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Title of Thesis: Susceptibility of Shellfish Aquaculture Species in the Chesapeake Bay and Maryland Coastal Bays to the Ostreid Herpesvirus-1 Microvariants

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ABSTRACT

Title of Document: Susceptibility of Shellfish Aquaculture Species in the Chesapeake Bay and Maryland Coastal Bays to the Ostreid Herpesvirus-1 Microvariants

Mariah Lynn Kachmar

Directed by: Dr. Colleen Burge and Dr. Harold Schreier

The Ostreid herpesvirus 1(OsHV-1) and its microvariants are highly virulent pathogens that cause mass mortalities of oysters and pose a threat to the shellfish aquaculture industry globally. OsHV-1 causes economically devastating mass mortality events up to 100% in the pacific oyster (*Crassostrea gigas*). However, OsHV-1 and its variants lack host specificity and are known to infect a range of bivalve species, such as bay scallops (*Argopecten irradians*), and be carried by the European green crab (*Carcinus maenas*). A recent laboratory study indicates that the eastern oyster (*Crassostrea virginica*) can experience infection and mortality from OsHV-1 which has significant implications for aquaculture species used in Maryland and globally, as eastern oysters have a wide geographic range spanning Northwest Atlantic to Panama and Venezuela. Therefore, determining the susceptibility of economically and ecologically important United States bivalve species to OsHV-1 is an essential step in improving biosecurity and disease management to protect the sustainability of the aquaculture industry. There is a lack of monitoring and research on OsHV-1 on the East coast of the United States, including in eastern oysters grown in the Chesapeake Bay, Virginia, and Maryland Coastal Bays where aquaculture is an important industry for food production,

job security, and restoration efforts. Chesapeake and Maryland Coastal Bay species are already threatened by various parasitic and viral diseases, indicating that they may be vulnerable to OsHV-1. Surveys were conducted in June-August 2021 in the Maryland portion of the Chesapeake Bay to determine the prevalence and viral load of OsHV-1 at five aquaculture farms. Using quantitative PCR, OsHV-1 was not detected at any sites. However, continuous surveillance is crucial in mitigating possible introductions to the area. Experiments conducted at the University of Arizona examined the susceptibility and horizontal transmission of eastern oysters and hard clams. Importantly, it has been shown that OsHV-1 microvariants did not cause mortality or infection in eastern oysters and hard clams through natural infection pathways. However, eastern oysters, when injected with OsHV-1, can transmit the virus. This creates implications for same or similar species cultivated throughout the East Coast and Gulf of Mexico Coasts as well as transport of bivalves to the West Coast. Further experimentation using various family lines and establishment of surveillance programs is necessary to fully manage the spread and impact of OsHV-1 related disease.

SUSCEPTIBILITY OF SHELLFISH AQUACULTURE SPECIES IN THE CHESAPEAKE BAY AND MARYLAND COASTAL BAYS TO THE OSTREID HERPESVIRUS-1 MICROVARIANTS

Mariah Lynn Kachmar
2022

Thesis submitted to the Faculty of the Graduate School of the University of Maryland, Baltimore County in partial fulfillment of the requirements for the degree of Master of Science in Marine Estuarine Environmental Science
2022

DEDICATIONS

I would like to dedicate this master's thesis to all past, present, and future women in science. May we continue to break glass ceilings.

ACKNOWLEDGEMENTS

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I would like to thank all the oyster farms and farmers for their collaboration during my surveys and experiments. It was truly an honor to work with and make connections with them, along with understanding the industry in a more personal way.

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Lastly, I am so grateful for my husband, Anthony Kane, for his constant support, patience, and sacrifices in my pursuit for an advanced degree in marine science. Thank you for standing by my side through this journey.

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CHAPTER 1: BACKGROUND/LITERATURE REVIEW

Historically, the spread and emergence of marine diseases have threatened the shellfish fisheries and aquaculture industries globally causing economically devasting mortality events (Lafferty et al. 2015). Despite this challenge, aquaculture has been growing rapidly in part due to shellfish production contributing greatly to global economies and ecosystems. The industry contributes 17.7 million tonnes of shellfish valued at USD 34.6 billion (FAO 2020). More specifically, on the East Coast of the United States the aquaculture industry has experienced a 97% increase in value since 2013 (USDA 2019). Marine diseases have the potential to negatively impact the economic value of a population by reducing the potential catch resulting from decreased biological productivity or possible risk to human health (Lafferty et al. 2015). The combination of marine disease effects and the desire to continue expanding this industry raises concern and a need for improved management and biosecurity practices (Carnegie et al. 2016; Groner et al. 2016). The highly virulent Ostreid herpesvirus 1 (OsHV-1) and its microvariants (μVars), have contributed to this threat within the shellfish aquaculture industry globally (Friedman et al. 2020; Burge et al. 2007). The shellfish industry in Maryland and more broadly on the US East Coast has been historically threatened by diseases such Dermo disease (caused by *Perkinsus marinus*), MSX disease (*Haplosporidium nelsoni*), and QPX disease (*Mucochytrium quahogi*; Geraci-Yee et al. 2021) in bivalve species. Scientists and members of the shellfish industry have expressed concern over the potential threats of the OsHV-1 μVars. The question has emerged whether OsHV-1 may have significant impact on the aquaculture industry in Maryland and the East Coast forming the focus of my master's thesis.

What is the Ostreid Herpesvirus-1?

In 1991, herpes-like viral particles associated with high mortality were first detected in larval Pacific oysters, *Crassostrea gigas*, in France and New Zealand due to mass mortality events (80-90%), followed by mortality of spat and juveniles in 1993 (Renault et al. 1994a, b).The OsHV-1 genome was later sequenced from infected larval Pacific oysters from France in 1994 and was classified within the *Malacoherpesviridae* family in the order *Herpesvirales* (Davison et al. 2005, 2009). This genomic sequence is known as the OsHV-1 reference genome (Segarra et al. 2010, OIE 2019). OsHV-1 outbreaks in Pacific oysters, have occurred in Europe (Nicolas et al. 1992, Renault et al. 1994), Australia (Hine and Thorne 1997), New Zealand (Hine et al. 1992), Asia, the United States (Tomales Bay, CA) (Friedman et al. 2005) and Mexico (Vásquez-Yeomans et al. 2004).

OsHV-1 μVar, a new genetic variant of OsHV-1, emerged in 2008 in France causing more severe and economically devastating mortalities, known in some locations as 'Pacific Oyster Mortality Syndrome' (Whittington et al. 2016; Segarra et al. 2010). OsHV-1 μVar is characterized by a deletion in the microsatellite locust upstream of open reading frame 4 (ORF4) and several polymorphisms in comparison to the reference genome (OIE 2019, Segarra et al. 2010). Similar variants are known as OsHV-1 microvariants or μVars. These microvariant induced mortality events have occurred in parts of Europe (Martenot et al. 2011, 2012; Roque et al. 2012; Dundon et al. 2011; Gittenberger et al. 2016; Batista et al. 2015; Mortensen et al. 2016), New Zealand (Bingham et al. 2013; Keeling et al. 2014), Australia (Jenkins et al. 2013; de Kantzow et

al. 2017), Asia (Renault et al. 2012; Shimahara et al. 2012; Bai et al. 2015), and more recently San Diego, CA (Burge et al. 2021)(Figure 1).

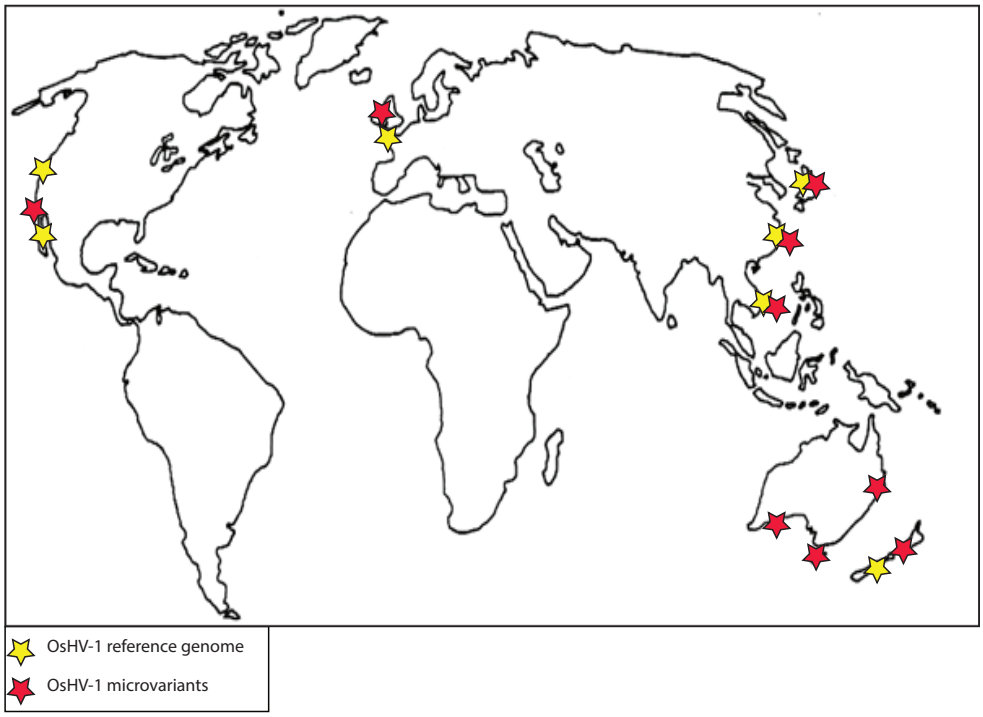

Figure 1: Map of OsHV-1 and OsHV-1 μVar detections globally. Image created by Dr. Colleen Burge.

Why Does OsHV-1 Matter?

OsHV-1 and its variants can cause 40-100% mortality within all life stages of Pacific oysters (Burge et al. 2017; Segarra et al. 2010; Burge et al. 2007). However, Pacific oysters are not the only species at risk. OsHV-1 and its µVars lack host specificity and are known to infect and kill at least seven bivalve species, including the bay scallop, *Argopecten irradians*, a native species to the Maryland coastal bays and the Chesapeake Bay (Arzul et al. 2001 a & b; Renault and Arzul 2001; Burge et al. 2006; Xia et al. 2015, Kim et al. 2019). It can also be carried and transmitted by the European green crab, *Carcinus maenas* (Bookelaar et al. 2018), an invasive species both on the West and East Coasts of the United States (Carlton and Cohen 2003 and Grosholz and Ruiz 2002).

A recent study, Friedman et al (2020), observed that some genotypes of the eastern oyster (*C. virginica*) appear to be susceptible to OsHV-1 µVars. The study examined three important US oyster species (*C. gigas, C. sikamea, and C. virginica*) and exposed them to two OsHV-1 µVars (the France µVar and the Australia µVar) via injection. Two important eastern oyster stocks on the US East Coast were used in this experiment that are selected for either high (DEBY) or low (LOLA) salinity performance and were acclimated to 20-21ppt. One of two eastern oyster stocks exposed, DEBY, experienced 10% mortality (Figure 2), and high viral load was detected in individuals that died (overall 1000X higher viral copy numbers were detected in dead oysters of all three species compared to survivors (Figure 3). The ability for microvariants to infect and induce mortality in eastern oysters has significant implications for native species in Maryland's growing aquaculture industry. However, the question remains if natural

infection, or transmission through water-exposure via filtration of the virus rather than through direct injection, and mortality can occur within eastern oysters.

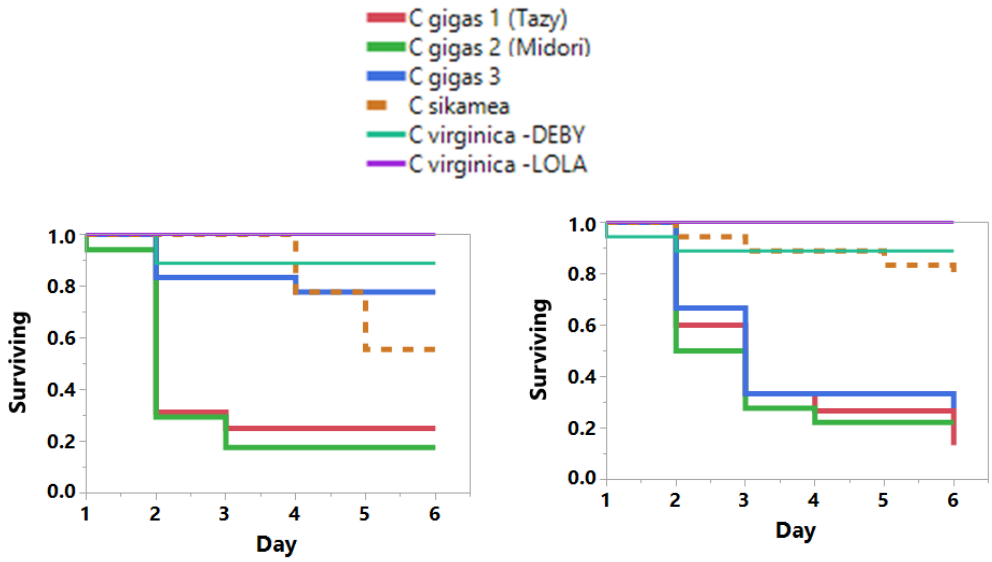

Figure 2: Survival of 6, U.S. oyster stocks and species to OsHV-1 µVar challenges from Friedman et al 2020. Left: OsHV-1 µVar French, Right: OsHV-1 µVar Australia. *Cg*=*C. gigas*, *Cs*=*C. sikamea*, *Cv*=*C. virginica*. *Cg* 1=Tazmania, *Cg* 2=Midori, *Cg* 3=Willapa, *Cv* 1=DEBY, *Cv* 2=LOLA. From Friedman et al 2020.

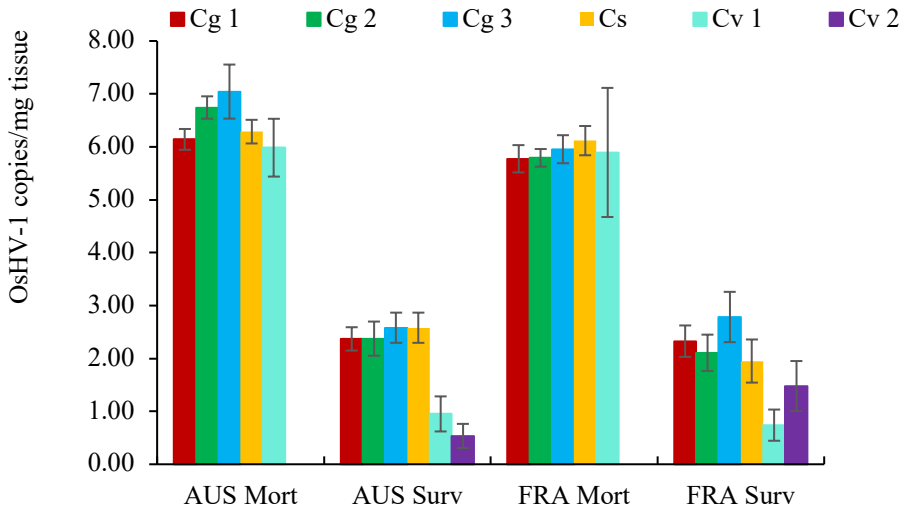

Figure 3: Log transformed qPCR copy numbers (±SE) for oysters injected with the Australian (AUS) or French (FRA) OsHV-µVar. *Cg=C. gigas*, *Cs=C. sikamea*, *Cv=C. virginica*. Cg 1=Tazmania, Cg 2=Midori, Cg 3=Willapa. From Friedman et al (2020). Surv = survivor. Mort = mortality/dead

Maryland Aquaculture and Disease

In Maryland, USA, shellfish aquaculture is an important and growing industry providing food, job security, and resources for reef restoration. Between 2013 and 2018, the average growth rate of the industry was 24%. As of December of 2020, 7,593 acres in the Maryland portion of the Chesapeake and Maryland Coastal Bays were used to cultivate oysters (Van Senten, et al. 2019; Hood et el. 2022) demonstrating the sheer scale of the industry. Eastern oysters, *Crassostrea virginica*, are the primary species cultivated in Maryland aquaculture with a value of USD 12.2 million in 2020 (MDDNR, Tarnowski 2022). The second most important aquaculture species in Maryland is the hard clam (*Mercenaria mercenaria*) valuing 5.5 million USD in 2018 (USDA 2018). Data specifically for Maryland was not disclosed in this report.

