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Susceptibility of shellfish aquaculture species in the Chesapeake Bay and Maryland coastal bays to ostreid herpesvirus 1 microvariants

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ABSTRACT: Ostreid herpesvirus 1 (OsHV-1) and its microvariants (µVars) cause economically devastating mass mortalities of oysters and pose a threat to the shellfish aquaculture industry globally. OsHV-1 outbreaks can cause up to 100% mortality in the Pacific oyster *Crassostrea gigas*. However, OsHV-1 and its variants have a broad host range and can infect at least 7 bivalve species, including bay scallops *Argopecten irradians* and eastern oysters *C. virginica*. Determining the susceptibility of economically and ecologically important bivalve species to OsHV-1 is critical for improving biosecurity and disease management to protect the aquaculture industry. Surveys of eastern oysters were conducted in June to August 2021 in the Maryland portion of the Chesapeake Bay to determine the prevalence and viral load of OsHV-1 at 5 aquaculture farms. Using quantitative PCR, OsHV-1 was not detected at any sites. Experiments examined the susceptibility of single stocks of eastern oysters and hard clams *Mercenaria mercenaria* to the virus and their ability to horizontally transmit it using OsHV-1 µVar SD (San Diego, California) and OsHV-1 µVar FRA (Marennes-Olreon, France). Results showed that OsHV-1 µVars did not cause mortality or symptomatic infection in the single stocks of eastern oysters and hard clams used in these experiments using natural infection pathways. However, the eastern oyster stock, when injected with OsHV-1, did transmit the virus to naïve Pacific oysters. Further experimentation using additional stocks and lines and establishment of surveillance programs along the east and Gulf coasts of the USA are necessary to prepare for the potential spread and impact of OsHV-1 related disease.

KEY WORDS: Eastern oysters · Hard clams · Aquaculture · Transmission · Infectious diseases

1. INTRODUCTION

Historically, the spread and emergence of marine diseases have threatened shellfish fisheries and aquaculture industries globally, causing economically devastating mortality events (Lafferty et al. 2015). Despite this challenge, aquaculture has been growing rapidly due to its great contribution to global economies and ecosystems. Shellfish aquaculture is a vital component to the economic and ecological success of the Chesapeake Bay and Maryland coastal bays spanning 7593 acres of Maryland waters, providing food, job security, and resources for restoration of native species and habitats (van Senten et al. 2019, Hood et al. 2022). Eastern oysters are the primary species cultivated in Maryland aquaculture, with a value of USD 12.2 mil-

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lion in 2020 (Tarnowski 2022). The second most important aquaculture species throughout the bays is the hard clam *Mercenaria mercenaria*, which was estimated to have a value of USD 55.9 million in the USA in 2018 (USDA 2019) and has continued to grow over the past 5 yr. Furthermore, the bivalve industry in Maryland and more broadly the US east coast has been historically threatened and greatly impacted by diseases such as Dermo disease (caused by *Perkinsus marinus*), MSX disease (*Haplosporidium nelsoni*), and QPX disease (*Mucochytrium quahogi*; Geraci-Yee et al. 2021). Marine diseases have the potential to negatively impact the economic value of a population by reducing the production 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).

Ostreid herpesvirus 1 (OsHV-1) was identified in 1991 in diseased Pacific oyster (*Crassostrea gigas*) spat and juveniles (Renault et al. 1994a,b) and has since emerged as a threat to the global shellfish industry (reviewed by Carnegie et al. 2016, Burge et al. 2018). The OsHV-1 genome was sequenced from infected larvae from France from 1994 (Davison et al. 2005), and this variant is referred to as the OsHV-1 reference variant. There are multiple genetic variants of OsHV-1 (Segarra et al. 2012, OIE 2019). Before 2008, the variants most closely related to the reference variant were detected in Pacific oysters in Europe (Nicolas et al. 1992, Renault et al. 1994b), Australia (Hine & Thorne 1997), New Zealand (Hine et al. 1992), Asia (Moss et al. 2007), the USA (Tomales Bay, California) (Friedman et al. 2005), and Mexico (Vásquez-Yeomans et al. 2010). A novel genetic variant, OsHV-1 microvariant (µVar), emerged in 2008 in France, causing more severe and economically devastating mortalities (Segarra et al. 2010). Similar µVar strains spread rapidly around the world and have been detected in parts of Europe, New Zealand, Australia, Asia (reviewed by Burge et al. 2018), and more recently San Diego, California (Burge et al. 2021).

