THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE PROGRESSION OF *HAPLOSPORIDIUM NELSONI* (MSX) IN *CRASSOSTREA VIRGINICA* (AMERICAN OYSTER) ON CAPE BRETON ISLAND, NOVA SCOTIA, CANADA

by

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I would like to dedicate this thesis to:

Curley, Mabel, and Willow – thank you for so many laughs.
Wendy, Leonard, and Eric – you taught me what matters most.
Janice, Austin, and Claire – you are who matters most.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 6

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

ABSTRACT

In the late 1950s, oyster populations in the Chesapeake and Delaware Bays suffered mortality from the parasite, *Haplosporidium* nelsoni that is now present from Florida to Maine. In 2001 and 2002, *H. nelsoni* caused oyster mortality in the Bras d'Or Lake, Cape Breton, N.S. Temperature and food availability are two factors thought to influence the development of *H. nelsoni* based on field observations from the U.S. and mathematical models developed for the parasite. This research investigated the role of environmental parameters that influence the progression of *H. nelsoni*. Field studies showed that (i) *H. nelsoni* prevalence differed among oysters within tens of metres, (ii) high prevalence does not always result in high mortality, (iii) detailed field studies could provide more valuable information than laboratory experiments, and (iv) current models for infection and disease progression could be tested and modified at a small scale in Cape Breton. To examine the role of temperature in the progression of the parasite, oysters were maintained at 5°C, 10°C, 15°C, 20°C, and 24°C. Surprisingly, the parasite prevalence was reduced under controlled conditions. Food availability treatments showed there was a reduction in the parasite burden of treated oysters compared to the field samples, but there was no clear pattern with respect to parasite progression or reduction and the amount of food available. These studies suggest that temperature and food availability may have been influencing some factor(s) outside of the host-parasite direct interaction such as the abundance of intermediate and/or reservoir host(s). Alternatively, it is possible that laboratory conditions had an effect on the results. Eighteen years after the initial oyster mortality in the Bras d'Or Lake, oysters from several locations within the Bras d'Or Lake having different histories of exposure to *H. nelsoni* were placed in an area known to have high *H. nelsoni* activity. Following 16 months exposure, high mortalities were observed from all originating oyster populations, providing evidence of a lack of resistance to mortality in any of the originating locations. The lack of mortality in some originating locations was likely due to low level or absent *H. nelsoni* in those areas. A potential model that includes the parasite, intermediate/reservoir hosts, and oyster mortality is proposed to explain some of these observations across Cape Breton.

LIST OF ABBREVIATIONS USED

ANOVA	Analysis of variance
bp	base pair
DFO	Department of Fisheries and Oceans
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
HCl	hydrogen chloride
N.B.	New Brunswick
N.S.	Nova Scotia
MSX	Multinucleated Spherical X (unknown)
nm	nanometres
OIE	World Organization for Animal Health
OsHV-1	Ostreid herpesvirus-1
PCR	Polymerase Chain Reaction
Tris	Tris(hydroxymethyl) aminomethane
U.S.	United States
UV	Ultraviolet

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CHAPTER 1 – INTRODUCTION

1. INTRODUCTION

Oysters play a critical role in maintaining ecosystem integrity by providing habitat for other species and feeding on suspended particles in the water column that exposes them to pathogens, some of which can have devastating effects. Their role in local and global economies has increased as traditional means of harvesting is replaced with more intense aquaculture operations (Marquis, et al. 2015). In 2002, the Bras d'Or Lake, Cape Breton, Nova Scotia suffered its first catastrophic oyster (*Crassostrea virginica*) losses on several private leases and on numerous public oyster beds. Within one year, it was determined that the causative agent for the mortalities was *Haplosporidium nelsoni* (Stephenson et al., 2003). *H. nelsoni* caused significant oyster losses in the Chesapeake and Delaware Bays in the late 1950s (Haskin and Ford, 1979).

Prior to 1957, there were no recorded oyster mortalities in Delaware Bay and mortalities that did occur were attributed to *Perkinsus marinus* (Andrews, 1968; Burreson et al., 2000). In 1957, *H. nelsoni* killed 90-95% of the oysters on the planting grounds and approximately 60% of the oysters on the seed-beds (where young oysters are placed to until maturity) in Delaware Bay (Ford and Haskin, 1982). In 1959, a similar mortality event occurred in the Chesapeake Bay where within two to three years, 90-95% of the oysters in the planting areas were lost. Losses up tributary rivers to the Chesapeake Bay (e.g. James River) were minimal (Burreson and Ford, 2004). A report by Kern (1976) identified a Haplosporidian with a similar appearance to *H. nelsoni* in *C. gigas* from Korea, leading Andrews (1980) to suggest that the parasite was introduced to the U.S eastern seaboard via imported *C. gigas*. *H. nelsoni* does not cause mortality in *C. gigas* and is maintained at a very low prevalence

(0.28% in Korea) as determined by histological methods (Kern, 1976), with PCR indicating a prevalence of 1.8% (Wang et al., 2010). *H. nelsoni* prevalence levels in *C. virginica* usually exceed 75%, and often exceed 89%, with corresponding mortality levels (Sunila et al. 1999, Burreson et al., 2000).

Even though it has been studied for over 50 years along the U.S. eastern seaboard, from Florida to Maine, there are still critical gaps in the knowledge of the biology of this organism. Of great importance is that the life cycle remains unknown. With little knowledge of the life cycle, including intermediate and/or reservoir hosts and details around the impact of environmental parameters, it is difficult to model and predict the transmission of this parasite from one organism to another and from one geographic area to another.

Co-evolution can be defined as the ongoing evolution of resistance-related genes in the host population in parallel with the ongoing evolution of genes associated with infectivity and/or virulence in the parasite population (Kaltz and Shykoff, 1998). A major influence on coevolution of host-parasite systems is their environment. Because the environment in which host-parasite relationships exist is dynamic, environmental conditions can arise that are advantageous to the host, the parasite, neither, or both at any given time. In addition to the influence of coevolution on host-parasite dynamics and disease development, environmental conditions can influence the relationship. To protect marine environments and aquaculture operations from introduced pathogens, a reduction in pathogen abundance must occur, as well as limiting the influence of factors such as reservoir hosts, environmental conditions, and other elements related to pathogen survival and disease outbreak. This will require a better understanding of these dynamic interactions (Harvell et al., 2004). The challenge lies with those pathogens, such as *H. nelsoni*, for which there is

little information despite repeated efforts to gather critical information, including life cycle patterns. Ecological factors such as habitat structure, host life history stages, and food all contribute to either the success or failure of infection (Krist et al., 2004). Most aquatic pathogens are ingested or have surface contact with potential hosts and initial resistance to infection is a function of the immunocompetence of the host (Poulin et al. 2010). Increased stress can reduce immune competence. In addition to causing increased stress on host organisms, Lenihan et al. (1999) suggest that stress-inducing environments can also have deleterious effects on pathogens that further complicates the interaction.

Resistance, as defined by Ayres and Schneider (2012) is the ability to clear pathogens; tolerance as the ability to reduce the impact on the health of a host for given level of pathogens. In simpler terms, "resistance (the ability to clear microbes)" and "tolerance (the ability to endure microbes)." Resistance is the term most often associated with the *H. nelsoni* and *C. virginica* relationship and when used throughout the following chapters it refers to the oyster's ability to resist mortality from the parasite, not resist infection. With respect to *H. nelsoni*, *C. gigas* is thought to have developed a tolerance of infection given the low infection levels and lack of mortality from the parasite (Wang et al., 2010). *C. virginica* has not developed the ability to prevent infection nor the ability to maintain a low parasite levels in most circumstances. Even for those oysters where significant efforts have been put forth to develop strains that are resistant to mortality, they will eventually succumb to infection and die, unlike *C. gigas*.

The relationship between *C. virginica* and some of its pathogens is complicated and not well understood. There are two reported incidences involving oysters where resistance to disease-induced mortality was present in the re-colonizing population (Ford and Haskin,

1987). The first incident occurred in Malpeque Bay, P.E.I., Canada in 1913, where approximately 90% of the oyster population died, but successive generations have not demonstrated any further mortality from the unknown agent that caused the earlier outbreak (Needler and Logie, 1947 in Burreson and Ford, 2004). Interestingly, despite the lack of mortality among native oysters in P.E.I. that are immune to Malpeque disease, naïve oysters imported to Malpeque Bay die approximately 18 months after transfer. The second incident involved *H. nelsoni* and *C. virginica,* where following the significant mortalities of the late 1950s in Delaware and Chesapeake Bays, some degree of resistance to mortality from the parasite developed (Haskin and Ford, 1979). In contrast to Malpeque disease, oysters bred for resistance to mortality from *H. nelsoni* still succumb to the parasite within six years despite artificial selection efforts over the past 50 years. These examples show the complicated dynamics between two lethal pathogens of *C. virginica* – one where those oysters that survived the initial infection have inherited immunity to the pathogen, and another, that despite 50 years of intensive selection efforts, oysters are still challenged to survive infection beyond 5-6 years.

The mortality in the late 1950s in the Delaware and Chesapeake Bays placed immediate, selection pressure on the native oyster population. However, little to no survival improvement was observed during the next 30 years because of limited selection pressure placed on the upper bay beds that released non-resistant seed into the lower bays that then suffered mortality (Burreson and Ford, 2004). It was not until the mid-1980s, during years of drought causing higher salinity in the upper bay areas, that heavy mortalities were observed again (Haskin and Ford, 1986). The elevated salinity was proposed to have allowed for *H. nelsoni* range expansion upriver thereby killing susceptible seed-producing oysters resulting in increased oyster resistance to the *H. nelsoni* parasite. Evidence for this

new resistance included: (i) imported stocks became heavily infected where wild stocks from the upper bay had fewer infections and (ii) infections detected with PCR in upper bay oysters did not progress to the disease state as seen via histology (Burreson and Ford, 2004). Despite the degree of resistance to proliferation of the parasite and subsequent disease and mortality, oysters were not resistant to the parasite and after 5-6 years of continual exposure, they died from advanced infections. Results of a selective breeding program for oysters that are resistant to both *H. nelsoni* and *P. marinus* have developed oysters with some resistance to *P. marinus* as demonstrated by the delay in the development of advanced infections and a high degree of resistance to *H. nelsoni* (Burreson and Ford, 2004).

1.2 ENVIRONMENTAL INFLUENCES

Environmental conditions play a significant role in the life cycle of many parasites, including *H. nelsoni*, but for some parasites, how these conditions affect different components of the life cycle is unknown. Of the numerous environmental factors involved in disease development, temperature is often critical for the introduction, transmission, and establishment of a potential pathogen in a marine area (Ford et al, 1999 and Harvell et al., 2004).

Barber et al. (1991) stated that the observed resistance to mortality was the result of an internal host environment that is not suitable for parasite for survival. This "resistance" is different from other types of resistance often referred to with *C. virginica*. For example, *C. virginica* that survived a disease outbreak in Malpeque Bay, Prince Edward Island in 1913 are either resistant to infection from the still unknown disease agent or able to live with an infection and show no increased morbidity or mortality. With respect to resistance to mortality observed in *C. virginica* infected with *H. nelsoni*, it is possible that there are

environmental conditions in which infected oysters can survive but are not suitable to support the growth and development, or even the survival, of *H. nelsoni*. An alternative hypothesis is that environmental conditions result in weakened infective particles, thereby causing infections to not reach lethal levels (Dr. S. Adamo, personal communication, 2018). For example, there are locations with freshwater influences that could cause a reduction in salinity that can be survived by oysters but are detrimental to parasites. Ford and Haskin (1988) showed that *H. nelsoni* plasmodia exposed to salinity lower than 15‰ experience loss of cell membrane integrity causing a reduction in parasite survival and removal of dead or damaged parasites by oyster haemocytes. Furthermore, while Andrews (1964) first identified the correlation between *H. nelsoni* infection reduction and salinity and Sprague et al. (1969) conducted laboratory experiments in which *H. nelsoni* disappeared (histologically) from infected oysters held at 10‰ or less for six weeks. Haskin and Ford (1982) demonstrated the susceptibility of *H. nelsoni* to low salinity environments during experiments where infected oysters were moved upriver to lower salinity regions. This resulted in a reduction in *H. nelsoni* prevalence and disease expression compared to those that remained in higher salinity environments. Additionally, under field conditions, *H. nelsoni* reached undetectable levels (histologically) when infected oysters were moved to locations where salinities went as low as 3-9 ‰. As stated by Haskin and Ford (1982), "… low salinity itself is unfavourable to MSX" (Multi-nucleated Spherical X-unknown). Ford et al. (1993) provided an explanation for the field observations when they showed that *C. virginica* hemocytes ingested *H. nelsoni* plasmodia with freshwater-induced cell membrane damage but were unable to ingest undamaged *H. nelsoni* plasmodia.

Haskin and Douglass (1971) suggested that temperature was influencing the effects of *H. nelsoni* and in the Delaware Bay area, the years of reduced disease were preceded by

unusually harsh winters and the years of high prevalence were preceded by unusually warm winters (Ford and Haskin, 1982). This demonstrated that air temperatures, which influence water temperatures, affected disease status the following year. The model developed by Hofmann et al. (2001) concluded that temperature is the most critical factor in the survival and spread of *H. nelsoni,* and it predicted that as winters continue to warm, *H. nelsoni* will remain established in its current locations and will continue to move northward. While increasing temperatures alone do not explain the entire pattern observed with *H. nelsoni* outbreaks (Hofmann et al., 2001), most epizootics observed in the northern U.S. have occurred during a warm period (Easterling et al., 1997). This supports their theory that climate change is a contributing factor to the northward movement of *H. nelsoni*.

Over the past decades as water temperatures increased, the number of *H. nelsoni* associated disease outbreaks also increased (Cook et al., 1998). Unlike the steady northward movement of *Perkinsus marinus* from the lower Chesapeake to Maine between 1985 and 1995 (Burreson and Ragone Calvo, 1996; Ford, 1996), the pattern for *H. nelsoni* had been much more irregular and unpredictable (Hofmann et al., 2001). One interesting fact is that the association between *H. nelsoni* and high temperature - disease outbreak pattern does not seem to exist for the Gulf of Mexico region (Hofmann et al., 2001). Outbreaks in the northern areas may be due to higher than normal temperatures and the 20-year delay in the outbreak in Long Island Sound reported by Sunila et al. (1999) may have been due to deeper waters staying below a critical temperature for a necessary period until the outbreak occurred. Sunila et al. (1999) proposed there is a 6-8 year cycle to *H. nelsoni* outbreaks because water temperatures in more northern areas periodically are warm enough for an intermediate host to establish itself. When water temperatures lower to normal temperatures for that area, the intermediate host retreats to more southern areas. Exactly what aspect(s) of

the life cycle are being affected by lower temperatures (i.e. number of infective particles, infectivity of infective particles, intermediate host factors, parasite development factors) is still unknown.

In circumstances such as reduced food availability, oysters may need to use energy reserves to maintain their metabolism and a disruption in their metabolism could result in systemic *H. nelsoni* infections and be the cause of mortality (Newell R., 1985). Haskin and Andrews (1988) suggested that a reduction in phytoplankton bloom could make oysters more susceptible to disease. The model proposed by Hofmann et al. (2001) included dry (high salinity), warm (winters above 3^{o}C), and low food (no spring algae bloom) conditions that generated an *H. nelsoni* prevalence comparable to warm conditions and dry, cold (lower than 3^{o}C), and low food conditions generated *H. nelsoni* prevalence similar to cold conditions. Combinations of factors that included high food conditions created prevalence results that differed compared to only changing only one other single factor, thus based on the model, the role of increased food is not clear. Powell et al. (1999) proposed that oyster filtration rate would decrease because of increased food availability, thus infective particle exposure and subsequent disease prevalence would decrease with lower filtration rates and that a small change in filtration rate could cause a change in disease prevalence.

Ford et al. (1999) stated that the quantity of food available to the parasite is a result of the quantity of food available to the oyster and the increase in nutrient availability favours rapid parasite proliferation. Removal of the spring algal bloom disrupted the annual infection cycle and because temperature and salinity alone cannot explain the observed annual infection cycles, other factors such as food availability are involved. Lenihan et al. (1999) conducted field studies by placing oysters in various environments and determining how

their location affected their exposure to *Perkinsus marinus*. They found that oysters in locations where food was abundant filtered less, thus had less exposure to *P. marinus* infective particles. Oysters in locations with reduced food availability fed more frequently and for longer, therefore increased their exposure to infective particles. While this study may not be directly applicable to *H. nelsoni* because *P. marinus* has a direct life cycle and we do not know the life cycle of *H. nelsoni*, the idea of exposure to infective particles has merit for investigating factors that influence *H. nelsoni* infections beyond temperature and salinity.

The following table (Table 1.1) provides an overview of studies carried out since the initial oyster mortality in Delaware Bay caused by *H. nelsoni* that investigated the effect of temperature and/or salinity on the prevalence of *H. nelsoni* and oyster mortality. It was not until Andrews (1968) reviewed prevalence and mortality data that it was recognized that salinity affected disease outbreaks and Farley (1968) demonstrated the influence of temperature and salinity. Studies that followed provided further evidence to support these observations.

Table 1.1

Author(s) (Year)	Factor(s)	Nature of Work, Suggestion, or Finding
Andrews (1968)	Salinity	• Report following 10 years of monitoring oyster diseases in Chesapeake Bay • Oysters upstream from the higher salinity bay areas did not suffer mortality from *H. nelsoni* outbreak
Farley (1968)	Salinity and Temperature	• Field investigation reporting disease status in three separate locations to follow the development of disease • Increases in *Minchinia nelsoni* (now *H. nelsoni*) prevalence with the return of increased salinity and temperature

Author(s) (Year)	Factor(s)	Nature of Work, Suggestion, or Finding
Sprague et al. (1969)	Salinity	• Held oysters at low salinity to determine effect of salinity • Infected oysters held in 10‰ salinity for six weeks showed no histological evidence of *H. nelsoni*
Ford and Haskin (1982)	Salinity and Temperature	• Review of research to date with specific interest in temperature and salinity effects • Colder temperatures could result in a lower number of intermediate or reservoir hosts, thus less infective material available • *H. nelsoni* may not be able to cope with cold temperatures • *H. nelsoni* is a highly aerobic organism (many mitochondria), may not be able to survive anaerobic conditions inside overwintering oyster • Delaware Bay had reduced *H. nelsoni* disease following unusually harsh winters (mid 1960s and 1972-1976) • Delaware Bay had high *H. nelsoni* prevalence years preceded by unusually warm winters (mid 1960s and 1972-1976) • Despite high water flow, and lower salinity during 1972-1976, *H. nelsoni* prevalence was high • Salinity is a factor, less influential than temperature with respect to *H. nelsoni* infection and oyster mortality
Haskin and Ford (1982)	Salinity	• Moved oysters upstream to investigate management strategy • *H. nelsoni* infected oysters moved upstream to lower salinity areas had a lower infection prevalence than those kept in higher salinity areas
Ford (1985)	Salinity	• Infected oysters were moved from high salinity waters to low salinity waters and back to high salinity waters to monitor for disease progression • *H. nelsoni* is lost from oysters exposed to low salinity • The lower the salinity, the more rapid the loss of parasite • Direct low salinity of killing of the parasite vs. indirect low salinity mediated removal by the host was not determined • *H. nelsoni* unlikely to survive any area where salinities are below 10‰ for two weeks or more during the summer
Haskin and Ford (1986)	Salinity	• Monitored progression of *H. nelsoni* upstream to new locations • Increased salinity due to drought created situation where *H. nelsoni* moved upstream
Ford and Haskin (1988)	Salinity	• Investigated possible management strategies for *H. nelsoni* in oysters • Salinity below 10‰ results in a loss of cell membrane integrity for *H. nelsoni*

Author(s) (Year)	Factor(s)	Nature of Work, Suggestion, or Finding
Haskin and Andrews (1988)	Salinity and Temperature	• Carried out a review of studies to profile possible intermediate or reservoir host • Above 20‰ *H. nelsoni* is not inhibited, below 15‰ *H. nelsoni* infections are rare, below 10‰ *H. nelsoni* cannot survive • *H. nelsoni* range expansion observed where low salinities had previously provided protection • Favourable salinities, but harsh winters were followed by reduced *H. nelsoni* disease suggesting temperature has a long-term impact on disease • Salinity may be affecting intermediate or reservoir host populations • Cold winters of the north thought to prevent *H. nelsoni* disease outbreaks
Cook et al. (1988)	Temperature	• Focus is on *P. marinus*, examined *H. nelsoni* outbreaks also • Increased water temperatures associated with increased *H. nelsoni* outbreaks
Ford et al. (1993)	Salinity	• Examined the ability of haemocytes from bivalves to determine their ability to recognize and ingest *H. nelsoni* • Haemocytes from *C. virginica* (selected or unselected for resistance) are unable to ingest *H. nelsoni* plasmodia unless the plasmodia are damaged using low salinity • Haemocytes from *C. gigas* are also unable to digest undamaged *H. nelsoni* plasmodia • Haemocytes from *Geukensia demissa* (ribbed mussel) are able to phagocytize *H. nelsoni* plasmodia
Ewart & Ford (1993)	Salinity and Temperature	• Tested for *H. nelsoni* in the water column • Moving oysters into different areas of the water column may result in exposure to different salinities and temperatures providing some degree of protection from mortality from *H. nelsoni*
Ford et al. (1999)	Salinity and Temperature	• Examined host-parasite parasite interactions – infection process and transmission • Salinity and temperature are two major driving factors in infection patterns with *H. nelsoni*
Paraso et al. (1999)	Salinity and Temperature	• Developed a model to explain salinity effects on infections • Salinity variation had a larger effect on *H. nelsoni* infections than food availability or temperature at 5°C or higher • Cold winters produce a reduction in infections and affect following annual *H. nelsoni* infection cycles
Powell et al. 1999	Salinity and Temperature	• Investigated local effect and transmission • Developed a model that predicts higher salinity will increase infective particle concentration • Cold winters have multi-year effect • High salinity may reduce the effect of cold winter • At low salinity, the host-parasite infection dynamic is more complicated than at high salinity

Author(s) (Year)	Factor(s)	Nature of Work, Suggestion, or Finding
Hofmann et al. (2001)	Salinity and Temperature	• Developed model to examine climate variability on *H. nelsoni* and *C. virginica* • Model predicts temperature and salinity are major *H. nelsoni* disease outbreak factors; temperature having long term effects with salinity effects being more short-term

Table 1.1 Brief description of studies that examined the effects of temperature and/or salinity since the initial outbreak in Delaware Bay in 1957.

1.3 HOST PARASITE INTRODUCTION AND INTERACTIONS

When a parasite or pathogen populates an area, the outcomes vary from devastating host populations and possible habitat destruction to long-term presence in reservoir host populations (Adlard et al., 2015). In the Bras d'Or Lake, there was host population devastation in Nyanza Bay, ongoing low-level infection in Potlotek with little recorded mortality, and cyclical events in Gillis Cove with reports of *H. nelsoni* appearing, disappearing, and then reappearing. Presumably, the cycling of events is a result of environmental factors that allow pathogen populations to increase in abundance or have deleterious effects on the host resulting in mortality. Increases in reservoir host numbers, intermediate host abundance, both, or neither may be advantageous for the parasite. Additionally, environmental factors can have detrimental effects on hosts that increase their susceptibility to infection and disease progression (Adlard et al., 2015).

When disease introductions occur, how the invader arrived is usually unknown (Stewart, 1991), such as how *H. nelsoni* was introduced to the U.S. eastern seaboard (Burreson and Ford, 2004), and how it was introduced into the Bras d'Or Lake. If we are going to be able to control/manage aquatic disease agents in changing environments, we must utilize conventional diagnostic identification of pathogens and their hosts and collect more information related to range, transmission, host specificity, and environmental limits of

these pathogens and their hosts (Adlard et al., 2015). We have observed the unpredictability of *H. nelsoni* in different locations throughout the Bras d'Or Lake and in small locations outside of the Bras d'Or Lake such as MacDonald's Pond (Beresford, unpublished observations). This irregularity and lack of baseline knowledge about its life cycle and the influence of biotic and abiotic factors present in different locations makes predictions about *H. nelsoni* difficult and form the basis for investigations that follow.

To date, *H. nelsoni* has been detected in various locations throughout the Bras d'Or Lake, as well as in MacDonald's Pond and Aspy Bay (both outside of the Bras d'Or Lake on the east coast of Cape Breton). It has not been detected using DNA analysis or microscopy in natural oyster beds and oyster aquaculture leases elsewhere in Nova Scotia (N.S.), Prince Edward Island (P.E.I.), and New Brunswick (N.B.). Historically, the Bras d'Or Lake oyster industry provided oyster seed for the rest of N.S., P.E.I., and N.B. Anecdotal information from local oyster growers and industry regulators indicates that oysters were transferred from the Bras d'Or Lake to locations in N.S., P.E.I., and N.B. prior to, during, and following the mortality events of early 2000s. Given the importance of the oyster industry in some rural locations in N.S., as well as P.E.I. and N.B., investigations into the potential factors and conditions associated with *H. nelsoni* disease outbreaks is important.

