CHARACTERIZATION OF GILL EPITHELIAL NUCLEAR HYPERTROPHY AND A HEALTH SURVEY OF WILD SOFT-SHELL CLAMS (*MYA ARENARIA*) IN MARYLAND'S CHESAPEAKE BAY

by

SUSAN KNOWLES

(Under the Direction of Alvin Camus and Elizabeth Howerth)

ABSTRACT

Abstract: Soft-shell clams, *Mya arenaria*, a commercially harvested and once economically important species in Maryland's Chesapeake Bay, have experienced a dramatic population decline since the peak harvests of the 1960s. Many factors have contributed to population decline, including overharvesting, environmental degradation, predation, and disease.

In 2000, the Maryland Department of Natural Resources began an annual disease surveillance program for soft-shell clams. In 2002, intranuclear inclusions were identified within hypertrophied gill epithelia of many clams, but the cause was unknown.

The principle objectives of this research were to (1) characterize the agent causing gill epithelial nuclear hypertrophy by light and electron microscopy, and molecular methods; (2) perform a retrospective histological health survey to determine the distribution and prevalence of this condition and other diseases, infections or conditions in archived samples of *Mya arenaria* collected from Maryland's Chesapeake Bay from

2005 to 2009; and (3) perform a metagenomic analysis of viral diversity in Chesapeake Bay soft-shell clams.

Light and transmission electron microscopy revealed Feulgen-positive, finely granular, amphophilic, intranuclear inclusions that marginated chromatin, and nonenveloped, moderately electron dense, icosahedral, 75–82 nm virus-like particles that occasionally formed paracrystalline arrays. To assess the overall health of this population, 630 wild soft-shell clams from 18 locations within Maryland's Chesapeake Bay collected from 2005 to 2009 were examined. Intranuclear virus-like inclusions, *Perkinsus* spp., unidentified pyriform ciliates, trichodinid ciliates, cestodes, copepods, rickettsia-like organisms, bacteria, disseminated neoplasia, a single polyp, renal concretions, hemocytic infiltration and pericardial gland concretions were identified.

The virome of three clams was analyzed using next-generation sequencing technology. Viral families represented included *Asfarviridae*, *Baculoviridae*, *Caulimovirdae*, *Circoviridae*, *Coronaviridae*, *Herpesviridae*, *Irodoviridae*, *Mimiviridae*, *Myoviridae*, *Nanoviridae*, *Nimaviridae*, *Parvoviridae*, *Phycodnaviridae*, *Polydnaviridae*, *Polyomaviridae*, *Poxviridae*, and *Retroviridae*. Sequences with identities to numerous unclassified viruses were also present. Phylogenetic analysis of sequences with identities to herpesvirus terminases showed genetic similarity with other molluskan herpesviruses and *in situ* hybridization using a digoxigenin-labeled DNA probe provided further evidence to support the presence of a novel herpesvirus in soft-shell clams.

Results from this study broaden our understanding of pathologic conditions which may be impacting this population and can be used for the management of this species.

INDEX WORDS: bacteria, Chesapeake Bay, clam, disease, histopathology, Illumina, *Mya arenaria*, neoplasia, parasite, transmission electron microscopy, virus

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DEDICATION

I dedicate this dissertation to Bert, Anne, Abby and Orangy, to my dear friends for their constant love and support, and especially to my parents for instilling in me the importance of hard work and higher education.

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CHAPTER 1

INTRODUCTION

Soft-shell clams, *Mya arenaria*, are eurythermal, euryhaline, benthic bivalves that occur along the western coast of the Atlantic Ocean from Labrador to South Carolina and in lesser numbers to Florida (Abraham & Dillon 1986, Newell & Hidu 1986). An introduced population of soft-shell clams occurs in the eastern Pacific Ocean from Alaska to California (Abraham & Dillon 1986, Newell & Hidu 1986). Soft-shell clams also populate the North Sea and European waters, including the Black, Baltic, Wadden, White, and Mediterranean Seas (Strasser & Barber 2008).

Soft-shell clam populations, once abundant in Maryland's Chesapeake Bay, are in dramatic and persistent decline, threatening the health of this estuarine ecosystem. These clams are an important prey item for jellyfish, oyster drills, lobed moon snails, whelk, flatworms, starfish, crabs, certain fish species, birds, and raccoons (Abraham & Dillon 1986). Soft-shell clams contribute to the overall water quality in the Chesapeake Bay by filtering phytoplankton which cleans and clears the water, and allows light to reach grassbeds enabling growth (Abraham & Dillon 1986). The burrowing of *Mya arenaria* permits aeration of benthic substrates, promoting root growth of submerged aquatic vegetation. This vegetation stabilizes substrate, oxygenates estuarine waters and provides protective nursery and spawning habitats for diverse marine species.

In addition to a recreational harvest, the soft-shell clam has also supported an important commercial fishery in the United States since the mid-1800s (Abraham & Dillon 1986). Commercial harvests in the United States peaked in 1969, when over 13 million pounds of clams were landed. Since that time, there has been a steady decline in landings, with just over 3.7 million pounds of clams landed in 2011 (http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html). Data from Maryland Chesapeake Bay bottoms mirror this trend, with more than 8 million pounds of clams landed in 1964 and just over 31,000 pounds in 2010 (http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html).

Potential reasons for the decline include predation, overharvesting, environmental degradation, and disease (Abraham & Dillon 1986). Known diseases, infections and conditions of soft-shell clams include virus, parasites, bacteria, neoplasia, and other pathologies (Sindermann & Rosenfield 1967). To ensure the future of this important species in the coastal ecosystem, there is a need to identify and assess the impacts of disease agents affecting both wild populations and those cultured for restoration efforts.

In 2000, the Maryland Department of Natural Resources initiated an annual disease surveillance program for soft-shell clams within Maryland's Chesapeake Bay. In 2002, massive gill epithelial nuclear hypertrophy was documented in this population of clams (E. Peters, personal communication). The agent causing the lesion and its significance to the clam population was undetermined.

The principle objectives of this research presented were to (1) characterize the responsible agent by light and electron microscopy, and molecular techniques; (2) conduct a retrospective survey of disease conditions and agents in Chesapeake Bay soft-

shell clams; and (3) perform a metagenomic analysis of viral diversity in Chesapeake Bay soft-shell clams.

The first objective was accomplished using histopathology and special staining techniques, lectin binding for glycogen localization, partial virus purification and negative staining, transmission electron microscopy, next-generation sequencing and *in situ* hybridization. The second objective was fulfilled using Ray's fluid thioglycollate medium assay for *Perkinsus* spp. detection, histopathology, and transmission electron microscopy to evaluate the disease status of 630 soft-shell clams collected using a commercial hydraulic escalator dredge from 18 locations within Maryland's Chesapeake Bay. For the third objective, Illumina next-generation sequencing was used to identify the virome present within 3 clams. Phylogenetic analysis of herpesviral terminase sequences allowed for the creation of a DNA oligonucleotide probe to detect the putative viral agent in affected clams via *in situ* hybridization.

Accomplishing these objectives, in general, broadened the current knowledge of diseases that affect this population of *Mya arenaria*, and also provided data on the current and historical prevalence of gill epithelial nuclear hypertrophy (GENH). More specifically, this research led to the creation of a DNA oligonucleotide probe which revealed an intranuclear signal localized to the nuclei of gill epithelial cells of clams with GENH. Once validated, this detection method can be utilized to identify disease-free seed stock for use in aquaculture and repopulation of natural clam beds.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Soft-shell clams, *Mya arenaria*, are an important member of the filter-feeding benthic infauna of the mesohaline portion of Chesapeake Bay. In addition to their ability to filter particulates from the water, they are also an important prey item for many species (Abraham & Dillon 1986). Populations once abundant in Maryland's Chesapeake Bay are in dramatic and persistent decline, which threatens the health of this estuarine ecosystem. Commercial harvests in the United States peaked in 1969, when over 13 million pounds of soft-shell clams were landed. Since then, landings have declined dramatically. Maryland landing data mirror that trend, with more than 8 million pounds harvested in 1964 and just over 30,000 pounds landed in 2010 (http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html). Potential reasons for this population decline include: overharvesting, environmental degradation, predation, lack of genetic diversity, and disease (Abraham & Dillon 1986, Strasser & Barber 2008).

It is important to have an understanding of diseases which may be contributing to the decline of this species. A review of diseases of commercially important bivalve mollusks by Sindermann and Rosenfield (1967) discusses ciliates, trematodes, and papillary neoplasia of *Mya arenaria*. This review expands upon that literature and adds

information on other parasitic infections, viruses, bacteria, neoplastic and hyperplastic conditions, and other pathologic conditions such as hemocytic infiltration, renal concretions and hermaphroditism, with a focus on epizootics that have occurred within the Chesapeake Bay population.

Nomenclature/Taxonomy

Common names of *Mya arenaria* include soft-shell clam, steamer clam, nannynose, sand gaper, and long-necked clam. They are classified in the Kingdom Animalia, Phylum Mollusca, Class Bivalvia, Subclass Heterodonta, Order Myoida, Superfamily Myoidea, Family Myidae, Genus *Mya*, Species a*renaria* (http://www.itis.gov).

Prehistoric and Modern Distribution

Mya arenaria likely originated in the middle Miocene in Japan and reached California by the late Miocene. By the early Pliocene, *M. arenaria* had spread to European and Atlantic waters and had become well established in California (Bernard 1979, Strasser & Barber 2008). Soft-shell clams ranged from Alaska to Oregon by the Pleistocene (Bernard 1979). *Mya arenaria* died out in Pacific and European waters in the early Pleistocene, leaving one surviving population in the Northwest Atlantic (Strasser & Barber 2008). Re-invasion of *M. arenaria* occurred in European waters in the seventeenth century and reintroduction occurred in the Pacific in the late nineteenth century (Strasser & Barber 2008). Today, soft-shell clams occur along the western coast of the Atlantic Ocean from Labrador to South Carolina and in lesser numbers to Florida. An introduced population exists along the coast of the eastern Pacific Ocean from Alaska to California (Abraham & Dillon 1986, Newell & Hidu 1986). Soft-shell clams are also

found in the North Sea and European waters, including the Black, Baltic, Wadden, White, and Mediterranean Seas (Strasser & Barber 2008).

Life History

Soft-shell clams are diecious and nonprotandrous with hermaphrodites seen rarely. Histologically, gonadal sex is indistinguishable in clams <25 mm in shell length, and the sex ratio in clams >25 mm is 1:1 (Brousseau 1978, Abraham & Dillon 1986). In females, body size and oocyte production are positively correlated (Brousseau 1978). In the Chesapeake Bay, spawning occurs in the spring when temperatures rise to 10°C and in the fall when temperatures decrease below 20°C. Within 12 hours of spawning, fertilized eggs develop into planktonic trochophore larvae, which feed on suspended particles. Within 36 hours, trochophore larvae develop into veliger larvae and use their ciliated velum to drift in currents and feed on phytoplankton. The late pedoveliger stage has developed a foot and by 6 weeks uses its byssus to anchor itself to substrate and become a juvenile clam. Juvenile clams temporarily retain their foot and are able to move. Eventually, the byssi are shed, the clams burrow several centimeters into the sediment, and mature to adulthood. Clams may reach 15 cm by 8 years of age, and are known to live up to 28 years (Abraham & Dillon 1986).

Environmental Requirements

Mya arenaria are widely euryhaline and eurythermal, but low salinity together with high temperatures can result in mass mortalities. Adult soft-shell clams bury up to 30 cm in the sediment in stiff sand and muds that will not collapse against their shell valves when closed. *Mya arenaria* are not affected by fluctuations in oxygen and can

survive long periods of anaerobiosis. Decreases in pH, resulting from long periods of shell closure, do not adversely affect digestion and absorption (Abraham & Dillon 1986).

Ecological Role

Soft-shell clams are an important prey item for jellyfish, oyster drills, lobed moon snails, whelk, flatworms, starfish, crabs, certain fish species, birds and raccoons (Abraham & Dillon 1986). They contribute to the overall water quality in the Chesapeake Bay by filtering phytoplankton, which cleans and clears the water, and allows light to reach grass beds, enabling growth (Abraham & Dillon 1986). Clam burrows also permit aeration of benthic substrates, promoting root growth of submerged aquatic vegetation. This vegetation stabilizes substrate, oxygenates estuarine waters and provides protective nursery and spawning habitats for diverse marine species.

Immune System

Bivalves do not have an adaptive immune system based on the clonal expansion of lymphocytes, and therefore rely entirely on the cellular and humoral components of innate immunity for defense against pathogens. The primary cellular response against pathogens is provided by hemocytes, which are capable of both encapsulation and phagocytosis, with destruction of pathogens within phagosomes. Following phagocytosis, destruction of pathogens occurs via phagosomal-lysosomal fusion, with subsequent release of lysosomal enzymes, reactive oxygen species, nitric oxide, and antimicrobial factors. Antibacterial effectors, opsonins, nonspecific hydrolysis, and toxic oxygen intermediates are also present in bivalve hemolymph and help to coordinate the humoral immune response (Song et al. 2010).

Diseases, infections, and other pathologic conditions

Known diseases, infections or pathologic conditions of soft-shell clams include parasites, viruses, bacteria, neoplasia and hyperplasia, and other pathologies.

Parasites

Parasites in soft-shell clams include protozoa, helminths and crustaceans.

Protozoa. Disease in invertebrates is most commonly caused by protozoa (Sparks 1985). The protozoan, *Perkinsus marinus* (formerly *Dermocystidium marinum* and *Labyrinthomixa marina*) was first identified in 1950 as the cause of large scale mortalities in *Crassostrea virginica* in the Gulf of Mexico (Mackin et al. 1950). *Perkinsus spp.* affect oysters, abalones, clams, scallops, cockles, and mussels from five continents (Villalba et al. 2004). McLaughlin et al. (2000) described a new species of *Perkinsus, Perkinsus chesapeaki,* from soft-shell clams from Maryland's Chesapeake Bay. Both *P. marinus* and *P. chesapeaki* are endemic parasites in multiple bivalve mollusks in the Chesapeake Bay, including the soft-shell clam (Andrews 1954, Reece et al. 2008).

Perkinsus spp. have traditionally been classified as Apicomplexan parasites, but new evidence has resulted in reclassification (Smolowitz 2013). Siddall et al. (1997) found that *Perkinsus* species lack a conoid, a feature present in Apicomplexa, and molecular evidence supported a phylogenetic affinity with the Dinoflagellida. However, some authors describe a conoid. Ultrastructural studies of *P. chesapeaki* showed that zoospores have two flagella, a basolateral nucleus, large anterior vacuole, and rudimentary apical complex composed of an open-sided conoid, rhoptries, micronemes and subpellicular microtubules (McLaughlin et al. 2000). The alveolates, a major

superphylum of protists, consist of the subgroups: ciliates, dinoflagellates and apicomplexans, with the latter two known as Myzozoa. *Perkinsus* spp. do not fit neatly into any of the three categories, having retained characteristics of Myzozoa and Alveolata as a whole (Hoppenrath & Leander 2009). Molecular evidence now shows that *P. marinus* belongs to the phylum Perkinsozoa that branched from the phylum Dinoflagellata shortly after dinoflagellates branched from the Apicomplexa (Smolowitz 2013).

Perkinsids have a free living motile stage (zoospore) and a non-motile vegetative stage (trophozoite) (Joseph et al. 2010, Mangot et al. 2011). Infection with *Perkinsus marinus* occurs when the zoospore is ingested by the host and phagocytosed by hemocytes, where it develops to a mature trophozoite. In the hemocyte, it divides by karyokinesis and cytokinesis to form a rosette, and develops into mature sporangia containing 8 to 32 trophozoites. Lysis of the sporangia and hemocyte releases trophozoites that infect additional naïve hemocytes (Joseph et al. 2010, Smolowitz 2013).

Gross signs of *Perkinsus marinus* infection are non-specific and include watery, thinned, soft tissues (Smolowitz 2013). Organisms can be found in all tissues (Mackin et al. 1950), and in heavily infected clams, milky white cysts or nodules form on the gills, foot, gut, digestive gland, kidney, gonad, and mantle that can interfere with respiration and reproduction (http://www.pac.dfo-mpo.gc.ca/science/species-especes/shellfish-coquillages/diseases-maladies/pages/perkincc-eng.htm).

Histopathology of *Perkinsus* spp. infected *Mya arenaria* shows 3.8 +/- 1.4 µm in diameter unicellular, circular, trophozoites with a large intracytoplasmic vacuole either free or within granulocytic hemocytes, and 17.8 +/-7.9 µm cysts composed of clusters of

trophozoites (McLaughlin & Faisal 1998). The host response to *Perkinsus* spp. involves hemocytosis, phagocytosis by hemocytes, and encapsulation (Sindermann 1990). Advanced infections can result in fusion of gill lamellae and loss of underlying tissues (McLaughlin & Faisal 1998).

Diagnostic methods for *Perkinsus* spp. include histopathology, Ray's fluid thioglycollate assay, and molecular diagnostic assays. Histopathology is not used commonly to detect *Perkinsus*, because in early infections small trophozoites are easily overlooked (Smolowitz 2013). Furthermore, false negatives are common, compared to gill or palp thioglycollate assays, in lightly infected clams (McLaughlin & Faisal 1999).

The Ray's fluid thioglycollate assay is the standard diagnostic method for *Perkinsus* spp. (Villaba et al. 2004, Carnegie 2009). Ray (1952) developed a technique in which mollusk tissues, typically heart, rectum, gill, and mantle, were incubated for a week or longer from 18°C to 25°C in antibiotic treated fluid thioglycollate medium. This allowed for the trophozoites to enlarge and develop into a new stage, the hypnospore, which could be detected after staining with Lugol's iodine. Ray (1963) modified the technique to include the addition of nystatin. In a study of *Mya arenaria*, McLaughlin and Faisal (1999) found gill and palps a more sensitive indicator than rectal tissue or hemolymph for the detection of light *Perkinsus* spp. infections and recommended the use of both gill and rectal tissue for routine diagnosis of *Perkinsus* spp. by thioglycollate assays.

Mackin (unpublished in Ray 1954) expanded the original procedure developing a numerical system to estimate *Perkinsus* infection intensity. Numerical values of one-half, one, two, three, four, and five, were assigned to very light, light, light to moderate,

moderate, moderate to heavy, and heavy infections, respectively. Choi et al. (1989) proposed a quantitative method for counting *P. marinus* hypnospores in order to determine the energetic cost of parasitism in oysters.

However, the thioglycollate assay is neither sensitive nor specific, as it does not examine all tissues from the mollusk, and stains all *Perkinsus* spp. and some *Perkinsus*-like species (Villaba et al. 2004, Smolowitz 2013). Similarly, histopathology cannot distinguish between species based on their morphology. Both polyclonal and monoclonal antibodies have been developed to detect various stages of *Perkinsus* spp., but they have been shown to cross react with some dinoflagellate species (Villaba et al. 2004, Carnegie 2009). Polymerase chain reaction (PCR) offers a rapid diagnostic method (Villaba et al. 2004) and both *Perkinsus* genus and *Perkinsus* species-specific assays have been developed (Carnegie 2009). Definitive diagnosis requires PCR and comparison of the internal transcribed spacers (ITS) region nucleotide sequences to those of reference sequences in the GenBank database (Carnegie 2009).

The prevalence and intensity of *Perkinsus marinus* infections increase with increasing temperature and salinity. In Chesapeake Bay oysters, *P. marinus* infections are most prevalent and intense when temperatures rise above 20°C and re-activate overwintering infections (Villaba et al. 2004). New infections peak in the summer with mortality concentrated in late August and September, then decline in winter. Historically, areas with salinities between 12 and 15 ppt were considered to be *P. marinus* free, but when salinities rose in drought years, *P. marinus* established itself and is now known to survive in salinities less than 5 ppt for up to three months (Villaba et al. 2004).

Surveys of Chesapeake Bay *Mya arenaria* indicate significant variability in the prevalence of *Perkinsus* spp. infections. Otto (1972) reported a single case of *Perkinsus* (formerly *Labyrinthomixa marina*) from the Potomac River in 1971. Soft-shell clams collected from 20 sites from 1965 to 1989, and examined by light microscopy, showed only rare occurrences. By 1991 and 1992, prevalences ranged from 3 to 53%, decreased to 3 to 17% in 1993 and zero in 1994. Prevalences peaked in the fall and declined in January through May, with early infections concentrated in the gills (McLaughlin et al. 1995). M. *arenaria* collected from four sites in the Chester River had a prevalence of 12%, as detected by histopathology, with parasites found predominately in gill tissue (McLaughlin & Faisal 1998). In a 2000 survey, eight populations were infected at prevalences ranging from 30 to 100% (Dungan et al. 2002). In 2008, Reece et al. reported prevalences ranging from 0 to 100% from seven populations.

Perkinsus spp. infections can cause death and mass mortalities in bivalve populations. Sublethal effects include reduction of condition, reduced host growth and decreased fecundity (Villaba et al. 2004). Disease caused by *P. marinus*, but not *P. chesapeaki*, is reportable to the Office International des Epizooties (Carnegie 2009).

Ciliates are another common group of protozoan parasites found in invertebrates, with effects ranging from benign to lethal (Sparks 1985). The genus *Ancistrocoma* was created in 1926 to include the ciliates *A. pelseneeri*, found on the palps and gills of *Macoma balthica*, and *A. pholadis*, found in *Barnea candida* (formerly *Pholas candida*) (Chatton & Lwoff 1926). In 1936, Kofoid and Bush described the ciliate *A. myae* (formerly *Parachaenia myae*), found in the pericardial cavity and excurrent siphon of *Mya arenaria* collected from San Francisco and Tomales Bay, California, and suggested

it was specific to *M. arenaria* because it was not found within other lamellibranchs in the same locations. However, in 1946, Kozloff declared the ciliate identical to *A. pelseneeri*, and stated that it is not specific to *M. arenaria*.

Ancistrocoma pelseneeri has been reported from soft-shell clams in Maryland's Chesapeake Bay (Otto 1972, Otto et al. 1977). *A. pelseneeri* ingests host cells and some pathological changes may be associated with the attachment site to the host, but these changes have not been widely studied (Cheng 1967).

Various bivalve mollusk hosts, including soft-shell clams, are infected by ciliates in the order *Rhynchodida* and genus *Sphenophrya*. Sphenophrya-like ciliates attach to the gills and palps of the host with no host response and cause little pathology other than at the attachment site of the suctorial tubule (http://www.pac.dfo-mpo.gc.ca/science/species-especes/shellfish-coquillages/diseases-maladies/pages/spccc-eng.htm). *Sphenophrya*-like ciliates have been reported from soft-shell clams in Maryland's Chesapeake Bay (Otto 1972, Otto et al. 1977).

In 1938, *Trichodina cardii* (formerly *Cyclochaeta cardii*), the first trichodinid to be reported from a marine lamellibranch, was found in the pallial cavity of *Cerastoderma edule* (formerly *Cardium edule*) in the Bay of Biscay. The second reported trichodinid was *T. myicola*, described in *Mya arenaria* from Sagadahoc Bay, Maine and Plum Island Sound, Massachusetts. The parasite is found in the oral region, especially on the outer face of the palps, and occasionally on the visceral body wall and internal face of the pallial muscles (Uzmann & Stickney 1954). Prevalence in endemic areas ranged from 0 to 62%, with the highest prevalence observed in late spring. *Trichodina myicola* is often found with the thigmotrich *Ancistrocoma myae*. There is little evidence that trichodinids

are significant parasites of invertebrates, and it is thought that most are commensals that simply benefit from the food gathering activities of the host (Uzmann & Stickney 1954).

Helminths. Known helminth parasites of *Mya arenaria* include trematodes, and nematodes. Stunkard (1938) demonstrated experimentally that cercariae of *Himasthla quissetensis* encyst within the mantle, gills and foot of soft-shell clams. Metacercariae of *H. quissetensis* were found predominantly in the palps and gills of 43% to 100% of *M. arenaria* collected in 1950 from Merrimack Bay, Plum Island Sound, and Annisquam River, Massachusetts, and in 90% of clams from St. Andrews, Canada (Uzmann 1951). *Himasthla leptosoma*, *H. compacta* and *H. littorinae* have also been described from *M. arenaria* (Cheng 1967).

A number of unidentified and incompletely characterized trematode stages have been noted in *Mya arenaria* including sporocysts, metacercariae and cercariae. Trematode sporocysts and 0.138 mm x 0.082 mm cercariae were reported in the venter of soft-shell clams from Gaspé Bay Canada in 1912; metacercariae measuring 0.078 mm x 0.056 mm were present on the mantle surface (Stafford 1912). Unidentified cercariae, referred to as *Cercaria myae*, were found in 0.33% of soft-shell clams from Massachusetts and were produced in sporocyts within the interfollicular spaces of the gonad, interlobular lymph spaces of the digestive gland and hemocoel. Sporocysts measured 0.21 mm x 0.60 mm and cercaria 0.12 mm x 0.25 mm (Uzmann 1952). Metacercariae, referred to as *Metacercaria I*, were found in 25% of soft-shell clams from Maine and Massachusetts (Cheng 1967).

Though not described in *Mya arenaria*, in various bivalve species trematodes are known to cause abnormal behavior, severe tissue damage, mortality, castration,

hyperplasia and metaplasia, calcium carbonate deposits, shell deformities, and pearls (http://www.pac.dfo-mpo.gc.ca/science/species-especes/shellfish-coquillages/diseases-maladies/pages/tremetcc-eng.htm).

Reports of nematode infections in *Mya arenaria* are few. The nematode *Malacobdella grossa* has been reported from the mantle cavities of clams from the Atlantic coast of North America and Europe (Cheng 1967).

The principal molluskan response to helminth parasites is encapsulation (Sindermann 1990). Nacrezation may also result if helminthes occur in the mantle (Sindermann 1990).

Crustaceans. Crustacean parasites are not significant pathogens of marine mollusks (Sindermann 1990). Several copepod species have been described in *Mya arenaria* with no reported pathology. Wright (1885) described the first copepod parasite in mollusks, *Myicola metisiensis*, based on specimens collected from within gill tubes of *Mya arenaria* from the Gulf of St. Lawrence, Québec. The copepod was later reported in the mantle cavity of *M. arenaria* from Massachusetts (Wilson 1932). In 1947, Medcof found *M. metisiensis* in soft-shell clams from Minister's Island, New Brunswick. Copepodids of *M. metisiensis* were present in the mantle cavity and gills of *M. arenaria* collected from New Brunswick and Québec from 1983 to 1985 (Humes 1986). Copepods resembling juvenile stages of *Leptinogaster major* (formerly *Myocheres major* and *Lichomolgus major*) were found commonly in the mantle cavities of *M. arenaria* collected in Rhode Island (Williams 1907, Cheng 1967). *Herrmannella rostrata* was first reported in the branchial chamber of *M. arenaria* from the Netherlands by Stock (1993).

Viruses

The study of molluskan virology is relatively new. Farley et al. 1972 were the first to report a virus in a bivalve mollusk when they described a herpes-type virus in *Crassostrea virginica* from Maine. Since that time, several viruses and virus-like particles have been reported in *Mya arenaria*, but few have been definitively identified due to a lack of continuous cell lines for in vitro virus propagation (Elston 1997) and a deficiency of molecular diagnostic assays. Most reports offer a presumptive diagnosis based on light and electron microscopic findings. Members of the virus families reported in soft-shell clams include *Papovaviridae*, *Papillomaviridae*, *Polyomaviridae*, *Paramyxoviridae*, and *Retroviridae*. In addition, a number of unknown virus-like particles have been observed.

Papovaviridae. In 1977, virus-like intranuclear inclusions were reported in atypical amoebocytes and germinoma cells in clams from an oil spill site in Maine. Particles in the atypical amoebocytes were 55 nm in diameter, nonenveloped and resembled papovavirus (Harshbarger et al. 1977a). However, the family *Papovaviridae* is no longer used and has been split into the families *Papillomaviridae* and *Polyomaviridae*. Due to 55 nm size, this reported papovavirus may represent a virus in the family *Papillomaviridae*.

Papillomaviridae. Farley (1978) described Feulgen positive, intranuclear inclusions, causing massive cellular hypertrophy, in gametogenic epithelia of *Crassostrea virginica*. Ultrastructurally, the icosahedral, 53 nm, nonenveloped particles were arranged in paracrystalline arrays. Similar inclusions have been observed in gill epithelia of *Mya arenaria*. In a population of clams from Upper New York Bay, New Jersey,

collected in 1980 from a site subject to sewage discharge and oil spills, 41% of the clams had virus particles in ciliated epithelial cells associated with the food groove and a presumptive identification of papillomavirus was made (Koepp 1984).

Polyomaviridae. Feulgen positive, intranuclear inclusions, causing some cell hypertrophy, were observed in gill epithelial cells, connective tissues, and hemocytes of soft-shell clams (Farley 1978). The icosahedral (6- and 5-sided), 40 to 45-nm, nonenveloped virions replicated and assembled in the nucleus and most closely resembled a polyomavirus.

Paramyxoviridae. Farley (1978) examined teratomatous glandular tissue from *Mya arenaria* and described Feulgen negative, intranuclear and intracytoplasmic inclusions. Transmission electron microscopy was not performed, but a presumptive diagnosis of paramyxovirus was made based on the histological findings.

Retroviridae. Retroviruses have been reported in association with disseminated neoplasia in soft-shell clams, and are discussed with neoplasia in this review (Oprandy et al. 1981, Oprandy & Chang 1983, House et al. 1998, Sunila 1994, AboElkhair et al. 2009 a,b, AboElkhair et al. 2012).

Virus-like particles. In a sample of 50 soft-shell clams collected from Jones Creek, Massachusetts in 1972, 20% had Feulgen positive, finely granular, intranuclear inclusions in gill epithelial cells (Farley 1976). In 2000, the Maryland Department of Natural Resources initiated an annual disease surveillance program for soft-shell clams within Maryland's Chesapeake Bay. One outcome of this program was the documentation of massive gill epithelial nuclear hypertrophy (GENH) in 2002 (E. Peters, personal communication). Knowles et al. (2010) described Feulgen-positive, finely

granular, amphophilic, intranuclear inclusions also within hypertrophied gill epithelia of soft shell-shell clams collected from the Chesapeake Bay. Ultrastructurally, the intranuclear particles were nonenveloped, moderately electron dense, icosahedral, 75–82 nm, and occasionally formed paracrystalline arrays. Inflammatory changes were not observed in gill tissues of adult soft-shell clams with viral nuclear inclusions. The intranuclear location, size, and morphology of the particles suggest a DNA virus belonging to the families *Adenoviridae*, *Herpesviridae*, or possibly an unidentified DNA virus family. The abundance of infected cells in some clams and their abnormal nuclear characteristics, suggest that normal feeding and respiratory functions may be compromised.

Bacteria

Vibriosis causes bacillary necrosis in larval and juvenile bivalve mollusks and is the most important disease of cultured bivalve larvae. Its effects and those of other bacteria on wild bivalve populations are less well understood (Sindermann 1990). In 1970 and 1971, large scale mortalities occurred in *Mya arenaria* within the Chesapeake Bay from Eastern Bay to the Wicomico River, in an area subject to sewage discharge. Members of the Enterobacteriaceae and a *Vibrio* sp. were identified as potential pathogenic agents after mortalities resulted from the feeding and injection of cultured bacteria (Kaneko et al. 1975). *Vibrio parahaemolyticus*, a cause of gastroenteritis in humans, was isolated from soft-shell clams collected in 1972 from Cape Cod, Massachusetts as part of a study to assess the human health risks associated with the consumption of undercooked shellfish (Earle & Crisley 1975). Specific effects on clams have not been reported.

