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Reproductive Investment in Crassostrea Virginica as an Indicator of a Tolerance Response to Perkinsus Marinus

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Reproductive Investment in *Crassostrea virginica* as an Indicator of a Tolerance Response to *Perkinsus marinus*

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ABSTRACT

The Chesapeake Bay region values oysters for the ecosystem services, lucrative fishery, and historical significance that the species embodies; however, over the last half century, oyster abundances have been reduced to historical lows. Two protozoan parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, have been major influences on oyster populations, especially in high-salinity regions. Today, the population is recovering; catches have increased and oysters have expanded spatially. To investigate the cause of the recovery, three measurements were made on slides of oysters from a histological archive collected during summer at Wreck Shoal in the James River from 1988–2017: oocyte diameter, oocyte density, and gonad area fraction. Gametogenic investment served as a proxy for the fitness of oysters; it was hypothesized that an outbreak of *P. marinus* in the 2000s led to a tolerance response that can be detected as an increase in reproduction. Oocyte diameter has remained variable yet steady overall, except for a decrease in 2001 and 2002. Oocyte density and gonad area fraction increased sharply around 2003. Mean oocyte densities increased by a factor of 2.05 and gonad area fraction by a factor of 2.04. Oocyte density has been maintained at these higher counts in recent years. The increase in gonadal area ratio is presently decreasing slowly yet significantly ($p=0.00429$). The cause of the increase is still not well understood, as a variety of environmental variables were significant predictors of reproduction as well as the hypothesized cause, *P. marinus* weighted prevalence.

Regardless of the cause, changes in reproductive patterns signify a positive change on the part of the oyster. The ability of the wild oyster population to adapt supports management strategies that protect old oysters, like sanctuaries and slot fisheries. These strategies allow fit oysters to grow to old age and pass on their beneficial traits to future generations. In the face of doubts about the efficacy of restoration, conservation emerges as a path forward.

GENERAL INTRODUCTION

Parasites are relevant to the ecology of virtually all species, both aquatic and terrestrial. One study in the Carpinteria Salt Marsh, California, discovered that parasites were involved in 78% of the links in the food web (Lafferty et al. 2006). Parasites are defined as having a prolonged negative interaction with their host, which distinguishes them from predators. They are an abundant and diverse group with a hypothesized 40% of known species belonging to this mode of living (Dobson et al. 2008)[1]. With a lifestyle so widespread across taxa, the benefits of parasitism must be significant. Combes (2001) listed the main benefits as habitat, mobility, and energy.

Parasites are successful at the cost of the host populations that they infect. It was hypothesized long ago (Hanson 1905) that parasitism could act as a control on host populations, similar to other trophic interactions such as predation, but concrete examples were few. One of the first well-characterized animal systems was red grouse populations in association with a nematode. Anthelmintic treatments reduced the normal population "boom-and-bust" cycling observed in untreated grouse populations (Hudson et al. 1998). A recent example is the decrease in North American bat populations with the introduction of the fungus *Pseudogymnoascus destructans* that causes white nose syndrome. The fungus has led to a precipitous decrease in bat abundance, with abundances in North America now matching the abundances of bats in Europe, where the fungus was already6

[1] Based on the analysis of known species from 25 major animal and protozoan taxa
[2] Weighted prevalence is infection prevalence weighted by intensity. Rare infections are weighted by 0.5,

present (Frick et al. 2015). The authors suggest that disease could play a role in determining many macroecological patterns that we have yet to explain.

Parasites are often well-studied if they affect hosts populations that humans are invested in. Obviously, parasitologists focus a great deal of effort on human parasites. The oldest written records of parasites discuss intestinal worms and Guinea worm disease in Egypt from about 1500 BC (Cox 2002). Research on the *Plasmodium* species that cause malaria has been ongoing since 1880 when scientists first discovered the parasite in a patient's blood (Cox 2002). Scientists initially focused on finding the vector and elucidating the lifecycle while researchers today are exploring gene drives to cause sterility in the vector, thus decreasing transmission and human illness (Hammond et al. 2016). Parasites of crop plants, like sugarcane, corn, and wheat, as well as livestock have been well-studied through time as well. With the importance of aquaculture as a way to feed a growing human population in the future (FAO 2016; Froehlich et al. 2018; Hudson 2017), more attention has turned towards aquatic parasites.

Oysters are both cultured and still harvested from wild populations. The importance of eastern oysters, *Crassostrea virginica*, to estuaries of the North American Atlantic and Gulf Coasts is particularly profound. Oysters provide ecosystem services, including but not limited to water filtration through feeding, increasing habitat complexity through reef building, and dampening erosive wave energy with their three-dimensional structure (Coen et al. 2007). Many of these ecosystem services provide additional economic benefits by also benefiting other commercially and recreationally valuable species, such as blue crabs and drum. Oysters have supported essential subsistence fisheries from pre-Columbian times and lucrative commercial fisheries along

the East and Gulf coasts of North America for centuries, with the Chesapeake Bay fishery becoming dominant in the 1800's as northern oyster stocks were depleted (Kirby 2004). Intensive oyster aquaculture in the Virginia region of the Chesapeake Bay alone is today worth tens of millions of dollars annually (Hudson 2017).

One of the greatest threats facing oyster populations around the world is disease. For at least the last six decades, there has been at least one major oyster epidemic every ten years. Some outbreaks, such *Bonamia ostreae* in *Ostrea chilensis* in New Zealand in recent years, are the result of novel pathogens emerging in naïve hosts (Lane et al. 2016). Other outbreaks are rooted in the emergence of a more virulent variant of a locally established pathogen, such as the microvariants of the oyster herpes virus in *C. gigas* (Martenot et al. 2012). In the Chesapeake Bay, there have been both endemic and emergent outbreaks. In the 1950s, invasive pathogen *Haplosporidium nelsoni* emerged and devastated oyster populations. Following this outbreak, the native parasite *Perkinsus marinus* intensified and nearly brought *C. virginica* to commercial irrelevance.

It is hypothesized that the *H. nelsoni,* belonging to the phylum Haplosporidia, came from Japan in *C. gigas* imported first to the western U.S. and then to the Chesapeake Bay (Burreson et al. 2000). The oysters in the Bay had never been exposed to anything like it. It thrives in salinities higher than 15 and is pathogenic in oysters most of the year. Oysters in high salinity waters experienced the most intense disease and mortality while oysters upstream in lower salinities were rarely affected (Andrews 1962). The effect was particularly severe for the oyster seed planting industry, which involved selling seed from lower salinity waters to be planted in higher salinity waters for grow-out.

When originally found in the Chesapeake Bay, *H. nelsoni* was called MSX, standing for Multinucleated Sphere Unknown. Although scientists have learned much about it, much remains unknown. The lifecycle of *H. nelsoni* beyond the oyster is a mystery. It is not directly transmissible from oyster to oyster and another host has yet to be identified, although current candidates include planktonic arthropods. Sporulation is also rarely seen in adult oysters and primarily occurs in oysters less than one year in age (Barber et al. 1991; Burreson 1994).

Despite the abundant unknowns, the impact of *H. nelsoni* on the Chesapeake Bay has declined over the last two decades without much human interference. Wild oysters in high salinity areas that have been exposed to *H. nelsoni* longest and most consistently have shown evidence of resistance (Carnegie and Burreson 2011). Resistance is defined as "the ability to avoid infection, eliminate parasites, or decrease parasite loads for a host in contact with a given parasite" (Thomas et al. 2009). Fewer *H. nelsoni* infections have been observed as time has passed since *H. nelsoni*'s introduction. The mechanism of this resistance is also an unknown.

The biology of *P. marinus* is better characterized than that of *H. nelsoni*. This is likely due to studies of *P. marinus* dating back to the 1940s (a decade before the first *H. nelsoni* infection was recorded) in addition to the capability of culturing *P. marinus* in the laboratory. *P. marinus* belongs to the phylum Perkinsozoa, a subunit of the superphylum Alveolata that also includes ciliates and dinoflagellates (Reňé et al. 2017). *P. marinus* has a wider salinity range than *H. nelsoni*, preferring salinities above 10 and, unlike *H. nelsoni*, tolerating lower salinities. *P. marinus* reproduces most rapidly at warmer temperatures. Unlike *H. nelsoni*, *P. marinus* is directly transmissible through the lifecycle

illustrated in Figure 1. It is likely that *P. marinus* is a native parasite to the Chesapeake Bay, as it was found in the 1940s when first sought (Mackin et al. 1950). *P. marinus* had developed what could be interpreted as a relatively stable and benign relationship with the oysters, suggesting coevolution. *P. marinus* caused annual mortality that was typically under 30% in wild oysters, which was manageable for the industry (Andrews and Hewatt 1957). The mortality was largely observed in older oysters, which had years to accumulate *P. marinus* infections (Paynter and Burreson 1991, Paynter et al. 2010). Under these conditions oysters were still able to have a lifespan of >3 years and reproduce annually, thus maintaining local populations.

In the 1980s, oyster disease caused by *P. marinus* increased dramatically during a period of multi-year drought (Burreson and Andrews 1988), with mortality in those years exceeding 70% annually and affecting oysters within months of initial exposure (Burreson and Ragone Calvo 1996). This disease pressure brought the oyster population and harvests in the Chesapeake Bay to its nadir of the 1990s and early 2000s (Figure 2). The change to a period of greatly intensified *P. marinus* parasitism was unexpected at the time. Since disease forms at the intersection of the pathogen, the host, and the environment, changes in any of these factors can lead to altered patterns of disease in oyster populations. Records of this " intersection" principle have already been described in this introduction, including increased disease in drought years and an increase in mortality causing a dramatic decrease in oyster populations with the introduction of a nonnative pathogen to the Chesapeake Bay system. In recent years, there have again been changes in oyster populations. An expansion of wild oyster populations, as interpreted through catch data from the Virginia Marine Resource Commission (Figure 2), began in

the mid-2000s. If disease is produced by an interaction between pathogen, host, and environmental factors, then which of these factors has played a role in the population expansion?

Interactions with parasites that would lead to increased host populations include a substantial decrease in infection, perhaps due to a substantial decrease in parasite populations, or a substantial decrease in virulence. With *P. marinus* arguably the "most important pathogen of the eastern oyster" (Burreson and Ragone Calvo 1996), it is the top suspect for initiating the increase in host populations. However, prevalence of *P. marinus* remains high (Figure 3a); therefore, there was not a marked decrease in infections that could explain an increase in host populations. Weighted prevalence[2] has also not decreased significantly over time (Figure 3b), indicating no major changes in infection intensity. Furthermore, naïve sentinel oysters brought to the York River in the spring every year since 1990 are still experiencing high mortality, indicating that the pathogens are still capable of causing high mortality and suggesting that there has been no decrease in pathogen virulence.

Another explanation for the increased oyster abundance is host adaptation. There are two pathways for the oyster host to adapt to a parasite: resistance and tolerance (Raberg et al. 2007; Roy and Kirchner 1999). Resistance is when a host is capable of limiting the parasite load through the prevention of infection, the limiting of parasite growth, or the expulsion of the parasite after infection. These routes all include direct limitation of the parasite by the host. Tolerance is defined as limiting the physiological consequences of infection without limiting the parasite. This means that a tolerant oyster

[2] Weighted prevalence is infection prevalence weighted by intensity. Rare infections are weighted by 0.5, light by 1, moderate by 3, and heavy by 5 as described in Ray (1954).

will perform better with a given parasite load than an oyster that is not tolerant through minimizing the impact of the infection, not the infection itself. Both of these pathways are possibilities for how oyster populations have recovered despite the continued high prevalence of *P. marinus*.

