SURVEY FOR THE OYSTER PARASITES BONAMIA, MSX, AND DERMO IN TEXAS BAY SYSTEMS

A Thesis

by

HAILEY M. BOECK

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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December 2018

ABSTRACT

Texas Parks and Wildlife Department restricts movement of American oysters (*Crassostrea virginica*) from one Texas bay system to another because of potential disease transfer and genetic differences in natural oyster stocks. Oyster diseases, such as Bonamiosis, which was found serendipitously in 2007 in Florida waters, and MSX (*Haplosporidium nelsoni*) have not been characterized in Texas bays. Therefore, it is prudent to periodically examine *Crassostrea virginica* and other species (e.g., *Ostrea equestris* and *Isognomon* sp.) from different Texas bays for the presence of the causative agents of these diseases, i.e., *Bonamia* spp. and *Haplosporidium nelsoni*, as well as known diseases such as *Perkinsus marinus*. American oysters (n=30/bay) were collected from October to December 2016 in Copano Bay, San Antonio Bay, Matagorda Bay, Galveston Bay, and Sabine Lake. In addition, 89 historical American oyster tissue samples collected from 2010 and 2011 in Aransas Bay and Copano Bay in Texas were assessed. Finally, 38 flat tree oysters (*Isognomon alatus*) were collected in December 2017 from Lower Laguna Madre in Port Isabel, Texas. All specimens were assessed by PCR and histology for the presence or absence of *Bonamia* sp., *Haplosporidium nelsoni*, and *Perkinsus marinus*. No *Bonamia* spp. or *H. nelsoni* was detected in any American or flat tree oyster, but on average 15% of the 2016 American oyster samples and 27% of the 2010-2011 American oyster samples contained *P. marinus*. Sanger sequencing of isolated DNA was performed on samples that were positive for Dermo as well as the positive *Bonamia* sp. and *H. nelsoni* controls, which confirmed results. These results serve as a point reference that indicate *Bonamia* spp. and *H. nelsoni* are still not currently present in Texas bays, but continued biennial monitoring is suggested.

ACKNOWLEDGMENTS

This research was supported in part by an Institutional Grant (NA14OAR4170102) to the Texas Sea Grant College Program from the National Sea Grant Office, National Oceanic and Atmospheric Administration, U.S. Department of Commerce. I would like to thank the TAMU-CC Center for Coastal Studies Hans and Patricia Suter Endowment, Millicent Quammen Memorial Endowment Fund, Texas Chapter of the American Fisheries Society, TAMU-CC College of Graduate Studies, and the TAMU-CC Marine Biology Department for supporting my research and travel to various conferences to present my findings.

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I received invaluable assistance in specimen collection through Texas Parks and Wildlife Department and cannot thank the following people enough for allowing me to join their boat crews, collect oysters, and offer their knowledge on the locations of some very elusive oysters: Dr. Mark Fisher, Dr. Chris Mace, Evan Pettis, Chas Downey, Norman Boyd, Christine Jensen, Dr. Carey Gelpi, and Leslie Hartman. I would also like to thank Jace Tunnel, Director of the

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INTRODUCTION

The American oyster, *Crassostrea virginica*, has been negatively affected by increasing natural and human-induced stressors in our oceans and estuaries (Grabowski et al. 2017, Keithly and Roberts 2017), resulting in an 85% loss of functional oyster reefs from global historical estimates (Beck et al. 2011). Gulf of Mexico oyster reefs have decreased by 50-80% in the last 100 years but are not stressed to the point of being functionally extinct (i.e., >99% loss; Mackenzie 2007, Beck et al. 2011). Extreme weather events, such as hurricanes Katrina (2005) and Ike (2008), as well as human induced stressors, such as the Deepwater Horizon oil spill (2010), other oil pollution, and freshwater run-off have dramatically increased mortality (Keithly and Roberts 2017). Following the DWH oil spill, Texas oyster landings in 2010 and 2011 declined by 25% (Fig. 1) and Mississippi oyster landings were reduced by 78% (NOAA NMFS 2016). These declines resulted in a direct economic loss of $9.7 million for Gulf of Mexico communities (NOAA NMFS 2017).

Currently, natural *C. virginica* reefs along the Gulf of Mexico coast are categorized as "fair" (Beck et al. 2011), but these reefs produced 56% of total U.S. commercial oyster landings (28.04 million lbs. meat) in 2016 (NOAA NMFS 2017). U.S. commercial landings have not kept up with national demand, and to compensate, importation and aquaculture have increased, up 1.7% from 2016-2017, to satisfy U.S. consumer demand (NOAA Fisheries 2017). Texas has an actively managed natural oyster fishery that has varied in annual production from 1.59 (2015) to 6.81 (2003) million pounds per year over the past two decades (NOAA NMFS 2017, Fig. 1).

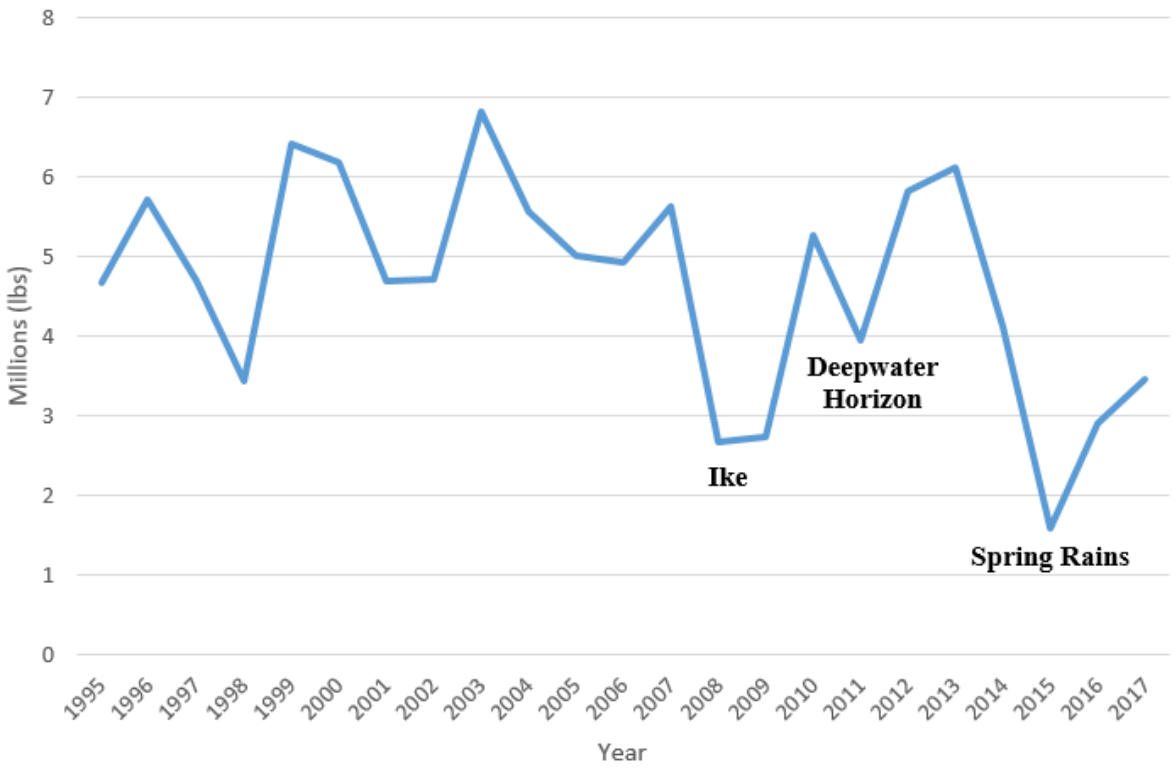


Figure 1: Texas fisheries oyster landings from 1995-2017 (data from NOAA NMFS 2017). The largest decreases in landings were caused by natural and human disasters such as Hurricane Ike in 2008, the Deepwater Horizon oil spill in 2010, and spring rains in 2015.

Despite the need for aquaculture to satisfy consumer demands, Texas has no regulatory policy for commercial oyster aquaculture (Texas Parks and Wildlife Department 2017a). The state may periodically restore oyster reefs by placing cultch, which provides a foundation for oysters to attach to, in Texas bays if substrate has been lost during a natural disaster such as a hurricane (Texas Parks and Wildlife Department 2017b). Existing policy focuses on genetics and diseases (e.g., Dermo for shellfish) for use of fish and shellfish from hatcheries for stock enhancement efforts (Texas Parks and Wildlife Code 2001). In 2002, Texas Parks and Wildlife Department (TPWD) initiated 15-year private leases for oyster management and harvest in

Galveston Bay (Texas Parks and Wildlife Code 2001) that were renewed in 2017. In 2016 TPWD initiated a plan to expand the existing lease program and develop an Oyster Aquaculture Plan (OAP, Texas Parks and Wildlife Department 2016).

Oyster aquaculture is self-sustaining when the production of oyster seed is from a hatchery; however, there are no oyster hatcheries in Texas, thus no oyster seed is available. If the OAP is adopted, TPWD would allow for oyster "spat-on-shell" production (i.e., a specialized seed type consisting of newly settled oysters on cultch material) and placement in Texas bays (Texas Parks and Wildlife Department 2017), which indicates policy for the management of oyster hatcheries would be forthcoming. Movement of oysters from one Texas bay system to another, such as Galveston Bay to Matagorda Bay, is not allowed because of potential genetic differences in oyster stocks and potential disease transfer (King et al. 1994, Texas Parks and Wildlife Code 1997, Anderson et al. 2014).

There are numerous known oyster diseases, but only Dermo is regularly monitored (Powell et al. 1992, Reece et al. 2001, Aguirre-Macedo et al. 2007) in the Gulf of Mexico. The oyster disease Dermo is caused by the parasite *Perkinsus marinus* (originally *Dermocystidium marinum*, Mackin et al. 1950) originally discovered in the late 1940s and early 1950s (Mackin et al. 1950). This parasite leads to high mortality of oysters, especially its primary host, *C. virginica* (Mackin et al. 1950, Andrews and Hewatt 1957, Ray 1966) but is not pathogenic to humans. It is endemic to the Gulf of Mexico and has since spread to the Atlantic Coast of the U.S. (Ford 1996). This protistan parasite is transmitted by a motile zoospore stage (Robledo et al. 2018). Once the host ingests a zoospore, it matures into a trophozoite, which absorbs nutrients from the host and multiplies by binary fission until the host cell ruptures (Fig. 2). *P. marinus* causes the infected host tissues to be severely degraded, which can be observed via pale

coloration, slow growth, and mantle regression (Smolowitz 2013, Arzul and Carnegie 2015). *P. marinus* is most abundant where there is low fresh-water inflow, high salinity, and high temperatures (Cook et al. 1998, Aguiree-Macedo et al. 2007).

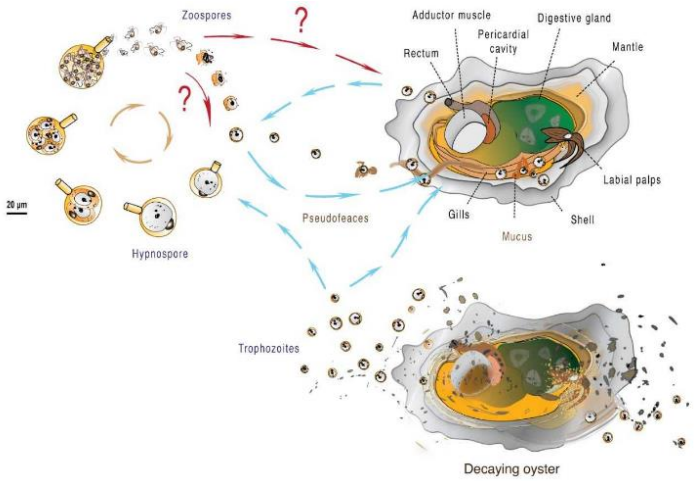


Figure 2: *Perkinsus* spp. life cycle (from Robledo et al. 2018).

Another oyster parasite, *Bonamia* spp., which is the etiological agent for Bonamiosis, is a microcell haplosporidian parasite that infects the hemocytes of several oyster species and may result in death (Gollasch et al. 2005). *Bonamia* sp. enters the bivalve host through ingestion as it filter-feeds, then hemocytes ingest the *Bonamia* sp. by endocytosis, causing the infection. The parasite then multiplies by binary fission until the host cell ruptures; the released parasites continue infecting other hemocytes, replicating, and rupturing hosts cells (Fig. 3). Infected bivalves may not always show visible symptoms such as tissue degradation, but in some cases, *Bonamia* sp. can cause gill erosion or lesions in connective tissues eventually leading to death (Culloty and Mulcahy 2007). There are at least three described *Bonamia* species (*B. ostreae*, Balouet et al. 1983, *B. exitiosa*, Hine et al. 2001, and *B. perspora*, Carnegie et al. 2016). *B. roughleyi* is sometimes considered a fourth species, but its status is debated (Cochennec-Laureau et al. 2003). The genus *Bonamia* was first described in 1979 during a mass mortality event of

European flat oysters (*Ostrea edulis*) in French waters (Pichot et al. 1979). *Bonamia* range has expanded through human-assisted movement (Hine 1996). Surveys and research have been conducted worldwide following its initial outbreak in France (Pichot et al. 1979), including in New Zealand (Hine 1996, Hine et al. 2001, Lane et al. 2016), Ireland (McArdle et al. 1991, Culloty 2007), Portugal (Batista et al. 2016), and Brazil (Suhnel et al. 2016). *Bonamia* spp. has been found in European flat oysters (*O. edulis*) along the coast of California (Friedman et al. 1989) and the Atlantic coast of the U.S. in the Damariscotta River in Maine (Friedman and Perkins 1994), as well as in *C. virginica* in south Florida (Dungan et al. 2012). Other bivalves, such as *Geukensia demissa*, *Isognomon* sp., and *Brachiodontes extusus*, were found to be PCR positive for *Bonamia* spp. along the east coast of Florida as well, with the highest prevalence in *Ostrea equestris* and *Isognomon* sp. (Laramore et al. 2014). The highest prevalence in Florida occurred in the fall, from September to November (Laramore et al. 2017). Because Florida has similar water temperature and salinity as Texas, it is possible that *Bonamia* spp. is present in Texas bays and estuaries. Despite the global attention on this parasite, *Bonamia* spp. has not been surveyed for in the Gulf of Mexico.