Although the shellfish aquaculture industry is a vital component to the Chesapeake and Maryland Coastal Bay's economic and ecological success, historically and presently, it has been threatened by disease. Key species in the industry have been impacted by various diseases. Eastern oysters have been affected by two parasitic diseases, Dermo (*Perksinsus marinus*) and MSX disease (*Haplosporidium nelsoni*). Dermo was first reported in the late 1940s (Andrews and Hewatt 1957; Burresson et al. 1996) but caused great devastation to the population spreading throughout the Chesapeake Bay in the late 1980s due to a prolonged drought (Andrews 1988; Burreson et al. 1996). MSX, believed to be an introduced pathogen (Burreson et al. 2000), impacted the industry in the late 1950s through the 1960s causing ~70% decrease in oyster harvest (Andrews and Burreson 1988). These diseases are heavily driven by environmental conditions. MSX disease infections are acquired at temperatures

exceeding 20°C and can persist at temperatures between 5-20°C and salinities of 15-20ppt. Dermo disease persists at temperatures exceeding 20°C and salinities above 12-15ppt (VIMS, 2022). Dermo and MSX persist significantly throughout oyster reefs and tributaries in the Chesapeake Bay today. Both Maryland and Virginia monitor the diseases through yearly fall surveys that are published to share with managers, industry, and the public (MDDNR, Tarnowski 2022; Carnegie and Burreson 2009). Hard clams have faced losses of 80-95% due to QPX disease (caused by a parasitic protozoan) and was first described in the Chesapeake and Coastal Bays in 1996 (Ragone Calvo et al. 1998, Smolowitz et al. 1998).

Disease Emergence and Spread

Emerging and re-emerging infectious disease outbreaks progress and persist through disruptions within the host-pathogen-environment relationship. Changes or disruptions occur through natural ecological and evolutionary processes or anthropogenic influences. (Reviewed by Harvell et al.1999, Burge et al. 2018, and Glidden et al. 2021). Disease emergence can occur due to various factors such as pathogen transmission (horizontal or vertical), population density and diversity of host or pathogen, host range or pathogen specificity, and the ability to live or spread within an environment (Roy et al. 2009). Movement of host organisms or equipment, water intake, feed, or infected broodstock are a common cause of infectious disease emergence creating increased "microbial traffic" (Morse, 2004, Harvell et al. 1999, Lafferty et al. 2015, Ben-Horin et al. 2015), allowing pathogens to persist in new environments if conditions are favorable (temperature, salinity, pH, etc.) (reviewed by Burge et al. 2017). Mutations within the pathogen also may occur allowing for them to widen host range or become more virulent

(Alonso et al. 2001; Gillings, 2016). For example, a hypervirulent *Perkinsus marinus* phenotype that is believed to have emerged sometime between 1983 and 1990 was associated with the prevalence and intensity of disease and mortality during major historical Dermo disease outbreaks. This phenotypic change is hypothesized to be an ecosystem response due to reduced oyster abundance and competition with Haplosporidium nelson (MSX). Although it is hypothesized that genetic differences are the basis of the phenotypes, these do not represent species level differences (Carnegie et al. 2021).

Climate changes can influence the productivity and health of marine ecosystems and basic biological properties of marine populations, therefore, increasing their susceptibility to infectious diseases (Harvell et al. 1999, Burge et al. 2014). Climate related drivers of marine disease include temperature, salinity, ocean acidification, and storm frequency (Burge et al. 2014). Compared to previous reports, Maryland experienced a 22% lower harvest of cultured oyster bushels in 2018 due to dramatic decreases in salinity in the Chesapeake Bay. Decreases in salinity are inherently stressful to oysters, increasing mortality and lowering growth rates, prolonging oysters' exposure to infection before they can reach market size (van Senton et al. 2019). Additionally, changing salinities heavily influence MSX and Dermo disease which can become severe during drought where salinities are high (Ford et al. 1985). Higher salinities are also proposed to be associated with higher mortality in Pacific oysters exposed to OsHV-1 (Fuhrmann et al. 2016).

Mortality events caused by OsHV-1 have been primarily dependent on life stage of the host organism and seawater temperatures (Renault et al. 2014; Petton et al. 2013).

OsHV-1 and its variants are particularly virulent when seawater temperatures exceed 16°C (Petton et al. 2013; Burge et al. 2006; Renault et al. 2014, Martenot et al. 2015), and recent laboratory studies indicated 18°C may be necessary for virus replication (de Kantzow et al. 2019); most laboratory studies are conducted at 22°C (de Kantzow et al. 2016). Subclinical or latent infections of OsHV-1 can persist in the host (Segarra et al. 2014). OsHV-1 is shed from infected organisms allowing it to be transmittable through trophic interactions and dispersed through currents (Bookelar et al. 2018; Paul-Pont et al. 2013, Schikorski et al. 2011, Pernet et al. 2012, Evans et al. 2014). OsHV-1 transmission is heavily promoted through cohabitation, as aquacultured oysters are typically cultured in close proximity (Burge et al. 2011, Evans et al. 2015).

Combining the current threats of disease, pollution, habitat degradation, shellfish culture conditions, and changing conditions in the bays, with the potential mortality events of OsHV-1, significant declines in native populations and aquaculture production may occur. Resource managers of the Chesapeake and Maryland coastal bays have interest in continuous expansion of the aquaculture industry, especially eastern oysters (National Center for Ocean Science, 2015); however, OsHV-1 may pose a threat to the economic and ecological success of the industry in Maryland and the Mid-Atlantic. Additionally, hard clams are also a native species of interest and given the broad host range of OsHV-1 variants, it is necessary to understand potential impacts to this species.

Significance of Disease Mitigation, Management, and Biosecurity

When diseases become severe or threatening, policy and management become important contributors to the aquaculture industry by implementing science-driven

surveillance, mitigation, and biosecurity practices (Groner et al. 2016, Carnegie et al. 2016). The World Organization of Animal Health (WOAH, formerly OIE) provides standards to control and handle aquatic animal diseases. Marine diseases, like OsHV-1, are classified as an emergency when there are significant disruptions of ecosystem functions, such as removal of keystone species, reduction of ecosystem services, biodiversity, or response to stressors (Groner et al. 2016). They also can be considered an emergency if they affect cultural and social systems (Lafferty et al. 2015)

Managing the health of shellfish populations ideally prevents the spread of pathogens with the goal of restoring, sustaining, or increasing shellfish abundance (Carnegie et al. 2016). Surveillance or monitoring programs are an important piece within management to respond to marine disease outbreaks (Burge et al. 2014, Glidden et al. 2021). Surveillance, which typically involves application of various diagnostic tools in a spatio-temporal sampling protocol, can identify pathogens in clinically unhealthy individuals or determine prevalence of pathogens in both the host and environment. Surveillance is also critical in early detection when clinical signs of infection or disease are not present or when the pathogen is unknown. This can result in pathogen impacts being mitigated before or at the beginning of an outbreak where delays in diagnosis can make management difficult or lead to significant production losses (Groner et al. 2016). Establishment of surveillance programs can also help reveal the distribution of pathogens within host populations that are at high risk of infection (Carnegie et al. 2016). To aid surveillance, increasing the coordination and communication among people studying pathogens impacting marine animal health, farmers, and managers and creating databases can improve effectiveness (Glidden et al. 2021, Groner et al. 2016, Burge et al. 2014).

Mitigation practices involve targeting the host-pathogen-environment relationship, which can be complex, and requires knowledge of the basic biology and ecology of the interactions (Groner et al. 2016, Carnegie et al. 2016, Glidden et al. 2021). Scientific studies focusing on ecology and evolution of pathogens can close knowledge gaps to aid in mitigation and avoiding economic consequences of marine diseases (Burge et al. 2014, Lafferty et al. 2015, Glidden et al. 2021). Some other mitigation examples include but are not limited to culling, biosecurity measures such as proper disposal of infected organisms or prohibiting transport or import of sick or non- native animals to new locations and promoting resistance to diseases through selective breeding programs (Groner et al. 2016, Lafferty et al. 2015, Glidden et al. 2021).

Surveillance for OsHV-1 in the United States, specifically on the East Coast, has historically been lacking. One study to date was published nearly 20 years ago, Friedman et al. (2005), surveying for OsHV-1 in eastern oysters in various locations along the East Coast. The study was conducted in 2002-2003 collecting samples from Louisiana, Florida, Virginia, and Maine and concluded that OsHV-1 was not detected in eastern oysters at any of the sites. While this survey has significance, methods that were available for detection have since improved and continuous surveillance is more effective in mitigation strategies. Further exploration or establishment of a monitoring program may benefit and protect the crucial resource of eastern oysters and hard clams on the Eastern seaboard.

Purpose

The purpose of this master's thesis was to 1) identify aquaculture farms within Maryland willing to test for OsHV-1 and perform testing, in hopes to continue surveillance 2) determine if economically and ecologically important species, including eastern oysters and hard clams, are susceptible to OsHV-1 infection and mortality, and 3) determine if they can also horizontally transmit the virus to naïve *Crassostrea gigas,* a highly susceptible species. The overarching goals of this study were to determine if OsHV-1 has the potential to significantly impact the aquaculture industry in the Chesapeake Bay and generally on the East Coast of the United States and to illustrate the need for enhanced biosecurity and management of marine disease.

CHAPTER 2: METHODOLOGY

OsHV-1 Surveys

Collection

Eastern oyster larvae (n = ~10,000) or juveniles (n=60) were collected during June 7-11th, July 8-15th, and August 23-27th of 2021. See Table 1 for age and size ranges of eastern oysters collected during survey times. Oysters were collected from five aquaculture farms located in the Maryland portion of the Chesapeake Bay: the Potomac River (PT), St. Jerome's Creek (SJ), Patuxent River (PA), Honga River (HR), and Choptank River (CO) (Figure 4). Where feasible, the same broods were followed through the summer; however, mortality events did occur at two sites during July (CO and PA), therefore, a new brood was sampled with any remaining animals from their stocks if possible. PA sent animals of the same age from an adjacent farm and this brood was followed July and August. Mortality events were not disease related in the stocks through disease screenings according to communications with the farms.

Table 1: Size and quantity of eastern oysters collected from 2021 OsHV-1 survey sites in Maryland. * Farms that experienced mortality events.

Date	*Location*	*Size range*	*n*
June 7-11th, 2021	Choptank River	7-day old larvae	~10,000
	Honga River	7-day old larvae	~10,000
	Patuxant River	7-day old larvae	~10,000
	Potomac River	5-10 mm	60
	St. Jerome's Creek	5-10 mm	60
July 8-15th, 2021	Choptank River*	Spat on shell 2-5 mm	36
		7-day old larvae	~10,000
	Honga River	1-2 mm	60
	Patuxant River*	2-5 mm	60
	Potomac River	10-25 mm	60
	St. Jerome's Creek	10-15 mm	60
August 23rd-27th, 2021	Choptank River	Spat on shell 2-5mm	60
	Honga River	5-10 mm	60
	Patuxant River	5-10 mm	60
	Potomac River	15-30 mm	60
	St. Jerome's Creek	25-30 mm	60

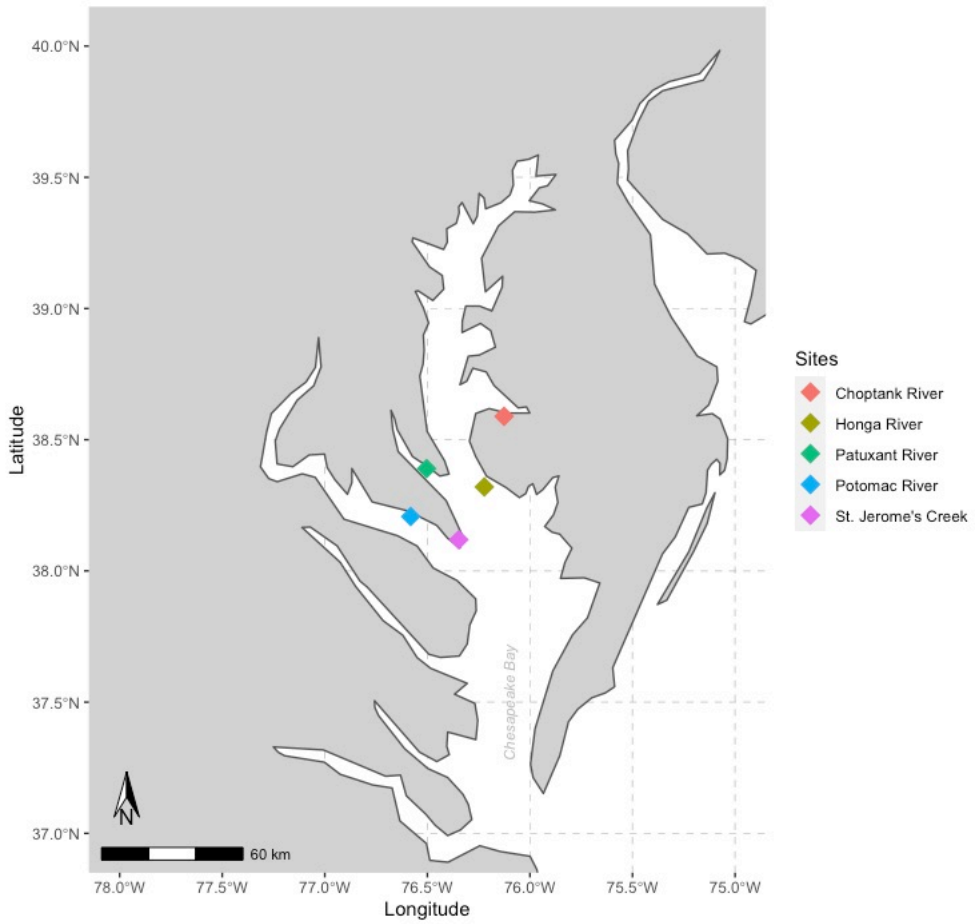

Figure 4: Survey sample sites in the Maryland portion of the Chesapeake Bay.