Pacific oysters are not the only species at risk of mortality or infection, as OsHV-1 and its µVars have a broad host range, being detected in 43 species (Shukla 2023), with the ability to infect and kill at least 7 bivalve species, including (but not limited to) *Pecten maximus* (Arzul et al. 2001a,b), *Ruditapes philippinarum* (Renault et al. 2001), *Scapharca broughtonii* (Xia et al. 2015), and *Argopecten irradians* (Kim et al. 2019). Friedman et al. (2020) recently showed high viral loads in tissues of 2 eastern oyster (*C. virginica*) lines when injected with OsHV-1 µVars, with 1 line experiencing ~11% mortality. The ability for µVars to infect and induce mortality in eastern oysters has significant implications for native species in the Chesapeake Bay and the east coast aquaculture industry. Members of the shellfish industry have expressed concern over the potential threats of the OsHV-1 µVars due to the previous vulnerability of the industry to other pathogens. With a historical lack of research and surveillance, the question has emerged whether OsHV-1 may have a significant impact on the aquaculture industry in Maryland and the east coast.

The purpose of this study was to (1) establish surveillance locations within Maryland, USA, to test for OsHV-1 prevalence; (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 these species can also horizontally transmit the virus to naïve *C. gigas*, a highly susceptible species. The overarching goals of this study were to determine if OsHV-1 can have the potential to significantly impact the aquaculture industry in the Chesapeake Bay and generally on the east coast of the USA and to emphasize biosecurity and management of marine disease.

2. MATERIALS AND METHODS

2.1. OsHV-1 field surveys

Eastern oyster larvae (n = ~10 000) or juveniles (n = 60) were collected during June 7–11, July 8–15, and August 23–27, 2021, from 5 aquaculture farms located along tributaries to the Maryland portion of the Chesapeake Bay: Potomac River, St. Jerome's Creek, Patuxent River, Honga River, and Choptank River (Fig. 1). See Table 1 for age and size ranges of eastern oysters collected during survey times. Where feasible, the same broods were followed through the summer. Mortality events occurred at 2 sites during July (Choptank River and Patuxent River); therefore, a new brood was sampled with any remaining animals from their stocks if possible. The Patuxent River site sent animals of the same age from an adjacent farm, and this brood was followed during July and August.

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

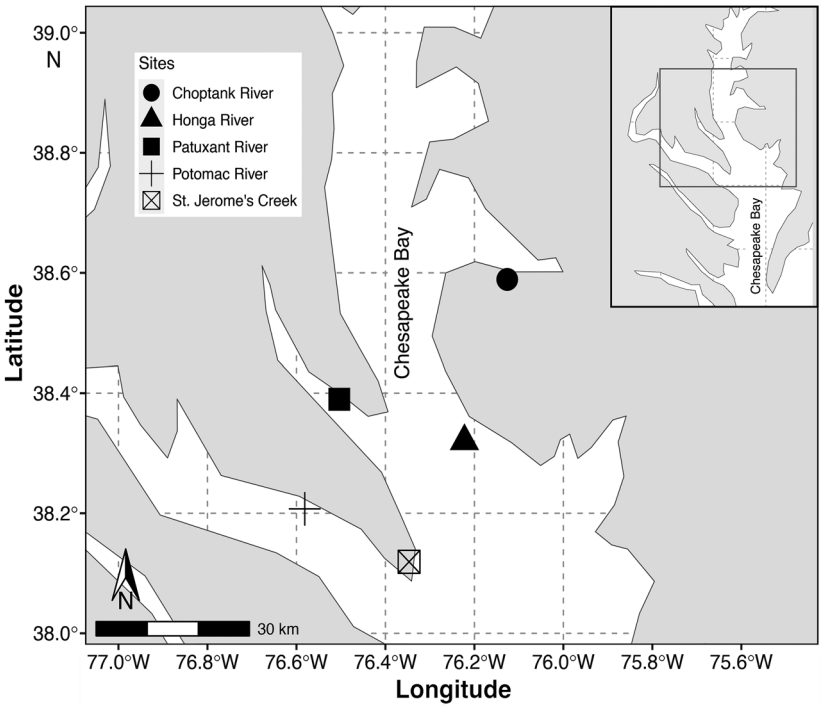

Fig. 1. Survey sample sites in the Maryland portion of the Chesapeake Bay, USA

Table 1. Size and quantity of eastern oysters collected from 2021 OsHV-1 survey sites in Maryland

