from WCC and WB were positive for either *B. exitiosa* or *B. persopra*. The winter NC PCR positive samples also were not positive for either *B. exitiosa* or *B. persopra*. Furthermore, about 37% of the PCR positive samples from NC fall were not positive for either *B. exitiosa* or *B. persopra*. This indicates that the *Bonamia* species for these samples is either *B. ostreae* or it is a species that has yet to be described.

Overall *Bonamia* prevalence levels in the bivalve species were consistent at each site. Every site that contained *C. virginica, G. granosissima, I. alatus,* and *I. bicolor* displayed high prevalence levels within those species. Every site that contained *O. equestris, B. exustus,* and *M. mercenaria* displayed low prevalence levels within those species. So it seems that there were no differences in bivalve species *Bonamia* spp. infections caused by geographical location.

FISH

Samples were examined for a specific shape of fluorescence, small, green rings, any other shapes were considered spurious fluorescence. Parasites in the gut lumen or on the surface of the gills were not indicative of an infection, since these parasites would have been passed out of the bivalve if it had lived longer.

For Wild Cat Cove summer all samples that were PCR positive for *Bonamia* spp. were histologically positive, but since that data represented only two samples I decided to examine all 24 *C. virginica* samples using FISH. Of all the summer samples examined, 70.93% (or 17 samples) were found to be histologically positive. This indicates that we

were receiving false negatives from PCR or false positives from FISH. I believe it may be a combination of the two and not exclusively one or the other. One way to validate the FISH results is to run a competitive exclusion FISH assay. Due to time constraints I was unable to do so for this project but the assay will be performed for the summer samples in the future. The overall intensity for this season was slightly below a light intensity level (0.85), which leads me to believe that it is likely that light spurious fluorescence may have caused false positives.

Of the fall Wild Cat Cove samples that were PCR positive for *Bonamia* spp., 76% were histologically positive. The samples that were histologically negative are not indicative of false positives from PCR or false negatives from FISH. Many of the samples of these FISH negative samples were observed to contain *Bonamia* spp., but the parasites were either located in the gut lumen or on the surface of the gills. In the samples that no parasites were observed it is possible that there were parasites present but they were in the initial stages of infection and not yet detectable histologically. The overall infection intensity for this season was in between a light and moderate infection level (2.08), which shows an increase in infection intensity compared to the summer. This was expected since PCR data had already shown that prevalence increased from summer to fall.

In the winter Wild Cat Cove sampling 86.96% of the PCR *Bonamia* spp. positive samples were histologically positive. The overall infection intensity for this season was just above a light infection level (1.24) indicating that there was a decrease in infection

intensity from fall to winter. Once again this was expected since PCR data had shown a drop in prevalence from fall to winter.

I hypothesized that the infection intensity would follow the same trend that was observed with the prevalence of the samples, which was highest in the fall and lowest in the summer and winter. The results I received validated my hypothesis. The infection intensity of most of the bivalve species also followed this trend. *I. alatus* is the only species that displayed a moderate intensity level (3.05), this indicates that the parasite may be well established within this host species.

Overall, the bivalves that I examined in the Indian River Lagoon followed the same seasonal trend seen by Carnegie *et al*. (2008) and Hine (1991): low prevalence/intensity in early summer, highest prevalence/intensity in late summer/fall, and declining prevalence/intensity in early winter. Therefore it seems that the warmer water temperatures caused by Florida's tropical climate does not allow the parasite to persist longer.