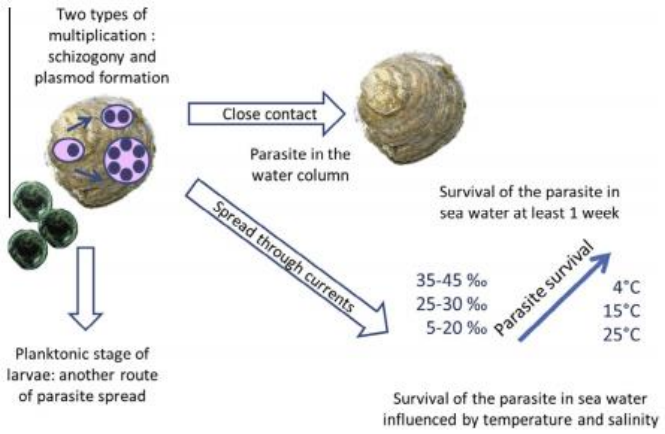


Figure 3: Infection dynamics of *Bonamia ostreae* in *Ostrea edulis* (from Arzul and Carnegie 2015).

Another parasite of concern is *Haplosporidium nelsoni*, the causative agent of MSX disease. This parasite can infect via spores and harms its host by reducing its feeding rate, stored carbohydrates, and fecundity (Arzul and Carnegie 2015, Fig. 4). MSX is endemic to the Northeastern U.S. seaboard and has been examined extensively in the United States along the east coast (e.g., Stokes and Burreson 1995, Dungan et al. 2012, Proestou et al. 2016, Laramore et al. 2017, Robledo et al. 2018). Its presence in the Gulf of Mexico, however, has not been confirmed (Ford et al. 2011, Dungan et al. 2012). MSX was detected by PCR in *C. virginica* from the Gulf of Mexico and the Caribbean Sea (Ulrich et al. 2007), but these results were never confirmed histologically or by genetic sequencing (Ford et al. 2011). Burreson (2008) suggested misuse of PCR for diagnosis of infections and stated that PCR results must be validated by histology. This is because PCR can result in false positives due to unspecific binding, primer dimers, using too much primer, or contamination. Following Ulrich's study (Ulrich et al. 2007), 32 sites in the Gulf of Mexico were surveyed for MSX, but no evidence for the parasite was found, indicating that the Gulf of Mexico should be "considered free of MSX" (Ford et al. 2011).

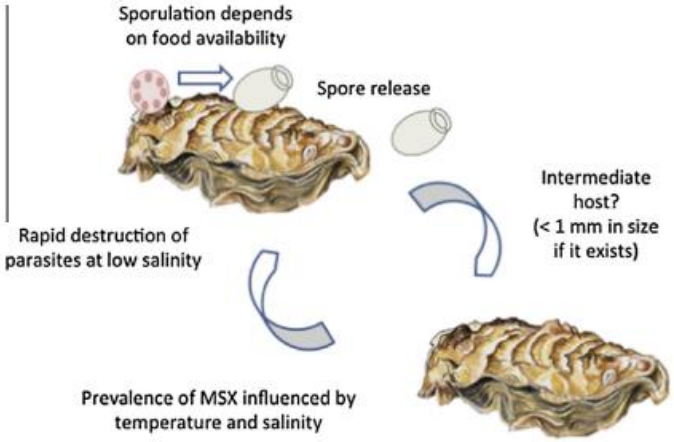


Figure 4: Life cycle of *Haplosporidium nelsoni* in *Crassostrea virginica*. The potential for an unknown intermediate host or hosts is also included (from Arzul and Carnegie 2015).

To determine if the Gulf of Mexico remains free of MSX and is free of *Bonamia,* it is necessary to survey presumptive hosts as well as other bivalves that may act as reservoirs of these parasites. The Gulf of Mexico is home to several hundred different species of bivalves, however, the current study focused on only three: *Crassostrea virginica*, *Ostrea equestris*, and *Isognomon alatus* (Tunnell 2010). *Ostrea equestris*, also known as the crested oyster, can be found in the Gulf of Mexico and along the Atlantic Coast of North America from Virginia to Texas and in the Caribbean and Brazil (Tunnell, 2010). *O. equestris* has been observed in hypersaline (35ppt or higher), intertidal waters such as the waters of Lower Laguna Madre, and frequently attaches itself to mangroves, hard surfaces, and *C. virginica*. They are generally small, ranging from 25-76 mm in length. *O. equestris* can be easily misidentified as juvenile *C. virginica*, but they are distinctly different with ridges seen along the lip of the oyster and the absence of the purple muscle scar found within the shell of *C. virginica* (Fig. 5, Tunnell, 2010).

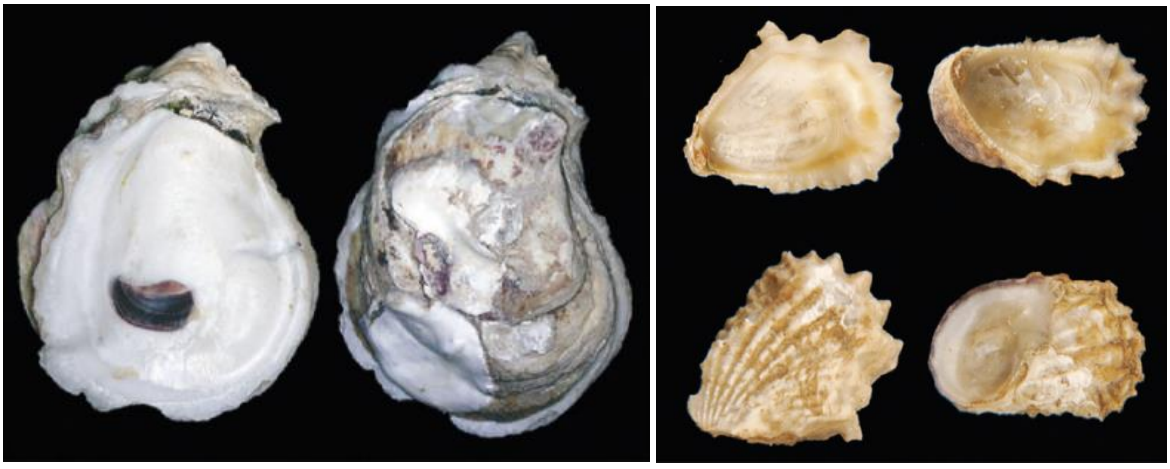


Figure 5: Morphological comparison of *Crassostrea virginica* (left) and *Ostrea equestris* (right) (from Tunnell 2010).

The flat tree oyster, *Isognomon alatus*, can be found in subtidal and intertidal waters attached to mangrove roots in the Gulf of Mexico, southern Florida, the Caribbean Sea, and the coast of Brazil (Tunnell 2010). In Texas, *I. alatus* is generally found as a single bivalve rather than in clusters, attached with byssal threads to hard surfaces. Laramore et al. (2014) reported a similar prevalence of *Bonamia* sp. in the fall and winter seasons in *O. equestris* (36%) and in *I. alatus* (58%) samples in eastern Florida waters by PCR; therefore, the abundance of parasites in *O. equestris* is lower than what is seen in *I. alatus*, which makes it potentially more likely to find *Bonamia* sp. in *I. alatus*.

Due to ever-increasing U.S. demand for oysters, the potential for oyster aquaculture in Texas, and the limited knowledge of oyster parasites, other than *P. marinus*, in Texas waters, the objective of the present study was to examine *C. virginica*, *O. equestris*, and *I. alatus* from Texas bay systems to determine if the parasites *Bonamia* spp. and *Haplosporidium nelsoni* were present.

METHODS AND MATERIALS

Collection and Initial Measurements

Thirty adult *Crassostrea virginica* (American oyster) were sampled from each of the northern five bay systems (Fig. 6) of the Texas coast. The northernmost site was Sabine Lake near Port Arthur, Texas and the southernmost site was Copano Bay near Port Aransas, TX. Oysters were collected from Copano Bay on October 13, 2016; San Antonio Bay on November 2, 2016; Galveston Bay on November 10, 2016; Sabine Lake on November 17, 2016; and Matagorda Bay on December 6, 2016. Oysters were collected from late-October to early-December as the fall season showed the highest *Bonamia* sp. prevalence (30-47%) in all species in waters off Florida (Laramore et al. 2014). Sites were chosen following Texas Parks and Wildlife Department (TPWD) regular oyster fishery management surveying schedule for these bays. All *C. virginica* were taken from the center of bays to remove tidal action and inundation periods as variables. TPWD collected *C. virginica* from each of the northern five bays with a 14" dredge that was pulled by boat for 30 seconds; the first 30 oysters from each dredge were collected for this study. At each collection location, hydrological data (i.e., temperature, salinity, dissolved oxygen, and pH) was collected with a YSI Multi-Parameter Water Quality Meter (Yellow Springs Instrument, Yellow Spring, Ohio: Model 11783217).

Isognomon alatus (flat tree oyster) was sampled from Lower Laguna Madre near Port Isabel on December 5, 2017 (Fig. 6). TPWD does not routinely sample for this species, but it has been reported in South Texas near Laguna Madre and Port Isabel (Tunnell 2010). *O. equestris* has also been reported to be in Lower Laguna Madre (Tunnell 2010), but this species could not be found during the current study. Because research indicated a similar prevalence of *Bonamia* in

I. alatus as in *O. equestris*, *I. alatus* is a likely host for *Bonamia* sp. and a viable bivalve to study for the presence of parasites.

A specimen collection permit was obtained from TPWD (SPR-1017-196). Flat tree oysters were removed from concrete structures at the shallowest, intertidal locations on the shorelines of Port Isabel (26.0757 N, -97.2224 E). *I. alatus* were seen mixed in clusters of *C. virginica* and collected individually by pulling or scraping them off the concrete substrate to which they were anchored. A total of 37 flat tree oysters were collected. Redfish Bay in Aransas Pass, Texas (27.900206 N, -97.134950 E) was also surveyed, but no *I. alatus* were found.

To determine if the parasites of interest were prevalent at other times, 85 *C. virginica* tissue samples from Aransas Bay and Copano Bay (Fig. 7) that had been collected in 2010 and 2011 were included in the current study. These samples were collected by Dr. Jennifer Pollack (TAMU-CC) for a population genetics study. On December 16, 2010, 19 oysters were collected from Causeway South in Copano Bay and 13 oysters were collected from Lap Reef in Copano Bay. On April 12, 2011, 28 oysters were collected from Causeway South in Copano Bay and 29 oysters were collected from Halfmoon Reef in Aransas Bay. The samples were preserved in 95% ethanol and stored in the laboratory of Dr. Susan Laramore at Harbor Branch Oceanographic Institute in Ft. Pierce, Florida, until they were made available for use in this study.

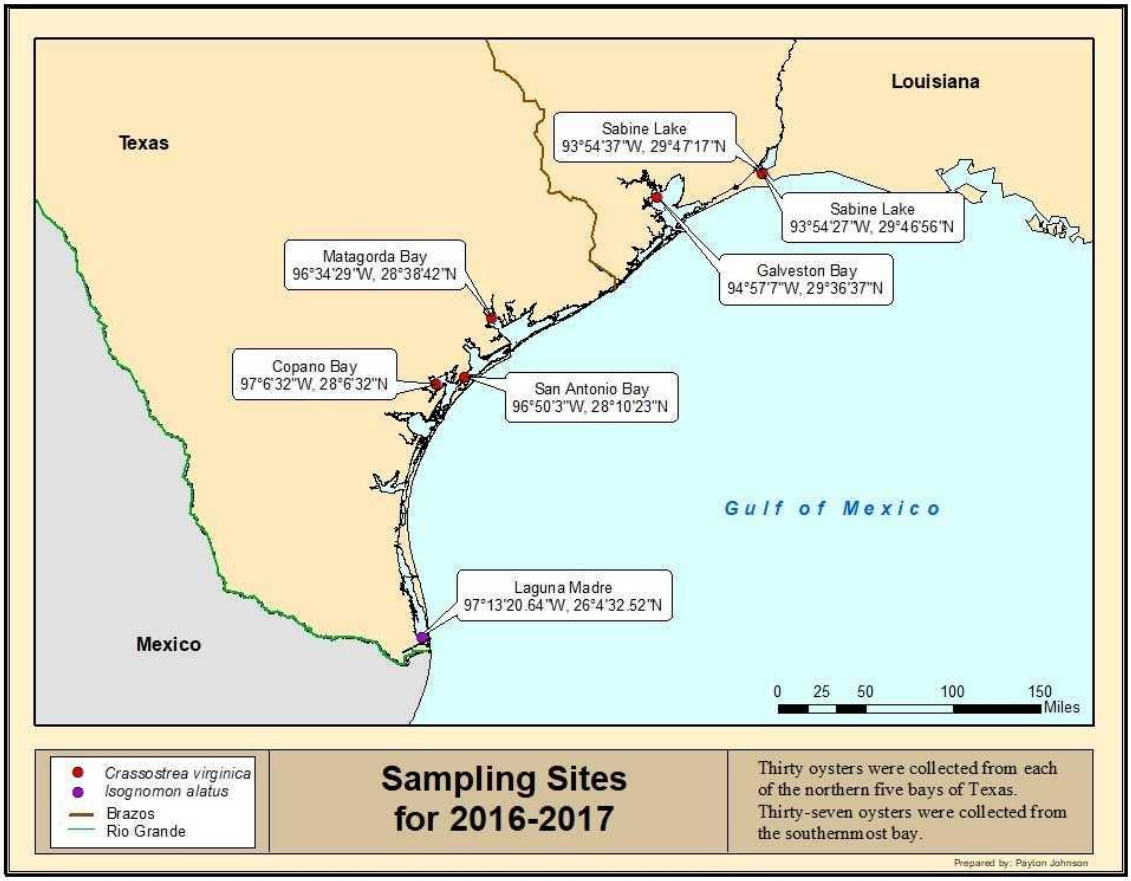


Figure 6: Sampling sites for *Crassostrea virginica* (red dots) and *Isognomon alatus* (purple dot). Thirty *C. virginica* were collected at each of the northern five bays and 37 *I. alatus* were collected from the southernmost bay (i.e., Lower Laguna Madre).