Date (2021)	Location	Size range	n
Jun 7–11	Choptank River	7 d old larvae	~10000
	Honga River	7 d old larvae	~10000
	Patuxant River	7 d old larvae	~10000
	Potomac River	5–10 mm	60
	St. Jerome's Creek	5–10 mm	60
Jul 8–15	Choptank River[a]	Spat on shell 2–5 mm	36
		7 d old larvae	~10000
	Honga River	1–2 mm	60
	Patuxant River[a]	2–5 mm	60
	Potomac River	10–25 mm	60
	St. Jerome's Creek	10–15 mm	60
Aug 23–27	Choptank River	Spat on shell 2–5 mm	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

[a]Farms that experienced mortality events

2.2. Experimental challenges

2.2.1. Animal husbandry

Animals for experiments were provided from various sources (see Appendix 1). All animals were shipped overnight on ice to the University of Arizona, Aquaculture Pathology Laboratory (UA APL) in Tucson, Arizona. For both studies, all animals were allowed a ~24 h acclimation period prior to injection or exposure in 4 l tanks containing 2 l of 32 ppt 50% natural seawater (NSW) and 50% artificial seawater (ASW). All ASW was made with Crystal Sea Marine Mix (150 gallon [~568 l] mixture dissolved in distilled water) and 0.22 µm filtered. NSW was collected from Bodega Bay (~35 ppt) and was UV sterilized, filtered (0.22 µm), and transported from the University of California, Davis, Bodega Marine Laboratory (BML). Laboratory temperatures were kept at 22°C. All seed animals were fed ad libitum *Nannochloropsis* sp., provided by the BML, upon arrival and daily prior to their exposure.

2.2.2. Inoculums

Viral inoculums used for the dose response and vector study included the French (FRA) µVar (Burge et al. 2020) and OsHV-1 San Diego (SD) µVar (Burge et al. 2021) preparations. Viral homogenates from the FRA µVar were previously created as described in Burge et al. (2020); cryopreserved stocks were held at the UA APL at −80°C following methods of Kirkland et al. (2015). For the SD µVar, no cryopreserved inoculums were available. Therefore, an inoculum was created from moribund Pacific oysters held at −80°C collected from the initial transmission study described in Burge et al. (2021). For the vector study, a new OsHV-1 SD µVar homogenate was prepared from Pacific oysters that were moribund 2 d post injection with OsHV-1 SD µVar (70% mortality observed).

Standard methods were used to create viral inoculum as described in Burge & Friedman (2012) and Burge et al. (2020). The resulting filtrate (referred to as the inoculum) was stored at 4°C until use. OsHV-1 viral copy numbers were quantified by extracting 200 µl of inoculum using the ZYMO Research Quick-DNA Miniprep Plus kit (biological fluids method). DNA was amplified using the OsHV-1 specific quan-

Table 2. Mean seawater temperature (°C) and 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

Month	Honga River		St. Jerome's Creek		Potomac River		Choptank River		Patuxent River	
	°C	ppt	°C	ppt	°C	ppt	°C	ppt	°C	ppt
May	15.8	13	16.7	15.34	16.6	9.4	16.8	10.3	17.9	10.2
June	22.5	11.5	22.5	14.59	24.6	9.3	24.8	10.3	25.3	10
July	27.5	13	26.9	15.81	27.2	10.7	27.9	11.7	28.8	11.2
August	26.8	12.9	27.1	17.11	26.5	12.6	27.3	11.4	28.5	12.7

titative PCR (qPCR) described in Section 2.5. To test the infectious nature of this inoculum, susceptible Pacific oysters were injected and monitored for mortality for 72 h. The inoculum was cryopreserved for future experiments following procedures outlined by Kirkland et al. (2015).