Rickettsial organisms were first reported in soft-shell clams from the Chesapeake Bay by Harshbarger et al. (1977b) who described amorphous, finely granular, basophilic, intracytoplasmic inclusions within digestive tubule epithelial cells containing 300 nm x 2500 nm, ribosome-rich, undulating rods. The same year, Otto et al. (1977) reported a 20.2% prevalence of similar Feulgen positive, 100 µm, round rickettsial inclusions in epithelial cell cytoplasm and the lumina of digestive diverticula of 2,401 *Mya arenaria* sampled over a seven-year period from Maryland's Chesapeake Bay. The presence of inclusions did not appear to be related to salinity, water temperature, industrial or domestic sewage, or proximity to population centers (Otto et al. 1977). Harshbarger and Hoover (unpublished) found inclusions in digestive diverticula of soft-shell clams from Searsport, Maine (Otto et al. 1977). Rickettsia have also been reported in the cytoplasm of ciliated gill epithelial cells of *M. arenaria* collected from drainage ditches south of Rehobeth Beach, Delaware. Ultrastructural examination showed 1.09 +/- 0.10 µm x 3.56 +/- 0.44 µm organisms with a rippled cell wall surrounded by a halo. The halo was not investigated chemically and its significance to virulence is undetermined. The author did not describe pathology caused by this organism (Fries et al. 1991).

Neoplasia

Neoplastic and hyperplastic conditions reported in *Mya arenaria* include disseminated neoplasia, germinoma, papillary tumors, mesothelioma, teratoma, siphon tumors, and atypical hyperplasia.

Disseminated neoplasia. Disseminated neoplasia, also known as hematopoietic neoplasia, hemocytic neoplasia, hemic neoplasia, sarcomatoid proliferative disease, disseminated sarcoma, and leukemia, is a progressive and often fatal condition of

circulating neoplastic cells (Elston & Moore 1992, Barber 2004). It was first described by Jones and Sparks (1969), who reported unusual cells the size of large hemocytes, in the Leydig organ of *Ostrea conchaphila* (formerly *Ostrea lurida*) from Yaquina Bay, Oregon. Farley (1969) reported a proliferative disease of likely hematopoietic system origin in *Crassostrea virginica* from Maryland and New York and *Crassostrea gigas* from Japan. Disseminated neoplasia was first reported in *Mya arenaria* in 1976 from clams collected at the Brunswick or Harpswell oil spill site in Freeport, Maine, a site contaminated by oil and jet fuel (Yevich & Barszcz 1976).

On histopathology, soft-shell clams with disseminated neoplasia have large anaplastic cells with a high nuclear to cytoplasmic ratio within connective tissue, blood vessels, and sinuses of the visceral mass, muscle and mantle tissue. Individual cells have hyperchromatic, lobed, or pleomorphic nuclei with one or more prominent nucleoli and occasional fine cytoplasmic granules. Numerous and bizarre mitotic figures are often present (Yevich & Barszcz 1976, Barber 2004). As neoplastic cells spread throughout the clam, fibrosis, displacement, compression, degeneration, and necrosis of normal gill, gonad and connective tissues can occur along with arrested gametogenesis and gonadal atrophy (Barber 2004).

Brown et al. (1977) examined the ultrastructure of the neoplastic cells and described anaplastic cells with large oval nuclei that were often lobed or binucleate, a single prominent nucleolus, clumped chromatin, and scant cytoplasm. When placed on a glass slide, normal hemocytes of *Mya arenaria* flatten, form pseudopods and move in an ameboid fashion. In contrast, neoplastic cells retain their spherical shape, have anaplastic characteristics, and are nonmotile (Brown et al. 1977). Moore et al. (1992) demonstrated

that diseased hemocytes have almost no phagocytic capabilities, fail to adhere to glass, and lose their ability to clump, likely due to disrupted cytoskeletal structure.

Although widely speculated to be of hemocytic origin, the progenitor cell for this neoplasm has not been definitively determined (Barber 2004). Several studies have examined their antigenic and chromosomal properties and contrasted them with those of normal hemocytes. Reinisch et al. (1983) were the first to generate monoclonal antibodies to marine invertebrate neoplastic cells. Of the created antibodies, 3H5c, 5A9, 5A6, 5A4, 5A5, ID7A, and IE7, all reacted with neoplastic cells, while only 3H5c reacted with neoplastic cells and normal hemocytes, suggesting separate ontogenic pathways of cell differentiation (Reinisch et al. 1983).

In 1994, Reno et al. investigated the DNA content and genomic characteristics of neoplastic cells in disseminated neoplasia and confirmed quantitative differences in normal versus neoplastic cells. Normal clam cells had chromosome numbers ranging from 26 to 39, while the chromosome number of the neoplastic cell population ranged from 44 to 80 and had 1.25 to 2.05 times more DNA than normal clam cells.

Disseminated neoplasia can be diagnosed by histopathology, histocytology, indirect peroxidase staining, and flow cytometry. Early cases of disseminated neoplasia were first detected by histopathology (Jones & Sparks 1969, Farley 1969). While this technique allowed for the evaluation of disease severity and dissemination to various tissues, it was costly, time consuming, required special equipment and was lethal to the bivalve (Howard & Smith 2004, Barber 2004).

Farley et al. 1986 described a non-lethal approach to diagnosing disseminated neoplasia, termed “histocytology,” which involved collecting hemolymph from the

posterior adductor muscle, diluting it with seawater, placing it on a poly-L-lysine coated glass slide, fixing it in an aldehyde fixative, and staining with Feulgen picromethyl blue. This approach was faster, allowed for a large number of animals to be examined, and was used to track the severity and progression of the disease in an animal over time (Barber 2004). Smolowitz and Reinisch (1986) improved the sensitivity and accuracy of this method by developing an indirect peroxidase staining method for hemocytes treated with the monoclonal antibody IE7, which is specific for neoplastic cells. Moore et al. (1991) used flow cytometry to rapidly detect and quantify DAPI-stained neoplastic cells collected from hemolymph of bay mussels, *Mytilus* sp.

Disseminated neoplasia was not documented in *Mya arenaria* from the Chesapeake Bay until 1980, when a single case was reported in a clam used as a control in a research study (Brown 1980). One case was seen in Chesapeake Bay *M.* arenaria in 1979, two cases in 1981, and one case in January 1983. By the winter of 1983 epizootic levels were observed and prevalence reached as high as 65% in some areas by 1985 (Farley et al. 1986). Farley et al. (1991) reported three major epizootics of disseminated neoplasia in Maryland soft-shell clams from 1984 to 1988 with prevalence reaching 90% in some areas. Seven of eight populations of *M. arenaria* sampled from Chesapeake Bay in 2000 had prevalences ranging from 3% to 37% (Dungan et al. 2002).

Prevalence and infection intensities of disseminated neoplasia have been examined in a number of studies outside of the Chesapeake Bay, involving various locations, ages and sizes of clams. In a population of soft-shell clams from New Jersey, prevalence and intensity were inversely related, with peak prevalence occurring in December and May, and peak intensity in October and April (Barber 1990). In soft-shell

clams from Long Island Sound, prevalence peaked in the late fall, females were less affected, and there was a prevalence pattern related to age (Brousseau 1987, Brousseau & Baglivo 1994). In *Mya arenaria* from Massachusetts, Leavitt et al. (1990) found the highest prevalence in the fall and in 3 to 4 year old clams. In a field experiment in Massachusetts, control soft-shell clams had the highest prevalence of disseminated neoplasia in the summer in larger size classes (Weinberg et al. 1997).

While, the etiology of disseminated neoplasia remains undetermined, an infectious agent is suspected (Barber 2004). The neoplasm can be transmitted between soft-shell clams by the injection of hemolymph (Weinberg et al. 1997, McLaughlin 1994) and ova from diseased clams (Sunila 1994). An investigation by Kent et al. (1991) showed that disseminated neoplasia could not be transferred from *Mytilus trossulus* to *Mya arenaria*, suggesting that the proposed infectious agent is species specific.

More specifically, several studies provide evidence for a retroviral etiology. Brown (1980) found that only clams with disseminated neoplasia had indications of reverse transcriptase activity, an enzyme necessary for retroviral replication, and visualized virus-like particles in negatively stained preparations by electron microscopy. A virus similar to a B-type retrovirus was purified from diseased soft-shell clams, that when inoculated into the pericardial area was able to induce neoplasia in nonneoplastic clams using a cell-free filtrate (Oprandy et al. 1981). Oprandy and Chang (1983) induced neoplasia in *M. arenaria* by exposing them to 5-bromodeoxyuridine, a synthetic nucleoside used to induce neoplasia and viral replication. Retrovirus-like particles were isolated from these clams and later shown to induce neoplasia in healthy specimens (Oprandy & Chang 1983). An icosahedral, 100 nm virus was isolated from ova of soft-

shell clams with disseminated neoplasia. Injection of suspected virus-infected ova, without neoplastic cells, induced disease in healthy clams, suggesting possible viral spread during normal spawning. Ultracentrifugation and fractionation of homogenized samples showed a visible band with peak ultraviolet absorbance at 260 nm which mirrored results of Oprandy et al. 1981, suggesting the presence of a retrovirus (Sunila 1994).

Other investigators have found no evidence to support a retroviral etiology. Studies by Oprandy et al. 1981 and Oprandy and Chang (1983) have been challenged due to a lack of repeatability (Elston et al. 1992). Furthermore, a study by House et al. (1998) showed that only whole neoplastic cells, and not cell free filtrates, caused neoplasia when injected into healthy soft-shell clams. This rules out the likelihood of a retroviral etiology unless the virus is associated with cell membranes and was removed during ultracentrifugation (House et al. 1998).

More recently, AboElkhair et al. (2009a) found a positive correlation between the amount of reverse transcriptase activity and the percentage of circulating tetraploid cells in clam hemolymph, which supported the fact that transformed cells express high levels of non-telomeric reverse transcriptase. However, reverse transcriptase activity is not unique to retroviruses. It is also found in hepadnaviruses, some bacteria, and in rapidly dividing cells (AboElkhair et al. 2009a). An additional investigation using electron microscopy, RNA analysis, protein analysis, and PCR targeting of the retroviral *pol* gene failed to detect a putative retrovirus in soft-shell clams with disseminated neoplasia (AboElkhair et al. 2012).

Taraska & Böttger (2013) induced disseminated neoplasia in soft-shell clams using 5-bromodeoxyuridine and found that animals from areas with the highest natural prevalence of the disease developed neoplasia more rapidly than clams from areas with lower prevalences leading them to conclude that a dormant infectious agent could be involved. When injected with filtered neoplastic hemolymph, only *Mya arenaria* between 40 and 80 mm developed neoplasia, while clams smaller than 20 mm or larger than 80 mm did not, suggesting that only certain size classes are susceptible.

Other potential causes of disseminated neoplasia include environmental pollutants and naturally occurring toxins. While several studies suggest a link between pollution and the development of disseminated neoplasia in *Mya arenaria* (Yevich & Barszcz 1976, Yevich & Barszcz 1977, Brown 1980, Farley et al. 1991), this is disputed by others (Brown et al. 1977, Smolowitz & Leavitt 1996). Appledoorn et al. (1984) suggested that while hydrocarbon pollution may be related to the frequency of neoplasms in soft-shell clams, it is not the only causative factor. Lansberg (1996) noted a similar spatial and temporal distribution of northeast populations of soft-shell clams and blooms of toxic dinoflagellates, suggesting that sublethal toxin effects could increase susceptibility to disseminated neoplasia.

Germinomas. Germinomas, also known as gonadal neoplasia, were first reported in three *Mercenaria* spp. from Rhode Island, where germinal epithelium had proliferated and extended into the lumina of ovarian follicles and invaded the kidney (Yevich & Berry 1969). The first reported cases of germinoma in *Mya arenaria* occurred in both males and females collected from an oil-contaminated site in Maine (Barry & Yevich 1975).

Reports of germinomas in soft-shell clams have remained restricted to Maine (Barber et al. 2002), with none reported from the Chesapeake Bay.

The etiology of gonadal neoplasia is undetermined. While Harshbarger et al. (1977a) found intranuclear inclusion bodies in neoplastic cells and 55 nm in diameter, nonenveloped virus-like particles using transmission electron microscopy, a viral etiology was not confirmed. Later transmission studies by Barber et al. (2002) suggest that gonadal neoplasia does not have an infectious etiology.

While early cases of germinomas cannot be grossly detected in *Mya arenaria*, more advanced cases can be roughly predicted when the visceral mass is shrunken, darkened, or has an uneven appearance (Barber 2004). Histologically, gonadal follicles are filled with monomorphic, basophilic, undifferentiated germ cells with eccentric nuclei, indistinct nucleoli, clumped chromatin, and frequent mitoses (Brown et al. 1977, Gardner et al. 1991). Neoplasms are malignant and can disseminate to other organs (Barber 2004).

In a survey of *Mya arenaria* from Long Cove, Searsport, Maine, from 1971 to 1974 Barry and Yevich (1975) examined over 2000 specimens and found 1 to 26.6% had gonadal neoplasia. They noted that the site with the highest tumor prevalence correlated to the most impacted site of a 1971 oil spill. Yevich and Barszcz (1977) examined *M. arenaria* collected in 1974 and 1975, also from Long Cove, in an area contaminated with no. 2 fuel oil and JP-5, a jet fuel, and found that 1 to 13% of the clams had gonadal neoplasms. Contrary to this, Gardner et al. (1991) concluded that petroleum products did not cause germinomas in soft-shell clams. Instead, they suggested that germinomas from Searsport, as well as germinomas found at 3% prevalence in Roque Bluffs, Maine and at

35% prevalence in Dennysville, Maine were associated with application of the herbicide, Tordon 101.

In a 1994 study of soft-shell clams from Whiting Bay, Maine, the average prevalence of gonadal neoplasia was 19.4%, with females more affected than males. There was no correlation between size, when clams measured between 45.7 mm and 60.7 mm mean shell length, and the presence of neoplasia (Barber 1996). Barber et al. (2002) examined *M. arenaria* from Atlantic Canada and the entire Maine coast and found prevalences of gonadal neoplasia ranging from 3.3 to 50%. Neoplasia occurred more commonly in females, and there was no correlation between neoplasia and mean clam size.

Barber et al. (2002) has suggested that gonadal neoplasia progresses slowly and causes little mortality. The most significant sublethal effect is reduced fecundity due to replacement of normal gametes with neoplastic cells, which has serious implications for *Mya arenaria* populations (Barber 2004).

Other neoplasms. Papillary tumors around the rectum were found in 2% of *Mya arenaria* collected from the Chesapeake Bay (Hueper 1963). However, evaluation of histological sections collected by Hueper (1963) and accessioned by the Registry of Tumors in Lower Animals, in conjunction with Hueper's published gross photographs, revealed papillary epithelial proliferation at the pedal orifice of the mantle and not the rectum (Pauley 1969). Mesotheliomas with both solid and glandular patterns were described from heart auricular and ventricular epicardium in two soft-shell clams from Maine. The neoplasms were encapsulated and pedunculated and projected into the pericardial cavity (Gardner et al. 1991). Harshbarger et al. (1977a) reported a teratoid

anomaly in a soft-shell clam from the Maryland portion of the Chesapeake Bay composed of muscle, nerve and glandular epithelium in which nuclear and cytoplasmic inclusions were present. Farley (1978) described Feulgen negative intranuclear and intracytoplasmic inclusions from teratomatous glandular tissue in a soft-shell clam with a presumptive diagnosis of paramyxovirus.

Benign masses, unidentified masses and hyperplastic lesions have also been described from *Mya arenaria*. A fungiform, wrinkled swelling was found on the basal portion of the siphon of a soft-shell clam collected from the Chesapeake Bay, Tred Avon River in 1965 (Pauley & Cheng 1968). Histologically, the benign mass was covered by highly convoluted columnar epithelium with deep crypts. The stoma was composed of smooth muscle, which blended with the normal siphonal smooth muscle. A large mass was found extending from the mantle of a soft-shell clam, but could not be identified microscopically (Pauley 1969). Atypical hyperplasia was observed in the gills and kidneys of 38% of 940 *M. arenaria* collected from Maine, Rhode Island, Maryland, and California. Histologically, hyperplastic cells were basophilic with large vesicular nuclei, prominent nucleoli, and clumped chromatin. Mitotic figures were abundant. Renal epithelial hyperplasia was extensive and made normal renal function unlikely (Barry et al. 1971).

Other pathologies

Other pathologic conditions in soft-shell clams include hemocytic infiltration, renal concretions and hermaphroditism.

Hemocytic infiltration in bivalves is thought to indicate stress, unrecognized injury or agents not visible by light microscopy. It has been reported in association with

starvation, spawning stress, shell damage, and exposure to hydrocarbons and metals in various bivalve species (Garmendia et al. 2011).

Solid renal concretions composed of purines, melanin and calcium phosphate are prevalent in the kidney cells and urine (Potts 1967), and concretion formation may represent a normal process in mollusks under reproductive, environmental, or pollution-induced stress (Doyle et al. 1978). Their significance is unknown. Rounded and concentrically layered, predominantly brown to black, calcium phosphorite concretions, as large as 250 µm and 30 µm, respectively, have been observed within renal epithelial cells of *Mercenaria mercenaria* and *Argopecten irradians*, as well as lesser numbers of off-white to gray, yellow, orange, beige and ochre concretions (Doyle et al. 1978). Amorphous calcium phosphate concretions have also been found found in *A. irradians*, *A. gibbus*, *Macrocallista nimbosa*, *Pecten maximus*, and *M. mercenaria* (Carmichael et al. 1979, Tiffany 1979, George et al. 1980, Gold et al. 1982). Tiffany (1979) reported <0.1 mm to 2.5 mm calculi with a layered structure within renal tubule lumina of *M. nimbosa*.

In an unpublished 5-year health survey of soft-shell clams in Maryland's Chesapeake Bay, variably-sized, granular, brown to gray to black concretions up to 387.5 µm in diameter were present within the lumina of kidney tubules. The granular material was occasionally surrounded by hemocytes. Concretions were observed in 33.62% of 577 clams. The composition of concretions was not analyzed and their significance to the host is unknown.

Soft-shell clams are diecious and nonprotandrous with a 1:1 sex ratio, although hermaphrodites occur rarely (Abraham & Dillon 1986). Hermaphrodites can be of either

the mixed type, in which each alveolus contains both male and female components, or the bilateral type in which male and female components occur in separate alveoli (Shaw 1970). Coe and Turner (1938) reported three hermaphrodites in a sample of 1,000 soft-shell clams collected near New Haven, Connecticut. One clam exhibited bilateral hermaphroditism and two showed mixed hermaphroditism. No hermaphrodites were observed in over 800 soft-shell clams collected from the Tred Avon River, Chesapeake Bay, Maryland from 1961 to 1963 (Shaw 1965). Hermaphroditism in *Mya arenaria* from the Chesapeake Bay was first reported in 1972, when the condition was observed in five clams from the Chester, Potomac, and Corsica Rivers. Four of the clams were bilateral hermaphrodites and one was a mixed hermaphrodite (Otto 1972). Cross et al. (2012) found no hermaphrodites in 432 soft-shell clams examined from Bannow Bay, Ireland. While the cause and significance of hermaphroditism is not known, the association of pollution and endocrine disrupting compounds with this condition has been suggested in other bivalve species (Villalobos et. al 2010). Future studies are needed to explore this link.

Conclusions

The decline in landings of *Mya arenaria* is certainly multi-factorial with overharvesting, environmental degradation, predation, lack of genetic diversity and disease all contributing. The development of molecular diagnostic techniques has expanded the knowledge base of agents that infect bivalves and will lead to the discovery of novel agents in the future. This information can be used in management plans to mitigate the spread of infectious diseases and to aid in the recovery of this periled species.

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CHAPTER 3

VIRUS-LIKE PARTICLES IN THE GILLS OF SOFT-SHELL CLAMS, *MYA ARENARIA*, FROM MARYLAND'S CHESAPEAKE BAY

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Abstract

Soft-shell clams, *Mya arenaria*, historically supported an important commercial fishery in the United States, but since the 1960s, landings have been in persistent and dramatic decline. In 2000, the Maryland Department of Natural Resources began an annual disease surveillance program for soft-shell clams to increase the understanding of the impact of diseases and infections on the Chesapeake Bay population. Adult *M. arenaria* were sampled from multiple sites within the Maryland portion of the Chesapeake Bay for histopathology and transmission electron microscopy (TEM). Feulgen-positive, finely granular, amphophilic, intranuclear inclusions that marginated chromatin were identified within hypertrophied gill epithelia of many clams. Transmission electron microscopy of gill tissue revealed nonenveloped, moderately electron dense, icosahedral, 75–82 nm virus-like particles that occasionally formed paracrystalline arrays. Following partial purification by sucrose gradient ultracentrifugation, negatively stained, moderately electron dense, 78 nm icosahedral virus-like particles were observed. The intranuclear location, size, and morphology of the particles suggest a DNA virus belonging to the families *Adenoviridae, Herpesviridae*, or possibly an unidentified DNA virus family. Inflammatory changes were not observed in tissues of adult soft-shell clams with viral nuclear inclusions. Abundant infected cells in some clams, and their abnormal nuclear characteristics by TEM, suggest that normal feeding and respiratory functions may be compromised. The potential impact of this agent on juvenile clams of this species or on other bivalve species within the Chesapeake Bay remains undetermined, but worthy of further investigation.

Introduction

Soft-shell clams, *Mya arenaria,* are benthic, saltwater bivalves found in oceans and estuaries in the Atlantic Ocean from the Subarctic to South Carolina and in the Pacific Ocean from Alaska to San Francisco (Abraham & Dillon 1986). In addition to their vital role in the ecosystem as filterers and prey for many species, the soft-shell clam fishery is the third most important commercial clam fishery in the United States (Abraham & Dillon 1986). The commercial harvest in the United States peaked in 1969 and since then has declined dramatically. Maryland landings data mirror that trend with more than 8 million pounds harvested in 1964 and just over 30,000 pounds landed in 2010 (http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html). Potential reasons for this decline include overharvesting, environmental degradation, predation, diseases and infections (Abraham & Dillon 1986).

Although the study of viruses infecting bivalve mollusks is relatively new, viruses within nine families have been reported. Since the first reports of viruses in the families *Herpesviridae* (Farley et al. 1972), P*apovaviridae* (Farley 1976a,b), *Iridoviridae* (Comps et al. 1976), *Reoviridae* (Hill 1976a), *Retroviridae* (Farley 1978), *Paramyxoviridae* (Farley 1978), *Togaviridae* (Farley 1978), *Birnaviridae* (Hill 1976b) and *Picornaviridae* (Rasmussen 1986), and an unidentified akoya-virus (Miyazaki et al. 1999), the literature has greatly expanded. While many reports are limited to histological descriptions and presumptive identification of the virus based on ultrastructural characteristics, molecular techniques have allowed for further virus characterization. There are multiple excellent reviews of viruses in bivalve mollusks by Farley (1978), Johnson (1984), Sparks (1985), Sindermann (1990), Elston (1997), McGladdery (1999), and Renault and Novoa (2004).

Members of the virus families *Papovaviridae*, *Papillomaviridae*, *Polyomaviridae*, *Paramyxoviridae,* and *Retroviridae* have been described from soft-shell clams. In addition, virus-like particles have been reported.

Papovaviridae. The family Papovaviridae is no longer used and has been split into the families *Papillomaviridae* and *Polyomaviridae*. In 1977, 55 nm, intranuclear, nonenveloped, virus-like particles resembling papovavirus were reported in atypical amoebocytes in soft-shell clams from Searsport Bay, Maine at the site of a prior oil spill. Intranuclear inclusions, which filled less than 50% of the nuclear space, were also present in germinoma cells (Harshbarger et al. 1977).

Papillomaviridae. Feulgen positive intranuclear inclusions were identified in massively hypertrophied gametogenic epithelium of *Crassostrea virginica* (Farley 1978). The icosahedral, 53 nm, nonenveloped particles arranged in paracrystalline arrays. Similar inclusions were observed in gill epithelium of *Mya arenaria*. Forty-one percent of soft-shell clams collected in 1980 from Upper New York Bay, New Jersey, in an area subjected to sewage discharge and frequent oil spills, had Feulgen-positive nuclei within gill epithelial cells accompanied by pronounced inflammation and necrosis (Koepp 1984). Virus particles were limited to ciliated gill epithelial cells associated with the food groove. In a reference sample of *M. arenaria* from Chesapeake Bay, Maryland, 2% of clams showed Feulgen-positive nuclei. Ultrastructurally, virus-like particles were arranged in paracrystalline arrays and a presumptive diagnosis of papillomavirus was made (Koepp 1984).

Polyomaviridae. Farley (1978) reported Feulgen positive, intranuclear inclusions, which caused some hypertrophy of the cell, within connective tissues, hemocytes, and

gill epithelium of *Mya arenaria*. Ultrastructurally, viral particles were icosahedral (5- and 6-sided), 40–45 nm, nonenveloped, and most closely resembled polyomavirus (Farley 1978).

Paramyxoviridae. Farley (1978) described a paramyxovirus from teratomatous glandular tissue of *M. arenaria* in which Feulgen-negative, intranuclear and intracytoplasmic inclusions were observed.

Retroviridae. Enveloped, 120 nm viral particles with eccentric or central nucleoids that resembled B-type retrovirus were first observed in *Mya arenaria* with disseminated neoplasia from Allen Harbor, Rhode Island (Oprandy et al. 1981). Since that time, there have been additional reports of retroviruses in association with disseminated neoplasia (Oprandy & Chang 1983, House et al. 1998, Sunila 1994, AboElkhair et al. 2009, AboElkhair et al. 2012).

Virus-like particles. In 1972, in a sample of *M. arenaria* from Jones Creek, Massachusetts, 10% of clams had gill hyperplasia and 20% had intranuclear, finely granular, Feulgen-positive inclusions within gill epithelial cells (Farley 1976b).

In 2002 (E. Peters, personal communication), soft-shell clams from Maryland's Chesapeake Bay were found with massive nuclear hypertrophy of gill epithelial cells associated with intranuclear inclusion bodies. Prevalence of inclusions was documented at 70.21% in 2005, and by 2009, 93.26%, with prevalence as high as 100% in some collection areas. The current study investigates the cause of this lesion, and presents histologic findings and an ultrastructural description of virus-like particles observed within affected gill tissues.

Materials and Methods

Clam specimens

Adult *Mya arenaria* were collected by hydraulic escalator dredge from multiple locations within Maryland's Chesapeake Bay as part of an annual disease surveillance program by the Maryland Department of Natural Resources that began in 2000.

Histology

Cross sections of clams that included gill tissue were fixed in Davidson's solution, processed routinely, embedded in paraffin, sectioned at approximately 5 µm, and stained with Mayer's hematoxylin and eosin (H&E). Selected sections were stained by the Feulgen reaction to detect deoxyribonucleic acid, the periodic acid–Schiff reaction (PAS) to identify mucopolysaccharides, especially glycogen, Giemsa and Gimenez to visualize bacteria, and Ziehl-Neelsen acid-fast methods to identify lead inclusions, lipofuschin and ceroid pigments, or acid-fast parasites (http://www.dako.com/us/index/knowledgecenter/kc_publications/kc_publications_edu/special_stains.htm).

Transmission electron microscopy

Gill samples were fixed in a primary fixative containing 2.5% (v/v) glutaraldehyde in 0.2M phosphate-buffered, 430 mOsm kg^{-1} artificial seawater, and post-fixed in 0.2M phosphate-buffered 1% (w/v) osmium tetroxide. Tissue samples were dehydrated with ethanol prior to infiltration and embedding with Spurr's epoxy resin. Thin sections (60 nm) were placed on 200-mesh copper grids, and stained with 5% methanolic uranyl acetate and Reynold's lead citrate for examination with a JEOL JEM-1210 transmission electron microscope.

Partial virus purification and negative staining

Frozen gill tissues were selected for virus purification and negative staining from clams that showed abundant intranuclear inclusions by histology and intranuclear virus-like particles by transmission electron microscopy (TEM). Filtered sea water at 24 ppt (Instant Ocean) was autoclaved and used as a buffer. For purification, 0.8 g of fresh frozen *Mya arenaria* gill tissue was added to 5 ml of sea water and disrupted in a tissue grinder. The resulting suspension was diluted with an additional 5 ml of sea water, ground again, then clarified at 250 x g, 1000 x g and 4000 x g at 4°C for 30 min each, saving the supernatant after each centrifugation step. The supernatant was centrifuged for 90 min at 139,445 x g at 5°C in a Beckman Type 35 rotor, and the pellet resuspended in 20 ml of seawater using a magnetic stirrer at 4°C. A discontinuous sucrose gradient of five fractions, 60% (3 ml), 50% (2 ml), 40% (3 ml), 30% (3 ml) and 10% (2 ml) sucrose (w/w) was prepared, and 5 ml of the suspension was layered on top of the gradient. The gradient was centrifuged at 80,000 x g at 5°C for 30 min in a Beckman Type 28 rotor. Fractions were collected with an electronic pipette. A formvar-carbon coated 400-mesh copper grid was floated on 40 µl of each sample. After 30 min, the grids were removed and blotted, and were then floated on a drop of 3% aqueous phosphotungstic acid pH 7.0 for 30 sec. Once blotted and dried, grids were viewed with a JEOL JEM-1210 transmission electron microscope.

Lectin binding for glycogen localization

Because intranuclear localization of glycogen particles can mimic viral inclusions, lectin binding was performed to rule out the presence of intranuclear glycogen. Paraffin-embedded clam tissues were sectioned at 5 µm, deparaffinized and rehydrated. Slides

were immersed in a 0.01M sodium citrate buffer at pH 6.0, and heated in a steamer for 35 min for heat-induced antigen retrieval. Slides were covered with 200 µl of fluorescein isothiocyanate (FITC)-conjugated concanavalin A (ConA) (20 ug/ml) (Vector Laboratories, Inc.), which has specific affinity for α-D-glucose, α-D-mannose and glycogen; or 200 µl of antibody diluent (DAKO) as a negative control for 60 min at room temperature. A section of canine liver with glycogenosis was used as a positive control. Slides were rinsed with PBS. Slides were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) for nuclear staining and coverslipped with Prolong Gold Antifade Reagent with DAPI (Invitrogen) (Jung et al. 2011). Sections were examined with an Olympus BX41 fluorescence microscope, using ultraviolet and blue excitation filters. FITC-conjugated ConA is excited at 495 nm and emits at 515 nm (green). DAPI bound to DNA is excited at 358 nm and emits at 461 nm (blue).

Results

Histology

Gills of *Mya arenaria* consist of two pairs of demibranchs, each with an inner descending and outer ascending lamella composed of filaments (Fig. 1A). Frontal, latero-frontal, and lateral epithelial cells are ciliated (Fig. 1B).

