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Development of Hemic Neoplasia in the Soft-Shell Clam (Mya arenaria) Along the East Coast of the United States

Nicole B. Seitz
West Chester University of Pennsylvania, ns822796@wcupa.edu

Development of Hemic Neoplasia in the Soft-Shell Clam (*Mya arenaria*) Along the East Coast of the United States

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ABSTRACT

Hemic neoplasia (HN) is a bivalve disease characterized by uncontrolled proliferation of abnormal hemocytes that invade solid tissue, similar to vertebrate leukemia, ultimately leading to death. High disease prevalence has been reported in many bivalve populations, yet disease etiology and development remain minimally understood. In this study, nine East Coast populations of soft-shell clams (*Mya arenaria*) were evaluated between 2011-2012 to identify environmental and health factors associated with HN. PCA analysis and statistical testing were used to compare data at and between each population based on HN status. The percentage of organisms with terminal stage 4 HN (76-100% neoplastic cells) was used to group sites into those of high and low disease incidence; with average terminal HN of 6.46% and 28.15% and organismal survival of 60.8% and 20.2% detected for low and high HN sites, respectively. Analysis revealed pollutants including PCBs (Aroclor 1221), PAHs (pyrene and fluoranthene), organochlorinated pesticides (methoxychlor, toxaphene, endrin, BHCs, aldrin, Endosulfan, and heptachlor) and heavy metals (iron, aluminum, chromium, copper, nickel, and zinc); as well as sediment organic content were strongly associated with HN prevalence and survival. On average, high HN sites displayed 1422 mg/kg total pollutants and 49.2% organic content, while low HN sites displayed 388 mg/kg and 36.5%, respectively. Water temperature was also associated with HN and mortality, emphasizing concerns about rising ocean temperatures associated with climate change. Overall, higher pollutant levels or elevated water temperatures increased HN incidence indicating disease transmission through viral infection is likely due to reduced immunity in *M. arenaria*.

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INTRODUCTION

Overview

Mya arenaria is a marine bivalve commonly known as the soft-shell or steamer clam. Native to the East and invasive on the West coast of the United States, these organisms are economically important only along the East Coast, while a larger bivalve species *(Panopea abrupta* or the geoduck clam) is harvested along the West Coast (Goodwin and Pease 1989; Emmett 1991; Strasser 1998). While the Northeast and Mid-Atlantic were the main harvest grounds for *M. arenaria* until the 1970's, due to overfishing of the latter only New England populations are currently being harvested (Homer et al. 2011). In addition to overharvesting, population declines have been linked to predators, such as green crabs, protist diseases like Dermo and MSX (caused by *Perkinsus marinus* and *Haplosporidium nelson,* respectively) and a leukemia-like disease or neoplasia leading to uncontrolled hemocyte proliferation (Newell et al. 1986; Dungan et al. 2002; Jesse et al. 2021). Neoplasia affects at least fifteen bivalve species worldwide and has been observed in populations of *M. arenaria* from Prince Edward Island to the Chesapeake Bay (Peters 1988; Barber 2004).

Considered to be analogous to vertebrate leukemia, this neoplasm is commonly known as disseminated neoplasia, disseminated sarcoma, hemopoietic or hemic neoplasia (Farley 1969a: Miosky et al. 1989; Barber 2004). Due to disease impact specifically on the hemic or circulatory system, including the uncontrolled proliferation of hemocytes, this research project will refer to the disease as hemic neoplasia (HN). In disease onset neoplastic hemocytes are initially located in the sinuses and organs of the circulatory system, indicating that abnormal cells are most likely of hemic origin (Farley 1969b).

Neoplastic hemocytes are enlarged, anaplastic cells lacking functional pseudopodia, therefore creating their characteristic rounded appearance. Due to the lack of pseudopodia, used for movement and adhesion, these hemocytes are visually distinct from normal hemocytes (Moore et al. 1992; Delaporte et al. 2008a). Neoplastic hemocytes may also be characterized by an elevated nuclear to cytoplasmic ratio, and pleiomorphic nuclei with prominent nucleoli (Leavitt et al. 1990; Elston et al. 1992; Carballal et al. 2015). Due to the uncontrolled mitosis in neoplastic hemocytes, cellular DNA content increases leading to changes in ploidy status from normal diploid to neoplastic polyploidy cells (Reno et al. 1994). In addition, neoplastic hemocytes also share morphological/genetic traits with neoplastic cells in vertebrates including altered Golgi complexes as well as alteration or inactivation of the tumor suppressor gene p53 (Auffret and Poder 1986; Brown et al. 1977; Mix et al. 1979; Elston et al. 1988a; Walker et al. 2009).

The severity of disease, divided into four progressive stages, is determined using a count of normal to neoplastic hemocytes in a sample of hemolymph evaluated for the presence or absence of pseudopodia. The four stages are as follows: 0-25% (stage 1, healthy disease-free animals), 26-50% (stage 2, early incipient), 51-75% (stage 3, late incipient), and 76-100% (stage 4, fully neoplastic) (Taraska and Boettger 2013).

With disease progression, neoplastic hemocytes in circulation will eventually invade all sinuses within the solid tissue, including the visceral mass and mantle (Auffret and Poder 1986; Smolowitz et al. 1989; AboElkhair et al. 2009b). A chronic state will eventually be reached as neoplastic hemocytes, incapable of apoptosis, continue to proliferate (Walker and Boettger 2008). Due to the accumulation of these nonfunctional neoplastic hemocytes associated with HN the animal's oxygen requirements will increase until the dissolved oxygen content of the water is

no longer able to keep up with the growing oxygen requirements of the animal, leading to mortality (Cooper et al. 1982; Leavitt et al. 1990; Sunila 1991). In some intermittent cases of HN remission has been observed, which never occurs in severe cases (Cooper et al. 1982; Elston et al. 1988a; Brousseau and Baglivo 1991). Healthy hemocytes capable of innate immune response by phagocytosis are believed to be able to phagocytize and destroy neoplastic hemocytes only in early disease stages (Cooper et al. 1982; Appeldoorn et al. 1984; Pipe and Coles 1995).

While the exact disease etiology for HN has not been determined, research suggests involvement of an infectious agent or retrovirus, in combination with contributing environmental factors such as pollution and temperature changes, or direct disease transmission through exchange of infected cells (Farley et al. 1972; Brown et al. 1979; Oprandy et al. 1981; Oprandy and Chang 1983; Farley et al. 1991; Elston et al. 1992). Studies have shown transmission of HN is possible by the cohabitation of animals (Brown 1980; Elston et al. 1988b; Sunila and Farley 1989), transmission of diseased cells (Appeldoorn et al. 1984; McLaughlin et al. 1992; Weinberg et al. 1997; House et al. 1998), or initiation by injection of cell free neoplastic hemolymph between animals (Oprandy et al. 1981; Collins and Mulcahy 2003; Taraska and Boettger 2013). Further, possible transmission of an infectious agent or direct exchange of infected cells is supported by studies of populations in both wild, and laboratory settings due to HN being able to rapidly spread through a population within close proximity to each other, and cause mass mortalities (Brousseau and Baglivo 1991; Mateo et al. 2016a).

The research organism in this study, *M. arenaria,* is one of the most susceptible species to the development of HN based on laboratory and field experimentation. The disease is prevalent in many Atlantic populations of *M. arenaria* and has even been reported at epizootic levels (> 1% of the entire population) in the North Atlantic region (Brown et al. 1979; Leavitt et

al. 1990; Brousseau and Baglivo 1991). Information on HN such as annual occurrence, temperature, and pollutant effects relies on studies conducted in *M. arenaria*, making it an excellent sample organism to understand disease progression. In this research project statistical analysis of data sets including environmental conditions (sediment composition, pollution, and water temperature), as well as health conditions (seasonal prevalence of HN, and mortality rate) will be utilized to evaluate effect of local conditions at each site on HN development in different populations of *M. arenaria* on the East coast of the United States.

Hemic Neoplasia

"Leukemia like" cells identifying a neoplastic disease were first reported in 1969 in the tissues and organs of *Mytilus edulis* (bay or blue mussel) as atypical immature hyaline hemocytes associated with mortality in affected animals (Farley 1969b). Development of the same disease in *Mya arenaria* was later discovered in its native habitat along the East coast of North America in 1977 (Yevich and Barszcz 1977). In addition to *M. arenaria* and *M. edulis*, other bivalve species effected by this disease include three additional species of mussels *Mytilus trossulus, M. galloprovincialis, M. chilensis,* six additional species of clams *Ruditapes decussatus, Solen marginatus, Ensis siliqua, Tagelus plebius, Macoma balthica, Venerupis aurea, Cerastoderma edule, C. glaucum,* and seven species of oysters *Crassostrea gigas, C. virginica, C. iredalei, Saccostrea glomerate, Ostrea lurida, O. edulis, and O. chilenis* (Brown et al. 1977; Peters 1988, Carballal et al. 2015). Development and progression of HN is facilitated by the semi-closed circulatory system of these bivalves where hemolymph is pumped into large sinuses in the structurally weak connective tissue allowing neoplastic cells to rapidly disseminate in the animal (Farley and Sparks 1970).

Two hypotheses have been suggested for development of HN based on origin of neoplastic cells, from either a single cell or cell clusters. The clonal hypothesis states that one single morphologically transformed cell, likely of hemopoietic stem cell origin, will replicate to initiate disease progression. The synchronous transition hypothesis suggests that a large portion of hemocytes simultaneously transform into cells that remain in a transitional state between host stem cell and a neoplastic cell. Though development of HN could differ between animal species, the synchronous transition hypothesis is most consistent with current knowledge, especially when considering a possible infectious agent involved in disease etiology (Elston et al. 1988a).

Continued proliferation of diffuse neoplastic cells decreases the amount of functional hemocytes, and increases tissue necrosis throughout the organism (Farley 1969a; Brown et al. 1977; Peters 1988). Disease progression of later stages leads to physiological changes, such as rapidly increased oxygen requirements, that will ultimately lead to mortality (Leavitt et al. 1990; Sunila 1991). Heavily diseased animals display a lower dry tissue weight and signs of emaciation that are evident physiological changes due to increased metabolic demands (Farley 1969a,b; Farley and Sparks 1970; Leavitt et al. 1990). Normal tissues such as the gill and digestive gland are infiltrated by circulating neoplastic cells, impairing normal function and leading to reduced metabolic processes, causing the animal to become increasingly cachexic (Brown et al. 1979; Leavitt et al. 1990).

Molecular mechanisms involved in HN development are important to understand disease etiology. Current research focuses heavily on molecular mechanisms of mortalin, a highly conserved mitochondrial heat shock protein 70 family member, and Steamer, a retrotransposon first isolated from *M. arenaria*. The tumor suppressor gene, p53, is sequestered in the cytoplasm by mortalin, which acts as a p53 tether, preventing it from reaching the nucleus where it would

originate apoptosis or programmed cell death (Walker et al. 2006; Siah et al. 2008). The Steamer retrotransposon inserts mutations into the genome that are associated with disease progression due to creation of genetic breaks and instability that may initiate or advance HN development (Arriagada et al. 2014; Metzger et al. 2018). In diseased bivalves mortalin and Steamer are found at high levels of amplification and expression, indicating that these proteins and RNA intermediaries are involved in HN development (Walker et al. 2006; Siah et al. 2008; Arriagada et al. 2014; Metzger et al. 2018).

Animal remission may be evident in early disease stages, while it is not generally reported in later disease development (Cooper et al. 1982; Elston et al. 1988a; Brousseau and Baglivo 1991). Remission is facilitated through healthy hemocytes by the process of either secreting an extracellular matrix that entraps neoplastic hemocytes, phagocytosis, or production of a hemolytic cytotoxin all of which ultimately will destroy neoplastic hemocytes and halt disease progression (Elston et al. 1988a; Leippe and Renwrantz 1988; Brousseau and Baglivo 1991).