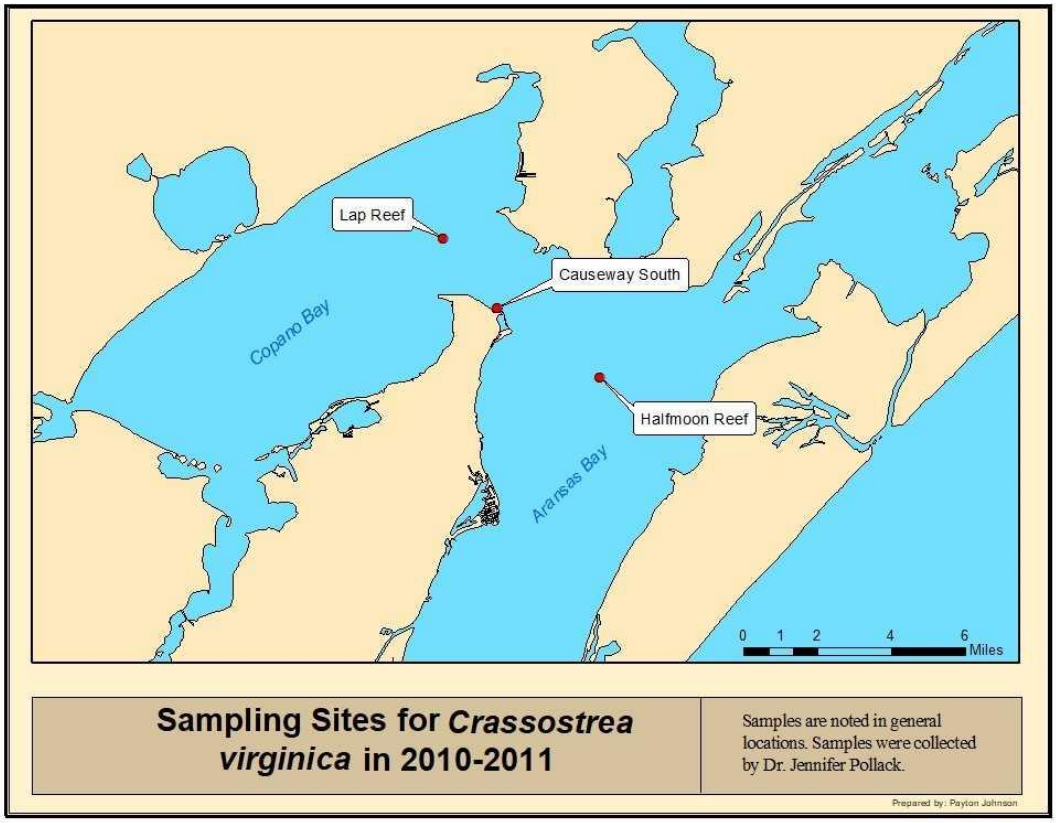


Figure 7: Sampling sites for 2010-2011 oyster tissue samples. Red dots indicate locations where *Crassostrea virginica* were collected by Dr. Jennifer Pollack in 2010 and 2011. In 2010, 19 oysters were collected from Causeway South and 13 oysters were collected from Lap Reef. In 2011, 28 oysters were collected from Causeway South and 29 oysters were collected from Halfmoon Reef.

All newly sampled species were placed in Ziplock™ bags and transported dry in a cooler with ice packs to maintain 4-10℃, to Texas A&M University - Corpus Christi. Specimens were kept in coolers for up to 5 hours depending on the distance from the sampling location to the university. Specimens were stored in a refrigerator at 4℃ for up to 48 hours before being processed for PCR and histology. All specimens were cleaned with cold water, dried with paper towels, and weighed to the nearest gram on a balance (Mettler Toledo, Columbus, Ohio: Model

30317515). Shell measurements (Fig. 8) were taken to the nearest 0.01 mm with a Vernier caliper (Control Company, Beaverton, OR: Model 14-648-17). Specimens were shucked and the meat weight (to nearest gram) was recorded. Meat content as a percentage of total weight was calculated by dividing the weight of the meat by the total weight of the bivalve and then multiplying by 100.

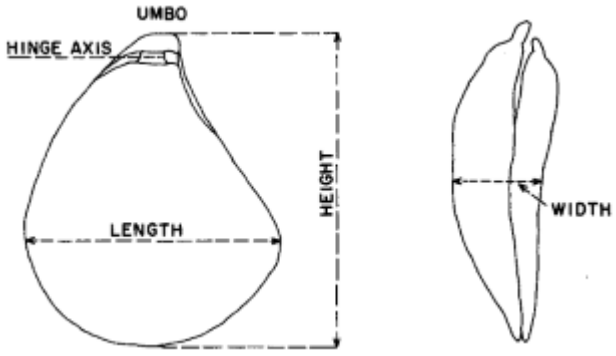


Figure 8: Diagram showing height, length, and width measurement for *Crassostrea virginica* shells (from Galtsoff 1964).

PCR Analysis and Duplex Assay Design

The presence of *Bonamia* sp., *Haplosporidium nelsoni*, and *Perkinsus marinus* in gill and mantle tissue from the sampled species was initially determined by PCR assays. *P. marinus* was included as it is a well-established oyster parasite in Texas and Gulf of Mexico and acted as a reference for the techniques used to screen for *Bonamia* sp. and *H. nelsoni*. All tissue samples were preserved in 95% molecular grade ethanol (Fisher BioReagents, Pittsburgh, PA) until extraction. Briefly, DNA was extracted from a 5-10 mm^2 piece of gill and mantle tissue cut with a disposable scalpel, using a Wizard Genomic purification kit (kit A1120, Promega Corp., Madison, WI). Extracted DNA was amplified using a general *Bonamia* primer (BoF/BoaR, Cochennec et al. 2000), an MSX primer (MSXA/MSXB, Stokes and Burrenson 1995), and a

Dermo primer (PmF/PmR, Marsh et al. 1995). For all PCR analysis, a total reaction volume of 30 µL was used: 1.0 µL of DNA extracted material, 11 µL of molecular grade water, 15 µL of 2X GoTaq Master mix solution (Promenga Corp., Madison, WI), 1 µL BSA, and 1 µL of forward and reverse primers (25 pmol). Positive and negative controls were included in each PCR run and consisted of 1.0 µL DNA extract from infected oyster tissues or molecular grade water, respectively. Thermocycler conditions for *Bonamia* sp. were as follows: 5 minutes at 94℃; 30 cycles at 94℃ for one minute, 55℃ for 1 minute, and 72℃ for 1 minute; and one final 10-minute elongation at 72℃. Thermocycler conditions for *Haplosporidium nelsoni* were as follows: 2 minutes at 94℃; 35 cycles at 94℃ for thirty seconds, 55℃ for 1 minute, and 72℃ for 1.5 minutes; and one final 5-minute elongation at 72℃. Thermocycler conditions for *Perkinsus marinus* were as follows: 3 minutes at 91℃; 35 cycles at 91℃ for one minute, 58℃ for 1 minute, and 72℃ for 1 minute; and one final 10-minute elongation at 72℃. Amplified products were kept at 4℃ and then separated on a 1.5% agarose gel along with a 50 bp ladder (Omega Bio-Tek). Gel bands 304 bp long are indicative of *Bonamia* spp. (Cochennec et al. 2000), bands 573 bp long are indicative of *H. nelsoni* (Stokes and Burrenson 1995), and 306 bp long are indicative of *P. marinus* (Marsh et al. 1995). Tissue samples that were PCR positive for *Bonamia* sp., *H. nelsoni*, or *P. marinus* were noted. Samples positive for *Bonamia* sp. or *H. nelsoni* were processed for fluorescent *in situ* hybridization (FISH) by using their corresponding tissue cross sections, histology, and Sanger sequencing to confirm or refute the presence of these parasites.

A duplex PCR assay was designed to detect the presence of *Bonamia* sp. and *P. marinus* simultaneously, targeting the small subunit of ribosomal DNA genes. The duplex PCR amplified DNA fragments near 350 and 200 bp from *Bonamia* sp. and *P. marinus*, respectively, in one

reaction. This PCR method was used to evaluate the same 150 *C. virginica* tissue samples collected in 2016 that were analyzed by single primer assays as described above. PCR was performed in 32 µL reactions consisting of 11 µL molecular grade water, 15 µL Hot Start *Taq* 2X Master Mix (New England Bio Labs), 1 µL BSA, 4µL *Bonamia* spp. and Dermo primers (1 µL BoF, 1 µL BoR, 1 µL PmF, 1 µL PmR), and 1 µL of target DNA. All primers were 25 pmol. A temperature gradient was tested for the middle annealing step in 1℃ increments from 54℃ to 62℃, with 55℃ yielding the clearest and brightest band. Hot Start *Taq* was used, which required a 2-minute cycle at 94℃. This was followed by 35 cycles of 94℃ for 30 seconds, 55℃ for 1 minute, and 72℃ for 1.5 minutes. The final elongation was completed in 5 minutes at 72℃.

Histological Analysis

Tissue from samples that were PCR positive for *Bonamia* sp. or *H. nelsoni* were processed for both histological analysis and fluorescent *in situ* hybridization (FISH; Carnegie et al. 2003). For histology, a 5-10 mm cross section of the body was cut to include the gills, mantle, and digestive tissue. This cross section was placed in a Shandon histology cassette (Thermo Fisher: Model number 1000957, Hanover Park, IL) and placed in Davidson's fixative (Poly Scientific, Pittsburgh, PA) for 48-72 hours, after which the tissue samples were rinsed once with de-ionized water and transferred to 70% ethanol for storage. Utilizing an Excelsior AS Tissue Processor (Thermo Fisher: Model A82300001, Hanover Park, IL), tissue samples were then dehydrated through ethanol baths of increasing strength (70, 80, 90, 100% for 10 minutes each) and then infiltrated with melted paraffin wax. Paraffin-infiltrated tissue samples were then set in paraffin utilizing a HistoStar Embedding Workstation (Thermo Fisher: Model A81000001, Hanover Park, IL). Paraffin-embedded tissue was cut to 4-micrometer-thick slices using a

microtome (HM355S Automatic, Thermo Fisher: Model 905200A, Hanover Park, IL). Slices were mounted on glass microscope slides, stained with hematoxylin (Shandon Harris Scientific, Hanover Park, IL) and counterstained with eosin (Shandon Y Scientific, Hanover Park, IL) utilizing a Gemini AS autostainer (Thermo Fisher: Model A81500001, Hanover Park, IL).

Fluorescent *In Situ* Hybridization (*FISH*) Analysis

If samples were PCR positive for *Bonamia* sp. or *H. nelsoni*, histological sections would be processed for FISH analysis to confirm results. Since no positives for *Bonamia* sp. or *H. nelsoni* were found, this method was not used. However, the following brief description follows briefly for the benefit of future studies. Any positive tissue samples would have been mailed to Dr. Ryan Carnegie at the Shellfish Pathology Lab at the Virginia Institute of Marine Science (Gloucester Point, VA) for FISH processing and analysis (Carnegie et al. 2003). Briefly, xylene is used to remove paraffin from prepared histological slides and the tissue rehydrated with decreasing ethanol solutions (100, 95, 80, and 70% for 10 minutes each). Prepared slides are then placed in a pre-warmed (37ºC) 50 µg/mL proteinase K solution for 10 minutes, transferred to a phosphate buffered saline (PBS) solution, then an acetic anhydride solution (5%), and finally rinsed in PBS and 5X SET (750mM NaCl, 6.4 mM EDTA, 100mM Tris base). The appropriate *Bonamia* sp. or *H. nelsoni* fluorescent probe (UME-BO-1, UME-BO-2, and UME-BO-3; UME-MSX-1) is placed on each slide with pre-hybridization buffer, which is then left in a humid chamber overnight. Slides are then washed in 0.2X SET and covered with mounting medium (Richard-Allan Scientific, Hanover Park, IL) and glass coverslips. An Olympus epi-fluorescent microscope with a universal blue-green filter is used to observe slides. *Bonamia* sp. or *H. nelsoni* when present would be visible as small green rings (Carnegie et al. 2003).