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APPENDIX A

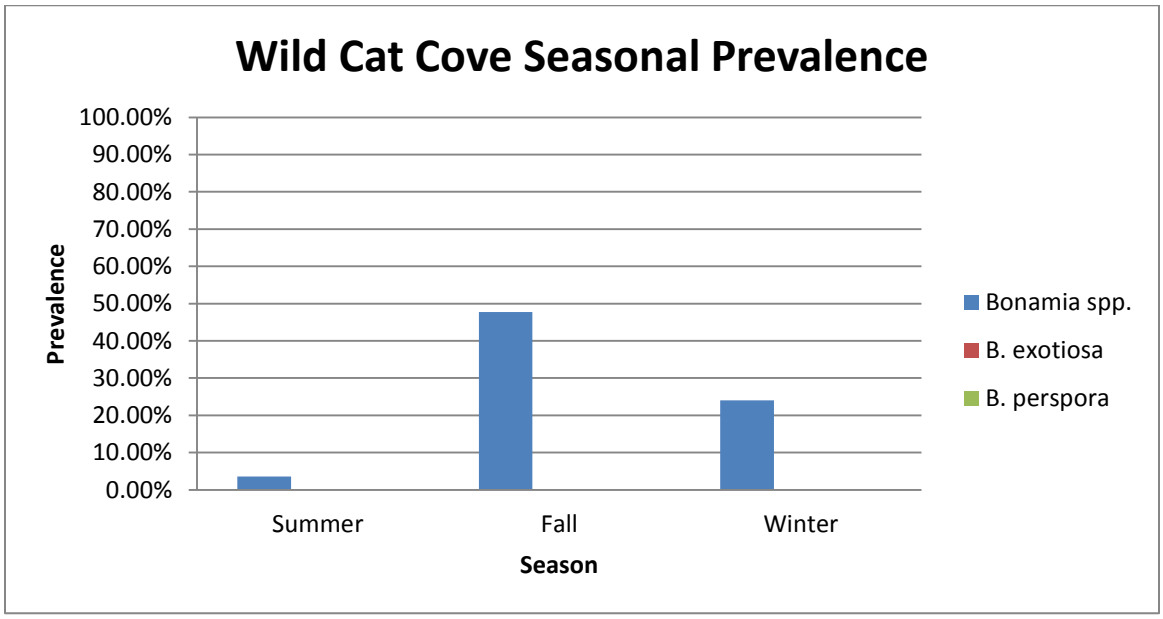

Figure 5. Prevalence of *Bonamia* spp., *B. exitiosa*, and *B. perspora* at Wild Cat Cove by season