There are many factors associated with *H. nelsoni* infections in *C. virginica*. Host density is usually an important factor related to parasite transmission and as the density of hosts increases so does the likelihood for parasite transmission. Spatial factors can influence the spread of disease and because filter feeders accumulate particles, including pathogens, from large distances (depending on water currents and feeding rates) they increase their likelihood for infection and disease progression (Bidegain et al., 2016). Depending on the nature of the

host, it may act as an unaffected reservoir for the pathogen thereby reducing dosage to disease susceptible hosts (Bidegain et al., 2016). Another possibility that could reduce available dosage is the presence of organisms in the locale that act as dead-end hosts of the parasite. Both reservoir hosts and dead-end hosts could result in immediate reduction in exposure of susceptible hosts to pathogens. Dead-end hosts would remove pathogens from the area while reservoir hosts would contribute to sustained pathogen presence in a given location. While spatial factors related to host density may be important for some aquatic diseases such as *P. marinus*, it has been shown that host density is not a factor for *H. nelsoni* infection (Ford, 1985), thus caution must be taken with such generalizations.

Macro-scale trends are observed in the U.S., but there have been few reports of laboratory experiments on environmental factors believed to affect disease progression. The focus of this thesis was to investigate the influence that environmental conditions have on the progression of *H. nelsoni* in oysters under controlled laboratory conditions and by using field studies. The field study in MacDonald's Pond examined the presence of small-scale prevalence differences. The first series of laboratory experiments investigated the influence of temperature on parasite progression. The second series of laboratory experiments investigated the role of food availability on parasite progression. The Nyanza Bay field study examined the possible development of natural resistance to mortality in Bras d'Or Lake oysters from *H. nelsoni* infection.

The purpose for the field studies carried out in MacDonald's Pond was to investigate the presence of microscale differences in a relatively small body of water where *H. nelsoni* was initially detected in oysters in 2007. Previous studies and mathematical models based on the U.S. based data did not consider the possibility that *H. nelsoni* infections may be different

from one location to another in small, connected water bodies such as MacDonald's Pond where one would expect that infection prevalence would be similar throughout the pond due to its small size (0.2 km^2).

The laboratory-based temperature experiments were designed to determine if *H. nelsoni* will progress more rapidly at higher temperatures than at lower temperatures. Given the assumed role of temperature based on observations in the United States, the role of temperature in the direct relationship between the parasite and the oyster host was investigated. Two models (Ford et al., 1999 and Hofmann et al., 2001) proposed that food availability was an important factor; furthermore, oyster mortalities in the Bras d'Or Lake occurred in areas of high food availability. The feeding experiments were designed to determine if food availability played a direct role in the progression of the parasite through the oyster hosts. Two different hypotheses with contrasting predictions were tested: (i) well-fed oysters would provide more energy to the parasites and so would succumb to the infection, or (ii) oysters that had less resources available to them would have weakened immune systems and so would develop advanced infections more rapidly.

The final study was designed to determine if there has been development of resistance to mortality from *H. nelsoni* in Bras d'Or Lake oyster populations that previously suffered mortality during the initial years of infection. In some of these locations there has been little to no mortality observed recently and there was an assumption by oyster leaseholders and government regulators that this lack of mortality was due to resistance of mortality from the parasite. The field experiment was carried out in an area known to have high *H. nelsoni* infection levels and oyster mortality. The experiment was designed to assess parasite activity at the transfer site using known susceptible oysters. The known susceptible oysters

were placed alongside those from locations that had previously suffered catastrophic losses from *H. nelsoni* but were recently showing little mortality at their source site, to determine if there has been development of resistance in any of these groups of oysters.

1.4 REFERENCES

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CHAPTER 2 - ANNUAL VARIATION AND SITE-SPECIFIC VARIATION OF *HAPLOSPORIDIUM NELSONI* PREVALENCE IN *CRASSOSTREA VIRGINICA* IN A SMALL BARACHOIS POND, IN CAPE BRETON, NOVA SCOTIA, CANADA

ABSTRACT

Field studies from the United States and mathematical models have shown that the three most important factors influencing *Haplosporidium nelsoni* infections in *Crassostrea virginica* are temperature, salinity, and food availability. These conclusions were derived from wide scale infection and disease trends observed in the United States since *H. nelsoni* arrived in the late 1950s and subsequent mathematical models. This chapter focused on small-scale observations from a small *H. nelsoni*-positive pond, MacDonald's Pond (~0.2 km^2), which has water movement due to wind and tidal action, thus it was expected that sampling sites would have similar *H. nelsoni* prevalence at a given time. The prevalence of *H. nelsoni* changed from year to year at a given location within the pond and this could be caused by changes in temperature and/or salinity from year to year. Furthermore, there were differences in *H. nelsoni* prevalence from site to site within the pond at a given sampling time. Given the differences in *H. nelsoni* prevalence among the sites during a given year, investigators will have to consider small-scale differences in sample locations when monitoring for *H. nelsoni* prevalence in the future. To determine the influence of temperature, salinity, and food availability, detailed field monitoring of these and other previously unconsidered factors along with disease progression will be necessary.

2.1 INTRODUCTION

In 1957, the Delaware Bay area suffered oyster mortalities in specific locations and over the next two years oyster mortalities expanded to other areas of Delaware Bay (Haskin et al. 1966). In 1959, similar events occurred in lower Chesapeake Bay (Andrews and Wood, 1967). The disease-causing organism known as multinucleated spherical "x" (x for unknown), now commonly called MSX, was identified as *Minchinia nelsoni* and is now classified as *Haplosporidium nelsoni* (Haskin et al. 1996).

From the late 1950s until the 1980s, *H. nelsoni*-linked oyster mortality seemed to be limited to mid-Atlantic estuaries (Ford and Tripp, 1996). Outbreaks occurred in the northeast through the 1980s and 1990s (Haskin and Andrews, 1988; Barber et al., 1997; Sunila et al., 1999) and in 2002 there were heavy mortalities at various sites in the Bras d'Or Lake, Cape Breton, Nova Scotia (Stephenson et al. 2003 and personal communication, Lorne Penny, Department of Fisheries and Oceans). Over the five years following the detection of *H. nelsoni* in the Bras d'Or Lake, two other sites along the coast of Cape Breton, Nova Scotia tested positive for *H. nelsoni*. In the more northern water body, South Harbour, within Aspy Bay, mortalities occurred, and the oyster population has been slow to recover. In the second water body, MacDonald's Pond, near the mouth of the Mira River, no mortalities have been observed despite variable seasonal and annual prevalence and variable expression of disease.

The models prepared by Ford et al. (1999) and Hofmann et al. (2001) suggested that changes in environmental conditions were a factor for the spread of *H. nelsoni*, and Ford et al. (1999) suggested that host density and host infection levels were not factors. Both models predicted that the greatest influences on the *H. nelsoni – C. virginica* interaction are: (i) temperature, (ii) food, and (iii) salinity.

It has been shown that temperature plays a critical role in the *H. nelsoni – C. virginica* interaction (Haskin and Douglass, 1971; Ford and Haskin, 1982) and this may be in part because, as ectotherms, many oyster metabolic processes are influenced heavily by temperature (Galtsoff, 1964; Newell, 1983), including circulation and removal of foreign substances. In the Delaware Bay area, years of reduced disease were preceded by unusually harsh winters and years of high prevalence were preceded by years of moderate or unusually warm winters (Ford and Haskin, 1982). The Hofmann et al. (2001) model implicated temperature as the most critical factor in the survival and spread of *H. nelsoni* and it predicted that as winters continue to warm *H. nelsoni* will remain established in existing locations and move northward. Over the past decades, as water temperatures increased, so have *H. nelsoni* associated disease outbreaks (Cook et al., 1998). One interesting fact is the association with *H. nelsoni* and high temperature followed by disease outbreak pattern does not seem to exist in the Gulf of Mexico region (Hofmann et al., 2001). Outbreaks in the northern areas may be due to higher than normal temperatures and the 20-year delay in the outbreak in Long Island Sound reported by Sunila et al. (1999) may have been due to deeper waters staying below a critical temperature for a necessary period until the outbreak occurred. They proposed that there is a 6-8 year cycle to *H. nelsoni* outbreaks due to water temperatures being warm enough for an intermediate host to establish itself in northern locations. When water temperatures lower to normal temperatures for that area, the intermediate host retreats to more southern areas. Following the initial mortalities in Delaware Bay, detection of *H. nelsoni* usually occurred after an oyster mortality in a given location. The difference between when *H. nelsoni* was detected and when a mortality occurred, if one occurred, is quite variable. Interestingly, Andrews (1968), as cited by

Burreson et al. (2000), observed possible *H. nelsoni* plasmodia in 1953, four years before the initial mortality associated with *H. nelsoni* was reported.

MacDonald's Pond, Cape Breton, Nova Scotia (Canada) is a small barachois pond on the east side of Cape Breton with direct exposure to the Atlantic Ocean resulting in water exchange during tidal changes. A barachois pond is a water body separated from the ocean, or, in some cases, the Bras d'Or Lake, by a barrier of sand and/or gravel. It is susceptible to periodic changes through the seasons, but usually remains open to the larger water body to which it is connected (Smith and Rushton, 1963). Oysters were transferred in and out of MacDonald's Pond for decades prior to the *H. nelsoni* outbreak in the Bras d'Or Lake in 2002. Based on oyster movement restrictions immediately following the mortality events of 2002, it is reasonable to assume that *H. nelsoni* was transferred into this pond sometime prior to the 2002 mortalities seen in the Bras d'Or Lake. Oysters in this pond have tested positive for *H. nelsoni* since 2007, but there had been no observed mortality.

The purpose of this study was to examine *H. nelsoni* prevalence patterns over time and over small distances (~70 m between sites on one side and ~300 m to the opposite side) within the pond, also, to see if the area would be suitable for the application of existing models (Ford et al., 1999 and Hofmann, et al., 2001) to explain observations. To do so, the following studies were carried out: (i) examination of the annual prevalence variation in MacDonald's Pond, Cape Breton, Nova Scotia, and (ii) determination of the prevalence of *H. nelsoni* infections in 4-5 areas of the pond. MacDonald's Pond is a relatively small sized (approximately ~0.2kms^2) water body that can have high *H. nelsoni* infection prevalence and variable expression of disease, but no observable mortality based on the absence of empty oyster shells over 10 years (R. Beresford, personal observation). This allowed for the

investigation of differences over time of localized *H. nelsoni* prevalence and expression of disease in a small water body.

2.2 METHODS AND MATERIALS

2.2.1 SAMPLING FOR EXAMINING ANNUAL VARIATION IN *H. NELSONI* PREVALENCE AND EXPRESSION OF DISEASE

Oysters (6.4 cm to 7.6 cm) were collected from one location (Site 2 Fig. 2.1) (*n*=30) within MacDonald's Pond, Cape Breton, Nova Scotia (Canada) (46°1'12.65"N; 59°57'56.43"W) (~0.2 kms^2) in November over six years (2008 – 2013) and processed for Polymerase Chain Reaction (PCR) and histological analysis.

2.2.2 SAMPLING FOR SITE-SPECIFIC VARIATION (2010 AND 2011)

Oysters (6.4 cm to 7.6 cm) were collected from five (2010) and four (2011) different locations within MacDonald's Pond on a single day in October and processed as stated previously (Figures 2.1 and 2.3). The locations of collections within the pond were chosen based on possible temperature and salinity influences within MacDonald's Pond based on their proximity to the open-ocean and freshwater inputs of the water body; however, temperature and salinity measurements prior to and in between collection dates did not indicate differences among any of the sites chosen.

2.2.3 OYSTER PROCESSING

Individual oysters were washed free of fouling organisms upon sampling. Using a knife sterilized by submersion in 95% ethanol and subsequent flaming, oysters were shucked, and their tissues removed from the shell onto a sterile bench covering. Sterilized forceps and scalpel were used to isolate three cross sections of mantle gill and digestive gland in

succession, with the first section being placed in a labeled cassette and then into Fisher's Histoprep™ for histology and a second section being placed in a sterile 1.5 ml microcentrifuge tube containing 95% ethanol. Remaining tissues were placed in sterile Whirl-pack™ bags and frozen at -80°C to preserve the remainder of each oyster. Tissues (*n*=30 unless otherwise indicated in figure captions) for PCR were analyzed at the Virginia Institute of Marine Sciences and histological specimens were processed at the Nova Scotia Department of Agriculture in Truro, Nova Scotia.

2.2.4 PCR ANALYSIS

DNA was extracted from tissue samples stored in ethanol using a Qiagen DNeasy™ tissue extraction kit using the manufacturer's protocol. Total genomic DNA concentration was determined using a GeneQuant Pro spectrophotometer (Fisher) read at 260 nm. Template DNA was added to a PCR mixture containing 25 µL AmpliTaq Gold PCR Master Mix (AmpliTaq Gold DNA Polymerase 0.05 U/µL, GeneAmp PCR Gold Buffer, (30 mM Tris/HCl, pH 8.05, 100 mM KCl) dNTP, 400 µM each, $MgCl_2$ 5 mM), 2.5 µL (1.0 µM) of each forward and reverse primer (MSX-A (5'-GCATTAGGTTTCAGACC-3') and MSX-B (5'-ATGTGTTGGTGACGCTAACCG-3')), 10 µL (500 ng) Template DNA, and 15 µL molecular biology grade water. This mixture was then subjected to a temperature cycling protocol of initial denaturation of 94°C for 4 min, 35 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 1.5 min, and final extension at 72°C for 5 min (as per the World Organization for Animal Health diagnostic protocol for the detection of *H. nelsoni* - http://www.oie.int/standard-setting/aquatic-manual/) using a TECHNE TC-412 thermocycler (Fisher). Amplified DNA was electrophoresed adjacent to a molecular weight standard along with positive and negative controls on a 1% agarose gel containing

0.5 μg/mL ethidium bromide and subsequently viewed under UV light using an Alpha Innotech imager (Fisher Scientific). Photographs of each gel were annotated with sample numbers and amplicons of appropriate size (500 bp) compiled as positive diagnostic results for each sampling locality group.

2.2.5 HISTOLOGY ANALYSIS

Preserved samples (in 10% Buffered Formalin) were dehydrated using increasing alcohol concentrations and cleared with xylene, followed by embedding with paraffin wax (OIE diagnostic protocol). Embedded samples were cut at 5 μm, mounted on glass slides, dried, and stained with hematoxylin and eosin. Samples were then examined using light microscopy for *H. nelsoni* presence and scored using a 0-4 scale as described in Carnegie and Burreson (2011). While a 0-4 scale was used for histological analysis, due to the most common score being "1", a score of "0" and ">0" was used as an indicator of disease expression.

2.2.6 STATISTICAL ANALYSIS

For PCR analysis, samples were grouped as *H. nelsoni*-positive or *H. nelsoni*-negative for comparison. For histological analysis, samples were grouped as 0 or >0. Differences in *H. nelsoni* prevalence (by both PCR and histology) were analyzed using Fisher's Exact Probability test; differences were considered significant at $p<0.05$ https://udel.edu/~mcdonald/statfishers.xls

2.3 RESULTS

2.3.1 BIOLOGICAL DATA

Infection prevalence assessed using PCR analysis at five different sites within MacDonald's Pond in 2010 revealed no differences among sites (Fig. 2.2; $p>0.15$) except between Site 3 (100%) and Site F (50%) (Fig. 2.2; $p<0.04$).

Infection prevalence assessed using PCR analysis at four different sites within MacDonald's Pond in 2011 revealed a difference Site F and all other sites (<27% and 60%) (Fig. 2.4A; $p<0.02$), but no differences between all other sites (7% to 27%) (Fig. 2.4A; $p>0.08$). Infection prevalence assessed by histology revealed no differences among all sites in 2011 when compared to each other (Fig. 2.4B; $p=1$).

A year-to-year infection prevalence comparison when assessed by PCR from four different locations in MacDonald's' Pond revealed a significant difference from 2010 to 2011 at each location (Fig. 2.5; $p\leq0.01$) except for Site F (Fig. 2.5, $p=0.72$), nearest the freshwater input.

The infection prevalence assessed by PCR over a six-year period showed peaks in 2008, 2011, and 2013. The lowest prevalence, in 2009 and 2012, followed each peak. Analysis revealed that prevalence in 2008 (78%), 2011 (60%) and 2013 (59%) were not significantly different from each other (Fig. 2.6A; $p\geq0.17$), but were significantly higher than 2009, 2010, and 2012 (13% to 47%) (Fig. 2.6A; $p\leq0.02$).

Histological examination revealed a similar cyclical trend to the PCR analysis, however the only significant differences were detected between 2008 and 2012, as well as 2012 and 2013 (Fig. 2.6 B; $p=<0.05$); all other years showed no difference (Fig. 2.6B; $p\geq0.14$).

Figure 2.1

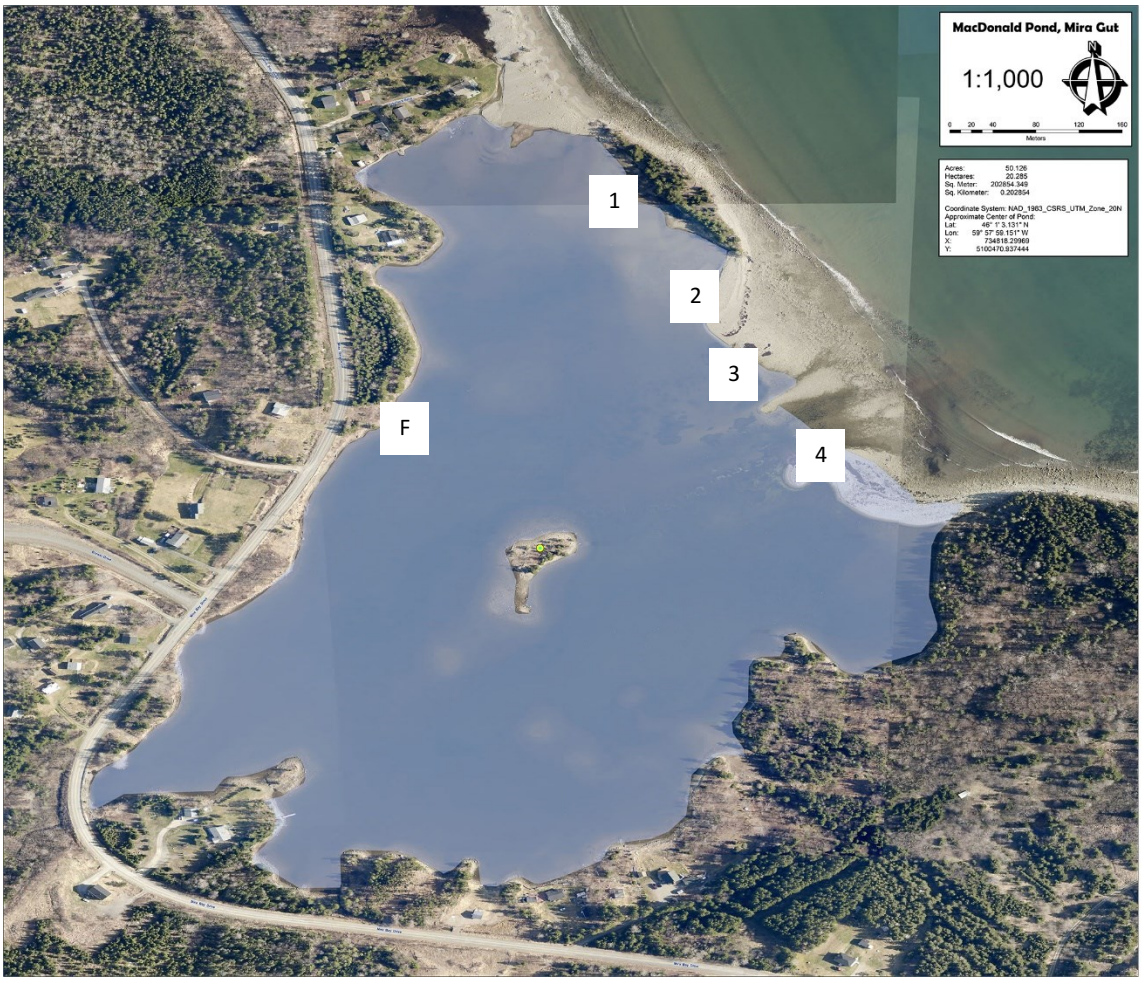

Figure 2.1. 2010 MacDonald's Pond Sampling Sites – five locations. Site F is near a freshwater input (brook); numbers refer to sampling site locations.

Figure 2.2

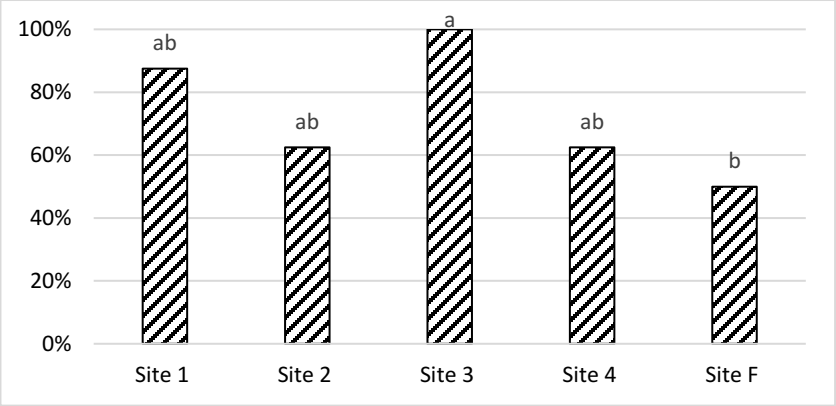

Figure 2.2. *H. nelsoni* prevalence in *C. virginica* from MacDonald's Pond in 2010 as assessed by PCR. n=8 for all sites except *n*=10 for Site F. Common letters denote no significant difference between sites at sample collection time as assessed by Fisher's Exact Probability Test.

Figure 2.3

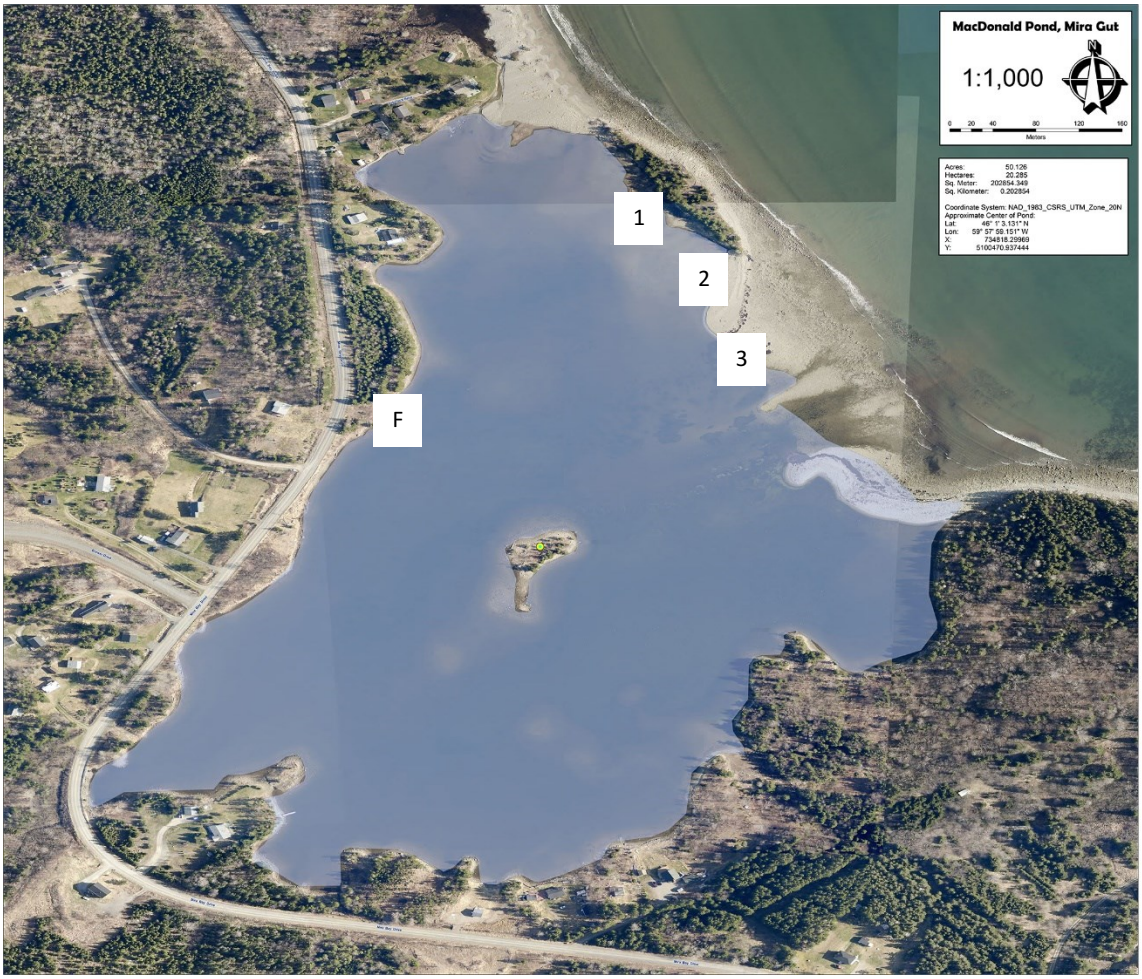

Figure 2.3. 2011 MacDonald's Pond Sampling Sites – four locations. Site F is near a freshwater input (brook); numbers refer to sampling site locations.