2.2.3. Dose response

A dose response experiment was conducted to determine viral concentrations required to promote infection and mortality. Pacific oysters, ~30 mm in size, were used to create exposed seawater (hereafter 'ExpSW') (Agnew et al. 2020) in the following manner: Post acclimation, animal shells were notched close to the location of the adductor muscle using a metal file to prepare for injection. Inoculum (1×10^6 OsHV-1 DNA copy numbers) was injected into the adductor muscle of oysters using a 28 gauge needle (Agnew et al. 2020, Friedman et al. 2020). After 10 min on the bench top, animals were placed back into their appropriate tanks, and an approximately 24 h incubation was allowed for animals to shed virus (Agnew et al. 2020). OsHV-1 specific qPCR was carried out post 24 h incubation to determine viral concentration (load) in the tank water. Each variant was handled separately; ExpSW was collected on separate days to reduce cross-contamination.

The OsHV-1 specific qPCR determined that the initial viral concentration of both SD and FRA variants in the seawater used for exposures was 1×10^6 viral particles (or copies) ml^{-1} (i.e. DNA copies detected in the qPCR assay). Tenfold serial dilutions were created from 1×10^6 to 1×10^4 viral copies ml^{-1} of the ExpSW. Standard antibiotic concentrations (150 U ml^{-1}) of penicillin and streptomycin were added to each viral concentration solution prior to exposing to the animals. Species exposed to OsHV-1 were eastern oysters, hard clams, and Pacific oysters (positive control), all 5 to 8 mm in size. For each µVar (SD and FRA), animals were exposed in 125 mm deep well petri dishes in replicates of 3 (n = 60 total, n = 20 per petri dish) per viral concentration. ExpSW (60 ml) of the appropriate viral concentration containing 150 U antibiotics ml^{-1} was added to each dish. Hard clams had 2 replicates for the 1×10^4 viral copies ml^{-1} due to shortage of available animals. Controls were in replicates of 3 (n = 60 total, n = 20 per petri dish) and were placed in filtered NSW containing antibiotics (see Fig. 2).

Mortalities were counted starting at 72 h post exposure (Days 3–7) by carefully opening petri dishes and probing animals that were gaping following methods described by Divilov et al. (2019) and Agnew et al. (2020). All dead animals were removed, placed into labeled whirl packs, and frozen at −20°C for at least 7 d and then stored at −80°C. On Day 2 of the experiment, all animals were fed 1 ml of algae (*Isochrysis galbana*) per petri dish. On Day 3, all animals received a water change of 60 ml of 50:50 NSW:ASW, with a final concentration of 150 U penicillin and streptomycin ml^{-1}. At the conclusion of the experiment on Day 7, all animals from exposures and controls (both mortalities and live) were collected and stored at −20°C.

2.2.4. Vector study

Pacific oysters (20–25 mm), hard clams (~40 mm), and eastern oyster juveniles (40–50 mm) were used to create ExpSW (Agnew et al. 2020) to test the ability of these species to horizontally transmit the virus. Post acclimation, oyster shells were notched and injected with 1×10^6 total viral copies as described in Section 2.2.3. Hard clams were injected into the umbo perpendicular to vascular tissue. Animals were given 10 min after injection to acclimate prior to placing them into their appropriate tanks. Animals were then placed into 4 l tanks containing 2 l of 50:50 NSW:ASW and a final concentration of 150 U penicillin and streptomycin ml^{-1} 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 smaller size. Approximately 24 h incubation was allowed for animals to shed virus. Post 24 h

incubation, OsHV-1 specific qPCR was run as a proxy for viral concentration of water from Pacific oysters, eastern oysters, and hard clams for both SD and FRA µVars separately. Control animals were inoculated with filtered seawater, with 2 replicate control tanks per species.

Naïve Pacific oyster spat (5–8 mm) were used to test 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 (n = 180) per dish for each ExpSW species (eastern oysters, hard clams, and Pacific oysters). Test animals were exposed immediately after determining the viral concentration of the ExpSW treatments from adult Pacific oysters, eastern oysters, and hard clams. Control animals were in replicates of 3, with 20 animals per dish (n = 60) for each control vector species. Controls were sham exposed using water from the control tanks. Seawater from each replicate tank was combined per species and added to the appropriate petri dishes.

Mortalities were counted and stored in the same manner as described (Section 2.2.3). On Day 3 of the experiment, all animals were fed in their petri dishes 1 ml of algae (*Nannochloropsis* sp.). On Day 4, all animals received a water change of 60 ml of 50:50 NSW:ASW, with a final concentration of 150 U ml^{-1} penicillin and streptomycin.