Disease Detection

Detection of HN cannot be conducted simply by gross morphological observation or comparisons and can be difficult to diagnose in early stages of disease. Hemocytology and histopathology are commonly used in order to diagnose HN (Farley 1969b; Barber 2004; Delaporte et al. 2008b). Hemocytology requires the analysis of hemolymph to determine the ratio of neoplastic to normal cells in a sample. Hemolymph can also be visually analyzed to distinguish between cell types by characteristic morphologies (House et al. 2006; Delaporte et al. 2008a). Cell counts are used for staging of disease, while the slide method relies on observation

of cells on a glass slide in order to qualitatively determine the presence of neoplastic cells in an animal (Farley et al. 1986; Elston et al. 1992). When neoplastic cells are placed on a glass slide, they will appear round due to lack of pseudopodia that adhere in normal hemocytes (Brown et al. 1976; Moore et al. 1992; Delaporte et al. 2008a). Hemocytology is favorable for watching progression of disease over time in the same animal by allowing the organism to remain viable (Farley et al. 1986; Peters 1988).

Histopathology, usually coupled with hemocytology, allows for comparison between healthy and diseased tissues. Tissues are removed from the animal, preserved using a fixative such as Bouin's, dehydrated, embedded in paraffin, cut into sections, stained, and then placed on a slide to be viewed microscopically in order to visualize how stage and progression of disease effects tissues (Mix 1983; Barber 2004; Delaporte et al. 2008b). Staging can be determined with this method but is more commonly done by hemocytology. The early stages of HN are characterized by individual invasive neoplastic hemocytes seen in the connective tissue and vascular spaces around the digestive gland. As disease progresses enlarged portions of tissue, due to neoplastic cell infiltration and accumulation, can be visualized around digestive organs (stomach, intestine, and style sac). In more advanced and terminal stages of HN the vascular spaces of the bivalve will be completely filled with neoplastic cells (House et al. 2006). This method requires the sampled organism to be sacrificed, so changes in tissue cannot be observed over time in the same animal (Elston et al. 1988a).

In addition, more rapid and accurate methods of detection have been developed recently. Flow cytometry is utilized to visualize ploidy changes, and more generally DNA changes, due to differences in DNA content between neoplastic and normal cells in a sample. This method uses cell cycle analysis and relative amounts of DNA varying between healthy and diseased cells in

order to determine total number of neoplastic cells in a sample (Reno et al. 1994; Delaporte et al. 2008a). Immunoassays use monoclonal antibodies (MAb) and indirect peroxidase (IP) staining in order to test for presence and amount of HN cells in a sample, by evaluating reactivity of cells to either a known antibody or stain. Monoclonal antibodies, such as MAb 1E10 specific for *M. arenaria*, have been developed to detect and attach to an antigen on the surface of neoplastic cells, and IP can then be used to detect and tag a specific antibody and stain cells containing that antibody in the sample (Smolowitz and Reinisch 1986; Delaporte et al. 2008a).

Ontogeny

The origin of neoplastic cells is currently unknown, though research suggests they are sarcomas of mesodermal tissue origin (Elston et al. 1992; Peters 1988; Carballal et al. 2015). HN cells are considered to be analogous to vertebrate leukemia cells, and could therefore be of hemocytic origin or having originated from normal hemocytes or hemopoietic tissue (Farley 1969a). In order for a disease to be considered leukemia, or a cancer of the blood-forming tissues, neoplastic cells must be derived from hemopoietic stem cells that normally mature into hemocytes (Reinisch et al. 1984). Neoplastic hemocytes first occur in the circulatory system of bivalves, and are morphologically similar in their round appearance to normal hemocytes suggesting they are derived from hemocytes (Elston et al. 1988a; Peters 1988; Carballal et al. 2015).

Hemopoiesis is poorly understood in bivalves but will be crucial information to understand the origin of neoplastic cells (Elston et al. 1992; Peters 1988). Several sites have been suggested for origin of hemopoietic cell development including the connective tissue, due to healthy hemocytes being normally present there, and the primordial epithelium of the

mesodermal germ layer, specifically the undifferentiated primordial layer of the gonad, because it is known to develop into hemopoietic tissue in vertebrates and displays high mitotic activity (Farley 1969a; Smolowitz et al. 1989).

Several authors have proposed possible ontogenies to consider for neoplastic cell development in bivalves. Neoplastic cells are reported to be atypical precursors of the granular amebocytes or to have developed from undifferentiated blastoid cells of the hemolymph system (Farley 1969b; Yevich and Barszcz 1977). It is also reported the origin of neoplastic hemocytes in the body may be in the digestive gland, based on high reverse transcriptase levels as well as histological observation of neoplastic cells being first seen in this location; or in the gonad, due to possible hemopoiesis site and proximity of neoplastic cells to this area in early disease stages as well (Farley 1969a; AboElkhair et al. 2009b). Overall most researchers favor the idea that neoplastic cells are related to granular or hyaline hemocytes, and originate from connective tissue (Barber 2004).

Disease Etiology

While the exact etiology of HN is unknown, there are many factors that contribute to disease development. Being indiscriminate filter feeders, bivalves have the potential to absorb pollutants from their environment that are believed to contribute to development of HN without being a direct cause (Brown et al. 1979; Cooper et al. 1982; Barber 2004). Clams from oil impacted sites, and sites with high contamination of pollutants such as polychlorinated biphenyls (PCB) were found to have increased prevalence of HN when compared to non-polluted sites, but no direct link between pollution and disease was established, rather an association was suggested (Yevich and Barszcz 1977; Reinisch et al. 1984; Harper et al. 1994). Pristine sites have been

found to display prevalence of HN, while other polluted sites have been found to be free of disease, further suggesting that pollution is not the direct causative agent of this disease (Brown et al. 1977; Smolowitz and Leavitt 1996; Krishnakumar et al. 1999). Similarly, the level of chlordane, a known mammalian carcinogen, in tissues of bivalves has been correlated to disease development; however, rather than direct cause the contaminant likely acts synergistically in the body to produce disease by affecting defense mechanisms of normal immune function (Farley et al. 1991).

Pollutants in the water and sediment have been correlated to disease development and may only contribute due to stress imposed on the immune system. The weakened immune system could allow initiation of disease by another factor such as a viral agent (Dyrynda et al. 1998; St-Jean et al. 2005; Muttray et al. 2012; Boettger et al. 2013; Arriagada et al. 2014). When hemocytes were exposed in vivo to an alkylating agent or genotoxic materials, such as ethyl methane sulfonate (EMS), micronuclei formation was detected as a measure of chromosome damaging effects and found to develop in response to exposure. Chromosome damage can lead to DNA changes seen in neoplastic cells and therefore are linked to development of HN (Dopp et al. 1996). Particular pollutants such as heavy metals and polycyclic aromatic hydrocarbons (PAH) have been linked to suppression of the innate immune defenses including decreased phagocytosis and decreased number of binding sites that allow hemocytes to recognize invaders, respectively (Pipe and Coles 1995). However, no one pollutant has been directly linked to disease development. In addition, environmental toxins such as harmful biotoxins produced from dinoflagellate algal blooms may also be linked to stress related susceptibility to an infectious agent such as a retroviral particle (Landsberg 1996).

Retroviral particles are one likely causative agent of HN development (Brown et al. 1979; Cooper et al. 1982; Oprandy and Chang 1983; House et al. 1998; Boettger et al. 2013; Taraska and Boettger 2013; Arriagada et al. 2014; Mateo et al. 2016b). 5-Bromodeoxyuridine (BrDU), a compound shown to induce leukemia in murine models, was used to induce HN as well as a retroviral like particle in healthy animals, suggesting possible retrovirus link to HN (Oprandy and Chang 1983; Taraska and Boettger 2013). Although reported to lack repeatability by other researchers, successful transmission of HN by injection of a type-B retrovirus has also been reported, and is important to consider with other preliminary transmission studies, and isolations of retrovirus like particles (Oprandy et al. 1981; Cooper and Chang 1982; Oprandy and Chang 1983; Elston et al. 1992). Due to nature of disease transmission infectious etiology involving retroviral particles has been widely accepted.

Evidence of retroviral etiology has also been supported by detection of reverse transcriptase (RT) activity in neoplastic cells (Medina et al. 1993). HN positive cells were found to have RT activity, believed to be linked to the infectious process of the disease, while healthy cells were lacking in RT activity (House et al. 1998). Increased RT activity in diseased cells was positively correlated to severity of disease, but contrary to the latter, was also reported in healthy cells at lower background levels and suggested to develop in the digestive gland rather than through disease progression. Without isolation of a retroviral particle, it is likely RT activity detection occurs from an endogenous source, and therefore retrovirus etiology cannot be confirmed (AboElkhair et al. 2009a,b,2012).

Transmission of disease has also been achieved by injection of whole neoplastic cells, supporting an infectious agent etiology (McLaughlin et al. 1992; House et al. 1998). Hemolymph filtered to the size of viral particles and injected into healthy clams showed transmission of HN

was still possible. In the same experiment BrDU was shown to induce HN in healthy animals, but initiation of disease depended on the native location of the clam, suggesting infectious agent could be viral and remain dormant at different levels (site dependent) until activated by a compound such as BrDU (Taraska and Boettger 2013). BrDU induced activation of disease suggests involvement of some endogenous retrovirus, or retrotransposon. The activation of the Steamer retrotransposon leading to genetic instability discussed above is believed to be triggered by pollution exposure, and be an endogenous agent for HN (Arriagada et al. 2014).

In addition, direct disease transmission through exchange of infected cells has also been considered as a causative agent. The genotypes of neoplastic hemocytes are almost identical to one another, regardless of the host, suggesting HN is transmissible by infected cells which likely originated from one individual (Metzger et al. 2015). HN therefore arises from transmissible independent cancer lineages which can spread in the marine environment, but there are cases where cross species transmission between bivalves is seen (Metzger et al. 2016). The Steamer retrotransposon was found to be horizontally transferred between different species of bivalves, suggesting that Steamer may move between organisms to insert into their genome, much like a retrovirus (Metzger et al. 2018).

Epidemiology

Prevalence of HN is site dependent and seasonally differs, in addition to other contributing factors such as population size and age (Brousseau 1987; Barber 2004; House et al. 2006; Boettger et al. 2013). HN in most native populations of clams occurs at low prevalence, though mass mortalities (greater than 95%) have occurred for example Prince Edward Island, a site known to be polluted with agricultural run-off, in 1999 and again in 2001 (Muttray et al.

2012; Mateo et al. 2016a,b). The largest concern for mortality though is farmed or laboratory populations due to the close proximity environment (Brousseau and Baglivo 1991; House et al. 2006; Mateo et al. 2016a). Once disease has been established in a population mortalities as high as 40-100% may occur (Boettger et al. 2013).

Seasonality reported for natural populations varies based on specific location. The highest prevalence usually seen is in late fall and winter months, with a decline in late spring time when water temperatures increase, especially at the northern distribution limit of *M. arenaria* (Brousseau 1987). Biphasic yearly prevalence is also reported with two yearly maximums of disease in winter and spring (Cooper et al. 1982; Potts 1993; Boettger et al. 2013). Sites of low HN prevalence have one yearly maximum of disease, while sites of high HN prevalence have two yearly maxima. Seasonality of disease prevalence is controlled by changing water temperatures that are believed to influence levels of HN (Leavitt et al. 1990). Colder water temperatures in the winter months, during which gametogenesis occurs in temperate bivalve species, in combination with decreased food availability creates oxidative and reproductive stress that could be linked to high prevalence of HN. In addition, there may be a decrease in prevalence during warmer water temperatures due increased food/ATP availability, and associated mortalities that occur after the high prevalence of HN in winter months (Cooper et al. 1982; Leavitt et al. 1990; Brousseau and Baglivo 1991; Minier et al. 2000; Boettger et al. 2013).

Susceptibility of clams to HN is also influenced by size/age of the animal. HN is reported to be most prevalent in New Bedford Harbor (NBH) in clams size 30-70mm which corresponds to 1- 2.5 years old, and greater than 4 years old, and similarly in Rhode Island around 2 years of age (Cooper et al. 1982; Leavitt et al. 1990; Boettger et al. 2013). Exact ages may differ but results overall suggest that HN is found most frequently in mature animals during the first onset

of reproduction. The physiological stress imposed on the animal during gametogenesis may be linked to increased susceptibility to disease at this stage (Boettger et al. 2013).