Figure 2.4

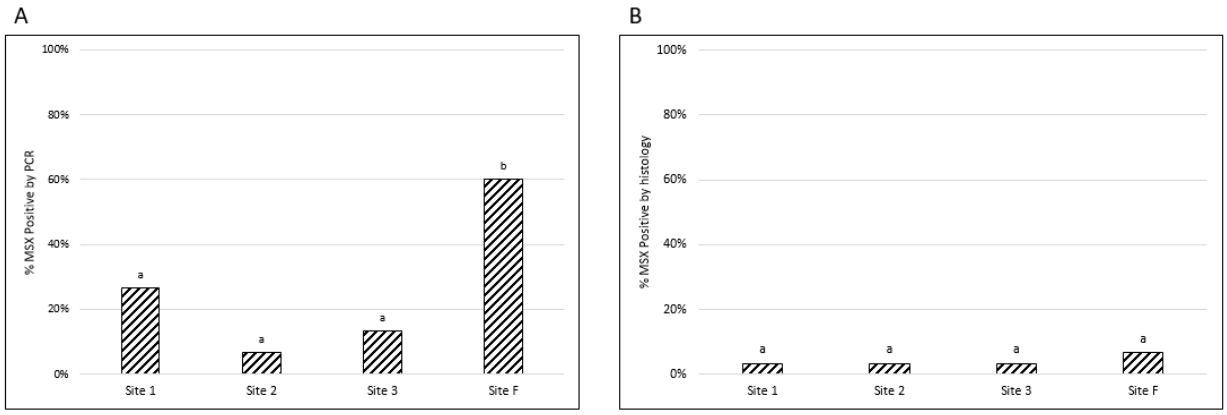

Figure 2.4. *H. nelsoni* prevalence in *C. virginica* from four sites in MacDonald's Pond in 2011 as assessed by (A) PCR and (B) histology. *n*=30 for all samples. Common letters denote no significant difference between sites at sample collection time as assessed by Fisher's Exact Probability Test.

Figure 2.5

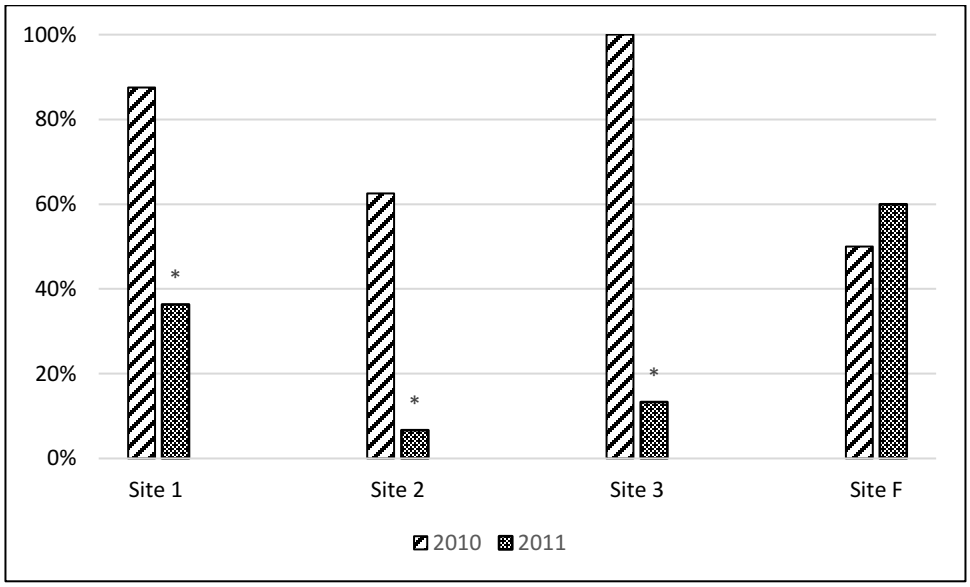

Figure 2.5 *H. nelsoni* prevalence in *C. virginica* from four sites in MacDonald's Pond in 2010 and 2011 as determined by PCR. *n*=8 for all sites except Site F (*n*=10) in 2010. *n*=30 for all sites in 2011. * denotes a significant difference for that specific site from 2010 to 2011 as assessed by Fisher's Exact Probability Test.

Figure 2.6

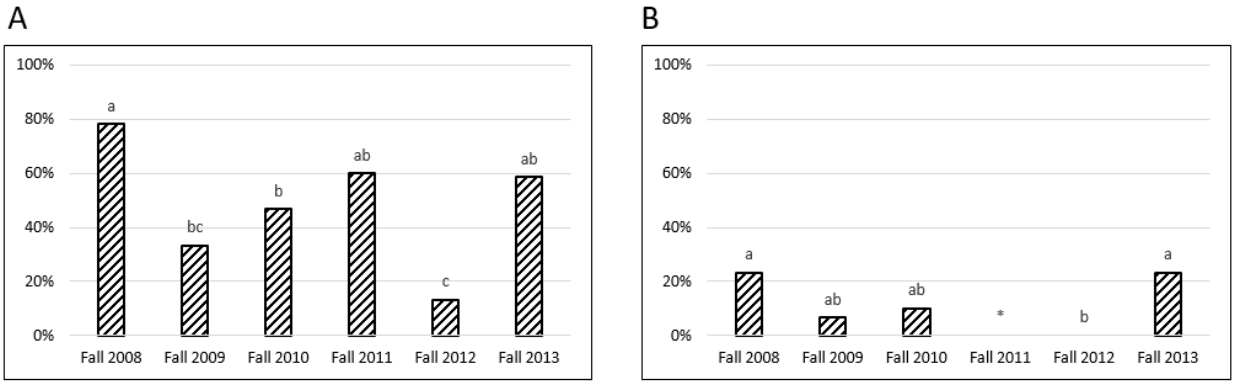

Figure 2.3.6. *H. nelsoni* prevalence in *C. virginica* over six years from a single location (Site 2) in MacDonald's Pond as determined by (A) PCR and (B) histology. *n*=30 for all samples except *n*=32 for 2008 and *n*=29 for 2013. * = samples not examined histologically. Common letters denote no significant difference between samples as assessed by Fisher's Exact Probability Test.

2.4 DISCUSSION

H. nelsoni had been present along Long Island and several New England states since the 1960s with no reported mass mortalities, and Ford and Haskin (1982) suggested that the colder winters in northern areas prevented mass mortality events associated with *H. nelsoni*. They suggested that the winter temperatures in the Chesapeake Bay area were never low enough to reduce *H. nelsoni* activity. However, there are locations that appear to be near the northern limit of *H. nelsoni*, specifically MacDonald's Pond, where this study was conducted, where *H. nelsoni* can occur at high prevalence and show different expression of disease, but with no mortality observed.

MacDonald's Pond initially tested positive for *H. nelsoni* in 2007 (DFO, personal communication, L. Penny) and samples from this pond have tested positive since that time ranging from 5% to 90% annually and from 7% to 100% at site-specific locations (L. Penny,

DFO, personal communication and R. Beresford, personal observation). Water temperature during summer ranged from 10-15ºC for at least four weeks in the Spring and above 20ºC for at least four weeks during the months of July and August (personal observation). Despite occurrences of high prevalence based on PCR analysis and different expression of disease based on histological examination, no signs of morbidity or mortality have been observed. PCR analysis was used to determine the presence or absence of the parasite whereas histology was used to determine the expression of disease and whether the parasite could be observed in the infected oysters.

The year-to-year variability observed in MacDonald's Pond may be due to the many factors that affect the life cycle and seasonal activity of this parasite that can vary from one year to the next. Factors such as temperature, food availability, potential intermediate and/or reservoir host abundance, and/or their activity could all vary from year to year. It is reasonable to assume that these factors will vary, but our understanding of the influence any of them is still not well established. It is possible that factors act in tandem and different combinations of factors result in different outcomes. One possibility to account for the difference in infections was variation among individual oysters. While it is not known for certain, anecdotal evidence from oyster fishers and regulators suggested that oysters in MacDonald's Pond originated from the Bras d'Or Lake. Based on the results from a mortality study (Chapter 5) with Bras d'Or Lake oysters, there is little reason to think there is wide variation in their likelihood for infection. This research provides some insight into the variability of this host-parasite relationship and some of the challenges that present when conducting further research into the life cycle or other natural history of this relationship.

The trends associated with temperature in the U.S. indicated that broad temperature changes have multi-year effects. A colder than normal winter when the temperature was below 3ºC

for multiple weeks was associated with reduced infection prevalence and oyster mortality for the succeeding 2-3 years. A milder than normal winter was associated with increased infection prevalence and oyster mortality for the succeeding 2-3 years (Burreson and Ford, 2004). What was not understood was whether these observations were the result of average temperatures or if they were the result of short-term changes within the timespan. The presence of an *H. nelsoni*-positive location such as MacDonald's Pond, because of its size, provides an interesting study area to monitor temperature, salinity, food, and disease status and this information could be used in mathematical models or used to refine the models to more accurately predict the outcome of various environmental parameters.

The prevalence of *H. nelsoni* in MacDonald's Pond, but lack of oyster mortality, provides an interesting contrast to some areas of the Bras d'Or Lake because the Bras d'Or Lake was likely the source of oysters and parasites now in MacDonald's Pond (DFO, personal communication, L. Penny). In the Bras d'Or Lake, at some locations, there has been oyster mortality and very slow or no recovery. For example, in Gillis Cove oysters (n=30) sampled in 2015, the parasite was no longer detectable, even using PCR. In other areas, such as Potlotek (n=30) in 2015, the parasite was detectable by PCR (13%) with no observed oyster mortality (Savoie-Swan, 2012). In contrast, MacDonald's Pond *H. nelsoni* infections were quite variable and sometimes hade high prevalence and differing expression of disease. In all likelihood, environmental factors played a critical role in the development of the infection to systemic levels but prevent mortality. The annual variation and site variation of *H. nelsoni* infections in MacDonald's Pond makes determining the factors involved challenging, but also presents a unique opportunity to try to determine what aspects of the parasite-host relationship and life history are affected by temperature or other environmental factors.

The temperatures in Cape Breton waters, including the Bras d'Or Lake and MacDonald's Pond, reach low temperatures associated with reduced parasite prevalence and associated low oyster mortality in the U.S. waters. This pattern has not been observed in MacDonald's Pond where mortality levels are low, but infection prevalence can reach high levels even after cold winters. If low temperature was the sole cause for lack of mortality in areas such as Delaware and Chesapeake Bays following cold winters, one would expect mortalities in more southern locations where the parasite is present, and temperatures seldom go below 20°C. However, no mortalities associated with *H. nelsoni* have occurred in some locations exhibiting temperature patterns where there are no cold winters, such as the Gulf Coast (Lewis et al., 1992; Bobo et al., 1996; Burreson and Ford, 2004).

Anecdotal information suggests that the oysters in MacDonald's Pond were originally from the Bras d'Or Lake and that *H. nelsoni* exists in MacDonald's Pond as a result of transfers of infected oysters from the Bras d'Or Lake. Temperature-salinity conditions in MacDonald's Pond reach levels and durations associated with mortality from *H. nelsoni* in the U.S., but do not result in mortality in this small pond; therefore, it would appear that temperature alone is not the only factor causing *H. nelsoni* infections to reach disease status and cause oyster mortality.

The Bras d'Or Lake is a complex system with varying environmental and biological parameters from location to location, so observed differences in *H. nelsoni* prevalence should not be surprising. Bras d'Or Lake oysters from many locations developed lethal infections under various environmental conditions. This study shows the temporal and spatial variability of *H. nelsoni* in this small barachois pond that provides an excellent situation to employ and possibly refine the predictive models developed by Ford et al. (1999) and Hofmann et al. (2001). It could be some unknown factor that was preventing the

usual observed mortality at such a high prevalence. One possibility for the lack of mortality is the development of resistance to mortality in the MacDonald Pond oyster population. While this may be possible, *H. nelsoni* was first detected in MacDonald's Pond in the fall of 2007, thus this would appear to be a relatively short time for resistance to develop based on *H. nelsoni* and *C. virginica* coevolution in other locations, such as along the United States Eastern seaboard (Haskin and Andrews, 1988). The lack of any noticeable mortality during the early detection of *H. nelsoni*, suggesting some degree of selection pressure in this pond is indicative that other factors may be contributing to the lack of mortality. It is unlikely that most of the oysters placed in MacDonald's Pond prior to its detection carried some ability to resist mortality from the parasite. Because of movement restrictions placed on oysters in the region, it is not permissible to transfer known susceptible oysters into MacDonald's Pond with which to compare the current population of oysters in MacDonald's Pond. Moreover, based on the same regulatory restrictions, oysters from MacDonald's Pond cannot be transferred to locations that are known to cause high mortality from *H. nelsoni* to assess the level, if any, of resistance to mortality.

Even though *H. nelsoni* has been studied since the late 1950s, there are still critical gaps in knowledge of its life cycle. With little knowledge of intermediate and/or reservoir hosts, or vectors, it is difficult to model and predict the movement of this parasite from one organism to another and from one geographic area to another. Further detailed research examining factors such as temperature and salinity and various biological factors such as abundance of candidate intermediate hosts, possible detection of *H. nelsoni* in candidate intermediate hosts, prevalence and expression of disease in oyster hosts, and likely other unknown factors is required to address some of the unknowns associated with this parasite. One explanation could be that if temperature influences *H. nelsoni* prevalence, it is due to short-term

temperature changes that have immediate effects on some aspect of the life cycle at critical times. Future studies should monitor temperature, salinity, and food, on a fine scale, perhaps hourly, daily, or weekly, at critical times during the year where they are predicted to have a substantial impact, so that predictive models can incorporate this information and be adjusted accordingly.

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CHAPTER 3 – THE EFFECT OF TEMPERATURE ON *HAPLOSPORIDIUM NELSONI* SURVIVAL IN *CRASSOSTREA VIRGINICA* UNDER CONTROLLED LABORATORY CONDITIONS

ABSTRACT - Temperature, salinity, and food availability are the three most influential factors related to the progression of *Haplosporidium nelsoni* in *Crassostrea virginica*. Since late1950s, observations from the United States have demonstrated that cold winters were usually followed by low levels of *H. nelsoni* infection prevalence and reduced oyster mortality. Conversely, mild winters and/or warm summers often resulted in both increased *H. nelsoni* prevalence and increased mortality of *C. virginica*. This study examined the influence of temperature on the progression of *H. nelsoni* in *C. virginica* by maintaining infected oysters for 4-12 weeks at temperatures between 5°C and 20°C under controlled conditions. The progression of the parasite in oyster hosts was monitored for both prevalence (by PCR) and expression of disease (by histology). The prevalence and expression of disease was reduced under all treatment conditions, in some instances reaching undetectable levels. Given that field studies from the U.S. and mathematical models suggest that temperature is a major factor in the development of *H. nelsoni* infections and oyster mortality, the reduction of parasite prevalence at warm temperatures in the current studies suggests that temperature influences some life cycle factor outside of the oyster-*H. nelsoni* interface, possibly potential intermediate host abundance or the number and/or pathogenicity of infectious agents. Alternatively, it is possible that conditions created within laboratory experiments influenced the results; therefore, to assess the effect of temperature, detailed field studies will be required.

3.1 INTRODUCTION

Increasing temperatures in marine environments due to climate change can benefit parasites by leading to range expansion into areas occupied by naïve hosts and reduce reproduction time by increasing growth and development rates, thus reducing parasite life cycle completion time (Adlard et al., 2015). Temperature change can also affect the parasites' ability to infect a host, or alternatively the host's susceptibility, and so research into the effect of temperature needs to go beyond measuring parasite or host abundance and must include broader investigations around host-parasite interactions with their changing environment (Adlard et al., 2015; Barber et al., 2016).

Environmental factors are thought to play a major role in the *H. nelsoni – C. virginica* interaction (Haskin and Douglass, 1971, Ford and Haskin 1982, Ford 1988). The Ford et al. (1991) and Hofmann et al. (2001) models suggest that the greatest influences on the *H. nelsoni – C. virginica* interaction are: (i) temperature, (ii) salinity, and (iii) food, but temperature is the most influential environmental factor in the field and in the model. Historically, in the United States, colder winters were associated with a reduction in prevalence and intensity of parasite infections followed by a reduction in oyster mortality in subsequent years. Meanwhile, milder winters and/or warmer, dryer summers were associated with increased parasite prevalence and intensity followed by increased oyster mortality. It is believed that colder winters reduce the abundance of intermediate host population and warmer winters allow intermediate host populations to increase. Hot, dry summers resulted in salinity increases up rivers allowing the parasite to survive in areas that previously were unsuitable for parasite survival because of low salinity (Ford and Haskin, 1982; Ford, 1988; Sunila et al., 1999). The Hofmann et al. (2001) model indicated that both

low temperature and low salinity can impede *H. nelsoni* activity, with low temperature having a broader and longer lasting effect while low salinity has a much more acute effect and Ford et al. (1999) indicated that neither temperature nor salinity alone can explain the multiple years of field observations.

While field data and computer models predict that temperature plays a critical role in the survival and activity of *H. nelsoni*, the specific role that temperature plays in the progression of the parasite in individual oyster hosts is not understood. Temperature could be affecting the parasites, the oyster hosts, intermediate hosts, or some combination of these.

This series of experiments assessed the direct effect of temperature on the development of the parasite in the oyster host over time using different temperature treatments, followed by periodic monitoring of the prevalence and expression of disease in oysters. Based on the conclusions from environmental data and associated models that temperature plays a critical role the progression of *H. nelsoni* infections, the hypothesis is that the expression of disease would increase with increasing temperature and not be influenced by the introduction of new parasites into the system. The first experiment investigated the progression of *H. nelsoni* infection at higher temperatures associated with increasing disease levels and lower temperatures associated with decreasing disease levels. A 12-week treatment period was used to simulate 12 weeks of summer temperatures (15˚C or 20˚C) or 12 weeks of winter temperatures (5˚C or 10˚C). The second experiment investigated the timing of parasite reduction over a 12-week period following treatment at 20ºC for 12 weeks. The third experiment investigated the effect of temperature treatment greater than 20ºC over four weeks.

3.2 METHODS AND MATERIALS

3.2.1 COLLECTION AND SAMPLING

Oysters (6.4cm to 7.6cm) were collected from MacDonald's Pond, Cape Breton, Nova Scotia in November (2008-2010). Water temperature and salinity measurements were taken at the sampling location at the collection time (10^{o}C and 24‰). A sample of 30 oysters was collected to determine the initial disease prevalence and expression of disease. Immediately following collection, experimental oysters were transported to a quarantine lab facility and distributed on three trays within three tanks per temperature (30 oysters per 200 L of water) in Instant Ocean (24‰) at 10^{o}C to avoid heat shock or cold shock. Over the following seven days, water temperature was raised or lowered 1-2^{o}C per day until the experimental temperatures were reached. Oysters were placed on trays that were rotated from top to bottom within tanks and between tanks on a regular basis for a given treatment. Water was maintained at a constant temperature and salinity for the duration of each experiment following acclimation and aerated/circulated using air stones. Oysters were batch fed maintenance diet according to Reed Mariculture™ instructions daily (3.6 mL/ 100 g tissue). Tanks were cleaned and water was changed once per week.

Experiment 1 (Figure 3.1) – Oysters were maintained for 12 weeks at four different temperatures and sampled at 12 weeks. Experiment 2 (Figure 3.2) – Oysters were maintained for 12 weeks at 20^{o}C and sampled at 4, 8, and 12 weeks. Experiment 3 (Figure 3.3) - Oysters were maintained for four weeks at 20^{o}C and 24^{o}C and sampled at four weeks.

3.2.2 OYSTER PROCESSING

Individual oysters were washed free of fouling organisms upon sampling. Using a shucking knife sterilized by submersion in 95% ethanol and subsequent flaming, oysters were shucked, and their tissues removed from the shell onto a sterile bench covering. Sterilized forceps and scalpel were used to isolate cross sections of mantle gill and digestive gland in succession, with the first section being placed in a labeled cassette and then into Fisher's Histoprep™ for histology and the second section being placed in a sterile 1.5 mL microcentrifuge tube containing 95% ethanol. Remaining tissues were placed in sterile Whirl-pack™ bags and frozen at -80°C to preserve the remainder of each oyster. For treatment at 5°C to 20°C, 30 oysters were sacrificed at 12 weeks and processed for PCR analysis, histological analysis, and remaining tissues were frozen at -80°C in Whirl-Pak® bags.

3.2.3 PCR ANALYSIS

DNA was extracted from tissue samples stored in ethanol using a Qiagen DNeasy™ tissue extraction kit using the manufacturer's protocol. Total genomic DNA concentration was determined using a GeneQuant Pro spectrophotometer (Fisher) read at 260 nm. 10 µL Template DNA (500 ng) was added to a PCR mixture containing 25 µL AmpliTaq Gold PCR Master Mix (AmpliTaq Gold DNA Polymerase 0.05 U/µL, GeneAmp PCR Gold Buffer (30 mM Tris/HCl, pH 8.05, 100 mM KCl), dNTP, 400 µM each, 5 mM $MgCl_2$), 2.5 µL (1.0 µM) of each forward and reverse primer (MSX-A (5'-GCATTAGGTTTCAGACC-3') and MSX-B (5'-ATGTGTTGGTGACGCTAACCG-3') (Stokes and Burreson, 1995) and 15 µL molecular biology grade water. This mixture was then subjected to a temperature cycling protocol of initial denaturation of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 1.5 min, and final extension at 72°C for 5 min (as per the

World Organization for Animal Health diagnostic protocol for the detection of *H. nelsoni* - http://www.oie.int/standard-setting/aquatic-manual/) using a TECHNE TC-412 thermocycler (Fisher). Amplified DNA was electrophoresed on a 1% agarose gel containing 0.5 μg/mL ethidium bromide adjacent to a molecular weight standard along with positive and negative controls and subsequently viewed under UV light using an Alpha Innotech imager (Fisher Scientific, Waltham, Massachusetts, U.S.A). Images were taken for each gel and annotated with sample numbers and amplicons of appropriate size (500 bp) compiled as positive diagnostic results for each sampling locality group. Samples analyzed by PCR were categorized as positive or negative for *H. nelsoni*.

3.2.4 HISTOLOGY ANALYSIS

Paraffin embedded blocks were prepared from tissues in Fisher's Histoprep™ fixative and sections prepared according to the procedure as laid out in the OIE Manual for Diagnostic Tests for Aquatic Animals (2003) by the Nova Scotia Department of Agriculture in Truro, N.S. Two 5 μm sections were stained with hematoxylin and eosin and examined for the presence and absence of plasmodia and then assigning individuals based on the intensity rating system 0 – 4 as described in Carnegie and Burreson (2011). While a 0-4 scale was used for histological analysis, due to the most common score being "1", a score of "0" and ">0" was used as an indicator of disease expression. Any signs of pathology or unhealthy parasites were noted for each organism.

3.2.5 STATISTICAL ANALYSIS

For PCR analysis, samples were grouped as *H. nelsoni*-positive or *H. nelsoni*-negative for comparison. For histological analysis, samples were grouped as 0 or >0. Differences in *H. nelsoni* prevalence (by both PCR and histology) were analyzed using Fisher's Exact

Probability test; differences were considered significant at $p<0.05$.

https://udel.edu/~mcdonald/statfishers.xls

3.3 RESULTS

Infection prevalence assessed using PCR analysis revealed a minimum 65% reduction in *H. nelsoni* prevalence for all incubated oysters relative to the field sample (Fig. 3.1A; $p\leq0.001$). Lab treatments showed no difference between each other (13% to 0% prevalence) (Fig. 3.1A; $p\geq0.112$), with the 20ºC group showing no presence of *H. nelsoni*. Histological examination revealed a decrease in positive samples from 25% in the field sample to 0% in lab-exposed oysters that were held at 15ºC (Fig. 3.1B; $p=0.005$) and 20ºC (Fig. 3.1B; $p=0.005$); whereas, those oysters treated at 5ºC decreased to 7%, and 10ºC treated oysters were only 10% positive by histology. Neither the 5ºC nor the 10ºC differed significantly from the field sample (Fig. 3.1B; $p\geq0.082$). Histological examination did not show any evidence of disease or dead/dying parasites.