2.3. DNA extraction and qPCR

For the survey, gill and mantle tissues were dissected from individual juvenile oysters and pooled (n = 5 oysters per pool, n = 12 pools per site and date). Larvae were distributed among 3 pools containing ~3000 larvae per pool. Total DNA was extracted from approximately 25 to 30 mg of juvenile oyster tissue and 30 to 50 mg of whole larvae. Whole larvae were ground using a pestle to aid in breakdown of the tissue.

For the dose response experiment, OsHV-1 DNA was extracted from tissues of random surviving animals (n = 15), all mortalities from all doses, and random controls (n = 15) for both FRA and SD exposures. Gill and mantle tissue were dissected from individual oysters and hard clams. For the vector study, gill and mantle tissue (20–35 mg) were dissected from random individual oyster samples exposed to the eastern oyster, Pacific oyster, and hard clam ExpSW. Approximately 20% of dead and surviving animals were sampled and dissected due to the large sample size. Control samples were pooled (n = 5 oysters per pool, n = 3 pools per treatment). Total DNA was extracted for all tissue samples using the ZYMO Research Quick-DNA Miniprep Plus kit following the manufacturer's protocol (solid tissues method). For the vector study, water samples of 200 µl taken from injected eastern oyster, Pacific oyster, and hard clam tanks were extracted using the ZYMO Research Quick-DNA Miniprep Plus kit following the manufacturer's protocol (biological fluids method).

OsHV-1 DNA from survey and experimental challenges was quantified using an OsHV-1 specific qPCR assay as a proxy for viral infection. We employed a qPCR assay to determine OsHV-1 presence and viral load using the method described in Burge & Friedman (2012) modified by Burge et al. (2020) (see Appendix 2). The melt temperature was an average of 75.6 ± 0.06°C.We aimed for reaction efficiency between 90 and 110% and $R^2 > 0.998$.

For survey samples, an additional OsHV-1 specific qPCR assay that included an internal control reaction was run. The method is a modification of Martenot et al. (2011), targeting the B region of OsHV-1 using a modified TaqMan probe (5'/-56-FAM/TGC CCC TGT/ZEN/CAT CTT GAG GTA TAG ACA ATC/3IABkFQ/-3'; C. A. Burge et al. unpubl., R. A. Elston & K. L. Humphrey unpubl.), primers OsHV-1 BF (5'-GTC GCA TCT TTG GAT TTA ACA A-3') and OsHV-1 BR (5'-ACT GGG ATC CGA CTG ACA AC-3'), and a gBlocks™ standard curve from 20 to 2×10^7 copies per reaction, 20 copies per reaction being the detection limit. See Appendix 3 for gBlocks™ sequence. No-template controls using UltraPure DNase/RNase-free distilled water in place of DNA were added to each plate (n = 3). Each 20 µl reaction contained 10 µl of TaqMan Fast Universal PCR Master Mix, 2 µl of 10× Exo PIC Mix, 0.4 µl of 50× Exo IPC DNA, 15 µg of BSA (12 mg^{-1}), 0.4 pmol of each primer, 250 nm of probe, and 2 µl of sample in a 20 µl volume. All standard curves were run in triplicate and samples in duplicate using QuantStudio5, with a limit of detection of 20 copies per reaction. Cycling conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. We aimed for reaction efficiency between 90 and 110% and $R^2 > 0.998$.

2.4. DNA sequence analysis

To confirm the identity of OsHV-1 variants in the dose response experiments, PCR was employed followed by direct sequencing of the OsHV-1 open reading frame 4 (ORF4), which is the method accepted for determining variants (OIE 2019). PCR amplification of the OsHV-1 ORF4 region with primers C2

(5′-CTC TTT ACC ATG AAG ATA CCC ACC-3′) and C6 (5′-GTG CAC GGC TTA CCA TTT TT-3′) was used for amplifying OsHV-1 fragments sequenced from hard clam samples from the dose response experiment yielding 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 run in duplicate with a negative control reaction following previously described methods (Segarra et al. 2010, OIE 2019, Burge et al. 2021). Samples were visualized using gel electrophoresis to confirm the amplification of a band of the expected size (Segarra et al. 2010, Burge et al. 2020). PCR products were submitted to the Institute of Marine and Environmental Technology BioAnalytical Services Laboratory in Baltimore, Maryland, for Sanger sequencing.