While resistance has been described as the impetus for the declining impact of *H. nelsoni* in the Chesapeake Bay (Carnegie and Burreson 2011), evidence for resistance to *P. marinus* is limited. Returning to Figure 3a, prevalence of *P. marinus* is still high, suggesting that oysters are not preventing or eliminating infection, and the continued high weighted prevalence (Figure 3b) suggests that oysters are also not preventing the parasite from growing.

For this work, the role of oyster tolerance and environmental changes will be investigated for their relevance to the oyster population recovery, with the hypothesis that the heavy *P. marinus* outbreak in the early 2000s acted as a selection event that favored increased gametogenic investment in oysters.

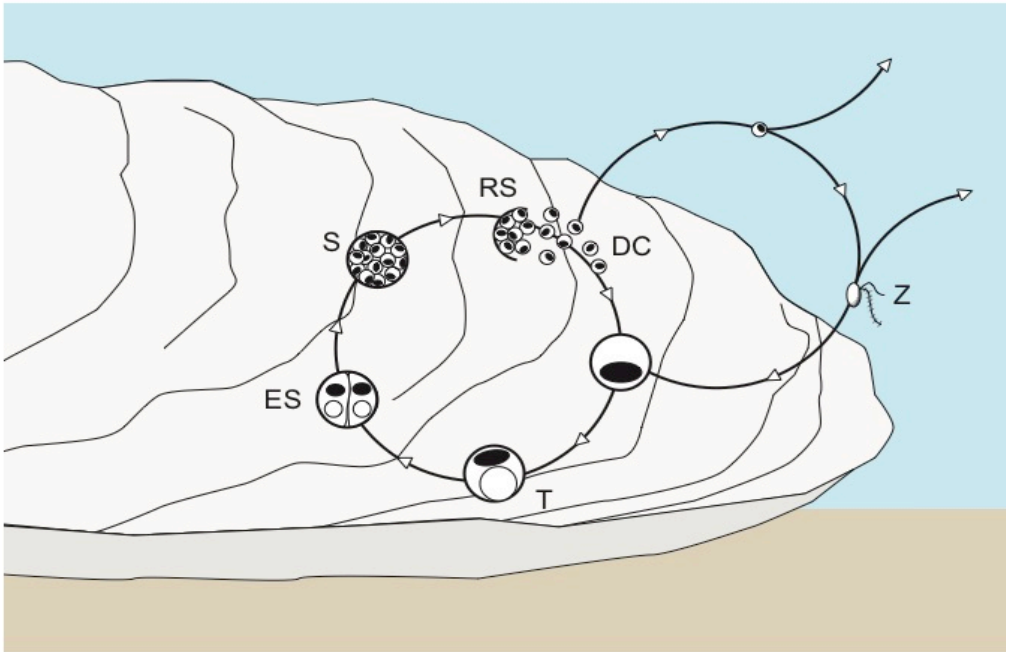

Figure 1. The traditional life cycle of *Perkinsus marinus*. ES= early schizont, S= schizont, RS= ruptured schizont, DC= daughter cells, Z= zoospore, and T= trophozoite. Adapted from Sunila et al. 2001. Not drawn to scale.

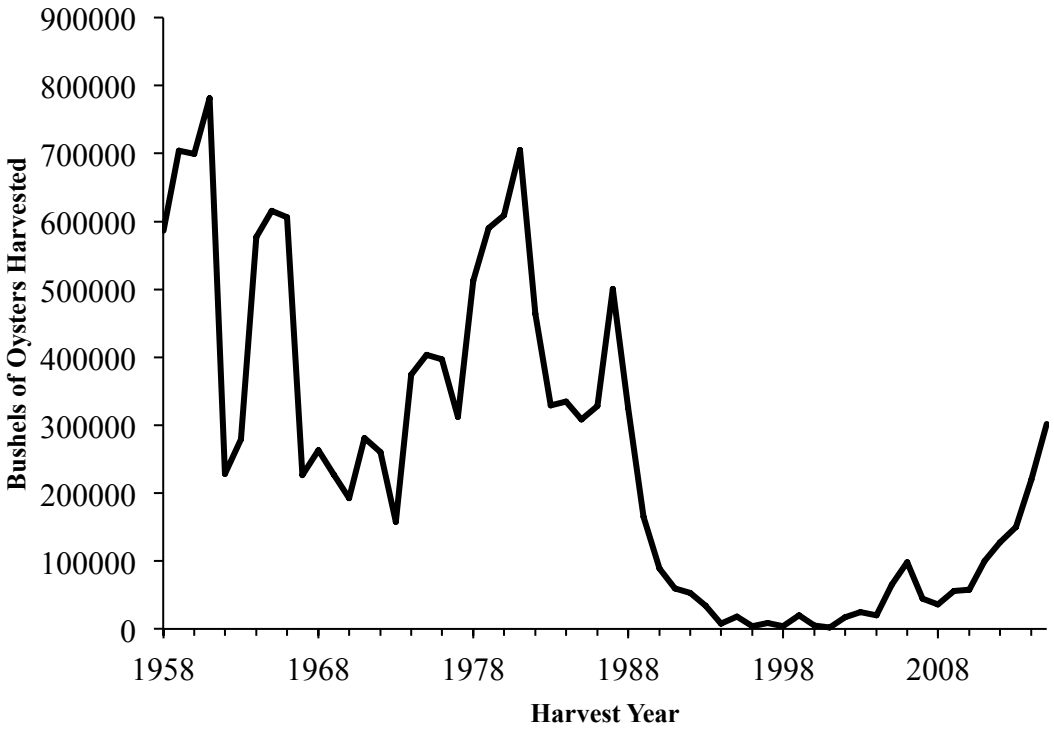

Figure 2. Bushels of oysters harvested from public reefs in Virginia over time. Data from the Virginia Marine Resource Commission.

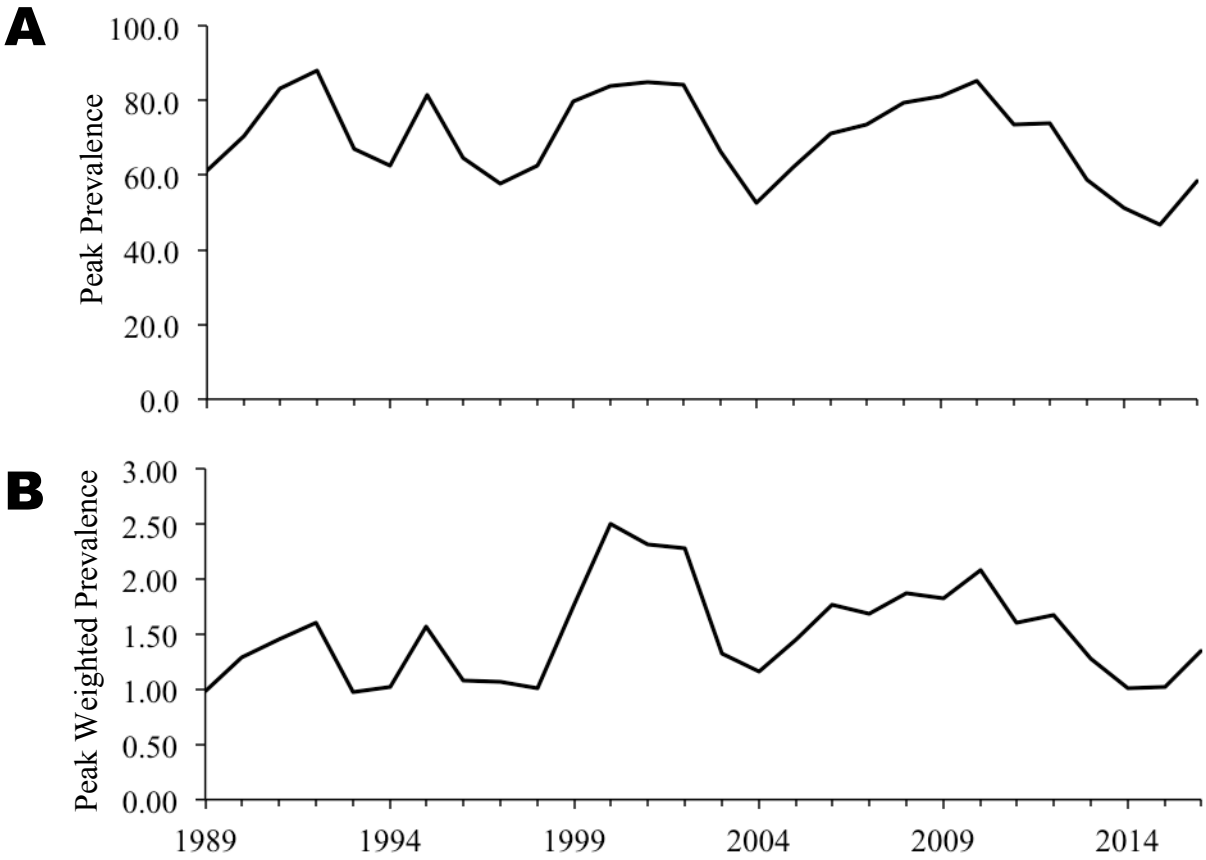

Figure 3. A) Peak annual prevalence of *P. marinus* over time averaged across 30 sites in the Chesapeake Bay. B) Peak weighted annual prevalence of *P. marinus* at the same 30 sites over time.

CHAPTER ONE: PATTERNS IN REPRODUCTIVE INVESTMENT OVER TIME

Introduction

Heterotrophic organisms such as oysters must eat to provide energy to the pathways of maintenance, growth, or reproduction (Alunno-Bruscia et al. 2011). In theory, the relative distribution of energy to different pathways is altered in a diseased animal as compared to a healthy animal, with more energy diverted to maintenance to mount an immune response. Parasites force an energetic cost on the host by consuming host metabolic products, including lipids and proteins (Choi et al. 1989). Most parasites also disrupt host tissues. This disruption can occur through proliferation within the tissues causing sloughing of cells (Carnegie and Burreson 2012) and/or through the secretion of enzymes by the parasite. The parasite of interest here, *P. marinus*, is a good example of these effects. Cells of *P. marinus* produce a lytic substance that disrupts the structure and function of epithelial gut cells (Mackin 1951). Especially in the gut epithelium, the tissue damage from a parasite can reduce the feeding efficiency of the host and can reduce energy intake on top of costing the host energy after assimilation of the food. Parasites can also indirectly cost the host energy through the establishment of an immune response. During an immune response, an oyster produces an abundance of hemocytes that must migrate to the site of infection. These hemocytes then phagocytose and degrade the non-self material. The process of phagocytosis requires the input of energy in the form of

glycolysis, reducing the oyster's overall glycogen stores (Cheng 1996). In *P. marinus* infections, phagocytosis leads to increased parasitic load as opposed to a decrease, since *P. marinus* cells replicate within and are transported by hemocytes (Alvarez et al. 1992). So, this expenditure of energy to phagocytose the parasite cells creates the need to expend further energy once the infected hemocyte bursts, creating a cycle of increasing energy expenditure.