DNA Sequencing

DNA from samples that were PCR positive for *Bonamia* sp. or *H. nelsoni*, as well as from positive controls, were purified by column with a QIAquick PCR Purification Kit (Qiagen: #28104). DNA concentration was then determined using a Qubit 2.0 fluorometer (Invitrogen: Q32866). DNA solutions were diluted to specifications of Genewiz. Purified, premixed PCR products were sent to Genewiz (115 Corporate Boulevard, South Plainfield, NJ 07080) for Sanger Sequencing. *Bonamia* sp. positive samples were sequenced with BoR primer 5'-CTG-ATC-GTC-TTC-GAT-CCC-CC-3' (Cochennec et al. 2000). *H. nelsoni* positive samples were sequenced with MSXB primer 5'-ATG-TGT-TGG-TGA-CGC-TAA-CCG-3' (Stokes and Burrenson 1995). *P. marinus* positive samples were sequenced with PmF primer 5'-CAC-TTG-TAT-TGT-GAA-GCA-CCC-3' (Marsh et al. 1995). Sequences from Genewiz were then aligned via MEGA7: Molecular Evolutionary Genetic Analysis version 7.0 for bigger datasets (Kumar et al. 2016). The FASTA files for each sequence were then analyzed via BLAST: Basic Local Alignment Search Tool (NCBI 1988) to confirm or refute the positive PCR results of *Bonamia* sp., *H. nelsoni*, *P. marinus*, and all positive controls.

Statistical Analysis

Prior to collecting bivalve specimens, a power analysis was performed to determine the sample size required to detect *Bonamia* and MSX (RStudio 2009). This power analysis was a two-tailed test; alpha was assumed to be 0.05, beta was 20%, and the standard deviation was a nominal variable ($p<0.0001$). The effect size chosen was 3%, as that is the average prevalence of *Bonamia* and MSX when they have been present in other waters (Wang et al. 2012, Laramore et al. 2014).

After collection, separate ANOVAs using SAS (SAS Institute Inc 2013) were performed to test for differences in height, length, width, and total weight among bay systems. To observe the response variable correlation, a protected ANOVA, or MANOVA, using SAS (SAS Institute Inc 2013) was performed. The response variables were height, length, width, and weight of the oysters. This specimen data was collected to consider the possibility if any of these variables might influence the presence of a specific parasite or if the specimen among bays were different. Each bay was a fixed variable in order to easily compare the specimens from each bay. The aim of this analysis was to look at how factors affected variability, not comparing means, which is why bays were not considered a random variable. Now that this perspective has been analyzed in the current study, in future similar collection studies, it may be beneficial to look at bays as a random variable.

Prevalence for *Bonamia* spp., *H. nelsoni,* and *P. marinus* in *C. virginica* was calculated for each of the five bays sampled in 2016 and samples from 2010-2011, as well as for the *I. alatus* from 2017, by dividing the number of infected specimens by the total number of sampled bivalves.

RESULTS

Water Quality

The 2016 *C. virginica* samples were collected in October and November. At Copano Bay, the water temperature was 27.2 ℃, dissolved oxygen was 6.9 ppm, and salinity was 15.6. At San Antonio Bay, water temperature was 27.2 ℃, dissolved oxygen was 7.3 ppm, and salinity was 17.5. At Galveston Bay, water temperature was 22.6 ℃, dissolved oxygen was 5.9ppm, and salinity was 17.9. At Sabine Lake, water temperature was 20.7 ℃, dissolved oxygen was 7.6 ppm, and salinity was 16.7. At Matagorda Bay, water temperature was 14.6 ℃, dissolved oxygen was 9.8 ppm, and salinity was 18.6 (Fig. 9). The *I. alatus* samples were collected in December 2017. At Lower Laguna Madre, the water temperature was 9.3 ℃, dissolved oxygen was 9.1 ppm, and salinity was 33.2 (Fig. 9). As fall approached winter, temperature decreased. Because of this, temperatures were lowest during December 2016 collections of *C. virginica* and December 2017 collections of *I. alatus*. Where collections were made at cooler temperatures, waters had higher salinity (Fig. 9).

Hydrological data was not available from the 2010-2011 collection times, so data was obtained from the Texas Parks and Wildlife Department (TPWD) database (access provided by Dr. Chris Mace). Past TPWD oyster surveys were performed and hydrological data collected near the locations where the 2010-2011 samples were obtained. The 2010 samples were collected in December near Lap Reef in Copano Bay where the water temperature was 15.9 ℃, dissolved oxygen was 9.3 ppm, and salinity was 9.4. At Halfmoon Reef in Aransas Bay, water temperature was 15.3 ℃, dissolved oxygen was 8.6 ppm, and salinity was 10.9. The 2011 samples were collected in April at Causeway South in Copano Bay where water temperature was

22.3℃, dissolved oxygen was 6.5ppm, and salinity was 18.4. At Lap Reef in Copano Bay, the water temperature was 21.5 ℃, dissolved oxygen was 6.6 ppm, and salinity was 16.9.

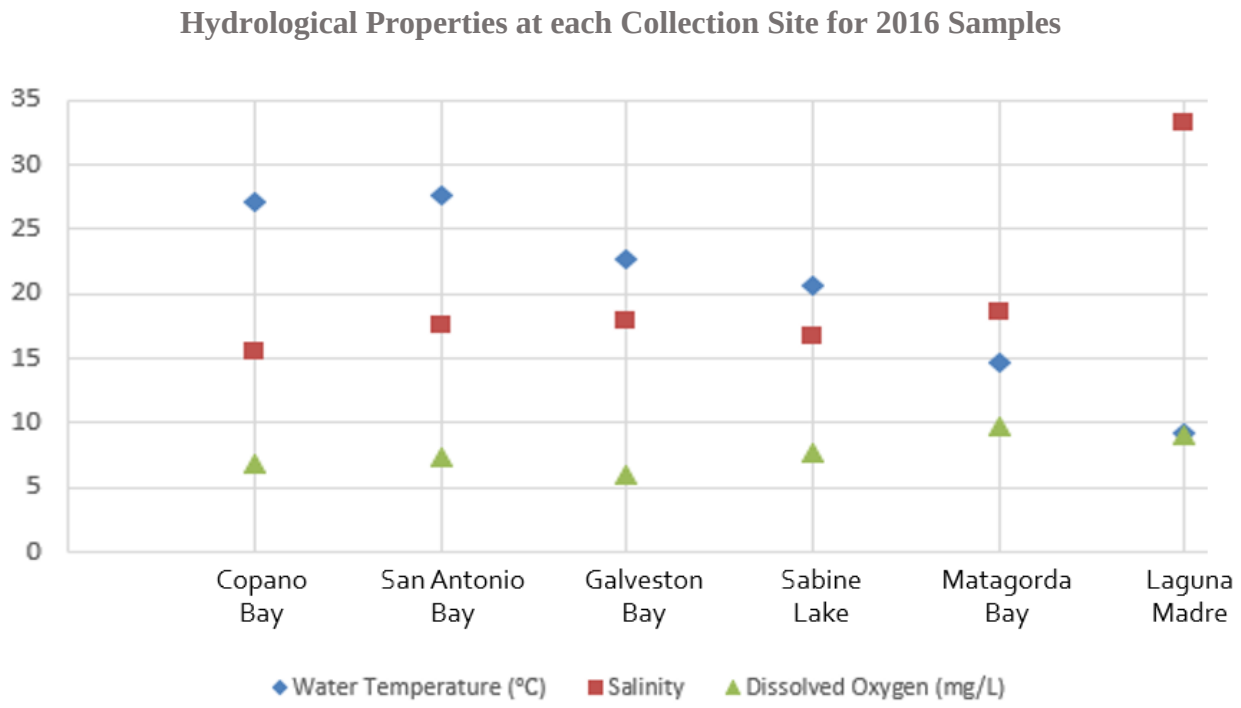
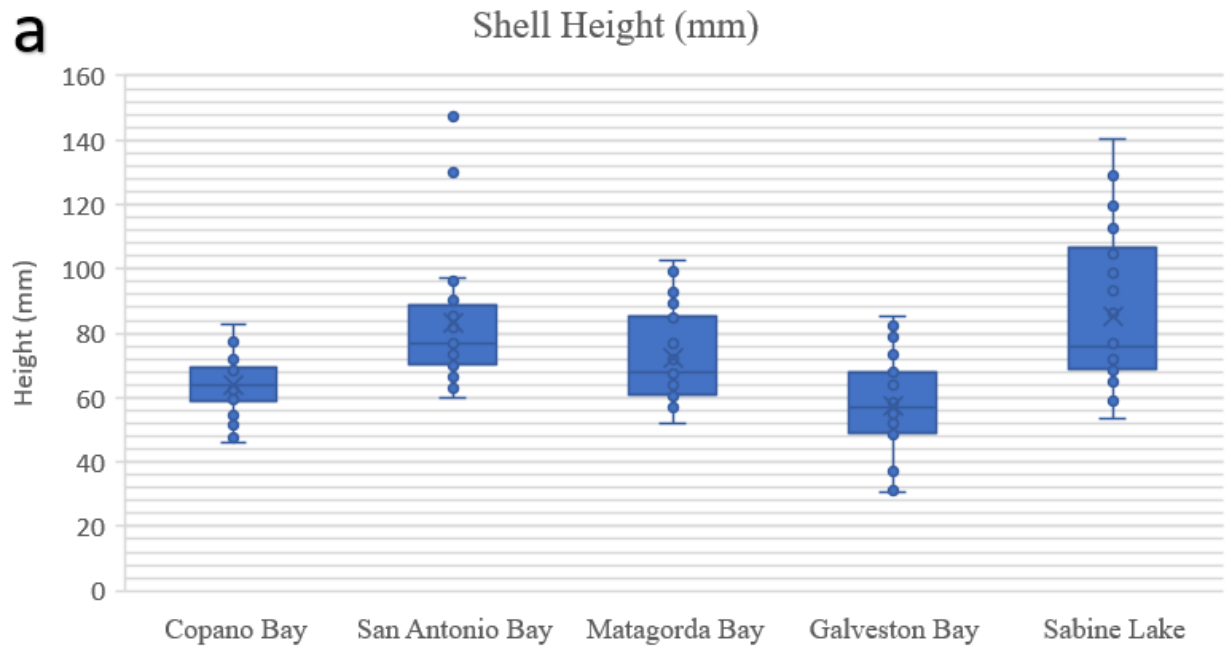


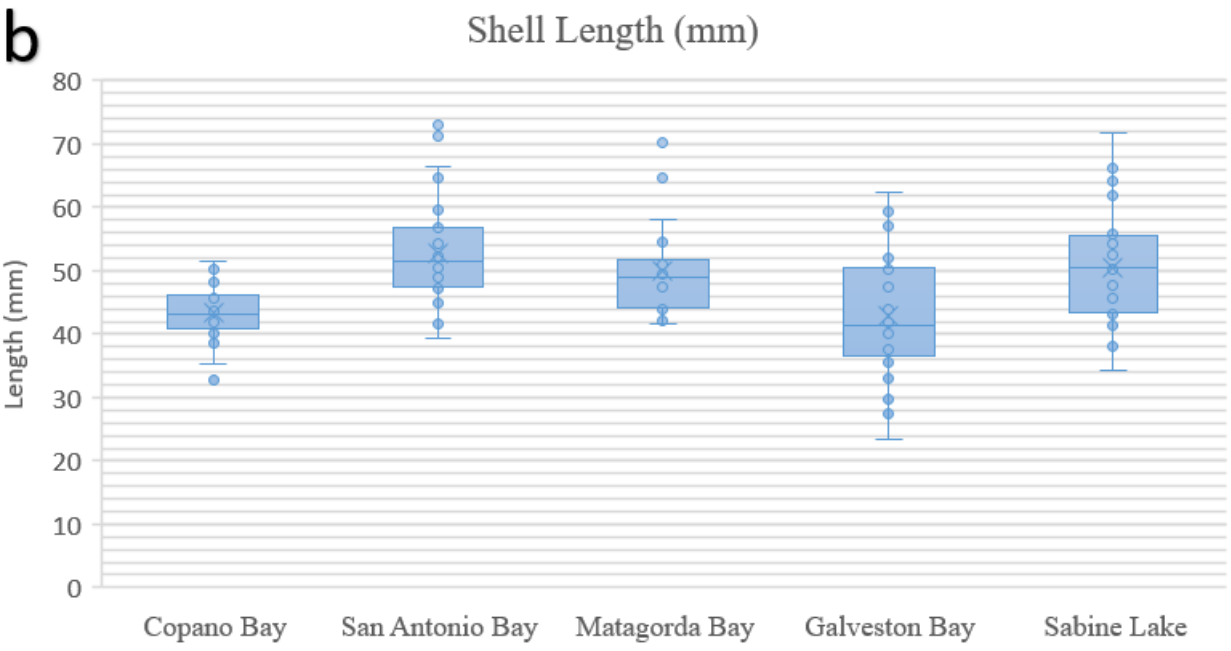
Figure 9: Water temperature, salinity, and dissolved oxygen of each of the six collection sites. X-axis is labeled for each bay listed in order of collection date.