Inclusions within hypertrophied epithelial cell nuclei were most commonly observed near the attachment of the gill to the suspensory ligament and at gill tips (Fig. 2A). In moderately and heavily infected clams, intranuclear inclusions were found throughout the gills. Finely granular, amphophilic, intranuclear inclusions that marginated nuclear chromatin were observed within hypertrophied ciliated gill epithelial cells (Fig. 2B). Inclusions were Feulgen-positive, varied in size, and were round to oval

to irregular and occasionally only partially filled hypertrophied nuclei (Fig. 2C). In lightly infected clams, inclusions most commonly occurred at the junction of gill and suspensory ligament and within frontal ciliated cells at gill tips (Fig. 2A,D-F). Intranuclear inclusions failed to stain with the periodic acid–Schiff reaction (PAS), Giemsa, Gimenez or Ziehl-Neelsen acid-fast stains.

Ultrastructural Examination

Ultrastructurally, virus-like particles which marginated nuclear chromatin were observed within hypertrophied nuclei of ciliated epithelial cells (Fig. 3A). Occasionally, particles formed paracrystalline arrays (Fig. 3B,C). Nuclear particles were nonenveloped, moderately electron dense, and 75–82 nm in diameter with surface projections (Fig. 3D). The cytoplasm of many gill epithelial cells contained numerous single, irregular, granular, moderately electron dense 25–30 nm particles or particles arranged in 50–100 nm rosettes (Fig. 3E). Fractions from the partial virus purification contained cellular debris and low numbers of virus-like particles of varying sizes. With negative staining, some of the observed virus-like particles were icosahedral and measured approximately 78 nm in diameter, consistent in size with virions observed in tissue (Fig. 3F).

Lectin Binding

Nuclei of gill epithelial cells, as well as nuclear inclusion bodies, were readily differentiated with DAPI localization (Fig. 4A). Lectin ConA was detected within the cytoplasm of gill epithelial cells and not within gill nuclei or nuclear inclusion bodies (Fig. 4B). In the positive control, lectin ConA was detected within the cytoplasm of hepatocytes, which contain glycogen, and none was detected in the negative control.

Discussion

Light and transmission electron microscopy, coupled with the results of lectin binding assays, showed that the observed inclusions within hypertrophied gill nuclei are likely of viral origin. Inclusions stained negative for rickettsia and bacteria with Giemsa and Gimenez stains, and were non-acid fast with the Ziehl-Neelsen stain, ruling out lead inclusions, lipofuscin and ceroid pigments, and acid-fast parasites. Positive Feulgen staining indicated that inclusions contained deoxyribonucleic acid. DNA viruses in the families *Herpesviridae, Adenoviridae*, *Papillomaviridae* and *Polyomaviridae* can produce intranuclear inclusion bodies similar to those observed in the current investigation. The size and morphology of the virus-like particles observed with TEM is consistent with viruses in the families *Herpesviridae* and *Adenoviridae*, or an unknown virus taxon. The intense fluorescence of nuclear inclusions with DAPI indicated the presence of DNA.

Glycogen has been reported to produce intranuclear inclusions; however, in the current study these inclusions stained negative for mucopolysaccharides with PAS, and ConA failed to bind to them, ruling out the presence of intranuclear glycogen (Cheville 2009). Small particles observed within the cytoplasm of epithelial cells are consistent with monoparticular glycogen or β-particles, which are typically 10–30 nm and occur free in the cytoplasm as dense granules. Larger cytoplasmic particles are consistent with glycogen rosettes or α-particles, which are aggregates of monoparticular glycogen and measure 50–200 nm in diameter (Cheville 2009).

Reports of viruses in *Mya arenaria* by Farley (1976b, 1978) and Koepp (1984) show some similarities to findings in this report. Histologic findings in the current case

are similar to those of Farley (1976b) in that Feulgen-positive, intranuclear inclusions were observed within gill epithelial cells. While inclusion bodies were limited to gill epithelial cells in our samples, Farley (1978) also observed intranuclear inclusions in connective tissue and hemocytes. In the current report, virus-like particles were icosahedral and nonenveloped with surface projections, and ranged in size from 75–82 nm. Particles reported by Farley were also icosahedral and nonenveloped, but particle size was considerably smaller at 40–45 nm; particles reportedly resembled polyomavirus (Farley 1978). Farley did not describe surface projections on the viral particles. While Koepp (1984) found Feulgen-positive gill epithelial nuclei, it is unclear from the report if inclusion bodies were observed by light microscopy. Inflammation and necrosis of gill epithelium as reported by Koepp (1984) was not a feature noted in the current study. Koepp (1984) described paracrystalline arrays, and while the report did not give particle size, the presumptive diagnosis of papillomavirus suggests a smaller particle than those observed in the current study.

In conclusion, observed virus-like particles differ from those previously reported in *Mya arenaria*. Based on histological and ultrastructural findings, the virus-like particles described potentially belong to the virus families *Adenoviridae, Herpesviridae* or an unknown virus taxon. The impact of this agent on *M. arenaria* and other bivalve mollusks is undetermined. Although unconfirmed, the hypertrophy of gill epithelial cells, presence of intranuclear inclusions, and abnormal nuclear morphology may be associated with decreased feeding and respiratory functions.

Historically, the study of molluskan viruses has been hindered by the lack of continuous cell lines for in vitro virus replication, but the application of new molecular

techniques offers promise for further characterization of such viruses (Elston 1997). Future work includes determining distribution and prevalence of virus infection in archived samples of *Mya arenaria*, sequencing the agent, and development of molecular tests for detection by polymerase chain reaction and *in situ* hybridization. Future studies should include cohabitation studies of infected and non-infected clams with observations on mortality and disease pathogenesis.

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Fig. 1. *Mya arenaria*. Representative section of a normal gill. (A) Gills of *M. arenaria* consist of 2 pairs of demibranchs one on each side of the visceral mass, each consisting of an inner descending lamella and an outer ascending lamella (double-headed arrows), separated by the interlamellar cavity (also known as water tube) (*) and joined by interlamellar junctions (il). Demibranchs are composed of filaments arranged in folds called plicae, which consist of a principal filament at the base of the fold, a transitional filament and numerous ordinary filaments (arrow). Individual filaments are fused laterally by interfilamentary junctions (if). H&E. Scale bar = 200 µm. (B) Each ordinary filament is composed of simple epithelium with cilia present on the frontal (f), latero-frontal (lf) and lateral (l) epithelial cells. Thin, pavement respiratory epithelium cells (arrowhead) are present in abfrontal areas of the lamellae. Also shown is a hemolymph sinus (h) and hemocyte (arrow). H&E. Scale bar = 50 µm.

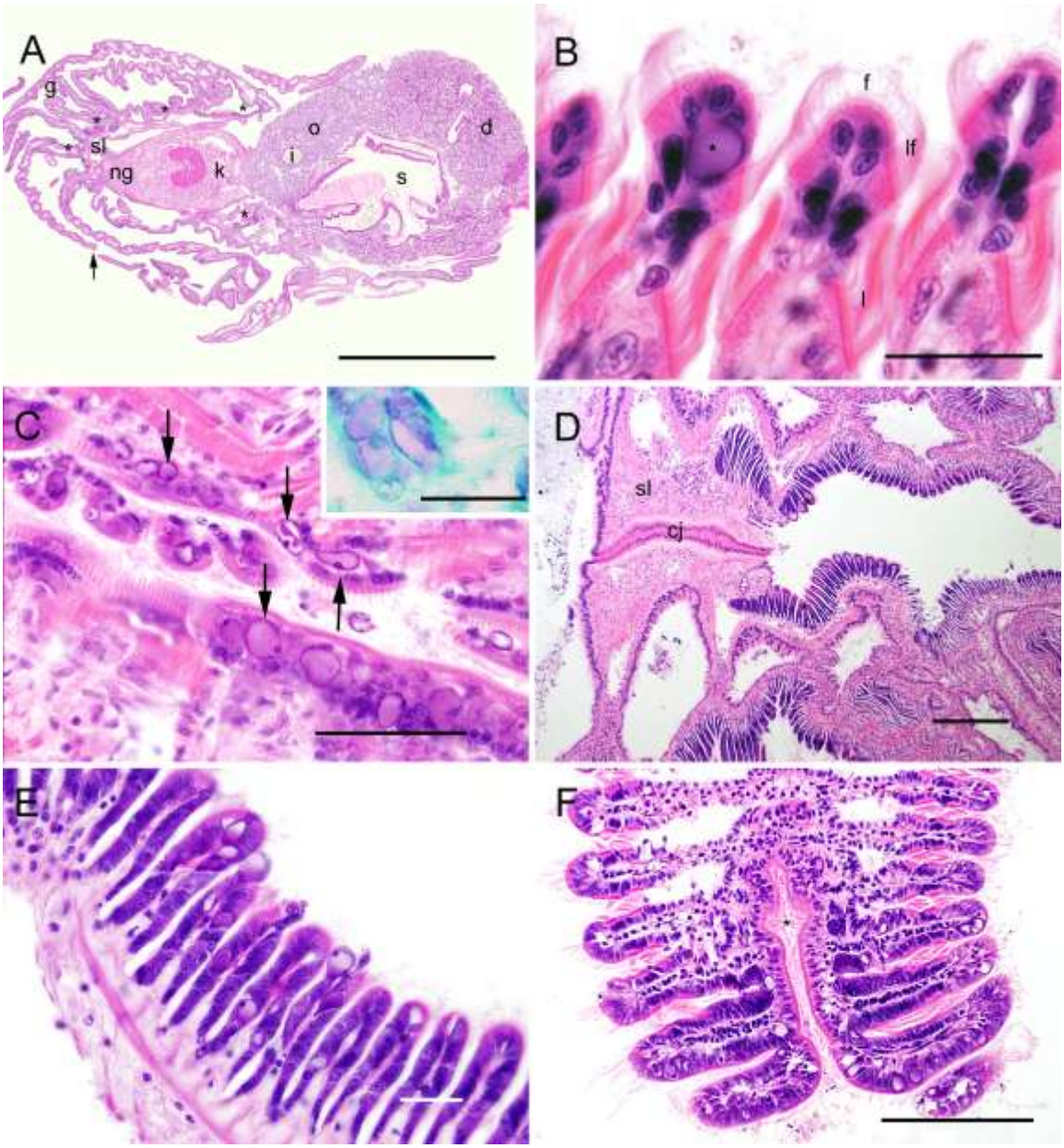

Fig. 2. *Mya arenaria*. (A) Low magnification image of a cross section through a clam, showing gills (g); more deeply basophilic areas (*) are the attachment site of gill to the suspensory ligament (sl) and the arrow shows a gill tip; neural ganglia (ng), kidney (k), intestine (i), ovary (o), stomach (s), and digestive gland (d). H&E. Scale bar = 0.3 cm. (B) Cross section of gill showing granular, amphophilic, intranuclear inclusion body (*) within the hypertrophied nucleus of a ciliated gill epithelial cell; frontal (f), latero-frontal

(lf) and lateral (l) ciliated epithelial cells. H&E. Scale bar = 100 µm. (C) Cross section of gill showing variably sized and shaped inclusion bodies (arrows) within hypertrophied nuclei. H&E. Scale bar = 50 µm; Inset shows Feulgen-positive intranuclear inclusions. Feulgen stain. Scale bar = 200 µm. (D) Cross section of gill through the suspensory ligament (sl) showing a ciliary junction (cj) and the deeply basophilic proximal gills (*). H&E. Scale bar = 200 µm. (E) Higher magnification through deeply basophilic proximal gills with numerous intranuclear inclusion bodies. H&E. Scale bar = 20 µm. (F) Cross section of distal gill tip at level of marginal food groove (*) with numerous intranuclear inclusion bodies. H&E. Scale bar = 100 µm.

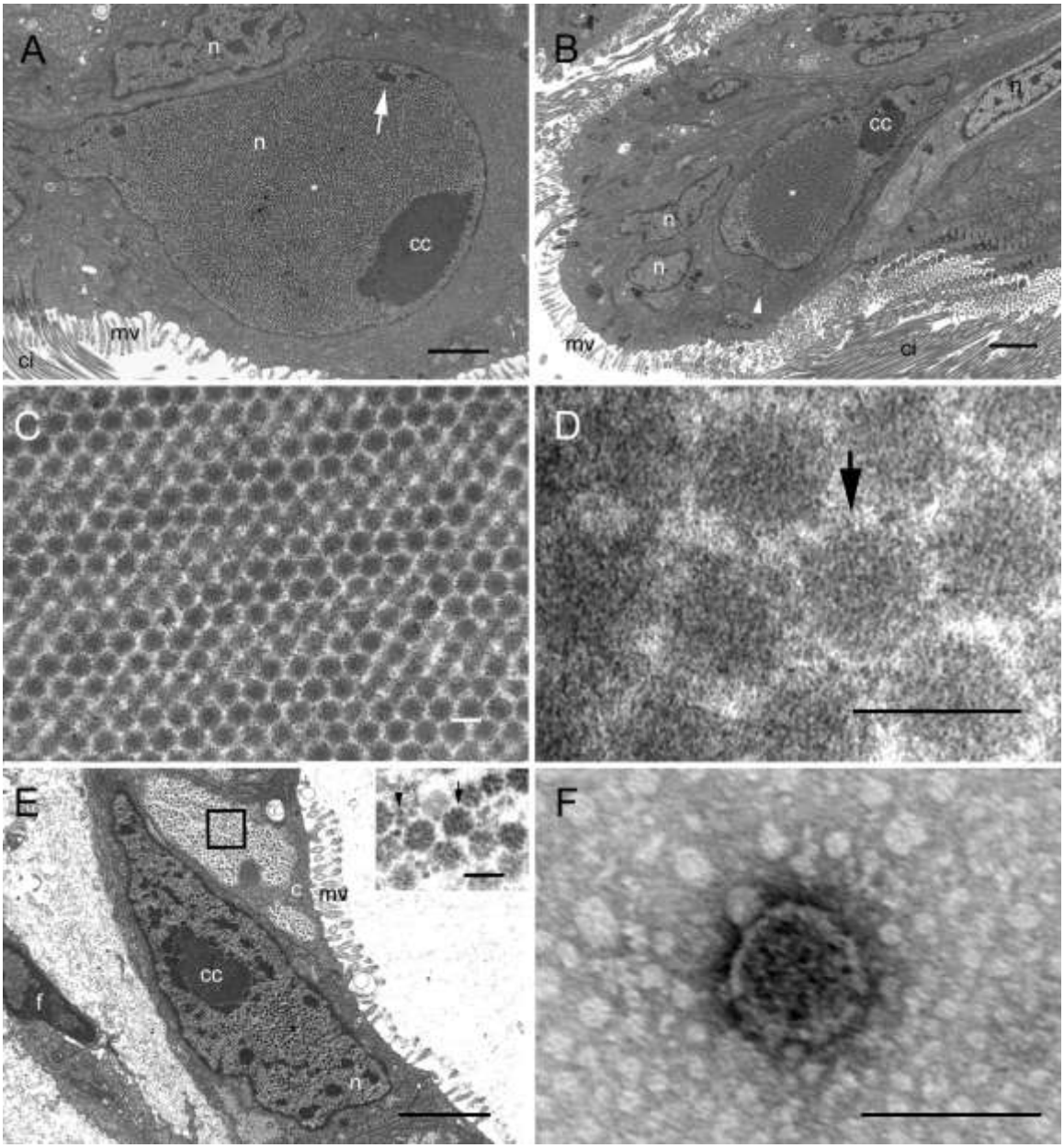

Fig. 3. *Mya arenaria*. (A) Transmission electron microscopy of hypertrophied ciliated gill epithelial cell with condensed chromatin (cc), marginated chromatin (arrow) and numerous intranuclear virus-like particles (*); nucleus (n), cilia (ci), microvilli (mv). Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 2 μm. (B) Transmission electron microscopy of hypertrophied ciliated gill epithelial cell with numerous intranuclear virus-like particles (*) arranged in a paracrystalline array;

mitochondria are present in the cytoplasm (arrowhead); nucleus (n), condensed chromatin (cc), cilia (ci), microvilli (mv). Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 2 µm. (C) Higher magnification of hexagonal and pentagonal virus-like particles arranged in a paracrystalline array. Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 100 nm. (D) High magnification of nonenveloped, moderately electron dense virus-like particle with surface projections (arrow). Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 100 nm. (E) Gill epithelial cell with scattered intranuclear virus-like particles; cytoplasm contains granular particles (box); nucleus (n), condensed chromatin (cc), cytoplasm (c), microvilli (mv), fibroblast (f). Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 2 µm. Inset: higher magnification of cytoplasmic particles which occur singly (arrowhead) or in rosettes (arrow). Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 100 nm. (F) Negative stain transmission electron microscopy of partially purified virus-like particle with icosahedral symmetry. Phosphotungstic acid. Scale bar = 100 nm.

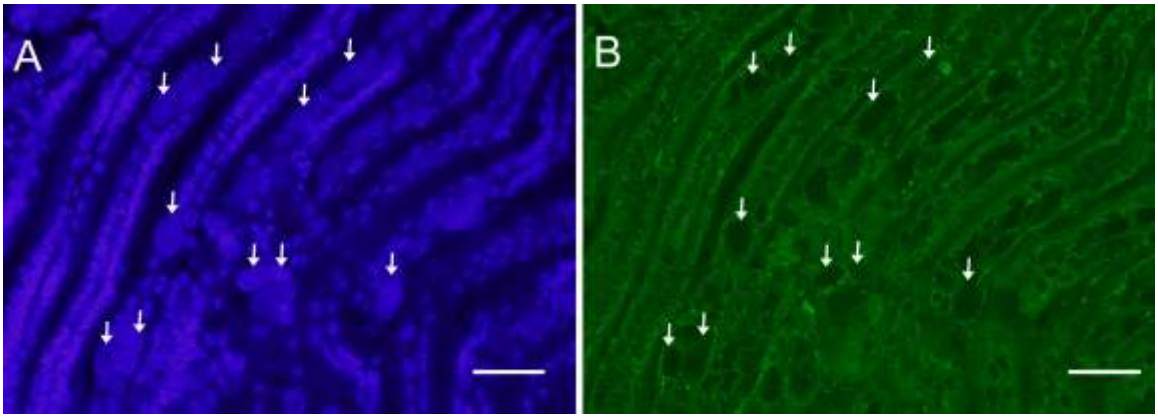

Fig. 4. *Mya arenaria*. Fluorescence micrographs of affected gills. (A) Nuclei of gill epithelial cells and nuclear inclusion bodies (arrows) fluoresce blue with 4, 6-diamidino-2-phenylindole. (B) Nuclear inclusion bodies (arrows) fail to fluoresce with FITC-conjugated concanavalin A. There is positive cytoplasmic staining. Scale bar = 100 nm.

CHAPTER 4

A HISTOLOGICAL HEALTH SURVEY OF WILD SOFT-SHELL CLAMS, *MYA ARENARIA*, FROM MARYLAND'S CHESAPEAKE BAY 2005 TO 2009

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Abstract

Soft-shell clams, *Mya arenaria*, a commercially harvested and once economically important species in Maryland's Chesapeake Bay, have experienced a dramatic and persistent population decline since the peak harvest in the 1960s. To assess the health of this population, 630 wild soft-shell clams from 18 locations within Maryland's Chesapeake Bay were collected from 2005 to 2009 and examined for evidence of viral, parasitic, bacterial, neoplastic, or other pathological conditions by histopathology, special staining techniques, and transmission electron microscopy. Intranuclear virus-like inclusions, present within gill epithelial cells, were observed in 84.53% of examined clams. *Perkinsus* spp. were detected by histopathology in 20.19% of clams and in 54.13% of clams by Ray's thioglycollate test. Unidentified pyriform ciliates and trichodinid ciliates were present in the gills at prevalences of 48.64% and 15.63%, respectively. Cestodes were observed in 1.43% of examined clams, and copepods in 0.16%. Rickettsia-like organisms were commonly observed in the digestive gland, with a prevalence of 75.49%. Bacteria were present in 10.97% of clams and occurred most commonly in the connective tissue around the rectum. Disseminated neoplasia was diagnosed in 2.23% of clams and a single polyp (0.16%) was observed extending from gill epithelium. Renal concretions were present in 33.62% of clams, hemocytic infiltration in 4.94% and pericardial gland concretions in 0.32%. The observed virus-like inclusions were present in a large number of animals during this 5-year period and may be significantly impacting this population. *Perkinsus marinus* is OIE notifiable and can cause high levels of mortality in bivalve hosts.

Introduction

Soft-shell clams, *Mya arenaria*, are eurythermal, euryhaline, benthic bivalves that in North America occur in marine and estuarine waters, both subtidally and intertidally, from Labrador to South Carolina and in lower numbers south to Florida and in the Pacific Ocean from Alaska to California (Abraham & Dillon 1986, Newell & Hidu 1986). In addition to their major role in the ecosystem as filterers and prey for many species, they support the third most important commercial clam fishery in the United States (Abraham & Dillon 1986). The hydraulic dredge escalator, introduced in 1951, allowed for commercial harvesting of soft-shell clams in Maryland's Chesapeake Bay (Ritchie 1976). Commercial harvests in both the United States and Maryland peaked in the 1960s and since then soft-shell clam populations have been in dramatic and persistent decline (http://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html).

Potential reasons for this population decline include predation, overharvesting, environmental degradation, and disease (Abraham & Dillon 1986). When in abundance, predators of soft-shell clams including diving ducks, certain fish species, blue crabs, horseshoe crabs, cownose rays, snails, and starfish can reduce clam populations (Abraham & Dillon 1986, Ritchie 1976). Population decline due to overfishing was evident by the early 1970s in Maryland clam beds (Ritchie 1976). Soft-shell clams are also affected by environmental degradation from pollution and extreme weather events such as tropical storms, hurricanes and blizzards. Industrial, domestic, and agricultural pollutants, large concentrations of waterfowl, oil and chemical spills, and biotoxins are known to reduce clam populations (Ritchie 1976). In 1972, tropical storm Agnes decimated Maryland clam beds with mortality as high as 90% in some upper bay regions

(Ritchie 1976, Abraham & Dillon 1986). Known diseases, infections or conditions of soft-shell clams include viruses, parasites, bacteria, metabolic disease processes, and neoplastic and hyperplastic conditions (Sindermann 1990). Several of these diseases have caused or have the potential to cause large scale mortalities in soft-shell clam populations.

In 1970 and 1971, large scale mortalities of soft-shell clams occurred within the Maryland portion of the Chesapeake Bay from Eastern Bay south to the Wicomico River (Kaneko et al. 1975). While there was no direct evidence of pathogenicity, bacteriological studies isolated members of the Enterobacteriaceae and a *Vibrio* sp. as potential pathogens (Kaneko et al. 1975).

Disseminated neoplasia, first reported by Jones and Sparks (1969) in the Leydig organ of *Ostrea conchaphila*, is a progressive and lethal condition of circulating cells characterized by enlarged nuclei and frequent mitotic figures (Elston & Moore 1992, House 1998). First described from *Mya arenaria* in Maine (Yevich & Barszcz 1976), the condition was not reported in *M. arenaria* from the Chesapeake Bay until 1980 (Brown 1980). Epizootic levels of disseminated neoplasia were documented by the winter of 1983 (Farley et al. 1986), and three major epizootics had occurred by 1988, with prevalence reaching 90% in some areas (Farley et al. 1991). In *M. arenaria* sampled from Chesapeake Bay in 2000, 7 of 8 populations had prevalences of disseminated neoplasia ranging from 3 to 37% (Dungan et al. 2002).

The parasitic dinoflagellate, *Perkinsus marinus* (formerly *Dermocystidium marinum*) was first described in 1950 in association with dead or dying oysters, *Crassostrea virginica*, from Louisiana (Gulf of Mexico, USA) (Mackin et al. 1950).

Perkinsus marinus was found in the Chesapeake Bay in 1950 (Andrews 1996) and has been reported in multiple bivalve species from this region, including the soft-shell clam (Andrews 1954). A single case was reported in *Mya arenaria* from the Potomac River in 1971 (Otto 1972) and a survey of 5,000 clams from 20 sites within the Maryland portion of the Chesapeake Bay showed only rare occurrences between 1965 and 1989 (McLaughlin et al. 1995). Since that time, prevalence has varied markedly, from 0 to 100%, in different areas of the Bay and its tributaries (McLaughlin et al. 1995, McLaughlin & Faisal 1998, Dungan et al. 2002, Reece et al. 2008). In 2000, McLaughlin et al. described a new *Perkinsus sp.*, *Perkinsus chesapeaki*, from soft-shell clams from Maryland's Chesapeake Bay.

This study reports results of a histopathological health survey of wild soft-shell clams collected from 18 locations within Maryland's Chesapeake Bay from 2005 to 2009 as part of a diseases surveillance program conducted by the Maryland Department of Natural Resources.

Materials and Methods

Study site

The Chesapeake Bay lies off of the Atlantic Ocean and is surrounded by Maryland and Virginia. It is the largest estuary in North America, measuring 320 km long by 55 km wide, and has an average depth of approximately 6.5 m (USEPA 2002). Salinity is lowest at the head of the bay, where water is fresh, and highest at its mouth, where it mixes with water from the Atlantic Ocean.

Samples

Six hundred and thirty soft-shell clams were collected from 18 locations within Maryland's Chesapeake Bay from 2005 to 2009 as part of a disease surveillance program administered by the Maryland Department of Natural Resources (Fig. 1). Clams were excavated from the bottom sediment using a commercial hydraulic escalator dredge. Collection date, sample number, sample code, tributary/region site, geographic coordinates, water salinity, and water temperature were recorded for each sampling site (Table 1). Each clam was measured from the most anterior to posterior portion of the shell and then shucked from its shell. Not all data were available for each of the 630 clams, and is reflected in the statistical analysis.

RFTM Perkinsus spp. assay

Clams were held in flow-through tanks for 24 to 72 h before processing to allow for purging of sand and mud. Labial palps were aseptically collected, placed in 3 ml of tubed Ray's fluid thioglycollate medium (RFTM), and incubated at 28°C for 96 h to allow for enlargement of *Perkinsus* spp. hypnospores (Ray 1963, McLaughlin and Faisal 1999). Incubated palps were stained with Lugol's iodine solution and examined microscopically for blue-black hypnospores (Ray 1952). Hypnospore counts were recorded as absent (0), or light (1) to heavy (5) (Ray 1954).

Histopathology

Cross sections of clams were collected as described by Howard and Smith (1983), fixed in Davidson's solution, processed routinely, embedded in paraffin, sectioned at approximately 5 μm, and stained with Mayer's hematoxylin and eosin (H&E). Selected sections were stained by the Feulgen reaction, Giemsa, Lillie-Twort, Gimenez, and Ziehl-

Neelsen acid-fast stains. All intranuclear viral inclusions present in gill epithelial cells in 10 HPF's at 400x were counted. All parasites present within an organ were counted at 200x. For *Perkinsus* spp., clusters and individual trophozoites were counted once. For rickettsial inclusions in the digestive gland, 10 HPF's were counted at 200x. Not all organs were present on every slide and in that event, data for that individual clam for that organ was excluded from the statistical analysis.

Transmission electron microscopy

Gill samples were fixed in a primary fixative containing 2.5% (v/v) glutaraldehyde in 0.2M phosphate-buffered, 430 mOsm kg^{-1} artificial seawater, and post-fixed in 0.2M phosphate-buffered 1% (w/v) osmium tetroxide. Tissue samples were dehydrated with ethanol prior to infiltration and embedding in Spurr's epoxy resin. Thin sections (60 nm) were placed on 200-mesh copper grids, and stained with 5% methanolic uranyl acetate and Reynold's lead citrate for examination with a JEOL JEM-1210 transmission electron microscope. Selected clam tissues, with histologically identified bacteria, were cored from paraffin blocks, deparaffined in xylene for 24 h, rehydrated in 2 changes of 100%, 90%, 70% ethanol, and post-fixed and stained as above.

Statistical analysis

Mean intensities were calculated by summing the numerical counts and dividing them by the observed number of values, and standard errors of the means were reported.

Results

Temperature, Salinity, Shell length

Temperature. The mean monthly temperature at all collection sites was highest in June at 23°C and decreased to 14°C in November (Table 2). The highest recorded

temperature at any collection site was in September 2007 at 25°C and the lowest was 14°C in November of 2006 (Table 2).

Salinity. The mean salinity was highest in the Chester River at 13.28 ppt and the lowest in the Wye River at 6 ppt (Table 3). The highest recorded salinity was in the Choptank River at 16 ppt and the lowest was 6 ppt in the Wye River.

Shell length. Of 629 clams, the average shell length was 51.42 mm +/- 0.51 mm with the smallest measuring 24 mm and the largest 89 mm. The average length of males was 51.38 mm +/- 0.71 mm, females 52.77 mm +/- 0.77 mm, hermaphrodites 34.75 mm +/- 2.78 mm, and clams of undetermined sex 40.42 mm +/- 0.90 mm. The largest males and females were collected from the Choptank River, Benoni Point and averaged 68.07 mm +/- 2.74 and 71.31 mm +/- 2.52, respectively (Fig. 2). The smallest males and females averaged 34.17 mm +/- 1.38 and 37 mm +/- 0.00, respectively, and were collected from the Chester River, Buoy Rock (Fig. 2).

Virus

Intranuclear, Feulgen positive, virus-like inclusions were commonly observed within hypertrophied, ciliated gill epithelial cells. Inclusions were variably sized and shaped, granular, amphophilic and marginated nuclear chromatin (Fig. 3A). Ultrastructurally, virus-like particles filled hypertrophied nuclei, marginated chromatin and occasionally formed paracrystalline arrays (Fig. 3B).

Of 627 clams, intranuclear virus-like inclusions were present in 84.53%. Yearly prevalence of inclusions in all clams varied from a low of 70.21% in 2005 to a high of 93.26% in 2009 (Table 4). At any individual site, the lowest prevalence of 40% was in clams collected in 2007 from the Wye River, Shawn's Wharf. Prevalences of 100% were

documented from the Chester River, Bouy Rock in 2006, Chester River, Old Field in 2006, Choptank River, Benoni Point in 2009, Patuxent River, Cuckold Creek in 2008, and Patuxent River, Helen Creek in 2009. The mean intensity for inclusions in all clams was 55.17 +/-2.71 per 10 400x fields with the lowest count at 0 and the highest at 458. The lowest annual mean intensity was in 2005 at 32.74 +/-4.97 per 10 400x fields and the highest was 69.79 +/-6.36 per 10 400x fields in 2008 (Table 4). The lowest mean intensity for site was 15.73 +/-4.67 per 10 400x fields in clams collected in 2005 from Chester River, Buoy Rock and the highest was 117.13 +/-18.98 per 10 400x fields from Eastern Bay, Parson's Island in 2006 (Fig. 4).

Parasites

Protozoa

*Perkinus RFTM***.** From labial palps incubated in Ray's fluid thioglycollate, hypnospores were observed in 54.13% of the 617 examined clams. Annual prevalence for hypnospores in all clams ranged from 0% in 2009 to 92.55% in 2005 (Table 4). For individual sites, no hypnospores were observed in clams from Choptank River, Benoni Point in 2008 and 2009, Choptank River, Bolingbroke Sands in 2008, 2009, Choptank River, Castle Haven in 2008 and Patuxent River, Helen Creek in 2009. Prevalences of 100% were observed in clams from Chester River, Old Field in 2006, Choptank River, Chlora Point in 2005 and Eastern Bay, Upper Hill in 2006. The mean intensity for *Perkinsus* spp. hypnospores in all clams was 0.88 +/-0.05, with the lowest intensity at 0 and the highest at 5. The lowest annual mean intensity was in 2009 at 0 and the highest was 1.54 +/-0.13 in 2006 (Table 4). The highest mean intensity by site was 2.17 +/-0.25 from Choptank River, Bolingbroke Sands in 2005.