Many laboratory and field experiments support the notion that diseased animals have less energy for pathways beyond maintenance than healthy animals, with evidence that oyster growth and reproduction stall during infection (Andrews 1961; Dittman et al. 2001; Kennedy et al. 1995; Mackin 1962; Menzel and Hopkins 1955; Newell et al. 1994; Paynter and Burreson 1991). The growth and reproduction pathways of an oyster are therefore interesting proxies for quantifying changes in energy budgeting under disease pressure, with applications in tolerance studies.

Reproduction is arguably a more applicable and interesting parameter than growth for investigating disease tolerance. Reproduction directly affects host population sizes and host fitness. The number of eggs that a female produces will increase her chances of successfully passing on her genes, with the added complication of egg size. Egg size is often an indicator of how well-provisioned an egg is, and therefore how well it will survive (Jaeckel 1995; Bayne 1978). On the contrary, an egg that has more energy than an oyster embryo needs to get it to its feeding phase is wasteful; there is an ideal egg size for oysters that maximizes survival while minimizing the female's investment. Together, oyster fecundity and size of eggs can indicate both fitness and a female's investment, since *C. virginica* do not brood or engage in other care of offspring. Size and fecundity

are related in *C. virginica* (Cox and Mann 1992; Dame 1976), so an oyster that is growing more will have measurably higher reproductive investment as well. Lastly, measuring the growth of an individual requires more than one measurement. This necessitates holding animals to take multiple measurements over time, as each animal will start at a different size. Oysters in temperate regions have an indifferent or inactive stage after spawning and resorption, which removes some (but not all) of the variability[3] (Eble & Scro 1996). Snapshots of peak gametogenesis can be taken via histology and archived for decades, giving some insight on the energy availability and possible tolerance adaptation of oysters.. Therefore, histological archives containing slides of oysters at peak reproduction can be used as window into the past to measure the adaptive response of a host as it was occurring, year-by-year.

In this study, the histological archive at the Shellfish Pathology Laboratory at the Virginia Institute of Marine Science was used to quantify reproduction annually and examine increases in reproductive production in mature female oysters as a proxy for a tolerance response.

Materials & Methods

Reproduction was quantified in terms of oocyte density, oocyte size, and gonadal area ratio for the years 1988-2017. These years represent regular monitoring following the intensification of *P. marinus* in the mid-1980s (Burreson and Ragone Calvo 1996). Histological slides of female oysters from Wreck Shoal in the James River collected between June and August were reviewed for maturity, and those determined to be mature

[3] The histological snapshots yield "standing stock" measurements without factoring in rates of production, resorption, spawning, or any other process that alters the number of mature eggs in an oyster at any given time.

were retained for analysis. For each reproductive variable, regression analyses or an ANOVA performed in R were used to assess change over time.

Before reproductive analyses could occur, preliminary validations of the methods were made. Due to the nature of archived material, comparability of samples over decades had to be demonstrated, especially because the frequency of sampling changed throughout the time period. Samples were taken monthly from 1988-2001; for years with monthly data, mature females sampled in June, July, and August were included in the analyses. From 2001-2016, oysters were only sampled in July. To allay concern that peak oyster reproductive maturity was not being captured in the July samples, oysters were collected every month from May through September at Horsehead Rock (HH) and Wreck Shoal (WS) in the James River during the summer of 2017. The proportion of mature females was then recorded for each month for the 2017 samples as well as the archived samples from 1988 and 1989 (WS and HH, respectively). A polynomial curve was then fitted to the data in R, with the curve of best fit selected by the Akaike Information Criterion (AIC), and peak reproductive maturity was calculated based on the polynomial equation for each site and each year.

Study Site. Wreck Shoal was chosen for retrospective analysis because of the site's regular monitoring and moderate salinity. There are four oyster reefs, all in the James River, that have been monitored regularly for the time period of interest: Deepwater Shoal, Point of Shoal, Horsehead Rock, and Wreck Shoal. Wreck Shoal is closest to the mouth of the James River, and therefore has the highest salinity of all four sites. The salinity of Wreck Shoal made it the only suitable site because *P. marinus* is a high-salinity parasite; the disease pressure at the upriver sites was likely to be

inconsistent according to precipitation patterns within the James watershed. The salinity recorded at Wreck Shoal during summer oyster sampling ranged from about 8 to 19. This is sufficient for *P. marinus*, while oysters upriver may have experienced lower salinities that may have provided a temporal refuge from disease. Since the purpose of this study is to elucidate host adaptation to *P. marinus*, Wreck Shoal was chosen to minimize these inconsistencies.

Density of Oocytes. Using the program CellSens (Entry 1.13, Build 13479) and an Olympus BX51 microscope with an attached Olympus DP73 color camera, images were taken of a field of view using the 10x objective with a 10x ocular magnification. Mature oocytes were then counted in this field of view. In order to be included in the count, the oocyte must have a clear nucleus and not be attached to the wall of the follicle as well as being completely within the field of view. Although the field of view was 1.1 mm by 838 μm, oocyte densities are reported as the count per field of view rather than a discrete area, as it is a more intuitive metric.

In performing oocyte density counts, one field of view was chosen based on visual assessment of uniform density through the field of view, to represent each individual. To validate that one field of view would be representative, 50 individuals were subsampled with one individual from each of the 30 years in the time series and an additional 20 individuals randomly selected from all individuals. The oocytes in three fields of view were counted, each one in the gonadal tissue of a randomly selected section of the slide. For slides from 2004-2017, the slide was divided into 8 tracts as shown in Figure 4. For slides from 1988-2003, the slide was divided into 4 tracts due to their small size as shown in Figure 5. Random selection of tract was performed by random number

generation in Microsoft Excel. The subsampled oocyte densities were then compared to the original means through a T-test to determine if their mean was significantly different from the mean for that year using the original method.

A discontinuous piecewise regression over time was applied to the oocyte density measurements in R. A piecewise regression was chosen based on the hypothesis that there had been an increase in gametogenesis. The piecewise regression would allow a change in slope over the course of the time series, with discontinuity allowing the two pieces of the regression to not meet at the breakpoint. The year 2003 was used as the breakpoint based on an iterative search code that isolated the breakpoint with the lowest residual error. The code compared the years preceding 2003 and those following 2003 and suggested that they were more similar within those categories than between the categories. The results of the iterative search were supported by hypothesized disease interactions in 2003. The fit of the piecewise regression was compared to the fit of a standard regression using AIC.

Gonadal Area Ratio. A Nikon D200 camera with an AF Micro-Nikkor 60mm f/2.8D lens was used to record an image of the entire slide because such an image could not be taken even at the lowest magnification by the Olympus microscope and camera combination. The slides were placed on a bright, white screen to enhance the quality of the image. Images were then imported into the CellSens program. The gonadal tissue was outlined using the "closed polygon" tool, followed by the visceral mass, with the areas recorded. The ratio was then calculated by dividing the area of the gonadal tissue polygon by the visceral mass polygon. Although a gondosomatic index is a more standard metric, it cannot be calculated from histological slides (Anderson and Gutreuter 1983).

Therefore, instead of using gonadal mass/total mass, gonadal area/total visceral area was calculated.

For years 1988 through 2003, the slides in the archive contained four separate individuals, with each individual represented by a quarter of a standard histological section (cut through the center of the gill filaments and at a right angle to that line, as seen in Figure 6a). Occasionally, the section was not a true quarter and was missing an additional part of the body wall. These irregular sections were excluded from areal analysis. To allow comparison of the older slides to the slides of whole sections made from 2004 through 2017, the whole sections were quartered. To do this, the "ellipse" tool in CellSens was used to encompass the body of the slide (Figure 6b). Using the "perpendicular line" tool, a line was drawn from the center point of the ellipse through the center of the gills to mimic the cutting of the older slides, with a second line at a right angle to this (Figure 6c). All area measurements were then taken within this quarter of the entire slide to allow comparison between old and new slides.

The area ratios were analyzed via a discontinuous piecewise regression over time in R for the same reasons as stated above. The year 2003 was used as the breakpoint based on hypothesized disease interactions.

Size of Oocytes. To evaluate the size of oocytes, the diameter was measured for five oocytes in each mature female. These oocytes were selected based on maturity, with a clear nucleus and no attachment to the follicle wall as criteria for selection. Using the 40x objective with a 10x ocular magnification, an image was taken with the same camera and image processing program as used for density counts. The "arbitrary line" tool in CellSens was used to record two cross-sectional measurements. One cross-section was

measured through the center of the nucleus at the longest distance from end to end while the second was measured at the shortest distance. The non-spherical, irregular shape of the eggs within the follicles made two measurements necessary to calculate a single, representative average diameter for each oocyte.

To evaluate how oocyte diameter changed with the increased parasitism, the measurements from the high disease years 2000-2003 were compared to all other years using orthogonal contrasts in an ANOVA statement. Based on preliminary data visualization, a piecewise regression did not seem appropriate. The hypothesis tested was that oocyte diameter decreased in the early 2000s due to heavy disease rather than testing if there was an increase over time.

Results

Validation of Methods. A polynomial of the second degree was fit to the Wreck Shoal 2017 maturity data with a multiple R^2 of 0.949. The peak of the polynomial equation was calculated to be 7.3. corresponding to approximately July 10th. The 1988 maturity polynomial had a multiple R^2 of 0.806 and a peak at 7.6, corresponding to July 18th. The Horsehead models had multiple R^2 values of 0.946 for the 2017 data and 0.984 for 1989. The peak of the 2017 polynomial was 8.13 while the 1989 peak was 7.14. The peaks correspond to August 4th and July 4th, respectively. The data and functions are plotted in Figure 7 for both sites and years. Polynomials applied and their AIC values can be viewed in Table 1.

Density of Oocytes. Mean oocyte density has increased over time at Wreck Shoal since 1988 with a sharp increase from a mean density of 103 in 2002 to 211.3 in 2003 (Figure 8). The piecewise regression model with a discontinuous break at 2003 was significant ($F=58.62$, $p<2.2e-16$) while explaining little of the variation in density

(R^2=0.2601). The first function, from the year 1988 to 2003, has a slightly negative though not significant (p=0.0705) slope of -0.7214. The second function from 2003 onwards has a slightly positive and not significant slope of 1.7782 (p=0.0732). At 2003, the first function predicts 153 oocytes per field of view while the second predicts 233 oocytes per field of view (Figure 9). The piecewise regression had a lower AIC than the standard regression model (5808 and 5846, respectively).

Of the 50 subsampled individuals, only 6% had p-values that suggested the mean of the three random subsampled densities were significantly different from the original count for the individual. None of the subsampled individuals showed a significant p-value when compared to the mean for the year the individuals were sampled from.