Many bivalve species of commercial interest are affected by HN, but some species are more vulnerable than others suggesting that genetics may play a role in susceptibility (Frierman and Andrews 1976; Brown et al. 1979; Barber 2004; Carballal et al. 2015). Although the exact reason is not known, research is geared towards levels of p53 tumor suppressor gene expression, similar to human cancer research. Mortalin sequestration of p53 in cytoplasm is found to control levels of p53 expressed and therefore is believed to be an important factor in individual susceptibility to HN based on amount of mortalin expression. Neoplastic hemocytes have overexpression of mortalin and when cells are treated with a mortalin inhibitor, such as the cationic inhibitor MKT-077, translocation of p53 to the nucleus and subsequent apoptosis will occur (Walker et al. 2006; Boettger et al. 2008). The role of molecular mechanisms involving p53 in HN development indicates application of research to human cancer models.

Many aspects of HN remain to be fully understood, in particular etiology, which is a vital component in order to fully understand this bivalve disease. This current research compares incidence in geographically separate populations of *M. arenaria* to determine whether a link exists between environmental differences (temperature, contaminants, sediment characteristics) and organismal health and therefore disease development. Identification of these factors provides a better understanding of HN, especially in terms of evaluating potential disease etiologies, as well as aids in determining individuals or populations of increased susceptibility to development of HN.

METHODS

Experimental Setup

Mya arenaria spat (5-10mm) were purchased from the Downeast Institute for Applied Marine Research and Education in Machias, ME. To ensure a disease-free population of spat, twenty clams were sacrificed and screened for hemic neoplasia (HN). Remaining spat clams were cultured in raceways with 2 inches of fine sand, and seawater flow of 15 GPM at a density of 24 clams/ft^2 until clams were 10-15mm or greater. Animals were transplanted to mudflats in Maine, New Hampshire, Massachusetts, Connecticut, and New York in the fall of 2011 (Figure 1). They were placed in 12-inch flowerpots containing local sediment in groups of ten, covered with plastic bird netting (1/4-inch opening) to avoid green crab predation and secured with 10-inch rubber bands. Containers were positioned with tops flush with sediment surface and locations marked with PVC pipes. At the time of transplant, sediment and water samples were collected in triplicates for analysis. Once every three months three containers were collected at each location and animals analyzed to determine HN development, growth, and rate of mortality. At the same time thirty adult native clams (>5cm diameter) were collected and screened for HN. Local temperatures were recorded, and sediments were evaluated regarding their components (grain sizes to determine sediment composition), amount of organic matter, and contaminants.

Growth and Mortality Rate

Transplanted clams (n=30) were collected once every three months and measured lengthwise to the nearest 0.1 mm using calipers. Clam numbers in transplant containers were also counted and mortalities expressed as a percentage compared to the number of spat clams that were transplanted.

Hemic Neoplasia Diagnosis

50 μl of hemolymph was removed from the pericardial sinus of each animal using a 21-gauge hypodermic needle, placed into 96-well plate, incubated for 2 hours at 8 degrees Celsius, and staged using an Olympus inverted microscope. Animals were staged based on counts of neoplastic to normal hemocytes in hemolymph sample.

Neoplastic hemocytes are visually distinguishable from normal hemocytes due to their rounded appearance, in addition to enlarged pleiomorphic nuclei, prominent nucleoli, and increased nuclear to cytoplasmic ratio (Farley 1976; Leavitt et al. 1990; Carballal et al. 2015). Stage one is characterized by the presence of 0-25% neoplastic hemocytes per sample, and the animal is considered healthy. Stages two and three, early and late incipient HN, are intermittent disease stages classified by 26-50%, and 51-75% neoplastic hemocytes respectively. At stage two and three animals may either revert to stage one via remission or progress to stage four, classified by 76-100% neoplastic hemocytes. At this terminal stage hemolymph is characteristically opaque due to overabundance of neoplastic hemocytes, and animals will appear emaciated due to increased metabolic demands that will ultimately lead to mortality within fourteen days (Appeldoorn et al. 1984; Farley 1969b; Leavitt et al. 1990; Walker et al. 2009; Taraska and Boettger 2013).

Site Description and Analysis

Twenty randomly selected native clams from each site were collected in spring and fall of 2011-12 and measured for maximum length (to the nearest 0.1 mm) using calipers. Animals were shucked to separate the soft tissue from the shell, placed in an acetone cleaned jar, frozen at -15 degrees Celsius, and shipped to Columbia Analytical Services for analysis of contaminants

including toxic chemicals (pesticides, PCB, and PAH) and heavy metals (Ag, Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Sn, Zn). The same procedure was followed to determine contaminant uptake in transplanted clams after twelve months.

Sediment samples were collected in triplicate core samples (5cm x 5cm) at each site and sent to Geotesting in Boxborough, Massachusetts. Grain size (% clay, silt, gravel, cobble, and sand) was determined using standard sedimentology techniques, and organic content by loss on ignition in a muffle furnace for 4 hours at 600 degrees Celsius and expressed as percent volatile. Temperatures were collected hourly over the 12-month period by placing a Hobo temperature gauge into a transplant container at each location. Temperature gauges failed at Jockey Creek (NY), Guilford (CT), Hampton Harbor (NH), and Ogunquit (MA) and missing temperature data from the National Oceanic and Atmospheric Administration (NOAA) National Data Buoy Center (NOAA 2012) was used instead. Temperature data included average temperatures throughout each season (winter, spring, summer, fall).

Natural clam densities at each site were also recorded using 0.25 m^2 quadrates randomly placed 15 times. Each quadrate was excavated to 30cm in depth and clams were counted, measured (in mm), and returned back to sediment.

During August of 2011 Hurricane Irene destroyed sites in Port Norris, New Jersey and Edgewater, Maryland that were originally included in analysis to cover full Eastern United States distribution of *M. arenaria.*

Statistical Analysis

Collected data was evaluated in R Studio using multivariate statistical analysis. Principal component analysis (PCA) was utilized to evaluate trends in environmental conditions including

temperature, sediment characteristics, contaminants, and population density; and health conditions including size, growth, and mortality in relation to HN development for both native, and transplanted clams. PCA, also known as multidimensional scaling, allows data to be evaluated for significant correlations, as well as reduce the large data set to include only significant information that reflects trends of entire data set.

Each variable of interest was analyzed per site as well as compared between all nine sites. Trends were then visualized using R studio, Microsoft excel, and SPSS to show relationships between X and Y variables as well as trends of significance.

The nominal dependent variable was development of HN, and the numerical independent variables included sediment/water characteristics, growth/mortality rate, density of population, local pollution or contamination, stage of disease, and temperature. The goal was to determine significant relationships and trends between each factor and HN development at and between each site.

Two datasets were created for PCA analysis: 1) mean data and 2) seasonal data. Datasets were imported into R studio and transformed into numeric variables in order to compensate for any unknown variables (NA) present. Data was then centered and scaled for each column of data to ensure mean was equal to 0 and the standard deviation was equal to 1. PCAs were run using "pcaMethods" R studio package for 10 principal components (PC), which accounted for over 99% of the data. The data was analyzed using loadings and scores functions for the first two principal components, which explained 89.7% of mean data, and 93.8% of seasonal data.

PCA plots were prepared for mean data as well as seasonal data using the plot function and colored according to site location and level of terminal stage 4 HN. Sites with high levels of HN were colored red. The four sites that were chosen to represent areas of high HN were Jockey

Creek, NY; Guilford, CT; Boston Wood Island, MA; and Freeport, ME. These sites displayed average level of stage 4 HN over 5% for native populations and over 20% for transplanted populations.

For individual PCA analyses the top six loading variables on each PC were evaluated. Transplant growth, native clam size, and water in sediment were not evaluated due irrelevance of data to the study. Data were grouped by type (PAHs, OPs, PCBs, heavy metals, sediment composition, and transplant survival) and presented as mean with standard error (SE). SE was calculated using 95% confidence interval.

Statistical analysis was completed using SPSS for variables identified in 2011-2012 PCA analysis and HN levels. Statistical analysis was conducted after assessment of assumptions of normality (Shapiro-Wilks) and homoscedacity (Levene's Test) were checked, with transformations used as necessary. Data did not meet assumptions; therefore, non-parametric Kruskal-Wallis test followed by pairwise comparisons were used to analyze variance. A p value less than 0.05 was considered significant.

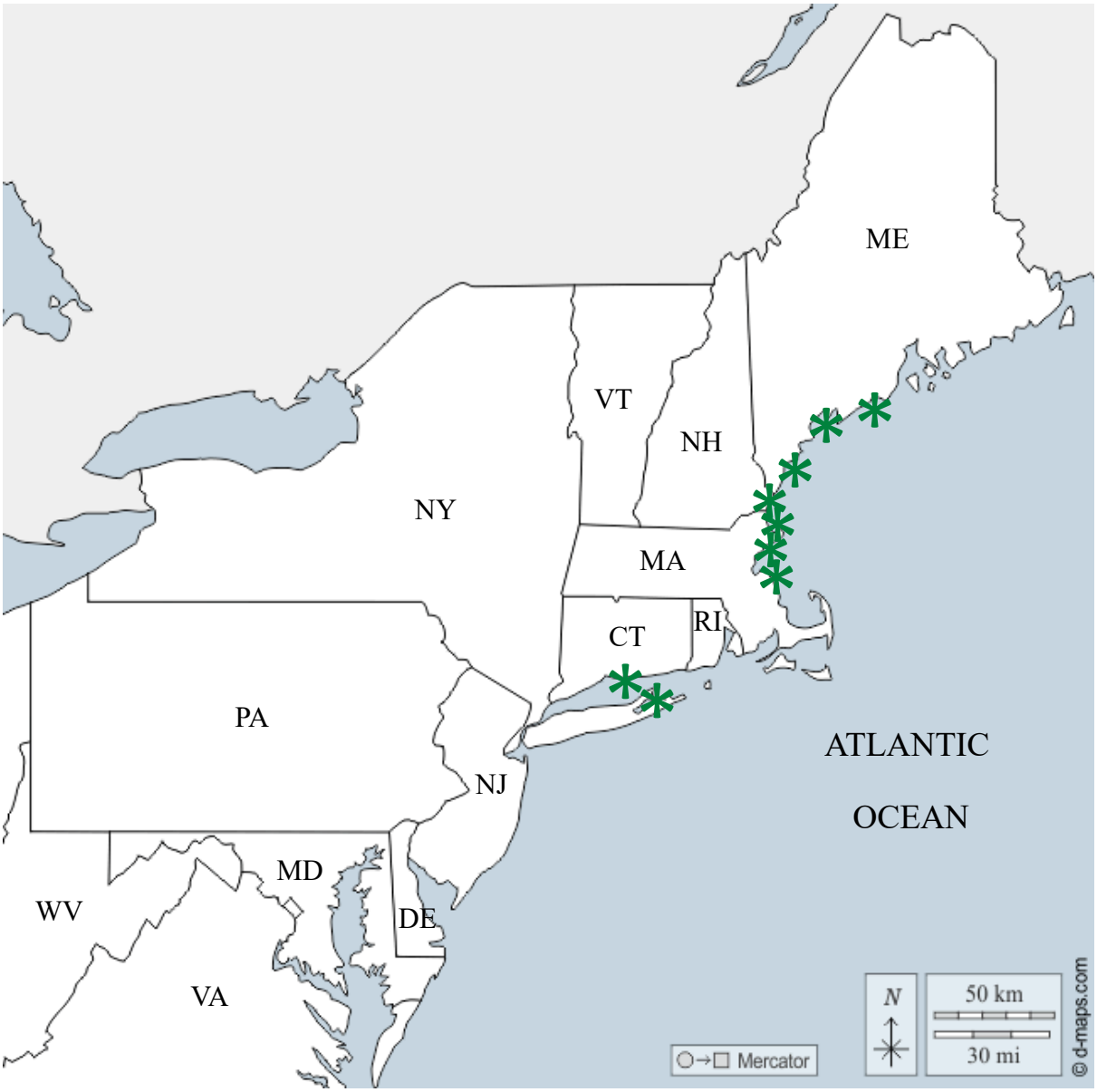

Figure 1. Map of study site locations along the East coast of the United States (Northeast into Mid-Atlantic). The range of study sites reflects the range of *Mya arenaria* distribution along the East Coast. Each study site was marked with an *. The nine sites were Jockey Creek, NY; Guilford, CT; Boston Greater Boston Harbor (GBH) 5.2 (Mudflat 5.2), MA; Boston Wood Island, MA; Gloucester, MA; Hampton Harbor, NH; Ogunquit, ME; Freeport, ME; and Damariscotta River, ME (specifically Walpole, ME).