Perkinsus. *Perkinsus* spp. trophozoites were 3.40 to 11.32 µm in diameter and contained large, clear, round, eccentrically located vacuoles which peripheralized nuclei (Fig. 5A). Perkinsus trophozoites occurred singly or in variably sized clusters up to 88.22 µm. Organisms were often rimmed by an eosinophilic, acellular material and were further surrounded by large numbers of hemocytes (Fig. 5A). In heavily affected gills, lamellae were often fused. Eosinophilic laminated structures, occasionally containing highly degraded *Perkinsus* spp. trophozoites rimmed by attenuated hemocytes, were observed in multiple tissues (Fig. 5B).

Of 629 clams, *Perkinsus* spp. were observed by histopathology in 20.19%. Yearly prevalence of *Perkinsus* spp. in all clams varied from a low of 6.67% in 2009 to a yearly high of 41.22% in 2006 (Table 4). At individual sites, the lowest prevalence of 0% was in clams collected from Chester River, Old Field, in 2006, Choptank River, Benoni Point in 2008, Choptank River, Castle Haven in 2008, and the Wye River, Shawn's Wharf in 2007. The highest prevalence at any one site was 62.50% from Chester River, Buoy Rock in 2006. *Perkinsus* spp. were most commonly observed in the gills (19%), digestive gland (4.55%), and gonad (3.18%) and less commonly in the kidney (2.77%), pericardial gland (1.90%), ganglia (0.91%), heart and intestine (0.80%), and stomach (0.64%). The mean *Perkinsus* spp. intensity for all years was highest in gills at 7.79 +/-2.11. The lowest annual mean intensity in gills was in 2009 at 0.17 +/-0.09 and the highest was 11.81 +/-7.17 in 2007 (Table 4). The highest mean intensity was 34.27 +/-33.31 Choptank River, Bolingbroke Sands in 2007 (Fig. 6).

Ciliates. Holociliated, pyriform trophozoites, were observed in gill water tubes and on gill surfaces (Fig. 5C). The largest measured 25.09 µm by 16.46 µm, and

contained a 7.53 µm basophilic macronucleus and micronucleus. Food vacuoles were present in the posterior portion of the ciliate.

Pyriform ciliates were present in 48.64% of 627 clams. Annual prevalence of ciliates for all clams varied from 13.85% in 2006 to 87.64% in 2009 (Table 4). For individual sites, no ciliates were observed in clams collected in 2006 from Chester River, Buoy Rock, in 2006 from Chester River, Old Field, in 2005 from Choptank River, Chlora Point, in 2006 from Eastern Bay, Upper Hill, and in 2007 from Wye River, Shawn's Wharf. Ciliates were present in 100% of clams from Choptank River, Bolingbroke Sands in 2008. The mean intensity for ciliates was 13.29 +/-1.47 with the lowest count at 0 and the highest at 424. The lowest annual mean intensity was in 2007 at 1.36 +/-0.30 and the highest was 30.19 +/-4.75 in 2009 (Table 4). The highest mean intensity was 59.77 +/-19.30 from Choptank River, Benoni Point in 2008 (Fig. 6).

Trichodinid ciliates up to 68.56 µm by 29.81 µm were present on gill surfaces. Trichodinids had ciliary fringes, eosinophilic denticles, and a slender, horse-shoe shaped macronucleus (Fig. 5D).

Of 627 clams, trichodinid ciliates were present in 15.63%. Annual prevalence from all clams varied from 4.26% in 2005 to 37.08% in 2009 (Table 4). For individual sites, no trichodinids were observed in clams collected in 2006 from Chester River, Buoy Rock and Chester River, Old Field, in 2007 from Chester River, Piney Point, in 2008 from Choptank River, Castle Haven, in 2005 from Choptank River, Chlora Point, in 2006 from Eastern Bay, Upper Hill, in 2007 from Upper Bay, Tolley Point and in 2007 from Wye River, Shawn's Wharf. The highest prevalence was in 2009 from clams at Choptank River, Bolingbroke Sands. The mean intensity was 0.89 +/-0.17 with a range

of 0 to 70. The lowest annual mean intensity was 0.85 +/-1.69 in 2005 and the highest 1.68 +/-0.62 in 2009 (Table 4). The highest mean intensity was 3.76 +/-1.81 from Choptank River, Bolingbroke Sands in 2009 (Fig. 6).

Helminths

Cestodes. Multiple cross sections of cestodes up to 422.70 µm by 239.41 µm were observed within intestinal lumina, digestive gland tubules and the stomach. Cestodes had a thin eosinophilic tegument and parenchymous body. Their presence elicited no host reaction (Fig. 5E).

Cestodes were observed in 1.43% of 629 clams at a prevalence of 2.13% in 2005 and 4.70% in 2007. They were not observed in other years (Table 4). The highest prevalence at any individual site was 20% in 2007 from Choptank River, Bolingbroke Sands. Cestodes were most common in the intestine (1.11%), and less common in the digestive gland (0.16%) and stomach (0.16%). The mean intensity for all years was highest in intestines at 0.01 +/-0.01. The highest annual mean intensity in intestines was in 2007 at 0.05 +/-0.02 (Table 4). The highest mean intensity for an individual site was 0.27 +/-0.12 in 2007 from the Choptank River, Bolingbroke Sands (Fig 6).

Crustaceans

Copepods. Several cross sections of copepods up to 259.23 µm by 222.51 µm were present within intestinal lumina. Copepods had a thin eosinophilic cuticle, body cavity, skeletal muscles, and intestines (Fig. 5F).

Copepods were observed in 0.16% of 628 clams and only in 2008 at a prevalence of 0.61% (Table 4). Copepods were observed in 3.85% of clams collected from

Choptank River, Todd's Point in 2008. The mean intensity for 2008 was 0.01 +/-0.01 (Table 4).

Bacteria

Rickettsia. Basophilic, finely granular, 3.36 µm to 32.32 µm colonies of gram-negative, Gimenez-positive rickettsia-like organisms were commonly observed in the cytoplasm of digestive gland epithelial cells or within lumina of digestive diverticula (Fig. 7A,B). Colonies were also present within epithelial cells of the gills, stomach, intestine and pericardial gland. Ultrastructurally, rickettsia appeared as 300 nm by 1100 nm undulating rods (Fig. 7C).

Of 629 soft-shell clams, rickettsia were present in 74.09%. Yearly prevalence for all clams varied from 61.82% in 2008 to a yearly high of 91.49% in 2005 (Table 4). The lowest prevalence for an individual site was 25% in clams from Chester River, Buoy Rock in 2006 and the highest 100% from Choptank River, Bolingbroke Sands in 2007. Rickettsia were most common in the digestive gland (75.49%), and less frequent in the gill (0.80%), stomach (0.32%), intestine (0.32%) and pericardial gland (0.32%). The mean intensity for all years was highest in the digestive gland at 20.03 +/-1.88 per 10 200x fields ranging from 0 to 727. The lowest annual mean intensity in the digestive gland was in 2008 at 4.78 +/-0.59 per 10 200x fields and the highest was 41.55 +/-8.79 per 10 200x fields in 2005 (Table 4). The highest mean intensity was 77.17 +/-25.78 per 10 200x fields from Choptank River, Bolingbroke Sands in 2005.

B*acteria*. Giemsa- and gram-positive, non-acid fast bacterial rods were present in the connective tissue surrounding the rectum and intestine (Fig. 7D). Giemsa-positive and gram-negative bacteria in the gill were found free or within hemocytes or

multinucleated giant cells (Fig. 7E). In some samples, numerous bacteria lined the stomach (Fig. 7F). Bacteria were also occasionally observed within the intestinal lumen.

Bacteria were present in 10.97% of 629 clams. Yearly prevalence varied from 1.11% in 2009 to 26.60% in 2005 (Table 4). The highest prevalence for an individual site was 66.67% in clams from Chester River, Spaniard Point in 2005. Bacteria were most commonly observed in connective tissue surrounding the rectum (5.56%), and less commonly in the stomach (3.83%), gill (3.35%), connective tissue surrounding the intestine (2.71%), and intestinal lumen (0.16%).

Gram-positive, non-acid fast bacterial rods were found in the connective tissue surrounding the rectum and intestine at prevalences of 5.56% and 2.71%, respectively. The highest annual prevalences for bacteria associated with the rectum and intestine were 8% in 2006 and 5.38% in 2005, respectively. The highest prevalences by site for bacteria in the rectum and intestine were 25% in clams from Choptank River, Bolingbroke Sands and 40% from Choptank River, Chlora Point in 2005, respectively. The highest annual mean intensity for bacteria in the rectum and intestine were 0.14 +/-0.10 in 2007 and 0.14 +/0.09 in 2005, respectively.

Gram-negative bacteria in the gill were found only in 2005 and 2007 at prevalences of 21.28% and 0.67%, respectively. Bacteria in the gill were found at only 2 sites, at a prevalence of 66.67% in clams from Chester River, Spaniard Point in 2005, and at a prevalence of 3.33% in clams from Chester River, Piney Point in 2007.

Bacteria were found in the stomach in 3 locations, in clams from the Choptank River, Benoni Point in 2008 at a prevalence of 56.67%, in clams from Upper Bay, Matapeake Hill in 2006 at a prevalence of 17.14%, and in clams from Wye River,

Shawn's Wharf in 2007 at a prevalence of 3.33%. Bacteria were present in the intestinal lumen of 2.33% of clams from Chester River, Buoy Rock in 2008.

Neoplasia

Disseminated neoplasia. Large anaplastic cells up to 23.67 µm in diameter were present within hemolymph channels in the gills or disseminated throughout the visceral mass (Fig. 8A). The neoplastic cells had distinct cell borders and lightly eosinophilic cytoplasm with a high nuclear to cytoplasmic ratio. Nuclei were round to oval, pleomorphic or lobed, with coarsely stippled chromatin and one or more prominent nucleoli. Numerous, often bizarre, mitotic figures were common.

Disseminated neoplasia was observed in 2.23% of 628 clams. It was absent in all clams collected in 2005. The highest prevalence in all clams was 5.37% in 2007 (Table 4). The highest prevalence at any one site was 27.59% in clams collected from Upper Bay, Tolley Point in 2007.

Hyperplasia. A focal polyp was observed extending from gill epithelium. It was composed of hyperplastic epithelial cells up to 2 cell layers thick with deeply basophilic nuclei (Fig. 8B).

A single polyp (0.16%) was observed in the 627 clams examined. It was present on the gill of a clam collected from the Chester River, Buoy Rock in 2007.

Other pathologies

Sex. In addition to clams with distinct male or female gonads, mixed or bilateral hermaphrodites were also observed in which both male and female gonadal tissue was present in the same individual (Fig. 9A). The sex of some could not be determined due to immaturity or post-spawning gonads.

Of the 628 clams examined, 319 (50.80%) were male, 279 (44.43%) were female, 4 (0.64%) were hermaphrodites and for 26 (4.14%) sex could not be determined. The overall female:male sex ratio was 1:1.14. Hermaphrodites were collected from Chester River, Buoy Rock in 2006 and 2008, Chester River, Piney Point in 2007 and Eastern Bay, Bodkin Island in 2006.

Kidney concretions. Variably-sized, granular, brown to gray to black concretions up to 387.5 µm in diameter were present within the lumina of kidney tubules (Fig. 9B). The granular material was occasionally surrounded by hemocytes.

Concretions were observed in 33.62% of 577 kidneys. The annual prevalence of concretions for all clams was lowest in 2009 at 13.48% and highest in 2007 at 48.95% (Table 4). Concretions were not present in any clams from Patuxent River, Cuckold Creek in 2008, Patuxent River, Helen Creek in 2009, and Upper Bay, Sandy Point N. in 2006. The highest prevalence at any one site was 75% in clams from Wye River, Shawn's Wharf in 2007.

Hemocytic infiltration. Large collections of hemocytes, which obscured or replaced normal tissues, were observed within multiple organs or tissues (Fig. 9C).

Hemocytic infiltration was observed in 4.94% of 628 clams and occasionally was present in multiple locations in a single clam. Prevalence for all clams was lowest in 2007 at 0.67% and was highest in 2009 at 12.22% (Table 4). The highest prevalence at any one site was 36.67% from Choptank River, Benoni Point in 2009. Hemocytic infiltration was most commonly observed in the digestive gland (2.23%), connective tissue (1.11%), gill (0.80%), ganglia (0.48%), labial palps (0.32%), mantle (0.32%),

pericardial gland (0.16%), gonad (0.16%), heart (0.16%), intestines (0.16%), and kidney (0.16%).

Pericardial gland concretions. Large, round to oval, deeply basophilic concretions were present within the cytoplasm of pericardial gland epithelial cells (Fig. 9D).

Pericardial gland concretions occurred in one (0.32%) of 316 clams collected from Upper Bay, Sandy Point N. in 2006.

Discussion

In this 5-year health assessment, 630 soft-shell clams from 18 locations in the Chesapeake Bay were examined for evidence of disease. Intranuclear virus-like inclusions were observed in 84.53% of clams. Clams at all 18 collection sites were affected indicating the condition is widespread in the Chesapeake Bay. While the significance of the lesion is undetermined, the hypertrophy of affected gill epithelial cells, presence of intranuclear inclusions, and abnormal nuclear morphology may be associated with decreased feeding and compromised respiratory function.

Using histopathology, *Perkinsus* spp. were observed most commonly in the gill, consistent with earlier findings from the Chester River (McLaughlin & Faisal 1998). *Perkinsus* spp. were observed in 20.19% of clams histologically and in 54.13% of clam palps incubated in Ray's fluid thioglycollate. As reflected in our findings, McLaughlin & Faisal (1999) found that histological examination of lightly infected clams resulted in false negatives compared to gill or palp thioglycollate assays. Trophozoites were often surrounded by hemocytes and high numbers of trophozoites resulted in distortion of gill architecture and occasional lamellar fusion. In addition to sublethal effects, such as

reduced condition, reduced growth and decreased fecundity, *Perkinsus* spp. can result in death and mass mortalities in many bivalve hosts (Villaba et al. 2004). Disease caused by *P. marinus* is reportable to the Office International des Epizooties (Carnegie 2009).

Unidentified pyriform ciliates were present in 48.64% of clams and trichodinid ciliates in 15.63%, with none observed at many collection sites. No host reaction was observed. Ciliates associate with marine bivalves as filter-feeding commensals utilizing food particles collected in ciliary currents, particle feeders on gills or mantle epithelia, and as parasites that consume contents of gill epithelial cells. While most ciliates do not cause disease, they can cause epithelial erosion, facilitating entry by microbial invaders and interfere with respiration. Some *Trichodina* spp. are thought to cause large-scale mortalities in various bivalve species (Lauckner 1983).

Uncommon parasites included cestodes and copepods. Cestodes were observed in less than 2% of all clams. While the specific taxa is undetermined, larval tapeworms of the orders Trypanorhyncha (or Tetrarhynchidea), Lecanicephalidea, Tetraphyllidea, and Diphyllidea are known to utilize bivalves as intermediate hosts (Lauckner 1983). The impact of cestodes on their host is unknown, but they can cause damage by distending digestive gland tubules and competing for nutrients. Heavy infestations cause physiological stress, which may affect growth and reproduction (Lauckner 1983). Copepods were found within the intestine of one clam and do not likely represent a significant parasite in this population of *Mya arenaria*.

First documented in Maryland soft-shell clams, rickettsia-like organisms observed in this study are similar to those reported by Harshbarger et al. (1977). The overall prevalence of 74.09% is significantly higher than the 20.2% reported from 1967 to 1974

by Otto (1977). Otto (1977) found no relation between infection and salinity levels, water temperature, industrial or domestic sewage, or proximity to population centers. While both prevalence and intensity were high in our study, there was no host response to the organism and their significance is unclear. Although rickettsia may not cause mortality, they may reduce metabolic efficiency of the digestive gland and may compete with the host for nutrients (Otto 1977).

Gram-positive bacterial rods in connective tissue of the rectum and intestine were found in highest prevalence in the Choptank River at Bolingbroke Sands and Chlora Point in 2005. Cultures could not be performed, however, histologically the bacteria are non-acid fast, ruling out *Mycobacterium* spp. Hemocytes were not observed responding to the bacteria and their significance is unknown.

The majority of gram-negative bacteria were observed in one Chester River, Spaniard Point sample in September of 2005 where 66.67% of clams were affected. Infections were intense, with large numbers of bacteria occurring free and within hemocytes and multinucleated giant cells infiltrating gill tissue. Large areas of gill tissue were obscured by bacteria, likely compromising respiratory function. While the etiology is undetermined, the Maryland Department of the Environment reported high fecal coliform concentrations in the Chester River in 2005, with the highest loads occurring in September and November (www.mde.state.md.us/assets/document/TMDL_Chester_River_060908_final.pdf). The source was not determined; however, in the Chester River basin, livestock are the predominant source of coliform bacteria followed by pet, wildlife, and human sources (www.mde.state.md.us/assets/document/TMDL_Chester_River_060908_final.pdf).

Disseminated neoplasia reached epizootic levels as high as 90% in some areas of the Chesapeake Bay in the mid-1980s and early 1990s (Farley et al. 1986, 1991). However, Dungan et al. (2002) later reported a maximum prevalence of 37%. While only 2.23% of all clams were affected, prevalence in some areas reached 27.59%.

Atypical gill hyperplasia is considered a pre-neoplastic lesion in *Mya arenaria*. Barry et al. (1971) examined clams from Maine, Rhode Island, Maryland and California and found hyperplastic changes in 22.45% of 940 clams. A single focus was observed in one clam from 100 collected in Maryland. Although the cause is uncertain, Barry et al. (1971) found a higher prevalence in larger clams, and suggested that increased age could play a role due to chronic exposure to sub-lethal levels of environmental stressors. The only affected clam in this study was an adult measuring 67 mm.

The overall female:male sex ratio of 1:1.14 is similar to the 1:1 ratio observed in 25 to 95 mm soft-shell clams from the Annisquam River, Massachusetts (Brousseau 1978). Soft-shell clams are diecious and nonprotandrous with hermaphrodites occurring rarely (Abraham & Dillon 1986). Only 4 hermaphrodites were present in this study, including both mixed type, in which male and female components are present in each alveolus, and bilateral hermaphrodites, in which male and female components occur in separate alveoli (Shaw 1970). Hermaphroditism was first documented in Chesapeake Bay *Mya arenaria* in 1972, and both mixed and bilateral types were reported (Otto 1972). Of the five hermaphrodites reported by Otto (1972) four were collected from the Chester River or its tributary, the Corsica River. Three of the four in this study were also collected from the Chester River.

While the cause of the hermaphroditic condition is undetermined, it could be related to endocrine disruption. The Chester River is on the impaired waters list maintained by the Maryland Department of the Environment and has been designated a priority area for water pollution control by the Maryland Department of Agriculture. For many years, wastewater containing phthalates and other organic chemicals was discharged by a chemical plant in Chestertown, Maryland into unlined ponds that emptied into a tributary flowing into the Chester River (www.oag.state.md.us/reports/2008EnvironmentalAudit.pdf). Phthalates are esters of phthalic acid used to enhance the plasticity of industrial polymers and are a known endocrine disruptor (Mankidy et al. 2013). It is not known if phthalate exposure induced the hermaphroditism observed in this study, but phthalates are known to cause reproductive tract anomalies in humans and wildlife (Mankidy et al. 2013).

Kidney concretions are a common occurrence in bivalve mollusks (Potts 1967) and were observed in 33.62% of clams in this study. The cause is unknown, but their formation may be a normal process in mollusks under reproductive, environmental, or pollution-induced stress (Doyle et al. 1978). Although concretion composition has not been determined in soft-shell clams, calcium phosphate has been identified in other bivalves (Carmichael et al. 1979, Tiffany 1979, George et al. 1980, Gold et al. 1982).

Hemocytic infiltration was observed in multiple organs at a prevalence of less than 5%. While the cause is not known, it is thought to indicate stress, injury or the presence of agents not visible by light microscopy. It has been associated with spawning stress, shell damage, starvation and exposure to hydrocarbons and metals (Garmendia et al. 2011).

The pericardial gland in bivalves is thought to be involved in the ultrafiltration of hemolymph to produce urine (Khan et al. 1988). A study of the cytoplasmic granules of the pericardial gland cells suggested they are composed of ferritin particles and may be involved in iron homeostasis (Khan et al. 1988). The cause of the observed pericardial gland concretions is undetermined.

Conclusions

Viral, parasitic, bacterial, neoplastic, and other pathological conditions were observed in this study of soft-shell clams from Maryland's Chesapeake Bay. While some may represent benign lesions, others have the potential to cause disease and mortality in this host. The observed gill virus-like inclusions were present at epizootic levels and may be significantly impacting this population by interfering with respiratory function and feeding. *Perkinsus marinus* is an OIE notifiable disease known to cause mortality in bivalve hosts. The finding of hypnospores in over 50% of clams examined by the thioglycollate assay demonstrates that this parasite has the potential to cause widespread mortality in the Bay. While only a few hermaphrodites were observed, 4 out of 5 of the cases occurred in the Chester River or its tributary. Further study on the potential effects of endocrine disrupters in this river may be warranted. Because *Mya arenaria* in Maryland's Chesapeake Bay are in a persistent decline, it is important to continue to broaden our understanding of pathologic conditions that may be impacting this population.

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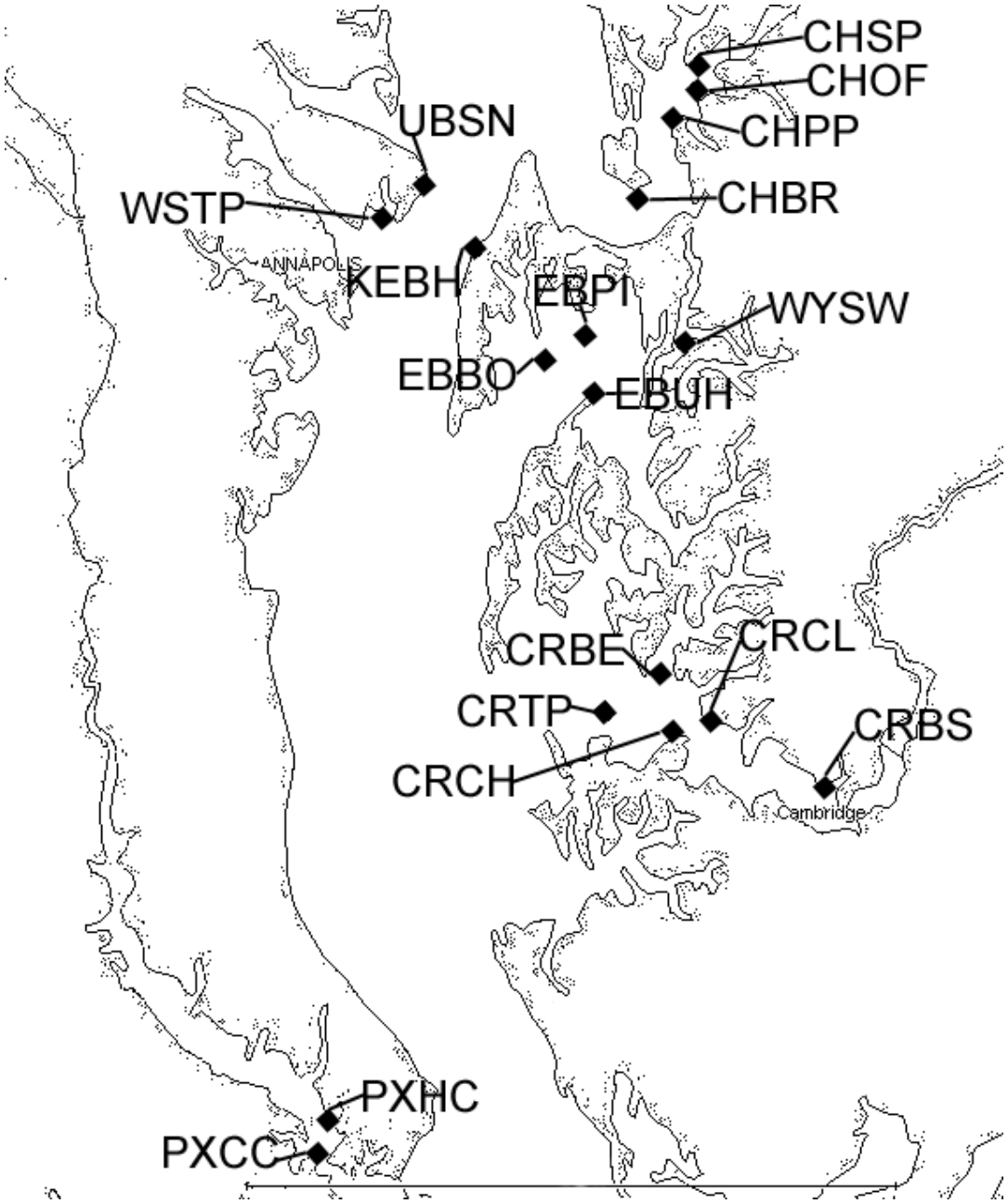

Fig. 1. Collection sites within Maryland's Chesapeake Bay for 2005 to 2009 Maryland Department of Natural Resources clam disease surveillance; Chester River, Spaniard Point (CHSP), Chester River, Old Field (CHOF), Chester River, Piney Point (CHPP), Chester River, Buoy Rock (CHBR), Upper Bay, Sandy Point N. (UBSN), Upper Bay, Tolley Point (WSTP), Upper Bay, Matapeake Hill (KEBH), Eastern Bay, Parsons Island

(EBPI), Eastern Bay, Bodkin Island (EBBO), Eastern Bay, Upper Hill (EBUH), Wye River, Shawns Wharf (WYSW), Choptank River, Benoni Point (CRBE), Choptank River, Todds Point (CRTP), Choptank River, Castle Haven (CRCH), Choptank River, Chlora Point (CRCL), Choptank River, Bolingbroke Sands (CRBS), Patuxent River, Helen Creek (PXHC), Patuxent River, Cuckold Creek (PXCC). Scale bar = 50 km.

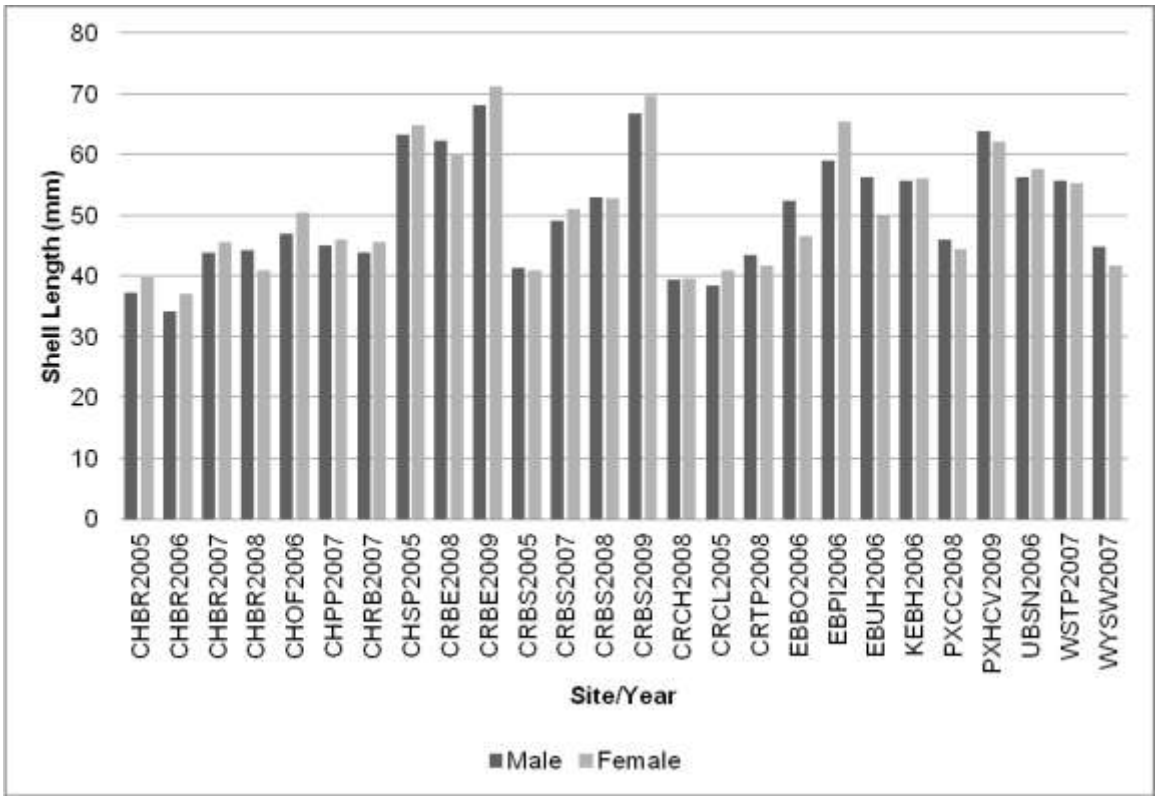

Fig. 2. *Mya arenaria.* Mean shell length in mm of male and female clams by collection site and year.