Gonadal Area Ratio. Mean gonadal area ratio has increased over time (Figure 11). Similar to density, a shift from 0.1098in 2002 to 0.2241 in 2003 was observed. The piecewise regression model with a discontinuous break in 2003 was again significant (F=45.38, p<2.2e-16) while describing less than one-third of the variation (R^2=0.276). The first segment has a slope of 0.0053 (p=0.00437) with a predicted gonadal area ratio of 0.1626 at the 2003 breakpoint. The slope of the second segment is -0.0044 (p=0.00429) with a value of 0.2918 at the breakpoint (Figure 12). Once again, the piecewise regression had a lower AIC (-2152) than the standard regression (-2097).

Size of Oocytes. The diameter of oocytes was variable over time (Figure 10). The ANOVA showed a significant impact of year on oocyte size (F=6.511,p<2.2e-16). Orthogonal contrasts revealed that the mean oocyte sizes for the years 2001 and 2002 were significantly different from all other years (F=23.893, p=1.27e-6 and

F=41.470,p=2.24e-10, respectively). The other contrasts (2000 and 2003) were not significant to an alpha of 0.05.

Discussion

A tolerance adaptation is defined as a lessening of the impact on host fitness of a certain intensity of infection (Roy and Kirchner 2000; Schafer 1971). While infection intensity was fairly constant, positive changes were observed in the reproductive effort of oysters over the three decades from 1988-2017.The changes may represent an improvement in host fitness for a given infection level. The period of greatest change encompassed the early 2000's for all three reproductive parameters. The piecewise regressions of oocyte density and gonadal area ratio showed a discontinuous break in reproductive investment in 2003 while oocyte diameter showed the greatest change in 2001 and 2002. The timing of the heavy *P. marinus* outbreak, with a weighted prevalence close to 3 in 2000, 2001, and 2002, lines up with these changes in gametogenesis.

The slopes for both segments of the oocyte density regression are not significantly different from zero, suggesting that oocyte density remained stable from 1988-2002 and from 2004-2017[4]. Therefore, the sudden increase in reproduction noted in 2003 has been maintained to the present. This observation supports the hypothesis that a selection event caused a long-term change in oyster energy budgeting and fitness. If attributed to the intense *P. marinus* outbreak and heavy mortality from 2000-2002, it would mean that an acute disease event changed the reproductive investment lasting to the present, and possibly beyond. A long-term change in an oyster population due to disease is not unprecedented. Similar selection events to the one proposed here have occurred in the Delaware Bay in response to *H. nelsoni* (Ford and Bushek 2012). In the 1950s and again

[4] Since 2003 is where the breakpoint occurs, assumptions cannot be made about density in that year.

in the 1980s, severe MSX disease outbreaks caused heavy mortality and ultimately a decrease in prevalence. The decrease in prevalence suggested that surviving Delaware Bay oysters were more resistant to *H. nelsoni*, while the surviving oysters in this study are hypothesized to be more tolerant of *P. marinus*.

The pattern for gonadal area ratio is slightly different than the oocyte density regression. The slope of the first segment of the gonadal area ratio regression is marginally positive (0.005) but significantly different from zero. The slope of the latter segment is marginally negative (-0.004) but significantly different from zero. According to the regression model, the ratio of gonad to visceral tissue was increasing slowly but significantly from 1988-2002 with a marked increase in 2003. Since 2003, the gonadal area ratio has been slowly but significantly decreasing. Unlike oocyte density, which appears to be at a stable, higher mean from 2003 to 2017, gonadal area ratio has been decreasing since 2003. It is possible that the mean gonadal area ratio could either decrease back to pre-2003 levels or stabilize at higher investment, but only continued monitoring over time will tell.

While both segmented regression models fit the data, little variation was explained in either dependent variable. This suggests that year alone cannot explain all of the variation in oocyte density or gonadal area ratio, which would be expected. The question of what predictors are significant is addressed in Chapter 2.

The oocyte diameter trends were different than those already described. The change in oocyte diameter was acute, with the years 2001 and 2002 differing from all other years and 2000 and 2003 not being significantly different. Therefore, the change in oocyte diameter occurred before the shifts in density and gonadal area ratio and during

the disease outbreak. Oocyte diameter may be a short-term, more immediate response than the other two parameters with diameter increasing again once disease pressure has decreased. It is of interest to note that Kennedy et al. (1995) found that oocyte diameter did not vary with *P. marinus* infection intensity; however, their measurements were based on oocytes that were released by the female, unlike the pre-spawned oocytes investigated here. Kennedy et al. (1995) also used a small number of individuals for their size analyses (34 oysters over two years). It is possible that the oysters in the Maryland study were below the disease threshold at which oocyte size is affected.

In terms of a potential tolerance response, oocyte density and gonadal area ratio increases represent an increase in oyster fitness. The change in oocyte size is too transient to label a tolerance adaptation. Conceivably, the physiological damage avoided by tolerance could allow higher egg production. Higher egg production could benefit the overall oyster population, as more eggs could translate to more larvae, spat, juveniles and adults. Such a response supports management strategies that provide oysters time and space to adapt. These strategies include sanctuaries from harvest and market size limits. Sanctuaries allow selective pressures to act upon the oyster population, producing adaptation to locally important stressors. Size restrictions on harvest, including slot fisheries that include a lower limit and an upper limit on harvest size, allow oysters to attain an older age. Older oysters have survived for longer, and are more likely to have valuable adaptive traits that allowed them to reach old age. In addition, larger oysters are more fecund, which means that an older, larger oyster is both better adapted to the local environment and better able to spread its adaptive genes than younger oysters.

This study adds to the limited research on tolerance in marine disease systems. Tolerance is commonly discussed in plant pathogen literature, dating back to 1894 with the observation of "rust-enduring" wheat (Cobb 1894). Oysters were a logical progression from crop plants, since they are also farmed with economic consequences to disease. In other animal systems in which tolerance has been measured, reproduction is usually not used as the proxy for host fitness. Body weight has been used for sheep and mice, extent of anemia for mice, and survival in fruit flies (Råberg et al. 2007; Hayward et al. 2014; Louie et al. 2016). Apart from these parameters not being measurable over time from the oyster archive, overall oyster populations available for harvest and to provide ecosystem services are the main interest. Therefore, similar to crop plants, it makes sense to investigate reproduction, like the production of grain by wheat plants, with oysters.

Method of sampling density and timing of reproduction are not likely to have influenced the trends reported in this study. The subsampling showed no extreme discrepancies between consciously picking a field of view to count and random assignment. According to the polynomials fitted to the reproductive maturity data, July still captures peak reproduction. The peak in Wreck Shoal oysters shifted from 8.1, which would be early August, to 7.3, which is early to mid-July. This shift echoes the findings of Roger Mann and colleagues (Mann et al. 2014). Through long-term monitoring, they detected a shift in recruitment to earlier summer months and hypothesized that the increase in disease intensity selected for oysters that reproduced earlier before disease pressure peaked. At Horsehead Rock, peak gametogenesis appears to have moved later in the summer, from 7.1 to 8.1, or from early July to early August. This is contrary to what

has been observed in recruitment timing patterns. It is possible that the timing of peak ripeness in females has not changed significantly, but perhaps fertilization and survival of larvae to recruitment are higher earlier in the summer. Since only two years were analyzed for peak reproduction at only two sites, and the polynomials fitted to just five data points per year and site, more work remains to be done to elucidate whether there has been a shift in the timing of reproduction that matches the shift in recruitment.

Table 1. Polynomials of degrees one through three tested for best fit to reproductive timing data. Site, year, degree of polynomial, and AIC value given. Bold text indicates the model chosen, with accompanying R^2 value.

Site and Year	Degree of Polynomial	AIC Value	R^2 Value
Wreck Shoal 2017	1	-4.924265	
	2	**-17.76562**	**0.9492**
	3	-16.07236	
Wreck Shoal 1988	1	-12.69364	
	2	-18.65341	
	3	**-25.35248**	**0.9659**
Horsehead Rock 2017	1	-8.156302	
	2	-10.49785	
	3	**-18.2258**	**0.9458**
Horsehead Rock 1989	1	-8.655147	
	2	-18.10208	
	3	**-25.4778**	**0.9845**

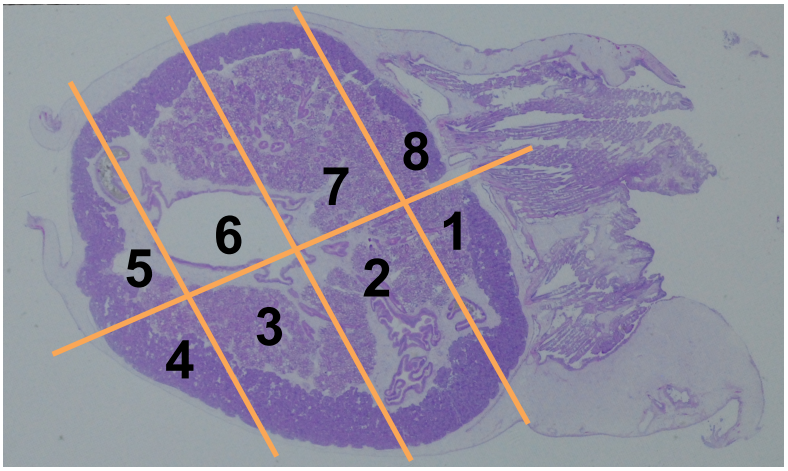

Figure 4. A digitally rendered example of how the tracts were delineated for subsampling in a whole slide. In the lab, a coverslip with marker lines was placed on top of the slide during subsampling.

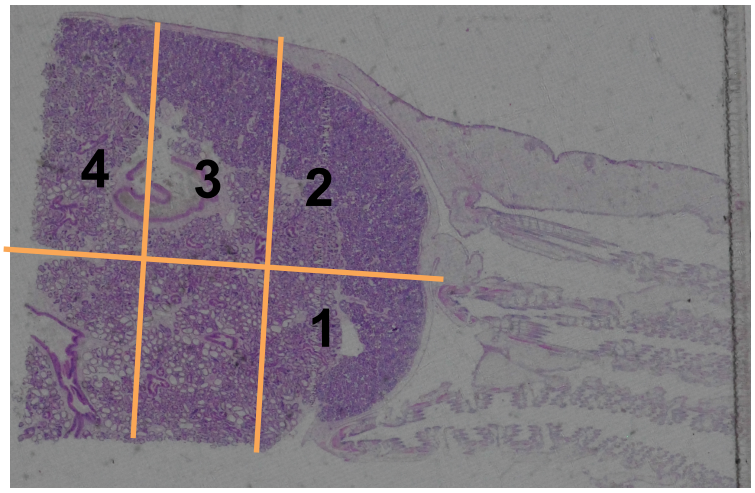

Figure 5. A digitally rendered example of how the tracts were delineated for subsampling in a quarter slide. In the lab, a coverslip with marker lines was placed on top of the slide during subsampling.