2.5. Statistical analysis

Statistical analyses were performed using R version 4.1.2. Kaplan-Meier survivorship curves with log-rank chi-square tests and Cox proportional hazard ratios were generated using the survival (Therneau & Grambsch 2000, Therneau 2022) and survminer (Kassambara et al. 2021) packages. Statistics for the dose response were used to investigate differences in survival probability between species exposed to each OsHV-1 variant and viral dose. Statistics for the vector study investigated differences in survival probability of Pacific oysters exposed to ExpSW from eastern oysters, hard clams, and Pacific oysters injected with the SD µVar or FRA µVar.

Differences among dose, virus, and species for tissue concentrations for the dose response experiment were tested individually with a Kruskal-Wallis test ($p < 0.05$) followed by a multiple comparison using the Wilcoxon sum rank test with a Holm probability adjustment (Kabacoff 2015, Agnew et al. 2020). Differences among treatment, virus, and species for tissue concentrations were tested for the vector study using the same models. All data and statistical code are available on Figshare (Kachmar 2023).

3. RESULTS

3.1. OsHV-1 field surveys

OsHV-1 was not detected (i.e. below the 3 copies mg^{-1} of tissue for the SYBR Green protocol and 20 copies mg^{-1} of tissue for the TaqMan protocol limits of detection) in oysters from any of the 5 sites during the 3 sample periods. For the SYBR Green assay, reaction efficiency for the 10 plates run for this analysis was 91.64 ± 1.39% SE and R^2 = 0.993 ± 0.01 SE. For the TaqMan assay, reaction efficiency for the 5 plates run for this analysis was 100.27 ± 0.86% SE and $R^2 = 0.999 \pm 3 \times 10^{-4}$ SE. Additional analyses conducted with the TaqMan qPCR assay achieved 97.94 ± 0.37% SE efficiency and $0.999 \pm 2 \times 10^{-5}$ SE R^2 across 15 plates (triplicate standard curves) with 100% detection of the 20-copy standard (C. A. Burge et al. unpubl.). Internal positive control reactions using the TaqMan assay indicated no inhibition occurred, yielding mean values of 25.65 ± 0.05, 25.74 ± 0.022, and 25.87 ± 0.05 SE for the no-template control, samples, and standards, respectively.

3.2. Dose response experiments

3.2.1. Mortality

Survival probability was observed for each species individually and at separate viral doses. Survival analysis based on mortality counts from Day 3 through Day 7 of the experimental challenge indicated that survival differed among all 3 species (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d157p113_supp.pdf; log-rank test, chisq = 88.9, df = 2, $p < 0.0001$). Pacific oysters had significantly decreased survival probability (87.3–84.3%) when exposed to OsHV-1 compared to controls across all viral doses and variants (Fig. 2a,d, Table S1). Eastern oysters had the highest survival probability (100%) followed by hard clams (96.6–100%) across all variants and doses (Fig. 2b,e, Table S1). Throughout the experiment, only 1 hard clam control individual experienced mortality (Fig. 2c,f, Table S1).

Differences in survival probability between variants and viral doses were analyzed by comparing only Pacific oysters due to a lack of or low mortality in eastern oysters or hard clams, respectively. Survival probability of Pacific oysters when exposed to either the FRA µVar or SD µVar (87.3 and 84.3% survival, respectively) was significantly lower compared to controls (100% survival) across all doses (log-rank test, chisq = 18.3, df = 2, $p < 0.001$) and was not significantly different between µVars (pairwise comparison using log-rank test, $p = 0.480$). Higher viral doses resulted in a significant decrease in survival probability compared to lower doses and the controls, where doses of