RESULTS

Hemic Neoplasia Status and Seasonality

Terminal stage 4 hemic neoplasia (HN) levels were significantly higher at Jockey Creek, Guilford, Boston Wood Island, and Freeport (high HN sites) compared to Boston GBH 5.2 (Greater Boston Harbor Mudflat 5.2), Gloucester, Hampton Harbor, Ogunquit, and Damariscotta River (low HN sites) in both native ($p<0.001$) and transplanted ($p<0.001$) clams (Figure 2). There was no significant difference in terminal stage 4 HN between seasons (data not shown) for either native ($p=0.478$) or transplanted ($p=0.308$) clams**.**

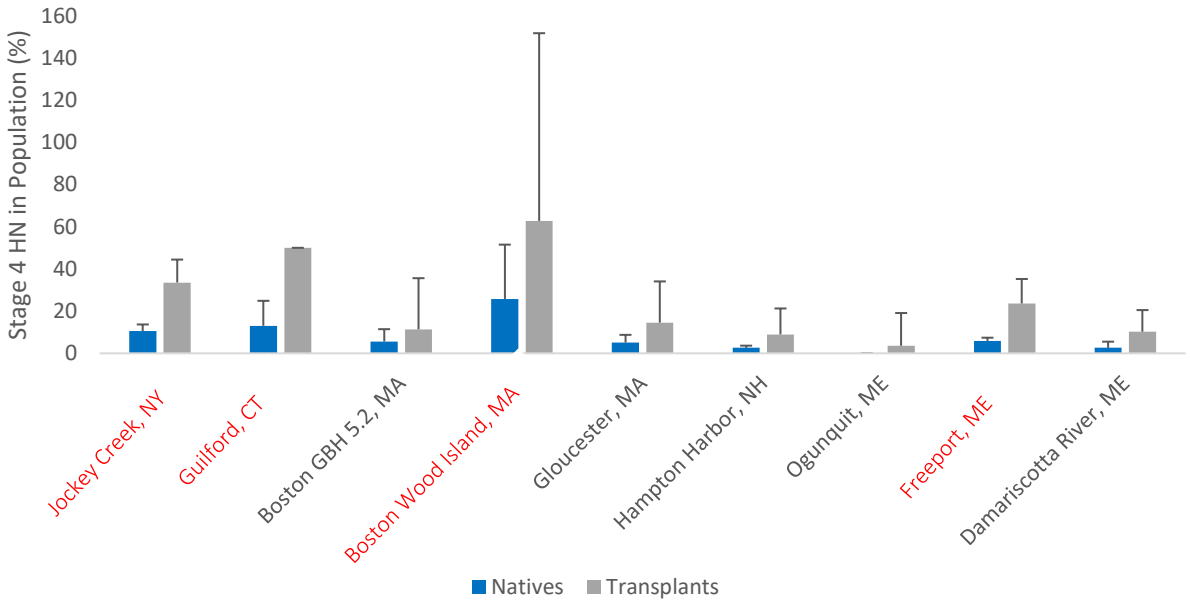

Figure 2. Stage 4 HN data (mean + SE) in transplanted and native clams. Transplanted and adult native clams were collected at each location and analyzed using microscopy to determine HN development. Average level of stage 4 HN (76-100% neoplastic hemocytes) was shown for each of the sites. High HN sites were highlighted red.

Data Analysis of Mean Data

Site locations on PCA plots identified factors that loaded most heavily at each site influencing the position of that site in relationship to others. Factors loading highest for each

principal component (PC) during PCA analysis were separated by positive or negative indications (Tables 1&2). To read PCA plots, site locations relative to each axis (positive/negative) and each other site (proximity) were used to determine factors of the highest importance at each site. For example, a site that was located negative relative to the PC1 axis was associated with factors loading negative for PC1, and sites located close together on plots were more similar than sites spread apart.

Sites with high levels of HN loaded differently on each principal component than sites with low levels of HN (Figure 3). High HN sites, with the exception of Jockey Creek, were correlated with increased levels of specific polycyclic aromatic hydrocarbons (PAH), pesticides, and heavy metals; as well as increased levels of organic content (Table 1).

Table 1. Principal component loadings from analysis of mean data. The top six negative and positive loading factors for each principal component were displayed in decreasing order. Data for this table came from PCA analysis of mean data. Positive or negative loading indications are determined by site location on Figure 3. Sites located above (PC1)/to the right of (PC2) the dotted line were associated with factors in the positive (+) loading column, and sites located below (PC1)/to the left of (PC2) the dotted line were associated with factors in the negative (-) loading column. Pesticides were highlighted in purple, heavy metals in blue, and PAHs in green. Boxes crossed out represented factors identified in PCA that were irrelevant to the study.

Principal Component 1		Principal Component 2	
(+) Loadings	(–) Loadings	(+) Loadings	(–) Loadings
~~Water in Sediment~~	Gravel	Sand	Gravel
Alpha BHC	Inorganic Content	Inorganic Content	Total Pesticides
Gamma BHC	Sand	Organic Content	Methoxychlor
Pyrene	Organic Content	Copper	Toxaphene
Fluoranthene	Copper	Chromium	Endrin Ketone
Beta BHC	Nickel	Zinc	Endrin Aldehyde

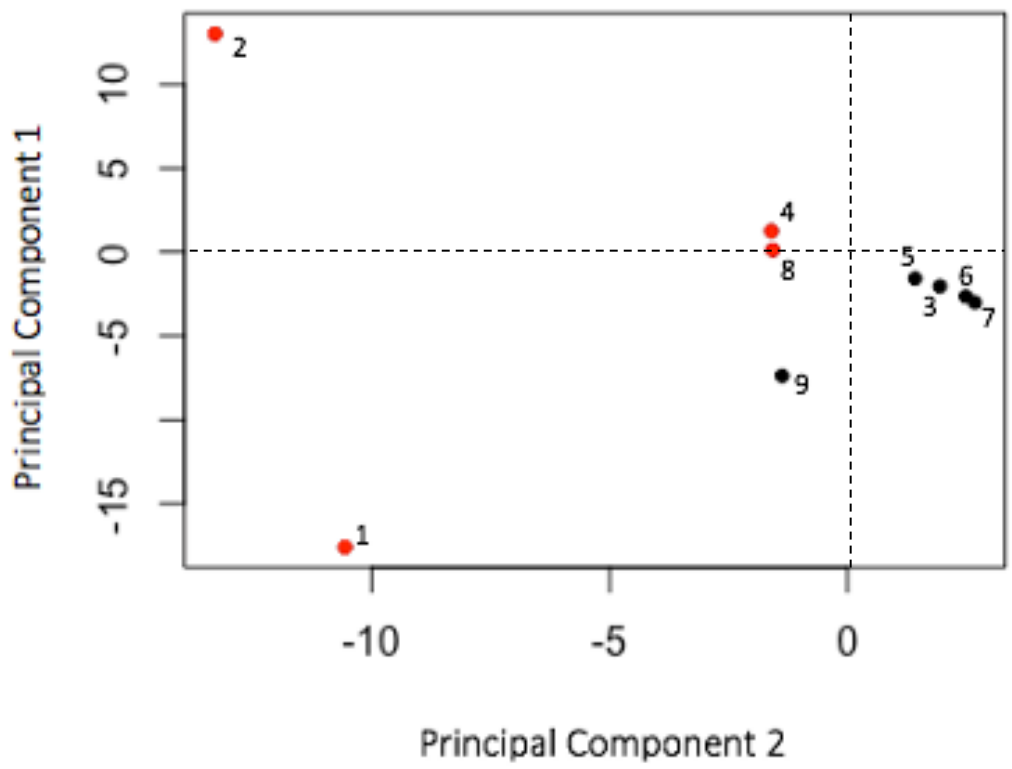

Figure 3. PCA plot for analysis of mean data (PC1 vs PC2). Data for this plot came from PCA analysis of mean data. Dotted lines represent change from negative to positive loadings for each principal component (Table 1). Each dot represents 2011-2012 average for one of the nine sites. Data points were numbered by site and high HN sites highlighted in red: Jockey Creek, NY = site 1; Guilford, CT = site 2; Boston GBH 5.2, MA = site 3; Boston Wood Island, MA = site 4; Gloucester, MA = site 5; Hampton Harbor, NH = site 6; Ogunquit, ME = site 7; Freeport, ME = site 8; and Damariscotta River, ME = site 9.

High HN sites Guilford, Boston Wood Island, and Freeport were correlated with increased levels of two out of sixteen PAHs (fluoranthene and pyrene) (Figure 4); total pesticides (Figure 4); and seven out of twenty-one organochlorinated pesticides (OP) (alpha (α) benzene hexachloride (BHC), beta (β) BHC, gamma (γ) BHC (Lindan), methoxychlor, toxaphene, endrin ketone, and endrin aldehyde) (Figure 5). Lower levels of pollutants at Boston Wood Island and Freeport compared to Guilford (Figures 4&5); as well as increased levels of gravel at Freeport and decreased organic content at Boston Wood Island (Figure 6) were reflected by proximity of these sites to low HN sites on plots (Figure 3) indicating similar factor loadings.

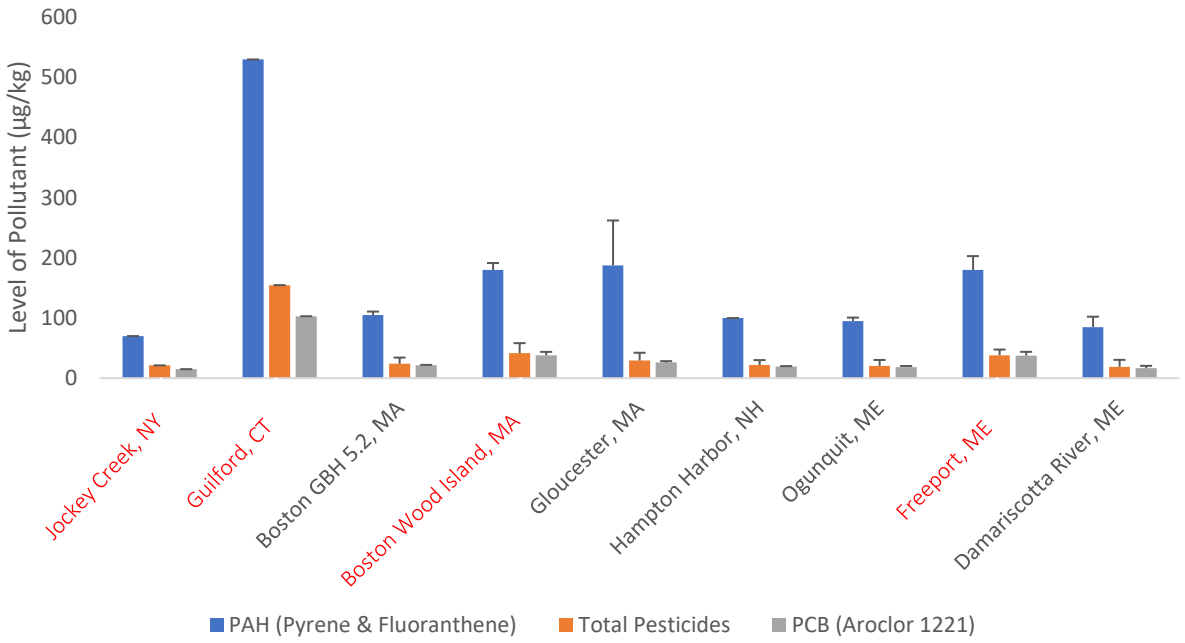

Figure 4. Pollutant data excluding heavy metals (mean + SE). Heavy metal data was excluded due to dramatically increased levels relative to PAHs, PCBs, and pesticides. Native and transplanted clams were collected and analyzed for contaminants including toxic chemicals and heavy metals. Pollutants were identified in PCA analysis and shown as an average for each site. PAHs were represented by average levels of pyrene and fluoranthene, pesticides by total pesticides, and PCBs by Aroclor 1221. High HN sites were highlighted red.