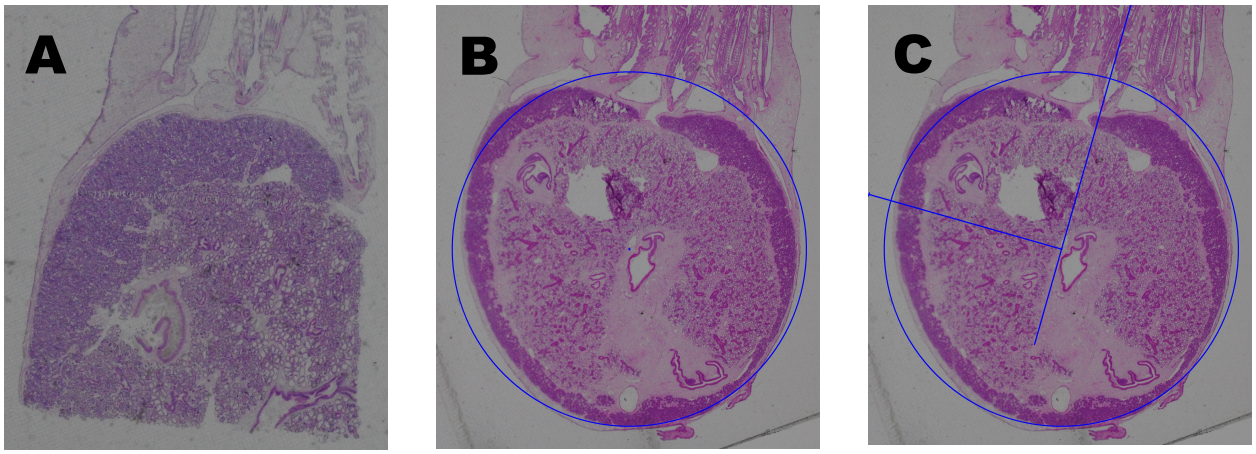

Figure 6. Quartering method for whole slides. A) quarter slide from the archive. B) ellipse overlaid on the whole slide. C) perpendicular lines added to delineate the quarter.

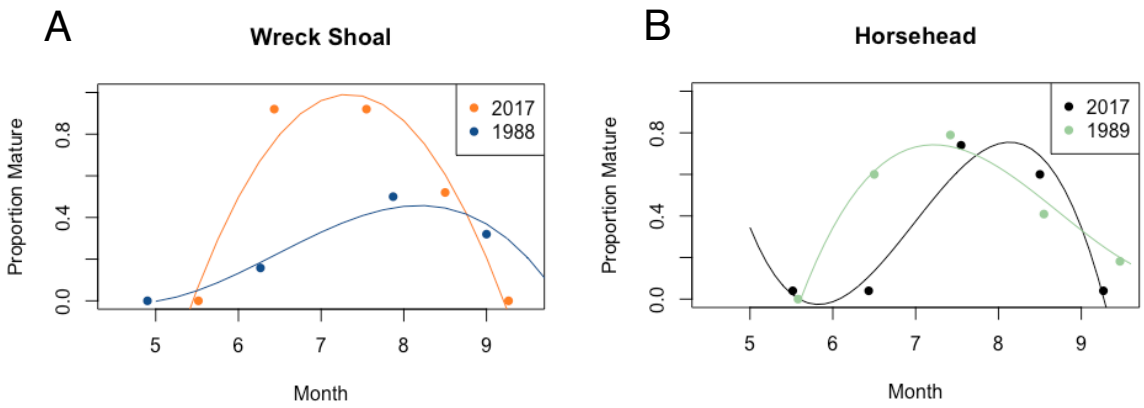

Figure 7. Proportion of oysters at sexual maturity for each month from May to September. A) compares 1988 to 2017 at Wreck Shoal while B) compares 1989 to 2017 at Horsehead Rock.

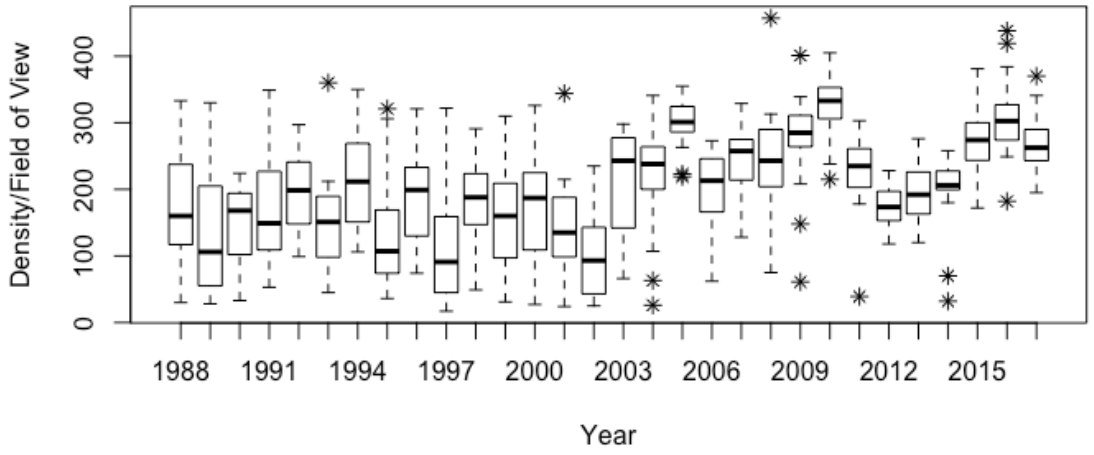

Figure 8. Boxplot of the oocyte density measurements over time (n=672). The box represents the first and third quartile, with the line within the box representing the median. The dotted lines signify the maximum and minimum values with the stars representing outliers.

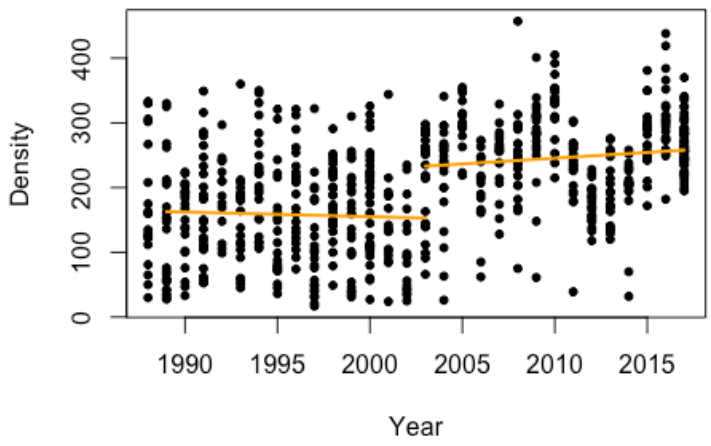

Figure 9. Oocyte density counts per field of view over time at Wreck Shoal. Points represent single individuals/observations. Orange lines show the predicted values from the two segments of the piecewise regression performed, discontinuous at the 2003 breakpoint.

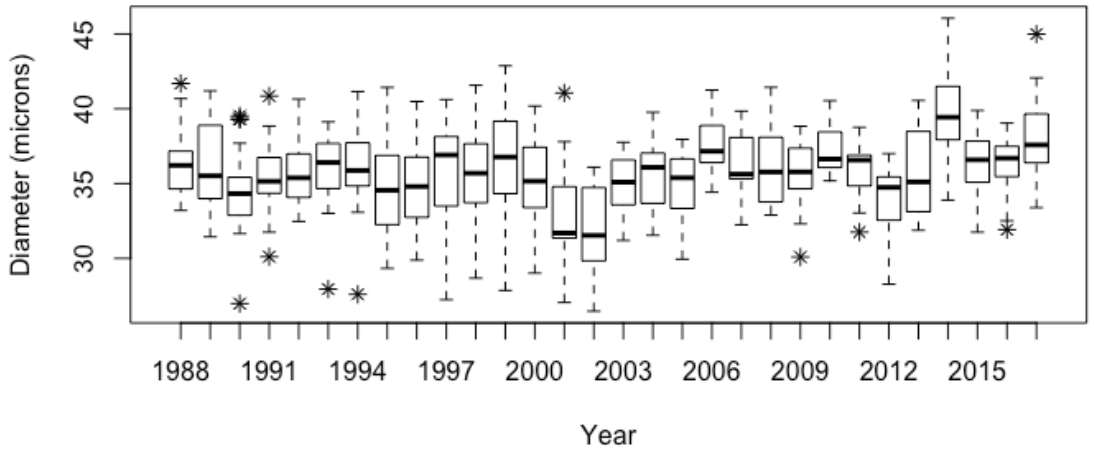

Figure 10. Boxplot of the diameter of oocytes over time (n=724 individuals). The box represents the first and third quartile, with the line within the box representing the median. The dotted lines signify the maximum and minimum values with the stars representing outliers.

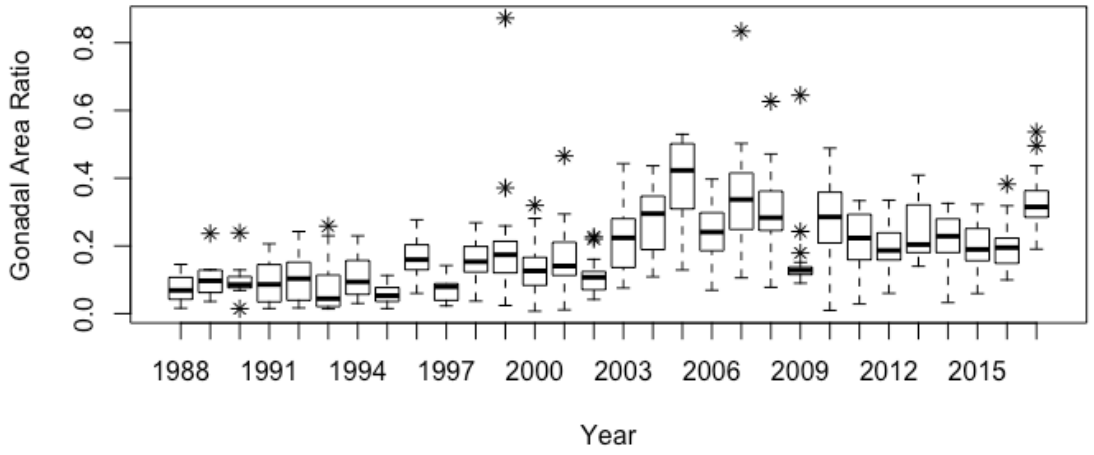

Figure 11. Boxplot of the gonad area ratio over time (n=481). The box represents the first and third quartile, with the line within the box representing the median. The dotted lines signify the maximum and minimum values with the stars representing outliers.

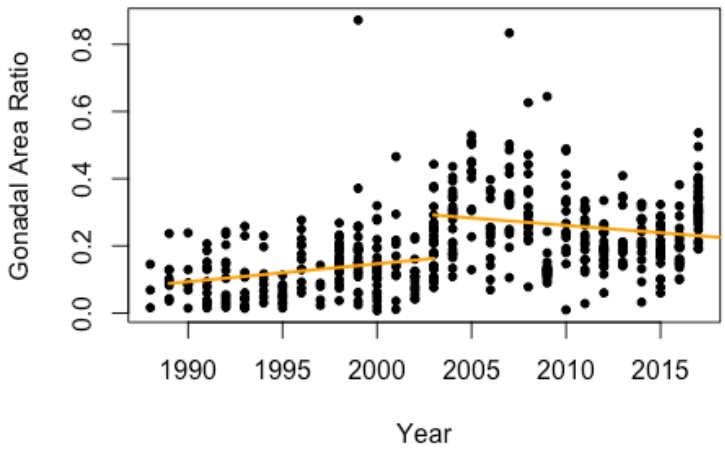

Figure 12. Gonadal area ratio over time at Wreck Shoal. Points represent single individuals/observations. Orange lines show the predicted values from the two segments of the piecewise regression performed, discontinuous at the 2003 breakpoint.