DNA Extraction and Quantification

Gill and mantle tissue were dissected from individual juvenile oysters and pooled (n= 5 oysters per pool, n= 12 pools per site and date). Larval samples were distributed among three pools containing ~3,000 larvae per pool. Total DNA was extracted from approximately 25-30 mg of juvenile oyster tissue and 30-50 mg of whole larvae using the ZYMO research Quick-DNA Miniprep Plus kit following the manufacturer's protocol (Solid Tissues method). Whole larvae were ground using a mortar and pestle to help the breakdown of the tissue.

The quantity of OsHV-1 DNA from all dates and sites were quantified using an OsHV-1-specific qPCR assay as a proxy for viral infection. Using the method described in Burge & Friedman (2012) modified by Burge et al. (2020), OsHV-1 ORF100 was targeted using primers ORF 100F (5′-TGA TGG ATT GTT GGA CGA GA-3′) and ORF 100R (5′-ATC ACA TCC CTG GAC GCT AC-3′) and a standard curve from 3 to $3x10^7$ copies was used to quantify viral DNA. 'No template' controls using UltraPure DNase/RNase Free distilled water in place of a DNA sample were added to each plate in replicates of three. A 20 µl reaction containing 10 µl of Fast SYBR Green Master Mix, 15 µg of BSA, 0.4 pmol of each primer, and 2 µl of DNA was used per sample. All standard curves were done in triplicate and samples were processed in duplicate using the QuantStudio 3 Real-Time PCR system with a limit of detection of 3 copies per reaction. Cycling conditions for each qPCR reaction included: 95°C for 20s followed by 40 cycles of 95°C for 3s and 60°C for 30s.

'Positive controls' were made by selecting random samples from each time and location and inoculating the samples with $3x10^4$ copies/2µl of OsHV-1 DNA that was

cloned in bacterial plasmid to show that OsHV-1 is amplifiable and assess any amplification inhibition during qPCR reactions. If inhibition occurred, 3 random samples with the greatest inhibition were purified using Promega Wizard DNA clean-up system (REF A7280) following the protocol DNA purification without a vacuum manifold. These samples were then inoculated with varying concentrations of plasmid ($3x10^2$ copies/2µl ,$3x10^4$ copies/2µl, $3x10^7$ copies/2µl) to determine if inhibition still occurred and to what percentage during qPCR reaction.

Environmental Data

Monthly mean temperatures and salinities for the sample site regions were collected using the Maryland Eyes on the Bay long term data monitoring program data https://eyesonthebay.dnr.maryland.gov/). Stations are CB5.1, LE2.3, LE2.2, EE2.1, and LE1.2.

Dose Response Challenge

Oysters and Husbandry

Animals in the dose response challenges were provided from various sources. Pacific oysters (~30 mm) were produced and provided by an oyster farm in Humboldt Bay, California area, where OsHV-1 has never been detected (Elston unpub data, Burge unpub data). Seed Pacific oysters (~5-8 mm) were produced by the University of California Davis, Bodega Marine Laboratory (BML) from a spawn from the Molluscan Broodstock Program. Eastern oysters (5-8mm) were produced by Rutgers University (NJ); animals were first shipped to the UC Davis BML where they were housed for two weeks on ambient seawater (~35 ppt, 15ºC). Hard clams (5-8mm) were produced by

Virginia Institute of Marine Science (VIMS), Eastern Shore Laboratory. All animals were shipped overnight on ice to the University of Arizona, Aquaculture Pathology Laboratory (UA APL) in Tucson, Arizona. All animals were allowed a ~24-hour acclimation period prior to injection or exposure in 4L tanks containing 2L of 32ppt 50% natural seawater and 50% artificial sea water. All artificial seawater was made with Crystal Sea Marine Mix, 150-gallon mix dissolved in distilled water available at the UA APL (Tuscon, AZ). Natural seawater (~35ppt) was UV sterilized, filtered, and transported from BML. Laboratory conditions were kept at 22ºC. Seed animals were fed *ad limitum* *Nannochloropsis sp*., provided by BML, upon arrival and daily prior to their exposure.

OsHV-1 Inoculum

Viral inoculums were used from the French µVar (Burge et al. 2020) and OsHV-1 San Deigo µVar (SD) (Burge et al. 2021). Viral homogenates from the French µVar were previously created as described in Burge et al. 2020; cryopreserved stocks were held at the UA APL at -80ºC following methods of Kirkland et al. (2015). For the SD µVar, no cryopreserved inoculums were available. Therefore, inoculum was created from moribund Pacific oysters (stored at -80ºC) collected from the initial transmission study described in Burge et al. (2021).

Standard methods were used to create viral inoculum. Briefly, gill and mantle tissue from 8-10 oysters were excised, homogenized, and passed through 0.22 µm filters to create inoculum as described in Burge and Freidman (2012) and Burge et al. (2020). Specifically, while on ice, ~1 g of gill and mantle tissue were added to 50 mL of filtered natural seawater and homogenized using a handheld tissue homogenizer for ~1 minute and then centrifuged at 150 x g for 10 min. The homogenate was then filtered through a

20µm screen followed by filtration through a 0.22µm Millipore Express ® PLUS Membrane to the "viral size" preparation. The inoculum was stored at 4ºC until oysters were ready for injection. OsHV-1 viral copy numbers were quantified by extracting 200 µl of inoculum or homogenate using the ZYMO Research Quick DNA Miniprep plus kit (Biological fluids method). DNA was amplified using the OsHV-1 specific qPCR (described above) to determine the viral load of the inoculum. To ensure the infectious nature of this inoculum, susceptible Pacific oysters were injected and monitored for 72 hours to observe mortality.

Exposed Seawater

Pacific oysters (*C. gigas*) ~30 mm in size were used to create 'exposed or exposure seawater' (Agnew et al. 2020) in the following manner. Post acclimation, animal shells were notched near their adductor muscle using a metal file to prepare for injection. Oysters were injected using inoculum ($1x10^6$ total OsHV-1 viral copies) into the adductor muscle using a 28-gauge needle. Animals were given 10-15 min to acclimate prior to placing them back into their appropriate tanks. Approximately 24 hr incubation was allowed for animals to shed the virus (Agnew et al. 2020). OsHV-1 specific qPCR was carried out post 24 hr incubation to determine the viral concentration/load in the water (Figure 5). This was done for both SD and FRA variants separately; exposures were done on separate days to reduce cross contamination.

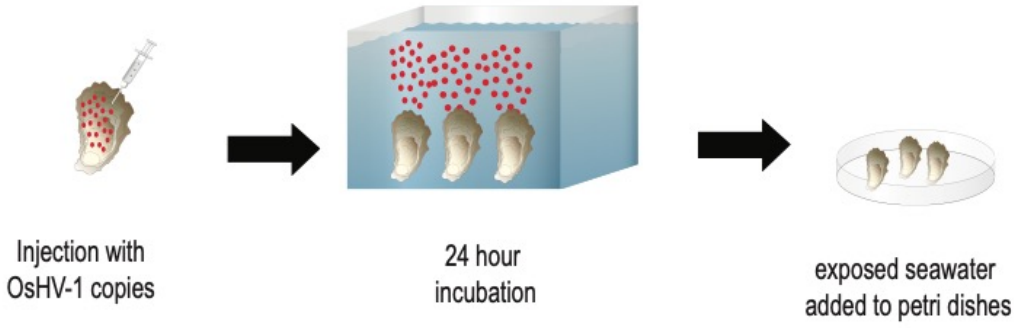

Figure 5: Creation and application of virus-exposed seawater. Animals injected with OsHV-1 filtrates were placed into a tank for 24hr incubation. This water was then used to expose naïve spat bivalves in petri-dish plates.

Lethal Dose 50% (LD_{50})

A lethal dose 50% or LD_{50} was done to determine which viral concentrations would cause mortality in 50% of the sample population and how much virus was required to promote infection and mortality. OsHV-1 qPCR assays showed that viral concentrations in exposed seawater were at $1X10^6$ viral copies/mL for both the SD and FRA variants. Tenfold serial dilutions were created from $1X10^6$ viral copies/mL to $1X10^4$ viral copies/mL of the exposed seawater. Standard antibiotic concentrations of 15 mL antibiotics /L (Penicillin-Streptomycin- 10,000 units penicillin and 10mg streptomycin/mL, 0.1 µm filtered) were added to each viral concentration prior to exposing animals. Volumes of 1L were made for each concentration, therefore, 15mL was added to each equating to 0.9mL per deep well petri-dish.

Species exposed to the FRA and SD OsHV-1 microvariants were eastern oysters (*C. virginica*, NJ), hard clams (*M. mercenaria*), and Pacific oysters (*C. gigas*, BML, positive control) all 5-8 mm in size. Approximately 180 animals total for each species were exposed to each OsHV-1 variant. For each variant (SD and FRA), animals were exposed in 125 mm deep well petri dishes in replicates of 3 (60 n total, 20 n each dish) per viral concentration. 60 mL of exposed seawater of the appropriate concentration containing 15mL antibiotics /L (0.9mL per petri dish) was added to each dish. Hard clams had 2 replicates for the $1X10^4$ viral copies/mL due to shortage of animals available. Controls were in replicates of 3 (20 n per dish) and were placed in filtered natural seawater containing antibiotics (Figure 6).

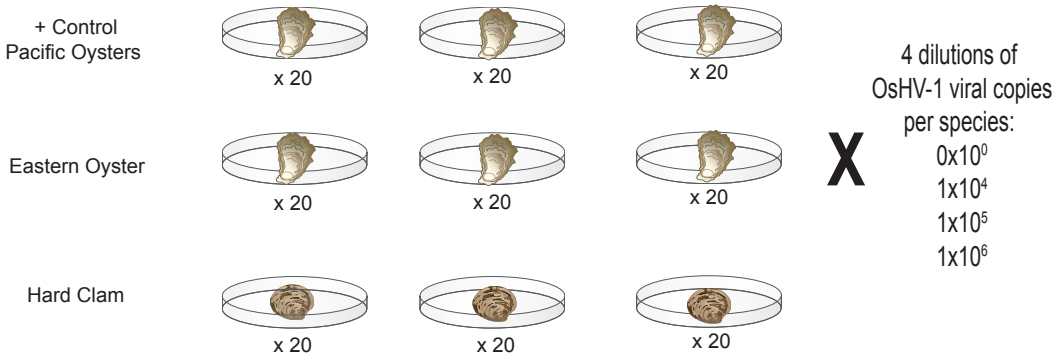

Figure 6: Petri-dish experimental design for the Dose response challenge/Lethal Dose 50% (LD50) study.

Sampling

Following standard methods, mortalities were counted starting at 72hrs post exposure (days 3-7) by carefully opening the petri dishes and probing to assess which animals that were gaping following standard methods of Divilov et al. (2019) and Agnew et al. (2020). All dead animals were removed from dishes, placed into clearly labeled whirl packs, and frozen at -80°C. On day 2 of the experiment all animals were fed in their petri dishes 1mL of algae (C-ISO or *Isochrysis sp*.). On day 3 all animals received a water change of 60mL of 50/50 natural/artificial seawater with standard concentrations of antibiotics. On day 7, mortalities, all remaining surviving animals, and controls were sampled to terminate the experiment.

DNA Extraction and OsHV-1 Quantification

DNA from random exposed surviving animals exposed to $1x10^6$ viral copies/mL (n=15), all mortalities across all viral doses ($1x10^6$, $1x10^5$, $1x10^4$), and controls (n=15) for both FRA and SD exposures was extracted using the ZYMO research Quick-DNA Miniprep Plus kit following the manufacturer's protocol (Solid Tissues method). Gill and mantle tissue were dissected from individual oysters. Total DNA was extracted from approximately 10-30 mg of oyster or hard clam tissue. DNA was amplified and quantified using OsHV-1 specific qPCR as described above.

Characterization

Two PCR assays targeting ORF4 using primers C2 (5′-CTC TTT ACC ATG AAG ATA CCC ACC-3′) and C6 (5′-GTG CAC GGC TTA CCA TTT TT-3′)and OFR100 using primers targeted using primers ORF 100F (5′-TGA TGG ATT GTT GGA CGA GA-3′) and ORF 100R (5′-ATC ACA TCC CTG GAC GCT AC-3′) were used for DNA sequence analysis

of OsHV-1 detected in hard clam samples from the DOSE RESPONSE with amplified copies of OsHV-1 DNA greater than 3 copies for both FRA (n=3) and SD (n=1) exposure trials. Dead Pacific oysters with high OsHV-1 copy numbers from each exposure (FRA and SD) were used as positive controls. Each sample was done in duplicate with a negative control reaction. A 50 µl reaction was used per sample containing (Promega GoTaq 9PIM300), 1X PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.3 pmol of each primer, 2.5 U DNA polymerase and 4 µl of DNA (Segarra et al., 2010; OIE, 2019; Burge et al 2021). Cycling conditions for ORF100 assay included: 95°C for 2 minutes followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 30 sec with a final extension of 72°C for 5 min ending in 4°C indefinitely. Cycling conditions for ORF4 (C2/C6) assay included: 95°C for 2 minutes followed by 35 cycles of 95°C for 1 min, 60C for 1 min, and 72°C for 1 min with a final extension of 72°C for 5 min ending in 4°C indefinitely. Samples were visualized using gel electrophoresis to ensure one band of expected size was amplified (Segarra et al. 2010, Burge et al. 2020). All PCR products were submitted to the Institute of Marine and Environmental Technology Bioanalytical Services Laboratory in Baltimore, MD for Sanger sequencing.

Statistical Analysis

Statistical analyses were performed using R version 4.1.2. Kaplan-Meier curves with Log-rank chi-square tests and Cox proportional hazard ratios were generated using the survival (Therneau T 2022, Therneau and Grambsch 2000) and survminer (Kassambara et al. 2021) packages to investigate differences in survival probability between species exposed to each OsHV-1 variant, virus, and viral dose. Figures were generated using the ggplot2 (Wickham 2016) and ggpubr (Kassambara 2020) package.

Differences among dose, virus, and species for tissue concentrations were tested individually with a Kruskal-Wallis test ($p < 0.05$) followed by a multiple comparison using the Wilcoxon Rank Sum Test with a "holm" probability adjustment (Kabacoff 2015, Agnew et al 2020). All data and statistical code are available on Figshare (Kachmar et al. 2022)

Vector study

Oysters and husbandry

Bivalves used in the vector study were provided from various sources. Pacific oysters (~20 mm and spat 5-8 mm) were produced by a farm in Humboldt Bay, California. Eastern oysters (30-40 mm) were provided by the NJ Aquaculture Innovation Center at Rutgers University. Hard clams were provided by an aquaculture farm which wishes to remain anonymous. All animals were allowed a 24 hr acclimation period upon arrival to the laboratory prior to injection or exposure. Animals were shipped to UA APL in Tucson, Arizona. Juvenile animals were placed in separate 4 L tanks containing 2 L of 30 ppt artificial sea water made using Crystal Sea Marine Mix dissolved in deionized water available at UA APL. Spat were placed into 4 L tanks containing 2 L of 30 ppt 50% natural and 50% artificial seawater. Laboratory conditions were held at 22°C. Pre experimental samples (60 n) were taken per species for further analysis post experiment. Spat were fed *Nannochloropsis sp*. upon arrival.