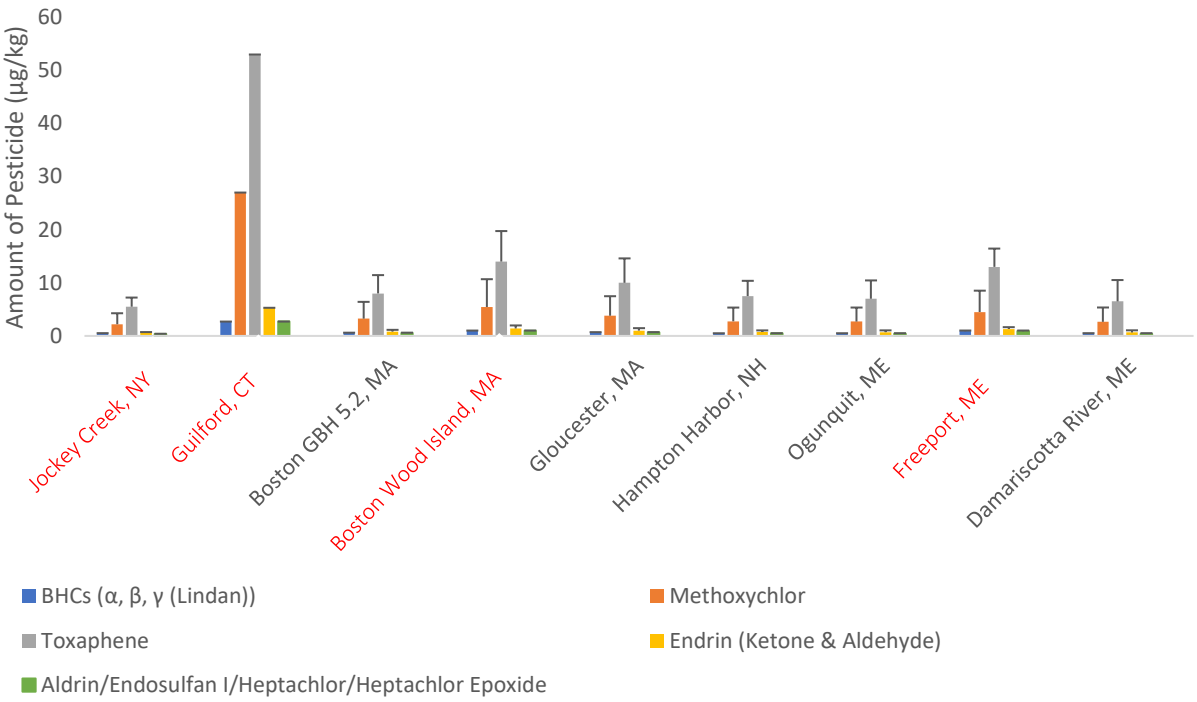

Figure 5. Specific organochlorinated pesticide (OP) data (mean + SE). Native and transplanted clams were collected and analyzed for contaminants including toxic chemicals and heavy metals. Specific OPs were identified in PCA analysis and shown as an average for each site. Aldrin, Endosulfan I, heptachlor, and heptachlor epoxide levels were identical at each site and therefore shown as one data point. High HN sites were highlighted red.

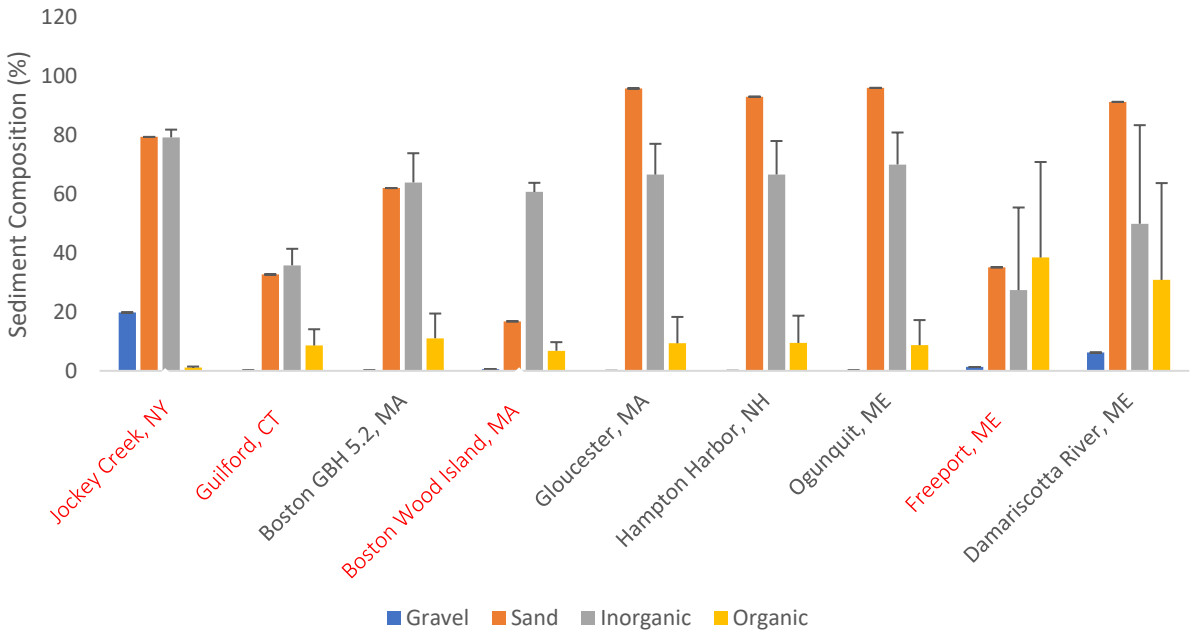

Figure 6. Sediment composition data (mean + SE). Sediment samples were collected in triplicate then grain size was determined using standard sedimentology techniques and organic content by loss on ignition. Sediment composition factors were identified in PCA analysis and shown as an average for each site. High HN sites were highlighted red.

Low HN sites Boston GBH 5.2, Gloucester, Hampton Harbor, and Ogunquit were correlated with decreased levels of organic content, increased levels of sand, and gravel (Figure 6); and decreased levels of four out of ten heavy metals (copper, nickel, chromium, and zinc) (Figure 7).

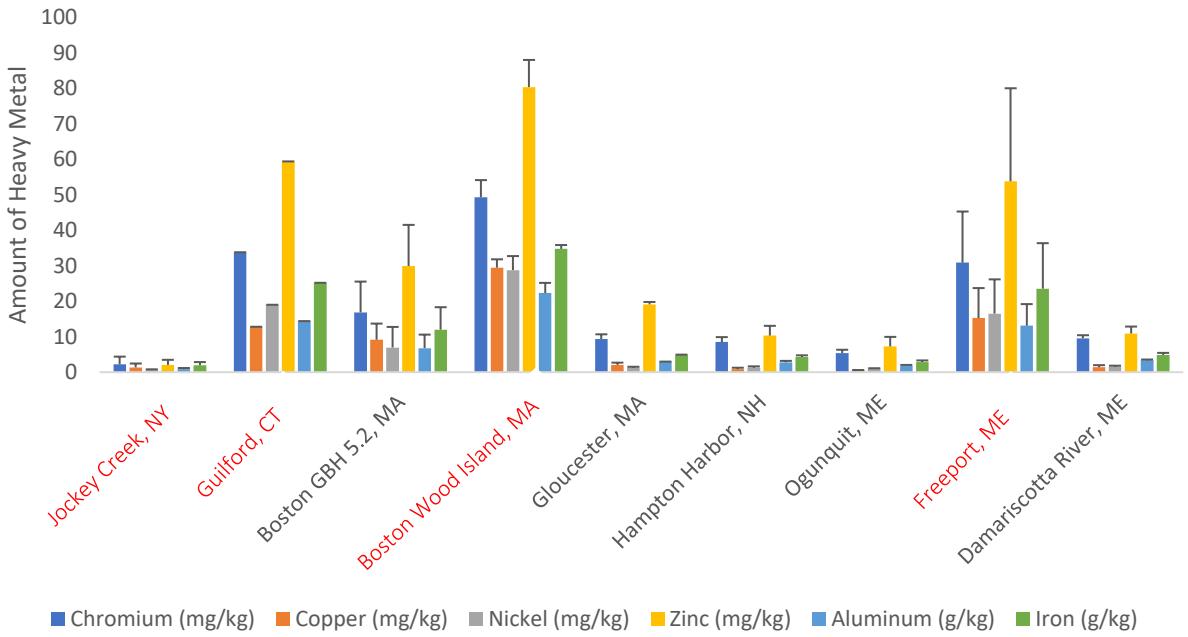

Figure 7. Heavy metal data (mean + SE). Native and transplanted clams were collected and analyzed for contaminants including toxic chemicals and heavy metals. Heavy metals were identified in PCA analysis and shown as an average for each site. High HN sites were highlighted red.

Outliers Damariscotta River and Jockey Creek were located differently on plots than sites sharing their respective HN status (Figure 3) due to drastically increased levels of gravel at both sites (Figure 6). Damariscotta River shared similar sediment composition (Figure 6) and decreased levels of heavy metals (copper, nickel, chromium, and zinc) with other low HN sites (Figure 7). Jockey Creek did not share increased level of PAHs (fluoranthene and pyrene) (Figures 4), total or specific OPs (alpha (α) BHC, beta (β) BHC, gamma (γ) BHC (Lindan), methoxychlor, toxaphene, endrin ketone, and endrin aldehyde) (Figure 4&5), or heavy metals (copper, nickel, chromium, and zinc) (Figures 7) with other high HN sites; but had increased levels of sand and gravel as well as decreased organic content (Figure 6) similar to low HN sites.

Data Analysis of Seasonal Data

Low HN sites were located in similar locations on PCA plots, suggesting similar loadings of factors, with variable locations of high HN sites indicative of most important factors at each site (Figures 8&9). High HN sites, with the exception of Jockey Creek, were comparable in increased levels of specific polychlorinated biphenyls (PCB), OPs, heavy metals, and organic content; as well as decreased survival of transplanted organisms (Table 2).

Table 2. Principal component loadings from analysis of seasonal data. The top six negative and positive loading factors for each principal component were displayed in decreasing order. Data for this table came from PCA analysis of seasonal data. Positive or negative loading indications are determined by site location on Figure 8 or Figure 9. Sites located above (PC1)/to the right of (PC2) the dotted line were associated with factors in the positive (+) loading column, and sites located below (PC1)/to the left of (PC2) the dotted line were associated with factors in the negative (-) loading column. Pesticides were highlighted in purple, heavy metals in blue, and PCBs in yellow. Boxes crossed out represented factors identified in PCA that were irrelevant to this study.

Principal Component 1		Principal Component 2	
(+) Loadings	(–) Loadings	(+) Loadings	(–) Loadings
Aroclor 1221	Sand	Copper	Sand
Beta BHC	Inorganic Content	Aluminum	~~Native Size~~
Aldrin	~~Transplant Growth~~	Chromium	Methoxychlor
Endosulfan I	Gravel	Nickel	Transplant Survival
Heptachlor	Transplant Survival	Iron	Gravel
Heptachlor Epoxide	~~Native Size~~	Zinc	Total Pesticides

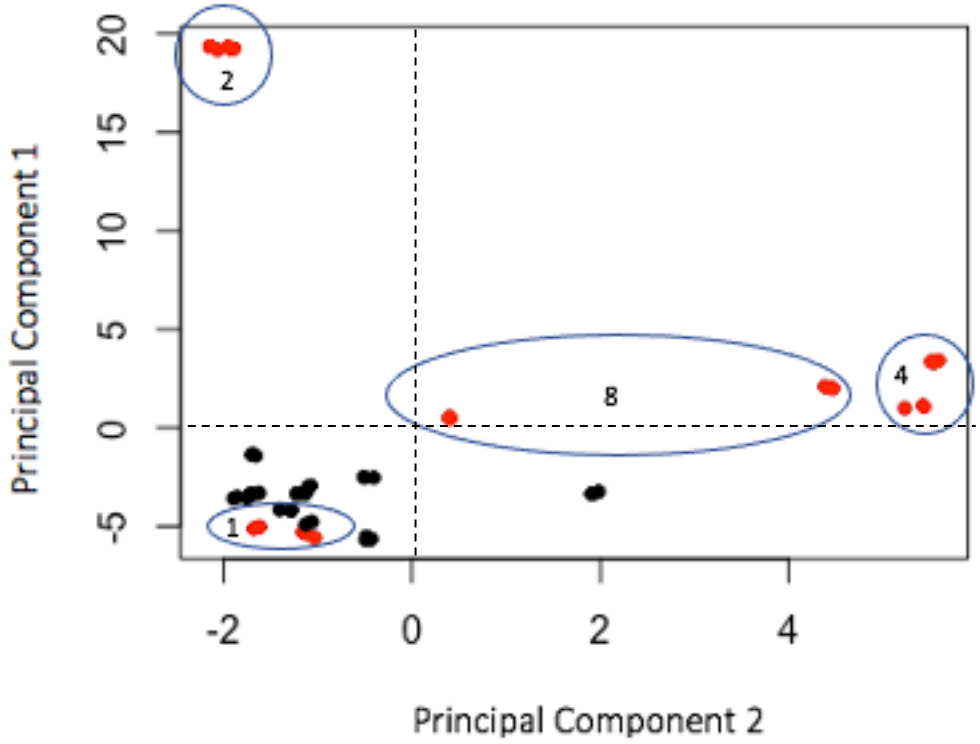

Figure 8. PCA plot for seasonal data analysis colored by HN status. Data for this plot came from PCA analysis of seasonal data (PC1 vs PC2). Raw data was separated and averaged by season (winter, spring, summer, fall) for each site (n=4, for each site). Dotted lines represent change from negative to positive loadings for each principal component (Table 2). Data points for high HN sites were numbered by site and highlighted red: Jockey Creek, NY= site 1; Guilford, CT = site 2; Boston Wood Island, MA = site 4; and Freeport, ME = site 8.