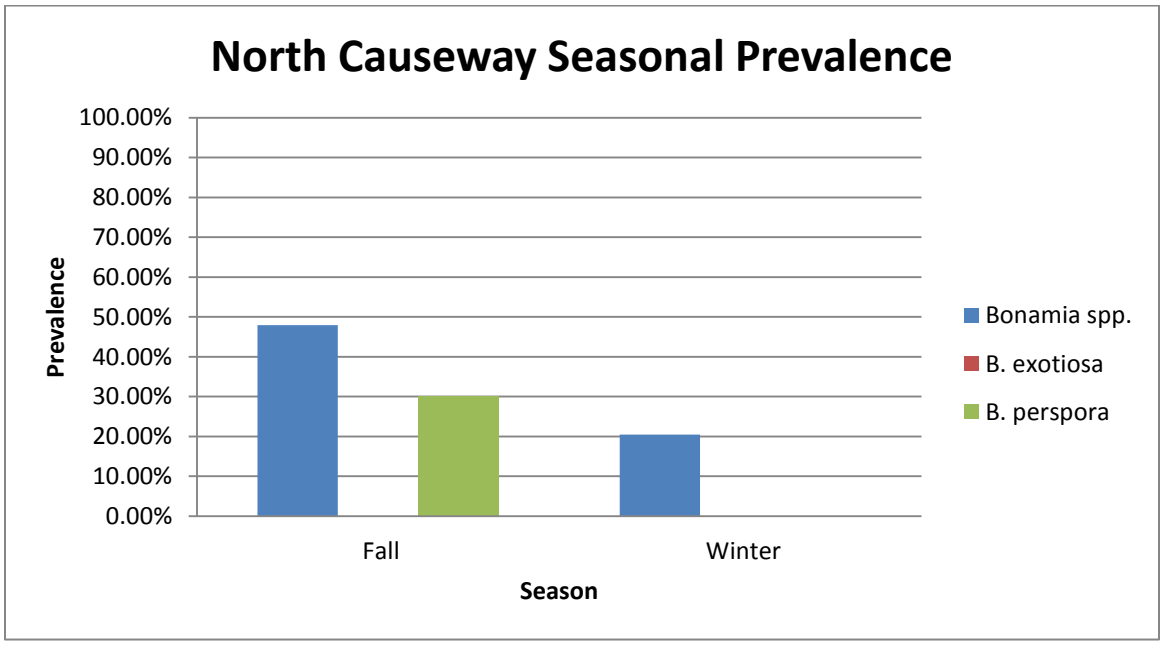

Figure 6. Prevalence of *Bonamia* spp., *B. exitiosa*, and *B. perspora* at North Causeway by season

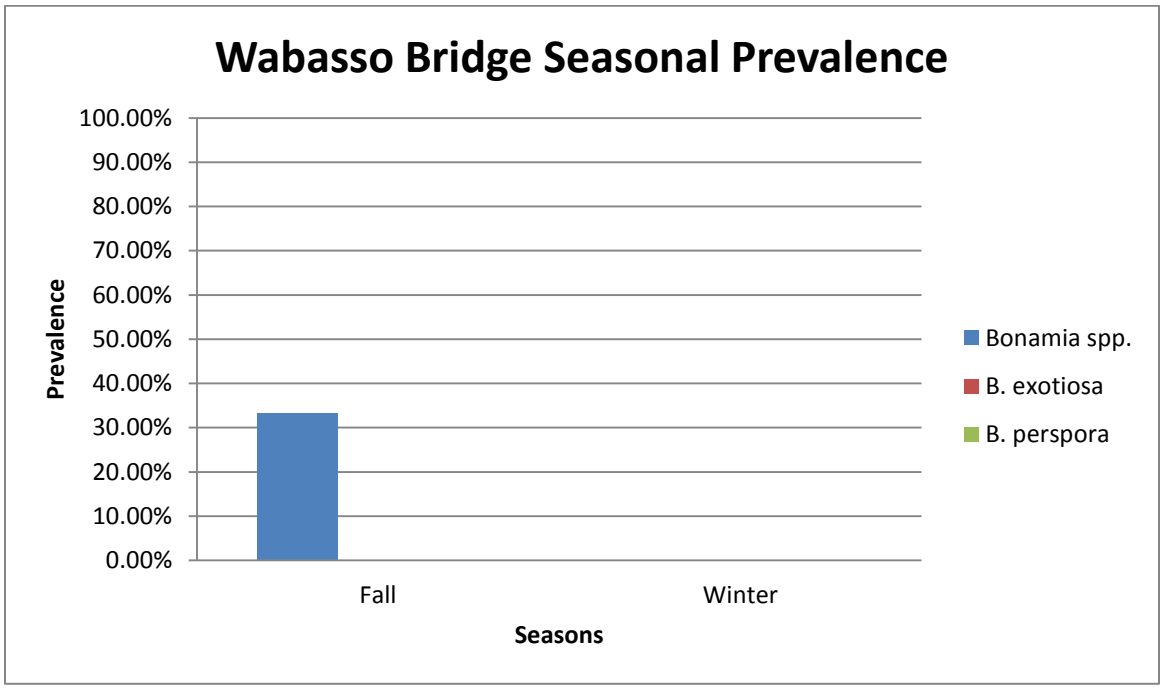

Figure 7. Prevalence of *Bonamia* spp., *B.* exotiosa, and *B. perspora* at Wabasso Bridge by season