Bivalve Morphology

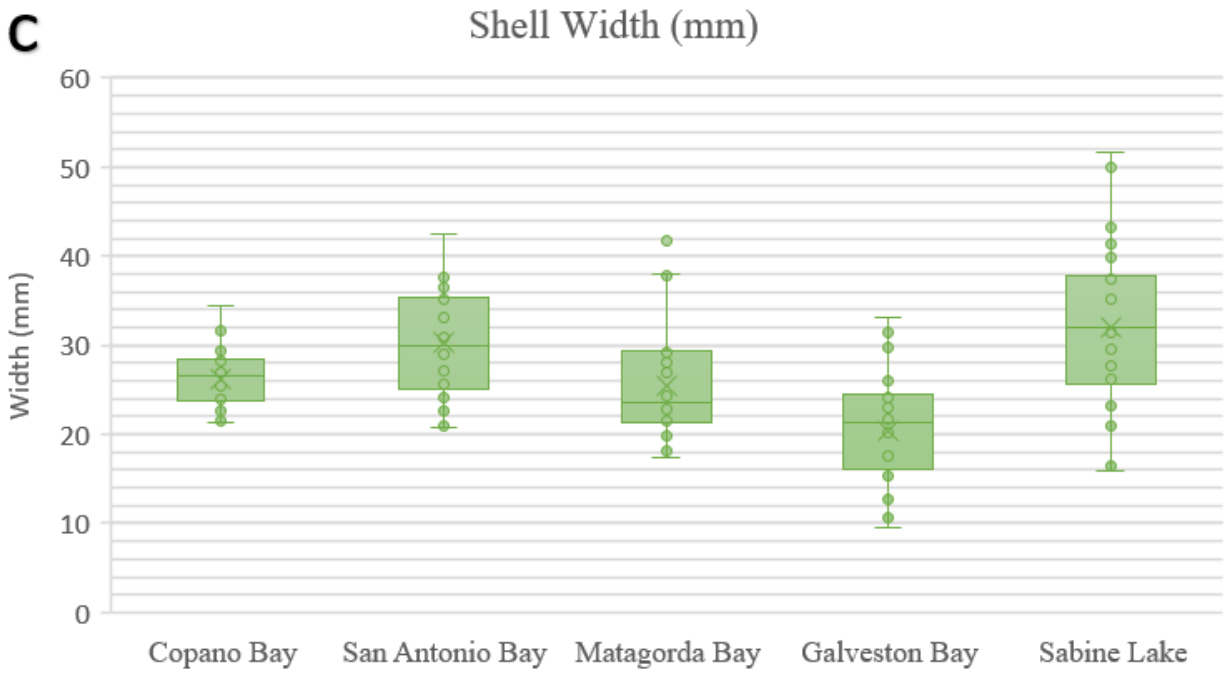
No crested oysters (*Ostrea equestris*) were found during any field sampling; therefore, results are reported for only *C. virginica* and *I. alatus* oysters. There were significant differences in mean *C. virginica* shell height ($p<0.0001$), shell length ($p<0.0001$), shell width ($p<0.0001$), and total weight ($p<0.0001$) among the five different bays sampled (Fig. 10a-d, Table 1, Appendix 1-4). Average percent meat content (0.08% to 0.12%) was not significantly different

(p=0.122) among *C. virginica* from the five different bays sampled, but Matagorda Bay oysters had a slightly higher meat percent content (Fig. 10e). Oyster morphology was not homogenous.

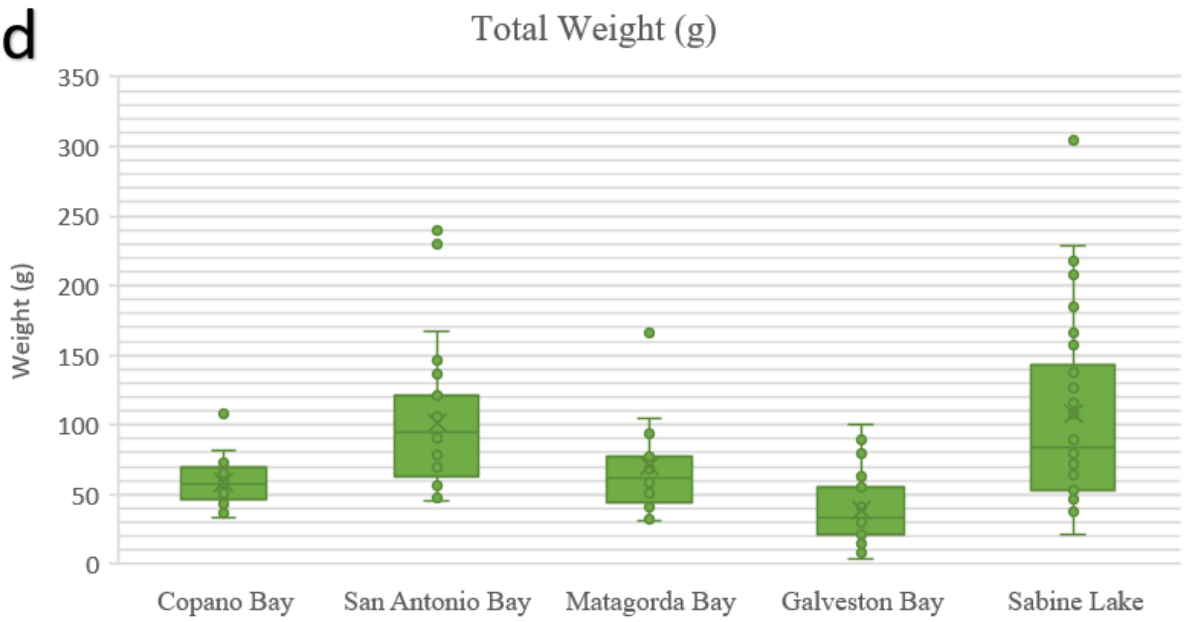




c


Shell Width (mm)
Width (mm)
60
50
40
30
20
10
0
Copano Bay
San Antonio Bay
Matagorda Bay
Galveston Bay
Sabine Lake

d


Total Weight (g)
Weight (g)
350
300
250
200
150
100
50
0
Copano Bay
San Antonio Bay
Matagorda Bay
Galveston Bay
Sabine Lake

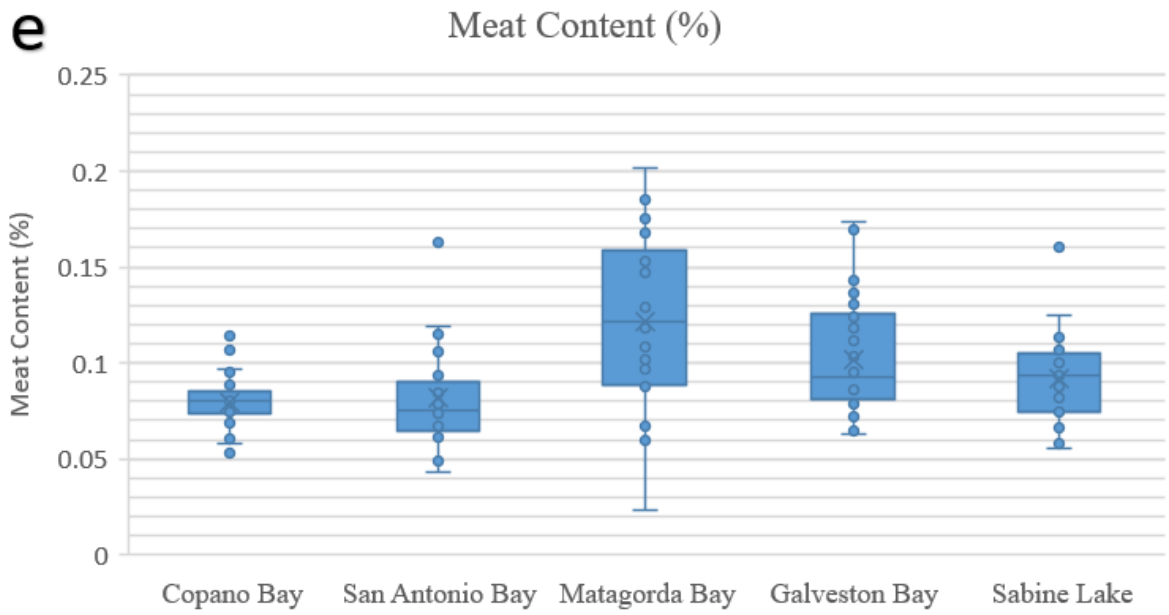


Figure 10. Box and whisker plots of shell height (a), shell length (b), shell width (c), total weight (d), and meat content (e) for *C. virginica* (n=30/bay) sampled from five Texas bays. Individual bays are listed on the x-axis, dots represent measurements of oysters collected in each bay, bars indicate the max and min points that are not outliers in each sample set, and the horizontal line in the box indicates the median.

Oysters from Sabine Lake were grouped into the largest subsection by Tukey comparisons in every category: shell height, shell length, shell width, and total weight. The next largest oysters were collected in San Antonio Bay, followed by Matagorda Bay, then Copano Bay. Oysters with the smallest sample size means were found in Galveston Bay (Table 1, Appendix 1-4).

Table 1: Mean (±s.d., n=30) shell height (mm), shell length (mm), shell width (mm), and total weight (g) of American oysters (*Crassostrea virginica*) sampled from five Texas bays (listed north to south). Means with different superscripts are significantly different ($p<0.05$).

	Shell height (mm)	Shell length (mm)	Shell width (mm)	Total weight (g)
Sabine Lake	85.5 (sd=3.21)^{A}	50.6 (sd=1.45)^{A}	32.0 (sd=1.13)^{A}	107.7 (sd=7.85)^{A}
Galveston Bay	57.6 (sd=3.21)^{C}	42.9 (sd=1.45)^{B}	20.4 (sd=1.13)^{D}	39.0 (sd=7.85)^{C}
Matagorda Bay	73.0 (sd=3.21)^{AB}	49.9 (sd=1.45)^{A}	25.4 (sd=1.13)^{C}	70.3 (sd=7.85)^{B}
San Antonio Bay	83.2 (sd=3.21)^{A}	52.9 (sd=1.45)^{A}	30.2 (sd=1.13)^{AB}	101.1 (sd=7.85)^{A}
Copano Bay	63.7 (sd=3.21)^{BC}	43.3 (sd=1.45)^{B}	26.2 (sd=1.13)^{BC}	58.7 (sd=7.85)^{BC}

Presence of Parasites by PCR

The average prevalence of *H. nelsoni* in the 2010-2011 *C. virginica* samples was 7% (i.e., 6 of 89, Table 2) and 4% (6 of 150) in the 2016 *C. virginica* samples (Table 3). *H. nelsoni* was not detected in *I. alatus* (Table 3). *Bonamia* sp. was not found in any *C. virginica* (Tables 2 & 3) but was found in 8% of the *I. alatus* sampled (i.e., 3 of 37 bivalves, Table 3). *P. marinus* was present on average in 29% (i.e., 26 of 89) of the 2010-2011 *C. virginica* samples (Table 2) and 15% (22 of 150) of the 2016 *C. virginica* samples (Table 3), but it was not detected in *Isognomon alatus* (Table 3).

Table 2: Prevalence (%) of *Bonamia* sp., *Haplosporidium nelsoni*, and *Perkinsus marinus* as determined via PCR in *Crassostrea virginica* samples from 2010-2011. In 2010, 19 oysters were collected from Causeway South, and 13 oysters were collected from Lap Reef. In 2011, 28 oysters were collected from Causeway South and 29 oysters were collected from Halfmoon Reef.

	Bonamia Prevalence	MSX Prevalence	Dermo Prevalence
Copano Bay 2010 (Causeway South)	0%	16%	42%
Copano Bay 2010 (Lap Reef)	0%	15%	8%
Copano Bay 2011 (Causeway South)	0%	4%	54%
Aransas Bay 2011 (Halfmoon Reef)	0%	0%	3%
AVERAGE 2010-2011	**0%**	**7%**	**29%**

Table 3: Prevalence (%) of *Bonamia* sp., *Haplosporidium nelsoni*, and *Perkinsus marinus* as determined via PCR in *Crassostrea virginica* (n=30/bay) and *Isognomon alatus* (n=37) from 2016 and 2017.

	Bonamia Prevalence	MSX Prevalence	Dermo Prevalence
Copano Bay (October 2016)	0%	7%	7%
San Antonio Bay (November 2016)	0%	10%	37%
Galveston Bay (November 2016)	0%	3%	27%
Sabine Lake (November 2016)	0%	0%	3%
Matagorda Bay (December 2016)	0%	3%	0%
AVERAGE *C. virginica*	**0%**	**5%**	**15%**
Port Isabel ***Isognomon alatus*** (December 2017)	8%	0%	0%

All oysters were processed for histology to examine for additional parasites and tissue degradation. Tissues from samples that were PCR positive for a parasite were processed for standard H&E histology and fluorescent *in situ* hybridization (FISH) to confirm or refute parasitic presence. Histological observation of specimen tissue stained with H&E revealed brown bodies (Figs. 11, 12, 14), hemocytic infiltration (Fig. 13), and a sporozoan parasite *Nematopsis* (Fig. 14), but no observations were made of *Bonamia* sp. or *H. nelsoni*. Without the ability to visually confirm *Bonamia* sp. or *H. nelsoni* via histology, the presence of these parasites could not be confirmed, therefore PCR product samples were sent to Genewiz for full Sanger sequencing to determine if the parasite DNA was present.

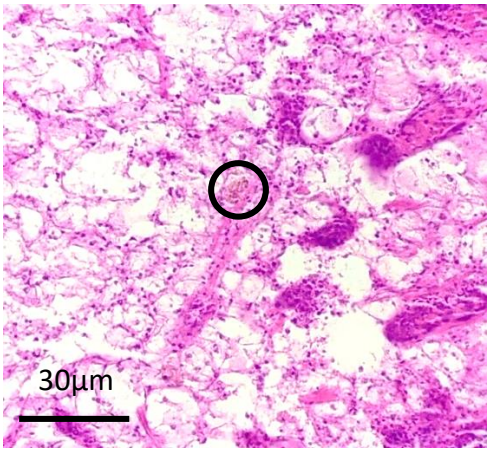


Figure 11: Histological section of connective tissue stained with H&E of an American oyster *Crassostrea virginica* from Copano Bay (sample C12) exhibiting brown bodies (circle). Objective power is 40x.