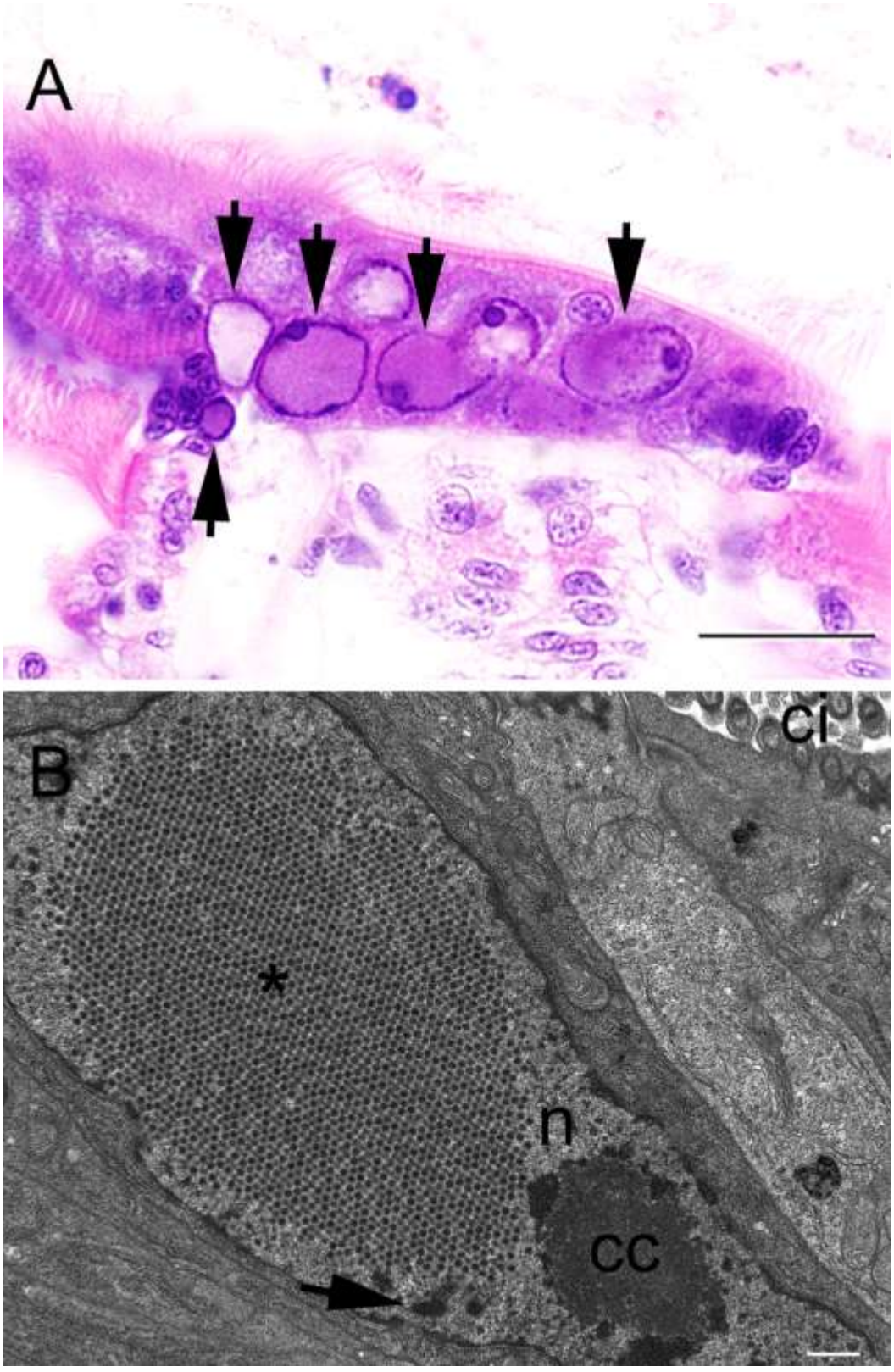
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Fig. 3. *Mya arenaria*. (A) Cross section of gill showing variably sized and shaped inclusion bodies (arrows) within hypertrophied nuclei. H&E. Scale bar = 20 µm. (B) Transmission electron microscopy of hypertrophied ciliated gill epithelial cell with condensed chromatin (cc), marginated chromatin (arrow) and numerous intranuclear virus-like particles (*) arranged in a paracrystalline array; nucleus (n), cilia (ci). Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 500 nm.

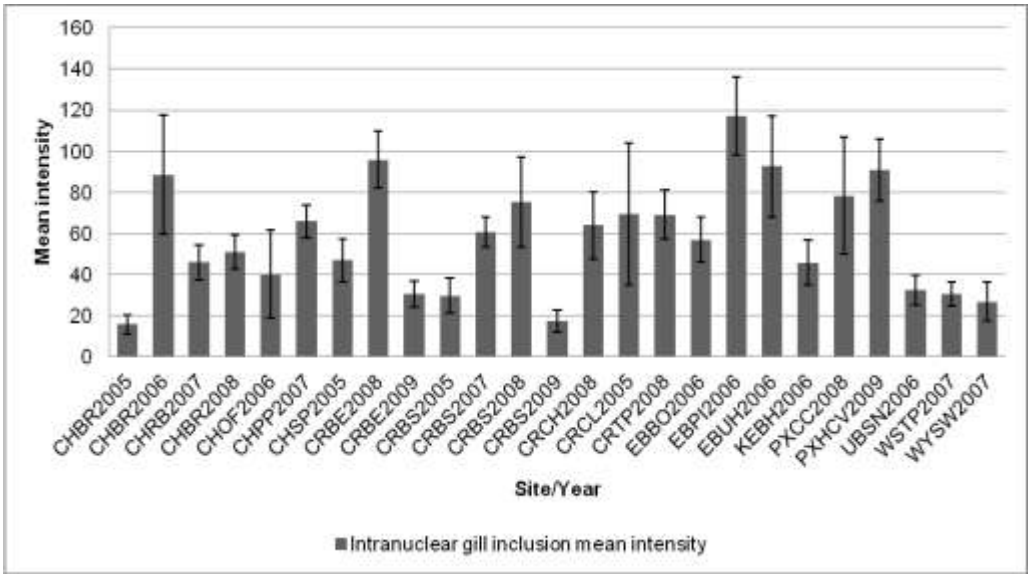

Fig. 4. *Mya arenaria*. Mean intensity of intranuclear gill inclusions by collection site and year.

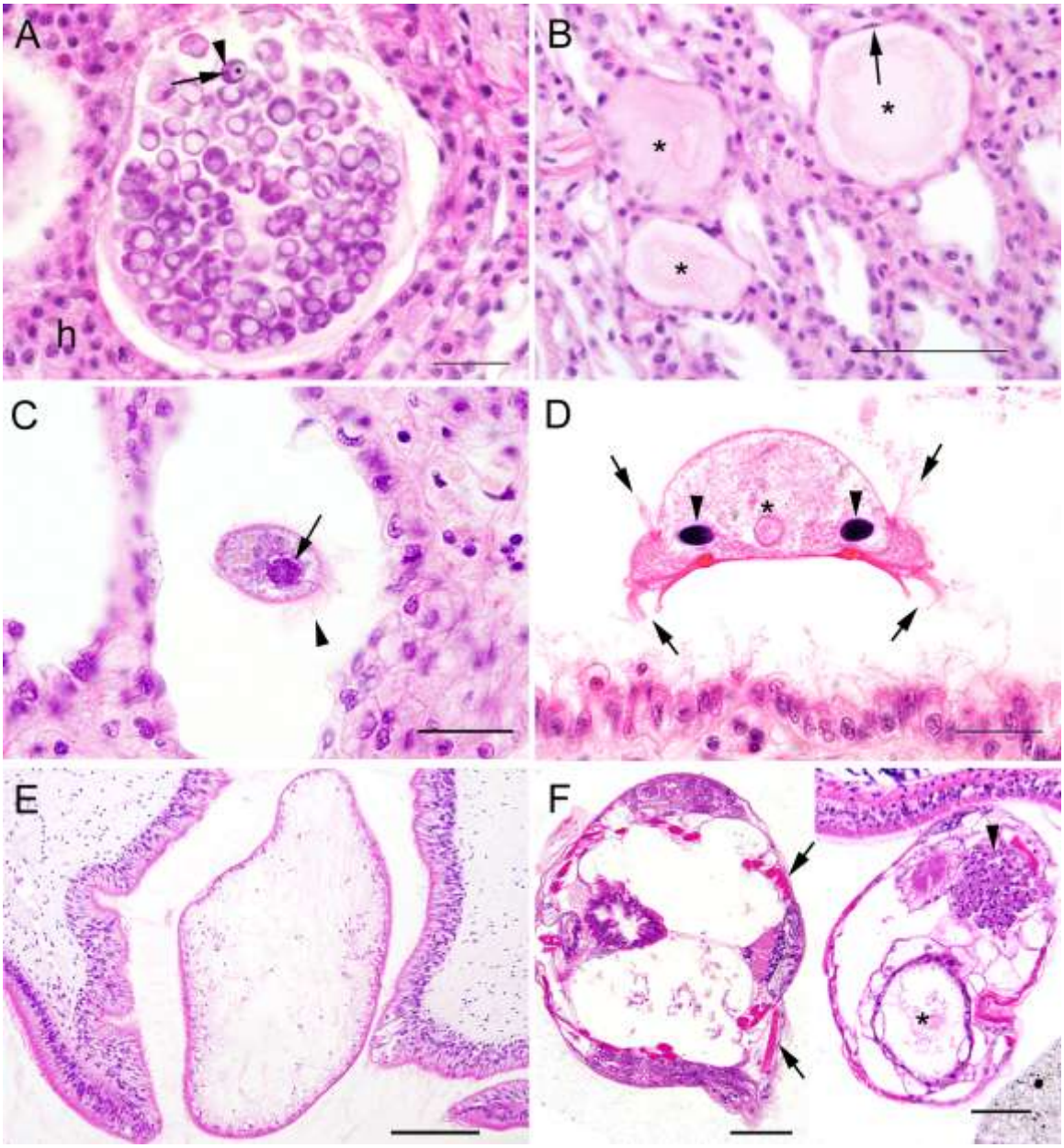

Fig. 5. *Mya arenaria*. (A) Gill with a cluster of *Perkinsus* spp. trophozoites rimmed by eosinophilic, acellular material and surrounded by a layer of hemocytes (h); nucleus (arrowhead), nucleolus (arrow), vacuole (*). H&E. Scale bar = 20 µm. (B) Gill with multiple foci of lightly eosinophilic laminated material (*) which contains rare, highly degraded *Perkinsus* spp. trophozoites rimmed by attenuated hemocytes (arrow). H&E. Scale bar = 50 µm. (C) Pyriform ciliate with large, densely basophilic nucleus (arrow)

and cilia (arrowhead) within the water tube of the gill. H&E. Scale bar = 20 µm. (D) Partial cross section of *Trichodina* within water tube in gill showing macronucleus (arrowhead), cilia (black arrows) and contractile vacuole (*). Scale bar = 20 µm. (E) Cross section of cestode within digestive gland. Scale bar = 100 µm. (F) Cross section of copepod within intestine showing skeletal muscles (arrows), intestine (*), and gonad (arrowhead). Scale bars = 50 um.

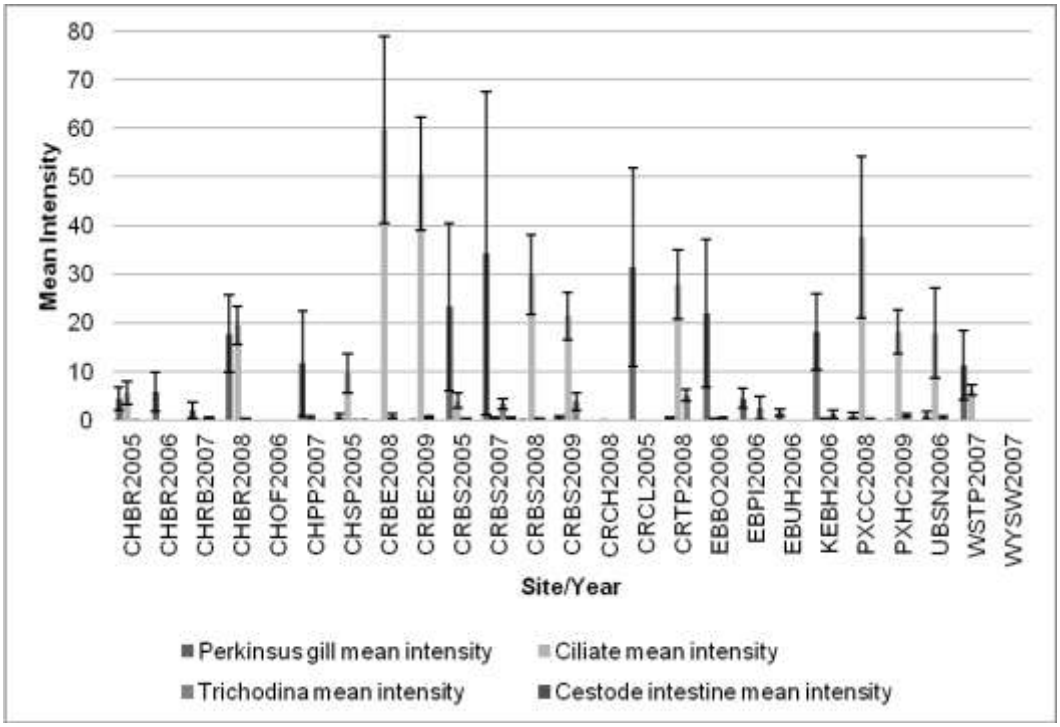

Fig. 6. *Mya arenaria*. Mean intensity of gill *Perkinsus* spp., ciliates, trichodina and cestodes by collection site and year.

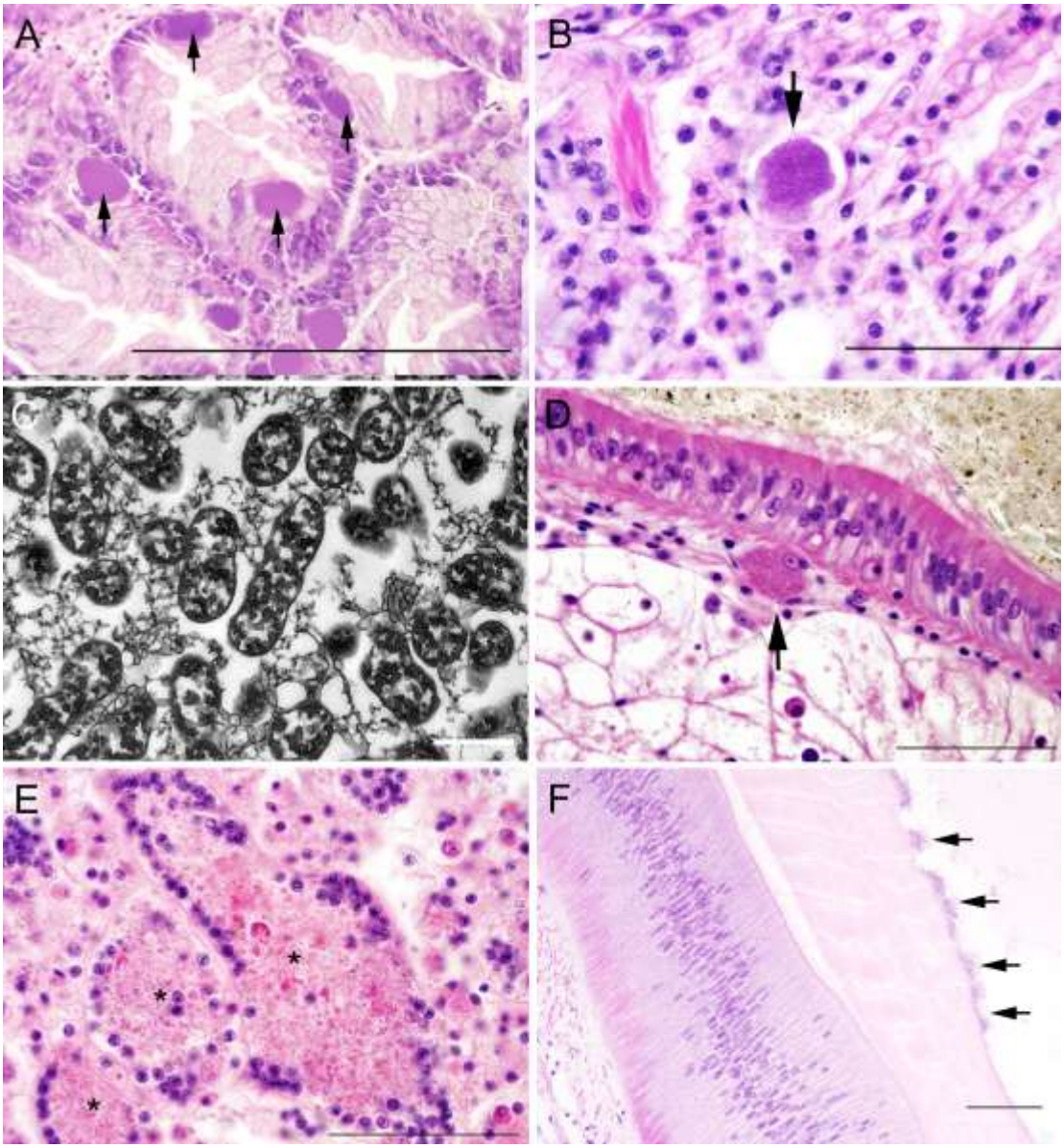

Fig. 7. *Mya arenaria*. (A) Digestive gland epithelial cells with intracytoplasmic basophilic and finely granular bacterial colonies (arrows). H&E. Scale bar = 200 µm. (B) Finely granular bacterial inclusion (arrow) within gill epithelial cell. H&E. Scale bar = 50 µm. (C) Undulating bacterial rods within digestive gland epithelial cell cytoplasm. Methanolic uranyl acetate and Reynold's lead citrate stain. Scale bar = 500 nm. (D) Cluster of bacterial rods (arrow) within connective tissue surrounding the intestine. H&E. Scale bar = 50 µm. (E) Bacteria (*) within the gill are free or within the

cytoplasm of hemocytes and multinucleated giant cells. H&E. Scale bar = 50 µm. (F) Numerous bacteria lining stomach. H&E. Scale bar = 50 µm.

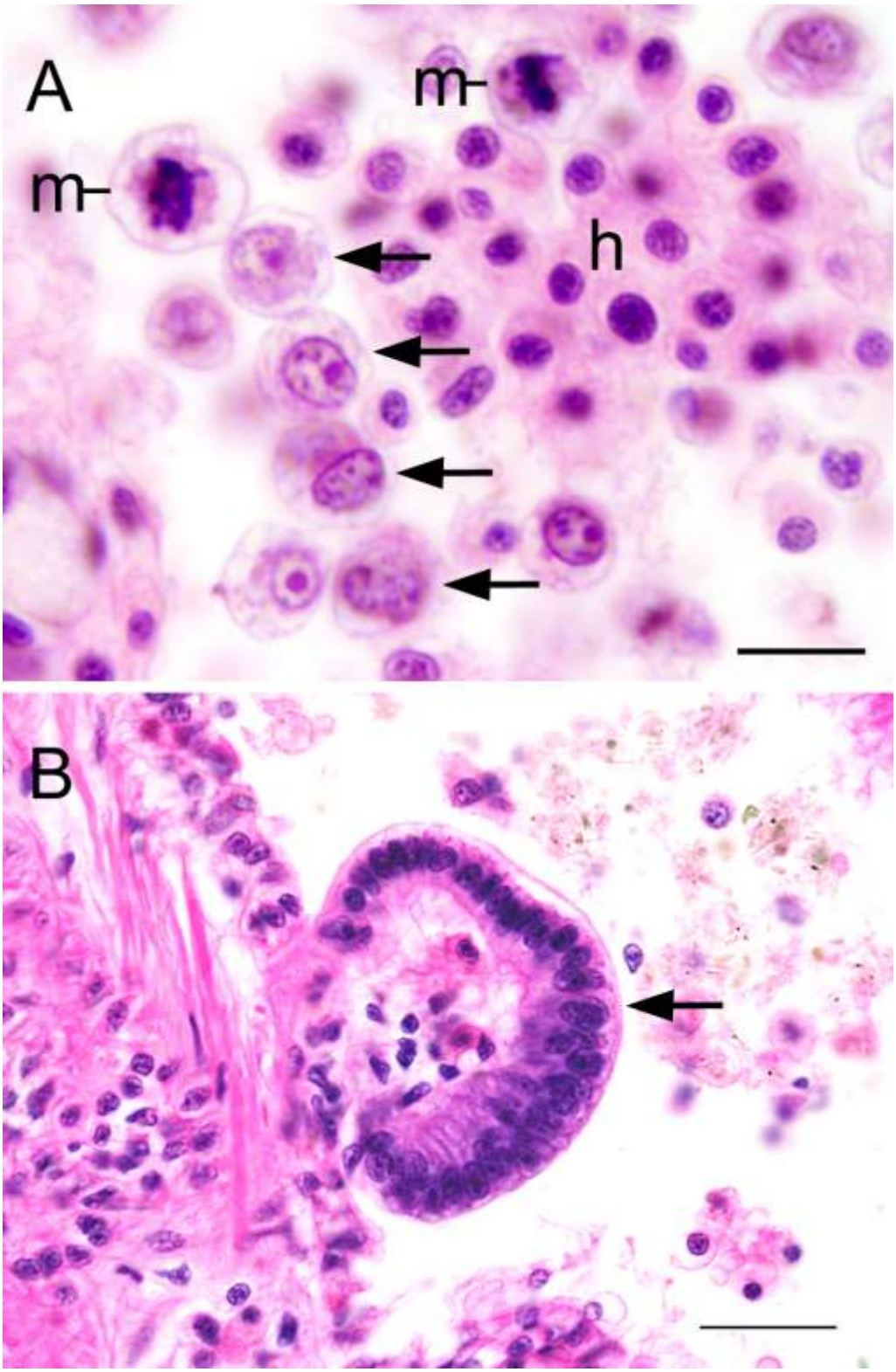

Fig. 8. *Mya arenaria*. Disseminated neoplasia with (A) Neoplastic cells within gill hemolymph channel (arrows), mitotic figures (m) and normal hemocytes (h). H&E.

Scale bar = 10 µm. (B) Focal polyp (arrow) extending from gill epithelial surface. H&E. Scale bar = 20 µm.

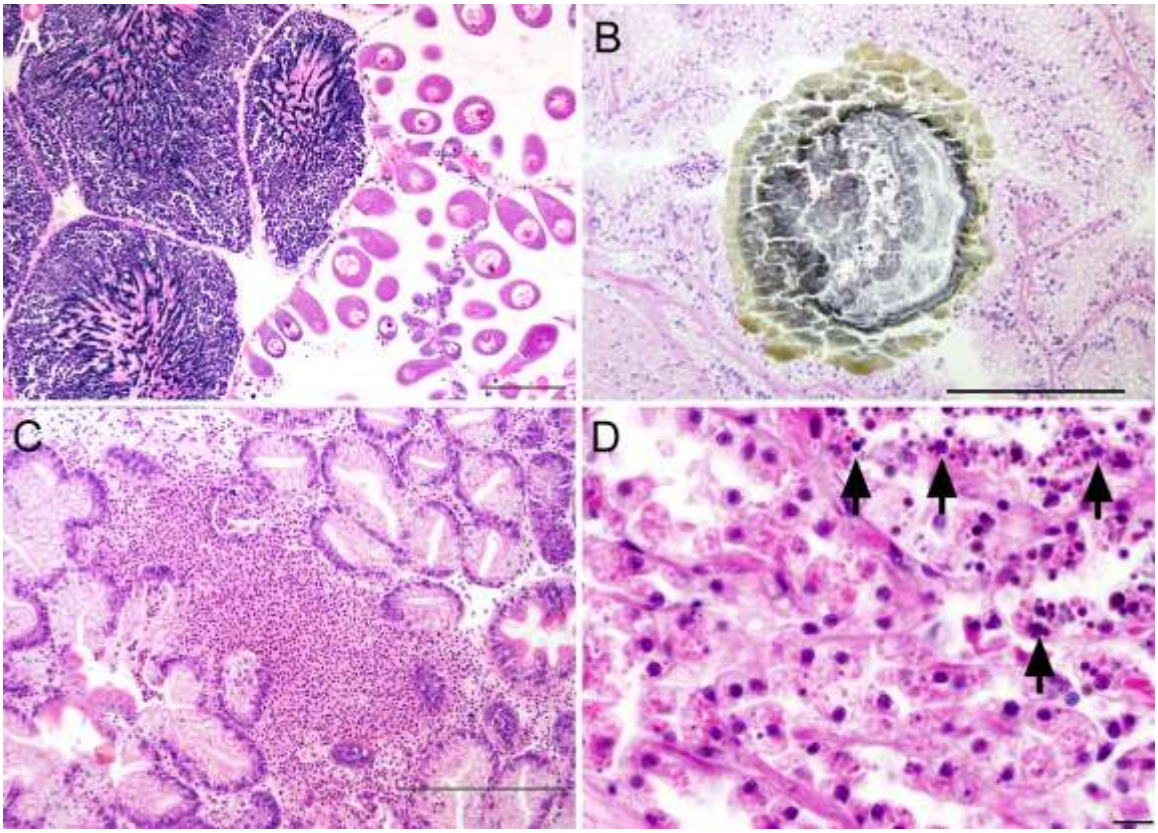

Fig. 9. *Mya arenaria*. (A) Bilateral hermaphrodite with male gonad on left and female gonad on right. H&E. Scale bar = 100 µm. (B) Focal laminated concretion within kidney tubule. H&E. Scale bar = 200 µm. (C) Hemocytic infiltration within digestive gland. H&E. Scale bar = 200 µm. (D) Deeply basophilic, round concretions (top right) within pericardial gland. H&E. Scale bar = 10 µm.

Collection date (mm/dd/yy)	Sample number	Sample code	Tributary/ Region Site	Latitude	Longitude	Water salinity (ppt)	Water temperature (°C)
06/01/09	30	PXHC	Patuxent River, Helen Creek	38.358333	76.485000	11.5	23
04/16/09	30	CRBS	Choptank River, Bolingbroke Sands	38.587500	76.046167	NA	NA
05/13/09	30	CRBE	Choptank River, Benoni Point	38.667500	76.190667	NA	NA
09/30/08	30	CHBR	Chester River, Buoy Rock	38.996667	76.210167	15	22
09/30/08	13	CHBR	Chester River, Buoy Rock	38.996667	76.210167	15	22
10/07/08	7	PXCC	Patuxent River, Cuckold Creek	38.336333	76.493333	14	20
05/29/08	30	CRBE	Choptank River, Benoni Point	38.667500	76.190667	10	20
05/29/08	30	CRBS	Choptank River, Bolingbroke Sands	38.587500	76.046167	9.0	21
05/29/08	29	CRCH	Choptank River, Castle Haven	38.627500	76.180000	10.0	20
10/02/08	26	CRTP	Choptank River, Todds Point	38.638333	76.240000	16	20
09/20/07	30	CHBR	Chester River, Buoy Rock	38.996667	76.210167	14.5	23
09/20/07	30	CHPP	Chester River,	39.051000	76.180833	13.0	22

			Piney Point				
09/27/07	30	CRBS	Choptank River, Bolingbroke Sands	38.587500	76.046167	13.0	24
09/13/07	30	WSTP	Upper Bay, Tolley Point	38.983667	76.436167	13.0	25
06/13/07	30	WYSW	Wye River, Shawns Wharf	38.896333	76.171833	6.0	NA
09/27/06	23	EBBO	Eastern Bay, Bodkin Island	38.884000	76.292667	12.5	22
09/28/06	8	CHBR	Chester River, Buoy Rock	38.996667	76.210167	11.6	22
11/14/06	35	KEBH	Upper Bay, Matapeake Hill	38.960000	76.354167	10.0	14
09/28/06	3	CHOF	Chester River, Old Field	39.073500	76.158500	11.5	22
09/27/06	30	EBPI	Eastern Bay, Parsons Island	38.901667	76.257000	13.0	22
11/14/06	21	UBSN	Upper Bay, Sandy Point N.	39.005833	76.401333	7.0	14
09/21/06	12	EBUH	Eastern Bay, Upper Hill	38.862000	76.250167	13.0	21
09/30/05	30	CHBR	Chester River, Buoy Rock	38.996667	76.210167	13.6	22.2
09/30/05	30	CHSP	Chester River, Spaniard Point	39.088167	76.155000	11.7	22
09/28/05	29	CRBS	Choptank River,	38.587500	76.046167	12.4	23

			Bolingbroke Sands				
09/28/05	5	CRCL	Choptank River, Chlora Point	38.635833	76.146500	13.9	23.2

Table 1. *Mya arenaria*. Collection date, sample number, sample code, collection site, latitude, longitude, water salinity, and water temperature data from Maryland Department of Natural Resources' clam disease surveillance 2005 to 2009; Not available (NA).

Month	Number of collection sites	Mean temperature °C	Minimum temperature °C	Maximum temperature °C
April	1	NA	NA	NA
May	4	20.33	20	21
June	2	23	23	23
September	15	22.49	22	25
October	2	20	20	20
November	2	14	14	14

Table 2. *Mya arenaria*. Mean, minimum, and maximum monthly water temperatures from Chesapeake Bay collection sites from September 2005 to June 2009; Not available (NA).

Tributary	Number of collection sites	Mean salinity ppt	Minimum salinity ppt	Maximum salinity ppt
Chester River	8	13.28	11.5	15
Choptank River	9	12.04	9	16
Eastern Bay	3	12.83	12.5	13
Patuxent River	2	12.75	11.5	14
Upper Bay, Matapeake Hill	3	10	7	13
Wye River	1	6	6	6

Table 3. *Mya arenaria*. Mean, minimum, and maximum water salinity from Chesapeake Bay tributary sites from September 2005 to June 2009.

Disease /Condition	**2005**		**2006**		**2007**		**2008**		**2009**	
	%	*int*	*%*	*int*	*%*	*int*	*%*	*int*	*%*	*int*
Virus										
GENH	70.21	32.74 +/-4.97	93.08	69.35 +/-6.81	77.18	45.96 +/- 3.77	87.88	69.79 +/-6.36	93.26	46.44 +/- 6.69
Parasite										
Perkinsus RFTM	92.55	1.52 +/- 0.11	77.10	1.54 +/-0.13	62.67	0.83 +/- 0.08	34.21	0.49 +/- 0.06	0.00	
Perkinsus all organs	20.79		41.22		15.44		9.70		6.67	
-gill	26.60	10.45 +/-5.52	40.77	10.37 +/-3.51	14.77	11.81 +/- 7.17	9.70	4.72 +/-2.16	5.62	0.17 +/- 0.09
-kidney	2.70	0.27 +/- 0.02	6.20	0.32 +/-0.14	2.10	0.07 +/- 0.05	1.41	0.26 +/-0.22	1.12	0.11 +/- 0.11
-heart	0.00		1.27	0.03 +/-0.03	0.00		3.33	0.03 +/-0.03	0.00	
- pericardial gland	4.17	0.13 +/- 0.13	4.65	0.21 +/-0.16	0.00		1.89	0.02 +/-0.02	0.00	
-ganglia	1.25	0.64 +/- 0.64	2.59	0.22 +/-0.16	0.78	0.09 +/- 0.09	0.00		0.00	
-gonad	5.38	0.65 +/- 0.42	5.34	7.86 +/-5.38	3.36	0.68 +/- 0.53	1.82	1.31 +/-1.21	0.00	
- stomach	2.15	0.60 +/- 0.59	0.77	0.04 +/-0.04	0.67	0.13 +/- 0.13	0.00		0.00	
- intestine	1.08	0.04 +/- 0.04	2.29	0.02 +/-0.1	0.67	0.01 +/- 0.01	0.00		0.00	
- digestive gland	8.60	2.29 +/- 2.09	7.94	0.37 +/-0.17	3.36	0.99 +/- 0.87	3.03	1.19 +/-0.68	0.00	
Spheno phrya-like ciliate	41.49	6.00 +/- 1.60	13.85	2.84 +/-1.51	28.86	1.35 +/- 0.30	76.97	27.34 +/-4.35	87.64	30.19 +/- 4.75
Trichodina	4.26	0.09 +/- 0.05	9.23	0.91 +/-0.58	13.42	0.73 +/- 0.24	17.58	1.01 +/-0.25	37.08	1.69 +/- 0.62
Cestode all	2.13		0.00		4.70		0.00		0.00	

organs										
- stomach	0.00		0.00		0.67	0.01 +/- 0.01	0.00		0.00	
- intestine	1.08	0.01 +/- 0.01	0.00		4.03	0.05 +/- 0.02	0.00		0.00	
- digestive gland	1.08	0.01 +/- 0.01	0.00		0.00		0.00		0.00	
Copepod	0.00		0.00		0.00		0.61	0.01 +/-0.01	0.00	
Bacteria										
Rickettsia all organs	91.49		72.52		74.50		61.82		80.00	
- digestive gland	91.40	41.55 +/-8.79	75.40	24.38 +/-4.66	74.50	18.72 +/- 2.19	61.82	4.78 +/-0.59	86.75	21.98 +/- 4.42
-gill	3.19	0.05 +/- 0.03	0.00		0.00		0.61	0.01 +/-0.01	1.12	0.01 +/- 0.01
- stomach	0.00		1.54	0.02 +/-0.01	0.00		0.00		0.00	
- intestine	0.00		0.76	0.01 +/-0.01	0.00		0.61	0.01 +/-0.01	0.00	
- pericardial gland	0.00		1.16	0.01 +/-0.01	0.00		0.00		0.00	
Bacteria all organs	26.60		11.45		6.71		10.91		1.11	
-rectum	0.00		8.00	0.12 +/-0.07	6.98	0.14 +/- 0.10	0.00		3.85	0.04 +/- 0.04
- intestine	5.38	0.14 +/- 0.09	5.34	0.10 +/-0.05	3.36	0.04 +/- 0.02	0.00		0.00	
-gill	21.28	NA	0.00	NA	0.67	NA	0.00	NA	0.00	NA
- stomach	0.00	NA	4.62	NA	0.67	NA	10.30	NA	0.00	NA
- intestine lumen	0.00	NA	0.00	NA	0.00	NA	0.61	NA	0.00	NA
Metabolic										
Kidney concretions	48.65	NA	26.36	NA	48.95	NA	29.58	NA	13.48	NA

Hemocytic infiltration	6.45	NA	8.40	NA	0.67	NA	1.21	NA	12.22	NA
Pericardial gland concretions										
Neoplasia										
Disseminated neoplasia	0.00	NA	2.29	NA	5.37	NA	1.21	NA	1.12	NA
Polyp gill	0.00	NA	0.00	NA	0.67	NA	0.00	NA	0.00	NA

Table 4. *Mya arenaria*. Prevalences, mean intensities and standard error of diseases, infections and conditions of soft-shell clams in Maryland's Chesapeake Bay 2005 to 2009; NA=Not applicable.