OsHV-1 Inoculum

The OsHV-1 µVar FRA inoculum used is described above. Due to low availability of the OsHV-1 µVar SD homogenate created above, a new homogenate was created from oysters, as described above (*Dose Response Challenge: OsHV-1 Inoculum*).

Briefly, Pacific oysters that were moribund two days post injection with OsHV-1 µVar SD were used following methods developed by Kirkland et al. (2015) and Burge et al. (2020). The methods for creation of the 0.22 µm tissue homogenate are described above.

Injections

Pacific oysters (20-25mm), hard clams (~40mm) and eastern oyster juveniles (40-50mm) were used to create 'exposed seawater' (Agnew et al. 2020) for mimicking horizontal transmission. Post acclimation, oyster shells were notched in approximation to their adductor muscle using a metal file to prepare for injection. Oysters were injected using inoculum ($1x10^6$ total viral copies) into the adductor muscle using a 28-gauge needle. Hard clams were injected into the umbo perpendicular to vascular tissue. Animals were given 10 min to acclimate prior to placing them into their appropriate tanks. Animals were then placed into 4 L tanks containing 2 L 50% natural seawater and 50% artificial seawater and 7.5 ml antibiotics /L (Penicillin-Streptomycin- 10,000 units penicillin and 10mg streptomycin/mL, 0.1um filtered) totaling 15mL per tank in replicates of 3 per species. Eastern oysters and hard clams had 10 individuals per replicate, while Pacific oysters had 20 individuals per replicate due to small size. Approximately 24-hr incubation was allowed for animals to shed the virus. OsHV-1 specific qPCR was run post 24-hr incubation to determine the viral concentration or load of the water from each Pacific oyster tank to confirm a positive control. Water samples were also taken from the tanks containing eastern oysters and hard clams for later analysis. This was done for both SD and FRA variants separately. Control animals were sham inoculated and were in replicates of 2 tanks per species. (Figure 7)

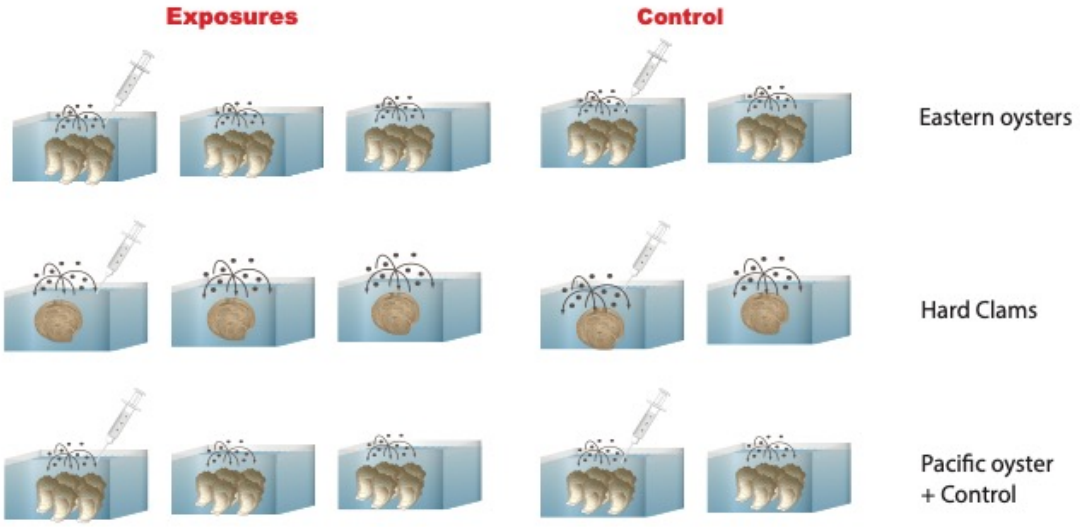

Figure 7: Vector study tank experimental design for injected animals.

Naïve Pacific oyster Petri-dish Exposures

Pacific oyster spat (5-8 mm) were used for naïve individuals as a proxy for horizontal transmission due to their high susceptibility to OsHV-1 infection. Oysters were placed into deep well petri dishes in replicates of 9 with 20 animals (180n) per dish for each 'exposed seawater' species (eastern oysters, hard clams, and Pacific oysters). Control animals were in replicates of 3 with 20 animals per dish (60n) for each control vector species. Controls were sham exposure using water from the control tanks. 60 ml of water was added to each petri dish with final concentrations of antibiotics of 15 ml antibiotics /L (Penicillin-Streptomycin- 10,000 units penicillin and 10mg streptomycin/mL, 0.1µm filtered) totaling 0.9mL per petri-dish. 60 ml of exposed seawater with standard concentrations of antibiotics from the injected animals was then added to the appropriate petri-dishes (Figure 8).

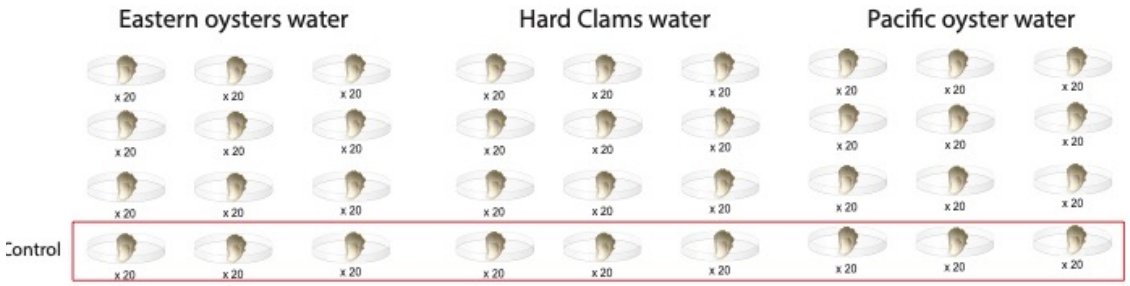

Figure 8: Vector study plate exposure experimental design using naïve Pacific oyster spat exposed to ‘exposed seawater’ from eastern oysters, hard clams, and Pacific oysters.

Sampling

72 hr post exposure mortalities were counted each day (days 3-7) by carefully opening the petri dishes and probing animals that were gaping. All dead animals were removed from the dishes, placed into clearly labeled whirl packs, and frozen at -80°C. On day 3 of the experiment all animals were fed in their petri dishes 1mL of algae (*Nannochloropsis sp.*). On day 4 all animals received a water change of 60mL of 50/50 seawater with 15mL antibiotics /L seawater. All remaining animals (both mortalities and survivors for exposures and controls) were sampled on day 7 to terminate the experiment. Vector animals or injected animals in tanks were sampled and monitored in the same manner daily (days 1-7).

DNA Extraction and Quantification

DNA was extracted from water samples of 200 µl taken from injected eastern oyster, Pacific oyster, and hard clam tanks using the ZYMO Research Quick-DNA Miniprep Plus kit following the manufacturer's protocol (Biological Fluids method). Gill and mantle tissue (20-35mg) was dissected from random individual oyster samples exposed to the eastern oyster, Pacific oyster, and hard clam 'exposed seawater'. Both dead and surviving animals were sampled and dissected (Table 2). Control samples were pooled (n= 5 oysters per pool, n= 3 pools per treatment). DNA was extracted using the ZYMO Research Quick-DNA Miniprep Plus kit following the manufacturer's protocol (Solid tissue method). DNA was amplified and quantified using OsHV-1 specific qPCR as described above.

Table 2: Quantity of animals sampled from the vector study for qPCR analysis. Mort = Mortality; Surv = Survivors

Virus	*Treatment*	*n*	*Mort*	*Sampled*	*Surv*	*Sampled*
SD control	*C. gigas*	60	0	0	60	15
	C. virginica	60	0	0	60	15
	M. mercenaria	60	0	0	60	15
FRA control	*C. gigas*	60	0	0	60	15
	C. virginica	60	0	0	60	15
	M. mercenaria	60	0	0	60	15
SD Exposure	*C. gigas*	182	178	45	4	2
	C. virginica	182	120	30	62	15
	M. mercenaria	180	1	1	179	45
FRA Exposure	*C. gigas*	180	180	45	0	0
	C. virginica	182	20	20	162	40
	M. mercenaria	180	0	0	180	45

Statistical analysis

Statistical analysis and generation of graphs were conducted as described above. Differences in survival probability of Pacific oysters exposed to 'exposed seawater' from eastern oysters, hard clams, and Pacific oyster injected with the SD variant or FRA variant were examined. Differences among treatment, virus, and species for tissue concentrations were tested, as described above.

CHAPTER 3: RESULTS

OsHV-1 Field Surveys

OsHV-1 could not be detected (i.e., below a level of detection of 3 copies/mg tissue) for any of the five sites during the three sample periods. The possibility that the absence of OsHV-1 amplification may have been due to qPCR inhibitors was tested by supplementing selected samples with a known concentration of OsHV-1 DNA (see Methodology). While 26-44% inhibition of amplification occurred for individual samples (Figure 9), this could not account for the absence of OsHV-1 amplification in any sample.

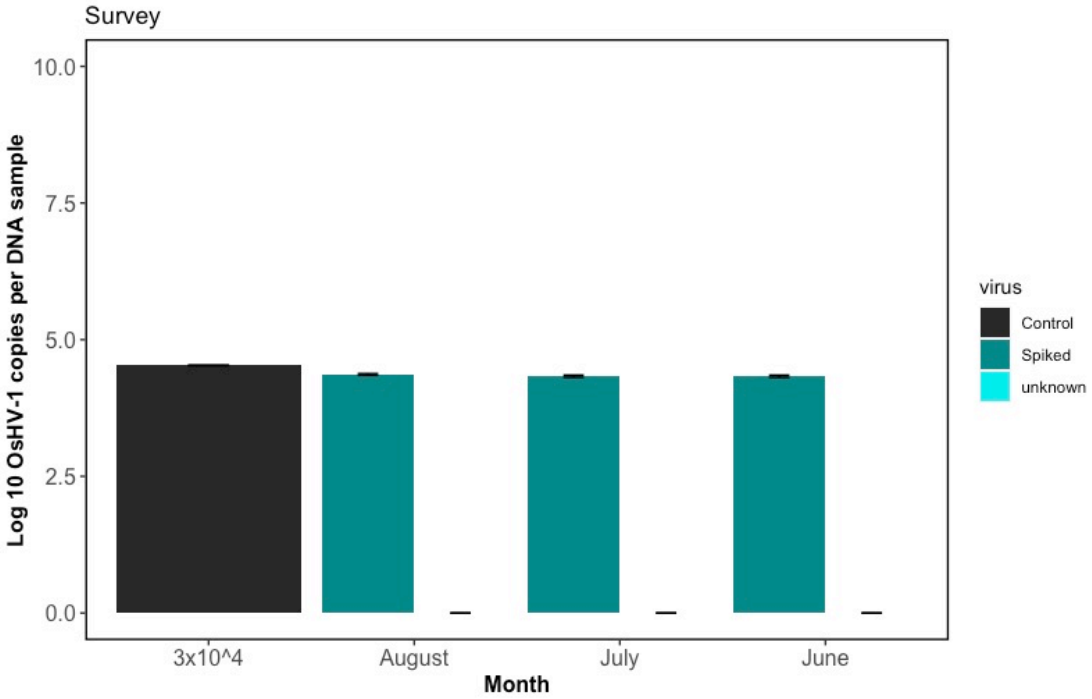

Figure 9: Top: Log10 transformed OsHV-1 copies/DNA sample for survey samples by month; Control = $3x10^4$ OsHV-1 ORF100 plasmid, Spiked = DNA template + $3x10^4$ OsHV-1 ORF100 plasmid, unknown = DNA samples from collected oysters

Environmental data

Table 3: Mean water temperature for 2021 obtained from Maryland Eyes on the Bay long term monitoring program data for the Honga River, St. Jerome's Creek, Potomac River, Choptank River, and Patuxent River.

	Honga River	St. Jerome's Creek	Potomac River	Choptank River	Patuxent River
May	15.8°C	16.7°C	16.6°C	16.8°C	17.9°C
June	22.5°C	22.5°C	24.6°C	24.8°C	25.3°C
July	27.5°C	26.9°C	27.2°C	27.9°C	28.8°C
August	26.8°C	27.1°C	26.5°C	27.3°C	28.5°C

Table 4: Mean surface seawater salinity (ppt) for 2021 obtained from Maryland Eyes on the Bay long term monitoring program data for the Honga River, St. Jerome's Creek, Potomac River, Choptank River, and Patuxent River.

	Honga River	St. Jerome's Creek	Potomac River	Choptank River	Patuxent River
May	13	15.34	9.4	10.3	10.2
June	11.5	14.59	9.3	10.3	10
July	13	15.81	10.7	11.7	11.2
August	12.9	17.11	12.6	11.4	12.7

Dose Response Challenge

<u>Statistical analysis</u>

Survival analysis based on mortality counts from day 3 through day 7 of the experimental challenge indicated that survival among all three species was significantly different (Figure 10?; Chi sq = 88.9, df= 2, $p<0.001$). Survival probability was observed for each species individually and at separate viral doses. Among all three species, Pacific oysters were the only species to have significantly decreased survival probability when exposed to both microvariants at all viral doses (Figure 10a & d, Table 5) Eastern oysters had the highest survival probability (100%) for both microvariants and across all viral doses (Figure 10b& e). Hard clams experienced high survival probability for both microvariants and across all viral doses ranging from 100%-96.6%. Throughout the experiment only one individual control experienced mortality for the hard clams (Figure 10c & f). Survival probability and risk of mortality between exposure to either the FRA μvar or SD μvar was significantly different from controls (Figure 2; Ch sq = 16.9, df = 2, p $p<0.001$), along with a significant difference between viral doses (Figure 3; Chisq= 78.2, df= 4, $p<0.001$).

Hazard ratios indicated that exposure to the FRA μvar (20x) and SD μvar (15x) increased risk of mortality significantly compared to controls (Figure 11) and that exposure to viral doses of $1x10^6$ viral copies/mL (27x) had a significant effect on increasing the risk of mortality compared to viral doses of $1x10^5$ viral copies/mL (7.3x), $1x10^4$ viral copies/mL(0.68x), and controls (Figure 12).