The initial temperature investigations revealed a reduction in parasite prevalence and expression of disease over a 12-week period. The 20ºC thermal treatment revealed the greatest parasite prevalence reduction as measured by PCR occurred within the initial four weeks (33% to ≤13%) (Fig. 3.2A; $p\geq0.05$), with limited incremental changes (Fig. 3.2A; $p=1$) over additional exposure durations (eight weeks (13%) and 12 weeks (10%). Histological examination detected no differences (Fig. 3.2B; $p\geq0.491$) when the field sample (7%) was compared to the treated samples at 4, 8, and 12 weeks (3%, 0%, 0% respectively) or when treated samples were compared to each other (3% to 0%) (Fig. 3.2B; $p=1$).

Four- week laboratory treatments at 20°C and 24°C reduced PCR-detected *H. nelsoni* prevalence from 47% to 0% (Fig. 3.3A; $p<0.001$) relative to oysters sampled initially at the time of collection from the field, although the response did not differ between 20°C and 24°C treatments (0% for both) (Fig. 3.3A; $p=1$). Despite the reductions detected by PCR, because of the low prevalence of field samples showing *H. nelsoni* during histological examination, laboratory-exposed oysters undergoing histological examination did not differ significantly (11% to 0%) (Fig. 3.3B; $p=0.237$) from the field sample, nor between each other (0% for each) (Fig. 3.3B; $p=1$)

Temperature experiments showed that there was a reduction in parasite presence in oysters maintained under laboratory conditions when assessed by PCR; however, the degree of the reduction was not constant and not always significant. Three of the five treatments showed a significant reduction (Fig. 3.4; $p<0.01$) and two were not significant (Fig 3.4; $p>0.13$).

Figure 3.1

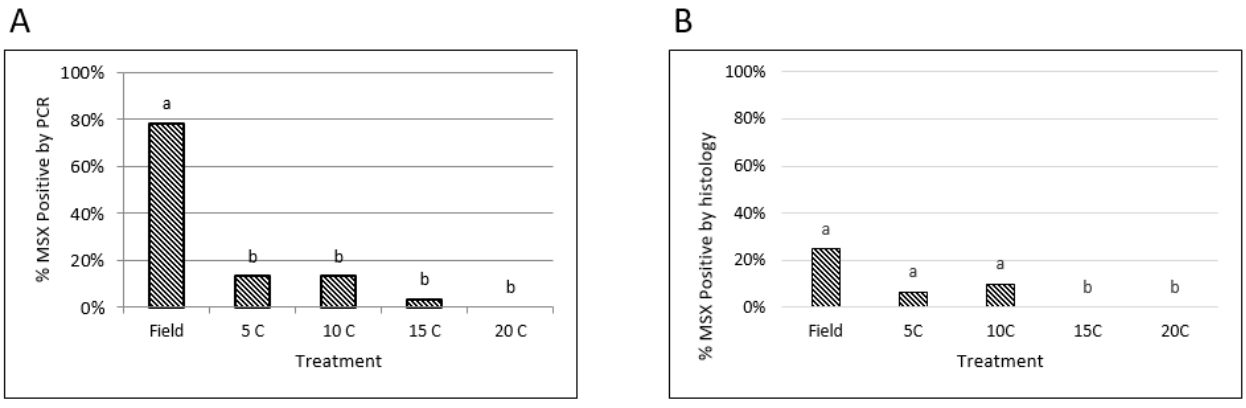

Figure 3.1. *H. nelsoni* prevalence in *C. virginica* (2008) by (A) PCR and (B) histology in following a 12-week incubation in the laboratory at various temperatures ($n=30$ for all samples). Field temperature was 12°C degrees when oysters collected. Common letters denote no significant difference between treatments as assessed by Fisher's Exact Probability Test.

Figure 3.2

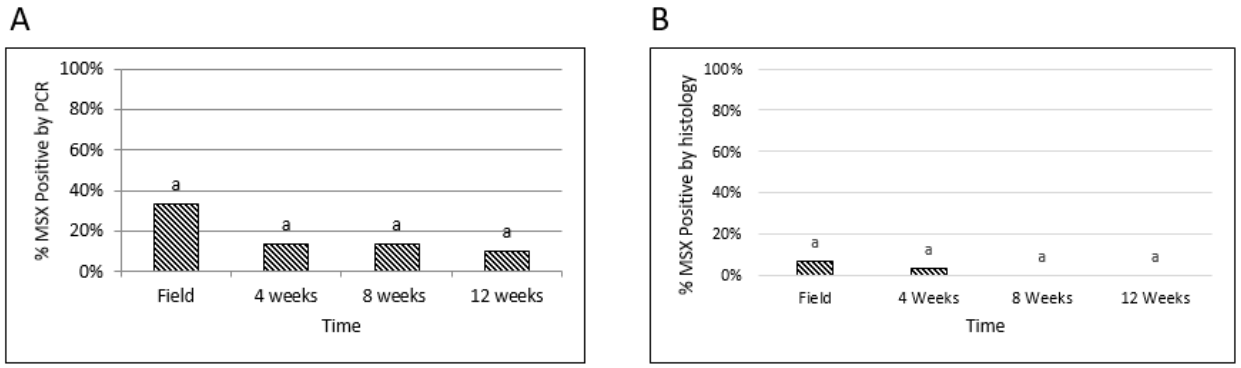

Figure 3.2. *H. nelsoni* prevalence in *C. virginica* (2009) by (A) PCR and (B) histology following 12 weeks at 20ºC temperatures (*n*=30 for all samples), sampled at 4 week intervals. Field temperature was 11ºC degrees when oysters were collected. Common letters denote no significant difference between treatment times as assessed by Fisher's Exact Probability Test.

Figure 3.3

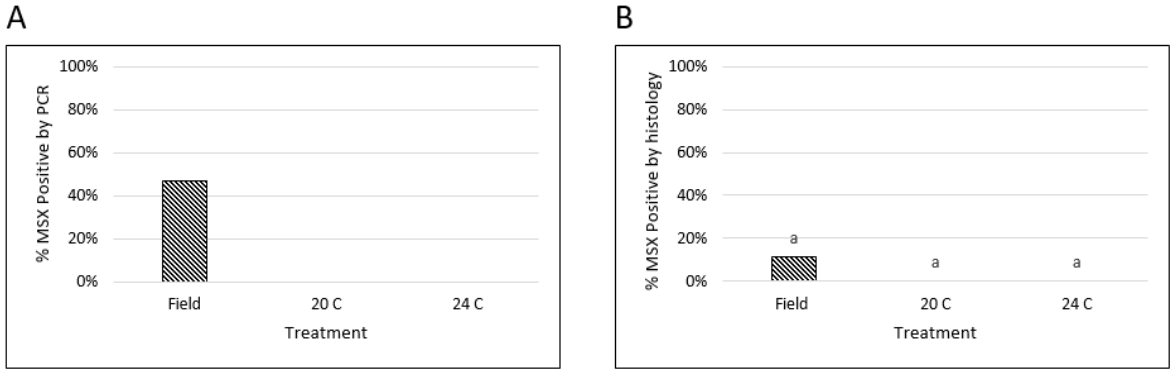

Figure 3.3. *H. nelsoni* prevalence in *C. virginica* (2010) by (A) PCR and (B) histology following a 4-week incubation at 20ºC and 24ºC (*n*=30 for all samples). Field temperature was 14ºC degrees when oysters collected. Common letters denote no significant difference between treatments as assessed by Fisher's Exact Probability Test.

Figure 3.4

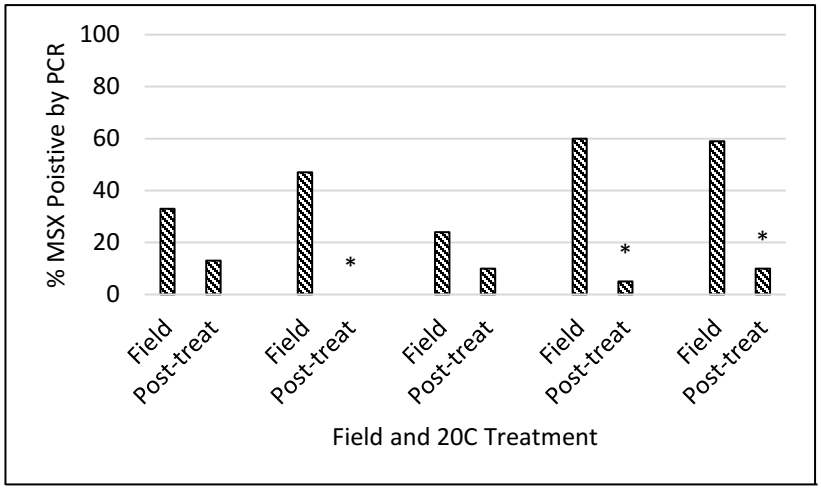

Figure 3.4. *H. nelsoni* prevalence in *C. virginica* by PCR in different collection samples before and following a 4-week incubation at 20°C (*n*=30 for each group). * indicates significant difference between field and post-treatment.

3.4 DISCUSSION

Warmer temperature is associated with large scale impacts on oyster mortality linked to *H. nelsoni* in the U.S. (Haskin and Douglass, 1971; Ford and Haskin 1982; Ford 1988). In the current study, oysters held at constant temperatures (5°C, 10°C, 15°C, 20°C, and 24°C) in controlled laboratory settings all revealed a reduction in parasite prevalence when assessed using PCR analysis for *H. nelsoni*. For each experiment, temperature, salinity, and food remained at levels that are associated with parasite progression and disease development, yet we observed no oyster mortality, reduction in parasite prevalence, and lower infection disease expression in oyster hosts.

Hofmann et al. (2001) noted that the high temperature and *H. nelsoni* disease pattern did not seem to exist in the Gulf of Mexico region. These laboratory results showed that at constant temperature in the range of 20°C, there was a reduction in *H. nelsoni* prevalence and

expression of disease and a lack of oyster mortality. Parasite prevalence was reduced under all treatments, 5°C, 10°C, 15°C, and 20°C, when assessed by PCR; however, the most notable reduction was observed histologically with oysters held at 15°C and 20°C. This is important because the histology results provide a better understanding of the degree of parasite removal. PCR results do not always provide clarity on what has occurred because some PCR positive samples may be the result of residual parasite DNA from dead or dying parasites. It is possible that the lack of *H. nelsoni* associated disease outbreaks in the Gulf of Mexico may have been due to temperatures consistently above 20°C for extended periods, thus creating an environment that was not suitable for parasite proliferation or for the development of an abundance of infective particles in a given location.

Most epizootics observed in the northern U.S. have occurred during a warming period and over a two-decade period, increased water temperature was associated with increased *H. nelsoni* disease outbreaks (Cook et al., 1998). *P. marinus* and *H. nelsoni* are known to have increased activity following what are referred to as "mild winters". The occurrence of mild winters might have caused *P. marinus* to resume its life cycle of oyster-to-oyster infection earlier in the spring because waters warmed up earlier (Cook et al., 1998), but what was affected by mild winters in the *H. nelsoni* life cycle is not known due to the lack of knowledge of how infections occur. While the steady, northward movement of *P. marinus* from the lower Chesapeake to Maine between 1985 and 1995 was described as predictable (Burreson and Ragone Calvo, 1996; Ford, 1996); *H. nelsoni* outbreaks were described as irregular and unpredictable. The Hofmann et al. (2001) model indicated that as winters continue to warm, *H. nelsoni* would remain established in locations where it is now present and would be able to move further northward as the warming water trend continues. The unpredictability of *H. nelsoni* in the wild was also observed in controlled laboratory settings.

Based on these results, it appeared that the previous history of the oyster hosts prior to transfer to experimental conditions may be a key influence on observations of host-parasite interaction. The length of time of the previous history may be a factor of days, weeks, or longer, but the inconsistency in response to a controlled treatment was likely due to some factor(s) that occurred prior to transfer into the laboratory setting or the conditions created when held in tanks in a laboratory. Oysters for the experiments were collected in November of each year because infection prevalence was highest. This timing coincided with oyster preparation for winter hibernation and so oysters would have had resources available to them. It would be interesting to investigate what happens to infection prevalence in the field over the same time course as the experiments and equally interesting to investigate the results under the same treatment conditions if oysters were collected at different times of the year (e.g. prior to spawning, immediately after spawning).

One notable difference between field conditions and laboratory conditions is that under controlled laboratory conditions there is no additional introduction of infective particles. The life cycle is unknown, so it is not possible to introduce infective particles via infected oysters in a laboratory setting. Additionally, while being held in tanks may introduce some degree and type of stress to oyster hosts and parasites, or alternatively remove certain stressors, it is not known how these types and degrees of stress compare with stressors encountered in the wild such as changing environments, the presence of predators, and other factors. The absence of inflammation or dead or damaged parasites observed, made it difficult to identify the mechanism of parasite removal under the laboratory conditions used in this study. Recent preliminary research, focused on parasite reduction following one week of exposure to 20^{o}C, showed an unusually high number of brown cells and no visible *H. nelsoni*. This observation suggests that parasite reduction occurred during the first week

of laboratory treatment. Further investigation and analysis is required before any conclusions can be drawn.

In some locations where *H. nelsoni* has been found, such as the Bras d'Or Lake, the presence of ice cover can result in an increase in water temperature because the presence of ice acts as a layer of insulation from cold winter air. However, in other locations in the Bras d'Or Lake the water temperature decreases during a cold winter (R. Stuart, personal communication). Therefore, the impact of colder or milder winters is not predictable with respect to parasite prevalence, expression of disease, or oyster mortality as it relates to *H. nelsoni.* There are areas in the Bras d'Or Lake, Nyanza Bay, for example, that did or did not freeze over in any given year since 2002, yet *H. nelsoni* maintained high infectivity levels and caused high oyster mortality every year when susceptible animals were transferred into that location (Beresford, unpublished data). In other locations in the Bras d'Or Lake, such as East Bay, with a similar intermittent freezing pattern there was no observable correlation between ice or lack thereof and oyster mortality (DFO, Lorne Penny, personal communication). Despite the trends observed by Cook et al. (1998) for *P. marinus* where disease outbreaks corresponded to increased temperatures, small-scale episodic events occurred whereby an increase in parasite prevalence was not always associated with increased temperature, thus other factors may be involved. These wide-scale trends and observations are not necessarily applicable for all circumstances and certainly not for *P. marinus* with a direct life cycle nor for *H. nelsoni* which is thought to have a complex life cycle.

The component of the *H. nelsoni* life cycle affected by temperatures, whether it be number of infective particles, infectivity of infective particles, intermediate host factors, or parasite

development factors, is unknown. For more northern areas of the U.S., it is possible that some of the outbreaks were due to higher than normal temperatures for critical periods of time. In contrast, the 20-year delay in the outbreak in Long Island Sound, reported by Sunila et al. (1999), may have been a result of deeper waters staying below a critical temperature for a required period. These observations are interesting when compared with laboratory experiment results in which infected oysters were held at temperatures normally associated with disease progression and high mortality were able to reduce the parasite burden and showed no mortality from the parasite.

Controlled temperature treatments produced a reduction in parasite prevalence with the greatest reduction in disease expression occurring at higher temperatures (15^{o}C and 20^{o}C). It is possible that the association of increased summer temperatures with increased *H. nelsoni* prevalence and oyster mortality in the U.S. could be the result of temperature affecting an intermediate host or hosts (abundance, development of the parasite within the intermediate hosts). Furthermore, colder temperatures that are associated with decreased prevalence and oyster mortality may have had a detrimental effect on intermediate hosts (abundance, development of the parasite within the intermediate host, or some unknown factor). The observations from these experiments suggest that the broader patterns associated with temperature observed in the U.S. since the 1960s were a result of the effects of temperature on something beyond direct infection progression in infected hosts, possibly intermediate host-parasite interactions. Alternatively, it is possible that the conditions created in tanks in the laboratory, such as uniform temperature or increasing abundance of some metabolite, resulted in morbidity or mortality of the parasite and subsequent removal of dead and dying parasites.

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CHAPTER 4 - THE EFFECT OF FOOD AVAILABILITY ON THE DEVELOPMENT OF *HAPLOSPORIDIUM NELSONI* IN *CRASSOSTREA VIRGINICA* UNDER CONTROLLED LABORATORY CONDITIONS

ABSTRACT - Field observations from the United States and mathematical models have suggested that temperature, salinity, and food availability are the three most important environmental factors that influence the development of disease in *Haplosporidium nelsoni* infections in Eastern oysters (*Crassostrea virginica*). The focus of the current experiments was to determine if there is a direct relationship between the progression of *H. nelsoni* in infected oysters and the availability of food. Oysters were held under controlled laboratory conditions for 28 to 33 days and provided various food treatments. Food availability ranged from no food to 300% of the standard manufacturer's recommended maintenance diet for hatchery-reared oysters. Treated animals were assessed using both PCR analysis for prevalence of the parasite and histological examination to determine the expression of disease at 28 and 33 days. Results showed that there was no direct relationship between food availability and progression of disease through infected oysters. Previous temperature treatment studies revealed a decrease in parasite prevalence and disease expression, but there was no clear relationship between *H. nelsoni* prevalence and expression of disease when infected oysters were provided different experimental food availability treatments. While treated oysters reduced the parasite burden during the experiment, there was no clear trend in the results from the food availability treatments; thus, it was difficult to arrive at conclusions from the conflicting results. Previous field observations and mathematical models suggested that food availability played a role in the progression of infection in the wild, but the results of this current study suggested that food availability may affect some

other component of the life cycle of the parasite and that further investigations of this nature are necessary to determine the role of food availability in the *H. nelsoni* - *C. virginica* interaction. Alternatively, it is possible that conditions created within laboratory experiments influenced the results; therefore, to assess the effect of food availability, detailed field studies would be required.

4.1 INTRODUCTION

It was suggested as early as 1968 that there are three factors involved in the eventual outcome of an infection from *Haplosporidium nelsoni* (formerly *Minchinia nelsoni*) – environmental, genetic, and physiological (Farley, 1968). Paraso et al. (1999) suggested that after winter, there were improved conditions for *H. nelsoni,* because the spring algae bloom provided better food availability leading to increased physiological activity resulting in the removal of waste products accumulated over the winter inside the oyster, possibly making the conditions more suitable for parasite growth. The *H. nelsoni* model developed by Hofmann et al. (2001) concurred with the hypothesis of Paraso et al. (1999) that increasing food supply in the spring was associated with an increase in growth rate of *H. nelsoni*. The model also suggested that the reduction or elimination of the spring algae bloom could limit the growth of the parasite resulting in a lower prevalence during the summer months.

Temperature plays a critical role in the development of *H. nelsoni* as discussed in Chapter 3, but there were also strong associations with increasing food supply and maximal salinity that influenced the growth phase of *H. nelsoni* such that they maximize parasite development allowing it to complete its life cycle (Ford et al., 1991). It is also plausible that different stresses placed on oyster hosts, such as rapidly changing temperature, excess or insufficient food, predation and additional parasite presence, may result in *H. nelsoni*-caused mortality

(Lenihan et al., 1999). Haskin and Andrews (1988) proposed that under less stressful circumstances, a given level of parasite infection may not cause mortality, but the presence of additional stresses in conjunction with parasitism can lead to oyster death. In a lysozyme study looking at the effects of environmental factors and parasitism in oysters, Chu and La Peyre (1989) suggested that the combined stresses of red tide, parasitism, and repeated bleeding contributed to the death of some oysters. Furthermore, a reduction in food availability or the redirection of resources/energy into gamete production may make oysters more susceptible to disease from the parasite. In contrast, oysters that obtains more energy in excess of maintenance requirements should be better able to withstand an infection from *H. nelsoni* and therefore have greater survival (Newell and Barber, 1988). Lenihan et al. (1999) summarized these two scenarios by stating that when a host suffers physiological stress it is more susceptible to parasitism and disease; alternatively, these same stressors may have a negative effect on parasites thereby reducing infection levels and disease expression in host populations.

Adaptive host exploitation is one strategy employed by parasites that is often determined by resources available to the host. Those parasites with inflexible host exploitation strategies are not able to modify their consumption of host resources that might result in host population decline (Jokela et al. 2005). To what extent *H. nelsoni* is able to modify its exploitation of its host is unknown, but given the usual high prevalence and mortality associated with *H. nelsoni* infections, it likely has not evolved an adaptive host exploitation process.

Ford et al. (1999) and Hofmann et al. (2001) models predicted that the greatest influences on the *H. nelsoni – C. virginica* interaction are: (1) temperature, (2) salinity, (3) food, but that

neither temperature nor salinity alone explain the multiple years of field observations. The model proposed by Ford et al. (1999) suggested that increasing food in the spring resulted in more energy available to *H. nelsoni* and allowed the parasite to grow at rates and densities beyond normal density thereby increasing prevalence as more infections reach detectable levels and increase parasite infection intensity.

Considering the predictions from the *H. nelsoni* models and other hypotheses, feeding experiments were designed to evaluate the role of food availability on the survival and progression of *H. nelsoni* infection in *C. virginica*. Under controlled laboratory conditions, environmental factors such as temperature and salinity were held constant to determine the effect of food availability. Experimental conditions ranged from providing no food to three times more food than the recommended maintenance diet from Reed Mariculture™. Treated oysters were sampled at 28 days for consistency with the treatment times in Chapter 3 and at 33 days to investigate the possibility that an extended food availability treatment could produce different results. There were multiple hypotheses under consideration: (i) increased food availability would result in healthier oysters that are able to combat the presence of the parasitic infection; (ii) increased food availability would provide an abundance of nutrients to the parasite resulting in infection progression; (iii) reduced food availability would result in reduced oyster host health resulting in parasite infection progression; or (iv) reduced food availability would not provide the adequate nutrients necessary for parasite infection progression.

4.2 METHODS AND MATERIALS

4.2.1 COLLECTION AND SAMPLING

Oysters (6.4 cm to 7.6 cm) were collected from MacDonald's Pond, Cape Breton, Nova Scotia in November 2011 and 2013. Water temperature and salinity measurements were taken at the sampling location at the collection time (10°C and 24‰). A sample of 30 oysters was collected to determine the initial disease prevalence and expression of disease. Immediately following collection, experimental oysters were transported to a quarantine lab facility and distributed among three trays within three tanks (30 oysters per 200L of water) per treatment in Instant Ocean (24‰) at 10°C to avoid heat shock or cold shock. Over the following seven days, water temperature was raised or lowered 2°C per day until 20°C was reached. Trays were rotated within and between treatment tanks on a regular basis for a given treatment. Water was maintained at constant temperature and salinity for 28 or 33 days. Water was aerated and circulated using air stones. In the first set of experiments, oysters and were batch fed daily with according to Reed Mariculture™ instructions (3.6 mL / 100 g tissue) at treatment quantities (no food, 50% maintenance diet, or 100% maintenance diet). In the second set of experiments, oysters were batch fed daily with maintenance diet according to Reed Mariculture™ instructions (3.6 mL / 100 g tissue) at treatment quantities (100% maintenance diet, 200% maintenance diet, or 300% maintenance diet). Tanks were cleaned and water was changed once per week. Prevalence and disease expression were assessed after 28 days and 33 days.

Experiments 1 and 2 – Consecutive treatments involved holding oysters at 20°C water for four weeks and treated with 0 food, 50% recommended maintenance diet, or 100% recommended maintenance diet and sampled at four weeks. Experiment 3 – Oysters were held in 20°C water and treated with 100% food, 200% recommended maintenance diet, or 300% recommended maintenance diet and sampled at 28 days. Experiment 4 – Oysters were

held in 20°C water and treated with 100% food, 200% recommended maintenance diet, or 300% recommended maintenance diet and sampled at 33 days

4.2.2 OYSTER PROCESSING

Individual oysters were washed free of fouling organisms upon sampling. Using a shucking knife sterilized by submersion in 95% ethanol and subsequent flaming, oysters were shucked, and their tissues removed from the shell onto a sterile bench covering. Sterilized forceps and scalpel were used to isolate three cross sections of mantle gill and digestive gland in succession, with the first section placed in a labeled cassette and then into Fisher's Histoprep™ for histology and a second section placed in a sterile 1.5 mL microcentrifuge tube containing 95% ethanol. Remaining tissues were placed in sterile Whirl-pack™ bags and frozen at -80°C to preserve the remainder of each oyster.

4.2.3 PCR ANALYSIS

DNA was extracted from tissue samples stored in ethanol using a Qiagen DNeasy™ tissue extraction kit using the manufacturer's protocol. Total genomic DNA concentration was determined using a GeneQuant Pro spectrophotometer (Fisher) read at 260 nm. Total genomic DNA concentration was determined using a GeneQuant Pro spectrophotometer (Fisher) read at 260 nm. 10 µL Template DNA (500 ng) was added to a PCR reaction mixture containing 25 µL AmpliTaq Gold PCR Master Mix (AmpliTaq Gold DNA Polymerase 0.05 U/µL, GeneAmp PCR Gold Buffer (30 mM Tris/HCL, pH 8.05, 100 mM KCl), dNTP, 400 µM each, 5 mM $MgCl_2$), 2.5 µL (1.0 µM) of each forward and reverse primer (MSX-A (5'-GCATTAGGTTTCAGACC-3') and MSX-B (5'-ATGTGTTGGTGACGCTAACCG-3') (Stokes and Burreson, 1995) and 15 µL molecular biology grade water. This mixture was then subjected to a temperature cycling protocol of initial denaturation of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 59°C for 30 sec and

72°C for 1.5 min, and final extension at 72°C for 5 min (as per the World Organization for Animal Health diagnostic protocol for the detection of *H. nelsoni* - http://www.oie.int/standard-setting/aquatic-manual/) using a TECHNE TC-412 thermocycler (Fisher). Amplified DNA was electrophoresed on a 1% agarose gel containing 0.5 μg/mL ethidium bromide adjacent to a molecular weight standard along with positive and negative controls and subsequently viewed under UV light using an Alpha Innotech imager (Fisher Scientific, Waltham, Massachusetts, U.S.A). Images were taken for each gel and annotated with sample numbers and amplicons of appropriate size (500 bp) compiled as positive diagnostic results for each sampling locality group. Samples analyzed by PCR were categorized as positive or negative for *H. nelsoni*.