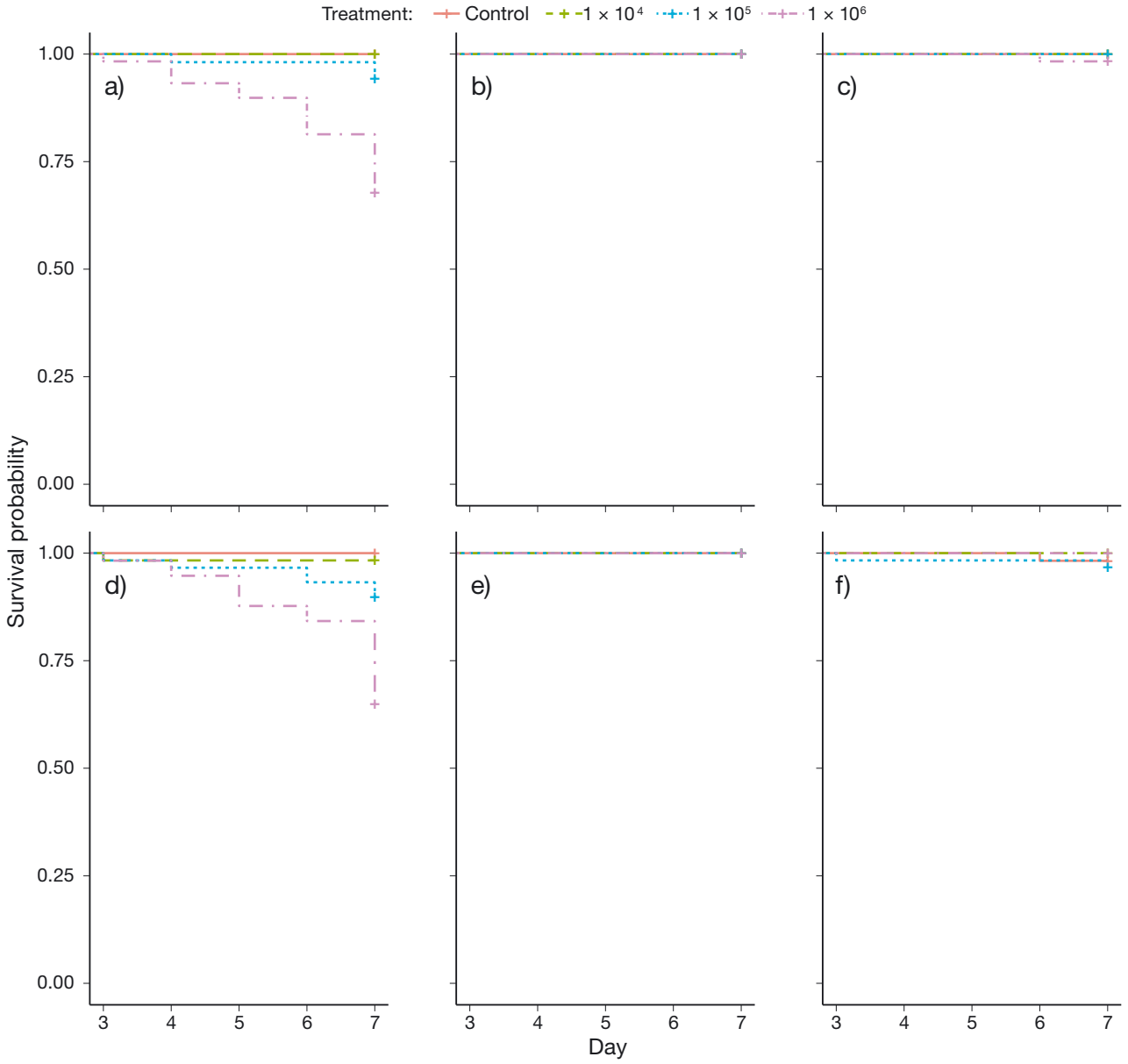

Fig. 2. Dose response of Pacific oysters, eastern oysters, and hard clams exposed to OsHV-1 doses of 10^4, 10^5, and 10^6 viral copies ml^{-1} and controls (no virus). (a–c) San Diego (SD) microvariant (μVar) Kaplan Meier survivorship curves of (a) Pacific oysters, (b) eastern oysters, and (c) hard clams; (d–f) French (FRA) μVar Kaplan Meier survivorship curves of (d) Pacific oysters, (e) eastern oysters, and (f) hard clams

1×10^6 viral copies had 42.5- and 4.3-fold greater mortality, respectively, compared to 1×10^4 and 1×10^5 viral copies (pairwise comparison using log-rank test, both $p < 0.0001$). Doses of 1×10^5 viral copies had 10-fold greater mortality compared to 1×10^4 viral copies (pairwise comparison using log-rank test, $p = 0.009$). Survival probability between viral doses of 1×10^6 (pairwise comparison using log-rank test, $p < 0.0001$) and 1×10^5 (pairwise comparison using log-rank test, $p = 0.003$) was significantly lower (85- and 20-fold greater mortality, respectively) than controls; however, a viral dose of 1×10^4 was not significantly different from controls (pairwise comparison using log-rank test, $p = 0.326$).