CHAPTER 5

METAGENOMIC ANALYSIS OF VIRAL DIVERSITY IN CHESAPEAKE BAY SOFT-SHELL CLAMS

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Abstract

Soft-shell clams, *Mya arenaria*, are filter-feeding marine bivalve mollusks that were once abundant in the Chesapeake Bay. They inhabit near shore habitats and are subject to pollution from human sewage discharge, agricultural livestock and farming run-off, and pollution from aggregating waterfowl. The uptake of viruses in bivalves is dynamic, and high virus titers can accumulate rapidly. Three clams from the Maryland portion of the Chesapeake Bay were collected and their virome analyzed using next-generation sequencing technology (Illumina Genome Analyzer IIx). More than 60 million raw reads were generated. A total of 410 singlets and 301 assembled contigs were obtained and compared to the GenBank non-redundant protein database using BLASTx. A total of 373 sequences, representing the viral families *Asfarviridae*, *Baculoviridae, Caulimovirdae, Circoviridae*, *Coronaviridae*, *Herpesviridae*, *Irodoviridae*, *Mimiviridae*, *Myoviridae*, *Nanoviridae*, *Nimaviridae*, *Parvoviridae*, *Phycodnaviridae*, *Polydnaviridae*, *Polyomaviridae*, *Poxviridae*, and *Retroviridae*, were identified. Sequences with similarity to unassigned viruses, unclassified viruses, unclassified ssDNA viruses, unclassified dsDNA viruses, an unclassified dsDNA phage and uncultured marine viruses were also present. Phylogenetic analysis of herpesvirus terminase sequences, identified by BLASTx, showed genetic similarity with those of other molluskan herpesviruses. *In situ* hybridization using a digoxigenin-labeled DNA probe provided further evidence to support the presence of a novel herpesvirus in soft-shell clams. Due to their niche as sedentary benthic filter feeders, *Mya arenaria* may serve as an important species for the monitoring of the aquatic virome.

Introduction

Soft-shell clams, *Mya arenaria,* are found in oceans and estuaries in the western Atlantic Ocean from the Subarctic to South Carolina and in the Pacific Ocean from Alaska to San Francisco (Abraham & Dillon 1986). Soft-shell clams also populate the North Sea and European waters, including the Black, Baltic, Wadden, White, and Mediterranean Seas (Strasser & Barber 2008). In the mesohaline portion of Chesapeake Bay, soft-shell clams are a major component of the benthic infauna, where adults burrow up to 30 cm into the sediment and extend their siphonal processes to the sediment surface (Abraham & Dillon 1986). To feed, soft-shell clams beat gill cilia to draw seawater in through their incurrent siphon (Newell & Hidu 1986). Seawater then passes through the gills where food particles are removed, trapped in mucus and swept into the mouth (Newell & Hidu 1986).

In addition to the collection of food particles, filter feeding in bivalves can also result in the accumulation of environmental pollutants, bacteria, and viruses (Song et al. 2010). Virus uptake occurs during filter feeding, when viral particles drawn in from the seawater bind to sulfate radicals on mucopolysaccharide moieties of gill mucus via an ionic bond. The uptake of viruses is dynamic, and high titers can accumulate rapidly (Di Girolamo et al. 1977). Because soft-shell clams inhabit near shore environments, they are also subject to pollution from human sewage discharges, agricultural livestock and farming run-off, and waste products from aggregating waterfowl (Ritchie 1976, Lees 2000). Many viruses are shed through feces, and sewage often contains many types and large numbers of viruses (Lees 2000). Human disease associated with the consumption of bivalves has been recognized since medieval times and is of international concern

today. As a result, bioaccumulation of human enteric viruses by bivalves has been widely studied (Lees 2000). Marine bivalves are also recognized as reservoirs of viral finfish pathogens (Meyers 1984). However, less is known about the bioaccumulation of viruses from other terrestrial and aquatic fauna and flora. Due to their niche as sedentary benthic filter feeders, *Mya arenaria* may serve as an important species for the monitoring of the aquatic virome.

Historically, the isolation of viruses from bivalves has been hindered by a lack of continuous molluskan cell lines (Elston 1997). While the polymerase chain reaction is useful for the detection of known viruses, detection of unknown or novel viruses is more challenging due to the need for virus-specific primers. This problem is circumvented in next-generation sequencing, which allows for the identification of viruses without prior viral sequence knowledge. This study reports results of a metagenomic analysis of DNA viral diversity in three soft-shell clams collected from the Maryland portion of the Chesapeake Bay. In addition, it provides evidence to support a herpesvirus etiology for the condition gill epithelial nuclear hypertrophy of soft-shell clams.

Materials and Methods

Clam specimens

Three adult soft-shell clams were collected by hydraulic escalator dredge from the Maryland portion of the Chesapeake Bay as part of an annual disease surveillance program by the Maryland Department of Natural Resources. Collection date, sample number, sample code, tributary/region site, geographic coordinates, water salinity, and water temperature were recorded for each site, and anterior to posterior shell length was recorded for each clam. Clam 1 measured 40 mm and was collected on May 29, 2008

from the Choptank River at Castle Haven (38.627500N/76.180000W). At the time of collection, salinity measured 10 ppt and water temperature 20°C. Clams 2 and 3, 71 mm and 65 mm, respectively, were collected on June 01, 2009 from the Patuxent River at Helen Creek (38.358333N/76.485000W). Salinity measured 11.5 ppt and water temperature 23°C.

Sample holding, histopathology, transmission electron microscopy, DNA extraction and metagenomic sequencing

Sample holding. Clams were held in flow-through tanks for 24 to 72 h before processing to allow for purging of sand and mud from tissues.

Histopathology. To evaluate clams for viral inclusions using histology, cross sections that included gill tissue were fixed in Davidson's solution, processed routinely, embedded in paraffin, sectioned at approximately 5 µm, and stained with Mayer's hematoxylin and eosin (H&E).

Transmission electron microscopy. Gill samples were fixed in a primary fixative containing 2.5% (v/v) glutaraldehyde in 0.2M phosphate-buffered, 430 mOsm kg-1 artificial seawater, and post-fixed in 0.2M phosphate-buffered 1% (w/v) osmium tetroxide in order to evaluate tissues for the presence of viral inclusions. Tissue samples were dehydrated with ethanol prior to infiltration and embedding with Spurr's epoxy resin. Thin sections (60 nm) were placed on 200-mesh copper grids, and stained with 5% methanolic uranyl acetate and Reynold's lead citrate for examination with a JEOL JEM-1210 transmission electron microscope.

DNA extraction and metagenomic sequencing. Gill tissue was aseptically collected, and placed in 3 ml of tubed 100% ethanol (EtOH). DNA templates from the

gill tissue for metagenomic analysis were prepared using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions for purification of total DNA from animal tissues. DNA sequence libraries were prepared by following the standard protocol of the paired-end DNA sample preparation kit (Illumina). Sequencing was performed on an Illumina Genome Analyzer IIx at Emory University, Atlanta, GA, USA with 100-bp paired-end reads according to the according to the manufacturer's instructions (Illumina).

Bioinformatics

More than 60 million raw reads were generated from each of the three clam samples. Metagenomic sequences were submitted to the high-throughput virus discovery pipeline in the Blood Systems Research Institute (Ng et al. 2012, Ng et al. 2013), where sequences were trimmed for quality and assembled into contigs using SOAP2 (Luo et al. 2012) for each sample. Assembled contigs and singlets were compared to the GenBank non-redundant protein database using BLASTx with an E-value cutoff of 10^{-2}. Potential virus hits were analyzed again individually with manual inspection of the first twenty BLAST alignments, to confirm correct blast hits.

Phylogenetic analysis

To determine if identified herpesvirus terminase sequences represented a virus responsible for previously identified intranuclear virus inclusions of unknown etiology in *Mya arenaria*, phylogenetic analysis of herpesvirus terminase sequences was performed using Clustal W, and the SEQBOOT, DNAML and CONSENSE programs of the Phylip package (Felsenstein 2004). Sequences were analyzed with a bootstrapped (n=500)

maximum likelihood analysis. Herpesvirus terminase proteins were selected from GenBank (Table 1).

Probe design

An oligonucleotide DNA probe (5′TTCACCGGCGATGTCAGAAA3′) based on the herpesvirus terminase sequences identified in the metagenomic analysis was designed using Primer-Blast (NCBI), and the 3′-end was labeled with digoxigenin (Sigma).

In situ hybridization

Paraffin embedded clam tissues were sectioned, dried at 60°C for 1 hr, deparaffinized and rehydrated to water. Tissues were digested with proteinase K (DAKO) and incubated for 10 min at 37°C. Digestion was stopped with 0.1M glycine in phosphate-buffered saline (PBS) for 5 min. Tissue sections were dehydrated, air dried and hybridization chambers (BioRad) applied. For hybridization, 2 µl of denatured probe were added to a hybridization solution containing 50% [v/v] deionized formamide, 0.25% blocking reagent, 10% [w/v] dextran sulphate, and 2x saline-sodium citrate buffer (SSC), then transferred to the chambers. Hybridization solution without probe was applied to a clam tissue section as a negative control. Slides were heated at 95°C for 10 min, cooled on ice for 1 min, and hybridized at 37°C for 24 hrs, then rinsed twice for 5 min in 2x SSC at room temperature, once for 10 min in 0.2x SSC 37°C, and twice in tris buffered saline with tween 20 (DAKO).

Tissues were blocked with Power Block (Biogenex) for 5 min at room temperature, then rinsed with tris buffered saline containing tween 20 (DAKO). For detection of labeled probe, anti-digoxigenin AP (1:5000) was applied for 1 hr at room temperature, followed by three washes for 5 min each with tris buffered saline containing

tween 20 (DAKO), and flooded with 1x detection buffer for 2 min. Slides were then covered in bromo-chloro-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT), prepared by adding 35 µl of BCIP to 45 µl of NBT in 10 ml of 1x detection buffer, and placed in the dark for 24 hrs. Slides were washed and flooded with fast green for 1 min, washed in tap water, and coverslipped using an aqueous mounting medium (DAKO).

Results

Histology and transmission electron microscopy

Light and transmission electron microscopy revealed Feulgen-positive, finely granular, amphophilic, intranuclear inclusions that marginated chromatin, and nonenveloped, moderately electron dense, icosahedral, 75–82 nm virus-like particles that occasionally formed paracrystalline arrays. There was no evidence of disseminated neoplasia, a condition suspected to have a retroviral etiology, in any of the clams.

Illumina singlets and contigs blast analysis

A total of 410 singlets and 301 assembled contigs were obtained and compared with GenBank. The longest was 3,548 bp and the shortest 99 bp. Twenty-four contigs and singlets were longer than 1,000 bp, 69 were between 400 and 999 bp, 202 were between 200 and 399 bp, and 416 were less than 200 bp. There were a total of 373 viral hits representing 11 dsDNA virus families, 1 ssDNA (-) virus family, 1 ssDNA (+) virus family, 1 ssDNA (+/-) virus family, 1 dsDNA reverse transcribing virus family, 1 ssRNA reverse transcribing virus family, and 1 ssRNA (+) virus family. Numerous sequences were also present from unassigned viruses, unclassified viruses, unclassified ssDNA viruses, unclassified dsDNA viruses, an unclassified dsDNA phage, and uncultured marine viruses (Table 2).

Contigs or singlets related to dsDNA viruses

Asfarviridae. Members of Asfarviridae are known to infect vertebrates and invertebrates and belong to the genus *Asfivirus*. A single contig was related to an *Asfarvirus* of the genus *Asfivirus* (Table 3). The contig had a low amino acid identity (32%) to a chaperone protein involved in the folding of the viral capsid. Representatives, including the African swine fever virus, are related to other other viruses in the nucleo-cytoplasmic large DNA virus superfamily that includes the *Poxviridae*, *Iridoviridae*, *Phycodnaviridae* and *Mimiviridae*.

Baculoviridae. Viruses in the family *Baculoviridae* infect invertebrates. Clam samples contained sequences with identity to six species in the genus *Alphabaculovirus* and 5 in the genus *Betabaculovirus*. Sequences had amino acid identities from 53% to 75% to inhibitors of apoptosis proteins. Baculoviruses share core genes with both nudiviruses and polydnaviruses.

Herpesvirales. Three families of herpesviruses exist within the order *Herpesvirales:* the *Alloherpesviridae* and *Herpesviridae* that infect vertebrates and the *Malacoherpesviridae* that infect invertebrates. A single sequence from the family *Alloherpesviridae*, genus *Cyprinivirus*, was present in clam samples and had 71% amino acid identity to ribonucleotide reductase subunit 1. Within the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, there were three sequences with identity to a single species in the genus *Mardivirus*, with 32% amino acid similarity to capsid maturation proteases. Four sequences, representing two species in the genus *Varicellovirus* had low amino acid similarity to DNA packing terminase subunit 1 proteins (26%) and DNA replication-origin binding protein (37%). Four sequences representing two species in the

subfamily *Betaherpesvirinae*, genus *Cytomegalovirus* were present; sequences shared less than 50% amino acid similarity to tumor necrosis factor receptor superfamily-like protein and glycoprotein B. Three sequences were present with homologies to members of the subfamily *Gammaherpesvirinae*. In the genus *Lymphocryptovirus*, a single sequence had 50% amino acid identity to Epstein Barr nuclear antigen. A single sequence had 25% amino acid identity to the DNA polymerase of a virus in the genus *Rhadinovirus* and a single sequence had 48% identity to a complement regulatory protein belonging to an unclassified genus within the subfamily. There were 36 sequence hits representing three virus species within the family *Malacoherpesviridae*. These included sequences with 22% to 25% amino acid identity with ORF102 of Abalone herpesvirus, 22% to 27% amino acid identity with ORF67 and 41% to 60% amino acid identity with ORF98 of Chlamys acute necrobiotic virus, and 24% identity with ORF68, 46% to 57% amino acid identity with ORF99, and 62% amino acid identity with ORF106 of Ostreid herpesvirus 1.

Iridoviridae. Iridoviruses infect both vertebrates and invertebrates. Clam samples contained sequences with identity to viruses within four genera of *Iridoviridae*: *Iridovirus*, *Chloriridovirus*, *Ranavirus* and *Lymphocystivirus* with 14 hits ranging in amino acid identity from to 43% to 79% for the RNA-dependent DNA polymerase of Lymphocystis disease virus. Iridoviruses share sequence similarities to other nucleo-cytoplasmic large DNA viruses.

Mimiviridae. Viruses in the family *Mimiviridae* infect protozoa. Only one genus, *Mimivirus*, is recognized in the family *Mimiviridae*. Sequences with identity to the genus

Mimivirus, representing six species, were identified in the clam samples and ranged in amino acid identity from 34% to 83%.

Myoviridae. Viruses in the family *Myoviridae* infect bacteria and archaea. A single sequence had 70% amino acid identity to the GP556 protein of Bacillus phage G, which is an unassigned species.

Nimaviridae. Nimaviruses infect a wide range of aquatic crustaceans including penaeids, crabs and crayfish. A single sequence with identity to the genus *Whispovirus* shared 81% amino acid similarity to chimeric thymidine kinase/thymidylate kinase.

Phycodnaviridae. Algae are infected by members of family *Phycodnaviridae*. Sequences with identity to the *Phycodnaviridae* were the most commonly found in the clam samples, with 120 hits representing four genera, as well as many unclassified species. Amino acid identities were generally high, with some as high as 100%.

Polydnaviridae. Polydnaviruses infect invertebrates. Sequences shared >40% amino acid identities with counterparts belonging to members of the genera *Bracovirus* and *Ichnovirus*.

Polyomaviridae. Viruses within the family *Polyomaviridae* affect vertebrates. Sequences corresponded to T antigens of two species of avian polyomaviruses with >50% amino acid identity. T antigens interfere with cell cycle regulation.

Poxviridae. Poxviruses infect both vertebrates and invertebrates. Sequences representing four genera were identified from the family *Chordopoxvirinae*. Five sequences representing one species had <50% amino acid identity with a putative RNA phosphatase belonging to a member of the genus *Avipoxvirus*. A single sequence had 81% amino acid identity to the MC066L protein of the genus *Molluscipoxvirus*. Six

sequences, representing two species, had 42% to 76% amino acid identity to proteins within the genus *Orthopoxvirus*. Three sequences, representing one species, had amino acid identities ranging from 36% to 64% to proteins within the genus *Yatapoxvirus*. Within the subfamily *Entomopoxvirinae*, 18 sequences had identity to a putative LINE reverse transcriptase, with amino acid identities of 33% to 58%. One sequence shared 65% identity with a putative inhibitor of apoptosis protein.

Contigs or singlets related to ssDNA (-) viruses

Circoviridae. Circoviruses infect vertebrates. Sequences sharing ≥48% similarity with replication proteins of two species within the genus *Circovirus* were identified. Greater than 50% amino acid identity was also shared with unclassified circoviruses. Members of the genus *Circovirus* show similarity to members of the *Nanoviridae* and *Gemniviridae*.

Contigs or singlets related to ssDNA (-) viruses

Nanoviridae. Nanoviruses infect plants. Sequences, representing two species, shared ≥48% amino acid identity to replication proteins in the genus *Nanovirus*.

Contigs or singlets related to ssDNA (+/-) viruses

Parvoviridae. Members of the family *Parvoviridae* infect both vertebrates and invertebrates. One sequence had low amino acid identity (29%) to a nonstructural protein from the subfamily *Parvovirinae*, genus *Parvovirus*. Within the genus *Dependovirus*, sequences had >50% amino acid identity to the structural protein VP1/VP2 and another to a capsid protein. An additional sequence had >50% amino acid identity to a structural protein of a single species within the subfamily *Densovirinae*, genus *Pefudensovirus*.

Sequences representing two species of unclassified parvoviruses had low amino acid identities (<35%) to non-structural proteins.

Contigs or singlets related to dsDNA-RT viruses

Caulimoviridae. Caulimoviruses infect plants. One sequence had 48% amino acid identity to a putative multifunctional pol protein belonging to a member of the genus *Soymovirus*. Sequences representing two species in the genus *Badnavirus* shared >50% amino acid identity to polyproteins.

Contigs or singlets related to ssRNA-RT (+) viruses

Retroviridae. Members of the family *Retroviridae* infect vertebrates. Sequences representing three genera of the subfamily *Orthoretrovirinae* were present. A single species was represented within the genus *Alpharetrovirus* and had 67% amino acid identity to the src protein. Sequences represented one species within the genus *Betaretrovirus* and had 59% amino acid identity with a gag protein. Sequences representing three species were present within the genus *Gammaretrovirus*, and had >50% amino acid identity to the identified reverse transcriptase, integrase, and polymerase proteins. Sequences represented a single species within the genus *Lentivirus* and had 45% amino acid identity to an RNase H protein. A single species represented an unclassified Retroviridae, which had 65% amino acid identity to a gag protein.

Contigs or singlets related to ssRNA (+) viruses

Coronaviridae. Coronaviruses infect vertebrates. Sequences representing two species within the subfamily *Coronavirinae* genus *Gammacoronavirus* had >50% amino acid identities to polyproteins and a putative papain-like protease.

Contigs or singlets related to unassigned viruses

One sequence had 61% amino acid identity with the ribonucleoside diphosphate reductase of the unassigned *Musca domestica* salivary gland hypertrophy virus that infects houseflies.

Contigs or singlets related to unclassified viruses

Two unclassified viruses were identified by sequence identity: Megavirus Iba which infects amoeba, and Rodent stool-associated circular genome virus that infects the house mouse, *Mus musculus*. For Megavirus Iba, sequences had 43% to 63% amino acid identity to hypothetical proteins and putative serine threonine-protein kinase receptors. For the Rodent stool-associated circular genome virus, the single sequence had 63% amino acid identity to a putative viral replication protein.

Contigs or singlets related to unclassified ssDNA viruses

Sequences shared partial homologies with proteins found in two unclassified ssDNA viruses. Sequences had 31% to 49% amino acid identity to a replication associated protein found in the Cyanoramphus nest associated circular X DNA virus of the yellow-crowned parakeet. A single sequence had 54% amino acid identity to a replication-associated protein belonging to the Dragonfly cyclicusvirus, infectious to the dragonfly, *Pantala flavescens*.

Contigs or singlets related to unclassified dsDNA viruses

Fifty to 100% homologies were shared with proteins of eight unclassified dsDNA viruses. These included Cafeteria roenbergensis virus BV-PW1, that infects marine zooplankton, Heliothis zea virus 1, that infects insects, Marseillevirus, infectious to

amoebae, and Micromonas pusilla virus 12T found in photosynthetic marine flagellates, as well as Ostreococcus lucimarinus viruses OIV4, OIV5, and OIV6 that infect algae.

Contigs or singlets related to unclassified dsDNA phage

A single sequence representing the unclassified dsDNA phage Cyanophage KBS-S-1A shared 73% amino acid identity to thymidylate synthase.

Contigs or singlets related to uncultured marine viruses

Eight sequences had 48% to 64% amino acid identity to replication proteins of uncultured marine viruses.

Herpesvirus DNA packaging terminase subunit 1 phylogeny

Phylogenetic analysis of herpesvirus terminase sequences 12_Contig_10, 14_2254470, and 15_Contig_9 showed closest identity with the *Malacoherpesviridae* (Fig. 1).

Herpesvirus in situ hybridization

Using the herpesvirus terminase 3′digoxigenin-labeled probe, *in situ* hybridization revealed an intranuclear signal localized to the nuclei of gill epithelial cells that had corresponding intranuclear virus inclusions on light microscopy and virus particles on transmission electron microscopy (Fig. 2). No hybridization signal was observed within unaffected gill epithelial cells or in the negative controls.

Discussion

Next-generation sequencing is a useful tool in the identification of the virome of an organism. Metagenomic analysis has been used to characterize viruses within seawater, near shore sediments, feces, serum, plasma, respiratory secretions, tissue samples and tumors (Delwart 2007, Ng et al. 2011). In addition to reducing the cost and

time involved in traditional sequencing, next-generation sequencing overcomes many of the limitations of traditional virus detection by eliminating the need for culture, cloning, sequence specific primers, and even knowledge of the organisms present in a sample.

A metagenomic analysis of three soft-shell clams from the Chesapeake Bay yielded 373 sequences representing 17 known viral families and unclassified virus families known to infect vertebrates, invertebrates, bacteria, protozoa, algae, archae, and plants. The most commonly identified sequences with identities to viruses belonged to the family *Phycodnaviridae* (120), followed by *Herpesviridae* (51), unclassified dsDNA viruses (37), *Poxviridae* (34), *Irodoviridae* (27), *Mimiviridae* (19), *Circoviridae* (14), unclassified ssDNA viruses (13), *Baculoviridae* (11), *Parvoviridae* and uncultured marine viruses (8), *Retroviridae* (7), unclassified viruses (5), *Polydnaviridae* (4), *Caulimovirdae* and *Coronaviridae* (3), *Nanoviridae* and *Polyomaviridae* (2), and *Asfarviridae*, *Myoviridae*, *Nimaviridae*, an unassigned virus and an unclassified dsDNA phage (1).

Sequences with similarity to three viruses known to infect mollusks were identified in the clam samples: Abalone herpesvirus, Chlamys acute necrobiotic virus and Ostreid herpesvirus 1. While many of these sequences shared amino acid identity with inhibitor of apoptosis proteins present in molluskan herpesviruses, it is important to note that these proteins are not herpesvirus-specific and are also present in other large DNA viruses of invertebrate hosts including *Baculoviridae*, *Ascoviridae*, *Poxviridae*, *Iridoviridae* and *Asfarviridae* (Davison et al. 2005). However, three proteins were identified that had 26% amino acid similarity to the DNA packing terminase subunit 1 of bovine herpesvirus 5. This ATPase subunit is herpesvirus-specific and is conserved in all

herpesviruses (King et al., 2012). A phylogenetic analysis of these sequences showed that they clustered with the known molluskan herpesviruses. Because intranuclear virus inclusions of unknown etiology had previously been observed in soft-shell clams by light and electron microscopy, *in situ* hybridization using a digoxigenin-labeled probe based on these terminase sequences was performed. The signal localized to the nuclei of gill epithelial cells, providing further evidence for a novel molluskan herpesviral etiology for these intranuclear gill inclusions.

While viral amino acid identity with many sequences was high, even 100% in some instances, in others it was <25%, suggesting the presence of novel virus sequences. This sequence information could be useful for future virus identification. Due to their sedentary nature and ability to rapidly accumulate viruses, soft-shell clams may serve as an important species to monitor the aquatic virome, and may contribute to the knowledge of the viral diversity present in the aquatic and terrestrial flora and fauna of the Chesapeake Bay region.

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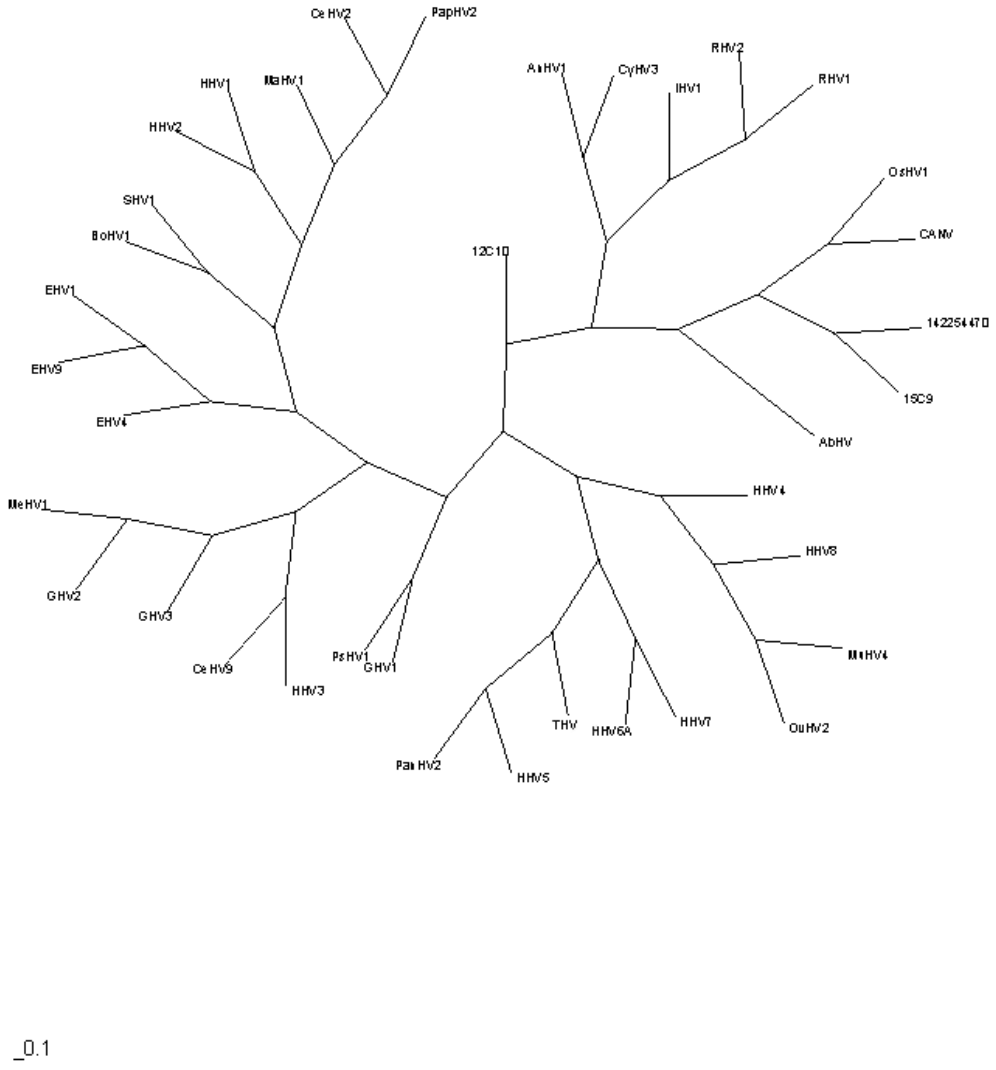

Fig. 1. *Mya arenaria*. Unrooted consensus tree of terminase genes of herpesviruses of representative vertebrate and invertebrate species.