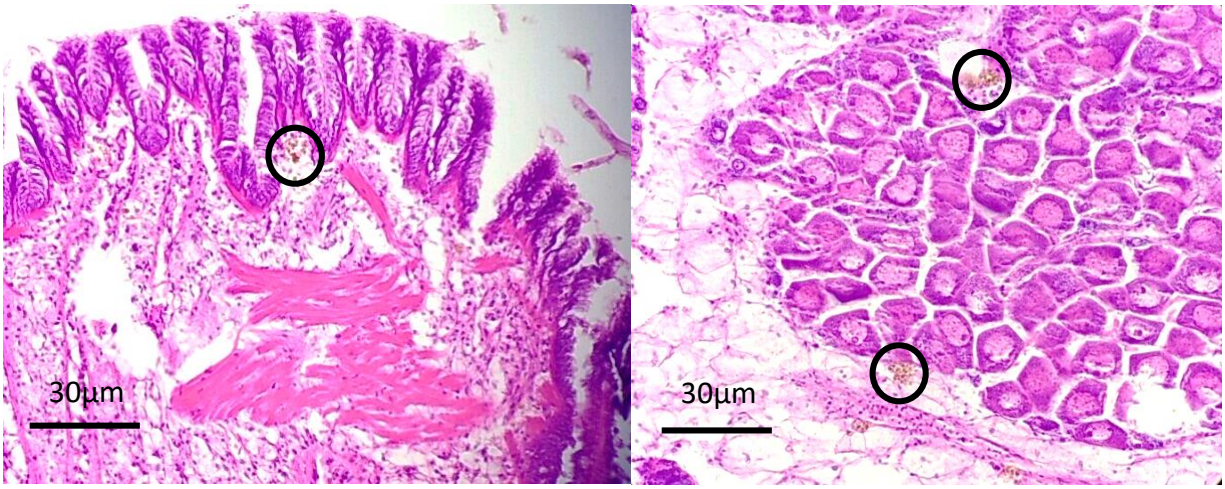


Figure 12: Histological section of mantle tissue (left) and connective tissue near ovaries (right) stained with H&E of an American oyster *Crassostrea virginica* from Copano Bay (sample C24) exhibiting brown bodies (circle). Objective power is 40x.

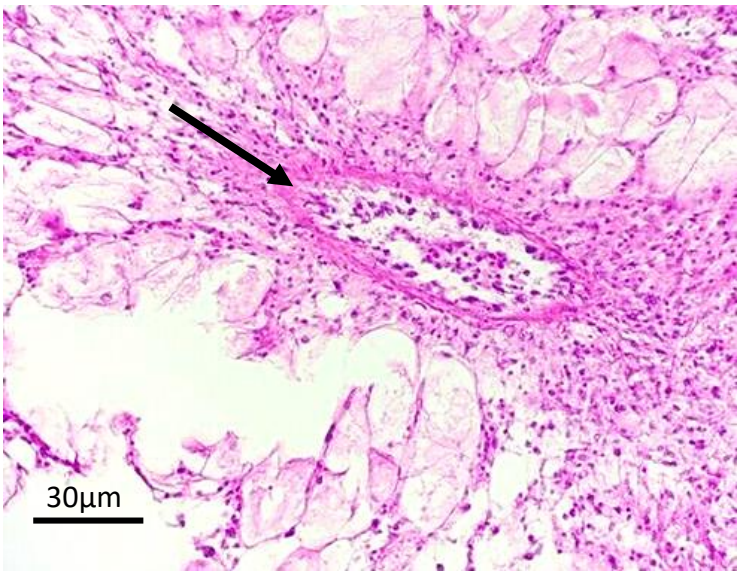


Figure 13: Histological section of connective tissue stained with H&E of an American oyster *Crassostrea virginica* from Matagorda Bay (sample T3) exhibiting hemocytic infiltration (arrow). Objective power is 40x.

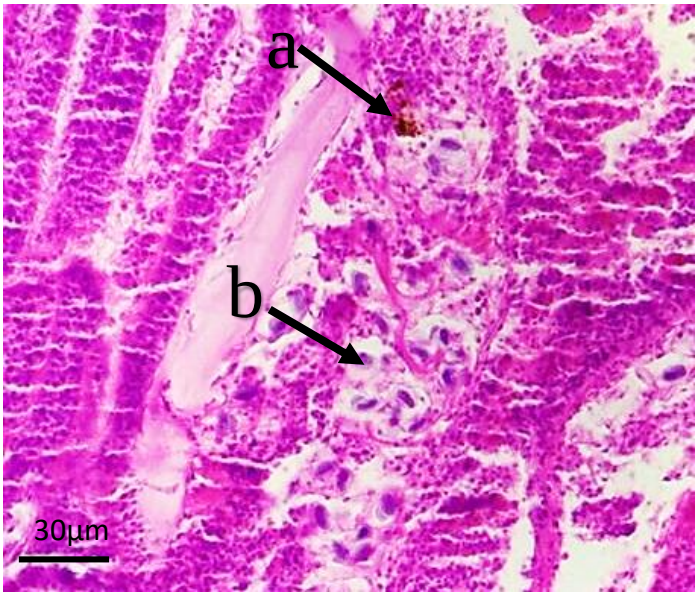


Figure 14: Histological section of connective tissue stained with H&E of an American oyster *Crassostrea virginica* from San Antonio Bay (sample M4) exhibiting brown bodies (a) and the parasite *Nematopsis* (b). This sample was confirmed PCR positive for Dermo. Objective power is 40x.

The DNA sequence of *Bonamia* sp. is 304 base pairs (Cochennec et al. 2000). When oyster samples that tested positive for *Bonamia* sp. were sequenced, the DNA found was 500 base pairs in length. This was larger than the expected sequence of *Bonamia* sp.. BLAST results were also inconsistent and did not reveal any organism that was genetically similar to the sequences from PCR products. Therefore, it was determined that PCR analysis resulted in a false-positive for *Bonamia* sp.. DNA sequencing of putative MSX infected tissue showed high background noise that resulted in the sequence not being able to be read. These sequence findings indicated that the PCR analysis resulted in false positives for both *Bonamia* sp. and *H. nelsoni*. It was believed that these false positive results could have been due to contaminated or incorrect primers. Therefore, new *Bonamia* sp. and *H. nelsoni* primers were ordered, and PCR

testing was repeated for the 239 *C. virginica* and 37 *I. alatus* samples. The re-analyzed samples showed presence of *P. marinus*, but no *Bonamia* sp. or *H. nelsoni*, in any past or recent samples of *Crassostrea virginica* and no presence of any parasite in *Isognomon alatus* (Tables 4 & 5).

Table 4: Prevalence of *Bonamia* sp., *Haplosporidium nelsoni*, and *Perkinsus marinus* as determined by PCR with new primers in *Crassostrea virginica* samples from 2010-2011. In 2010, 19 oysters were collected from Causeway South, and 13 oysters were collected from Lap Reef. In 2011, 28 oysters were collected from Causeway South and 29 oysters were collected from Halfmoon Reef.

	Bonamia Prevalence	MSX Prevalence	Dermo Prevalence
Copano Bay 2010 (Causeway South)	0%	0%	42%
Copano Bay 2010 (Lap Reef)	0%	0%	8%
Copano Bay 2011 (Causeway South)	0%	0%	54%
Aransas Bay 2011 (Halfmoon Reef)	0%	0%	3%
AVERAGE 2010-2011	**0%**	**0%**	**29%**

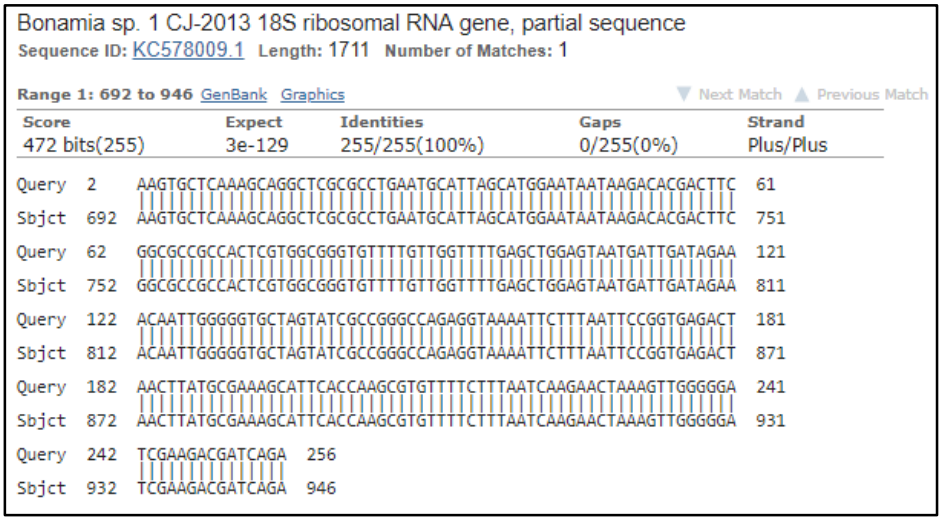
Table 5: Prevalence of *Bonamia* sp., *Haplosporidium nelsoni, and Perkinsus marinus* as determined by PCR with new primers in 2016 *Crassostrea virginica* (n=30/bay) and 2017 *Isognomon alatus* samples (n=37).

	***Bonamia* Prevalence**	**MSX Prevalence**	**Dermo Prevalence**
Copano Bay (October 2016)	0%	0%	7%
San Antonio Bay (November 2016)	0%	0%	37%
Galveston Bay (November 2016)	0%	0%	27%
Sabine Lake (November 2016)	0%	0%	3%
Matagorda Bay (December 2016)	0%	0%	0%
AVERAGE *C. virginica*	**0%**	**0%**	**15%**
Port Isabel ***Isognomon alatus*** (December 2017)	0%	0%	0%

PCR results are widely accepted to determine the presence of Dermo without the need to confirm with other methods such as histology, FISH, or genetic sequencing (Laramore et al. 2017). After finding false-positive results for *Bonamia* sp. and *H. nelsoni*, it was determined that tissue samples should also be re-analyzed by PCR for *P. marinus* and then confirmed by sequencing. Re-analysis of tissue samples by PCR for *P. marinus* yielded the same results as that found in the first analysis: average of 29% prevalence (n=25 of 89) in the 2010-2011 *C. virginica* samples (Tables 2 and 4) and 15% (n=24 of 150) in the 2016 samples (Tables 3 and 5) with no *P. marinus* found in *I. alatus* (Table 5). In 2010-2011 samples, *P. marinus* was found in eight of nineteen *Crassostrea virginica* from Causeway South in Copano Bay in 2010, one of thirteen samples from Lap Reef in Copano Bay in 2010, fifteen of twenty-eight samples from Causeway

South in Copano Bay in 2011, and one of twenty-nine from Halfmoon Reef in Aransas Bay in 2011. In the 2016 samples, *P. marinus* was found in two *Crassostrea virginica* from Copano Bay, eight from Galveston Bay, eleven from San Antonio Bay, and one from Sabine Lake. *P. marinus* was not observed in *C. virginica* from Matagorda Bay nor in *I. alatus* of Lower Laguna Madre.

New PCR products from re-analyzed tissue samples that indicated positive for *Bonamia* sp., *H. nelsoni*, or *P. marinus* were sent to Genewiz to be sequenced. Where there were no parasite-positive PCR samples, such as the case with *Bonamia* sp. and *H. nelsoni*, the positive controls were sequenced. *Bonamia* sp. and *H. nelsoni* positive controls showed 100% similarity in nucleotide sequences with sequences of 255 and 513 base pairs in length, respectively (Figs. 15 & 16). *P. marinus* samples showed 100% similarity in nucleotide sequences with sequences of 254 base pairs long (Fig. 17).


Bonamia sp. 1 CJ-2013 18S ribosomal RNA gene, partial sequence
Sequence ID: KC578009.1 Length: 1711 Number of Matches: 1

Range 1: 692 to 946 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
472 bits(255)	3e-129	255/255(100%)	0/255(0%)	Plus/Plus

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Query  62   GGCGCCGCCACTCGTGGCGGGTGTTTTGTTGGTTTTGAGCTGGAGTAATGATTGATAGAA  121
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Sbjct  752  GGCGCCGCCACTCGTGGCGGGTGTTTTGTTGGTTTTGAGCTGGAGTAATGATTGATAGAA  811

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Query  182  AACTTATGCGAAAGCATTCACCAAGCGTGTTTTCTTTAATCAAGAACTAAAGTTGGGGGA  241
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Query  242  TCGAAGACGATCAGA  256
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Sbjct  932  TCGAAGACGATCAGA  946
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Figure 15: BLAST results for *Bonamia* positive control shows 100% nucleotide match

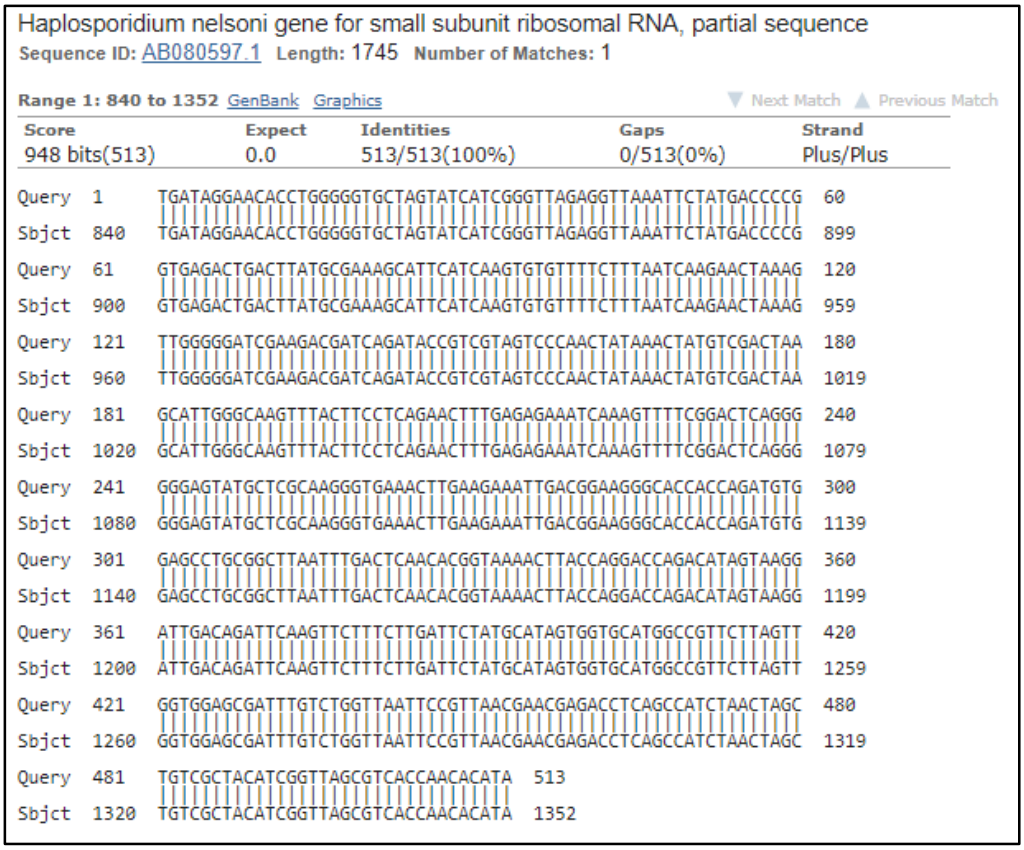

Figure 16: BLAST results for *Haplosporidium nelsoni* positive control shows 100% nucleotide match.