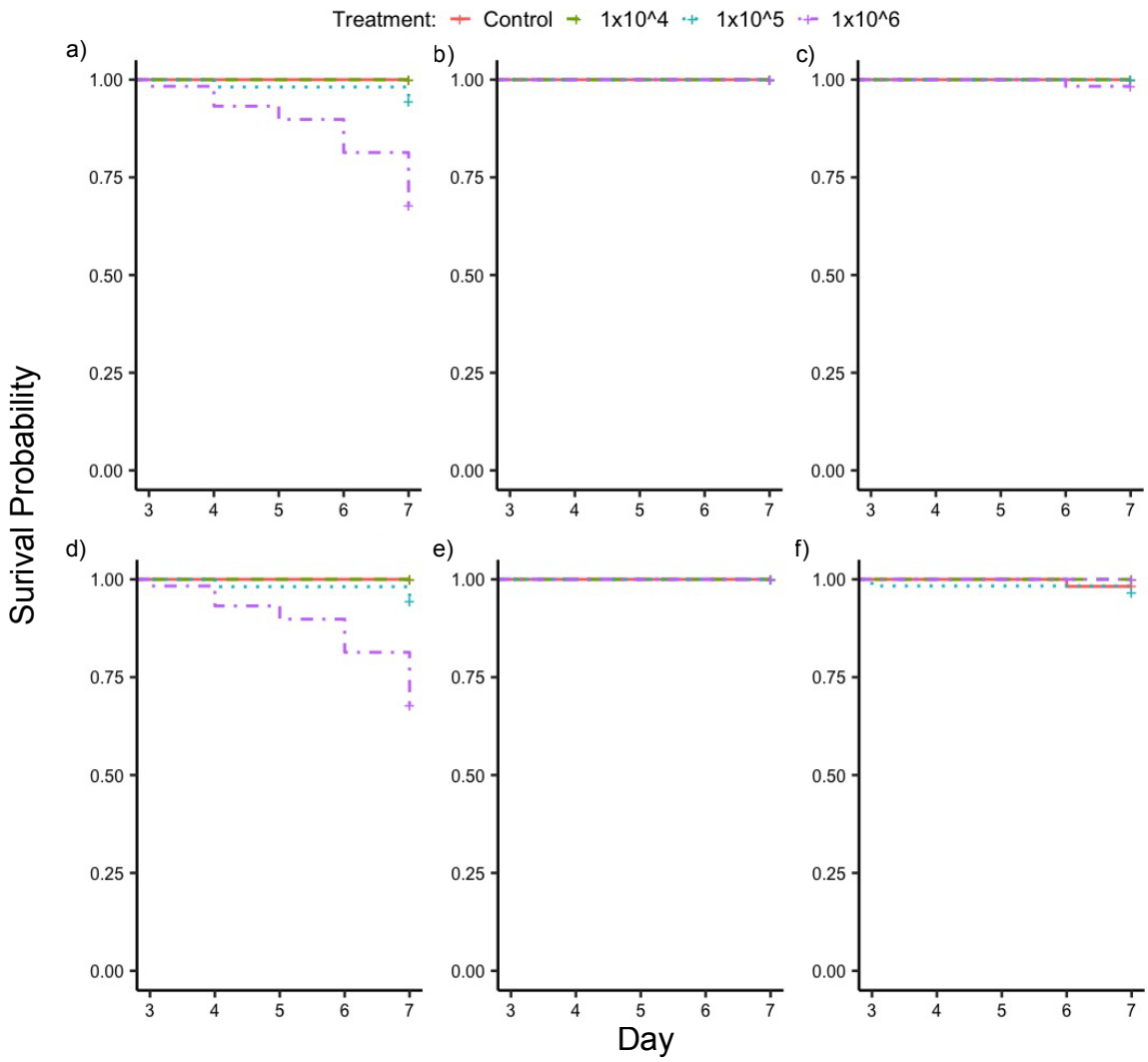

Figure 10: Top panel (a,b, and c): DOSE RESPONSE SD µVar Kaplan Meier survivorship curves of Pacific oysters (a), eastern oysters (b), and hard clams(c); Bottom panel (d, e, and f) DOSE RESPONSE FRA µVar Kaplan Meier survivorship curves of Pacific oysters (d), eastern oysters (e), and hard clams(f)

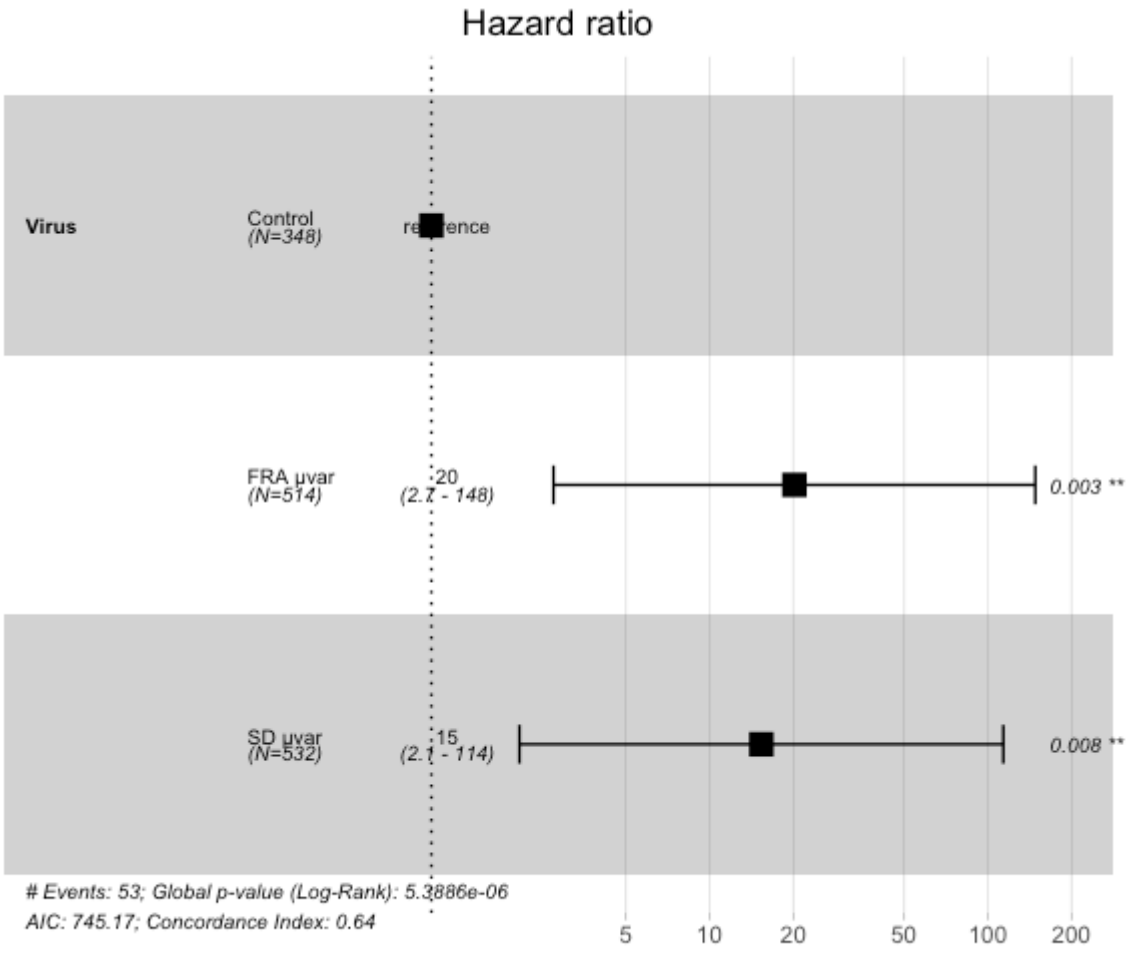

Figure 11: Cox proportional hazard showing that FRA & SD significantly increased risk of mortality compared to controls. *** $p < 0.001$

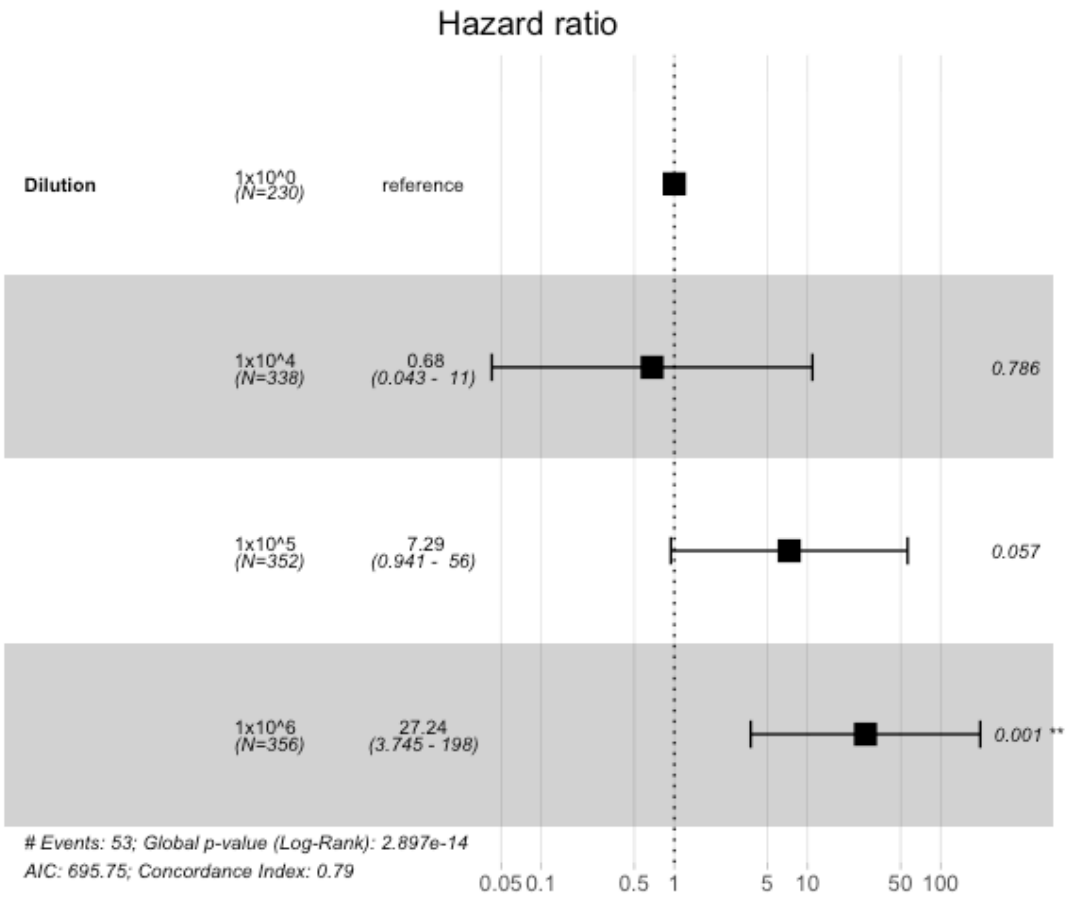

Figure 12: Cox proportional hazard ratio showing that exposure to viral dose of $1x10^6$ viral copies/mL significantly increases the risk of mortality than viral doses of $1x10^5$ viral copies/mL, $1x10^4$ viral copies/mL compared to controls. *** $p < 0.001$

Table 5: Kaplan Meier survival probability observed for each species individually and at separate viral doses.

Species	*Virus*	*Dose*	*Survival probability (%)*	*Std error (+/-)*	*n*	*Observed Mortality*
Pacific oyster (*C. gigas*)		*Control*	100	0	115	0
	SD µvar	$1x10^4$	100	0	60	0
	SD µvar	$1x10^5$	94.3	0.03	53	3
	SD µvar	$1x10^6$	67.8	0.06	59	19
	FRA µvar	$1x10^4$	98.3	0.02	59	1
	FRA µvar	$1x10^5$	89.8	0.04	59	6
	FRA µvar	$1x10^6$	64.9	0.06	57	20
Eastern oyster (*C. virginica*)		*Control*	100	0	119	0
	SD µvar	$1x10^4$	100	0	60	0
	SD µvar	$1x10^5$	100	0	61	0
	SD µvar	$1x10^6$	100	0	60	0
	FRA µvar	$1x10^4$	100	0	60	0
	FRA µvar	$1x10^5$	100	0	60	0
	FRA µvar	$1X10^6$	100	0	61	0
Hard Clam (*M. mercenaria*)		*Control*	99.1	0.01	114	1
	SD µvar	$1x10^4$	100	0	60	0
	SD µvar	$1x10^5$	100	0	60	0
	SD µvar	$1x10^6$	98.3	0.02	59	1
	FRA µvar	$1x10^4$	100	0	39	0
	FRA µvar	$1x10^5$	96.6	0.02	59	2
	FRA µvar	$1x10^6$	100	0	60	0

Viral load in exposed bivalves

SD exposure: Pacific oyster survivors had a mean of $9.7x10^5 \pm 8.9x10^5$ viral copies/mg of tissue. Pacific oyster mortalities had a mean of $1.3x10^8 \pm 2.3x10^7$ viral copies/mg of tissue. A mean of 11± 5 viral copies/mg of tissue (n=5) was detected in eastern oyster survivors and the remaining (n=10) with amplification undetectable. PCR amplification of controls for both the Pacific oysters and Eastern oysters did not yield a product, indicating that the presence of OsHV-1 DNA was below the limit of detection. The single hard clam mortality had a $2.5x10^2$ viral copies/mg of tissue. A mean of 9 ±3.5 viral copies/mg of tissue (n=5) was detected in hard clam survivors and the remaining (n=10) were below the limit of detection. One single hard clam control yield 12.3 copies/mg of tissue the remaining (n=14) were below the limit of detection.

FRA exposure: Pacific oyster survivors had a mean of $1.9x10^5 \pm 1.8x10^5$ viral copies/mg of tissue. Pacific oyster mortalities had a mean of $9.6x10^7 \pm 1.1x10^7$ viral copies/mg of tissue. One single Pacific oyster control had a mean of 3.5 copies/mg of tissue. Eastern oyster survivors had a mean of 12±7 viral copies/mg of tissue (n=2) and n=13 with undetectable quantification. Two eastern oyster controls had a mean of 4.9±1.4 copies/mg of tissue while n=13 had undetectable quantification. Hard clam exposed mortalities had a mean of 785.2± 18 viral copies/mg of tissue. Hard clam survivors had a mean of 8.1±4.1 viral copies/mg of tissue (n=3) and n=12 with undetectable quantification. The single hard clam control morality had $9.1x10^2$ viral copies/mg of tissue. No surviving hard clam controls had detectable quantification. (Figure 13)

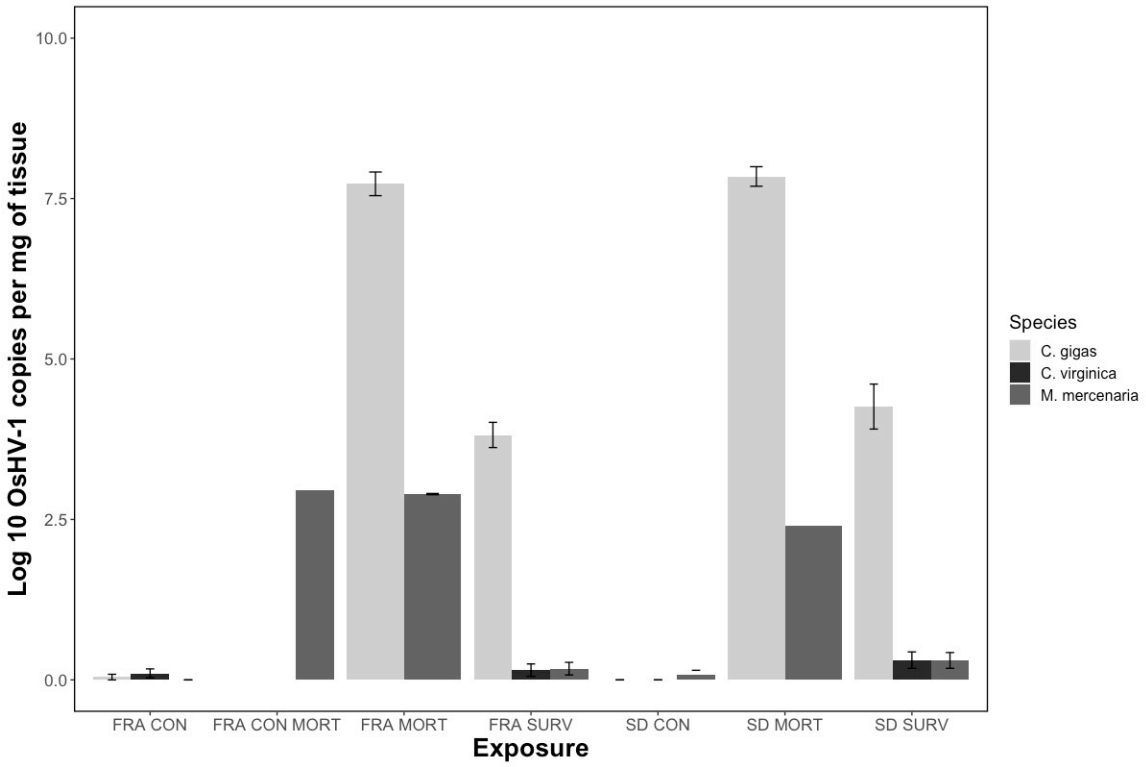

Figure 13: Dose Response Challenge OsHV-1 log transformed quantification for both viral dose response exposures of the SD and FRA microvariants. Note: standard error is absent when n<2.