CHAPTER TWO: ENVIRONMENTAL INFLUENCES ON REPRODUCTIVE PATTERNS

Introduction

As reproductive strategies go, broadcast spawning seems to be an uncertain strategy, albeit one that the oyster's sedentary lifestyle makes necessary. The first challenge is that timing of the release of gametes must align with other individuals. In order to be ready at a given time, oysters must start gametogenesis and progress to have mature gametes ready for spawning. Once released, sperm and oocytes must find each other. Oysters have developed chemical signals that attract sperm to the eggs and proteins that aid in recognition of gametes from the same species to tackle this barrier (Evans and Sherman 2013). After fertilization and development in the water column, which requires sufficient and appropriate food, larvae must find a suitable place to settle. With such a complex system, there are many factors that affect the reproductive potential of an oyster. Galtsoff (1964) claimed that temperature, salinity, food availability, depth and pollution are the most important influences on reproductive output. The first three parameters, along with disease, are emphasized here for their possible significance as predictor variables of the increase in reproductive investment of oysters observed in the early 2000s.

Temperature influences many physiological processes. Higher temperatures increase the rate of most biological reactions and metabolism/growth generally increases as well. Dame (1972) calculated that the Q10 temperature coefficient, or the change in the rate of a reaction when the temperature is raised 10˚ C, was roughly two for the growth of oysters during the warmer months. It is conceivable that temperature and an increase in the rate of reactions could increase the production of eggs as well, although most studies concerning reproduction and temperature have investigated temperature as a cue for the timing of gonadal development and spawning, rather than investigating how temperature influences total gamete production (Shpigel et al. 1992). Dalila Aldana Aranda and colleagues (2014) found that temperature was correlated with gametogenesis in oysters spawning in Mexico. Along the middle and south Atlantic coast, oysters begin to spawn between 16 and 20˚ (Lorio and Malone 1994). It is possible that warmer temperatures solely help oysters achieve peak maturity earlier without actually increasing the amount of eggs that are produced, which would be less relevant to this study than the alternative.

Salinity is similar to temperature in that it has been studied mainly as a cue for the initiation of reproduction. However, since salinities lower than 10 may stunt gonadal development, salinity could play a role in suboptimal oocyte production (Lorio and Malone 1994). An improvement to ideal salinity could then increase reproductive output.

Food availability has a logical impact on the energy-intensive process of gametogenesis. Gonadal index, defined as the gonadal thickness divided by the diameter of the adductor muscle, increases with increasing food index, defined as the sum of the lipid, protein, and carbohydrate content of the seston, in the spring and summer (Soniat &

Ray 1985); however, these seasonal changes may represent effects on timing as well, rather than increasing total reproductive output. In the mussel *Mytilus edulis*, differences in maximum reproductive condition and fecundity have been noted between different years, which have been attributed to differences in the abundance of food and the timing of peak energy availability (Thompson 1979; Newell et al. 1982). The total production of gametes in *C. virginica* may be similarly affected by food availability.

Depth and pollution are not considered here as predictors of reproductive change observed in oysters. The oysters at Wreck Shoal are dredged routinely by the Shellfish Pathology Lab from the same area, and a significant and consistent change in depth of sampling is not likely. Furthermore, pollution by "trade wastes" (Galtsoff 1964) does not seem a likely cause of reproductive change in the oysters. The most significant reductions in pollution, such as the banning of Kepone and the passing of the Clean Water Act, occurred in the 1970s, well before any change in reproduction was observed.

Fecundity in oysters is related to size. As oysters grow larger, they produce more oocytes per gram than younger oysters (Harding et al. 2008, see also Mann et al. 2014), however, this ontogenetic change in fecundity is irrelevant to this study because the oysters have been collected routinely by the same scientists or people trained by those scientists. We can be reasonably assured that a sudden increase in the mean size and/or age of oysters collected was not a cause of the shift in reproduction.

Therefore, temperature, salinity, food availability, and disease were selected as possible predictors of oyster reproduction (specifically oocyte density, gonad area ratio, and oocyte size). Each specific reproductive parameter was modeled with these predictors to identify which factors contributed significantly to overall gonadal production from

1988-2017. The purpose was to determine whether changes in the predictors could help explain the change in reproduction observed in the early 2000s, with the hypothesis that disease was a driving force.

Materials & Methods

Two multiple linear regressions were performed in R to determine the significance of temperature, salinity, peak chlorophyll-a (chl-a) concentration and the timing of the peak as proxies for food, disease, and any significant interactions as predictors of reproductive investment patterns in oysters. Separate models were run for gonadal area ratio and oocyte diameter. A negative binomial model was run in R with the same predictors for the oocyte density data, as they are counts per unit area. Assumptions of the models were tested through residuals plots against fitted values and against time, and quantile-quantile plots.

The temperature data was obtained from the Virginia Estuarine and Coastal Observing System (VECOS) stations 5.1, 5.2, and 5.3 for the years 1987-2017. While station 5.2 was closest to the Wreck Shoal collection site, stations upstream and downstream were included to increase the sample size and hopefully increase the accuracy of the environmental data (see VECOS map, Figure 13). Seasonal temperatures were calculated by averaging observations from June, July, and August as summer temperatures. The same seasonal averaging process was repeated for salinity.

The chl-a concentrations were collected from VECOS stations as well. The highest chlorophyll-a concentration from each site for each year were averaged. Phytoplankton blooms, including the spring bloom, are notoriously patchy and variable through time, so averaging values helped emulate what the oysters experienced that year.

The month that the peak was recorded was used as the timing predictor, coded as a factor with 4 levels equivalent to February, March, April, and May.

The annual average weighted prevalence of *P. marinus* came from the Shellfish Pathology Laboratory's regular monitoring program (Carnegie and Burreson 2009). Weighted prevalence was used to incorporate both intensity and prevalence.

Models were run in R with all interactions included preliminarily, with those with p-values less than 0.05 dropped for the subsequent, final model. The variance inflation factor (VIF) was calculated for each predictor to detect multicollinearity in the models. Coefficients were standardized (values of predictors had the mean value subtracted and then were divided by the standard deviation) to allow comparisons of magnitude between predictors.

Results

Density of Oocytes. All predictors in the model excluding the timing of the spring bloom were significant ($p<0.05$). The significant interactions were between summer temperature and chl-a concentration (p=1.99e-07), summer salinity:chl-a concentration (p=1.44e-06), summer temperature:summer salinity (p=2.69e-07), and a three-way interaction between summer salinity, summer temperature, and chl-a concentration (p=6.93e-07). Multicollinearity was present in summer temperature (VIF=302.5), summer salinity (VIF=6234.8), chl-a concentration (VIF=205577.5), and all interaction terms (Table 2). Multicollinearity was not present in timing of peak chl-a (VIF=1.87) and disease (VIF=1.56). Coefficient estimates are given in Table 2.

Gonadal Area Ratio. All predictors in the model were significant ($p<0.05$). The significant interactions included in the model were summer temperature:summer salinity (p=0.00399), summer temperature:timing (p=7.02e-05), summer salinity:timing

(p=3.37e-05), and a three-way interaction between summer temperature, summer salinity, and timing (p=2.43e-05). Multicollinearity was again observed in the majority of predictors (VIF values can be seen in Table 3). The multiple R^2 was 0.197. Coefficient estimates are included in Table 3.

Size of Oocytes. Multicollinearity was present in all predictors so the data were centered to reduce the impact. After centering, six of the variables still had VIFs above the threshold of 10 (see Table 3). Temperature, chl-a, and disease were significant ($p<0.05$) as well as the interactions between disease and chl-a, and all constituent two-way interactions of temperature:ch-a:salinity, temperature:chl-a:timing, and chl-a:salinity:timing except temperature:salinity. The multiple R^2 was 0.214. Coefficient estimates are given in Table 4.

Discussion

When considering oyster reproduction as a whole, the temperature and salinity during the reproductive season, peak spring bloom chlorophyll-a concentration, the timing of the peak of the bloom, and weighted prevalence of *P. marinus* are all important in determining gonadal quantity and quality at peak reproduction. Though timing of peak chlorophyll-a levels was not significant in predicting oocyte density, it was significant for predicting gonadal area ratio and oocyte diameter. This adds to the current knowledge (Thompson et al. 1996) that temperature, salinity, and phytoplankton are important cues for the timing of beginning gametogenesis and spawning. They are significant predictors of the quality and quantity of oocytes that will be present at peak maturity as well.

It must be noted that the interaction terms complicate interpretation of model results in the current study. A significant interaction between two or three terms means that the effect of each term is dependent on the values of the other term(s). Therefore, the

coefficients for summer temperature, summer salinity, and chl-a concentration in the density model cannot be accurately interpreted in isolation. Similarly, the coefficients for summer temperature, summer salinity, and timing of peak chl-a concentrations for the area model cannot be interpreted. For the oocyte size model, every predictor was involved in a significant interaction term, which severely limits the interpretation of individual coefficients.

The presence of multicollinearity in the current study results influences interpretation. Collinearity was expected from these environmental variables because they tend to be linearly related, with patterns changing over space and time (Dormann et al. 2013). For example, both temperature and chl-a concentration tend to be low in the winter and increase into spring and summer. The model employed herein cannot determine how much variance in reproduction is explained by the change in temperature versus how much is explained by the change in salinity because both temperature and salinity follow the same seasonal pattern. Since the hypothesis examined in this study concerned disease, I chose not to address multicollinearity in the models in which the disease variable had a VIF of less than ten. Because the multicollinearity remains present, the following interpretations will acknowledge that the collinear environmental predictors cannot be truly separated, while disease can be interpreted by itself.

The density of oocytes at maturity is not affected by timing of peak chl-a levels, but is affected positively by the concentration of chl-a during the peak. The importance of chl-a concentration emphasizes the potential role that storage of energy in forms such as glycogen could have on the production of oocytes. The latest peak chl-a levels in the dataset was observed in April, well before reproduction peaks in Wreck Shoal oysters.

Therefore, the timing of the peak chl-a may not have mattered because oysters are able to store abundant glycogen reserves and utilize it as they start producing gametes (Thompson et al. 1996). If peak chl-a occurred in February, the oysters would still be able to store the energy from the bloom to use later. The concentration of the peak chl-a still mattered because it determines how much energy there is to both store and use. Disease weighted prevalence from the previous year had a negative effect on oocyte density with a standardized coefficient estimate of -0.118. Although the coefficients for the environmental variables cannot be directly interpreted due to interactions and multicollinearity, the effect of disease is the same order of magnitude as the coefficient estimates for environmental variables as well as interactions. Therefore, disease is a significant predictor of oocyte density in a statistical as well as a practical sense; it exerts a similar magnitude of effect as the other significant predictors.