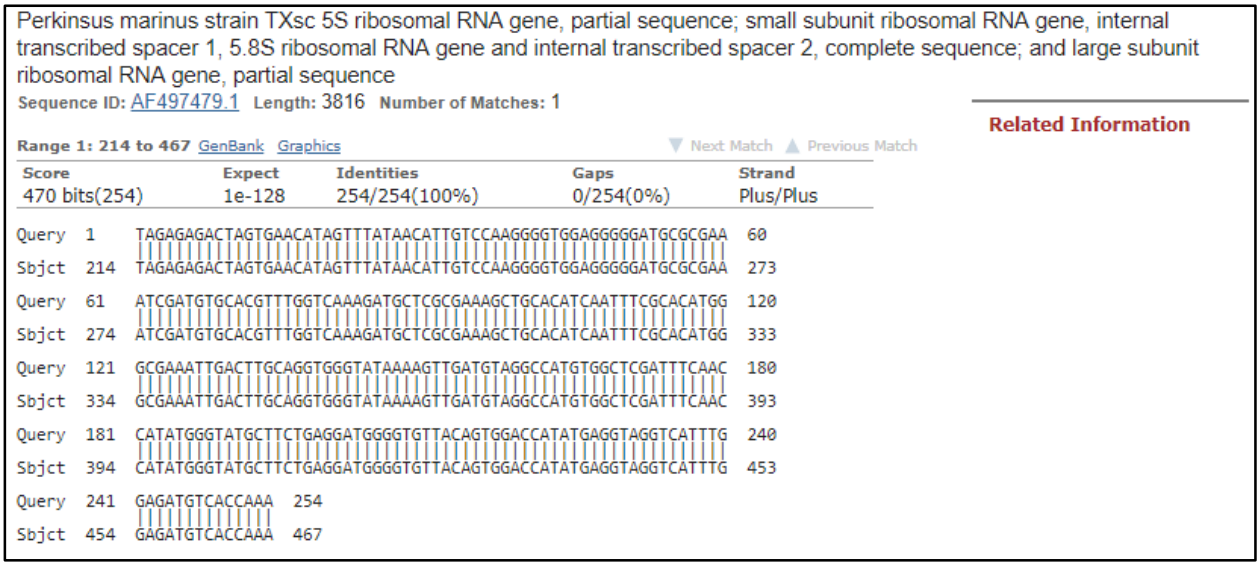

Figure 17: BLAST results for *Perkinsus marinus* positive sample shows 100% nucleotide match.

Duplex PCR Assay

A duplex PCR assay was designed for the detection of *Bonamia* spp. and *P. marinus* simultaneously. This technique provided a rapid and reliable assessment of the presence of *Bonamia* spp., *P. marinus*, or both in each specimen analyzed (Fig. 18). The 2016 *C. virginica* were all tested for *Bonamia* spp. and *P. marinus* individually and resulted in no positive *Bonamia* spp. except for the positive control and 15% *P. marinus* prevalence. Results from the duplex assay were the same as individual samples.

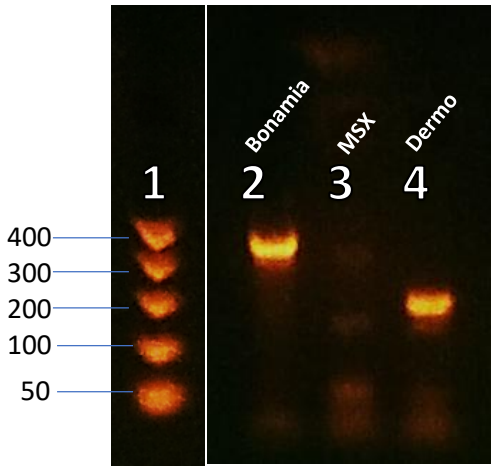

Figure 18: Duplex designed to detect *Bonamia* spp. and *Perkinsus marinus* was tested with the positive control of three parasites: *Bonamia* sp. (Lane 2), MSX (Lane 3), and *P. marinus* (Lane 4). Lane 1 shows the Omega Bio-Tek 50-bp DNA ladder for comparison: *Bonamia* sp. has a band present at approximately 350bp and *P. marinus* at approximately 200bp. These PCR positive results were not sequenced.

DISCUSSION

This was the first, to my knowledge, survey of *C. virginica* in Texas bays for *Bonamia* spp. The parasites *Bonamia* spp. and *Haplosporidium nelsoni* were not found, which suggests that these parasites were not likely present in Texas bays in the fall season of 2016.

Currently, in Texas waters the only prevalent parasite in *C. virginica* is *P. marinus*, which has been observed and monitored in the Gulf of Mexico since 1949 (e.g. Powell et al. 1992, Reece et al. 2001, Aguirre-Macedo et al. 2007); therefore, it was expected that *P. marinus* would be observed in the current study. *P. marinus* was found in *C. virginica* in four of the five bays sampled but was not found in Matagorda Bay, nor was it found in *I. alatus* in Laguna Madre. These two bays are also the locations with the highest salinities of all the collection sites. This was unexpected, as *P. marinus*is is more prevalent in higher salinities (Aguiree-Macedo et al. 2007). These collections, though, were made in December when water temperatures were low (9.3℃ and 14.6℃) and colder water is known to decrease the rate that *P. marinus* replicates (Powell et al. 1992, La Peyre et al. 2008, Vazquez et al. 2018), which may explain the absence of *P. marinus* in these two bays. With warming oceans, the range of *P. marinus* is expanding northward; a study performed in the northeast region of the United States from New Jersey to Maine found 82% of the samples were infected with *P. marinus* in areas where they had not been previously reported (Reece et al. 2001). This range expansion indicates the need for continued monitoring programs for *P. marinus* and periodic monitoring for other parasites such as *Bonamia* spp. and *H. nelsoni*. These parasites have likely increased prevalence in warmer waters, as current absence does not preclude future absence of these parasites in Texas waters. It is also notable that *P. marinus* was found at a fairly low prevalence in both historic (29%) and current (15%) tissue samples. This could be due to spring rains that caused a significant decrease in oyster population in 2015 (Fig. 1). In San Antonio Bay, the average number of market sized

oysters was higher when salinity was closer to 30 in 2011- 2014 than at lower salinity seen in 2015-2016 (Fig. 19). It could be argued that this is due to over-harvesting; however, the same pattern is also observed in Sabine Lake where harvest is closed due to metal and fecal contamination (Fig. 20). This decrease in market sized oysters is likely due to salinity stress; it is possible that this salinity stress is the reason parasite abundance, especially that of *P. marinus*, was lower than expected.

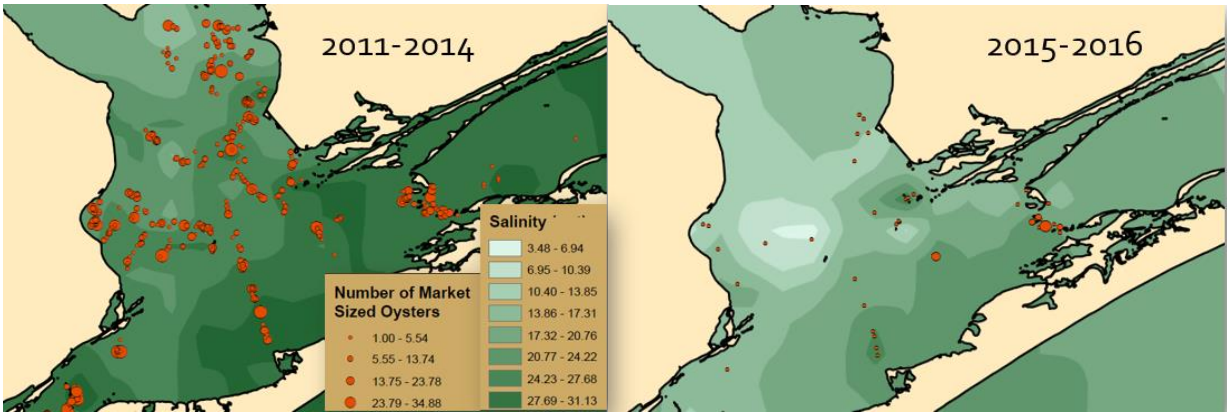

Figure 19: Comparison of average number of market sized oysters in San Antonio Bay from 2011-2014 to those in 2015-2016 in relation to salinity of the bay. Correlation is seen between lower salinity and fewer market sized oysters. Data from TPWD; maps made by Payton Johnson.

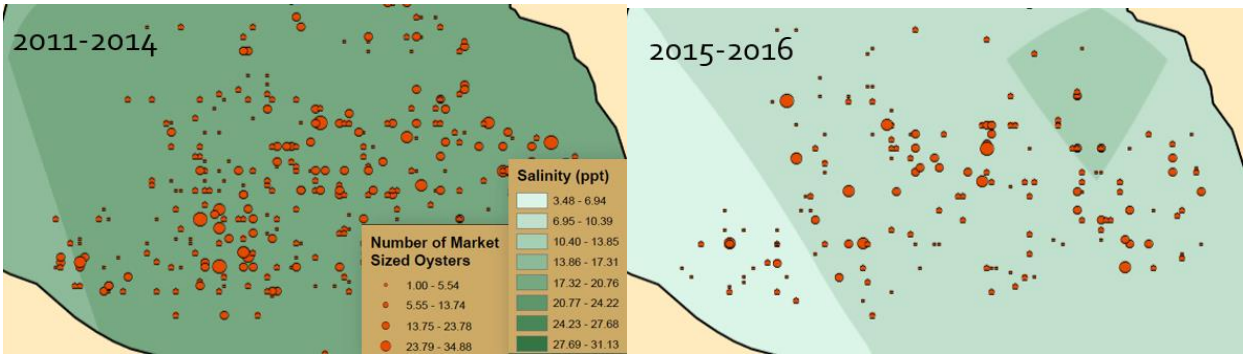

Figure 20: Comparison of average number of market sized oysters in Sabine Lake Bay from 2011-2014 to those in 2015-2016 in relation to salinity of the bay. Correlation is seen between lower salinity and fewer market sized oysters. Data from TPWD; maps made by Payton Johnson.

The primary goal of the current study was to survey *C. virginica* for *Bonamia* spp., which has been found on both the Pacific and Atlantic US coasts (Friedman et al. 1989, Friedman and Perkins 1994, Carnegie et al. 2003, Dungan et al. 2012, Proestoue et al. 2016, Robledo et al. 2018). Thus, it was hypothesized that *Bonamia spp.* could be present in Texas bivalves. *Bonamia*

spp. might not currently be present even in the Gulf of Mexico, and it is possible that it has not spread due to seasonal differences in water temperature, salinity, and dissolved oxygen. However, global climate change and increasing temperatures has made poleward regions more suitable for parasites (Ford 1996). In Maine, extreme winters keep *Bonamia* spp. populations low (Friedman and Perkins 1994); however, as oceans continue warming, there are fewer cold fronts. The Gulf of Mexico has the fastest rate of temperature change (+ 0.03℃ per year) on the planet and this has been occurring for over twenty-five years (Pershing Alexander et al. 2015). These higher temperatures and reduced frequency of cold fronts may lead to an increase in parasitic prevalence due to alleviation from cold-stress (Friedman and Perkins 1994). *Bonamia* spp. thrives at higher temperatures and salinity (Ford and Smolowitz, 2007; Chu and La Peyre, 1993; Chu et al., 1993), and sites with both high temperature (Copano Bay, 27.2℃; San Antonio Bay, 27.2℃) and high salinity (Lower Laguna Madre, 33.2) were sampled, though not on the same date. However, no *Bonamia* spp. was found; thus, the parasite does not appear to be present in Texas bay systems. It may be absent because the crested oyster *Ostrea equestris,* the primary host for *Bonamia* spp. (Pichot et al. 1979), was not present at the Texas bays collection sites or is present in very low densities. The crested oyster was noted to be at Seven and a Half Fathom Reef, Port Mansfield, Texas (Tunnell 2010), but when this area was examined by Dr. Kim Withers in 2017-2018 (26.873374 N, -97.344171 E) no crested oysters were found (K. Withers, personal communication, August 21, 2017). It is possible that this species' population has declined or is no longer present at Seven and a Half Fathom Reef.

There was a possible observation of *O. equestris* in 2010-2011 in Corpus Christi Bay at Mud Island (27.951610 N, -97.003130 E, J. Pollack, personal communication, August 28, 2017); however, upon Dr. Jennifer Pollack's examination in November and December 2017 this species

was not found. This species was also not found at Fish Pass (27.686050 N, -97.177315 E) or the John F. Kennedy Memorial Causeway (27.631452 N, -97.229832 E) on Padre Island. The only bivalves seen at these locations were *Crassostrea virginica*.