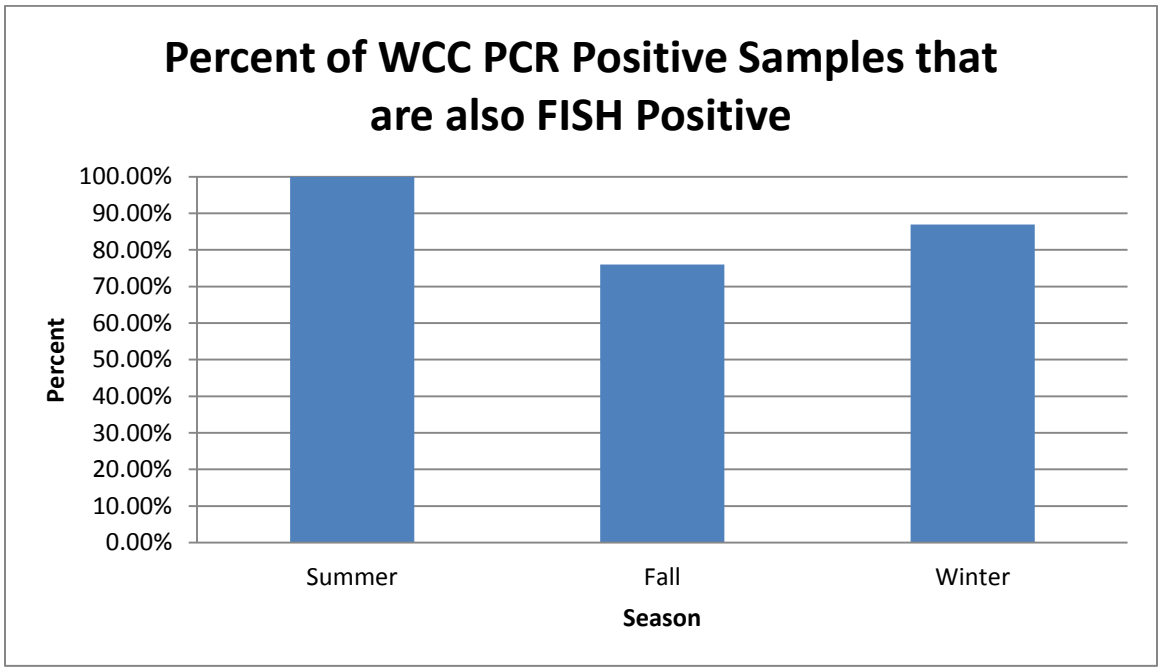

Figure 8. Percent of PCR *Bonamia* spp. positive samples that were found to be histologically using FISH for the site Wild Cat Cove