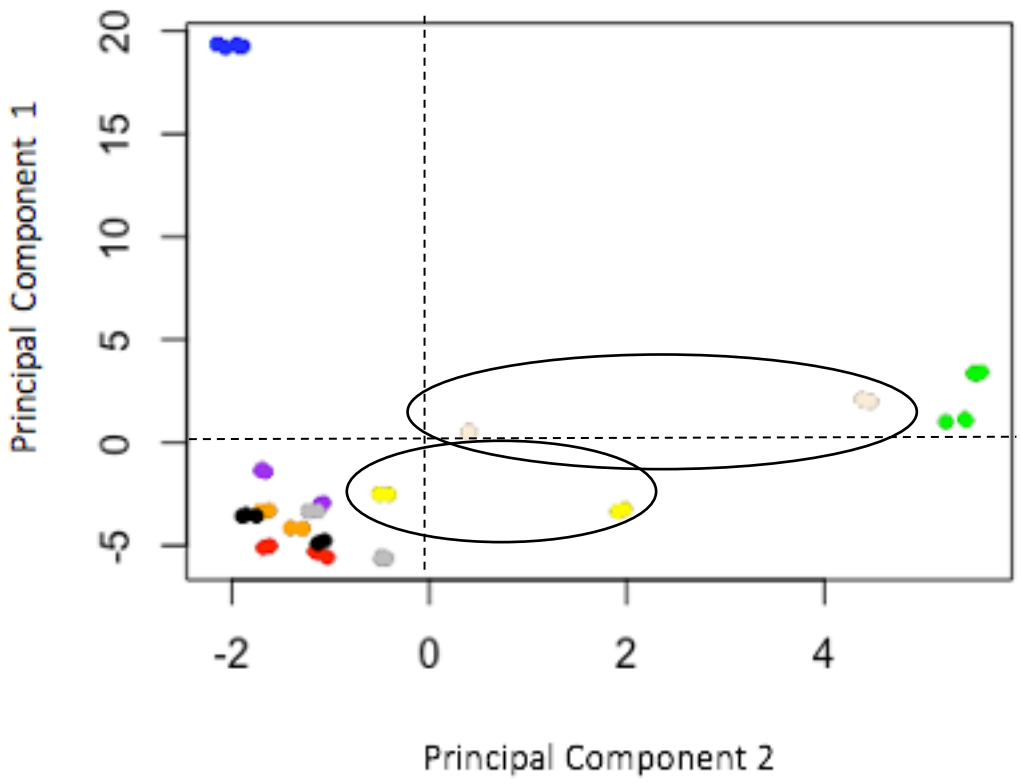

Figure 9. PCA plot for seasonal data analysis colored by site. Data for this plot came from PCA analysis of seasonal data (PC1 vs PC2). Raw data was separated and averaged by season (winter, spring, summer, fall) for each site (n=4, for each site). Dotted lines represent change from negative to positive loadings for each principal component (Table 2). Data points were colored by site: Jockey Creek, NY = red; Guilford, CT = blue; Boston GBH 5.2, MA = yellow; Boston Wood Island, MA = green; Gloucester, MA = purple; Hampton Harbor, NH = orange; Ogunquit, ME = black; Freeport, ME = white; and Damariscotta River, ME = grey. Sites displaying split data between seasons were circled.

High HN sites Guilford, Boston Wood Island, and Freeport were correlated with increased levels of one out of seven PCBs (Aroclor 1221) ($p<0.05$) (Figure 4), and five out of twenty-one OPs (β BHC, aldrin, Endosulfan I, heptachlor, and heptachlor epoxide) ($p<0.05$) (Figure 5). Aroclor 1221 levels were highest at Guilford (Figure 4), driving the separation of this site from Boston Wood Island, and Freeport on PCA plots (Figure 8). Boston Wood Island and Freeport were correlated with increased levels of six out of ten heavy metals (copper, aluminum, chromium, nickel, iron, and zinc) ($p<0.05$) (Figure 7) while Guilford was correlated with increased total pesticides ($p<0.05$) (Figure 4), and one OP (methoxychlor) ($p<0.05$) (Figure 5).

Boston Wood Island and Freeport also shared increased total pesticides ($p<0.05$) (Figure 4), and one OP (methoxychlor) (Figure 5); and Guilford increased levels of heavy metals (copper, aluminum, chromium, nickel, iron, and zinc) ($p<0.05$) (Figure 7).

Low HN sites Boston GBH 5.2, Gloucester, Hampton Harbor, Ogunquit, and Damariscotta River were correlated with increased levels of sand ($p<0.05$) and gravel ($p<0.05$) and decreased organic content ($p<0.05$) (Figure 6), as well as increased transplant survival ($p<0.05$, except Hampton Harbor) (Figure 10).

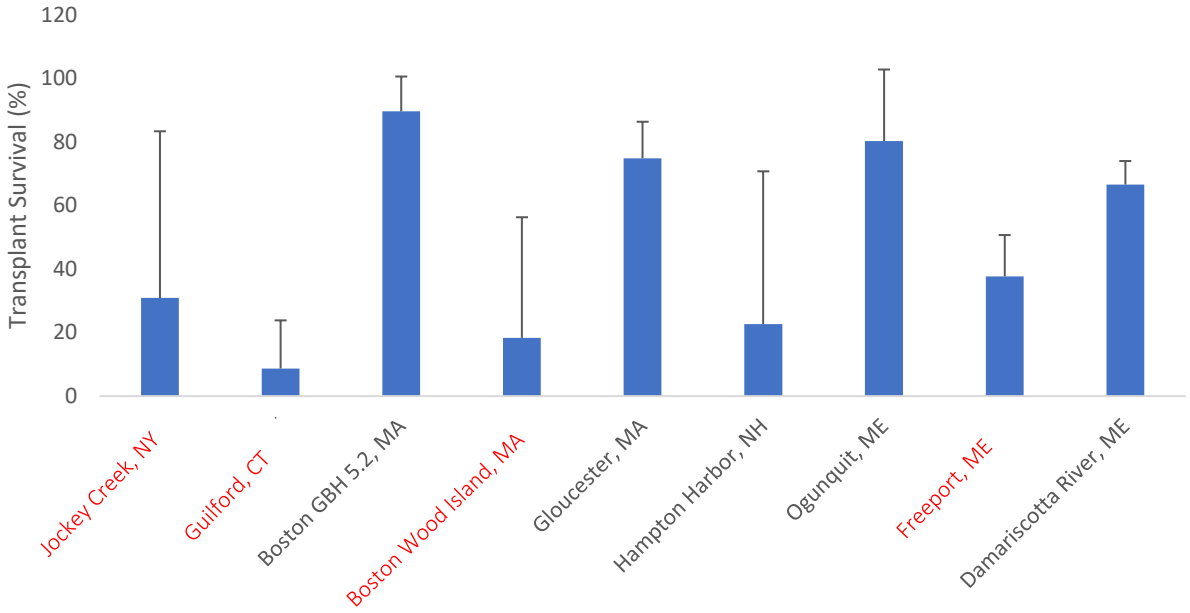

Figure 10. Transplant survival data (mean + SE). Transplanted clams were collected and counted quarterly for mortalities. Average level of transplant survival was shown for each site. High HN sites were highlighted red.

Outlier Jockey Creek, a high HN site, was located similarly to low HN sites on plots (Figure 4) due to increased levels of sand ($p<0.05$) and gravel ($p<0.05$) and decreased organic content ($p<0.05$) (Figure 8). Jockey Creek did not display increased levels of PCBs (Aroclor 1221) ($p>0.05$) (Figure 6), total and specific OPs (β BHC, aldrin, Endosulfan I, heptachlor, and heptachlor epoxide) ($p>0.05$) (Figure 6&7), or heavy metals (copper, aluminum, chromium,

nickel, iron, and zinc) ($p>0.05$) (Figure 9); but did display decreased transplant survival similar to other high HN sites ($p<0.05$) (Figure 10).

Freeport and Boston GBH 5.2, also outliers, displayed a split in data on plots due to increased levels of heavy metals at each site from 2011 to 2012 (Figure 9, Table 2). For both Freeport and Boston GBH 5.2 the left cluster of data points represented 2011 data, and the right cluster represented 2012 data (Figure 9). Between 2011-2012 Freeport displayed a one-fold increase in aluminum, chromium, and iron, a two-fold increase in zinc, and a three-fold increase in copper and nickel (data not shown); but terminal stage 4 HN did not increase significantly ($p=0.221$ for transplanted and $p=0.346$ for native clams) at this site (data not shown). Boston GBH 5.2 displayed an increase over two-fold in copper, aluminum, chromium, iron and zinc and an increase in nickel of more than six-fold (data not shown) without a significant increase in terminal stage 4 HN ($p=0.221$ for transplanted and $p=0.346$ for native clams) at this site (data not shown).

DISCUSSION

Hemic neoplasia (HN) is a molluscan disease that remains minimally understood, especially in terms of disease etiology and identification of factors involved in disease progression. Contamination (Brown et al. 1979; Reinisch et al. 1984; Harper et al. 1994), viral infection (Oprandy et al. 1981; Oprandy and Chang 1983; House et al. 1998; Boettger et al. 2013; Taraska and Boettger 2013), genetic alterations (Frierman and Andrews 1976; Barker et al. 1997; Walker et al. 2006; Siah et al. 2008; Muttray et al. 2012; Arriagada et al. 2014), or transmission of infected cells (McLaughlin et al. 1992; Weinberg et al. 1997) have been identified as possible etiologies, but remain unconfirmed. The current study evaluated HN incidence and development in *Mya arenaria* in nine East Coast populations (New York to Maine) to identify factors involved with disease development. Data was collected using native populations as well as spat clams transplanted to each site. Each site was assigned a group (high/low) based on terminal stage 4 HN (76-100% of cells being neoplastic) status, confirmed by pairwise comparisons. Average level of terminal, stage 4 HN was 6.46% for low HN sites and 28.15% for high HN sites (77% higher). Principal component analysis (PCA) was then utilized to evaluate temperature, sediment composition, population density, pollution, size, growth, and mortality data at and between each site. Analysis revealed sites sharing similar HN status were closely associated in terms of influencing factors, confirmed by pairwise comparisons. Results indicated sediment organic content and pollutant levels were major factors differentiating between high and low HN sites in terms of survival and incidence of HN with one exception identified at Jockey Creek, NY, where high HN and mortality was associated high temperatures instead. High levels of pollutants and temperatures are confirmed to decrease immunity and increase stress in *M. arenaria*, indicating stress level was strongly associated with HN status in

analysis. While it was not within the scope of the current study to evaluate transmission or genetic alterations in terms of etiology, data strongly supports a viral causative agent of HN.