4.2.4 HISTOLOGY ANALYSIS

Paraffin embedded blocks were prepared from tissues in Fisher's Histoprep™ fixative and sections prepared according to the procedure as laid out in the OIE Manual for Diagnostic Tests for Aquatic Animals (2003) by the Nova Scotia Department of Agriculture in Truro, N.S. Two 5 μm sections were stained with hematoxylin and eosin and examined for the presence and absence of plasmodia and then assigning individuals based on the intensity rating system 0 – 4 as described in Carnegie and Burreson (2011). While a 0-4 scale was used for histological analysis, due to the most common score being "1", a score of "0" and ">0" was used as an indicator of disease expression. Any signs of pathology or unhealthy parasites were noted for each organism.

4.2.5 STATISTICAL ANALYSIS

For PCR analysis, samples were grouped as *H. nelsoni*-positive or *H. nelsoni*-negative for comparison. For histological analysis, samples were grouped as 0 or >0. Differences in *H.*

nelsoni prevalence (by both PCR and histology) were analyzed using Fisher's Exact Probability test; differences were considered significant at $p<0.05$.

https://udel.edu/~mcdonald/statfishers.xls

4.3 RESULTS

The first and second experiment examined whether low food availability would affect the prevalence and expression of disease of *H. nelsoni* infections in *C. virginica.* In the first experiment, the field sample prevalence was 60% and prevalence in all treated samples was reduced (to 7% in oysters given no food, 30% in oysters fed 50% maintenance diet, and 10% in oysters fed 100% maintenance diet) (Fig. 4.1A). The second experiment, a repeat of the initial experiment, had an initial prevalence assessed by PCR of 37% with a reduction in overall prevalence (minimum of 27%) for all treated samples (Fig. 4.1B; $p<0.05$) with no difference between treated oysters when compared to each other (Fig. 4.1B; $p=1$). Infection assessment using histology showed no significant reduction when treated samples were compared to the field sample or to each other (Fig. 4.1C; $p=1$).

The third experiment examined if food availability greater than the recommended maintenance diet would influence the prevalence and/or expression of disease of *H. nelsoni* infection levels in *C. virginica*. Oysters were fed treatments of 100%, 200%, or 300% the recommended maintenance diet (28 days). The fourth experiment was a continuation of the third experiment with five additional days of treatment (33 days). For the first experiment, following 28 days of treatment, *H. nelsoni* prevalence was assessed using PCR and expression of disease evaluated using histological examination. Infection prevalence assessed using PCR showed a reduction in parasite presence for oysters fed full maintenance diet and 3x maintenance diet when compared to the field sample (Fig. 4.2A; $p<0.05$), but no

difference between the field sample and oysters fed 2x maintenance diet (Fig. 4.2A; p=0.196). There was a difference in infection prevalence between oysters fed 1x maintenance diet and those fed 200% maintenance diet and those fed 200% maintenance diet and 300% maintenance diet (Fig. 4.2A; $p \leq 0.05$), but no difference between those fed 100% maintenance diet and 300% maintenance diet (Fig. 4.2A; p=0.237). Expression of disease as assessed by histology showed no significant difference between the field sample and 100% maintenance diet (Fig. 4.2B; p=0.333), but there was a difference between the field sample and the 200% and 300% maintenance diet (Fig. 4.2B; $p<0.05$). No noticeable differences were observed with respect to tissue condition nor was there any evidence of degraded parasite, inflammation, or immune response. Oysters in the fourth experiment were fed treatments of 100%, 200%, or 300% the recommended maintenance diet. Following 33 days of treatment, *H. nelsoni* prevalence was assessed using PCR and disease expression evaluated using histological examination. Infection prevalence assessed by PCR showed a difference between the field sample and oysters fed 200% or 300% maintenance diet (Fig. 4.3A; $p \leq 0.008$), but no significant difference between the field sample and oysters fed 100% maintenance diet (Fig. 4.3A; p=0.438). Infection assessment using histology showed a reduction between the field sample and 100% maintenance diet and 300% maintenance diet (Fig. 4.3B; p=0.004), but no difference between the field sample and 200% maintenance diet (13%) (Fig. 4.3B; p=0.333). No noticeable differences were observed with respect to tissue condition nor was there any evidence of degraded parasite, inflammation, or immune response.

Figure 4.1

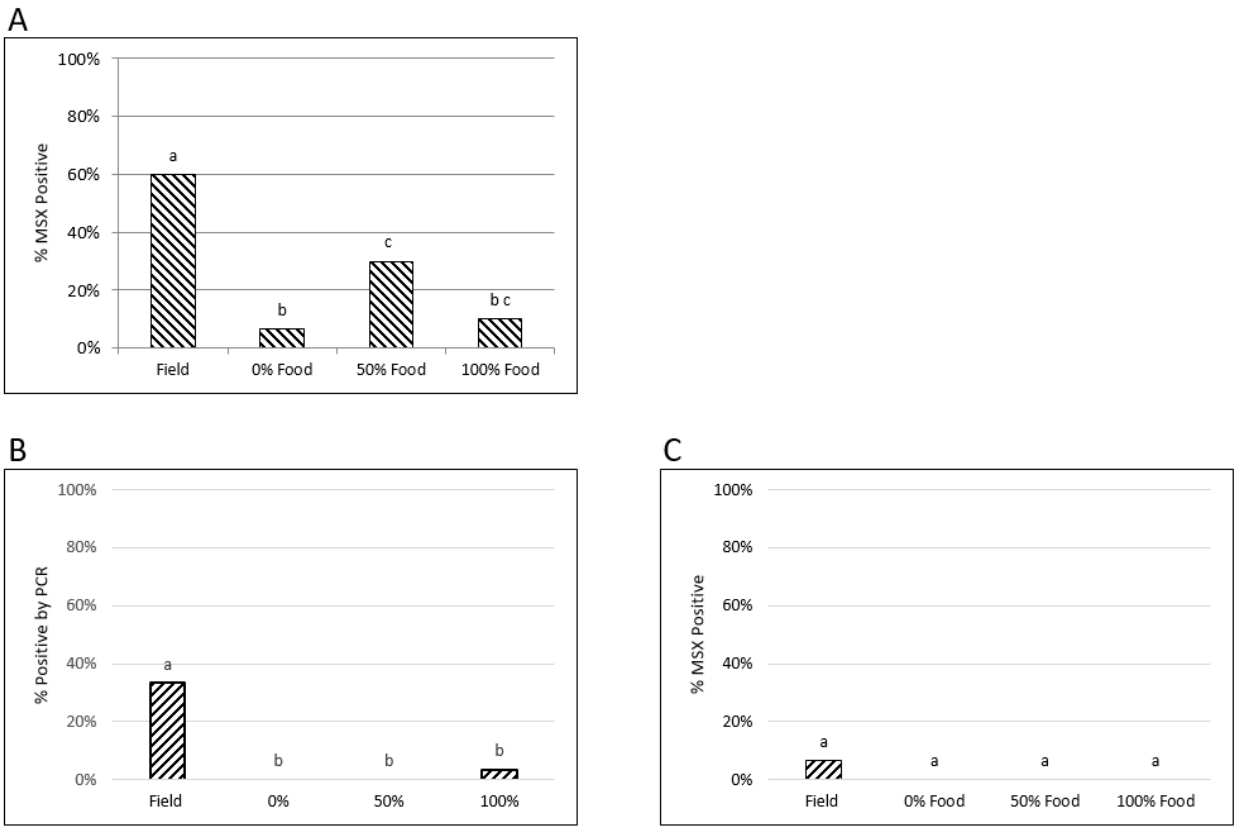

Figure 4.1. *H. nelsoni* prevalence in *C. virginica* by (2011) (A) PCR following various feeding treatments for 28 days at 20ºC. *H. nelsoni* prevalence in *C. virginica* by (B) PCR and (C) histology following various feeding treatments for 28 days at 20ºC. Oysters were fed maintenance diet (according to Reed Mariculture). 100% represents the manufacturer's recommended feed rate (3.6 mL / 100 g of wet tissue weight). *n*=30 for all samples. Common letters denote no significant difference between treatments as assessed by Fisher's Exact Probability Test.

Figure 4.2

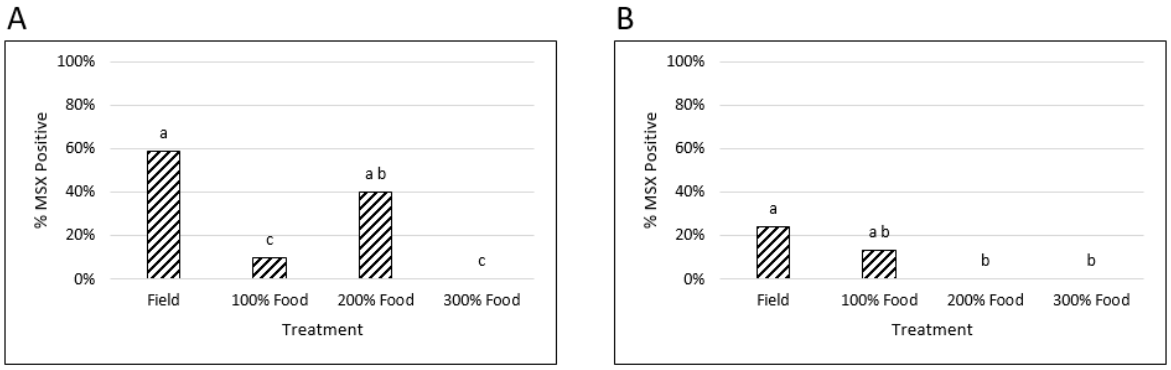

Figure 4.2. *H. nelsoni* prevalence in *C. virginica* (2013) by (A) PCR and (B) histology under different food treatments at 20ºC for 28 days as determined by PCR. Oysters were fed maintenance diet at 100% recommended dose according to Reed Mariculture (3.6 mL / 100 g of wet tissue weight) and up to 300% maintenance diet. *n*=30 for all samples. Common letters denote no significant difference between treatments as assessed by Fisher's Exact Probability Test.

Figure 4.3

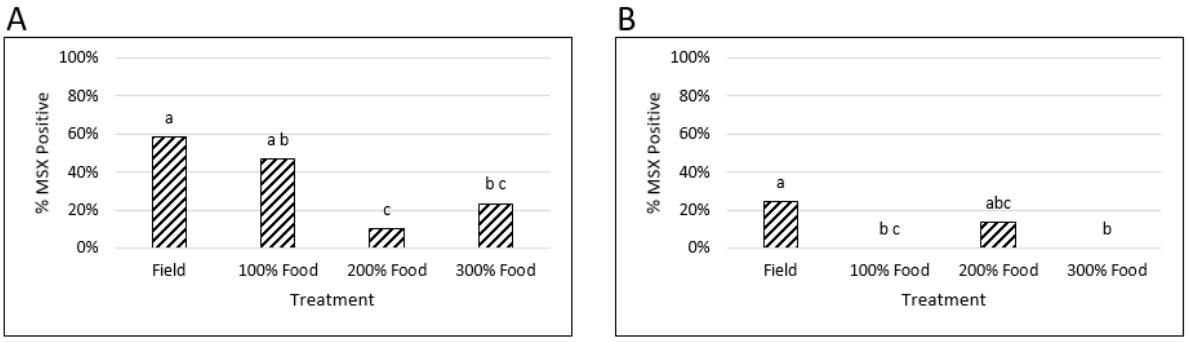

Figure 4.3. *H. nelsoni* prevalence in *C. virginica* (2013) by (A) PCR and (B) histology under different food treatments at 20ºC for 33 days as determined by PCR. Oysters were fed maintenance diet at 100% dose according to Reed Mariculture (3.6 mL / 100 g of wet tissue weight) and up to 300% maintenance diet. *n*=30 for all samples. Common letters denote no significant difference between treatments as assessed by Fisher's Exact Probability Test.

4.4 DISCUSSION

The results from the feeding trials with maximum food availability at 100% recommended food volume per oyster showed similar results to what was seen in previous temperature experiments when assessed using PCR and histology (Chapter 3 – Figs. 3.1 to 3.4). For each of the treatments with maximum food availability at 100% of manufacturer's maintenance diet, the prevalence of the parasite was reduced from the initial field sample in all treated samples, as determined by PCR and histology. Those oysters fed a reduced diet, no food or 50% of the recommended amount, showed a reduction when compared to the field sample, but the level of reduction was not consistent when the experiment was replicated. This may be a result of the history of the oysters prior to being treated. Without knowing the specific environmental conditions individual oysters were exposed to prior to collection (days, weeks, or months), the response to treatment could be very individualistic. Despite the fact that oysters were collected from a single location within a 10 m radius to control for environmental factors, their individual behaviour could result in differences among the sampled group. Possible prior factors that could affect individual oysters prior to field collection include food availability, temperature, salinity, predators, and overall health condition and any one of these factors individually or collectively could impact the response an individual oyster has to the treatment used. The reason for no food and reduced food was to investigate the possibility that the stress induced from a lack of food could be beneficial for the parasite if the oyster host was in a weakened state with respect to immunity; however, it is also possible that the lack of available resources could also negatively impact the parasite. Another possibility that could be a factor in these results was that oysters at this latitude have evolved to survive for months at a time over the winter when water temperatures are low and they do not feed. Thus, not feeding for four weeks may not have

created the intended stress that could affect the immune system of the oyster. To examine this possibility, oysters might have to be held with reduced or no food available to them for a longer period.

Feeding trials when oyster food availability was increased or decreased compared to the recommended diet from the manufacturer showed unpredictable results with no definitive pattern when PCR prevalence was compared to histological prevalence, and the infection pattern was different from 28 days of treatment compared to 33 days of treatment. Unlike when the maximum food available was 100% of the recommended diet, oysters with a greater amount of food available than the recommended diet showed differences in prevalence when assessed by PCR and/or histology. For example, after 28 days of 100% to 300% of the recommended diet, there was a reduction in PCR prevalence of only 19% for those fed 200% of the recommended diet, the lowest of the treatments; however, when assessed histologically, this same treatment had one of greatest reductions in parasite presence. In contrast, in this same treatment experiment, oysters fed 100% of the recommended diet had the second greatest reduction in parasite presence when assessed by PCR, but the least reduction when assessed by histology. For those oysters held for 33 days under the same conditions, the greatest reduction in parasite presence assessed by PCR was the group fed 200% recommended diet, which contrasted with the 28-day treatment group with the same food availability. Furthermore, the 200% treatment group that had the greatest reduction in parasite presence by PCR showed the least reduction in parasite presence when assessed by histology. The observed differences and lack of clear pattern in these results suggest that food availability may play some role in the development of the parasite inside the oyster. However, much more detailed and extensive studies are necessary to develop the relationship between food availability and parasite development.

The differences in the results of the 28 and 33 day treatments warrant further examination. All the oysters exposed to the 28 and 33 day treatments were collected from the same location and at the same time and in the same group of tanks per treatment. The only experimental difference between the two experiments was exposure time at 28 days and 33 days, with different oysters sampled at each time point (due to lethal sampling). In other treatment experiments, the trend was for parasite prevalence to decrease as treatment time increased. It is interesting to note that these food availability studies are the only experiments we conducted where the parasite prevalence increased following continued exposure to the treatment. Oysters exposed to 100% food availability after 28 days only showed a 10% prevalence in contrast to those after 33 days showing a 47% prevalence. For those oysters fed 300% of the recommended diet, at 28 days the PCR prevalence was reduced to 0% whereas those held for 33 days to 23% from the initial 59%. The one food treatment that showed results consistent with a previously observed trend (reduction of parasite presence over time) (Chapter 3) was the group fed 200% with a 40% PCR prevalence after 28 days and a 10% after 33 days; however, this group showed an increase in parasite presence from 28 to 33 days when analyzed histologically. These results suggested that factors other than food availability are responsible for the observed differences. Possibilities include differences in previous life history of oysters, individual variability in oysters, or multiple infections, but taking these differences into account it was important to note that differences did exist across treatments suggesting that further investigation into the role of food availability is worthwhile.

Pernet et al. (2015) suggested the use of predictive modeling for disease prevention and while this may be applicable for many pathogens, caution is advised, as there are diseases that are not well understood and for which the models were not accurate. Models developed

by Ford et al. (1991) and Hofmann et al. (2001) suggested that food plays a critical role in the development of disease. The results of the current feeding experiments indicate that food availability did not have a direct effect on the progression of *H. nelsoni* infections with respect to the host-parasite relationship under controlled laboratory conditions. However, laboratory conditions may have created conditions that affected the host-parasite response such that there are limits to the conclusions to be made. Previous laboratory experiments have shown that oysters held for four weeks at temperatures in the 20^{o}C range showed a reduction in *H. nelsoni* infection and this was in direct contrast with predictions based on the Ford et al. (1991) and Hofmann et al. (2001) models, as well as field observations from the U.S. with respect to *H. nelsoni* and temperature. As with temperature, food may be a significant factor in the development of *H. nelsoni* and progression to disease in oysters; however, the results of this study suggested that food availability did not affect the *H. nelson – C. virginica* interaction. Food availability may affect some other factor in the life cycle of *H. nelsoni* that these experiments did not include, such as the abundance and/or health of candidate intermediate hosts.

Environmental stressors exist in various forms and can have a negative impact on host immunity causing an increase the level of parasitism or they can cause a decrease in the level of parasitism because of negative impacts on the parasite (Lenihan et al., 1999). To evaluate the impact that water flow and subsequent food availability and feeding behaviour have on infection levels, Lenihan et al. (1999) assessed the interaction between *C. virginica* with *P. marinus* based on their position on the reef. They found that food availability, due to environmental conditions such as water flow, might result in greater or lesser exposure to infective particles because it can alter the feeding behaviour of oysters. Decreased food availability resulted increased feeding rates and increased infective particle exposure,

whereas increased food availability resulted in decreased feeding rates and decreased infective particle exposure. Haskin and Andrews (1988) referred to a lack of phytoplankton production as a possible factor in the progression of *H. nelsoni* to a disease state because the reduction in food availability could make the oysters more susceptible to disease. The model developed by Ford et al. (1999) suggested that the infection level of *H. nelsoni* is a function of the filtration rate of oyster hosts and the abundance of infective particles. The abundance of particles was influenced by salinity on large and small scales and by long-term fluctuations in temperature, but the model did not address feeding rates that, according to Lenihan et al. (1999), could be an important factor. Dose response curves have been determined for *P. marinus* (Ragone Calvo et al., 2003), but similar information is not yet available for *H. nelsoni* (Burreson and Ford, 2004). Isolating *H. nelsoni* so controlled dosages could be administered would provide valuable information in developing our understanding of the parasite-host relationship and allow for better experimental investigations. The current study could not examine the effect of food availability on changes in prevalence due to feeding behavior since no infective particles were present in the laboratory system.

The unknown previous history of the oyster hosts prior to undergoing the various feeding treatments must be considered as a potential complicating factor to any controlled laboratory experiments with *H. nelsoni*. With so little knowledge of *H. nelsoni*, there were challenges conducting controlled laboratory experiments when the organisms have unknown previous histories with respect to factors, such as temperature, food, salinity or other unknown variables that could have an impact on the outcome of the treatment. Haskin and Andrews (1988) pointed out that we do not know the infective stage of the parasite and that life cycle

elucidation would be the most important advance in our understanding of *H. nelsoni*. Their observations are as accurate today as they were 30 years ago.

It would be worthwhile to examine the role of food availability in the field and monitor *H. nelsoni* infection progression in oysters, both prevalence and disease expression. To date, this avenue of research has not been pursued with respect to *H. nelsoni*. Results of the current study indicated that food availability did not appear to have a direct effect on the progression of *H. nelsoni* in already infected oysters. However, this did not rule out the possibility that food availability could affect increased uptake of *H. nelsoni* or some other factor involved in the life cycle of the parasite such as the abundance or health of possible intermediate or reservoir hosts.

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CHAPTER 5 EVALUATION OF RESISTANCE TO MORTALITY FROM *HAPLOSPORIDIUM NELSONI* IN *CRASSOSTREA VIRGINICA* IN THE BRAS D'OR LAKE

ABSTRACT - The aim of this study evaluated the possibility that oysters previously exposed to *H. nelsoni* in the Bras d'Or Lake have developed resistance to mortality from *H. nelsoni*. In the U.S., 60 years of research on the development of resistance to mortality has resulted in oysters that can survive an *H. nelsoni* infection for up to six years before dying. The initial oyster mortality events in the Bras d'Or Lake occurred in 2001 and 2002, approximately 16 years prior to the current study. Since the initial mortality events, some heavily affected locations have had reductions in observed mortality of oysters. However, the level of *H. nelsoni* activity in those locations since the initial infection is unknown. The focus of this research was to determine if the observed lack of mortality was due to the development of resistance to mortality or if there could be some other factors. Oysters from three locations that suffered previous mortality from *H. nelsoni* were transferred to an area with known *H. nelsoni* activity along with oysters that are known to be susceptible to *H. nelsoni* infection and subsequent mortality to act as a control group to determine *H. nelsoni* activity at the transfer site. Oysters were assessed for *H. nelsoni* presence prior to transfer and oyster mortality and parasite presence was assessed three times (9, 11, & 16 months) following transfer. At nine months, there was a reduction in *H. nelsoni* infection and little mortality. After 11 months, there was infection and mortality among oysters from all sites. After 16 months, there were signs of infection and significant mortality of oysters from all sites. The 11-month mortality was not expected; thus oysters may have been be infected with an unknown agent that caused the mortality, caused premature mortality from *H. nelsoni*, or both. It is possible that the lack of mortality in the source sites of the transferred

oysters was due to a lack of *H. nelsoni* activity at those locations and not due to the development of resistance to mortality from *H. nelsoni*.

5.1 INTRODUCTION

In 1957, the Delaware Bay area suffered oyster mortalities (Haskin and Ford, 1979). The following two years, the mortality spread over a wider area of Delaware Bay and after a three-year period, it was estimated that 90-95% of the oyster population was killed in high salinity areas and 50-70% in lower salinity areas. The only areas "protected" from the mass mortalities were the tidal rivers and estuaries that consistently had very low salinity (Haskin et al. 1965). In 1966, Haskin et al. identified the disease-causing organism as *Minchinia nelsoni* (now called *Haplosporidium nelsoni*). In 1959, similar events occurred in lower Chesapeake Bay (Andrews and Wood, 1967).

Farley (1968) reported that oysters from previously infected populations showed some resistance, because those that had survived infection and showed signs of previous infections (such as mantle recession), were found to have localized infections contained to the gill region which suggested that a re-infection occurred but was confined within the gill area. These infections showed moribund parasites with little infiltration, suggesting that the parasite was not able to progress beyond the initial site of infection. There were no obvious signs of resistance in the introduced oyster populations (based on infections levels post exposure). As a result, Farley (1968) suggested that the difference in the state of the infection was a result of resident oyster populations' previous exposure to *H. nelsoni* and subsequent resistance development. Despite the evidence for some degree of resistance development, Farley et al. (1968) stated that it was too soon to determine if there is an immunity to *H. nelsoni* infection developing and if it would be similar to the immunity

observed with Malpeque disease resistant oysters (Logie et al., 1960). They demonstrated the development of resistance to Malpeque disease in surviving offspring of oysters that had survived the initial mortality event in Malpeque Bay, P.E.I., Canada in 1913 from an unknown etiology. The result of this mortality event was a natural development of inherited immunity (presumably) producing a population of oysters that was free from mortality from the unknown causative agent.

With respect to *H. nelsoni* infections, in 1979 there was evidence that an oyster population that had suffered previous devastation from *H. nelsoni* showed resistance to mortality (Haskin and Ford, 1979). The evidence to support this conclusion was the presence of spat from surviving oyster populations in Delaware Bay showed increased survival. From 1958-60, oysters were exposed to heavy infection from *H. nelsoni* with the following levels of survival: (1) 1957, 84% mortality; (2) 1958, 48% mortality; and (3) 1959, 29% mortality. In addition to increased survival year after year, oysters from Delaware Bay showed much lower mortality to *H. nelsoni* when compared to naïve stocks.