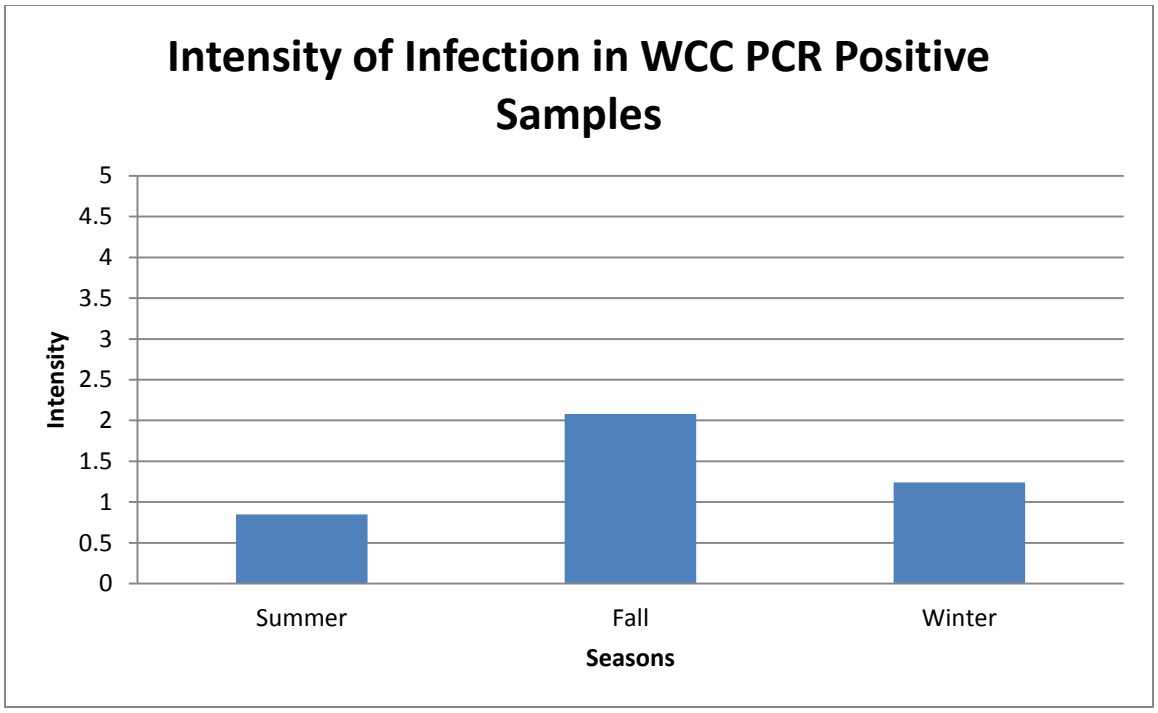

Figure 9. Overall infection intensity by season for the site Wild Cat Cove

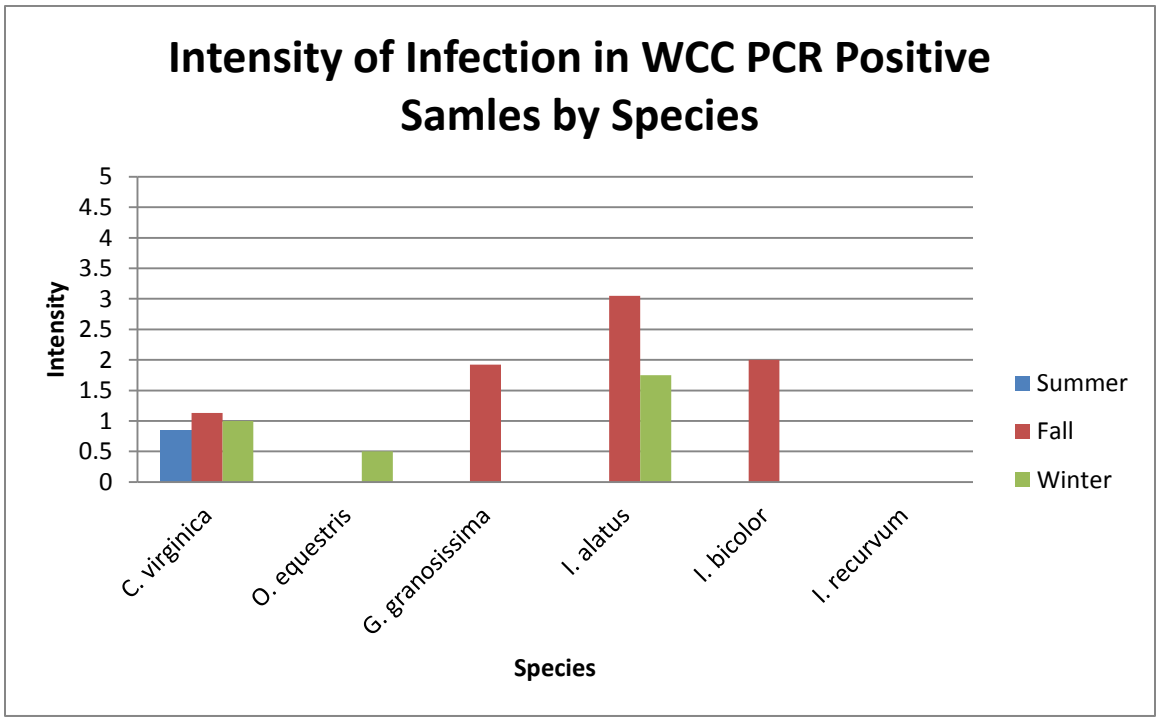

Figure 10. Intensity of infection in the PCR *Bonamia* spp. positive samples by species for the site Wild Cat Cove

Table 1. Prevalence by site and season			
	Bonamia sp.	*B. exotiosa*	*B. perspora*
WCC Summer	3.57%	0%	0%
WCC Fall	47.73%	0%	0%
WCC Winter	24%	0%	0%
NC Fall	47.92%	0%	30.21%
NC Winter	20.43%	0%	0%
WB Fall	33.33%	0%	0%
WB Winter	0%	0%	0%