Statistical analysis qPCR

OsHV-1 copy numbers accumulated in live and dead animals exposed to either the FRA or SD µVar did not significantly differ between viruses (Kruskal-Wallis chi-squared = 0.0016461, df = 1, $p > 0.05$, Wilcoxon sum test $p > 0.05$). However, copy numbers were significantly different between live and dead species exposed to either the FRA or SD µVar across all doses (Kruskal-Wallis chi-squared = 98.675, df = 2, $p < 0.001$; Wilcoxon sum test *M. mercenaria* to *C. virginica,* $p = 1.6x10^{-1}$; Wilcoxon sum test *M. mercenaria* to *C. gigas,* $p < 0.001$; Wilcoxon sum test *C. virginica to C. gigas,* $p < 0.001$). There is also a significant difference between copy numbers accumulated between mortalities, to survivors, and to controls (Kruskal-Wallis chi-squared = 130.59, df = 3, $p<0.001$). There is a significant difference in viral copy numbers accumulated in exposed animals among viral doses (Kruskal-Wallis chi-squared = 71.274, df = 3, $p < 0.001$). Viral copies accumulated from dead animals exposed to doses of $1x10^6$ viral copies/mL and $1x10^5$ viral copies/mL were significantly different from controls (Wilcoxon sum test $p < 0.001$). Viral copies accumulated in dead animals exposed to doses of $1x10^4$ were not significantly different to controls (Wilcoxon sum test $p = 0.27$). There is a significant difference in viral copies accumulated in dead animals exposed to doses of $1x10^6$ viral copies/mL compared to doses of $1x10^5$ viral copies/mL (Wilcoxon sum test $p = .00739$), however, there is no significant different between viral copies in dead animals exposed to doses of $1x10^4$ viral copies/mL compared to $1x10^6$ viral copies/mL or $1x10^5$ viral copies/mL (Wilcoxon sum test $p = 0.27$).

Characterization and Sequencing

Gel electrophoresis of the PCR targeting ORF4 (Figure 14) and ORF100 (Figure 15) indicated that products were obtained for the desired regions. The hard clam samples that experienced mortality in the SD µVar exposures had 100% identity of 634 nucleotides of the C2/C6 (ORF4) region to the SD µVar (GenBank Accession ID: MW504462). The hard clam samples that experienced mortality in the FRA µVar exposures had a 100% identity of 623 nucleotides of the C2/C6 (ORF4) region and the control hard clam mortality had 100% identity of 594 nucleotides to the FRA µVar (GenBank Accession ID: MT157287). Positive *C. gigas* mortalities exposed to the SD µVar (GenBank Accession ID: MW504462) had 100% identity of 636 nucleotides. Positive *C. gigas* mortalities exposed to the FRA µVar (GenBank Accession ID: MT157287) had 100% identity of 608 nucleotides.

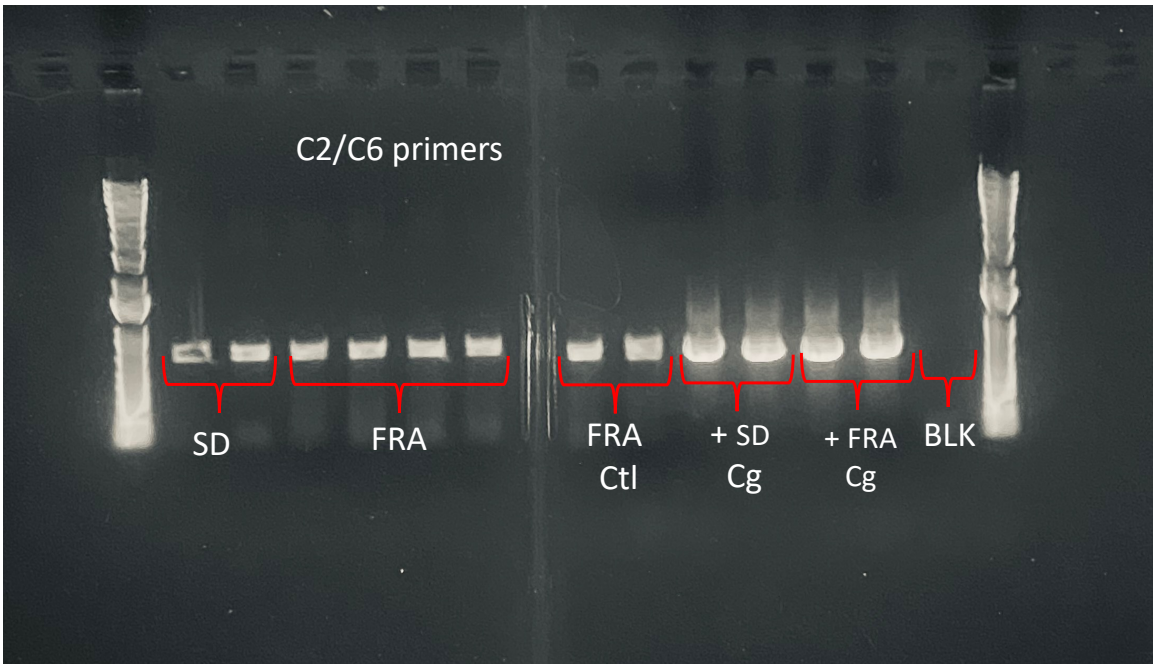

Figure 14: Gel electrophoresis of polymerase chain reaction targeting OsHV-1 open reading frame 4 using C2/C6 primers; SD = San Diego µVar exposed *M. mercenaria*; FRA = French µVar exposed *M. mercenaria*; +SD Cg = positive San Diego exposed Pacific oyster; +FRA Cg = positive control French exposed Pacific oyster; BLK= Blank; Ctl = Control.

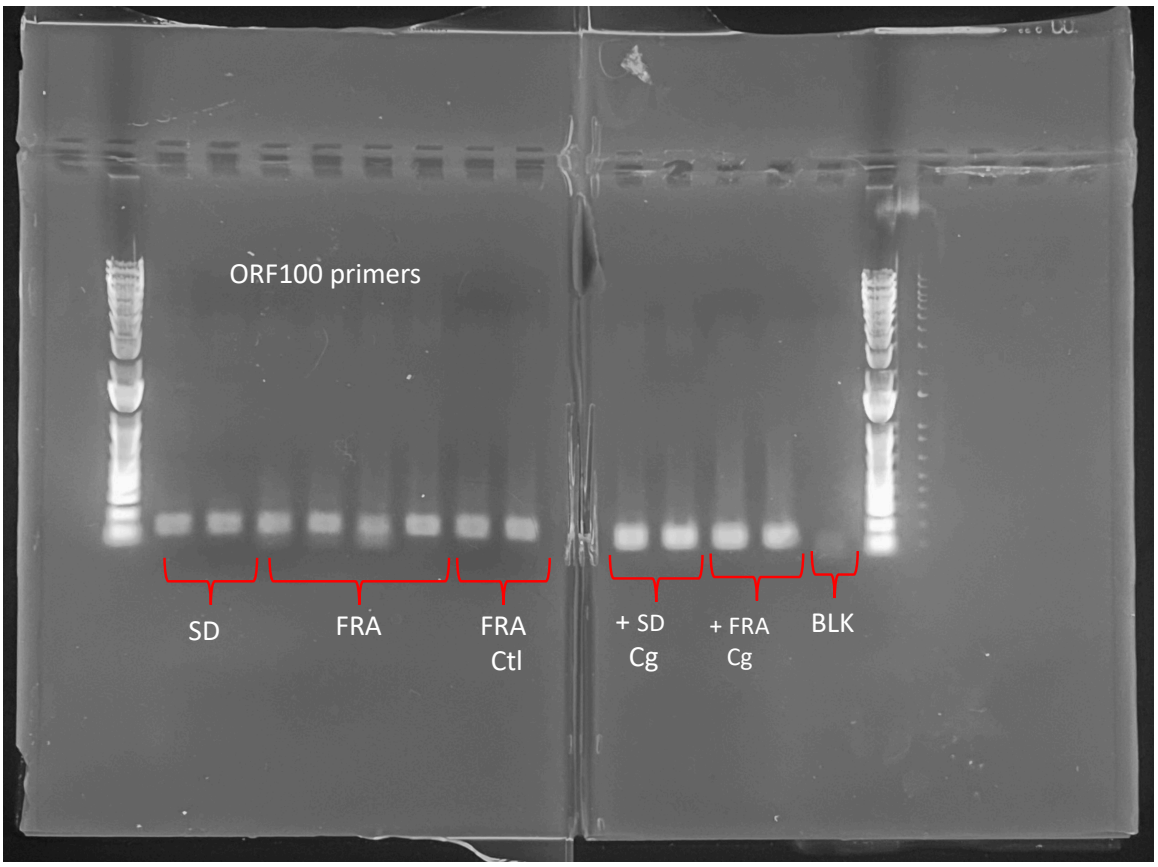

Figure 15: Gel electrophoresis of polymerase chain reaction using OsHV-1 open reading frame 100 (ORF100) forward and reverse primers. SD = San Diego µVar exposed *M. mercenaria*; FRA = French µVar exposed *M. mercenaria*; +SD Cg = positive San Diego exposed Pacific oyster; +FRA Cg = positive French exposed Pacific oyster; BLK= Blank; Ctl = Control.

Vector Study

<u>Injected Donors</u>

Water samples from injected bivalve species tanks were taken immediately before that water was used to expose spat to 'exposed seawater' treatments. Pacific oysters shed $2.9 \times 10^7 \pm 1.6 \times 10^4$ viral copies/ml of the SD variant and $8.9 \times 10^6 \pm 5.5 \times 10^3$ viral copies/ml of the FRA variant. All Pacific oysters died following injections of either the SD or FRA variant. Eastern oysters shed $5 \times 10^3 \pm 2.2$ viral copies/ml of the SD variant and $2 \times 10^3 \pm 0.3$ viral copies/ml of the FRA variant. Two of the thirty Eastern oysters injected with the SD variant died on days 1 and 5 post injection. Eastern oysters injected with the FRA variant had 1 individual die on day 3 post injection. Hard clams shed $4.8 \times 10^2 \pm 4.6$ viral copies/ml of the SD variant and $6.9 \times 10^2 \pm 3.1$ viral copies/ml of the FRA variant (Figure 16). No hard clams experienced mortality from injection of either variant. Data from donor animals were not statistically analyzed or sampled for viral quantification.

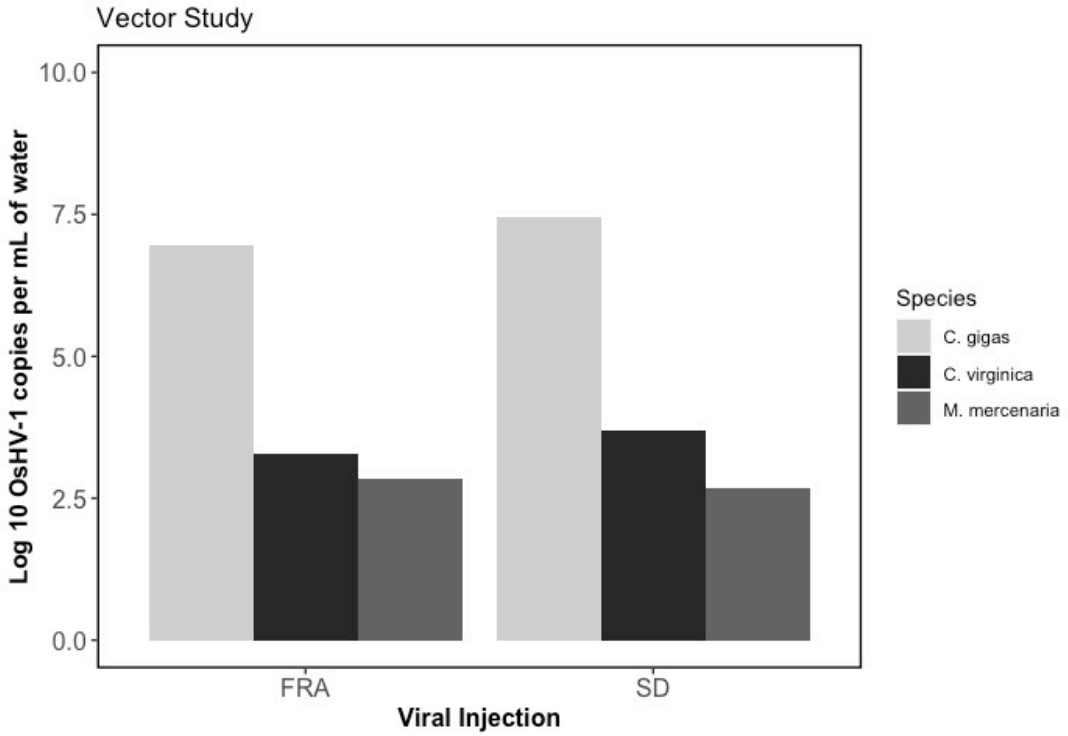

Figure 16: Log transformed quantification of 'exposed seawater' from Pacific oysters (*C. gigas*), eastern oysters (*C. virginica*), and hard clams (*M. mercenaria*).