Ostrea equestris was seen by C. Downey in 2006 in Port Isabel (C. Downey, personal communication, October 24, 2017). The shoreline (26.0757, -97.2224) had concrete structures that I examined in December 2017. Intertidal locations examined were covered in *C. virginica* with a few individual *I. alatus* observed within clumps of *C. virginica*. Downey (C. Downey, personal communication, October 24, 2017) noted that bulkheads were the best location to find *O. equestris*, but when searched for the current study in October, November, and December 2017, only dead shell of *C. virginica* was observed. The Arturo Galvan Coastal Park (26.0746 N, -97.2210 E) had only *C. virginica* and the East Shore had only sandy shores; no oysters were found. Laguna Vista (26.107968 N, -97.296633 E) and Port Mansfield (26.564092 N, -97.427396 E) were also searched in October and November of 2017, but no *O. equestris* were found; only *C. virginica* was present. It can be speculated that *O. equestris* was once present in Port Isabel but recent changes in the environment have favored the population of *C. virginica* over *O. equestris*. Regardless, *O. equestris* could not be observed in fall 2017 at the same locations it was observed in fall 2010.

Ostrea equestris could not be used in the current project simply because it could not be located. However, *Isognomon alatus*, which is also found in Texas, has also been reported to be positive for *Bonamia* spp. in Florida (Laramore et al. 2014) and is an equally viable host. Though this species was also challenging to locate, 37 *I. alatus* were collected at Lower Laguna Madre for this study in early December 2017. No *Bonamia* spp., *H. nelsoni*, or *P. marinus*, were found in these organisms. The absence of these parasites in *I. alatus* specifically, may be due to the

time of the year (winter) when the specimens were collected. The total sample size (n = 276) should have been large enough to detect *Bonamia* spp. if it was present in Texas bays at frequency of 0.3% or higher. A survey that would have included the primary host of this parasite, *O. equestris*, would strengthen the determination that *Bonamia* spp. is not currently present in Texas bays.

The third parasite of interest in the current study was *H. nelsoni*. It has been suggested that the Gulf of Mexico has favorable conditions for *H. nelsoni*; however, the prevalence of *H. nelsoni* may be too low to cause mass mortality of infected oysters (Ulrich 2007, Ford et al. 2011). *H. nelsoni* has been found in *C. virginica* in North Carolina and Rhode Island (Wilbur et al. 2012) and its highest prevalence was found in October and November in Delaware Bay (Wang et al. 2012). Salinities over 18 and temperatures over 24 ℃ typically increase infections by *H. nelsoni*, the causative agent of MSX (Wang et al. 2012). In the present study, collections of *C. virginica* were done during the months of October and November, which previously has been the time of the year to have high *H. nelsoni* incidents (Wang et al. 2012). The ranges of temperature for the northern bays sampled was 14.6-27.2℃, and the range of salinity was 15.6-18.6, but no *H. nelsoni* was found. *I. alatus* samples were collected from the southernmost bay in the first week of December, when temperature was 9.3℃ and salinity was 33.2. The cooler water may partially explain why there may not have been any parasites of interest in Lower Laguna Madre during the current study, but the high salinity would be optimal for *H. nelsoni*.

In the past, *H. nelsoni* (MSX) was detected in the Gulf of Mexico via PCR (Ulrich et al. 2007), but Burreson (2008) discussed the misuse of PCR for diagnosis of infections, pointing out that all samples PCR positive for a disease should be validated by histology. A later study found no evidence for the parasite and suggested that the Gulf of Mexico should be "considered free of

MSX" (Ford et al. 2011). Ulrich et al. (2007) did not confirm results histologically, but the study did attempt confirming the presence of MSX through DNA sequencing. Sequence lengths were 288 to 543 base pairs instead of the expected 573 base pairs, which could still confirm results with a high enough sequence similarity. However, Ford et al. (2011) found only 94.1% sequence similarity to a sister phylum of Haplosporidia (Cercozoa). This may have been what Ulrich et al. (2007) detected as they reported a 94% similarity. The DNA sequences reported by Ford et al. (2011) and Ulrich et al. (2007) were perhaps related to *H. nelsoni*, but they were not actually *H. nelsoni*. Similarity of alignments should be 99-100% similarity to confirm these specific species (NCBI).

In the initial analysis for the current study, PCR indicated the presence of *H. nelsoni* in one *C. virginica* sample from 2010 and five *C. virginica* samples from 2011, this was during the same time period that Ford's study indicated the Gulf of Mexico was considered "free of MSX." Initial PCR results also indicated the presence of *H. nelsoni* in seven *C. virginica* samples from 2016. Though PCR results from 2016 were consistent with what was indicated in 2010 and 2011 samples, it was imperative to confirm these results multiple ways to ensure accuracy in the current study. *Bonamia* sp. was also indicated in initial PCR results in the *I. alatus* samples from 2017. This could have meant that *Bonamia* spp. was very recently introduced into Texas bays, or that *Bonamia* spp. was found only because its primary host, *I. alatus*, was sampled.

When the samples were further assessed by histology and Sanger sequencing, there was no confirmation of either disease agent being present. Sequencing returned a 500 base-pair long fragment for *Bonamia* sp. BLAST results, when it was expected to be about 304 base pairs long. The DNA sequences of *H. nelsoni* showed high background noise that resulted in the sequence not being able to be read. The results indicated that PCR results were inaccurate for *Bonamia*

spp. and *H. nelsoni*. It is possible that the wrong primers were used, the primers had degraded over time, there was a contamination in the primers, or the primers no longer worked properly due to continual thawing and re-freezing. Regardless, the primers were considered unusable and all PCR results determined while using them were no longer credible. This highlights the importance of verifying independently (e.g., histologically, sequencing) all PCR positive results to confirm findings as suggested by Burreson (2008). Secondary analysis is essential to pair with PCR analysis, not only to confirm or refute results, but to provide quality control in methods. PCR may be a quick and efficient technique, but it is not infallible.

Histology did not indicate the presence of *Bonamia* spp. or *H. nelsoni*. Histology is generally not performed on *P. marinus* because the parasite is very difficult to see and easily missed in examination (Smolowitz 2013); however, histology was still performed in the current study as it can give additional valuable information on bivalve health to complement PCR results. Several instances of brown bodies were observed as well as hemocytic infiltration, both of which reflect poor bivalve health (Cáceres-Martínez et al. 2008). Most of the oysters that were seen with these brown bodies and hemocytic infiltration were not positive for any of the three parasites in the current study, but several other bacteria, parasites, or irritations in the oyster can result in these brown bodies (Cáceres-Martínez et al. 2008). Histological examination of one oyster that was PCR positive for *P. marinus* revealed brown bodies. While *P. marinus* is not easily viewed histologically, the presence of brown bodies indicates poor oyster health (Sühnel et al. 2016) and exhibits a strong host defensive response (Vazquez et al, 2018), which is consistent with the PCR positive finding of *P. marinus* (Smolowitz 2013). The *Nematopsis* parasite was also found in the oyster specimen, further explaining the presence of brown bodies (Sühnel et al.

2016). Because no *Bonamia* spp. or *H. nelsoni* was present in the oyster tissues, fluorescent *in situ* hybridization (i.e., FISH) was not performed on histological preparations.

Management of oyster populations has become challenging with multiple parasites expanding their ranges and becoming more prevalent (Cook et al. 1998, Reece et al. 2001, Williams and Boyko 2016, Robledo et al. 2018). This has increased the interest in developing techniques to identify parasites that are potential threats (Wilbur et al. 2012). In the current study, a duplex assay was designed to identify the presence of both *Bonamia* spp. and *P. marinus* simultaneously. This duplex PCR reaction allows diagnosis for two parasites to be completed faster and more efficiently. This technique would be beneficial if utilized in locations that are concerned about the presence of *Bonamia* spp. and *P. marinus* in their waters. The duplex assay will make it more time efficient and economically feasible by using only half the time and resources that traditional PCR analysis requires.

A power analysis was performed to determine the sample size required to detect the parasites of interest before the current study was initiated; a power of 80% resulted in a required sample size of 124 bivalves. There were two sample collections: samples from 2010-2011 that consisted of 89 *C. virginica* and samples from 2016-2017 that consisted of 150 *C. virginica* and 37 *I. alatus*. The effect size chosen for the analysis was 3%, as this was the previous observed prevalence for *Bonamia* spp. and *H. nelsoni* (Carnegie et al. 2003, Wilbur et al. 2012, Laramore et al. 2014). If the abundance of *Bonamia* spp. and *H. nelsoni* were half this value (i.e., 1.5%), then the sample size required would be 252, which is still lower than the combined sample size of both bivalve species examined in the current study (n=276). It is only if the parasite prevalence is one-third (effect size = 1%) that the sample size would have been too small as the required sample size would be 280; just four bivalves more than the 276 total in the current

study. A larger sample size would increase the possibility of finding the parasites; however, the total of 276 bivalve samples was more than double of what was required by the power analysis, and therefore considered a sufficient sample for the current study.

Collections of bivalves were also made when parasitic prevalence was historically highest. Samples were collected from late October through early December as fall is the most likely season in which these parasites would be present based on previous studies concerning seasonal prevalence (Wang et al. 2012, Smolowitz 2013). Prevalence of parasites may vary by season, due to changes in temperature; however, seasonality was not considered to be an essential variable in the sampling for this survey. The current study was aimed to be an assessment of the presence of specific parasites in Texas bivalves. *H. nelsoni* is found at its highest prevalence in warm and dry weather (Robledo et al. 2018). *P. marinus* thrives at high temperatures (>20℃) and high salinity (Chu et al. 1993, Ford and Smolowitz 2007). All three of these parasites are found at their highest prevalence at warm temperatures near 20℃, which historically is during the fall in Texas bays (NOAA NODC 2018). With these conditions, if *Bonamia* spp. or *H. nelsoni* were present in Texas waters, it should have been found in the present study.

Though not considering seasonality is a limitation in this study, this survey is still an accurate depiction of what parasites were present in Texas bays. In future studies, it is suggested that seasonality and multi-year monitoring be implemented to provide a more robust assessment of potential parasite presence.

The present study was the first survey for *Bonamia* spp. and *H. nelsoni* in *Crassostrea virginica* and *Isognomon alatus* in Texas bay systems. The survey found neither parasite, but as oceans continue warming, it should be expected to see parasite populations increase. Gulf of

Mexico oyster reefs produce more than half of the United States' commercial oyster landings (NOAA NMFS 2017). With recent decreases in oyster populations due to natural disasters and human-induced stressors (Beck et al. 2011, Keithly and Roberts 2017), it is essential that oyster production increases to keep up with consumer demands (NOAA Fisheries 2017). To accomplish this, oyster aquaculture should be initiated, but potential disease agents and their transfer must be determined. Though *Bonamia* spp. and *H. nelsoni* were not found in Texas bays in the current study, it is possible that these parasites may be introduced to Texas bays through natural or human-assisted range expansion. The results of the current study serve as a point reference that indicate *Bonamia* spp. and *H. nelsoni* have not been and are still not currently present in Texas bays, but continued biennial monitoring is suggested. The current study also indicted that PCR must be confirmed multiple ways to ensure results.

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LIST OF APPENDICES

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Appendix 1: Height ANOVA and Tukey Comparison

ANOVA Height of *C. virginica* from northern 5 bays

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	17489.40166	4372.35041	14.14	<0.0001
Error	145	44844.11829	309.26978		
Corrected Total	149	62333.51995			

TUKEY COMPARISON: Height of *C. virginica* from northern 5 bays

Tukey Grouping		Mean	N	Bay
	A	85.485	30	Sabine Lake
	A			
	A	83.171	30	San Antonio
	A			
B	A	72.992	30	Matagorda
B				
B	C	63.714	30	Copano
	C			
	C	57.564	30	Galveston

Appendix 2: Length ANOVA and Tukey Comparison

ANOVA Length of *C. virginica* from northern 5 bays

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2459.11439	614.77860	9.74	<0.0001
Error	145	9154.99637	63.13791		
Corrected Total	149	11614.11076			

TUKEY COMPARISON: Length of *C. virginica* from northern 5 bays

Tukey Grouping	Mean	N	Bay
A	52.863	30	San Antonio
A			
A	50.591	30	Sabine Lake
A			
A	49.912	30	Matagorda
B	43.341	30	Copano
B			
B	42.873	30	Galveston

Appendix 3: Width ANOVA and Tukey Comparison

ANOVA Width of *C. virginica* from northern 5 bays

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2450.525867	612.631467	15.89	<0.0001
Error	145	5590.721883	38.556703		
Corrected Total	149	8041.247750			

TUKEY COMPARISON: Width of *C. virginica* from northern 5 bays

Tukey Grouping		Mean	N	Bay
	A	31.993	30	Sabine Lake
	A			
B	A	30.241	30	San Antonio
B				
B	C	26.201	30	Copano
	C			
	C	25.412	30	Matagorda
	D	20.428	30	Galveston

Appendix 4: Total Weight ANOVA and Tukey Comparison

ANOVA Total Weight of *C. virginica* from northern 5 bays

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	100155.4101	25038.8525	13.53	<0.0001
Error	145	268326.8109	1850.5297		
Corrected Total	149	368482.2210			

TUKEY COMPARISON: Weight of *C. virginica* from northern 5 bays

Tukey Grouping		Mean	N	Bay
	A	107.72	30	Sabine Lake
	A			
	A	101.10	30	San Antonio
	B	70.26	30	Matagorda
	B			
C	B	58.71	30	Copano
C				
C		38.96	30	Galveston