Table 2. *Bonamia* sp. Prevalence by Species for Wild Cat Cove 2012			
Species	Summer	Fall	Winter
Crassostrea virginica	8.33%	30%	70%
Ostrea equestris	-	33.33%	100%
Geukensia granosissima	0%	42.10%	0%
Ischadium recurvum	-	40%	-
Brachiodontes exustus	-	-	0%
Mytilus citrinus	-	-	-
Isognomon alatus	0%	83.33%	45%
Isognomon bicolor	-	50%	0%
Mercenaria mercenaria	0%	-	0%

Table 3. *Bonamia* spp. & *B. perspora* Prevalence by Species for North Causeway 2012				
	Bonamia Fall	*Perspora* Fall	*Bonamia* Winter	*Perspora* Winter
Crassostrea virginica	73.33%	77.27%	30.00%	0.00%
Ostrea equestris	10.00%	100.00%	0.00%	0.00%
Geukensia granosissima	66.67%	100.00%	0.00%	0.00%
Ischadium recurvum	-	-	0.00%	0.00%
Brachiodontes exustus	22.72%	20.00%	14.29%	0.00%
Mytilus citrinus	-	-	16.67%	0.00%
Isognomon alatus	36.84%	42.86%	33.33%	0.00%
Isognomon bicolor	75.00%	55.56%	42.86%	0.00%
Mercenaria mercenaria	0.00%	0.00%	0.00%	0.00%

Table 4. *Bonamia* sp. Prevalence by Species for Wabasso Bridge 2012		
Species	Fall	Winter
Crassostrea virginica	62.07%	0.00%
Ostrea equestris	20.00%	0.00%
Geukensia granosissima	100.00%	0.00%
Ischadium recurvum	-	-
Brachiodontes exustus	5.56%	0.00%
Mytilus citrinus	-	-
Isognomon alatus	-	-
Isognomon bicolor	-	-
Mercenaria mercenaria	0.00%	0.00%

Table 5. Wild Cat Cove FISH Prevalence and Infection Intensity by Season		
Season	Prevalence	Intensity
Summer	70.83%	0.85
Fall	76.00%	2.08
Winter	86.96%	1.24

Table 6. FISH Prevalence & Infection Intensity for Species by Season for Wild Cat Cove 2012						
Season	Summer Prevalence	Summer Intensity	Fall Prevalence	Fall Intensity	Winter Prevalence	Winter Intensity
Crassostrea virginica	70.83%	0.85	73.33%	1.13	85.71%	1
Ostrea equestris	-	-	-	-	100%	0.5
Geukensia granosissima	-	-	69.23%	1.92	-	-
Isognomon alatus	-	-	84.21%	3.05	87.50%	1.75
Isognomon bicolor	-	-	100%	2	-	-
Ischadium recurvum	-	-	0%	0	-	-

Table 7. Number of Samples Examined by PCR for Each Site and Collection Period 2012							
	Wild Cat Cove			North Causeway		Wabasso Bridge	
Bivalve Species	Summer	Fall	Winter	Fall	Winter	Fall	Winter
Crassostrea virginica	24	30	20	30	20	29	20
Ostrea equestris	5	3	1	10	5	15	9
Geukensia granosissima	20	19	22	3	16	1	10
Ischadium recurvum	0	5	0	0	1	0	0
Brachiodontes exustus	0	0	10	22	7	18	8
Mytilus citrinus	0	0	0	0	6	0	0
Isognomon alatus	7	24	20	19	24	0	0
Isognomon bicolor	0	4	6	12	7	0	0
Mercenaria mercenaria	5	0	20	3	6	2	1

Table 8. Number of Samples Examined by Fish by Bivalve Species			
Bivalve Species	Summer	Fall	Winter
Crassostrea virginica	24	15	14
Ostrea equestris	0	0	1
Geukensia granosissima	0	13	0
Ischadium recurvum	0	1	0
Isognomon alatus	0	19	8
Isognomon bicolor	0	2	0