Unlike oocyte density, the gonad area ratio was affected by all predictors, including timing of peak chl-a concentrations. This could indicate that storage is not as efficient as sending energy directly from consumption into gamete production, or it is limited. Perhaps oysters are able to store enough energy from early blooms to reach optimal density, but later blooms allow oysters to send energy directly into expanding the gonad to be able to accommodate more eggs at the same density (Bayne et al. 1975). However, the magnitude of the effect is hard to interpret due to one three-way and two two-way interactions. The weighted prevalence of *P. marinus* again shows a negative effect with a standardized coefficient estimate of -0.049, which is within the range of magnitude of the environmental variables. An oddity of this model is that chlorophyll-a concentration is estimated to have a coefficient estimate of -0.016. This would suggest a

very slight, but still significant (p=0.013) negative influence of increasing chlorophyll-a on gonadal area fraction. While Rodríguez-Jaramillo and colleagues (2008) found a negative correlation between gonad coverage area and chlorophyll-a concentration in *Crassostrea corteziensis*, they hypothesized that the effect was due to high chlorophyll during the winter when females were in a resting stage. Since only peak chlorophyll and peak reproduction were modeled, this is not an applicable hypothesis to apply to this study. Perhaps further work should be done with more consistent chlorophyll sampling to address this question.

The oocyte diameter model is the hardest to interpret, as the independent variable of interest (disease) is wrapped up in an interaction, and multicollinearity remained a problem, even after centering. Perhaps modeling oocyte size with additional data would allow the variables to be teased apart and provide more useful information.

Ultimately, a great deal of environmental variables influence reproductive patterns in oysters and the connections between the variables make it hard to understand the system. Disease had a clear negative effect on reproduction when measuring oocyte density and gonadal area ratio. In the oocyte diameter model, the effect of disease (as well as many environmental variables) were obscured by interactions and collinearity. Although the goal of the study was to test the hypothesis that disease was driving the changes in reproduction, the hypothesis cannot be definitively supported or rejected because the significant influence of many environmental factors in reproduction as well. An analysis with more data might be able to focus in on the importance of each predictor over the years, but the limits of historical studies include limits on available data.

Table 2. Coefficient estimates, standardized coefficient estimates, standard error, z-values, p-values, and VIFs for all variables and interactions modeled to explain the variation in oocyte density.

	Estimate	Std. Estimate	Std. Error	z-value	p-value	VIF
(Intercept)	-54.353143	5.32759	11.507551	-4.723	2.32e-06	
Temperature	2.325658	-0.00149	0.434272	5.355	8.54e-08	302.5066
Salinity	4.059463	0.23963	0.807455	5.027	4.97e-07	6234.849
Chlorophyll-a	1.880162	-0.07593	0.371690	5.058	4.23e-07	205577.5
Timing	-0.044710	-0.03766	0.032971	-1.356	0.175	1.869770
Disease	-0.234085	-0.11761	0.050440	-4.641	3.47e-06	1.563872
Temp:Chl-a	-0.074170	-0.14727	0.014264	-5.200	1.99e-07	208563.7
Sal:Chl-a	-0.128526	0.22438	0.026668	-4.819	1.44e-06	200212.2
Temp:Sal	-0.156658	0.04476	0.030453	-5.144	2.69e-07	7216.631
Temp:Sal:Chl-a	0.005080	0.20257	0.001023	4.963	6.93e-07	203777.7

Table 3. Coefficient estimates, standardized coefficient estimates, standard error, z-values, p-values, and VIFs for all variables and interactions modeled to explain the variation in gonadal area ratio.

	Estimate	Std. Estimate	Std. Error	z-value	p-value	VIF
(Intercept)	-10.61	0.20959	4.192	-2.530	0.01184	
Temperature	0.4148	0.02947	0.1581	2.624	0.00905	549.052927
Salinity	0.825	0.06301	0. 3068	2.876	0.00426	11041.981957
Chlorophyll-a	-0.0006376	-0.01604	0.0002558	-2.493	0.01312	1.259867
Timing	7.373	-0.00164	1.863	3.957	9.14e-05	65689.707249
Disease	-0.09405	-0.04911	0.01641	-5.730	2.12e-08	2.235635
Temp:Sal	-0.03325	0.03597	0.01147	-2.897	0.00399	13058.404479
Temp:Timing	-0.2859	0.01790	0.07108	-4.022	7.02e-05	59478.202667
Sal:Timing	-0.6068	0.01434	0.1445	-4.200	3.37e-05	54753.681021
Temp:Sal:Timing	0.02349	0.03090	0.005492	4.277	2.43e-05	50385.409811

Table 4. Coefficient estimates, standardized coefficient estimates, standard error, t-values, p-values, and VIFs for all centered variables and interactions modeled to explain the variation in oocyte diameter.

	Estimate	Std. Estimates	Std. Error	t-value	p-value	VIF
(Intercept)	36.386273	36.38627	0.191977	189.534	< 2e-16	
Temperature	-1.835995	-1.4816	0.347082	-5.290	1.86e-07	6.407205
Salinity	-0.118202	-0.2309	0.190514	-0.620	0.53526	11.312134
Chlorophyll-a	0.025782	0.6364	0.009674	2.665	0.00796	4.657822
Timing	0.176113	0.1495	0.368086	0.478	0.63254	7.975487
Disease	2.873985	1.4255	0.575229	4.996	8.17e-07	6.648445
Disease:Chl-a	0.186620	2.2849	0.028075	6.647	8.05e-11	5.866701
Salinity:Temp	-0.011946	-0.0188	0.209377	-0.057	0.95452	8.421982
Salinity:Chl-a	-0.028780	-1.3878	0.012141	-2.371	0.01815	14.613666
Salinity:Timing	0.601564	0.9976	0.196912	3.055	0.00237	13.310504
Temp:Timing	0.913057	0.6255	0.320584	2.848	0.00458	5.650314
Temp:Chl-a	-0.063502	-1.2650	0.014671	-4.328	1.83e-05	5.823875
Chl-a:Timing	-0.098878	-2.0721	0.020517	-4.819	1.93e-06	8.703007
Temp:Chl-a:Salinity	0.107350	4.2593	0.014033	7.650	1.09e-13	19.036245
Temp:Chl-a:Timing	0.112568	1.9037	0.024302	4.632	4.66e-06	11.798200
Salinity:Chl-a:Timing	0.104046	4.1773	0.018182	5.722	1.84e-08	32.501403

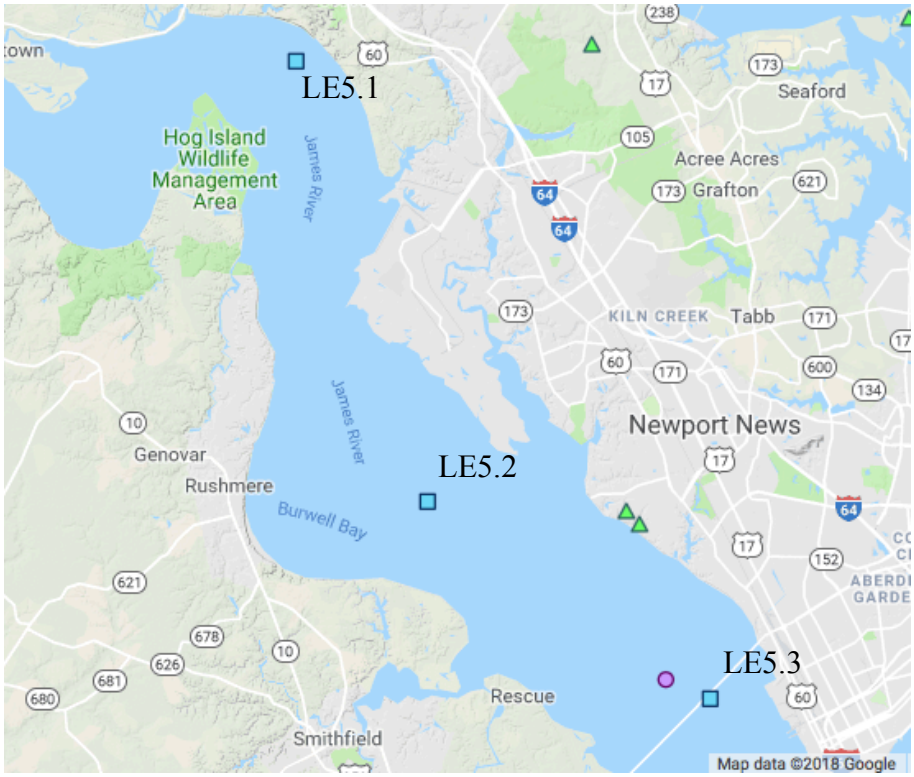

Figure 13. Stations LE5.1, LE5.2, and LE5.3 from the Virginia Estuarine and Coastal Observing System marked by blue squares. Image from Google Maps.

SUMMARY

The objectives of this study were to 1) quantify the change in reproduction since 1988 and to 2) determine the cause of the reproductive patterns observed. Using the histological archive at VIMS, oocyte counts per field of view (density) were recorded from 672 individuals, gonadal area ratios were calculated for 481 individuals, and two oocyte diameter measurements were made for five oocytes from 724 individuals. Segmented regressions were performed to look at changes over time with an ANOVA performed on the diameter data. The environmental variables summer temperature, summer salinity, peak recorded chlorophyll-a concentrations, the timing of the peak chlorophyll-a concentrations, and *Perkinsus marinus* weighted prevalence were then used to model each of the reproductive parameters.

In both oocyte density and gonadal area ratio, there was a sharp increase in 2003. The regression for oocyte density showed that the increase was stable, whereas gonad area ratios have been declining slightly but significantly since 2003. Oocyte size in the years 2001 and 2002 were significantly lower than the sizes for the years 2000 and 2003. All environmental variables that were not collinear were significant with the exception of timing of peak chl-a for oocyte density. For every model, interactions and multicollinearity limited the ability to make interpretations about individual predictors. However, disease was not collinear and did not have significant interactions in the

density or area ratio models, so it can be stated that disease had a significant, negative effect on oocyte density and gonadal area ratio.

Despite the cause of the increase in reproductive effort being clouded, the increase in reproduction combined with the recent increase in oyster populations Bay-wide should give us hope for the future of oysters in the area. With the knowledge that oysters are able to respond to environmental changes and increase their reproduction supports the use of management strategies such as sanctuaries from harvest. These sanctuaries allow oysters to develop local adaptation by leaving them exposed to the challenges of the area, rather than harvesting them before they experience those selective pressures.

LITERATURE CITED

Alvarez, M.R., Friedl, F.E., Hudson, C.M., & O'Neill, R.L. 1992. Uptake and tissue distribution of abiotic particles from the alimentary tract of the American oyster: A simulation of intracellular parasitism. *Journal of Invertebrate Pathology* 59(3): 290-294.

Andrews, J.D. 1962. Oyster mortality studies in Virginia: IV. MSX in James River public seed beds. *Proceedings of the National Shellfisheries Association* 53: 65-84.

Andrews, J.D. & Hewatt, W.G. 1957. Oyster mortality studies in Virginia. II. The fungus disease caused by *Dermocystidium marinum* in oysters of Chesapeake Bay. *Ecological Monographs* 27: 1-25.

Aranda, D. A., Díaz, M. E., Reynoso, F. L., Brulé, T., Montero, J., & Cárdenas, E. B. 2014. Reproductive strategies of the eastern oyster *Crassostrea virginica* (Gmelin 1791) in tropical lagoons of the Mexican Gulf of Mexico. *Journal of Shellfish Research* 33(1): 145-152.