Hemic Neoplasia Status and Seasonality

All sites in analysis, regardless of HN status, displayed higher HN incidence in transplanted populations compared to natives. On average, transplanted populations (24.3%) showed 67.5% higher incidence relative to native populations (7.9%), linking increased HN development to pollution susceptibility in juvenile clams (Johnson and Finley 1980; Ahrens et al. 2002; Chung et al. 2007; Lindsay et al. 2010) as well as bivalve size/age (Cooper et al. 1982; Leavitt et al. 1990; Boettger et al. 2013). Results indicate bivalve populations composed of large portions of spat clams (5-10mm) exposed to high pollutant levels are of concern for high HN prevalence. Stressors potentially affecting spat clams during transplantation, capable of impacting HN incidence (Lacoste et al. 2001), were controlled for by maintaining spat animals in refrigerated conditions prior to transplantation. In addition, disease seasonality was not indicated throughout the limited time of analysis though importance of HN incidence in relation to seasonality controlled by fluctuating water temperatures has been indicated in long term analyses over one year in length (Brousseau 1987; Farley et al. 1991; Barber 2004; Boettger et al. 2013). High HN levels are typically reported in winter months when stress is high (due to gametogenesis and limited food availability), associating stress to HN status, similar to this analysis (Cooper et al. 1982; Leavitt et al. 1990; Minier et al. 2000; Boettger et al. 2013). Consequently, seasonality would be expected to be displayed given a longer term of data collection.

Analysis of Sites with Low Levels of Hemic Neoplasia

Sites with low levels of HN (Boston GBH 5.2 [Greater Boston Harbor Mudflat 5.2], Gloucester, Hampton Harbor, Ogunquit, and Damariscotta River) also exhibited low organic content and heavy metals (copper, chromium, nickel, and zinc) which were therefore considered factors influencing local HN levels at these sites. On average, low HN sites displayed 1552 mg/kg of heavy metals, and high HN sites 5686 mg/kg (72.7% higher). Average organic content was 36.5% at low HN sites, and 49.2% at high HN sites (13% higher). Survival was also affected with an average 60.8% survival at low HN sites and 20.2% at high HN sites (66.8% higher); further indicating site conditions were associated with healthier populations overall due to mortalities associated with HN (Cooper et al. 1982).

High levels of inorganic content in sediments (sand and gravel), or low levels of organic content, were associated with healthier sites overall (low HN and mortality) due to negative associations with contaminant concentrations (EPA 2012; Hartwell et al. 2018; Nelson 2020). Low sediment organic content at Boston GBH 5.2, Gloucester, Hampton Harbor, Ogunquit, and Damariscotta River did not allow high loading of pollutants, which lowered exposure and associated HN development in animals associated with life inside these sediments (Hartwell et al. 2018). Sediment organic content is therefore an important factor to consider for identification of healthy/sustainable populations of bivalves to harvest from. Low HN sites were associated with only heavy metal levels due to high levels relative to PAHs, OPs, and PCBs in analysis. However, at high HN sites all levels of pollutants were associated with HN and organic content, discussed below.

At sites of elevated HN levels (Guilford, Boston Wood Island, and Freeport) high levels of organic content were associated with high loading of all pollutants, identified below, as well

as low survival. Organic content (silt and clay) originates from natural breakdown of phytoplankton, leaves, and dead organisms; but also increases via anthropogenic sources (organic industrial waste, agricultural run-off, or sewage) (EPA 2012) and is associated with poor sediment quality and low species richness and abundance (EPA 2012; Hartwell et al. 2018). Pollutants (particularly heavy metals) tend to bind to organic matter in sediment due to greater surface to area volume allowing for higher absorption of pollutants. High pollutant loading is especially problematic due to pollutant interactions with organic content that cause increased toxicity in sediment (Capuzzo and Sasner 1977; Swain 1983; Eisler 1986; EPA 2012). Proximity of *M. arenaria* to pollutants in sediment has a direct relationship to exposure concentration (Downs et al. 2002), therefore relationship between organic content and pollutant loading/toxicity is important to identify populations with high risk of developing HN or subsequent population declines.

Analysis of Sites with High Levels of Hemic Neoplasia

Sites with high levels of HN (Guilford, Boston Wood Island, and Freeport) were associated with high levels of specific polycyclic aromatic hydrocarbons (PAH) (fluoranthene and pyrene), organochlorinated pesticides (OP) (alpha (α) benzene hexachloride (BHC), beta (β) BHC, gamma (γ) BHC (Lindan), methoxychlor, toxaphene, aldrin, Endosulfan I, heptachlor, heptachlor epoxide, endrin ketone, and endrin aldehyde), polychlorinated biphenyls (PCB) (Aroclor 1221), and heavy metals (iron, aluminum, chromium, copper, nickel, iron, and zinc) as factors strongly influencing HN incidence. On average all pollutant levels at high HN sites were 72.7% higher compared to low HN sites, with PAH levels higher by 52.3%, OPs by 64.2%,

PCBs by 57.6%, and heavy metals by 72.7% relative to low HN sites. Results indicate high HN was associated with high levels of specific PAH's, OPs, PCB's and heavy metals.

All sites in analysis displayed higher pollution levels than Northeast Coast (Delaware to Maine) averages between 2011-2012 from Mussel Watch Program (NCCOS 2012) (Table 3). On average levels of all pollutants identified in analysis were 1422 mg/kg at high HN sites, 388 mg/kg at low HN sites, and 23.5 mg/kg for Mussel Watch averages (Table 3). Programs such as Mussel Watch utilize bivalves as indictors of local environmental pollution due to high lipid content which hydrophobic pollutants are attracted to, and limited ability to metabolize contaminants (Phillips 1977; O'Connor 2002; Grosell and Walsh 2006). Higher pollutant levels at low HN sites compared to Mussel Watch averages was not expected, but due to more sites being included in the comparatively large dataset collected by Mussel Watch (NCCOS 2012). Regardless, *M. arenaria* were exposed to pollutants levels above average, especially at Guilford, Boston Wood Island, and Freeport which displayed over 98.3% higher pollutant levels compared to averages for the same time period and location. Comparisons indicate pollutants were relevant to evaluate in terms of HN status in analysis.

Table 3. Comparison of pollutant averages for each pollutant group. Levels of specific pollutants were averaged together based on indicated group between all sites for high HN sites (Jockey Creek, Guilford, Boston Wood Island, and Freeport) and low HN sites (Boston GBH 5.2, Gloucester, Hampton Harbor, Ogunquit, and Damariscotta River). Averages were displayed compared to Mussel Watch Program averages for the Northeast Coast (Delaware to Maine) between 2011-2012 (NCCOS 2012)

Parameter	High HN Site Average	Low HN Site Average	Mussel Watch Average
PAHs	240 μg/kg	114.5 μg/kg	50.5 μg/kg
Ops	63.9 μg/kg	22.9 μg/kg	0.15 μg/kg
PCBs	48.4 μg/kg	20.4 μg/kg	7.3 μg/kg
Heavy Metals	5686 mg/kg	1552 mg/kg	93.9 mg/kg

All specific pollutants identified in analysis are classified into four groups. These include PAHs, which are commonly associated with oil spills in marine and coastal waters and originate from carbon and fossil fuels (EPA 2009; Girón-Pérez 2010). OPs, used in agriculture to kill pests via interference with normal metabolic function (Renault 2011,2015). PCBs, a group of anthropogenic industrial chemicals found in transformers, flame retardants, plasticizers, and heat transfer fluids (Hutzinger et al. 1974; EPA 1980). Lastly, heavy metals, which are naturally found in aquatic environments and important for normal function in bivalves. However, industrial waste or other anthropogenic sources can elevate levels above normal, causing cellular and physiological problems similar to PAHs, OPs, and PCBs (Trevisan and Dafre 2013; Yap et al. 2021). Associations between these pollutants groups and HN are common (Yevich and Barszcz 1977; Lowe and Moore 1978; Brown et al. 1979; Reinisch et al. 1984; Harper et al. 1994; Dyrynda et al. 1998; Barber 2004; St-Jean et al. 2005) due to nature of pollutants, discussed below.

PAHs, OPs, PCBs, and heavy metals are introduced into aquatic environments via run-off or atmospheric inputs (Kimbrough et al. 2008) and are especially problematic for *M. arenaria* which inhabit top 20cm of sediment closest to pollutants (Downs et al. 2002). These pollutants display long half-lives due to hydrophobicity, high liposolubility, low volatility and degradability, as well as ability to bioaccumulate (Jones and de Voogt 1999; Silva et al. 2008; Basso 2011). Chronic pollutant exposure is facilitated by long half-life as well as ability of hydrophobic pollutants to bioaccumulate in lipid rich tissues of *M. arenaria*. Bioaccumulation is important for any species above *M. arenaria* in the food chain, causing concern for chronic health effects in humans (Phillips 1977; Baker and Mann 1991; Grosell and Walsh 2006).

Nature of pollutants emphasizes importance of research in bivalves, relating to both HN and pollution, for applications to health in many other species.

Pollutant effects, specifically on bivalves, have not been fully elucidated; though studies indicate effects of short and long-term exposure to select pollutants (Table 4). Exact mechanism of HN influence differs for each individual or population studied due to many factors that modulate pollutant toxicity and physiological response such as interactions between natural habitat (including temperature, salinity, pH, oxygen levels, and UV exposure); effects of multiple pollutants; and differences in size, age, sex, and reproductive status of exposed bivalves (Rand and Petrocelli 1985; Girón-Pérez 2010; Breitwieser et al. 2016). Accordingly, lethal and effective pollutant doses are widely variable and dependent on each individual or population studied (Johnson and Finley 1980; Mackay et al. 2001; Parry and Pipe 2004; Renault 2011). Factors that can modulate toxicity and response were not able to be controlled for in analysis, or any natural environment, therefore doses of pollutants are irrelevant. Instead, significant differences in pollutant levels at Guilford, Boston Wood Island, and Freeport compared to low HN sites confirm significance relative to this analysis.

Table 4. Summary of pollutant effects on bivalves. Reports of immune or physiological effects for each group of pollutant (PAH, OP, PCB, heavy metal) identified in analysis were listed for any bivalve species as well as specifically for *Mya arenaria*. The left column displays reported effects of pollutant exposure in bivalves, and right column displays source(s) for each reported effect.

Reported Exposure Effects in Bivalves	Sources
PAHs	
1. Decreased hemocyte membrane stability	1. Hannam et al. 2009, 2010a, 2010b; Matozzo et al. 2009
2. Decreased hemocyte adhesion capability	2. Matozzo et al. 2009
3. Decreased digestive epithelial thickness	3. Weinstein 1997
4. Suppressed pathogen recognition and protein synthesis	4. Ertl et al. 2016
5. DNA damage such as DNA strand breaks	5. Wessel et al. 2007; Banni et al. 2010; Tian et al. 2020

6. Impairment of hemocyte mitochondrial function	6. Auffret et al. 2004
7. Micronuclei formation indicative of chromosomal damage	7. Siu et al. 2008; Banni et al. 2010
8. Gill morphological abnormalities and metabolic alterations	8. Cappello et al. 2013
9. Lysosome damage such as membrane destabilization	9. Grundy et al. 1996; Galloway et al. 2002; Wootton et al. 2003; Gagnaire et al. 2006; Matozzo et al. 2009; Tian et al. 2020
10. Reduced antioxidant levels	10. Hannam et al. 2010a, 2010b
11. Increased ROS induced cell damage	11. Hannam et al. 2010a, 2010b
12. Inhibition of hemocyte released enzymes	12. Coles et al. 1994; Matozzo et al. 2009
13. Alterations to actin cytoskeleton of hemocytes	13. Goméz-Mendikute et al. 2002
14. Increased number of hemocytes in circulation indicative of decreased function	14. Coles et al. 1994; Hannam et al. 2010a; Croxton et al. 2012
15. Decreased time of survival in air	15. Matozzo et al. 2009
***Mya arenaria* specifically**	***Mya arenaria* specifically**
1. Large reductions in carbon flux	1. Gilfillan et al. 1977
2. Increased production of heat shock proteins (HSP) 60 & 70 indicative DNA damage	2. Downs et al. 2002
3. Decreased phagocytosis	3. Frouin et al. 2007
4. Delayed gametogenesis	4. Frouin et al. 2007
5. Reduced growth	5. Appeldoorn 1981
6. Interference with hemocyte maturation or differentiation	6. Pichaud et al. 2008
7. Increased release of reactive oxygen species (ROS)	7. Pichaud et al. 2008
Pesticides	
1. Decreased hemocyte viability	1. Fries and Tripp 1980; Alvarez and Friedl 1992; Gagnaire et al. 2006; Anguiano et al. 2007
2. Increased release of ROS	2. Gagnaire et al. 2006; Trevisan and Dafre 2013
3. Increased susceptibility to bacteria or parasites	3. Gagnaire et al. 2007; Kim et al. 2008
4. Decreased phagocytosis	4. Alvarez and Friedl 1992; Gagnaire et al. 2007; Canty et al. 2007; Geret et al. 2013
5. Reduced filtration rates	5. Anguiano et al. 2007
6. Stagnation or decreased growth	6. His and Seaman 1993; Butler et al. 1960; Revankar and Shyama 2009
7. DNA damage such as DNA strand breaks	7. Anguiano et al. 2007; Wessel et al. 2007; Geret et al. 2013
8. Micronuclei formation indicative of chromosomal damage	8. Siu et al. 2008; Revankar and Shyama 2009
9. Down regulation of genes involved with hemocyte function	9. Gagnaire et al. 2007
10. Impairment of lysosome function	10. Lowe et al. 1995
***Mya arenaria* specifically**	***Mya arenaria* specifically**
1. Increased prevalence of HN	1. Farley et al. 1991; Muttray et al. 2012
2. Increased expression of the p53 tether mortalin	2. Muttray et al. 2012
3. Decreased hemocyte number	3. Greco et al. 2011