H. nelsoni infection levels in the wild population approached 100% prior to a mortality event; but laboratory reared stocks from brood stock that survived previous infections showed reduced mortality due to some inherited trait when transferred into *H. nelsoni* positive locations (Haskin and Ford, 1979). They proposed that the most susceptible oysters die after the initial infection, but after two to three infection cycles, the only remaining oysters are those that had some capacity to resist mortality; however, after three generations, *H. nelsoni* killed oysters that had survived previous infections. Ford and Haskin (1987) continued working with oysters that demonstrated increased survival over successive generations. However, despite the increased survival over three generations, the

continuation of increased survival slowed/stopped and the fourth and subsequent generations had a higher mortality level than expected. This observation was consistent with what Haskin and Ford (1979) predicted for the Delaware Bay area and Andrews (1968) and Farley (1975) for the Chesapeake Bay area. One conclusion from these observations was that resistant oysters become overwhelmed and eventually succumbed to the parasite during times of intense activity of the pathogen (infection pressure) (Haskin and Andrews, 1988). Ford and Haskin (1988) continued this work and proposed that the increased oyster mortality observed in the mid-1980s was not so much a failure of the disease resistant strains that had developed, as much as it was due to increased disease pressure not previously experienced.

The sites chosen for this field study included Potlotek, East Bay, Gillis Cove, and Waycobah. Oysters from Potlotek have tested positive for *H. nelsoni* for more than eight years with a prevalence by PCR between 10-30%, but the expression of disease was not observed histologically (L. Penny, DFO personal communication; Savoie-Swan, V. 2012). Oyster mortality has been low (A. Basque, Potlotek First Nation, personal communication) and recent testing showed a prevalence of 17% with no parasites observed histologically. The area is open to the larger Bras d'Or Lake and salinity is consistently in the 18-20‰. East Bay oysters have tested positive for *H. nelsoni* for 10 years and suffered mortalities on the order of 80% until 2015 (L. Penny, DFO, personal communication) when survivorship increased, but prevalence remained in the range of 60% by PCR (L. Penny, DFO, personal communication). The area is at the east end of the Bras d'Or Lake and salinity is in the range of 18-20‰. Gillis Cove oysters suffered losses from *H. nelsoni* in the early 2000s with losses approaching 95%. Since 2015 there was increased survivorship and in 2016 the parasite was undetectable and the oyster population in Gillis Cove appeared to be

recovering. Gillis Cove is connected to the larger Bras d'Or water body by a narrow, shallow channel approximately 5 m in width and 1-1.5 m deep, thus providing reduced water exchange with the larger Bras d'Or Lake when compared to more open areas such as Potlotek. The salinity of Gillis Cove is similar to Potlotek being in the range of 18-20‰. Waycobah oysters are from a location that had previously suffered losses near 90% (R. Stuart, personal communication) from *H. nelsoni* but showed reduced mortality levels possibly because of the manner in which they were contained in floating cages at the source site. The salinity rage in Waycobah is 18-20‰; however, periodic heavy rainfalls may cause a temporary reduction in salinity.

The current field study aimed to determine if the lack of mortality observed at two locations, East Bay and Gillis Cove, that had previously suffered losses from *H. nelsoni*, was due to: (i) some degree of resistance to mortality from the parasite, or (ii) if the reduced mortality was a result of a reduction in the activity of *H. nelsoni* at the source site. Furthermore, oysters from Waycobah had also shown very little mortality from *H. nelsoni,* so there was an interest to determine if those oysters showed some degree of resistance to mortality, or if there was a lack of *H. nelsoni* activity at that site. Potlotek oysters are susceptible to *H. nelsoni* infection and had previously suffered mortality when placed in Nyanza Bay in 2013 (L. Penny, DFO, personal communication), an area that is known to have active *H. nelsoni* infection activity. Potlotek oysters served as a control population for the assessment of the degree of *H. nelsoni* activity in Nyanza Bay. This field study was designed to determine if the lack of mortality observed in these locations was a result of the development of resistance to mortality or if it was due to lack of *H. nelsoni* activity at the source site.

5.2 METHODS AND MATERIALS

5.2.1 OYSTER COLLECTION AND TRANSFER

Oysters were collected from four different locations within the Bras d'Or Lake and transferred to Nyanza Bay, an area in the Bras d'Or Lake known to have active and lethal *H. nelsoni* activity, in September 2015. Oysters were monitored for *H. nelsoni* prevalence and oyster mortality until December 2016. The four sources of oysters included two locations that had previously suffered losses from *H. nelsoni* (East Bay and Gillis Cove), one location that was *H. nelsoni* positive where oysters are held on the surface (to prevent smothering) and show no mortality from *H. nelsoni* (Waycobah), and one location, Potlotek, that had low *H. nelsoni* prevalence, but was known to be susceptible to infection and subsequent mortality when placed in Nyanza Bay (DFO, personal communication, L. Penny).

Oysters of various sizes, but a minimum of three years old as determined by the supplier, from each location were placed in separate cages and located approximately 1.2 m below the surface of the water within a 4.5 m radius. Potlotek oysters were placed in two cages (approximately 94 / cage), East Bay oysters were distributed among five cages (approximately 132 /cage), Gillis Cove oysters were distributed between two cages (approximately 120 / cage), and Waycobah oysters were distributed between two cages approximately 150 / cage). An initial sample (n=30 from each site) was collected in September 2015 to determine the prevalence of each cohort of oysters prior to transfer into Nyanza Bay. Subsequent samples were taken in June 2016, August 2016 and December 2016 to determine *H. nelsoni* prevalence, periodic mortality, and total mortality was determined in December 2016.

Figure 5.1

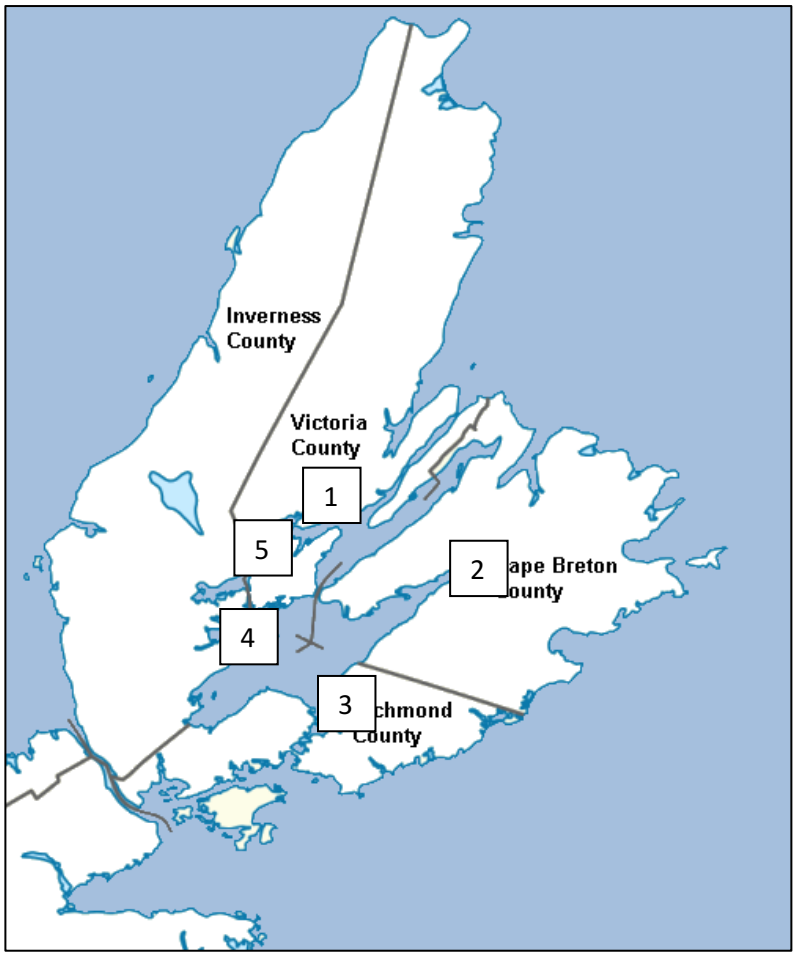

Fig. 5.1. Map of Cape Breton indicating oyster source sites (2, 3, 4, 5) and transfer location (1). 1=Nyanza Bay; 2=East Bay; 3=Potlotek; 4=Gillis Cove; 5=Waycobah.

5.2.2 OYSTER PROCESSING

Individual oysters were washed free of fouling organisms upon sampling. Oysters were shucked with a knife sterilized by submersion in 95% ethanol and subsequent flaming, and their tissues removed from the shell onto a sterile bench covering. Sterilized forceps and scalpel were used to isolate three cross sections of mantle gill and digestive gland in succession; the first section placed in a labeled cassette and then into Fisher's Histoprep™ for histology; the second section placed in a sterile 1.5 mL microcentrifuge tube containing 95% ethanol; and the third section wasplaced in a sterile 1.5 mL microcentrifuge and

transferred to -80°C storage. Remaining tissues were placed in sterile Whirl-pack™ bags and frozen at -80°C to preserve the remainder of each oyster.

5.2.3 PCR ANALYSIS

DNA was extracted from tissue samples stored in ethanol using a Qiagen DNeasy™ tissue extraction kit using the manufacture's protocol. Total genomic DNA concentration was determined using a GeneQuant Pro spectrophotometer (Fisher) at 260 nm. Template DNA was added to a PCR reaction mixture containing 25 µl AmpliTaq Gold PCR Master Mix (AmpliTaq Gold DNA Polymerase 0.05 U/µL, GeneAmp PCR Gold Buffer, (30 mM Tris/HCl, pH 8.05, 100 mM KCl) dNTP, 400 µM each, $MgCl_2$ 5 mM), 2.5 µL (1.0 µM) of each forward and reverse primer (MSX-A (5'-GCATTAGGTTTCAGACC-3') and MSX-B (5'-ATGTGTTGGTGACGCTA-ACCG-3') (Stokes and Burreson, 1995), 10 µL (500 ng) Template DNA, and 15 µL molecular biology grade water. This mixture was subjected to a temperature cycling protocol of initial denaturation of 94°C for 4 min, 35 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 1.5 min, and a final extension at 72°C for 5 min (as per the World Organization for Animal Health diagnostic protocol for the detection of *H. nelsoni* - http://www.oie.int/standard-setting/aquatic-manual/) using a TECHNE TC-412 thermocycler (Fisher). Amplified DNA was electrophoresed on a 1% agarose gel containing 0.5 µg/mL ethidium bromide adjacent to a molecular weight standard along with positive and negative controls and subsequently viewed under UV light using an Alpha Innotech imager (Fisher Scientific, Waltham, Massachusetts, U.S.A). Images were taken for each gel and annotated with sample numbers and amplicons of appropriate size (500 bp)

compiled as positive diagnostic results for each sampling locality group. Samples analysed by PCR were categorized as positive or negative for *H. nelsoni*.

5.2.4 HISTOLOGY ANALYSIS

Paraffin embedded blocks were prepared from tissues in Fisher's Histoprep™ fixative and sections prepared according to the procedure as laid out in the OIE Manual for Diagnostic Tests for Aquatic Animals (2003) by the Nova Scotia Department of Agriculture in Truro, N.S. Two 5 μm sections were stained with hematoxylin and eosin and examined for the presence and absence of plasmodia and then assigning individuals based on the intensity rating system 0 – 4 as described in Carnegie and Burreson (2011). While a 0-4 scale was used for histological analysis, due to the most common score being "1", a score of "0" and ">0" was used as an indicator of disease expression. Any signs of pathology or unhealthy parasites were noted for each organism.

5.2.5 MORTALITY CALCULATION

Periodic mortality was calculated by dividing the number of dead oysters by the total number of oysters in the cage at the time of the count. Adjustments were made for oysters collected during sampling.

Cumulative mortality was calculated by dividing the total number of dead oysters at the end of the field study by the total number of oysters at the beginning of the experiment. Adjustments were made for oysters collected during sampling.

5.2.6 STATISTICAL ANALYSIS

For PCR analysis, samples were grouped as *H. nelsoni*-positive or *H. nelsoni*-negative for comparison. For histological analysis, samples were grouped as 0 or >0. Differences in *H.*

nelsoni prevalence (by both PCR and histology) were analyzed using Fisher's Exact Probability test; differences were considered significant at $p<0.05$.

https://udel.edu/~mcdonald/statfishers.xls

5.3 RESULTS

5.3.1 PCR ANALYSIS

At each sampling time (June, August, December) there was an increase in *H. nelsoni* prevalence in surviving oysters as assessed by PCR, except for a decrease in East Bay, Gillis Cove, and Waycobah oysters from September to June, and no change in Waycobah in June and August (Figure 5.2). Overall, following June, there was a trend toward increasing *H. nelsoni* presence (as determined by PCR) in August and December, suggesting active infections occurring in the preceding months. Initial *H. nelsoni* prevalence (September) across oysters from all source sites revealed a prevalence ranging from 0% to 16% with no significant difference between oysters from any sites when compared to each other (Fig. 5.2A; $p\geq0.01$). Infection prevalence in oysters when assessed using PCR following 16 months (December) showed no difference between oysters from all sites when compared to each other (57% to 77%) (Fig. 5.2D; $p\geq0.17$).

5.3.2 HISTOLOGY ANALYSIS

Prior to transfer into Nyanza Bay in September, 27% of oysters from East Bay had visible parasite presence; oysters from other locations did not have visible parasites. In June, there was no histological evidence of the parasite in oysters transferred into Nyanza. The August and December oyster samples had observed parasites in oysters from three sites (Potlotek, East Bay, and Gillis Cove) with a lower parasite presence observed in December oyster samples compared to those oysters collected in August from all three sites. The exception

was Waycobah oysters where there was no evidence of parasite histologically. Initial examination (September) by histology across all source sites revealed a difference in oysters showing infections between East Bay samples (27%) and samples from all other sites (0%) (Fig. 5.3A; $p \leq 0.01$) and no difference between oysters from all other sites when compared to each other (0% at all remaining sites) (Fig. 5.3A; $p=1$). Histological examination of samples following nine months (June) in Nyanza Bay revealed no differences in infection presence when determined by histology between oysters from all sites when compared to each other (0% for all sites) (Fig. 5.3B; $p=1$). Samples collected in August after 11 months in Nyanza Bay showed that infection of oysters from East Bay (27%) were different from Waycobah oysters (0%) (Fig. 5.3C; $p<0.01$), but not different from Potlotek oysters (10%) or oysters from Gillis Cove (13%) (Fig. 5.3C; $p \geq 0.18$). Oysters from all other sites were not different from each other when their infection level was compared to one another (0% to 13%) (Fig. 5.3C; $p \geq 0.11$). Final examination of samples collected in December after 16 months in Nyanza Bay showed that the infection level in oysters from East Bay (20%) was different from Waycobah oysters (0%) (Fig. 5.3D, $p=0.02$), but oysters from other sites were not different from each other (0% to 7%) (Fig. 5.3D; $p \geq 0.10$).

5.3.3 PERIODIC MORTALITY ANALYSIS

There was evidence of oyster mortality at each sampling time (June, August, and September), with noticeable mortality evident during August and December. The highest oyster mortality for August was observed in the Potlotek oysters (64%) and the highest mortality in the December sample was East Bay oysters (63%). For both August and December, Waycobah oysters showed the second highest mortality levels (63% and 49%, respectively) and for each time of sampling, Gillis Cove had the lowest oyster mortality level (August = 47% and December 40%). Analysis of mortality levels in June, after nine

months in Nyanza Bay, showed that Potlotek oyster mortality (12%) was different from all other sites (≤6%) (Fig. 5.4A; p≤0.01), East Bay oyster mortality (6%) was different from Waycobah (3%) (Fig. 5.4A; p=0.02), and no difference between Gillis Cove (3%) when compared to East Bay (6%) or Waycobah mortalities (3%) (Fig. 5.4A; p≥0.06). In August, the initial mortality level was high and ranged from 47% to 69%. In August, after 11 months in Nyanza Bay, mortality levels of remaining oysters from Gillis Cove (47%) differed from all other sites (≥64%) (Fig. 5.4B; p≤0.01); mortality levels among other sites (64% to 69%) did not vary significantly (Fig. 5.4B; p≥0.08). In December, the mortality levels of remaining oysters ranged from 40% to 63%, a decrease from the mortality levels observed in August. Mortality levels of remaining oysters in December, following 16 months in Nyanza Bay, showed a difference between East Bay (63%) oysters and oysters from all other sites (≤49%) (Fig. 5.4C; p≤0.03), and no difference was detected in oyster mortalities in oysters from other sites when compared to each other (40% to 49%) (Fig. 5.4C; p≥0.24).

5.3.4 CUMULATIVE MORTALITY ANALYSIS

The cumulative mortality of oysters, which is a measure of the total number of mortalities compared to the number of oysters at the start of the study, from all source locations following a 16-month deployment in Nyanza Bay showed high mortality ranging from 63% for Gillis Cove oysters to 88% for East Bay oysters. Comparison of cumulative oyster mortality for all sites following 16 months in Nyanza Bay revealed differences between sites (63% to 88%) (Fig. 5.5; $p \leq 0.04$), except between Potlotek oysters and Waycobah oysters, 82% and 74% respectively (Fig. 5.5; $p = 0.467$).

Figure 5.2

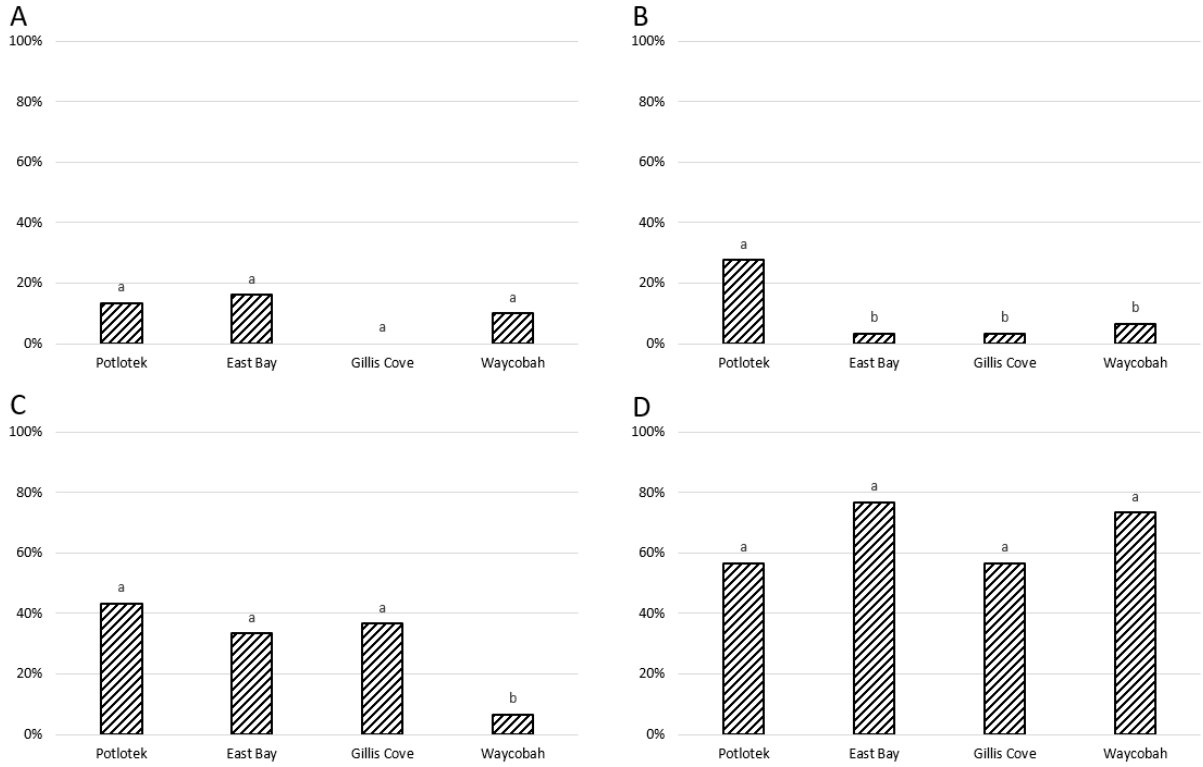

Fig. 5.2. *H. nelsoni* prevalence in oysters from different locations placed in Nyanza Bay in September 2015 assessed by PCR in *C. virginica* at time of sampling: (A) September 2015 (B) June 2016 (C) August 2016 (D) December 2016. *n*=30 for all samples except for *n*=31 East Bay in September (2015) and *n*=29 Potlotek in June (2016). Common letters denote no significant difference between oysters from different sites as assessed by Fisher's Exact Probability Test.

Figure 5.3

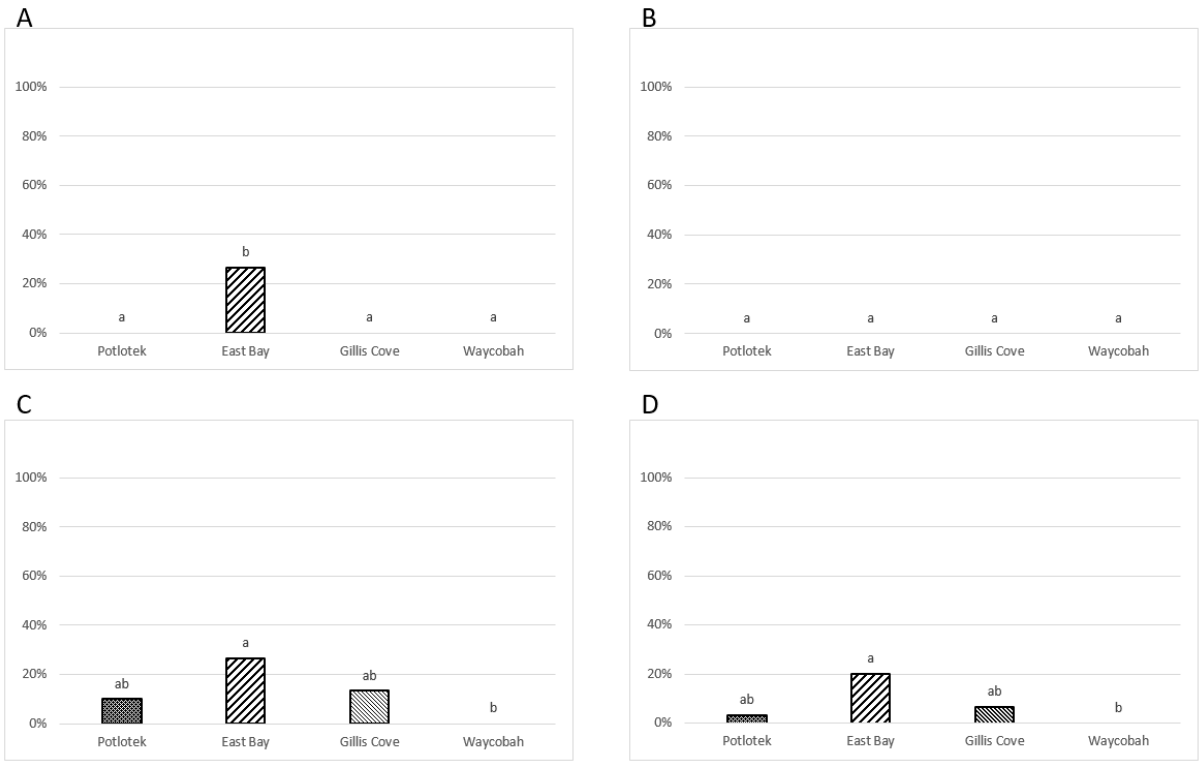

Fig. 5.3. *H. nelsoni* prevalence of oysters from different locations placed in Nyanza Bay in September 2015 assessed by histology in *C. virginica* at time of sampling: (A) September 2015 (B) June 2016 (C) August 2016 (D) December 2016. *n*=30 for all samples except for *n*=31 East Bay in September (2015) and *n*=29 Potlotek in June (2016). Common letters denote no significant difference between oysters from different sites as assessed by Fisher's Exact Probability Test.