Statistical analysis petri-dish exposures

Survival analysis based on mortality counts from day 3 through day 7 of the vector study indicated that there was a significant difference between treatments of 'exposed seawater' from Pacific oysters, eastern oysters, and hard clams (Chisq= 731 on 2 degrees of freedom, $p<0.001$). Survival probability was observed for all treatments combined with virus, FRA µVar or SD µVar. High survival probability was observed in Pacific oysters treated with 'exposed seawater' from hard clams (FRA 100%±0 and SD 99.4%±5.54e-03), compared to 'exposed seawater' from eastern oysters (FRA 88.9%5±2.34x10^{-2} and SD 21.9%±3.07 x10^{-2}) and from Pacific oysters (FRA 0% and SD 2.2%±1.09x10^{-2}), which experienced significantly lower survival (Figure 17 & 18). There was a significant difference between treatments of 'exposed seawater' combined with virus and when compared to controls (Chisq= 1991 on 8 degrees of freedom, $p<0.001$). There was no significant different between controls ($p=1$). There was a significant difference between treatments of 'exposed seawater' from Pacific oysters and eastern oysters for both FRA and SD µVars and when compared to controls ($p <0.001$). Survival probability was not significantly different between Pacific oysters exposed to hard clam 'exposed seawater' when compared to controls for either the FRA ($p = 0.49$) or SD µVar ($p = 1$). For all 'exposed seawater' treatments, Pacific oysters exposed to the FRA µVar ($p<0.001$) and SD µVar ($p<0.001$) had significantly lower survival compared to controls. However, there was a significant difference in survival between FRA and SD µVar exposures (Chisq= 276 on 2 degrees of freedom, $p<0.001$). Pacific oysters exposed to 'exposed seawater' treatments of the FRA µVar had survival probability of 63% , while

treatments of the SD µVar had a survival probability of 41%. Controls had a 100% survival probability.

Cox proportional hazard model indicated that due to the significant difference in survival between viruses, FRA and SD, and compared to controls the hazard ratio was numerically infinite (coef = 18.82 and 18.83; Likelihood ratio test= 408.3 on 2 df, $p<0.001$; Score (logrank) test = 263.9 on 2 df, $p<0.001$). Hazard ratios indicated that hard clams decreasing risk of mortality compared to eastern oysters and Pacific oysters which were significantly increasing the risk of mortality (Figure 19).

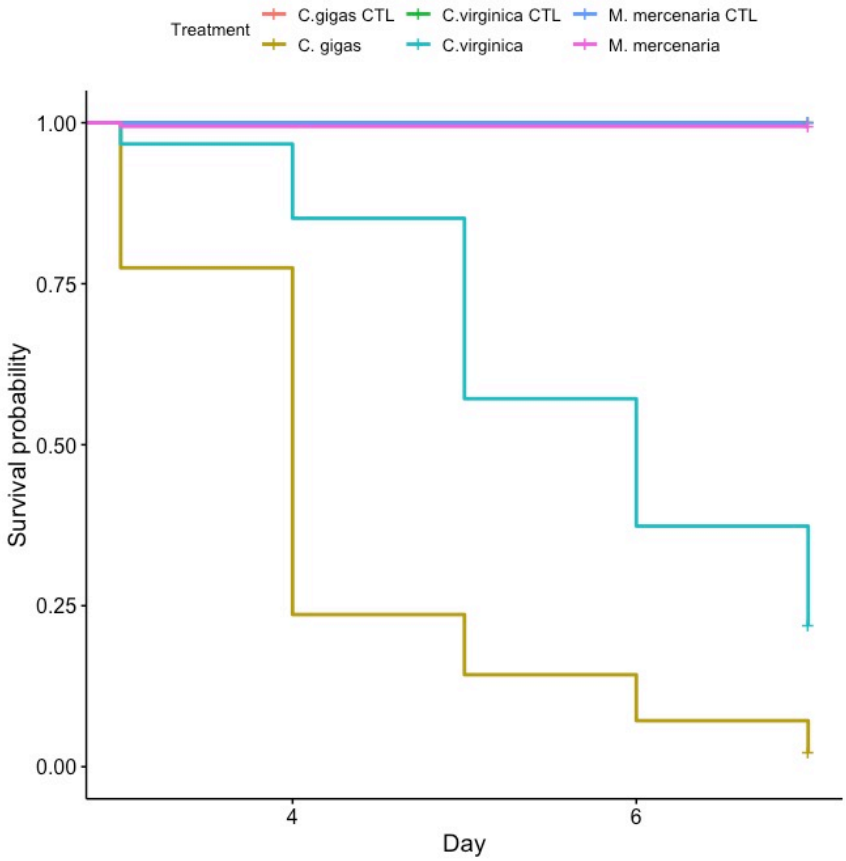

Figure 17: Kaplan Meier survivorship curves for Pacific oyster spat exposed to San Diego µVar 'exposed seawater' from eastern oysters (*C. virginica*), hard clams (*M. mercenaria*), and Pacific oysters (*C. gigas*). CTL = Control.

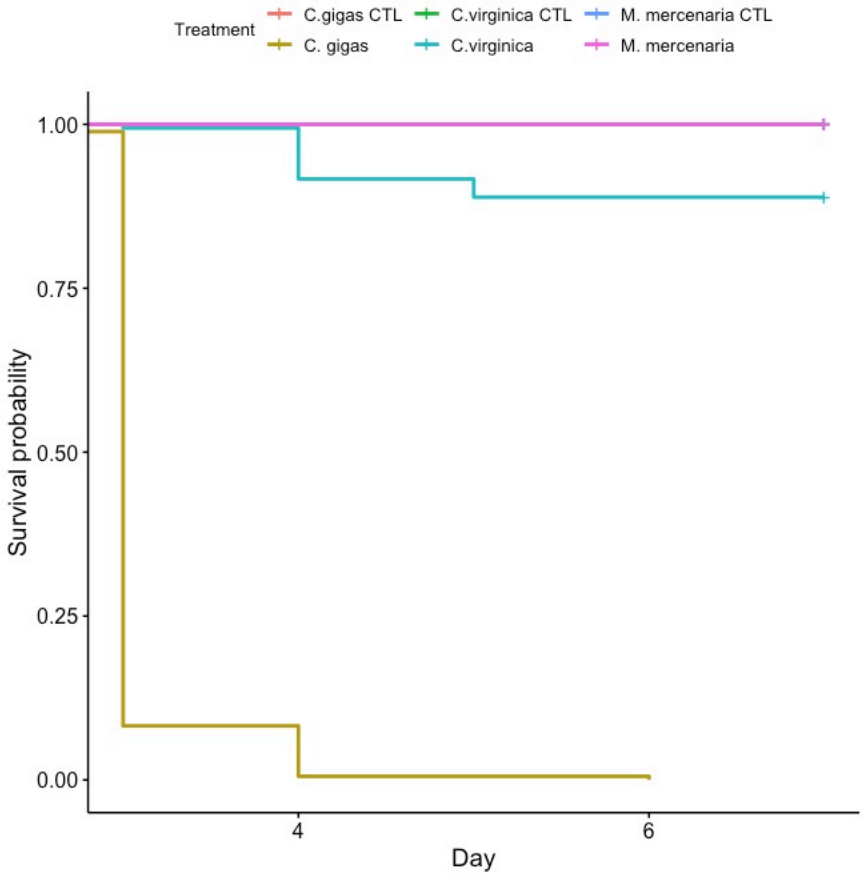

Figure 18: Kaplan Meier survivorship curves for Pacific oyster spat exposed to French µVar 'exposed seawater' from eastern oysters (*C. virginica*), hard clams (*M. mercenaria*), and Pacific oysters (*C. gigas*). CTL = Control.

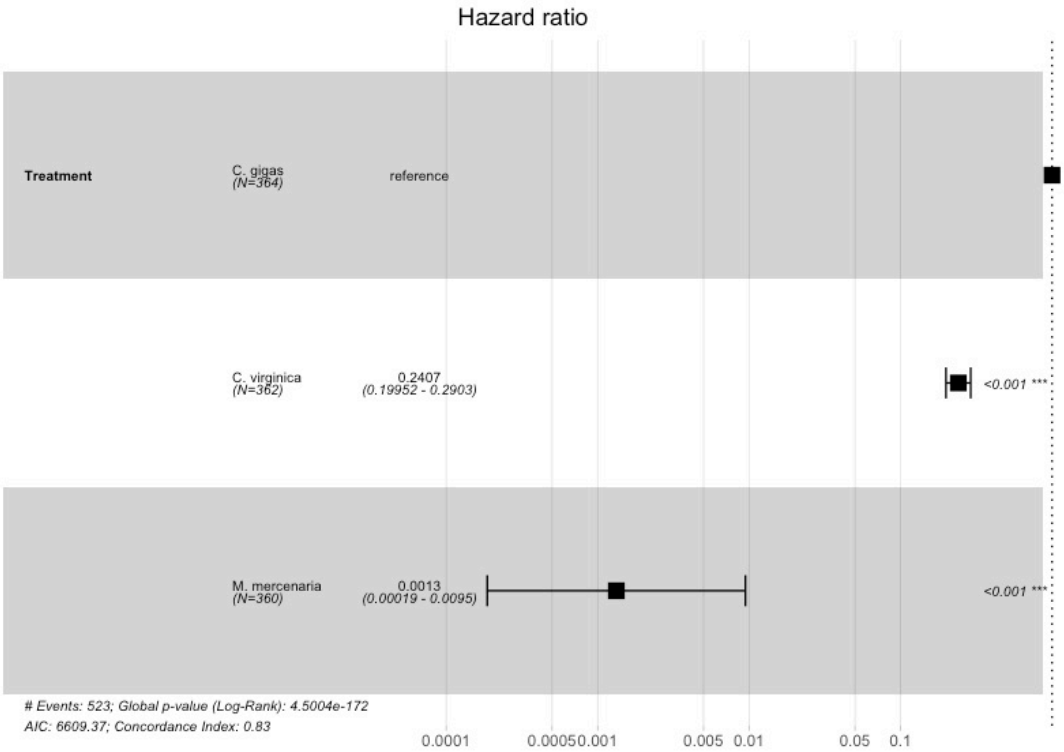

Figure 19: Cox proportional hazard ratio showing that hard clams are significantly decreasing risk of mortality compared to eastern oysters and Pacific oysters which are significantly increasing the risk of mortality in Pacific oyster spat. *** $p < 0.001$

Viral Load in Petri-dish Exposures

SD exposures: Pacific oyster mortalities treated with 'exposed seawater' from Pacific oysters had a mean of $2.9x10^7 \pm 2.3x10^6$ viral copies/mg of tissue; from eastern oysters had a mean of $3.5x10^7 \pm 3.5x10^6$ viral copies/mg of tissue; and the individual pacific oyster mortality exposed to hard clam water had $2.9x10^4$ viral copies/mg of tissue. Pacific oyster survivors treated with 'exposed seawater' from Pacific oysters had a mean of $4.5x10^7 \pm 1.1x10^6$ viral copies/mg of tissue; from eastern oysters had a mean of $4.9x10^6 \pm 3.5x10^6$ viral copies/mg of tissue; and from hard clams had a mean of 23±11 copies/mg of tissue (n=8) while the remaining (n= 37) were below the level of detection. Pacific oyster controls treated with eastern oyster seawater had one pool have 21 copies/mg of tissue while the remaining 2 pools did not have detectable quantification. (Figure 20).

FRA exposures: Pacific oyster mortalities treated with 'exposed seawater' from Pacific oysters had a mean of $1.6x10^8 \pm 2.4x10^7$ viral copies/mg of tissue and from eastern oysters had a mean of $8.4x10^7 \pm 1.9x10^7$ viral copies/mg of tissue. No hard clam treatments experienced mortality. Pacific oyster survivors treated with 'exposed seawater' from easter oyster has a mean of 11±2.3 viral copies/mg of tissue (n=9) while n=31 had undetectable quantification. Pacific oyster survivors treated with 'exposed seawater' from hard clams had 3.3 copies/mg of tissue (n=1) with the remaining 44 individuals below the level of detection. No Pacific oysters treated with 'exposed seawater' from Pacific oysters survived. Pacific oyster controls treated with eastern oyster seawater had a one pooled sample amplify with 18 viral copies/mg of tissue. Pacific oyster controls treated with hard clam seawater had one pooled sample amplify with 4.2 viral copies/mg of tissue. (Figure 20).

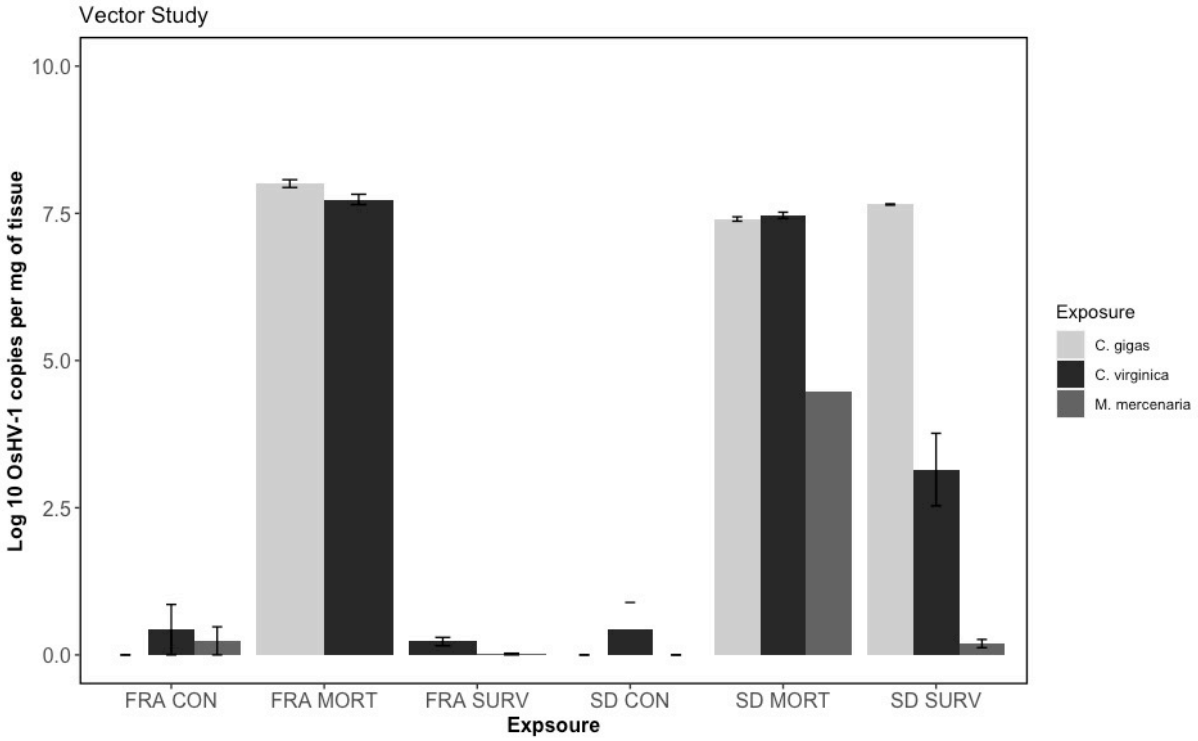

Figure 20: Log transformed quantification of Pacific oysters treated with 'exposed seawater' from Pacific oysters (*C. gigas*), eastern oysters (*C. virginica*), and hard clams (*M. mercenaria*). CON = control; SURV= survivors; MORT= mortalities. Note: standard error is absent when n<2.