4. Increased energy demands 5. Higher ROS induced cell damage 6. Altered antioxidant enzyme activity 7. Upregulation in p53 followed by significant down regulation of the gene that is found in most cancers	4. Greco et al. 2011 5. Greco et al. 2011 6. Greco et al. 2011 7. Pariseau et al. 2011
PCBs	
1. Decreased growth rate 2. DNA damage 3. Generation of ROS 4. Activation of stress related transcription factors 5. Lysosome damage such as membrane destabilization	1. Lowe et al. 1972 2. Liu et al. 2009 3. Liu et al. 2009 4. Canesi et al. 2003 5. Canesi et al. 2003; Gagnaire et al. 2006; Liu et al. 2009
***Mya arenaria* specifically**	***Mya arenaria* specifically**
1. Increased incidence of HN 2. Increased micronuclei formation indicative of chromosomal damage 3. Inhibition of phagocytosis and decreased hemocyte counts 4. Decreased hemocyte viability (PCB and PAH Mixture) 5. Decreased body condition (PCB and PAH Mixture) 6. Decreased heart rate (PCB and PAH Mixture) 7. Decreased hemocyte activity (PCB and PAH Mixture)	1. Reinisch et al. 1984; Strandberg et al. 1998 2. Dopp et al. 1996 3. Fournier et al. 2002 4. Galloway et al. 2002 5. Galloway et al. 2002 6. Galloway et al. 2002 7. Gagnaire et al. 2006
Heavy Metals	
1. Generation of ROS and ROS induced damage 2. Inflammatory reaction in the gills due to osmotic imbalance 3. DNA damage such as DNA strand breaks 4. Increased energy requirements and impairment of mitochondria in hemocytes 5. Decreased hemocyte counts 6. Lysosomal membrane damage 7. Increased vulnerability to infectious disease 8. Induction of HSPs and changes to transcription of immunity genes 9. Inhibition of hemocyte released enzymes 10. Deleterious effects on actin cytoskeleton of hemocytes 11. Reproductive toxicity 12. Respiratory and cardiovascular depression	1. Ringwood et al.1998; Fisher et al. 2000; Géret et al. 2002; Thiagarajan et al. 2006; Trevisan and Dafre 2013; Haberkorn et al. 2014 2. Hietanen et al. 1988 3. Tran et al. 2007; Trevisan et al. 2011 4. Matozzo et al. 2001 5. Suresh and Mohandas 1990; Ridha et al. 2015 6. Lowe et al. 1995; Regoli et al. 1998; Matozzo et al. 2001; Ciacci et al. 2011; Franzellitti et al. 2012 7. Parry and Pipe 2004 8. Ciacci et al. 2011; Franco et al. 2006; Franzellitti et al. 2012 9. Cheng 1990; Cheng 1989; Ciacci et al. 2011 10. Goméz-Mendikute and Cajaraville 2003 11. Myint and Tyler 1982; Guo et al. 2020 12. Scott and Major 1972
***Mya arenaria* specifically**	***Mya arenaria* specifically**
1. Decreased phagocytosis	1. Brousseau et al. 2000

2. Decreased hemocyte viability 3. Reduced growth 4. Severe oxidative stress leading to cellular damage and mortality 5. Inhibition of ciliary activity due to interference with energy metabolic pathways 6. Reduced filtration rates 7. Increased oxygen consumption	2. Brousseau et al. 2000 3. Appeldoorn 1981 4. González et al. 2010 5. Capuzzo and Sasner 1977 6. Capuzzo and Sasner 1977 7. Thurberg et al. 1974

Pollutants identified in analysis influence bivalve innate immune function (Table 4), which relies primarily on hemocytes to identify and destroy foreign entities through phagocytosis (Cheng 1983). Hemocyte alterations, reduced phagocytosis, altered hemocyte enzyme activity, DNA and gene alterations, and lysosome damage directly reduce immune capabilities and increase stress; whereas decreased growth, respiration, heart rate, gametogenesis, carbon flux, and ciliary activity, as well as increased oxygen consumption are indicative of stress produced by pollutant exposure (Table 4). Direct alterations to immune system and its components, as well as indirect effects indicative of stress support association identified at Guilford, Boston Wood Island, and Freeport. This analysis does not suggest pollution exposure directly causes HN, but rather contributes to HN development in combination with other factors, such as a virus.

Stress and lowered innate immunity caused by pollutant exposure (Table 4) leads to increased oxygen demands, as well as decreased energy and resources available for *M. arenaria* to defend against foreign invaders such as parasites, bacteria, or viruses (Beckmann et al. 1992; Girón-Pérez 2010; Burgos-Aceves and Faggio 2017). Thus, a retrovirus can easily initiate HN due to the compromised state of *M. arenaria*; which strongly supporting retroviral involvement of HN. Retroviral involvement has also been supported through HN initiation via exposure to BrDU (a compound known to induce leukemia in murine models) (Oprandy and Chang 1983), tranmission of HN with cell free hemolymph (Taraska and Boettger 2013), as well as detection

of reverse transcriptase acitivity (House et al. 1998; AboElkhair et al. 2009a). Despite strong support for involvement of a retrovirus in HN (Brown et al. 1979; Cooper et al. 1982; Oprandy and Chang 1983; House et al. 1998; Boettger et al. 2013; Taraska and Boettger 2013; Arriagada et al. 2014; Mateo et al. 2016b) isolation of a viral agent is needed in order to confirm this theory (AboElkhair et al. 2009b). Additionally, the relationship between stress and HN identified in analysis supports stress induced genetic alterations (p53/mortalin/Steamer) leading to HN (Barker et al. 1997; McGladdery et al. 2001; St-Jean et al. 2005; Walker et al. 2006; Boettger et al. 2008; Arriagada et al. 2014) which are of massive importance due to connections with cancers in other organisms, including humans.

Jockey Creek lacked factors statistically correlated with other high HN sites, which was an interesting finding relative to this analysis. On average, levels of organic content (20.74%) and pollutants (PAHs, OPs, PCBs, and heavy metals) (116.5 mg/kg) were lower than high HN site averages by 97.6% for pollutants and 57.9% for organic content. However, low survival (26.78% average) and high HN (22.05% average) at Jockey Creek indicated another factor was associated with HN. No identified factors could have influenced HN except water temperature, however temperature data for each site was collected from different sources, due to failure of multiple temperature probes in the field. Thus, collected data was not a reliable measure; instead, geographical location of Jockey Creek revealing it as the southernmost site of analysis and within range of the gulf stream (Church 1932) was used to evaluate temperature differences at this site compared to others.

Temperature tolerances for *M. arenaria* vary (generally -2 to 32 degrees Celsius) (Kennedy and Mihursky 1971; Feder and Paul 1974; Abraham and Dillon 1986; Strasser 1998), with thermal stress and mortality induced after 18 degrees Celsius (Newell et al. 1986; Abele et

al. 2002). Temperatures at Jockey Creek were on average 13.1 degrees Celsius during this study, however, there was a maximum temperature of 26.2 degrees Celsius, and temperatures consistently above 18 degrees Celsius during June-October in both 2011 and 2012 (NOAA 2012). High water temperatures displayed at Jockey Creek, also associated with climate change, are concerning due to resulting effects on thermal stress (Harvell et al. 1999; Scavia et al. 2002; Gagné et al. 2007; Schiedek et al. 2007; Fabbri et al. 2008; Greco et al. 2011; Zhang et al. 2020) as well as increased mortality (Farley et al. 1972; Pfitzenmeyer 1972; Anestis et al. 2007). Accordingly, *M. arenaria*, a cold-water species, were once reported in Cape Hatteras, North Carolina over 10 years ago, however, due to increasing water temperatures and associated mortalities (S. A. Boettger, personal communication) the southernmost population is currently found in northern Virginia within the Chesapeake Bay (Baker and Mann 1991). Similarly, *M. arenaria* at Jockey Creek displayed low survival due to reported water temperatures associated with thermal stress and mortality.

High water temperature at Jockey Creek also acted as a stressor for viral initiation of HN (Farley et al. 1972; Chou et al. 1994; Parry and Pipe 2004; Morley 2010) similar to pollutants found at Guilford, Boston Wood Island, and Freeport. Elevated temperatures induce mitochondrial reactive oxygen species (ROS) (Abele et al. 2002; Heise et al. 2003), expression of HSP23, 60, 70 & 90 (Buckley et al. 2001; Snyder et al. 2001; Fabbri et al. 2008; Zhang et al. 2020), increased energy requirements (Gagné et al. 2008), and oxidative stress (Abele et al. 2001); therefore, initiating HN via increased stress and decreased immunity. High temperatures at Jockey Creek also effect *M. arenaria* metabolic processes and physiology thus effecting toxicity of pollutants (Sjursen and Holmstrup 2004; Cherkasov et al. 2007; Min et al. 2015) and ability to respond to exposure (Eisler 1977; Greco et al. 2011). Although pollutants levels at

Jockey Creek were not statistically high in analysis, the possibility of increased pollutant toxicity due to high water temperatures cannot be ruled out and is important to consider at this site. Higher levels of HN and mortality will be seen in *M. arenaria* if water temperatures continue to rise as predicted with climate change (Harvell et al. 1999; Karvonen et al. 2010) which is concerning for the shell fishing industry, but more importantly future health and sustainability of bivalve populations.

Freeport and Boston GBH 5.2 displayed significant increases in heavy metals between 2011-2012, but statistical analysis revealed no resulting effects on HN. On average, Freeport displayed 61.9% higher levels of heavy metals, and Boston GBH 5.2 64.1% higher levels due to seasonality of pollutant loading and bioaccumulation influenced by biological sources such as reproduction and growth; or environmental sources such as temperature, and periodic use of anthropogenic sources (Amiard et al. 1986; Butler 1973; Bryan 1973; Amiard and Berthet 1996; Lee et al. 1996; Devier et al. 2005; Guéguen et al. 2011; Galvao et al. 2012; Breitwieser et al. 2016). Absence of increased HN incidence in response to increased heavy metal exposure may indicate threshold level of exposure that no longer effects *M. arenaria* in terms of HN development; but this cannot be confirmed within this analysis. Finally, *M. arenaria* are exposed to another source of stress by way of fluctuating pollutant levels that prevent leveling out of immune response due to chronic steady exposure. Stress is the main factor influencing levels of HN at all sites in analysis, therefore any additional sources of stress are important to account for.

CONCLUSIONS

Overall, hemic neoplasia (HN) development and incidence was influenced by stress, related to pollution and temperature, in *Mya arenaria*. Pollutant stress is known to compromise bivalve innate immune function which allows for HN initiation through infection from a viral agent; therefore, strongly supporting retroviral etiology. Organic content was also directly related to HN incidence, due to association with pollutant loading, further indicating the strong influence of pollution on HN development. Pollutants identified in analysis help to understand HN development, but also highlight importance of HN research for applications to health in other species, due to bioaccumulation. Additionally, deleterious effects associated with rising water temperatures suggest how climate change may affect future incidence of viral diseases, such as HN, as well as sustainability of future bivalve populations. This analysis contributes to knowledge gaps in current HN research by identifying sources of stress that contribute to development of HN.

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