Figure 5.4

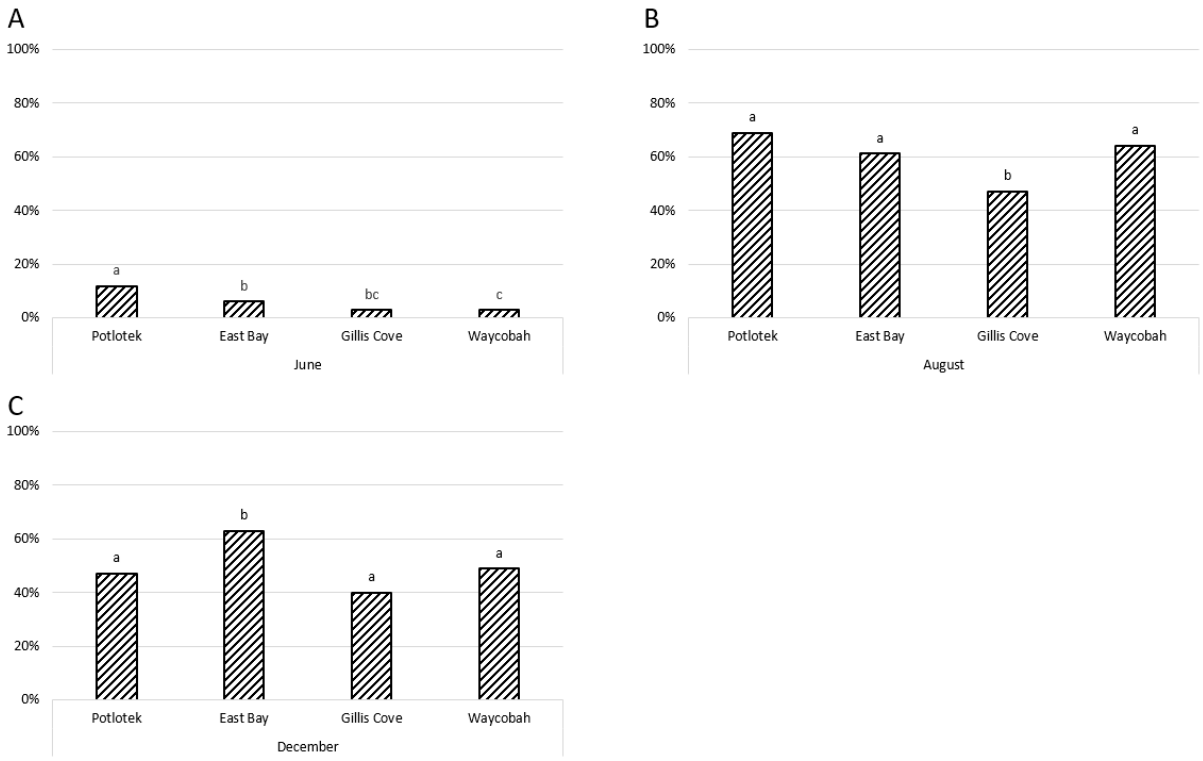

Fig. 5.4. Periodic oyster mortality of remaining oysters placed in Nyanza Bay from different source locations at sampling intervals (A) June 2016 (B) August 2016 (C) December 2016. June: Potlotek (*n* =188), East Bay (*n* =663), Gillis Cove (*n* =243), Waycobah (*n* =258). August: Potlotek (*n* =158), East Bay (*n* =633), Gillis Cove (*n* =213), Waycobah (*n* =228). December: Potlotek (*n* =49), East Bay (*n* =246), Gillis Cove (*n* =113), Waycobah (*n* =82). Periodic mortality was calculated from the percentage of oysters that were deceased compared to the total number in the cage at the time of sampling. Common letters denote no significant difference between oysters from different sites as assessed by Fisher's Exact Probability Test. *n* = number of surviving oysters.

Figure 5.5

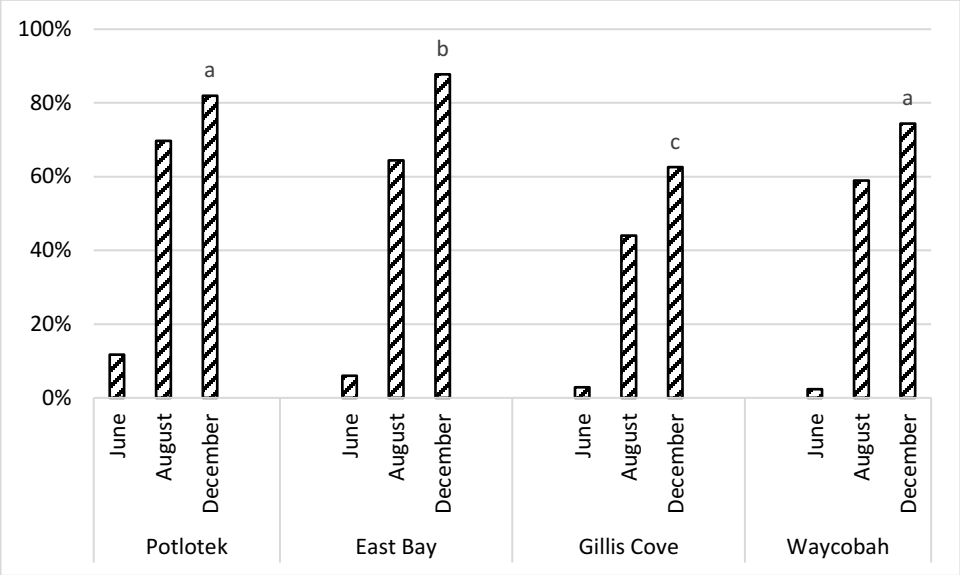

Figure 5.5 Cumulative *C. virginica* mortality from different sources following 16 months in Nyanza Bay. *n*=188 for Potlotek. *n*=663 for East Bay. *n*=243 for Gillis Cove. *n*=258 for Waycobah. Common letters denote no significant difference between oysters from different sites as assessed by Fisher's Exact Probability Test. *n* = number of oysters at the beginning of the field study.

5.4 DISCUSSION

Since 2008, oysters from locations in the Bras d'Or Lake that previously suffered losses were showed reduced mortality in their original locations. In the current study, when oysters from these locations were transferred to an area known to have *H. nelsoni* infection activity, significant infection prevalence and oyster mortality followed in oysters from all source locations. This suggested that the lack of mortality at their original locations was likely due to reduced *H. nelsoni* activity at those sites and not because of development of resistance to mortality from *H. nelsoni* infections. For example, in Gillis Cove, there was little oyster mortality in recent years, yet when oysters from Gillis Cove were placed in Nyanza Bay, cumulative mortality was 64% in 16 months. Infection levels increased from

June to December, so it was likely that infections took place in early spring, were detectable by June, and progressed through the summer and into the fall. This pattern was consistent with field observations in the U.S.; albeit, the timing was somewhat delayed in the Bras d'Or Lake which may be due to a difference in water temperature given the more northern location resulting in a later onset of temperatures that affect parasite life cycle factors that are yet to be determined.

H. nelsoni activity and presence may have changed over time in Gillis Cove. Oysters from that location suffered a die off when *H. nelsoni* first caused oyster mortalities in the Bras d'Or Lake, but the most recent sample collected from Gillis Cove showed no detectable level of *H. nelsoni* when assessed using PCR. One noticeable difference between Nyanza Bay and Gillis Cove is its physical location. Nyanza Bay, as described previously, is a large, wide-open area of the Bras d'Or Lake. Gillis Cove is a small, isolated region of the Bras d'Or Lake with a small opening to the larger Bras d'Or Lake water body resulting in less water exchange than Nyanza Bay. The reduced water exchange could reduce the possibility of recruitment of intermediate or reservoir hosts into Gillis Cove. Furthermore, Gillis Cove, because of its smaller size, may reach lower water temperatures in winter than Nyanza Bay. If environmental conditions were such that intermediate or reservoir hosts died, were reduced in abundance, or caused a reduction in the abundance of infective particles in the area by some other means, the lack of necessary life cycle components being recruited could explain the observed undetectable levels of *H. nelsoni* in oysters found in Gillis Cove. The fact that *H. nelsoni* was undetectable suggested that there was little to no *H. nelsoni* activity in Gillis Cove, thus no opportunity for natural resistance to the parasite to develop. Oysters that have levels of resistance to mortality develop infections, but are able to prevent the infection from becoming systemic. The oysters from Gillis Cove did not show

a similar infection pattern to what is associated most often with resistance to mortality development and Gillis Cove oysters had a mortality level of 60% following one year of exposure to a known *H. nelsoni* active location. The possible absence of *H. nelsoni* in Gillis Cove provided some explanation for the lack of mortality in Gillis Cove oysters and accounted for their susceptibility to *H. nelsoni* infection and mortality from infection when transferred to Nyanza Bay, at least in part. It is possible that the initial infections and mortalities in Gillis Cove resulted in oysters that were resistant to mortality, perhaps even infection; however, this was not consistent with observations from the U.S. with respect to the development of resistance to mortality from *H. nelsoni*.

Potlotek oysters have low infection prevalence (7% to 13%) when tested (L. Penny, DFO, personal communication) and there has never been a noticeable oyster mortality event associated with *H. nelsoni* in this area. When Potlotek oysters were transferred to Nyanza Bay, they suffered mortality (82% cumulative mortality after 16 months). Therefore, the sample from that population did not appear to have developed resistance to mortality from *H. nelsoni*. Oysters from the Potlotek area grow slowly, likely due to limited food availability (R. Stuart, personal communication). The lack of observed oyster mortality in Potlotek and the low *H. nelsoni* prevalence could be a result of limited nutrient availability that does not provide enough resources for the parasite to progress beyond low prevalence levels and light infections. Infections did occur and have been reported as high as 30% prevalence by PCR, but there was no histological evidence of infection and no obvious mortality observed (Savoie-Swan, 2012). This is consistent with observations in this study from the initial Potlotek sample collected in September prior to transfer to Nyanza Bay.

The fact that Potlotek oysters succumb to infection in Nyanza Bay could have numerous explanations. The historical lack of progression to lethal infection levels of *H. nelsoni* in Potlotek oysters would result in limited opportunities for the development of oysters resistant to mortality. The suspected lack of food, as indicated by slower oyster growth, could result in reduced abundance or health of intermediate or reservoir hosts, thereby resulting in lower doses of infective particles. Alternatively, the prevalence sometimes reaches 30% indicating that there was an abundance of infective particles reaching oysters, but for some reason the environment within the oyster did not support progression of the disease to lethal infection intensities or the environment was such that infective particles are not as virulent and so did not progress to lethal infections. It is also possible that there was a low abundance of infective particles accounting for a significant prevalence but the individual dosage threshold was not met, therefore infections failed to reach systemic levels within the oysters in that area. The lack of histological presence in samples collected from Potlotek oysters at their source site in this study can support any of these hypotheses and is consistent with histological observations when the prevalence of *H. nelsoni* by PCR reached 30% (Savoie-Swan, 2013).

Oysters from Waycobah showed no resistance to mortality from *H. nelsoni* but maintained one of the lowest infection levels by PCR and histology during the first 11 months of the study in Nyanza Bay. Waycobah oysters, unlike oysters from other sources, were grown in floating bags near the water surface for the last six years. These oysters showed low mortality and appeared to maintain an annual *H. nelsoni* prevalence at about 10% (R. Stuart, personal communication). The lack of mortality within these oysters at their source was of interest because unlike Potlotek oysters, they grew at a normal rate for the Bras d'Or Lake, therefore food availability was not likely to affect parasite progression. It was possible that

being located higher in the water column, where there are greater changes in temperature and salinity over short periods (hours/days) was providing some degree of protection from the parasite but this requires research to examine.

The transfer of Waycobah oysters into Nyanza Bay demonstrated that the lack of mortality observed in Waycobah oysters over the past six years at their source site was not due to development of resistance to mortality. The mortality levels from Waycobah oysters transferred into Nyanza Bay was significant following a single year of exposure. Furthermore, with an infection level of approximately 10% in Waycobah, which was comparable to oysters found in Potlotek, it was probable that Waycobah oysters were not in an environment whereby *H. nelsoni* activity was present to a great enough degree to select for *H. nelsoni* resistant oysters. The oysters from Waycobah showed a different infection pattern by PCR, with almost no incremental prevalence change until December. There was a lack of detectable *H. nelsoni* infection in Waycobah oysters and no histological evidence during the entire study, yet Waycobah oysters showed a similar mortality to other sites where prevalence, as assessed by PCR, increased and there was histological evidence of an infection. It was possible that the *H. nelsoni* rapidly killed infected oysters because these oysters were held at the surface for the past 3-5 years and may have had limited exposure to *H. nelsoni* infection thereby increasing their susceptibility to mortality from infection prior transfer into Nyanza Bay.

Of the four locations from which oysters were sourced and transferred into Nyanza Bay, East Bay appeared to be the location where there was likely to have been selection pressure for the development of a resistant oyster strain due to the consistent detection of *H. nelsoni* by PCR and histology over several years (DFO, Lorne Penny, personal communication).

East Bay oysters were the only group where *H. nelsoni* was observed histologically at three of the four sample times, the difference being that *H. nelsoni* was observed in the sample prior to transfer into Nyanza Bay. The initial infection of 2002 at East Bay resulted in approximately 60% mortality. Since that time, East Bay consistently has had an *H. nelsoni* infection level of approximately 60%. East Bay is at the eastern side of the Bras d'Or Lake and despite having higher infection levels than other locations in the Bras d'Or Lake, there has been a decrease in the degree of mortality observed from 2011-2016 (L. Penny, personal communication). East Bay is an open area like Nyanza Bay, but at the eastern end of the Bras d'Or Lake, so it could be a receiving and collecting area for potential intermediate or reservoir hosts. When oysters from East Bay were transferred into Nyanza Bay, they suffered high mortality (88%) following what was likely a single year of exposure to *H. nelsoni*. It is possible that other factors are involved, such as temperature and salinity changes and/or extremes, or other environmental factors. The abundance of necessary life cycle components may be different in East Bay compared to Nyanza Bay, such that it caused lethal infections in Nyanza Bay and non-lethal infections in East Bay.

The level of resistance to *H. nelsoni* mortality in an oyster population may be expected to reflect the rigor with which it has been selected through generations after exposure to *H. nelsoni* infections (Haskin and Andrews, 1988). The term "resistance" refers to resistance to mortality from an *H. nelsoni* infection not resistance to infection, which reflects the ability of the oyster to restrict the parasite numbers to tolerated levels (Myhre and Haskin, 1970; Ford and Haskin 1982, 1987; Ford and Figueras, 1988; and Ford, 1988). Valiulis and Haskin (1972) found that oysters that showed resistance for mortality from *H. nelsoni* infection were also more resistant to *P. marinus*. Ford and Haskin (1987) stated that the exact mechanism involved with resistance to mortality from *H. nelsoni* is not known, but it

could be that a non-specific defense mechanism was involved, or these oysters have a stronger physiological state that allows them to tolerate non-specific stress and could contribute to resistance to other infective agents. Two factors that are critical to survivorship are (i) the dosage, both initial and ongoing, neither of which can currently be measured accurately, and (ii) the kinds and numbers of additional stresses present (Haskin and Ford, 1979). Ford and Haskin (1987) further suggested that increased survival of selected strains of oysters resistant to mortality from *H. nelsoni* could be assisted by minimizing additional stressors. It is possible that neither the parasite nor the other stressor alone would be enough to cause mortality. However, when both occur together, they may be more than the oyster host is able to tolerate and mortality follows.

Oysters that were transferred into Nyanza Bay from four different locations all suffered mortality levels in excess of 47% from June to August. This was higher than expected as part of natural, annual oyster mortality in the Bras d'Or, especially for that time of year. Furthermore, the highest PCR prevalence at the time of the first mortality (August) was 43% which was lower than what is usually associated with mortality from *H. nelsoni*. It is possible that infection levels were initially higher, but mortality may have reduced the calculated prevalence amongst survivors. It is also possible that the observed infections were not new infections, but conditions in Nyanza Bay allowed for the proliferation of the existing infections. Alternatively, it is possible that there was some other cause of mortality that with the *H. nelsoni* parasite, resulted in premature death to infected oysters. Karvonen et al. (2018) stated that a single host may encounter multiple parasites during its life span and that the arrival sequence of the parasites can affect the infection success and virulence. In all likelihood, having multiple infections at the same time would also affect the host organism. Karvonen et al. (2018) also suggested that multiple infection interactions can be

antagonistic as they compete for similar resources or cause increased immune response from the host, or there could be an immune suppression occurring which would be of beneficial for infective agents.

One possible disease agent that could be present in the Nyanza Bay area is Malpeque disease. This disease is suspected to have caused significant oyster mortalities on leases near Orangedale in the Bras d'Or Lake in 2008 (R. Cusack NS DFA 2008, personal communication) which is not far from Nyanza Bay (approximately 37 kms). With no test to determine the presence or absence of Malpeque disease, there was no way to know if Malpeque disease contributed to the August mortality. Despite it being the suspected cause in a previous nearby mortality event, Malpeque disease causes oyster death 18 months following exposure of susceptible oysters to disease-associated waters. The unusual mortality observed in Nyanza Bay occurred somewhere between 10 and 11 months from the time of transfer. Furthermore, Malpeque disease is believed to be most lethal in high salinity waters (30ppt), not 18ppt as in Nyanza Bay. Thus, the evidence was stronger for *H. nelsoni* as the root cause of mortalities.

While investigating physiological comparisons of resistant versus susceptible oysters, Barber et al. (1991) demonstrated a delay of detectable infections in the resistant oysters. The susceptible oysters acquired noticeable infections shortly after exposure (June 1988), while the infection in resistant oysters did not appear until two months later. They found that susceptible oysters had an infection prevalence of 80%-90% in July through September while the resistant group showed a prevalence of 21%. Systemic infections in the susceptible group reached 70% in September while never exceeding 5% in the resistant group. The cumulative mortality of the susceptible group in the fall was as high as 82% and

reached 98% the following May, while the resistant group mortality was 3% in the fall and reached 15% in May (Barber et al., 1991). In the current study, oysters transferred into Nyanza Bay from other Bras d'Or Lake locations showed cumulative mortalities ranging from 63% to 88%, with three of the four groups having a mortality level ≥74%. In these oysters there were very few systemic infections, possibly due to mortality prior to any sample collection. Oyster cumulative mortality levels in the current study ranged from 63% to 88% following a single year of exposure to *H. nelsoni*. There appeared to be little evidence for resistance to mortality when compared to the results observed by Barber et al. (1991) at 15% following a single year of exposure to *H. nelsoni*. However, it is also important to consider that oysters in the Barber et al. (1991) study were not transferred from one location to another, thus, it is possible that the transfer of Bras d'Or oysters from their source location to Nyanza Bay (a potentially different environment) could have negatively affected their survival.

Nyanza Bay oysters suffered mortality during early 2001 and 2002 to the degree that in 2016 it was a challenge to find resident oysters in Nyanza Bay. In 2013, oysters from Potlotek were transferred into Nyanza Bay and high infection levels and high mortality were observed within four months of the transfer (June to September) (DFO, personal communication). Oysters that survived the exposure were removed from the area, thus no additional oysters were placed in Nyanza Bay since the initial mortality events almost two decades ago. Currently, Nyanza Bay has very few oysters due to previous mortality events and lack of natural recruitment to the area. However, as seen in the current study, *H. nelsoni* was active and did not appear to have reduced its capacity to cause mortality in oysters. The area itself is different from other locations in the Bras d'Or that have been affected by *H. nelsoni*. Most locations in the Bras d'Or Lake previously affected by *H. nelsoni* were

sheltered, cove-like water bodies. However, Nyanza Bay is an open water area exposed to the Bras d'Or Lake. It is possible that there was recruitment of life cycle components to the area such as potential intermediate or reservoir hosts each year. Another possibility was that necessary life cycle components may be present year-round. The presence of a small oyster population in Nyanza Bay along with active *H. nelsoni* infections still taking place suggested that there was some life cycle component present in Nyanza Bay, such as a reservoir host or intermediate host, that sustained the parasite. Alternatively, reservoir hosts or intermediate hosts carrying the parasite may be recruited into Nyanza Bay on a regular basis that allowed for regular release of infective particles. Another possibility, suggested by Andrews and Frierman (1974), is that a very small, infected oyster population might be all that is needed to maintain ongoing *H. nelsoni* infections in a larger oyster population.

This 16-month field study in Nyanza Bay using oysters from several locations across the Bras d'Or Lake revealed a decrease in *H. nelsoni* presence from September to June, possibly due to overwintering of the oysters. This was followed by an increase in parasite activity in the spring of the year as seen with the increase in *H. nelsoni* prevalence by PCR and histology in August in all samples. The one exception was Waycobah where there was no histological evidence of infection at any time during the study. The August increase in infection occurred concurrently with a mortality after only 11 months in Nyanza Bay and possibly one infection cycle. The PCR prevalence, histological evidence, and mortalities continued into December until the end of the study. Further investigation will be necessary to determine when infections occur in detail (days or weeks or under specific environmental conditions), as well as assessment of the parasite burden and abundance of potential intermediate or reservoir hosts.

Eutrophication is an environmental factor that can introduce stress into a host-parasite system because of increased nutrients causing decreased oxygen levels. For *H. nelsoni*, eutrophication is potentially important given that the parasite can be found in open areas with ongoing water exchange, such as Nyanza Bay, where one would expect the effects of eutrophication to be lower. In areas that have much less water flow, such as East Bay, one would expect the effects of eutrophication to be greater, but this may not be the case.

To examine the effects of eutrophication on pathogen transmission among several hosts in a complex life cycle (trematode-snail-amphibian), Johnson et al. (2007) compared parasite abundance in areas of ambient and high eutrophication. They found that increased eutrophication was associated with greater numbers and size of snails resulting in increased parasite egg production and cercaria. This increased parasite prevalence in the amphibian host. They suggested that overwintering behaviour in snail and amphibian hosts could affect the parasite population, but the experiment was not carried out long enough to assess that factor. From their research, it appears that greater eutrophication provides more nutrients resulting in an increase in snail host numbers and size, but there was no assessment of the effect of increased eutrophication on host immunity. Nutrient increase may favour greater parasite and snail population growth but could alter the immunity of the snail or amphibian hosts. This schistosome-trematode-amphibian experiment, where the life cycle is known, provided useful insight into the effects of eutrophication on host-parasite systems. It would be interesting to conduct similar experiments on parasites with direct, and known, life cycles such as *P. marinus*. A greater challenge may be to understand the impacts of eutrophication on those host-parasite systems where life cycle is unknown, such as *H. nelsoni*.

A detailed investigation into biological and non-biological factors in each oyster source location concurrent with a similar examination of biological and non-biological factors in Nyanza Bay is worthwhile to try and determine what factors may be playing a role in the infection and mortality patterns. Building the capacity to measure the dosage of infective particles in the water (or location) and that received by oyster hosts is a critical piece of missing information. This type of information would allow for an accurate assessment of the dosage in the water and the dosage to which oysters are exposed. Research of this nature would help to determine critical values associated with the progression of *H. nelsoni* infections to lethal degrees. The development of a resistant strain of oyster that shows increased survival from an *H. nelsoni* infection in the Bras d'Or Lake will require ongoing access to a location such as Nyanza Bay and decades of exposure to the parasite.

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CHAPTER 6 DISCUSSION

To investigate the role of environmental factors related to development of *H. nelsoni* disease in *Crassostrea virginica*, a combination of field and laboratory experiments were conducted. Based on the results of these experiments, it was apparent that determining the role of environmental factors in the development of *H. nelsoni* to a disease state will require detailed field studies of various biotic and abiotic factors. Locations such as MacDonald's Pond provided insight into the micro-scale variation that potentially exists with respect to *H. nelsoni* infections and demonstrated why micro-scale variation is an important consideration when studying *H. nelsoni*. Locations such as Nyanza Bay in the Bras d'Or Lake demonstrated how unpredictable *H. nelsoni* can be with respect to its presence in a location and ability to cause mortality in the absence of large oyster populations. This research showed where *H. nelsoni* reduced to undetectable levels in sampled oysters in an area that once suffered high oyster mortality such as Gillis Cove, but can exist at a prevalence that is normally associated with high mortality yet not cause mortality, as observed in MacDonald's Pond. Additionally, this research has revealed that the absence of mortality in a location previously associated with mortality caused by *H. nelsoni* should not necessarily be attributed to the development of resistance to mortality from an infection. Oysters in the Bras d'Or Lake in locations that suffered few losses in the last 6-8 years became heavily infected in less than 11 months and suffered significant losses when placed in Nyanza Bay. Therefore, it is important to assess the activity of *H. nelsoni* in water bodies where little oyster mortality is observed, using hosts that are known to be susceptible.

H. nelsoni showed more spatial variability than previously reported. Access to water bodies such as MacDonald's Pond and several locations in the Bras d'Or Lake provide a unique

opportunity to study *H. nelsoni* in the field. While *H. nelsoni* is unpredictable and different infection patterns have emerged in these areas, ongoing monitoring and future modeling would benefit from consideration of detailed infection patterns and environmental data from these locations. The lack of mortality in MacDonald's Pond despite high prevalence of infection was unusual for this parasite as is the high degree of oyster mortality of susceptible oysters when placed in Nyanza Bay with such a small resident oyster population. Both circumstances provide a unique opportunity to investigate this parasite further.

In discussion of the detection of Ostreid Herpesvirus type 1 (OsHV-1), Pernet et al. (2015) recommended sentinel oyster monitoring along with detailed habitat mapping, and better understanding of biotic and abiotic factors as a potential means to identify the risk factors associated with disease outbreaks from and to identify possible reservoir hosts. This is very similar to the type of monitoring needed in locations in the Bras d'Or Lake where *H. nelsoni* was causing mortality, such as Nyanza Bay, to determine the conditions and life cycle elements necessary for disease outbreaks. Pernet et al. (2015) suggested the use of predictive modeling for disease prevention and while this may be applicable for many pathogens, caution is advised, as there are diseases that are not well understood and for which the models are not accurate. They raised the point that most models assume at least one infected host in a population at all times. However, in aquatic systems pathogens may enter a system to initiate the infection cycle. Likewise, there are models for *H. nelsoni* prediction, yet the models do not consider the micro-scale differences that can occur with *H. nelsoni*, even within a relatively small, well-connected water body such as MacDonald's Pond. Existing models provide valuable information on pathogen movement and transmission; however, there must be ongoing modification of the models as new information becomes available. Carrying out this type of research in the Bras d'Or Lake

could lead to the determination of the life cycle of *H. nelsoni* and would add to our understanding of the conditions necessary for disease in currently unaffected areas of Nova Scotia as well as Prince Edward Island and New Brunswick.