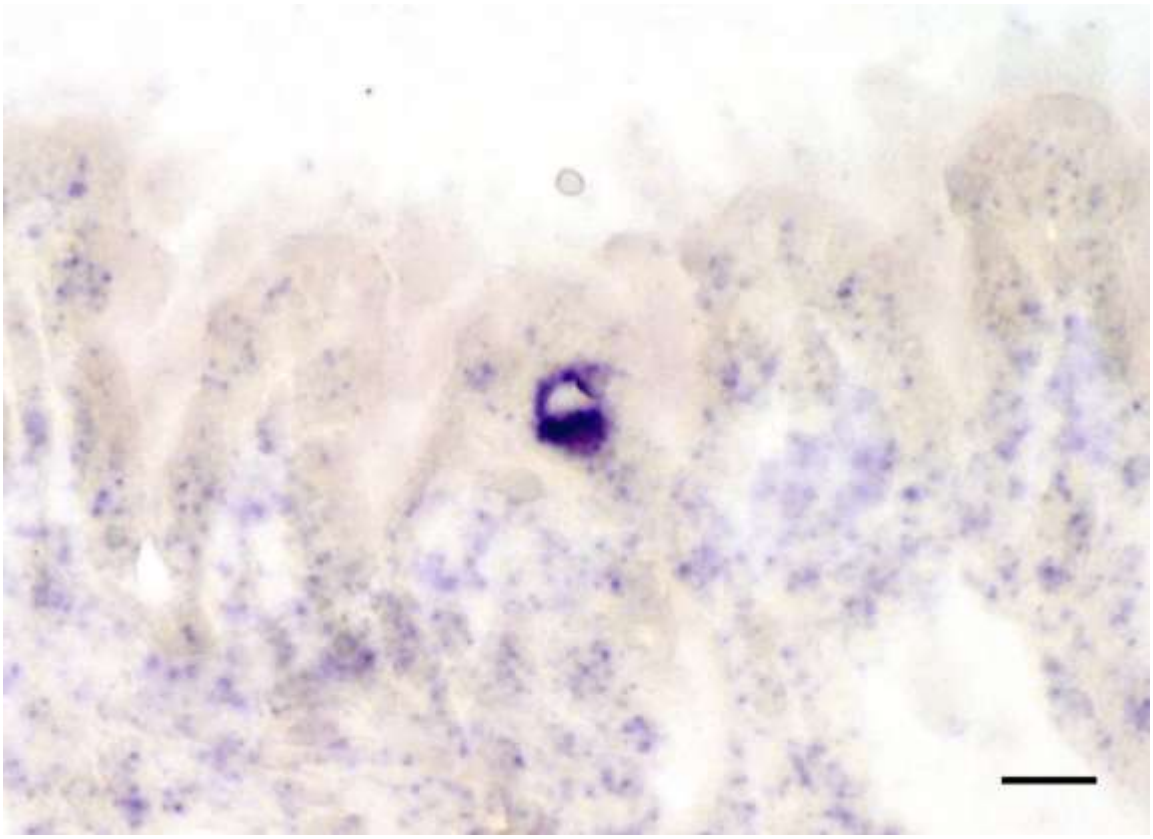

Fig. 2. *Mya arenaria*. Nucleus of gill epithelial cell showing strong hybridization signal using a probe based on novel herpesvirus terminase sequences. *In situ* hybridization; fast green counterstain. Scale bar = 10 µm.

Virus	**Terminase nucleotide**	**Corresponding terminase protein**
Abalone herpesvirus	HM631981.1	ADJ95315.1
Anguillid herpesvirus 1	NC_013668.3	YP_003358149.1
Bovine herpesvirus 1	NC_001847.1	NP_045342.1
Cercopithecine herpesvirus 2	NC_006560.1	YP_164457.1
Cercopithecine herpesvirus 9	NC_002686.2	NP_077457.1
Chlamys acute necrobiotic virus	GQ153938.1	ADD24834.1
Equid herpesvirus 1	NC_001491.2	YP_053090.1
Equid herpesvirus 4	NC_001844.1	NP_045262.1
Equid herpesvirus 9	NC_011644.1	YP_002333526.2
Gallid herpesvirus 1	NC_006623.1	YP_182378.2
Gallid herpesvirus 2	NC_002229.3	YP_001033943.1
Gallid herpesvirus 3	NC_002577.1	NP_066845.1
Human herpesvirus 1	NC_001806.1	NP_044616.1
Human herpesvirus 2	NC_001798.1	NP_044484.1
Human herpesvirus 3	NC_001348.1	NP_040165.1
Human herpesvirus 4	NC_007605.1	YP_401690.1
Human herpesvirus 5	FJ527563.1	ACL51158.1
Human herpesvirus 6A	NC_001664.2	NP_042953.2
Human herpesvirus 7	NC_001716.2	YP_073802.1
Human herpesvirus 8	NC_009333.1	YP_001129382.1
Ictalurid herpesvirus 1	NC_001493.1	NP_041153.2
Macacine herpesvirus 1	NC_004812.1	NP_851874.1
Meleagrid herpesvirus 1	NC_002641.1	NP_073308.1
Murid herpesvirus 4	NC_001826.2	NP_044866.2
Ostreid herpesvirus 1	NC_005881.1	YP_024647.1
Ovine herpesvirus 2	NC_007646.1	YP_438152.1
Panine herpesvirus 2	NC_003521.1	NP_612722.1
Papiine herpesvirus 2	NC_007653.1	YP_443861.1
Psittacid herpesvirus 1	NC_005264.1	NP_944422.2
Ranid herpesvirus 1	NC_008211.1	YP_656697.1
Ranid herpesvirus 2	NC_008210.1	YP_656576.1
Suid herpesvirus 1	NC_006151.1	YP_068358.1
Tupaiid herpesvirus 1	AF228035.1	AAK00707.1
Tupaiid herpesvirus 1	AF228035.1	AAK00707.1

Table 1. Herpesvirus terminase nucleotide sequence GenBank accessions used for phylogenetic analysis.

Virus Taxonomy	Protein hit	GenBank accession number	Contig or singlet number	Contig or singlet length	Max score	Total score	Query coverage	E-value	Max identity
dsDNA									
1) Asfarviridae									
Genus Asfivirus									
African swine fever virus	B602L protein	CAJ90766.1	15_Contig_101	207	49.7	264	98%	3e-06	32%
2) Baculoviridae									
Genus Alphabaculovirus									
Anticarsia gemmnatalis nucleopolyhedrovirus	IAP3	YP_803428.1	12_@s23553071_2	99	49.7	49.7	90%	4e-06	67%
Anticarsia gemmnatalis nucleopolyhedrovirus	IAP3	YP_803428.1	15_Contig_114	113	46.2	46.2	79%	7e-05	60%
Anticarsia gemmnatalis nucleopolyhedrovirus	IAP3	YP_803428.1	15_@s34478206_2	99	47.0	47.0	96%	6e-05	56%
Epiphyas postvittana nucleopolyhedrovirus	IAP-1	NP_203202.1	12_@s5019789_1	99	46.2	46.2	96%	6e-05	53%
Mamestra configurata nucleopolyhedrovirus A	IAP-3	NP_613222.1	14_@s14722018_2	99	47.0	47.0	84%	3e-05	61%
Spodoptera exigua multiple nucleopolyhedrovirus	ORF110 IAP-3	NP_037870.1	12_@s19495819_1	99	49.7	49.7	96%	4e-06	56%
Genus Betabaculovirus									
Agrotis segetum granulovirus	ORF53	YP_006291.1	12_@s21128335_1	99	45.4	45.4	60%	1e-04	75%
Cydia pomonella granulovirus	ORF94 IAP	NP_148878.1	15_@s17145221_1	99	44.3	44.3	96%	2e-04	53%
Epinotia aporema granulovirus	IAP-3	YP_006908519.1	15_Contig_74	113	45.1	45.1	76%	2e-04	66%

Phthorimaea operculella granulovirus	IAP1	NP_663251.1	12_Contig_76	101	44.3	44.3	86%	2e-04	59%
Phthorimaea operculella granulovirus	IAP1	NP_663251.1	15_@s23769395_1	99	44.3	44.3	87%	2e-04	59%
3) Order Herpesvirales									
Alloherpesviridae									
Genus Cyprinivirus									
Cyprinid herpesvirus 2	Ribonucleotide reductase subunit 1	YP_007003955.1	14_Contig_23	132	55.1	55.1	77%	2e-07	71%
Herpesviridae									
Subfamily Alphaherpesvirinae									
Genus Mardivirus									
Gallid herpesvirus 2	UL26 Capsid maturation protease	AAF66761.1	12_1941918	2123	56.2	56.2	20%	4e-05	32%
Gallid herpesvirus 2	UL26 Capsid maturation protease	AAF66761.1	14_2233387	535	54.7	54.7	82%	4e-06	32%
Gallid herpesvirus 2	UL26 Capsid maturation protease	AAF66761.1	15_3417543	2013	56.2	56.2	21%	4e-05	32%
Genus Varicellovirus									
Bovine herpesvirus 5	DNA packing terminase subunit 1	YP_003662508.1	12_Contig_10	2165	112.0	112.0	67%	1e-22	26%

Bovine herpesvirus 5	DNA packing terminase subunit 1	YP_003662508.1	14_2254470	1690	112.0	112.0	86%	6e-23	26%
Bovine herpesvirus 5	DNA packing terminase subunit 1	YP_003662508.1	15_Contig_9	2320	112.0	112.0	63%	2e-22	26%
Human herpesvirus 3	DNA replication origin-binding protein	2012335A	12_Contig_49	307	43.5	43.5	78%	0.006	37%
Subfamily Betaherpesvirinae									
Genus Cytomegalovirus									
Macacine herpesvirus 3	Tumor necrosis factor receptor superfamily-like protein	ABS84083.1	15_2256812	228	47.8	47.8	53%	2e-05	46%
Porcine cytomegalovirus	Glycoprotein B	ACM17140.1	12_1941010	1197	76.6	76.6	69%	5e-12	25%
Porcine cytomegalovirus	Glycoprotein B	ACM17140.1	14_2254910	3548	74.3	74.3	15%	2e-10	26%
Porcine cytomegalovirus	Glycoprotein B	ACM17140.1	15_3406869	758	74.4	74.3	73%	5e-12	26%
Subfamily Gammaherpesvirinae									
Genus Lymphocryptov									

irus									
Human herpesvirus 4 type 2	Epstein-Barr nuclear antigen	YP_001129465.1	12_1301564	215	47.8	85.1	78%	1e-04	50%
Genus Rhadinovirus									
Porcine lymphotropic herpesvirus 2	DNA polymerase	AAO12282.1	15_3416019	1208	60.1	60.1	68%	1e-06	25%
Unclassified genus									
Rodent herpesvirus Peru	Complement regulatory protein	YP_004207845.1	12_1690524	272	56.2	56.2	60%	2e-07	48%
Malacoherpesviridae									
Abalone herpesvirus	ORF102 hypothetical protein	YP_006908754.1	14_2249824	779	97.4	97.4	85%	1e-19	25%
Abalone herpesvirus	ORF102 hypothetical protein	YP_006908754.1	14_2254498	1731	92.0	92.0	56%	2e-16	22%
Chlamys acute necrobiotic virus	ORF67 class I membrane protein	ADD24796.1	12_1859115	355	57.8	57.8	99%	2e-07	27%
Chlamys acute necrobiotic virus	ORF67 class I membrane protein	ADD24796.1	14_Contig_59	834	86.7	86.7	79%	6e-16	24%

Chlamys acute necrobiotic virus	ORF67 class I membrane protein	ADD24796.1	15_3328038	469	44.7	44.7	81%	0.006	22%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	12_Contig_37	317	79.7	79.7	55%	3e-16	53%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	12_Contig_8	2341	86.3	86.3	9%	2e-15	49%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	12_Contig_66	125	53.1	53.1	98%	2e-07	54%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	12_Contig_67	121	57.0	57.0	99%	7e-09	60%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	12_1672506	268	62.0	62.0	69%	4e-10	45%
Chlamys acute necrobiotic virus	ORF98 BIR	ADD24825.1	14_Contig_6	308	93.2	93.2	69%	5e-21	54%

	protein-lacking RING finger								
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	14_Contig_9	614	69.7	69.7	28%	1e-11	48%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	14_Contig_48	171	76.3	76.3	96%	1e-15	53%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	14_Contig_74	144	59.7	59.7	93%	8e-10	51%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_2	314	85.9	85.9	72%	2e-18	49%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_3	1183	86.3	86.3	18%	3e-16	49%
Chlamys acute necrobiotic virus	ORF98 BIR protein-	ADD24825.1	15_Contig_17	1527	89.0	89.0	15%	9e-17	48%

	lacking RING finger								
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_28	327	83.2	83.2	56%	3e-17	52%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_33	597	85.9	85.9	51%	3e-17	41%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_49	143	54.3	54.3	81%	1e-07	51%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_70	229	58.9	58.9	58%	5e-09	53%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking RING finger	ADD24825.1	15_Contig_82	583	55.8	84.7	45%	1e-08	45%
Chlamys acute necrobiotic virus	ORF98 BIR protein-lacking	ADD24825.1	15_Contig_94	269	48.1	84.7	73%	9e-09	51%

	RING finger								
Chlamys acute necrobiotic virus	ORF105	ADD24831.1	12_1762239	295	47.8	47.8	65%	2e-04	41%
Genus Ostreavirus									
Ostreid herpesvirus 1	ORF68 class I membrane protein	YP_024608.1	15_Contig_80	1168	89.4	89.4	55%	3e-16	24%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	12_Contig_4	404	85.9	85.9	56%	7e-18	49%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	12_Contig_6	153	67.0	67.0	100%	2e-12	51%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	12_Contig_11	203	85.5	85.5	91%	9e-19	56%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	14_Contig_1	216	86.7	86.7	100%	4e-19	50%
Ostreid herpesvirus 1	ORF99 BIR protei	YP_024638.1	14_Contig_17	625	85.9	85.9	35%	3e-17	49%

	n lacking RING finger								
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	14_Contig_35	154	73.6	73.6	99%	9e-15	57%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	15_Contig_8	244	80.9	80.9	90%	6e-17	46%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	15_Contig_15	489	58.2	92.4	50%	4e-11	46%
Ostreid herpesvirus 1	ORF99 BIR protein lacking RING finger	YP_024638.1	15_Contig_22	159	67.4	67.4	86%	1e-12	57%
Ostreid herpesvirus 1	ORF106 BIR protein containing RING finger	YP_024644.1	12_Contig_70	104	52.0	52.0	98%	9e-07	62%
Ostreid herpesvirus 1	ORF106 BIR protein contai	YP_024644.1	14_Contig_51	125	57.0	57.0	88%	2e-08	62%

	ning RING finger								
4) Iridoviridae									
Genus Iridovirus									
Wiseana iridescent virus	Hypothetical protein	YP_004732967.1	15_3278584	421	45.4	45.4	54%	0.002	36%
Genus Chloriridovirus									
Invertebrate iridescent virus 3	Hypothetical protein MIV106R	YP_654678.1	15_Contig_77	108	46.6	46.6	94%	8e-05	65%
Genus Ranavirus									
Ambystoma tigrinum virus	Neurofilament triplet H1-like protein	ACB11435.1	12_1631353	259	43.1	75.9	92%	0.003	40%
Ambystoma tigrinum virus	Hypothetical protein	YP_003847.1	12_Contig_3	484	160	716	99%	1e-45	47%
Ambystoma tigrinum virus	Hypothetical protein	YP_003847.1	14_Contig_70	171	47.8	216	100%	4e-05	39%
Ambystoma tigrinum virus	Hypothetical protein	YP_003847.1	15_Contig_7	229	92.0	440	99%	6e-21	55%
European catfish virus	Hypothetical protein	YP_006347598.1	12_1419161	227	50.8	134	72%	8e-07	55%
European catfish virus	Hypothetical protein	YP_006347688.1	15_2417154	240	48.5	48.5	65%	5e-05	48%

European catfish virus	Hypothetical protein	YP_006347710.1	14_@s24077365_1	99	47.8	87.0	100%	4e-05	73%
Grouper iridovirus	Unknown protein	AAV91103.1	12_Contig_25	224	50.1	867	87%	2e-05	51%
Grouper iridovirus	Unknown protein	AAV91103.1	14_Contig_34	256	52.4	917	86%	6e-06	50%
Singapore grouper iridovirus	ORF062R Hypothetical protein	YP_164157.1	12_Contig_30	224	59.7	231	99%	3e-09	50%
Genus Lymphocystivirus									
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	12_Contig_20	121	55.5	55.5	91%	9e-08	70%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	12_Contig_23	368	80.1	80.1	79%	3e-15	79%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	12_Contig_53	227	40.8	67.8	92%	8e-04	46%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	12_@s7484893_1	99	43.5	43.5	96%	8e-04	53%
Lymphocystis disease virus	RNA-dependent DNA	YP_073558.1	12_@s15604175	99	43.9	43.9	96%	7e-04	66%

	poly merase								
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	14_Contig_13	118	53.5	53.5	91%	4e-07	69%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	14_Contig_18	229	60.8	60.8	74%	3e-09	49%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	15_Contig_23	123	58.9	58.9	95%	5e-09	69%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	15_Contig_30	246	86.7	86.7	98%	3e-18	51%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	15_Contig_43	111	45.4	45.4	89%	2e-04	61%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	15_1669907	199	58.2	58.2	97%	2e-08	44%
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	15_2125516	220	55.8	55.8	91%	2e-07	43%
Lymphocystis disease virus	RNA-dependent	YP_073558.1	15_@s13601883	99	47.0	47.0	100%	6e-05	67%

	DNA polymerase								
Lymphocystis disease virus	RNA-dependent DNA polymerase	YP_073558.1	15_@s43092247_2	99	44.3	44.3	96%	5e-04	53%
Lymphocystis disease virus	Hypothetical protein	YP_073684.1	12_Contig_78	100	45.5	45.5	84%	1e-04	64%
5) Mimiviridae									
Genus Mimivirus									
Acanthamoeba castellanii mamavirus	Hypothetical protein	AEQ60809.1	12_Contig_29	246	49.3	49.3	80%	2e-05	42%
Acanthamoeba polyphaga mimivirus	Hypothetical protein	YP_003987126.1	12_Contig_28	275	65.1	164	79%	8e-11	45%
Acanthamoeba polyphaga mimivirus	Hypothetical protein	YP_003987126.1	12_Contig_40	128	56.2	272	98%	2e-08	60%
Acanthamoeba polyphaga mimivirus	Hypothetical protein	YP_003987126.1	14_Contig_41	261	65.1	184	78%	9e-11	48%
Acanthamoeba polyphaga mimivirus	Hypothetical protein	YP_003987126.1	14_Contig_41	245	58.5	212	89%	2e-08	47%
Acanthamoeba polyphaga mimivirus	Hypothetical protein	YP_003987126.1	14_Contig_45	224	57.4	201	89%	3e-08	46%
Acanthamoeba polyphaga mimivirus	Hypothetical protein	YP_003987126.1	14_1021470	193	55.8	179	90%	7e-08	46%
Acanthamoeba	Hypot	YP_00398	15_1968	212	45.1	198	97%	6e-04	45%

polyphaga mimivirus	hetical protein	7126.1	761						
Acanthamoeba polyphaga mimivirus	Putative ankyrin repeat protein	YP_003987437.1	12_1157809	204	44.3	44.3	94%	0.001	34%
Acanthamoeba polyphaga moumouvirus	HSP70-like protein	YP_007354398.1	14_Contig_84	111	71.6	71.6	97%	2e-14	83%
Acanthamoeba polyphaga moumouvirus	HSP70-like protein	YP_007354398.1	12_@s15758968_2	99	65.9	65.9	100%	2e-12	82%
Acanthamoeba polyphaga moumouvirus	HSP70-like protein	YP_007354398.1	14_@s31394114	99	63.2	63.2	96%	1e-11	81%
Megavirus chiliensis	Mg597 gene product	YP_004894648.1	12_1360973	221	50.8	93.2	96%	2e-06	36%
Megavirus chiliensis	Mg861 gene product	YP_004894912.1	12_Contig_79	100	46.6	86.6	96%	5e-05	59%
Megavirus chiliensis	Mg1076 gene product	YP_004895127.1	15_Contig_87	320	64.7	338	64%	4e-11	46%
Mimivirus cher	B-family DNA polymerase	AFM52352.1	14_Contig_46	198	95.1	95.1	100%	7e-21	65%
Mimivirus cher	B-family DNA polymerase partial	AFM52363.1	14_Contig_82	122	64.7	64.7	98%	9e-11	73%
Moumouvirus	Enzy	AGF8517	15_Conti	288	71.6	71.6	72%	2e-13	54%

goulette	me E2	2.1	g_91						
Moumouvirus goulette	Hypothetical protein glt_00513	AGF85322.1	15_@s20480829_1	99	50.8	50.8	93%	6e-07	74%
6) Myoviridae									
Bacillus phage G	GP556	AEO93803.1	14_@s33618960_1	99	45.4	120	90%	2e-05	70%
7) Nimaviridae									
Genus Whispovirus									
White spot syndrome virus	Chimeric thymidine kinase/thymidylate kinase	ACX54176.1	15_Contig_120	102	51.6	51.6	79%	6e-07	81%
8) Phycodnaviridae									
Chlorovirus									
Chlorella virus	Protein kinase A248R	AAU06286.1	14_Contig_12	121	45.4	45.4	69%	4e-05	71%
Paramecium bursaria chlorella virus	DNA ligase	AGE55055.1	14_@s1569466_2	99	48.1	48.1	93%	1e-05	61%
Paramecium bursaria chlorella virus	Hypothetical protein FR483_N331R	YP_001425963.1	15_3337539	481	45.8	45.8	64%	0.002	28%
Paramecium bursaria chlorella virus	Hypothetical protein AR158_c499L	YP_001498580.1	15_3336538	480	65.1	475	74%	1e-10	63%

Genus Coccolithovirus									
Emiliania huxleyi virus 86	Hypothetical protein EhV141	YP_293894.1	15_@s20722517_1	99	43.5	43.5	84%	7e-04	64%
Emiliania huxleyi virus 86	Hypothetical protein EhV307	YP_294064.1	14_2117248	348	58.5	571	98%	3e-08	22%
Emiliania huxleyi virus 207	Ribonucleoside-diphosphate reductase	AEP15458.1	15_Contig_6	323	144	144	98%	4e-38	59%
Genus Prasinovirus									
Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_026	YP_004061456.1	12_@s13526657_1	99	64.7	64.7	96%	5e-12	78%
Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_043	YP_004061473.1	14_@s11020720_1	99	46.6	46.6	93%	3e-05	58%
Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_052	YP_004061482.1	14_@s32029915_2	99	50.4	50.4	96%	4e-06	69%
Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_122	YP_004061552.1	14_@s26596293_2	99	52.0	52.0	90%	3e-07	70%

Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_144c	YP_004061574.1	12_@s26215317_1	99	72.8	72.8	100%	3e-14	97%
Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_148	YP_004061578.1	12_@s2105734_1	99	63.2	63.2	96%	2e-11	88%
Bathycoccus sp. RCC1105 virus BpV1	Hypothetical protein BpV1_188c	YP_004061618.1	14_Contig_68	182	112	112	98%	5e-27	85%
Bathycoccus sp. RCC1105 virus BpV2	Hypothetical protein BpV2_126	ADQ91293.1	14_@s17628173_1	99	50.1	50.1	75%	2e-06	80%
Bathycoccus sp. RCC1105 virus BpV2	Hypothetical protein BpV2_176c	ADQ91343.1	14_@s28220425_1	99	50.4	50.4	93%	7e-07	75%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV1_013c	YP_004061896.1	14_@s12348216_2	99	61.2	61.2	100%	9e-10	79%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV1_036	YP_004061919.1	12_Contig_61	168	100	100	98%	6e-24	78%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein	YP_004061936.1	12_@s21882119_2	99	58.9	58.9	96%	9e-10	91%

	MpV 1_051								
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_055	YP_00406 1938.1	14_@s30 456632_ 1	99	55.1	55.1	100%	4e-08	76%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_072	YP_00406 1955.1	14_Contig_57	99	47.0	252	100%	8e-05	77%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_072	YP_00406 1955.1	15_Contig_51	121	58.9	302	96%	7e-09	81%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_078	YP_00406 1961.1	12_@s32 278339_ 2	99	52.4	52.4	93%	4e-07	68%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_091 c	YP_00406 1974.1	14_@s32 818712_ 1	99	47.0	47.0	96%	8e-06	69%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_120	YP_00406 2003.1	14_@s67 48679_1	99	51.2	51.2	93%	3e-07	68%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV 1_135	YP_00406 2018.1	12_@s33 429202_ 1	99	65.9	65.9	96%	2e-12	94%
Micromonas sp. RCC1109 virus MpV1	Hypothetical	YP_00406 2022.1	15_Contig_13	321	136	136	97%	3e-35	59%

	protein MpV1_139c								
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV1_169	YP_004062052.1	14_@s12713027_2	99	54.3	54.3	90%	1e-07	83%
Micromonas sp. RCC1109 virus MpV1	Hypothetical protein MpV1_224c	YP_004062107.1	14_@s9587126_1	99	51.6	51.6	100%	2e-06	65%
Micromonas pusilla virus PL1	Hypothetical protein MPWG_00028	AET43518.1	12_@s8289306_1	99	64.7	64.7	96%	3e-11	84%
Micromonas pusilla virus PL1	Hypothetical protein MPWG_00083	AET43572.1	14_Contig_78	129	87.4	87.4	100%	2e-19	88%
Micromonas pusilla virus PL1	Proliferating cell nuclear antigen	AET43634.1	12_@s25588682_2	99	50.4	50.4	100%	1e-06	70%
Micromonas pusilla virus PL1	Hypothetical protein MPWG_000156	AET43643.1	12_@s18252806_2	99	54.3	54.3	96%	1e-07	69%
Micromonas pusilla virus PL1	Hypothetical protei	AET43650.1	12_@s20193778_2	99	61.6	61.6	96%	5e-11	88%

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Micromonas pusilla virus PL1	Cell division protein	AET43698.1	14_@s5263375_1	99	53.1	53.1	96%	5e-07	75%
Micromonas pusilla virus SP1	Ribonucleotide reductase	AET84860.1	14_Contig_73	146	95.9	95.9	98%	9e-22	94%
Micromonas pusilla virus SP1	Hypothetical protein MPXG_00237	AET85035.1	14_@s15639035_1	99	57.8	57.8	100%	7e-09	79%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061636.1	12@s4334257_2	99	58.9	58.9	93%	3e-10	81%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061659.1	12_@s24622205_1	99	51.2	51.2	100%	1e-06	64%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061659.1	14@s17816736_2	99	55.8	55.8	100%	3e-08	76%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061659.1	14_@s20575019_1	99	62.0	62.0	96%	2e-10	88%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061781.1	14_@s19082136_1	99	62.0	62.0	96%	5e-11	97%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061793.1	12_@s17459155_1	99	62.8	62.8	100%	2e-11	88%
Ostreococcus lucimarinus virus OIV1	Hypothetical protein	YP_004061849.1	14_@s31629301_1	99	57.0	57.0	96%	1e-08	72%
Ostreococcus	Hypot	YP_00406	12_@s20	99	62.0	62.0	96%	2e-11	91%

lucimarinus virus OIV1	hetical protein	1857.1	385643_1						
Genus Phaeovirus									
Ectocarpus siliculosus virus 1	EsV-1-21	NP_077506.1	12_@s18215611_2	99	43.5	43.5	93%	0.001	58%
Unclassified genus									
Organic lake phycodnavirus 1	Putative thymidylate synthase	ADX05794.1	14_Contig_85	111	58.5	58.5	97%	1e-09	69%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648087.1	12_@s23562635_2	99	63.5	63.5	96%	3e-11	78%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648087.1	12_@s12322798_1	99	69.7	69.7	93%	2e-13	100%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648087.1	14_@s1878119_2	99	77.0	77.0	100%	4e-16	100%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648087.1	14_@s25788941_1	99	74.3	74.3	100%	4e-15	100%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648097.1	12_@s21819868_2	99	45.8	45.8	93%	1e-04	52%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648177.1	12_@s4768713_1	99	45.4	45.4	100%	2e-04	58%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648181.1	12_@s28492292_2	99	63.5	63.5	96%	4e-11	94%
Ostreococcus virus OsV5	Hypothetical	YP_001648182.1	12_@s13845671_1	99	65.1	65.1	96%	4e-12	91%

	protein								
Ostreococcus virus OsV5	Hypothetical protein	YP_001648188.1	14_Contig_83	115	55.1	55.1	99%	4e-08	74%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648207.1	12_@s19726605_1	99	62.0	62.0	100%	4e-11	85%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648212.1	12_@s936402_2	99	51.6	129	100%	2e-06	73%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648212.1	14_Contig_97	99	60.1	115	96%	2e-09	94%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648245.1	12_@S25569354_2	99	46.6	46.6	81%	6E-05	78%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648254.1	12_@S33642237_2	99	48.9	48.9	90%	8E-06	77%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648272.1	12_Contig_59	174	87.8	87.8	77%	2e-20	87%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648273.1	12_@s1264194_2	99	60.8	60.8	100%	3e-10	73%
Ostreococcus virus OsV5	Hypothetical protein	YP_001648322.1	12_@s16799505_2	99	57.0	57.0	90%	9e-09	87%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC34936.1	12_@s2145615_2	99	61.2	61.2	100%	7e-11	76%
Ostreococcus tauri virus RT-2011	Hypothetical	AFC34954.1	14_@s13800986_1	99	61.2	61.2	100%	5e-11	85%

	protein								
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC34980.1	12_@s1942451_1	99	55.5	55.5	87%	4e-08	86%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35007.1	12_@s13388671_1	99	51.6	51.6	100%	8e-07	70%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35039.1	15_@s19751511_1	99	66.6	66.6	96%	1e-12	97%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35043.1	14_Contig_3	595	248	248	96%	3e-74	60%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35052.1	14_@s23630468_2	99	74.7	74.7	96%	2e-15	97%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35064.1	12_@s6558571_2	99	72.4	72.4	100%	4e-14	94%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35139.1	12_@s14193014_2	99	48.5	48.5	96%	1e-05	69%
Ostreococcus tauri virus RT-2011	Hypothetical protein	AFC35140.1	14_@s7864533_2	99	60.8	60.8	93%	1e-09	90%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212825.1	12_Contig_81	99	60.5	60.5	96%	93-11	88%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212860.1	12_@s18377296_1	99	48.5	48.5	72%	2e-05	92%
Ostreococcus tauri virus 1	Hypothetical	YP_003212872.1	12_@s7684707_2	99	65.9	65.9	96%	9e-13	97%

	protein								
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212872.1	12_@s7692509_1	99	62.8	62.8	93%	1e-11	94%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212894.1	14_@s29409_1	99	50.4	50.4	96%	3e-06	69%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212917.1	12_@s16075151_2	99	49.7	49.7	96%	6e-06	66%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212934.1	14_@s3995011_2	99	57.4	57.4	81%	2e-09	93%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212938.1	12_@s33232481_1	99	55.5	55.5	96%	1e-07	81%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212939.1	15_@s37727564_2	99	66.6	66.6	96%	1e-12	91%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212939.1	15_@s41397470_1	99	70.9	70.9	96%	3e-14	91%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212949.1	14_@s12172053_2	99	70.5	70.5	96%	4e-14	100%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212949.1	14_@s15639312_1	99	65.5	65.5	96%	3e-12	97%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212950.1	12_@s28707435_1	99	62.8	62.8	87%	2e-10	93%
Ostreococcus tauri virus 1	Hypothetical	YP_003212968.1	12_@s18333003_1	99	59.7	59.7	96%	1e-09	75%

	protein								
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212965.1	14_@s30775447_1	99	66.2	66.2	100%	1e-12	91%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212979.1	12_@s22597104_2	99	71.2	71.2	100%	5e-14	94%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003212986.1	12_@s18414089_1	99	68.9	68.9	100%	1e-12	97%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213005.1	14_@s2627500_2	99	68.6	68.6	90%	2e-13	100%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213009.1	14_@s30983110_2	99	45.8	45.8	90%	2e-05	73%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213005.1	14_@s31423988_1	99	67.0	67.0	96%	8e-13	97%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213011.1	12_@s20186799_1	99	60.8	60.8	96%	4e-11	88%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213024.1	15_@s41931241_1	99	60.5	60.5	96%	9e-11	75%
Ostreococcus tauri virus 1	DNA topoisomerase II	YP_003213035.1	14_@s29788595_1	99	63.2	63.2	90%	2e-10	93%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213031.1	14_Contig_71	169	113	113	99%	2e-27	91%
Ostreococcus tauri virus 1	Hypothetical protein	YP_003213024.1	15_@s29248603_2	99	53.9	53.9	75%	2e-08	88%