Statistical analysis qPCR

OsHV-1 copy numbers accumulated in live and dead animals did not significantly differ between the FRA or SD µVar (Kruskal-Wallis chi-squared = 0.41893, df = 1, $p > 0.05$, Wilcoxon sum test $p > 0.05$). However, copy numbers were significantly different between 'exposed seawater' treatments for either the FRA or SD µVar (Kruskal-Wallis chi-squared = 149.75, df = 2, $p < 0.001$; Wilcoxon sum test *M. mercenaria* to *C. virginica*, $p < 0.001$; Wilcoxon sum test *M. mercenaria* to *C. gigas*, $p < 0.001$; Wilcoxon sum test *C. virginica* to *C. gigas*, $p < 0.001$). There is also a significant difference between copy numbers accumulated between mortalities, survivors, and controls between viruses (Kruskal-Wallis chi-squared = 217.08, df = 2, $p < 0.001$).

CHAPTER 4: DISCUSSION

This is the first study to directly expose eastern oysters and hard clams to OsHV-1 microvariants to determine their susceptibility to infection, mortality, and potential promotion of horizontal viral transmission. In this series of studies, eastern oysters and hard clams exposed to OsHV-1 SD or FRA μvars via bath exposure did not experience infection or mortality under conditions that led to 100% infection of the natural host, the Pacific oyster. However, eastern oysters, when injected directly with OsHV-1 microvariants (FRA and SD μVars), had the capability to support replication of the virus and shed virus into the surrounding water, therefore, having the potential to promote OsHV-1 microvariant outbreaks in susceptible species. This study also supports the importance of management and biosecurity. Establishment of a surveillance program and further experimentation can help support results or claims from this study and mitigate introduction of OsHV-1.

OsHV-1 was not detected in the Maryland portion of the Chesapeake Bay during the 2021 summer surveys. Due to viral replication being heavily influenced by environmental elements such as temperature (Martenot et al. 2015, de Kantzow et al. 2016), the environmental conditions in the Chesapeake may be unfavorable to viral replication and infection. Experimental temperatures typically between 18-26°C and field temperatures of 16.2 to 21.9°C are likely to increase the risk of spread and induce high viral replication in OsHV-1 μVars (de Kantzow et al. 2016, Petton et al. 2013). June through August, temperatures were ~26°C (± 2°C) (Table 3), which may exceed the threshold for viral replication. Delisle et al. (2020) investigated how temperature influences a host's (Pacific oysters) response to OsHV-1 by comparing transcriptional

profiles by using RNA sequencing during experimental infections at 21 and 29°C. The study found that temperature influenced the expression of genes related to immune processes, cell death, synaptic signaling, protein processes, metabolism, growth, and cell development which occur at all stages of infection. The results suggested that host susceptibility could be reduced at temperatures as high as 29°C because seawater temperatures may control the entry of OsHV-1 into the host cells by controlling the composition of the cell matrix and inhibiting the attachment of the virus. Furthermore, higher temperatures increased the expression of apoptotic genes at 29°C which can limit rapid early viral replication. Although OsHV-1 can persist in the environment for long periods, it's also apparent that high temperatures reduce its infectivity (Martenot et al. 2015). Considering the findings of this study, eastern oysters may be less susceptible or are releasing an immune response to OsHV-1 present in the water column due to higher temperatures that were seen during survey months.

Salinity can also play a role in the results of the summer survey. In the Maryland portion of the Chesapeake Bay and especially in tributaries salinities are low and variable due to freshwater input. Fuhrmann et al. (2016) studied mortality of Pacific oysters exposed to varying salinities and OsHV-1. Two experiments were conducted using salinity acclimated and non-acclimated oysters. Experiment 1 determining that the survival of oysters acclimated to higher salinities and OsHV-1 was between 43-73% compared to oysters in lower salinities having a survival rate of ~95%. Experiment 2 determined that non-acclimated oysters' survival was only 23% and it is possible that due to the salinity shock OsHV-1 was not the main cause of death (Figure 21). Therefore, low salinity may also reduce infectivity of OsHV-1. For survey sites, salinity had a maximum

of 17.1 ppt and a minimum of 10 ppt. Most of these sites ranged closer to the minimum salinity range (11-12ppt) (Table 4). OsHV-1 may not be able to induce infection in eastern oysters due to their acclimation to a lower salinity environment.

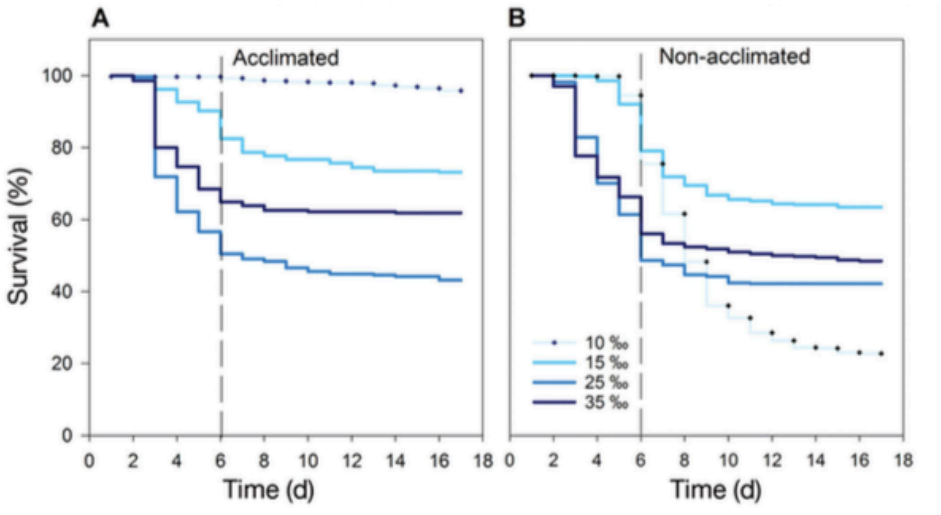

Figure 21: Survival of Pacific oysters *Crassostrea gigas* exposed to the source of infection at 4 salinities (A) with or (B) without prior acclimation. Survival time was measured as days from the onset of exposure to the source of infection. From Fuhrmann et al. (2016).

Although OsHV-1 DNA did amplify in the inoculated random samples for the survey 'positive controls', inhibition (26-44%) of qPCR amplification was detected. Since the survey samples are environmental samples, inhibitors can coextract with DNA. Due to this level of inhibition, three random samples exhibiting ~44% inhibition occurred were purified and spiked with varying concentrations of OsHV-1 DNA ($3x10^2$, $3x10^4$, $3x10^7$) to confirm the level of inhibition. Quantification indicated that no more than 50% inhibition occurred for all concentrations. It was concluded that since the OsHV-1 specific qPCR assay has a limit of detection of 3 viral copies/2 µl (Burge & Friedman 2012), if present, viral DNA would still be detectable even with 50% inhibition.

The dose response challenge strengthens the explanation to why OsHV-1 may not thrive in the Chesapeake Bay. Eastern oysters and hard clams experienced minimal to no mortality and low copy numbers when exposed to OsHV-1, indicating that infection did not occur in the dose response experiment. Eastern oysters did have amplifiable OsHV-1 DNA at mean copies/mg of tissue at 11±5 (n=5, SD µVar) and 12±7 (n=2, FRA µVar). Hard clams experienced two individual mortalities at exposure of $1x10^5$ viral copies/ml (FRA µVar) and had low viral copy numbers averaging ~ 785 copies/mg of tissue. Hard clam survivors had low amounts of amplifiable OsHV-1 DNA of 9± 3.5 (n=5, SD µVar) and 8.1± 4.1(n=3, FRA µVar). These low copy numbers are not typical or consistent with infection and mortality caused by OsHV-1 in previous studies and observations (Friedman et al. 2020, Agnew et al. 2020, Burge et al. 2020), which can suggest that stress may have been a contributor to the mortalities of the hard clams. It is possible that these species are resistant to OsHV-1 and its microvariants, however, only one stock was used for each species in these experiments which may be a factor in their resilience.

Additionally, only high salinity animals were used in these trials. Further experiments should be conducted using additional oyster and clam stocks to fully understand the susceptibility or resistance of eastern oysters and hard clams in the United States. One hard clam control died and had low viral copy numbers (~ 900 viral copies/mg of tissue). There were also low levels of amplifiable DNA in one single surviving hard clam from the SD variant exposure and two eastern oyster controls from the FRA variant exposures which could have been a result of aerosolization of viral DNA in the wet lab and stress.

Although understudied in eastern oysters, another factor that may influence disease progression or infection is the microbiome. The microbiome can shift under various stressors or conditions such as temperature, salinity, translocation, infectious disease, or antibiotics (Reviewed by King et al. 2019). Eastern oysters are believed to have seasonal variation with temperature having a significant relationship with the community structure of their microbiome. (Pierce et al. 2016). Additionally, eastern oysters and many other important species undergo selective breeding for disease resistance. However, there are research gaps on whether breeding can alter the microbiome composition, or if the microbiome is responsible for resistance (reviewed by Degremont et al. 2015 and King et al. 2019). A recent study, King et al. (2019), investigated how breeding for resistance to OsHV-1 microvar affects the Pacific oyster microbiome of various families with varying susceptibility to disease. The study found that the microbiomes of families with higher susceptibility to infection were significantly different to that of disease resistant families. This study also reveals there may be key bacterial taxa in the microbial composition that may provide protection or play a role in OsHV-1 microvar disease outbreaks. When considering the results of the field surveys

and the dose response challenge, the family lines tested may have had an advantage if they were bred for disease resistance. Further investigation into influence of disease resistance, environmental conditions, and infection on the eastern oyster microbiome may support the findings of this study.

Eastern oysters injected with OsHV-1 were able to transmit the virus showing that although this family line was not susceptible to mortality in the dose response, they can be vectors for the virus when exposed via injection. Eastern oysters shed 5 x 10^3 ± 2.2viral copies/ml of the SD variant causing 67% mortality in exposed Pacific oyster spat and 2 x 10^3 ± 0.3 viral copies/ml of the FRA variant causing 11% mortality in exposed Pacific oyster spat. This shows that small quantities of shed virus are enough to induce infection and mortality in directly exposed susceptible and naïve Pacific oysters. Biosecurity practices such as limiting transport of non-native species and performing disease screenings that include OsHV-1 are important to limit spread and emergence of disease to new locations. Note, the animals were injected with the virus which does not represent natural infection pathways through filtering. Animals naturally exposed may not produce the same amount of shed virus giving opportunity for further experimentation on shedding rates of naturally exposed eastern oysters or other bivalve species. Hard clams did not become infected, experience high mortality, or transmit the virus. This could indicate that hard clams are not susceptible to OsHV-1. In the vector study, hard clams shed 479 viral copies/ml of the SD variant and 693 viral copies/ml of the FRA variant. This viral load shed is ~10x lower than the viral load shed by the eastern oysters. One individual Pacific oyster died in the SD variant hard clam 'exposed seawater' treatment with 2.9x10^4 viral copies/mg of tissue. This viral load in tissue is

high enough to be associated with mortality in the field. The low viral load shed by the hard clams was not enough to induce high mortality rates in this experiment. However, this may not be the case on a larger scale or in the field where animals are in greater abundance. Further experimentation is needed to fully rule out that hard clams are not susceptible or vectors of OsHV-1. Controls for each treatment from the experiment were pooled in replicates of 3 with 5 individuals per pool (n=15). Low amounts of OsHV-1 were amplified in the samples which could be due to aerosolization of viral DNA in the wet lab. In all treatment controls only 1 pool amplified, while the 2 others had 0 copies/DNA sample. Due to the samples being pooled this accumulation of DNA could be less than 3 copies of viral DNA per animal.

These experiments provide better knowledge on the more recently detected San Diego OsHV-1 μVar (Burge et al. 2021). Both experiments showed that the variant had high virulence and infectivity like the historically potent French μVar. In the Dose Response challenge, the survival probabilities (Table 5) of Pacific oysters were similar among variants. However, note that SD μVar had a survival probability of 100% at an exposure concentration of $1x10^4$ viral copies/ml in Pacific oysters, while FRA μVar did induce mortality at this concentration. In the vector study, this slight variation in infectivity was also apparent for Pacific oyster water exposures. The SD μVar caused 98.8% mortality with only two individuals surviving on day 7 of the experiment. The FRA μVar had 100% mortality by day 6 of the experiment. The mortality rate was more gradual for SD μVar compared to the FRA μVar where 92% died by day 3. In other words, the mortality rates for both variants were significant. In the eastern oyster vector experiment exposing naïve Pacific oysters, high mortality was observed for animals

exposed to the SD μVar (66%) as compared to the FRA μVar (11%), which was concentrated to one specific replicate or petri-dish in the experiment. This plate had 100% mortality. If one animal becomes infected and sheds virus, it is likely that surrounding animals will become infected and experience mortality due to cohabitation or close proximity of the animals, and small volume of water. This could have been caused by the low viral concentration of the water and subsequent water change on day 4 of the experiment reducing stress and prohibiting fast viral replication. The Pacific oyster survivors in the eastern oyster vector experiment had a mean 0 viral copies/mg of tissue indicating that infection did not occur.

For the vector study, cox proportional hazard ratios were infinite between viruses due to such significant differences in survival probabilities. This value is not meaningful to describe the hazard ratio between variables. The reason the hazard ratio coefficient may be infinite is due to several reasons: 1) the FRA µVar Pacific oyster 'exposed seawater' treatment exemplified ~92% mortality by day 3 of the experiment, significantly decreasing or stopping the amount of events compared to the SD Pacific oyster 'exposed seawater' treatment where mortality events happened on days 3 through 7 of the experiment, 2) only 11% of the FRA eastern oyster 'exposed seawater' treatment animals died compared to 66% in the SD eastern oyster 'exposed seawater' treatment, and 3) little to no mortality events occurred for hard clams in either viral exposure. Therefore, many extreme events are being compared. This is also the case when comparing virus combined with treatment.

CHAPTER 5: CONCLUSION

The susceptibility and horizontal transmission of OsHV-1 microvariants to eastern oysters and hard clams was examined in this study. Importantly, it was shown that OsHV-1 did not cause mortality or infection in eastern oysters and hard clams cultivated in the Chesapeake Bay and Coastal Bays through experimental challenges. As well, infection was not observed in eastern oysters surveyed in the Maryland portion of the Chesapeake Bay. This may imply that these species, cultivated throughout the East Coast and Gulf of Mexico, may be safe from the threat of OsHV1-related mortality. However, this study should be followed by further experimentation using different eastern oyster family lines in varying environmental conditions. Furthermore, there is a need to establish a surveillance program to guard against the spread and impacts of OsHV-1 related disease. These findings support the importance of disease management and biosecurity practices in the aquaculture industry and testing oyster species against threatening diseases such as OsHV-1 and it's microvariants. This study also demonstrated how conducting testing with multiple OsHV-1 microvariants can help us understand varying virulence and potential spread of viral variants.

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