Environmental stressors occur in various forms and can have a negative impact on host immunity causing an increase the level of parasitism (Lenihan et al., 1999), or they can cause a decrease in the level of parasitism because of negative impacts on the parasite (Lenihan et al., 1999; Lafferty, 1997). To evaluate the impact water flow and subsequent food availability and feeding behaviour have on infection levels, Lenihan et al. (1999) carried out an experiment using *C. virginica* and *P. marinus* by placing oysters under different flow conditions based on their position on the reef. The placement resulted in differences in food quantity and quality, and presumably different exposure to *P. marinus*. They concluded that oysters in low flow rates (at the bottom of the reef) were exposed to greater concentration of infective particles, because lower food quality and quantity would cause an increase in their feeding rates and this would result in increased exposure to the parasite. In contrast, oysters located higher on the reef were exposed to higher flow rates and more food of better quality and would reduce their feeding rates because they can adjust their feeding behaviour, resulting in a reduced exposure to infective *P. marinus* particles. These observations are important as they show that habitat structure can influence infection levels of aquatic pathogens, even if we do not understand the details of the mechanisms involved, or whether they are affecting the parasite or the host. In contrast to what was observed about flow on infection levels for *P. marinus* above (Lenihan et al., 1999), *H. nelsoni* infection prevalence did not show a similar pattern (Beresford, unpublished observations). In MacDonald's Pond, the habitat is consistent in areas where oysters are found, and regardless of their position in the pond, infection prevalence can vary widely (10-

70%) over short distances (<50 metres). Therefore, despite the importance of some environmental factors, the conclusions made for host-parasite systems such as *C. virginica* and *P. marinus* with direct life cycles may not be applicable for those pathogens with more complicated life cycles such as *C. virginica* and *H. nelsoni*.

Laboratory studies are challenging with this parasite due to its inconsistent nature in the field and its unpredictable and/or unexplainable response to some laboratory conditions. The data from this research suggested that temperature alone does not have a direct effect on the progression of infection in the same way as has been observed in the wild in the U.S. It is possible that temperature may affect an unknown component (or components) of the life cycle. For example, increased temperature might influence intermediate host abundance, parasite development within intermediate hosts, or some other component.

The defense mechanism employed by oysters against *H. nelsoni* has not yet been clearly determined, although some factor(s) must be involved to cause the resulting light and localized infections (Ford and Haskin, 1987). Because the defense mechanisms involved are not well understood, it is possible that survival of an infection is less due to resistance to the parasite than to what Ford and Haskin (1987) refer to as "insusceptibility", meaning a failure of the host to satisfy the "life needs" of the parasite (Read, 1958). This may be a particular factor in laboratory conditions. It is possible that laboratory conditions are such that the internal environment of the oyster was not conducive to parasite survival and/or progression resulting in morbidity and mortality of the *H. nelsoni* parasite followed removal of dead or moribund parasites by the immune system of the oyster. Histological examination did not reveal the presence of damaged *H. nelsoni* plasmodia. It is possible they were present, but not recognized due to their condition, however there were no visible signs of inflammation

or accumulation of oyster immune cells indicative of an immune response to the presence of dead or dying plasmodia. Experimental salinity was maintained at 24‰; therefore, there was no osmotic pressures on *H. nelsoni* plasmodia causing parasite membrane disruption resulting in dead or dying parasites. For comparison, the salinity in the Bras d'Or Lake, Cape Breton, is 15-18‰ and *H. nelsoni* has caused significant mortality in various locations throughout the Bras d'Or Lake and continues to cause mortality in some locations in that salinity range. Thus, while 24‰ may be lower salinity than normal seawater, it was within the range at which *H. nelsoni* can be lethal to oysters based on the mortality events recorded in the Bras d'Or Lake.

It has been documented that damaged *H. nelsoni* plasmodia can be detected and removed by oyster haemocytes when the parasite plasmodia are damaged using reduced salinity (Ford and Haskin, 1988). If the internal environment of the oyster becomes unsuitable for parasite survival, experimental evidence suggested that the conditions inside the oyster become unsuitable during the initial four weeks of treatment that causes a reduction in prevalence detected by PCR and histological observation (Beresford, unpublished results). Furthermore, under experimental conditions, observations suggested that there was removal/reduction of parasite burden during the initial four-week period, but after that parasite removal/reduction diminished. It would be interesting to repeat this experiment and transfer oysters back into the wild determine if the parasite presence detected by PCR that remained in treated oysters was from viable parasites or if the PCR detected unviable parasites that not yet removed by the oysters' immune system. Such a transfer would have to occur in a location where there was no risk for the oysters to be exposed to new infections in the wild, otherwise there would be no way to differentiate a new infection from the development of an infection from those parasites that remained post-treatment.

These laboratory experiments, both temperature treatments and food availability treatments, have shown there was unpredictability in the response of the oyster, the parasite, or the oyster-parasite combination under controlled conditions. The immediate history of the oyster and/or parasite prior to treatments may play a role. One observation that has been consistent is that oysters held under laboratory conditions experience almost no mortality, regardless of the treatment (temperature or food availability) or length of treatment (weeks or months).

Ongoing monitoring of oysters in locations affected by *H. nelsoni* and are thought to have developed resistance will require the use of susceptible animals to determine parasite activity otherwise it might mistakenly be assumed that resistance to mortality developed, when in fact low mortality is due to low infection pressure. The Bras d'Or Lake provides an opportunity to begin the process of developing broodstock that could be used for distribution throughout Atlantic Canada if *H. nelsoni* causes mortalities in other locations in N.S., P.E.I., and N.B. For coevolution to occur, there needs to be evolving infectivity and virulence from the parasite to which the host progressively adapts. The information that can be inferred from increased survival or abundance of hosts in a host-parasite system is reduced when the degree of infectivity and virulence is not known. If infective particles (quantity and quality) are not measured, a reasonable method to collect information on parasite activity is to transfer susceptible hosts into the system under observation. Susceptible hosts provide a basis for comparison against those thought to have evolved resistance. An example of the importance of knowing the parasite activity in a given study was shown in Flannery et al. (2014). They conducted an extensive study in Ireland using stocks of *Ostrea edulis* with both short (5 years) and long (22 years) exposure to *Bonamia ostreae* and monitored for environmental factors (temperature and salinity) and other important factors such as year

class of oysters. They found a small difference in overall prevalence (<1%) between the two locations and concluded that natural resistance was slowly developing. Given that the two locations demonstrated similar resistance, it is possible that the resistance developed during the initial infection period in both locations. Despite the extensive monitoring (13 months), an assessment of parasite activity in each location was absent, such as the transfer of susceptible hosts into each area to determine the level of infective particles. Similar observations have occurred in the Bras d'Or Lake, Cape Breton, with respect to *H. nelsoni* and *C. virginica*. In East Bay and Gillis Cove there was little change in mortality levels since 2012 so it was assumed that resistance to mortality was developing. However, oysters from these two locations showed significant *H. nelsoni* infection prevalence and oyster mortality when placed in an area (Nyanza Bay) with known susceptible oysters. The presence of other pathogens in these locations was not confirmed but is a possibility. Thus, in circumstances where the activity of the parasite cannot be measured objectively, the lack of mortality or reduced parasite prevalence may not be an indication of developing resistance unless compared with a susceptible population to provide information around parasite activity.

The movement of farmed species that are not at risk of disease from a pathogen of concern, but raised in locations where the pathogen is present, presents a definite risk to spread of pathogens. The spread of OsHV-1 virus through water or possible long distance spread via plankton is an example presented by Thrush et al. (2017). They describe the mechanical movement of pathogens as anthropogenic or natural, and over long and short distances. Depending on the organism in question and the environmental conditions at the time, great unpredictability exists among combinations of these factors. Given that *H. nelsoni* was detected in the gut of tunicates (Messerman and Bowden, 2016), the reported distribution of

eelgrass in the Atlantic region, and where tunicates use eelgrass as a substrate (Caman et al., 2016), this presents an interesting challenge to control the movement of *H. nelsoni* associated organisms. Messerman and Bowden (2016) sampled biofouling organisms for *H. nelsoni* around oyster aquaculture sites using PCR analysis to determine if any organisms could possibly aid the dissemination of the parasite. They suggested that tunicates may aid the spread of *H. nelsoni* by allowing infective stages to pass through their digestive system and transferring them to new areas as the tunicates are transported by industrial or recreational activities or through natural means, but they acknowledge that the co-existence of *H. nelsoni* with tunicates and other biofouling organisms does not necessarily mean that they play a role in the life cycle of *H. nelsoni*. Furthermore, it is important to note the high specificity and sensitivity of PCR analysis and that the presence of *H. nelsoni* DNA does not necessarily indicate the presence of a viable organism, nor does it give any indication of the life stage, or its infectivity to an intermediate, reservoir, or definitive host. Among the many invaders to P.E.I. waters, are four tunicates (*Styela clava*, *Botryllus schlosseri*, *Botrylloides violaceus*, and *Ciona intestinalis* (Locke et al. 2009). Although the role of tunicates in the life cycle of *H. nelsoni* is unknown, it is worth stating that Cianco et al. (1999) have found *C. intestinalis* is a host for *H. ascidarium*. The fact that a tunicate plays a direct role in the life cycle of a Haplosporidian should be a concern with respect to the potential spread of *H. nelsoni*.

Rapid, accurate testing and detailed monitoring of environmental conditions are needed while testing for the presence of pathogens that may or may not develop into an unfavourable situation for host organisms (Groner et al., 2016). Recent improvements in molecular diagnostics and sampling techniques, such as eDNA technology (Pilliod et al. 2014) have reduced the detection time of invaders. In addition to testing for those

organisms associated with parasites, e.g. known intermediate or reservoir hosts, other organisms not normally associated with pathogens could be tested on a regular basis in the event that they have become reservoir hosts. This is especially critical for those organisms for which we do not know the life cycle. For *H. nelsoni* and other poorly understood pathogens and invaders, developing detailed understandings of what organisms are involved and what conditions are necessary (abundance of hosts, environmental factors, etc.), alone or in combination with each other, may be key to future mitigation measures and disease management strategies.

One approach to disease monitoring for *H. nelsoni* would be the ongoing survey of possible organisms that could harbour the parasite – as a developing stage or infective stage. It is possible that by the time *H. nelsoni* is detected in oysters, it has already become established in a region, and thus it is too late to intervene with potential mitigating measures (removal of oysters or other biotic/abiotic factors involved in the life cycle). To make this an effective approach, monitoring detailed environmental conditions and population structures in locations where *H. nelsoni* causes disease is necessary. A rise in population size of some previously unassociated organism may be linked directly or indirectly to the development of disease and subsequent mortality. If this was true, areas not yet affected by *H. nelsoni* could be monitored for environmental parameters associated with disease outbreaks along with candidate or sentinel organisms monitored for changes in their populations that are associated with disease outbreaks. Alternatively, based on the unpredictability of *H. nelsoni* thus far, there is no guarantee that the conditions in one location will cause the same outcome as conditions in another location. However, this would be a reasonable first approach to investigate and possibly predict future disease outbreaks.

Having temperature and salinity range conditions that are associated with *H. nelsoni* survival is not enough to make any confident predictions with respect to *H. nelsoni*. For example, it was initially believed that the waters of the Bras d'Or Lake were too cold and the salinity too low for the survival of *H. nelsoni* (similar to what was believed for green crab – *Carcinus maenus*) (Lorne Penny, DFO, personal communication). We now know that is not correct given the impact of *H. nelsoni* and presence of green crab in the Bras d'Or Lake. Similar assumptions were made with respect to different tunicates surviving in P.E.I. (Locke et al., 2009). In P.E.I. the mean water temperature and salinity, as well as temperature and salinity range, are well within the limits conducive to *H. nelsoni* survival and development (Sanchez et al. 2015). Our lack of understanding about the effects of other environmental factors such as the presence, absence, and duration of winter ice cover, episodes of freshwater run-off, and other weather-related events on life cycle need to be further investigated. Because the life cycle and elements of the life cycle are not known we cannot say that the environment in and around P.E.I. would support its presence. However, *H. nelsoni* completes its life cycle under very similar environmental conditions elsewhere so it is likely that those conditions would support missing components of the life cycle if introduced.

A recent model proposed by Ben-Horin et al. (2018) demonstrated that intensive aquaculture can remove substantial oyster parasite abundance from an area thereby reducing the infection prevalence in wild oyster populations. Their study animals were *C. virginica* and *P. marinus*, which has a direct life cycle, and if there are enough farmed oysters in an area, they will provide a sink for infective particles and reduce the pathogen burden on wild oyster populations. When considering the *P. marinus* and *C. virginica* relationship, the farmed oysters should be removed from the system prior to onset of mortalities or they could

serve as a source of infective particles. This model was considered in context of the *H. nelsoni* situation in Cape Breton, where low oyster levels in Nyanza Bay are associated with mortalities of transplanted oysters, but low mortalities are seen in the source locations. An important difference to consider when applying this model to *C. virginica* and *H. nelsoni* is that the life cycle is not known, thus there is the possibility of an intermediate/reservoir host in the infection cycle. Nevertheless, this model (Ben-Horin et al., 2018) could have application to three different infection patterns observed with *H. nelsoni* and *C. virginica* on Cape Breton. Table 6.1 below summarizes key parameters found at sites in Cape Breton.

Table 6.1

Location	Gillis Cove	Aspy Bay	Potlotek	Nyanza Bay	MacDonald's Pond
Early *H. nelsoni* presence	> 95%	> 95%	10-30%	> 95%	70-80%
Oyster mortality	>75%	>90%	Low	>90%	Low
Recent *H. nelsoni* presence	• Undetectable	• Undetectable	• Unchanged	• High in transplanted oysters	• Prevalence varies year to year (13%-90%) • Inconsistent across sample locations
Change in oyster population	Recovering / small	Recovering / small	Unchanged / unknown	• Too small to measure	Unchanged / very large
Connection to larger water body / water exchange	Limited – Bras d'Or Lake	Limited – Atlantic Ocean	Unlimited	Unlimited	Tidal exchange
Recruitment of parasite or intermediate/reservoir host	Unlikely – limited water exchange	Unlikely – connected to ocean	Likely – connected to Bras d'Or Lake	Likely – connected to Bras d'Or Lake	Unlikely – connected to ocean
Environmental insult	Temperature – very low in winter	Salinity – periodically very low	Unlikely – constant connection to open Bras d'Or Lake	Unlikely – constant connection to open Bras d'Or Lake	Unlikely – constant connection to open Atlantic Ocean

Table 6.1 Description of four different locations representing three scenarios where *H. nelsoni* has been detected in *C. virginica* in Cape Breton showing physical descriptions of each location and impact of *H. nelsoni* on *C. virginica* in the locations.

The diverse and changing circumstances around Cape Breton Island provide an opportunity to explore factors related to *H. nelsoni* presence, absence, and oyster mortality. A location such as MacDonald's Pond has revealed the unusual scenario whereby *H. nelsoni* prevalence can be quite high yet show no oyster mortality. In locations such as Potlotek, *H. nelsoni* prevalence remains low year after year with no oyster mortality. Aspy Bay and Gillis Cove, while different in location and environmental influences, showed that *H. nelsoni* can cause oyster mortality, and then become undetectable during subsequent sampling. Oysters transplanted to Nyanza Bay, where there are few oysters present, resulted in high *H. nelsoni* prevalence and subsequent mortality. Each of these locations is different in many ways yet provide an opportunity for detailed field studies to try and elucidate some of the unknowns associated with *H. nelsoni*. The figures below depict possible explanations for consideration in the development of a new model that would require detailed field studies for verification. The solid lines represent the oyster abundance, the dashed lines represent parasite abundance, and the "+" lines represent intermediate/reservoir host abundance. The size of the circle corresponds to the total abundance in the system at a given time.

Figure 6.1

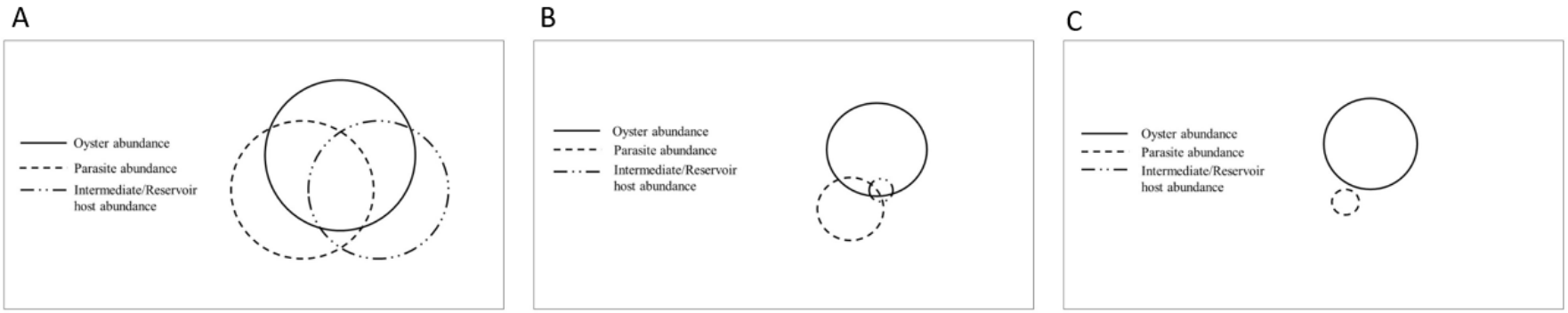

Figure 6.1 Depiction of one possible scenario to explain the observed events in Aspy Bay and Gillis Cove.

Figure 6.1A describes a possible explanation for the observations in Aspy Bay and Gillis Cove whereby in each location there was a large population of oysters, parasites, and intermediate/reservoir hosts resulting in a significant mortality over two to three years. An environmental event, such as a temperature or salinity, reduces the abundance of intermediate/reservoir hosts thereby causing a reduction in parasite abundance and fewer infected oysters (Fig. 6.1.B). The lack of recruitment of intermediate/reservoir hosts, due to limited water exchange, eventually results in no intermediate/reservoir host presence thereby causing the parasite population to fall to undetectable levels in the area and not infecting oyster hosts (Fig. 6.1.C).

Figure 6.2

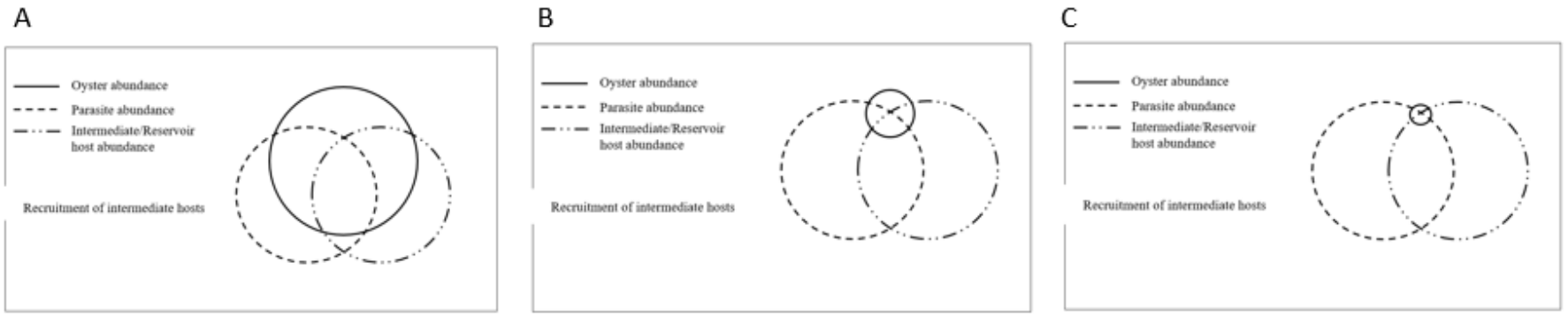

Figure 6.2 Depiction of one possible scenario to explain the observed events in Nyanza Bay.

Figure 6.2A shows the initial large population of oysters in Nyanza Bay along with a large intermediate/reservoir host population and parasite population causing significant oyster mortality. Following a significant oyster mortality (Fig. 6.2B), the intermediate/reservoir host and parasite population remained high due to the recruitment of new individuals into the area, largely due to its openness to the greater Bras d'Or Lake. The presence or absence of an environmental event may not have the same consequences as mentioned when describing Aspy Bay or Gillis Cove (Fig. 6.1) because of the ongoing recruitment of intermediate/reservoir hosts into the area. A subsequent oyster mortality results in few oysters hosts available, however, the parasite can to maintain its life cycle due to the presence of a suitable abundance of intermediate/reservoir hosts and possibly a small number of oyster hosts. Any oysters introduced into this system are exposed to a significant volume of infective particles resulting in high infection prevalence and subsequent oyster mortality.

Figure 6.3

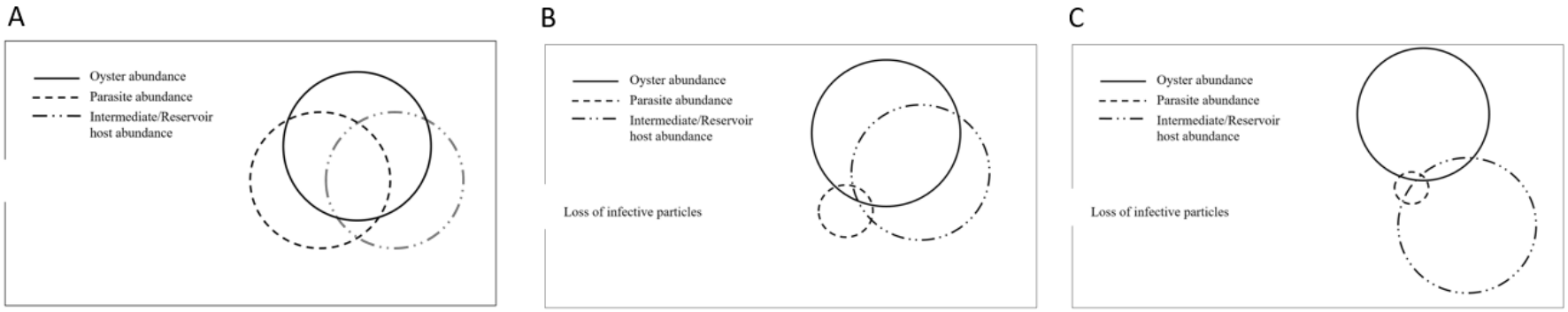

Figure 6.3 Depiction of one possible scenario to explain the observed events in MacDonald's Pond.

Oysters in MacDonald's pond were likely infected with *H. nelsoni* because of an oyster transfer from the Bras d'Or Lake. At some time, conditions were such that the intermediate/reservoir host population reached a critical abundance as did the parasite itself (Fig. 6.3.A). The broad connection to the ocean where there is substantial water flow at the changing of the tides could create a situation whereby the dosage of parasite per oyster is high enough that infection spreads throughout the oyster population, but the removal of infective particles at tidal changes reduces the overall exposure and dosage of parasite (Fig. 6.3.B). Furthermore, the large population of oysters in MacDonald's Pond, where infection prevalence can reach high levels, but has not resulted in oyster mortality, could be such that individual oysters do not receive lethal doses of parasite. Over time, the large oyster population continues to get infected, MacDonald's Pond sustains an intermediate/reservoir host population, and also a parasite population; however, the large volume of oysters and tidal exchange results in the available dosage of parasite being high enough to infect at a high prevalence, but not high enough to reach lethal doses (Fig. 6.3.C). The abundance of oysters in MacDonald's Pond may serve as a sink for *H. nelsoni,* reducing the parasite load per oyster. Oysters reduce the overall abundance of parasite in the pond during the summer followed by the parasite succumbing to the conditions in the oysters while the oysters are closed during the winter months, resulting in lack of mortalities.

This proposed model has multiple components that could change from year to year depending on environmental conditions, but because these locations are all located on Cape Breton Island, they collectively present a unique opportunity for investigation and comparison to one another. The insights gained from developing an understanding of the role of these factors and environmental parameters in the *H. nelsoni* life cycle could provide

valuable information to understand the risk of spread of this parasite to P.E.I., N.B., and the rest of N.S.

A general overview of the gaps in our knowledge of this life cycle indicates that we do not even know the source of *H. nelsoni* infections in *C. virginica* (Haskin and Andrews, 1988). The most important work to be done to develop our understanding of *H. nelsoni* biology would be to work out its life cycle so we may work toward a method for controlling *H. nelsoni* (Haskin and Andrews, 1988). In order to do so, detailed field observations will be necessary to determine the role of various environmental parameters and to understand what aspects of the life cycle are impacted by associated environmental conditions. Given that there are locations in Cape Breton where *H. nelsoni* is now pervasive and the development and application of new biological tools, there is an opportunity to discover all aspects of the life cycle of this parasite.

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