Barber, R. D., Kanaley, S. A., & Ford, S. E. 1991. Evidence for regular sporulation by *Haplosporidium nelsoni* (MSX)(Ascetospora; Haplosporidiidae) in spat of the American oyster, *Crassostrea virginica*. *Journal of Eukaryotic Microbiology* 38(4): 305-306.

Bayne, B. L., Holland, D. L., Moore, M. N., Lowe, D. M. and Widdows, J. 1978. Further studies on the effects of stress in the adult on the eggs of *Mytilus edulis*. *J. Mar. Biol. Ass. U. K.* 58: 825-841.

Burreson, E. M. 1994. Further evidence of regular sporulation by *Haplosporidium nelsoni* in small oysters, *Crassostrea virginica*. *The Journal of Parasitology* 80(6): 1036-1038.

Burreson, E. M., Stokes, N. A., & Friedman, C. S. 2000. Increased virulence in an introduced pathogen: *Haplosporidium nelsoni* (MSX) in the eastern oyster *Crassostrea virginica*. *Journal of Aquatic Animal Health* 12(1): 1-8.

Burreson, E. M. & Ragone Calvo, L. M. 1996. Epizootiology of *Perkinus marinus* disease of oysters in Chesapeake Bay with emphasis on data since 1985. *Journal of Shellfish Research* 15: 17-34.

Carnegie, R. B., & Burreson, E. M. 2009. Status of the Major Oyster Diseases in Virginia 2006-2008 A Summary of the Annual Oyster Disease Monitoring Program.

Carnegie, R. B. & Burreson, E. M. 2011. Declining impact of an introduced pathogen: *Haplosporidium nelsoni* in the oyster *Crassostrea virginica* in Chesapeake Bay. *Marine Ecology Progress Series* 432: 1-15.

Cobb, N. A. 1894. Contributions to an economic knowledge of Australian rusts (Uredineae) Chap. 10. Agr. Gas. N. S. W. 5: 239-50.

Coen, L.D., Brumbaugh, R.D., Bushek, D., Grizzle, R., Luckenbach, M.W., Posey, M.H., Powers, S.P. & Tolley, S.G. 2007. Ecosystem services related to oyster restoration. *Marine Ecology Progress Series* 341: 303-307.

Combes, C. 2001. *Parasitism: The ecology and evolution of intimate interactions*. University of Chicago Press.

Cox, F. E. 2002. History of human parasitology. *Clinical microbiology reviews* 15(4): 595-612.

Dame, R. F. 1972. The ecological energies of growth, respiration and assimilation in the intertidal American oyster *Crassostrea virginica*. *Marine biology* 17(3): 243-250.

Dobson, A., Lafferty, K. D., Kuris, A. M., Hechinger, R. F., & Jetz, W. 2008. Homage to Linnaeus: how many parasites? How many hosts?. *Proceedings of the National Academy of Sciences* 105(Supplement 1): 11482-11489.

Dormann, C. F., Elith, J., Bacher, S., Buchmann, C., Carl, G., Carré, G., Marquéz, J. R., Gruber, B., Lafourcade, B., Leitão, P. J., Münkemüller, T. 2013. Collinearity: a review of methods to deal with it and a simulation study evaluating their performance. *Ecography* 36(1): 27-46.

Eble, A. F. & Scro, R. 1996. General Anatomy. In V. Kennedy, R. Newell, & A. Eble (Eds.), The eastern oyster *Crassostrea virginica* (pp. 19–73). Maryland: Maryland Sea Grant College.

Evans, J. P., & Sherman, C. D. 2013. Sexual selection and the evolution of egg-sperm interactions in broadcast-spawning invertebrates. *The Biological Bulletin* 224(3): 166-183.

FAO (Food and Agriculture Organization). 2016. The State of World Fisheries and Aquaculture 2016. SOFIA, Rome.

Ford, S.E. & Bushek, D. 2012. Development of resistance to an introduced marine pathogen by a native host. *Journal of Marine Research* 70: 205-223.

Frick, W. F., Puechmaille, S. J., Hoyt, J. R., Nickel, B. A., Langwig, K. E., Foster, J. T., Barlow, K.E., Bartonička, T., Feller, D., Haarsma, A., Herzog, C., Horáček, I., van der Kooji, J., Mulkens, B., Petrov, B., Reynolds, R., Rodrigues, L., Stihler, C. W., Turner, G. G., & Kilpatrick, A.M. 2015. Disease alters macroecological patterns of North American bats. *Global Ecology and Biogeography* 24(7): 741-749.

Froehlich, H. E., Runge, C. A., Gentry, R. R., Gaines, S. D., & Halpern, B. S. 2018. Comparative terrestrial feed and land use of an aquaculture-dominant world. *PNAS* 115(20): 5295-5300.

Galtsoff, P. S. 1964. The American oyster Crassostrea virginica (Gmelin). *US Fish Wildlife Service Fisheries Bulletin* 64: 1-480.

Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M., Baker, D., Marois, E., Russell, S. & Burt, A., 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nature biotechnology* 34(1): 78.

Hanson, O. A. 1905. Insect Parasitism. Senior thesis, Kansas State Agricultural College.

Harding, J. M., Mann, R., & Southworth, M. J. 2008. Shell length-at-age relationships in James River, Virginia, oysters (*Crassostrea virginica*) collected four centuries apart. *Journal of Shellfish Research* 27(5): 1109-1115.

Hayward, A. D., Nussey, D. H., Wilson, A. J., Berenos, C., Pilkington, J. G., Watt, K. A., Pemberton, J. M. & Graham, A. L. 2014. Natural selection on individual variation in tolerance of gastrointestinal nematode infection. *PLoS biology* 12(7): e1001917.

Hudson, K. 2017. Virginia shellfish aquaculture situation and outlook report: Results of the 2016 Virginia shellfish aquaculture crop reporting survey. Virginia Sea Grant Marine Extension Program and Virginia Institute of Marine Science.

Hudson, P. J., Dobson, A. P., & Newborn, D. 1998. Prevention of population cycles by parasite removal. *Science* 282(5397): 2256-2258.

Jaeckle, W. B. 1995. Variation in the size, energy content, and biochemical composition of invertebrate eggs: correlates to the mode of larval development. In McEdward L. (Ed) Ecology of Marine Invertebrate Larvae (pp. 49-78). CRC Press.

Kirby, M.X. 2004. Fishing down the coast: Historical expansion and collapse of oyster fisheries along continental margins. *Proceedings of the National Academy of Science* 101: 13096-13099.

Lafferty, K. D., Dobson, A. P., & Kuris, A. M. 2006. Parasites dominate food web links. *Proceedings of the National Academy of Sciences*, 103(30): 11211-11216.

Lane, H. S., Webb, S. C., & Duncan, J. 2016. *Bonamia ostreae* in the New Zealand oyster *Ostrea chilensis*: a new host and geographic record for this haplosporidian parasite. *Diseases of aquatic organisms* 118(1): 55-63.

Lorio, J. W. & Malone, S. 1994. The cultivation of American oysters. *Crassostrea virginica*.

Louie, A., Song, K. H., Hotson, A., Tate, A. T., & Schneider, D. S. 2016. How many parameters does it take to describe disease tolerance?. *PLoS biology* 14(4): e1002435.

Mackin, J. G., Owen, H. M., & Collier, A. 1950. Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science* 111(2883): 328-329.

Martenot, C., Fourour, S., Oden, E., Jouaux, A., Travaille, E., Malas, J. P., & Houssin, M. 2012. Detection of the OsHV-1 µVar in the Pacific oyster *Crassostrea gigas* before 2008 in France and description of two new microvariants of the Ostreid Herpesvirus 1 (OsHV-1). *Aquaculture* 338: 293-296.

Newell, R.I.E., Hilbish, T.J., Koehn, R.K., & Newell, C.J. 1982. Temporal Variation in the Reproductive Cycle of *Mytilus edulis* L. (Bivalvia, Mytilidae) from Localities on the East Coast of the United States. *Biological Bulletin* 162(3): 299-310.

Paynter, K. T., & Burreson, E. M. 1991. Effects of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*: II. Disease development and impact on growth rate at different salinities. *Journal of Shellfish Research* 10(2): 425-431.

Paynter, K. T., Politano, V., Lane, H. A., Allen, S. M., & Meritt, D. 2010. Growth rates and prevalence of *Perkinsus marinus* in restored oyster populations in Maryland. *Journal of Shellfish Research* 29(2): 309-317.

Råberg, L., Sim, D., & Read, A. F. 2007. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science* 318(5851): 812-814.

Ray, S. M. 1954. Biological Studies of *Dermocystidium marinum*, A Fungus Parasite of Oysters. Doctoral thesis, The Rice Institute.

Reňé, A. Alacid, E., Ferrera, I., & Garcés, E. 2017. Evolutionary Trends of Perkinsozoa (Alveolata) Characters Based on Observations of Two New Genera of Parasitoids of

dinoflagellates, *Dinovorax*gen. nov. and *Snorkelia* gen. nov. *Frontiers in Microbiology* 8:1594.
Rodríguez-Jaramillo, C., Hurtado, M. A., Romero-Vivas, E., Ramírez, J. L., Manzano, M., & Palacios, E. 2008. Gonadal development and histochemistry of the tropical oyster, *Crassostrea corteziensis* (Hertlein, 1951) during an annual reproductive cycle. *Journal of Shellfish Research* 27(5): 1129-1141.
Roy, B. A. & Kirchner, J. W. 2000. Evolutionary dynamics of pathogen resistance and tolerance. *Evolution* 54(1): 51-63.
Schafer, J. F. 1971. Tolerance to plant disease. *Annual review of phytopathology* 9(1): 235-252.
Shpigel, M., Barber, B. J., & Mann, R. 1992. Effects of elevated temperature on growth, gametogenesis, physiology, and biochemical composition in diploid and triploid Pacific oysters, *Crassostrea gigas* Thunberg. *Journal of experimental marine biology and ecology* 161(1): 15-25.
Soniat, T.M. & Ray, S.M. 1985. Relationships between possible available food and the composition, condition and reproductive state of oysters from Galveston Bay, Texas. *Contributions in Marine Science* 28: 109-121.
Sunila, I., Hamilton, R. M., & Dungan, C. F. 2001. Ultrastructural Characteristics of the In Vitro Cell Cycle of the Protozoan Pathogen of Oysters, *Perkinsus marinus*. *Journal of Eukaryotic Microbiology* 48: 348-361.
Thomas, F., Guégan, J.F., & Renaud, F. (Eds.) 2009. Ecology and evolution of parasitism. Oxford University Press. New York, New York. 240 pp.
Thompson, R.J. 1979. Fecundity and reproductive effort in the blue mussel (*Mytilus edulis*), the sea urchin (*Strongylocentrotus droebachiensis*) and the snow crab (*Chionoecotes opilio*) from populations in Nova Scotia and Newfoundland. *Journal of the Fisheries Research Board of Canada* 36: 955-964.
Thompson, R., Newell, R., Kennedy, V., & Mann, R. 1996. Reproductive processes and early development. In V. Kennedy, R. Newell, & A. Eble (Eds.), The eastern oyster *Crassostrea virginica* (pp. 335–390). Maryland: Maryland Sea Grant College.