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Ostreococcus tauri virus 1	Hypothetical protein	YP_003213052.1	12_Contig_68	120	57.4	57.4	97%	2e-08	56%
Ostreococcus tauri virus 2	Cytosine-specific methyltransferase	YP_004063436.1	14_@s16138223_1	99	73.2	73.2	96%	9e-15	94%
Ostreococcus tauri virus 2	Putative glycosyl transferase group 1	YP_004063441.1	14_@s4250789_1	99	62.8	62.8	100%	4e-11	85%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063474.1	12_Contig_58	183	125	125	100%	3e-35	92%
Ostreococcus tauri virus 2	FAD-dependent thymidylate synthase ThyX	YP_004063474.1	14_@s4947136_2	99	68.2	68.2	100%	4e-13	94%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063514.1	12_@s16408863_1	99	54.3	54.3	96%	2e-08	75%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063514.1	12_@s26134930_1	99	59.3	59.3	100%	4e-10	85%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063515.1	15_@s34297550_2	99	45.1	45.1	96%	5e-05	63%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063522.1	14_Contig_79	127	85.5	85.5	99%	2e-20	95%

Ostreococcus tauri virus 2	Hypothetical protein	YP_004063523.1	14_Contig_67	184	82.8	82.8	92%	2e-17	74%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063527.1	14_@s14227004_1	99	59.3	59.3	96%	4e-10	88%
Ostreococcus tauri virus 2	Putative virus inclusion body	YP_004063542.1	12_@s8402827_1	99	57.8	57.8	96%	2e-08	84%
Ostreococcus tauri virus 2	Ribonuclease III	YP_004063550.1	12_@s24593100_1	99	64.7	64.7	96%	5e-12	94%
Ostreococcus tauri virus 2	Putative lambda-type exonuclease	YP_004063551.1	12_@s16331966_2	99	64.3	64.3	96%	9e-12	91%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063584.1	14_Contig_75	138	93.6	93.6	97%	2e-21	96%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063585.1	12_@s31476821_2	99	57.0	57.0	81%	1e-09	96%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063610.1	14_@s19110960_1	99	61.6	61.6	93%	4e-11	94%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063629.1	12_Contig_60	169	113	113	97%	1e-30	93%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063629.1	15_@s10180957_1	99	63.5	63.5	90%	8e-12	90%
Ostreococcus tauri virus 2	Hypothetical protein	YP_004063646.1	15_Contig_103	161	85.1	85.1	98%	1e-19	96%

Ostreococcus tauri virus 2	Predicted protein	YP_004063662.1	15_@s32485509_1	99	50.1	50.1	96%	8e-07	72%
9) Polydnaviridae									
Genus bracovirus									
Cotesia congregate bracovirus	Hypothetical protein CcBV_31.2	YP_184882.1	12_1155513	204	43.9	43.9	73%	8e-04	44%
Cotesia congregate bracovirus	Hypothetical protein CcBV_31.2	YP_184882.1	12_@s20698778_1	99	44.7	44.7	90%	1e-04	53%
Cotesia congregate bracovirus	Hypothetical protein CcBV_31.2	YP_184882.1	15_Contig_116	106	48.1	48.1	84%	9e-06	63%
Genus Ichnovirus									
Glypta fumiferanae ichnovirus	GfC-C8-ORF1	YP_001029442.1	12_1892634	400	43.9	43.9	33%	0.005	47%
10) Polyomaviridae									
Genus Polyomavirus									
Budgerigar fledgling disease polyomavirus	Large T and small t antigens	AAC33626.1	12_@s29872878_2	99	46.2	46.2	96%	7e-06	56%
Goose hemorrhagic polyomavirus	Putative large T antigen	NP_849170.1	15_@s33333268_1	99	45.4	45.4	93%	2e-04	61%
11) Poxviridae									
Subfamily Chordopoxvirinae									
Genus									

Avipoxvirus									
Canarypox virus	CNPV085 Putative RNA phosphatase	NP_955108.1	14_Contig_5	689	75.5	1027	99%	8e-13	38%
Canarypox virus	CNPV085 Putative RNA phosphatase	NP_955108.1	14_Contig_19	221	48.9	429	92%	4e-05	38%
Canarypox virus	CNPV085 Putative RNA phosphatase	NP_955108.1	15_Contig_25	267	38.1	517	96%	6e-04	37%
Canarypox virus	CNPV085 Putative RNA phosphatase	NP_955108.1	15_Contig_34	429	66.6	458	98%	2e-10	32%
Canarypox virus	CNPV085 Putative RNA phosphatase	NP_955172.1	12_1297062	215	45.8	45.8	58%	3e-04	45%
Genus Molluscipoxvirus									
Molluscum contagiosum virus	MC066L	NP_044017.2	12_@s14023253_2	99	57.8	57.8	93%	2e-09	81%
Genus Orthopoxvirus									
Cowpox virus	Secreted complement binding protein C3b/C4b	ADZ29790.1	14_1523805	225	48.5	48.5	73%	4e-05	42%
Cowpox virus	Ribon	ADZ3026	12_Conti	115	69.3	69.3	99%	1e-12	76%

	ucleotide reductase large subunit protein	6.1	g_41						
Cowpox virus	Thymidine kinase	CAA77152.1	14_@s5232959_2	99	55.5	55.5	90%	6e-09	73%
Cowpox virus	Thymidine kinase	CAA77153.1	15_@s7823437_2	99	52.4	52.4	90%	9e-08	70%
Cowpox virus	CPXV034	NP_619823.1	12_1047319	197	47.0	47.0	82%	8e-05	46%
Ectromelia virus	EVM025	NP_671543.1	15_Contig_5	590	120	1340	87%	5e-29	48%
Genus Yatapoxvirus									
Tanapox virus	ORFL4R	AAD46179.1	12_Contig_33	109	52.8	52.8	99%	2e-07	64%
Tanapox virus	ORFL4R	AAD46179.1	15_Contig_115	107	47.8	47.8	98%	1e-05	60%
Tanapox virus	Kelch-like protein	YP_001497136.1	14_2208426	448	72.8	72.8	81%	3e-12	36%
Subfamily Entomopoxvirinae									
Melanoplus sanguinipes entomopoxvirus	ORFMSV061 putative LINE reverse transcriptase	NP_048132.1	12_Contig_47	481	52.8	52.8	43%	1e-05	37%
Melanoplus sanguinipes entomopoxvirus	ORFMSV061 putative LINE reverse transcriptas	NP_048132.1	12_898894	169	47.8	47.8	95%	7e-05	40%

	e								
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	12_980924	193	53.9	53.9	87%	7e-07	48%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	12_1767729	298	60.8	60.8	85%	7e-09	35%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	12_1864671	361	46.6	46.6	59%	6e-04	39%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	12_1901712	418	88.2	88.2	98%	7e-18	33%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	12_@s32254671_2	99	43.9	43.9	93%	7e-04	52%

Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	14_Contig_66	198	56.2	56.2	95%	1e-07	44%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	14_Contig_69	178	56.6	56.6	82%	7e-08	58%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	14_1984441	294	51.6	51.6	54%	1e-05	51%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	14_2032933	309	50.4	50.4	67%	3e-05	35%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	15_Contig_64	294	38.5	90.9	95%	5e-05	50%
Melanoplus	ORF	NP_04813	15_Conti	319	42.0	72.8	60%	3e-05	55%

sanguinipes entomopoxvirus	MSV 061 putative LINE reverse transcriptase	2.1	g_88						
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	15_Contig_92	282	61.2	61.2	90%	4e-09	40%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	15_2804779	279	63.9	63.9	97%	5e-10	36%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	15_2926902	299	48.5	48.5	69%	1e-04	36%
Melanoplus sanguinipes entomopoxvirus	ORF MSV 061 putative LINE reverse transcriptase	NP_048132.1	15_2962628	306	52.8	52.8	73%	5e-06	43%
Melanoplus sanguinipes	ORF MSV	NP_048132.1	15_3335012	478	54.7	54.7	40%	3e-06	45%

entomopoxvirus	061 putative LINE reverse transcriptase								
Melanoplus sanguinipes entomopoxvirus	ORF MSV 248 Putative inhibitor of apoptosis protein	NP_048319.1	14_@s12585354_1	99	44.3	44.3	78%	7e-05	65%
ssDNA(-)									
12) Circoviridae									
Genus Circovirus									
Bat circovirus ZS/Yunnan-China/2009	Replication associated protein	AEL28791.1	12_Contig_15	313	85.1	85.1	79%	3e-19	49%
Bat circovirus ZS/Yunnan-China/2009	Replication associated protein	AEL28791.1	14_Contig_32	452	87.4	87.4	53%	2e-19	52%
Gull circovirus	Rep	AFJ93342.1	12_Contig_32	136	55.1	55.1	79%	8e-08	67%
Gull circovirus	Rep	AFJ93342.1	14_Contig_49	142	62.4	62.4	88%	2e-10	69%
Gull circovirus	Replication associated protein	YP_803546.1	15_Contig_76	110	46.2	46.2	87%	7e-05	63%
Gull circovirus	Replication associated protein	YP_803546.1	15_@s24577618_2	99	47.0	47.0	87%	4e-05	66%
Unclassified Genus									

Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	12_Contig_31	174	60.8	60.8	75%	3e-10	55%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	12_Contig_39	138	56.6	56.6	95%	7e-09	50%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	12_Contig_64	136	55.1	55.1	94%	3e-08	56%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	14_Contig_28	152	62.0	62.0	90%	9e-11	54%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	14_Contig_37	135	58.2	58.2	95%	2e-09	53%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	14_Contig_76	134	51.6	51.6	98%	6e-07	50%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	15_Contig_108	126	54.7	54.7	97%	3e-08	54%
Circoviridae pork NW2/USA/2009	Rep protein	ADU77001.1	15_Contig_113	114	53.1	53.1	97%	1e-07	59%
ssDNA(+)									
13) Nanoviridae									
Genus Nanovirus									
Faba bean necrotic yellows virus	Rep protein	NP_619567.1	15_Contig_109	124	56.6	56.6	99%	1e-08	56%
Subterranean clover stunt virus	Possible replication associated protein	NP_620700.1	12_1727094	283	50.4	50.4	46%	1e-05	48%
ssDNA (+/-)									
14) Parvoviridae									
Subfamily Parvovirinae									
Genus Parvovirus									

Rat minute virus 1c	Nonstructural protein 1	AAM93279.1	12_1716160	280	53.9	53.9	99%	2e-06	29%
Genus Dependovirus									
Goose parvovirus	Structural protein VP1/VP2	ABI20761.1	12_Contig_17	396	49.3	49.3	25%	2e-05	58%
Goose parvovirus	Structural protein VP1/VP2	ABI20761.1	14_Contig_42	250	55.5	55.5	45%	4e-08	58%
Serpentine adeno-associated virus 2	Capsid protein	ACJ66591.1	15_Contig_81	977	47.4	47.4	11%	0.002	53%
Subfamily Densovirinae									
Genus Pefudensovirus									
Periplaneta fuliginosa densovirus	Structural protein	NP_051016.1	15_Contig_58	922	44.7	44.7	10%	0.023	59%
Unclassified Parvoviridae									
Fox parvovirus	Non-structural polyprotein	AGK45548.1	15_3356085	511	57.8	57.8	66%	3e-07	32%
Parvovirus partridge/PA147/ITA/2008	Nonstructural protein	ADZ48579.1	14_1991663	296	48.9	48.9	65%	6e-05	35%
Parvovirus partridge/PA147/ITA/2008	Nonstructural protein	ADZ48579.1	15_3415631	1142	65.1	65.1	30%	1E-08	31%
dsDNA-RT									
15) Caulimoviridae									
Genus Soymovirus									
Cestrum yellow leaf curling	Putative	NP_861410.1	15_@s42299021_	99	43.9	43.9	100%	7e-04	48%

virus	multifunctional pol protein		1						
Genus Badnavirus									
Commelina yellow mottle virus	Polyprotein	NP_039820.1	15_@s40305812_2	99	47.4	47.4	93%	7e-05	65%
Piper yellow mottle virus	Polyprotein	ABI30239.1	14_@s5505039	99	46.2	46.2	90%	3e-05	57%
ssRNA-RT (+)									
16) Retroviridae									
Subfamily Orthoretrovirinae									
Genus Alpharetrovirus									
Rous sarcoma virus	Scr	CAA36154.1	15_@s18564527_2	99	48.1	48.1	100%	3e-05	67%
Genus Betaretrovirus									
Simian endogenous retrovirus vero ATCC CCL-81	Gag protein	AEJ22865.1	15_@s29226804_1	99	43.5	43.5	90%	9e-04	59%
Genus Gammaretrovirus									
Feline leukemia virus	Reverse transcriptase	NP_955579.1	12_@s12977749_2	99	44.7	44.7	93%	4e-04	52%
Moloney murine leukemia virus	Integrase	AAA46502.1	15_@s10237411_1	99	44.3	44.3	100%	1e-04	52%
Rat leukemia virus	Polymerase	AAC78249.1	14_@s16549923_2	99	51.6	51.6	96%	2e-06	63%
Genus Lentivirus									
Human immunodeficiency virus 1	RNase H	ABU62687.1	14_1906549	275	47.4	47.4	60%	3e-05	45%
Unclassified Retroviridae									
Human endogenous retrovirus K	Gag protein	CAA71418.1	15_@s25971669_2	99	47.4	47.4	93%	4e-05	65%
ssRNA (+)									

17) Coronaviridae									
Subfamily Coronavirinae									
Genus Gammacoronavirus									
Beluga whale coronoavirus SW1	ORF 1ab polyprotein	YP_001876435.1	14_Contig_47	193	50.1	50.1	55%	2e-05	61%
Beluga whale coronoavirus SW1	ORF 1ab polyprotein	YP_001876435.1	15_@S26998859_2	99	43.9	43.9	99%	0.001	61%
Turkey coronavirus	NSP3	YP_001941176.1	15_@s28261934_2	99	43.9	43.9	96%	0.001	56%
18) Unassigned viruses									
Musca domestica salivary gland hypertrophy virus	Ribonucleoside diphosphate reductase	YP_001883393.1	15_Contig_53	112	53.1	53.1	83%	7e-07	61%
19) Unclassified virus									
Megavirus Iba	Hypothetical protein LBA_00420	YP_007418314.1	15_Contig_102	168	61.2	525	100%	1e-10	43%
Megavirus Iba	Hypothetical protein LBA_00420	YP_007418314.1	14_Contig_81	126	50.1	99.7	97%	2e-06	63%
Megavirus Iba	Putative serine/threonine-proteinkinase/receptor	YP_007418797.1	14_Contig_88	107	50.4	92.0	98%	5e-06	56%
Megavirus Iba	Putati	YP_00741	15_Conti	103	47.4	87.4	96%	6e-05	57%

	ve serine /threo nine-protei nkina se/rec eptor	8797.1	g_118						
Rodent stool-associated circular genome virus	REP 1	AEM0581 0.1	14_@s55 66185_1	99	46.6	46.6	96%	1e-05	63%
20) Unclassified ssDNA viruses									
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated protei n	AGC5514 6.1	12_Conti g_14	921	157	157	80%	1e-41	35%
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated protei n	AGC5514 6.1	12_Conti g_35	708	130	130	79%	2e-32	37%
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated protei n	AGC5514 6.1	12_Conti g_69	117	53.9	53.9	100%	2e-07	49%
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated protei n	AGC5514 6.1	12_1632 817	259	65.9	65.9	89%	4e-11	45%
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated protei n	AGC5514 6.1	12_1937 244	721	88.6	136	65%	1e-17	40%
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated protei n	AGC5514 6.1	14_Conti g_11	1441	165	213	71%	3e-43	36%
Cyanoramphus nest associated circular X DNA virus	Repli cation associ ated	AGC5514 6.1	14_Conti g_14	704	110	110	88%	3e-25	31%

	protein								
Cyanoramphus nest associated circular X DNA virus	Replication associated protein	AGC55146.1	14_Contig_33	412	114	114	93%	8e-28	43%
Cyanoramphus nest associated circular X DNA virus	Replication associated protein	AGC55146.1	14_1514125	224	57.8	57.8	95%	2e-08	39%
Cyanoramphus nest associated circular X DNA virus	Replication associated protein	AGC55146.1	15_Contig_40	855	124	124	76%	1e-29	35%
Cyanoramphus nest associated circular X DNA virus	Replication associated protein	AGC55146.1	15_Contig_41	581	150	150	97%	9e-41	39%
Cyanoramphus nest associated circular X DNA virus	Replication associated protein	AGC55146.1	15_Contig_67	285	85.5	85.5	98%	3e-18	45%
Dragonfly cyclicusvirus	Replication-associated protein	AFS65303.1	12_1874202	372	69.7	69.7	41%	3e-12	54%
21) Unclassified dsDNA viruses									
Cafeteria roenbergensis virus BV-PW1	Putative superfamily II helicase	YP_003969704.1	12_@s2886810_1	99	45.4	45.4	93%	2e-04	52%
Cafeteria roenbergensis virus BV-PW1	Hypothetical protein	YP_003969940.1	15_2389057	237	50.1	369	99%	2e-06	50%
Heliothis zea	Rr1	AAN0438	14_@s85	99	48.1	48.1	87%	3e-05	72%

virus 1		9.1	16611_2						
Heliothis zea virus 1	Rr1	AAN04389.1	15_Contig_119	103	52.0	52.0	90%	1e-06	74%
Marseillevirus	Hypothetical protein	YP_003406820.1	14_949968	189	47.8	169	92%	6e-05	52%
Micromonas pusilla virus 12T	DNA topoisomerase IIB	YP_007676289.1	15_@s33837159_2	99	58.5	58.5	96%	7e-09	91%
Micromonas pusilla virus 12T	Hypothetical protein	YP_007676287.1	14_@s974064_2	99	46.6	46.6	78%	1e-05	73%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84472.1	12_Contig_65	134	79.7	79.7	98%	2e-17	80%
Ostreococcus lucimarinus virus OIV4	2-polyprenylphenol 6-hydroxylase	AET84494.1	12_@s29201258_1	99	61.2	61.2	96%	5e-10	78%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84543.1	12_@s22742604	99	52.8	52.8	96%	4e-08	75%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84572.1	14_@s25612488	99	59.3	59.3	81%	3e-10	96%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84573.1	14_@s16429166_1	99	66.6	66.6	96%	7e-13	88%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84597.1	12_@s7970968_1	99	74.3	74.3	100%	2e-15	94%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84597.1	14_@s14176938_1	99	68.2	68.2	100%	4e-13	94%

Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84605.1	12_@s13763500_2	99	48.9	48.9	93%	9e-06	77%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84637.1	15_@s34229702_2	99	53.9	53.9	96%	3e-07	72%
Ostreococcus lucimarinus virus OIV4	Helicase	AET84641.1	14_@s12561958_2	99	70.1	70.1	100%	4e-13	97%
Ostreococcus lucimarinus virus OIV4	Helicase	AET84641.1	14_@s22177011_1	99	68.9	68.9	96%	1e-12	100%
Ostreococcus lucimarinus virus OIV4	Hypothetical protein	AET84652.1	15_@s43175040_1	99	49.7	49.7	96%	9e-06	72%
Ostreococcus lucimarinus virus OIV6	6-phosphofructokinase	AFK65834.1	12_@s7050609_1	99	65.9	65.9	93%	3e-12	97%
Ostreococcus lucimarinus virus OIV6	Hypothetical protein	AFK65980.1	12_@s20633780_1	99	58.9	58.9	96%	5e-10	81%
Ostreococcus lucimarinus virus OIV3	Hypothetical protein	AFK66011.1	15_@s25006319_2	99	55.8	55.8	96%	7e-09	72%
Ostreococcus lucimarinus virus OIV3	Hypothetical protein	AFK66026.1	15_@s17156950_1	99	63.9	63.9	96%	6e-12	88%
Ostreococcus lucimarinus virus OIV3	Topoisomerase 2	AFK66029.1	15_Contig_104	160	104	104	97%	2e-24	98%
Ostreococcus lucimarinus virus OIV3	DNA ligase	AFK66079.1	12_@s31570043_1	99	62.8	62.8	93%	8e-11	97%
Ostreococcus lucimarinus virus OIV3	Hypothetical protein	AFK66113.1	12_@s14018125_1	99	59.7	59.7	100%	2e-10	85%
Ostreococcus lucimarinus virus OIV3	Hypothetical	AFK66129.1	12-_@s19598936_2	99	62.4	62.4	96%	3e-11	91%

	protein								
Ostreococcus lucimarinus virus OIV3	Ribonuclease III	AFK66134.1	14_@s23110089_2	99	62.8	62.8	96%	2e-11	97%
Ostreococcus lucimarinus virus OIV3	viral A-type inclusion protein	AFK66144.1	14_@s4603767_1	99	55.5	55.5	96%	1e-07	78%
Ostreococcus lucimarinus virus OIV3	GDP-mannose 4,6-dehydratase	AFK66218.1	12_@s17740516_2	99	63.2	63.2	96%	5e-11	84%
Ostreococcus lucimarinus virus OIV3	Cell division protein	AFK66240.1	14_@S13543361_2	99	58.9	58.9	96%	4e-09	81%
Ostreococcus lucimarinus virus OIV3	Cell division protein	AFK66240.1	15_Contig_106	149	83.6	83.6	98%	2e-17	75%
Ostreococcus lucimarinus virus OIV5	Topoisomerase 2	YP_007674675.1	15_@s30719476_1	99	67.8	67.8	100%	5e-12	97%
Ostreococcus lucimarinus virus OIV5	Hypothetical protein	YP_007674682.1	12_@s8444588_1	99	64.7	64.7	96%	1e-12	94%
Ostreococcus lucimarinus virus OIV5	Hypothetical protein	YP_007674764.1	12_@s6857218_1	99	57.4	57.4	87%	1e-09	93%
Ostreococcus lucimarinus virus OIV5	Hypothetical protein	YP_007674781.1	12_@s17600699_2	99	59.7	59.7	96%	2e-09	88%
Ostreococcus lucimarinus virus OIV5	Hypothetical protein	YP_007674868.1	12_@s4119250_1	99	62.0	62.0	96%	5e-11	88%
22) Unclassified dsDNA phage									
Cyanophage	Thym	AET7281	14_@s25	99	55.5	55.5	100%	1e-08	73%

KBS-S-1A	idylate synthase	2.1	289883_1						
23) Uncultured marine virus									
Uncultured marine virus	Replication protein	GAC77814.1	15_Contig_18	328	89.4	89.4	78%	4e-20	48%
Uncultured marine virus	Replication protein	GAC77854.1	12_@s21689081_1	99	50.1	50.1	96%	5e-07	63%
Uncultured marine virus	Replication protein	GAC77854.1	14_Contig_36	137	67.8	67.8	91%	2e-13	64%
Uncultured marine virus	Replication protein	GAC77854.1	14_Contig_61	321	70.1	70.1	57%	2e-13	52%
Uncultured marine virus	Replication protein	GAC77854.1	15_Contig_20	510	93.6	93.6	48%	2e-21	53%
Uncultured marine virus	Replication protein	GAC77854.1	15_Contig_21	488	100	100	49%	5e-24	57%
Uncultured marine virus	Replication protein	GAC77854.1	15_Contig_37	194	85.9	85.9	89%	6e-20	62%
Uncultured marine virus	Replication protein	GAC77854.1	15_Contig_56	1218	106	106	20%	1e-24	56%

Table 2. BLASTx similarities for singlets and contigs in the GenBank non-redundant database with an E-value $<10^{-2}$.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The goals of this research were to characterize the agent responsible for intranuclear inclusions observed within hypertrophied nuclei of gill epithelial cells in *Mya arenaria* by light and electron microscopy, next-generation sequencing and *in situ* hybridization, secondly to perform a retrospective health survey of this population to document the distribution and prevalence of this condition along with other diseases, infections or conditions, and finally to perform a metagenomic analysis of viral diversity in Chesapeake Bay soft-shell clams.

Adult *Mya arenaria* were sampled from multiple sites within the Maryland portion of the Chesapeake Bay for histopathology and transmission electron microscopy. On light microscopy, Feulgen-positive, finely granular, amphophilic, intranuclear inclusions that marginated chromatin were identified within hypertrophied gill epithelia of many clams. To rule out possible non-viral causes for the inclusions, the periodic acid–Schiff reaction was used to identify mucopolysaccharides, especially glycogen, Giemsa and Gimenez to visualize bacteria, and Ziehl-Neelsen acid-fast methods to identify lead inclusions, lipofuschin and ceroid pigments, or acid-fast parasites. All of these stains and reactions were negative.

Transmission electron microscopy revealed nonenveloped, moderately electron dense, icosahedral, 75–82 nm virus-like particles that occasionally formed paracrystalline

arrays. The cytoplasm contained 25-30 nm particles or particles arranged in 50-100 nm rosettes, consistent with glycogen. Following partial purification by sucrose gradient ultracentrifugation, negatively stained, moderately electron dense, 78 nm icosahedral virus-like particles were observed.

Due to the abundant glycogen observed in the cytoplasm of gill epithelial cells on transmission electron microscopy, and the propensity for glycogen to form intranuclear inclusions, further investigation was warranted. While the periodic acid–Schiff reaction did not stain the inclusions, we did not rule out the presence of intranuclear glycogen because traditional paraffin processing of tissues can result in glycogen loss. Lectin binding using ConA for glycogen localization confirmed the presence of cytoplasmic glycogen and the absence of intranuclear glycogen.

The positive Feulgen staining, intranuclear location, size, and morphology of the particles suggested a DNA virus belonging to the families *Adenoviridae*, *Herpesviridae*, or possibly an unidentified DNA virus family.

To assess the health of this population and to document the distribution and prevalence of gill epithelial nuclear hypertrophy, 630 wild soft-shell clams from 18 locations within Maryland's Chesapeake Bay collected from 2005 to 2009 were examined for evidence of viral, parasitic, bacterial, neoplastic, or other pathological conditions by histopathology, special staining techniques, and transmission electron microscopy.

Intranuclear virus-like inclusions, present within gill epithelial cells, were observed in 84.53% of examined clams. *Perkinsus* spp. were diagnosed by histopathology in 20.19% of clams and in 54.13% of clams by Ray's thioglycollate test.

Unidentified pyriform ciliates and trichodinid ciliates were present in the gills at prevalences of 48.64% and 15.63%, respectively. Cestodes were observed in 1.43% of examined clams and copepods in 0.16%. Rickettsia-like organisms were commonly observed in the digestive gland, with a prevalence of 74.09%. Bacteria were present in 10.97% of clams and occurred most commonly in the connective tissue around the rectum. Disseminated neoplasia was diagnosed in 2.23% of clams and a single polyp (0.16%) was observed extending from gill epithelium. Renal concretions were present in 33.62% of clams, hemocytic infiltration in 4.94% and pericardial gland concretions in 0.32%.

Prevalence of the virus-like inclusions was high at most collection sites, indicating that the condition is widespread. However, its significance is yet to be determined. Future studies including cohabitation of infected and non-infected clams with observations on mortality and disease pathogenesis are warranted.

Perkinsus spp. were the most significant parasites identified in this study and were detected in over half of the clams by the thioglycollate assay. In addition to numerous sublethal effects, this parasite can cause mass mortalities and is OIE reportable. Although present in high numbers, the observed ciliates likely do not result in disease. In contrast, cestodes were observed uncommonly, but do have the potential to cause damage to the host. No reports of cestodes in *Mya arenaria* were found in a literature search and these findings may represent the first report.

Rickettsia-like organisms, while commonly reported in *Mya arenaria*, were found at much higher prevalences in this survey compared to previous studies in the Chesapeake Bay. While these organisms are not known to cause mortality, they do likely

reduce the metabolic efficiency of the digestive gland and may compete with the host for nutrients. The gram-positive bacterial rods found in connective tissue around the rectum and intestine have not been previously documented and further studies to identify them and determine their significance are warranted. Large numbers of gram-negative bacteria were observed in the gills of clams from the Chester River during a time when high fecal coliform concentrations were reported, suggesting bacterial loads could possibly be used as an indicator of water quality in these areas.

Only low numbers of clams were affected by disseminated neoplasia in contrast to reports from the mid-1980s and early 1990s that showed prevalences as high as 90%. The reason for the decline in prevalence is not known.

While only four hermaphrodites were observed in this study, three were collected from the Chester River, a site contaminated with phthalates, a group of chemicals known to contain endocrine disrupting agents. Further studies for the presence of these chemicals in the Chester River and its effects on bivalve mollusks may be warranted.

Renal concretions were common and have likely been previously observed in *Mya arenaria*. However, no documented cases were found in a search of the literature. Similarly, no information was found concerning pericardial gland concretions in bivalves. The cause of these lesions and their significance are unknown.

To further characterize the agent causing gill epithelial nuclear hypertrophy, the virome of three clams was analyzed using next-generation sequencing technology (Illumina Genome Analyzer IIx). Next-generation sequencing was employed because it allowed for the identification of viruses present without prior viral sequence knowledge. More than 60 million raw reads were generated. A total of 410 singlets and 301

assembled contigs were obtained and compared to the GenBank non-redundant protein database using BLASTx. A total of 373 sequences with similarity to virus were identified , and represented the families *Asfarviridae*, *Baculoviridae*, *Caulimovirdae*, *Circoviridae*, *Coronaviridae*, *Herpesviridae*, *Irodoviridae*, *Mimiviridae*, *Myoviridae*, *Nanoviridae*, *Nimaviridae*, *Parvoviridae*, *Phycodnaviridae*, *Polydnaviridae*, *Polyomaviridae*, *Poxviridae*, and *Retroviridae*. Sequences with similarity to unassigned viruses, unclassified viruses, unclassified ssDNA viruses, unclassified dsDNA viruses, an unclassified dsDNA phage and uncultured marine viruses were also present. Due to their niche as sedentary benthic filter feeders, *Mya arenaria* may serve as an important species for the monitoring of the aquatic virome.

Because results of light and electron microscopy suggested a DNA virus belonging to either the families *Adenoviridae* or *Herpesviridae*, and because no adenoviruses were identified in the three clam samples, the sequences with identity to herpesviruses were closely examined. Three proteins were identified that had 26% amino acid similarity to the DNA packing terminase subunit 1 of bovine herpesvirus 5. This ATPase subunit is herpesvirus-specific and is conserved in all herpesviruses. A phylogenetic analysis of these herpesvirus sequences showed genetic similarity with other molluskan herpesviruses. An oligonucleotide DNA probe was designed to the DNA packing terminase subunit 1 of herpesvirus using Primer-Blast (NCBI) and the 3′-end labeled with digoxigenin. *In situ* hybridization using the digoxigenin-labeled probe showed localization to the nuclei of clams with GENH, providing further evidence to support the presence of a novel herpesvirus in soft-shell clams.

Results from this study broaden our understanding of pathologic conditions that may be impacting this population, and can be used for the future management of this species.