Hazard ratios indicated that exposure to the FRA μVar and the SD μVar both had a 20-fold increased risk of mortality in Pacific oysters compared to controls (Fig. S1) and that exposure to viral doses of 1×10^6 viral copies ml^{-1} had a significant effect on increasing the risk of mortality (27-fold) compared to viral doses of 1×10^5 viral copies ml^{-1} (7.3-fold), 1×10^4 viral copies ml^{-1} (0.68-fold), and controls (Fig. S2).

3.2.2. Viral load in exposed bivalves

OsHV-1 copy numbers accumulated in animals (both live and dead) exposed to either the FRA µVar or SD µVar did not significantly differ between viruses (Kruskal-Wallis chisq = 0.002, df = 1, p = 0.968, Wilcoxon sum rank test p = 0.968). There were no eastern oyster mortalities for the FRA µVar or SD µVar. A single hard clam control mortality from the FRA and SD experiments each had an average of 9.1×10^2 and 12.3 viral copies mg^{-1}, respectively. Four control survivors had a mean of 6.9 ± 1.4 SE OsHV-1 copies mg^{-1} of tissue (n = 4) detected, with no viral DNA amplification from the remaining tissue samples (n = 86) (Fig. 3, Table S2).

Viral copy numbers varied between species (Kruskal-Wallis chisq = 98.7, df = 2, p < 0.0001), with Pacific oyster mortalities and survivors having significantly higher copy numbers than eastern oysters survivors (9.5×10^6- and 5.0×10^4-fold) (Wilcoxon sum rank test *Crassostrea virginica* to *C. gigas*, $p = 1.56 \times 10^{-2}$) and hard clam mortalities (2.2×10^5-fold) and survivors (6.8×10^4-fold) (Wilcoxon sum rank test *Mercenaria mercenaria* to *C. gigas*, p < 0.0001). Viral copy numbers in eastern oysters and hard clams were not significantly different (Wilcoxon sum rank test *M. mercenaria* to *C. virginica*, $p = 1.56 \times 10^{-2}$). Across all species, dead animals accumulated significantly higher viral copy numbers than surviving animals (2.9×10^2-fold), which had significantly higher copy numbers than control animals (chisq = 130.6, df = 3, p < 0.0001; Wilcoxon sum rank test control to mortalities, p < 0.0001 ; Wilcoxon sum rank test control to survivors, p < 0.0001; Wilcoxon sum rank test survivors to mortalities, p < 0.0001).

Across all species, higher viral doses resulted in increased viral copy numbers compared to lower viral doses (Kruskal-Wallis chisq = 71.3, df = 3, p < 0.0001). Viral copies detected in dead animals exposed to doses of 1×10^6 viral copies ml^{-1} were higher (1.1-fold) than dead animals exposed to 1×10^5 viral copies ml^{-1} (Wilcoxon sum rank test $p = 7.39 \times 10^{-3}$), and dead animals at both doses had significantly higher (1.1×10^5-fold for both) viral loads than the controls, which had little amplifiable DNA (Wilcoxon sum rank test, both p < 0.0001). Viral copies detected in dead animals exposed to doses of 1×10^4 were not significantly different than those exposed to 1×10^6 or 1×10^5 viral copies ml^{-1} (Wilcoxon sum rank test p = 0.27) or to the controls (Wilcoxon sum rank test p = 0.27).

3.2.3. DNA sequencing analysis

PCR amplification of the ORF4 and ORF100 regions were of expected size (data not shown). DNA sequencing verified that viral DNA amplified from hard clam samples that experienced mortality in the SD µVar dose response exposures were 100% identical in the 634 nucleotides of the SD µVar C2/C6 (ORF4) region (GenBank accession ID: MW504462). Similarly, DNA amplified from hard clam samples that experienced mortality in the FRA µVar dose response exposures were 100% identical over 623 nucleotides of the FRA µVar C2/C6 (ORF4) region (GenBank accession ID: MT157287), with viral DNA amplified from the control hard clam mortality displaying 100% identity across

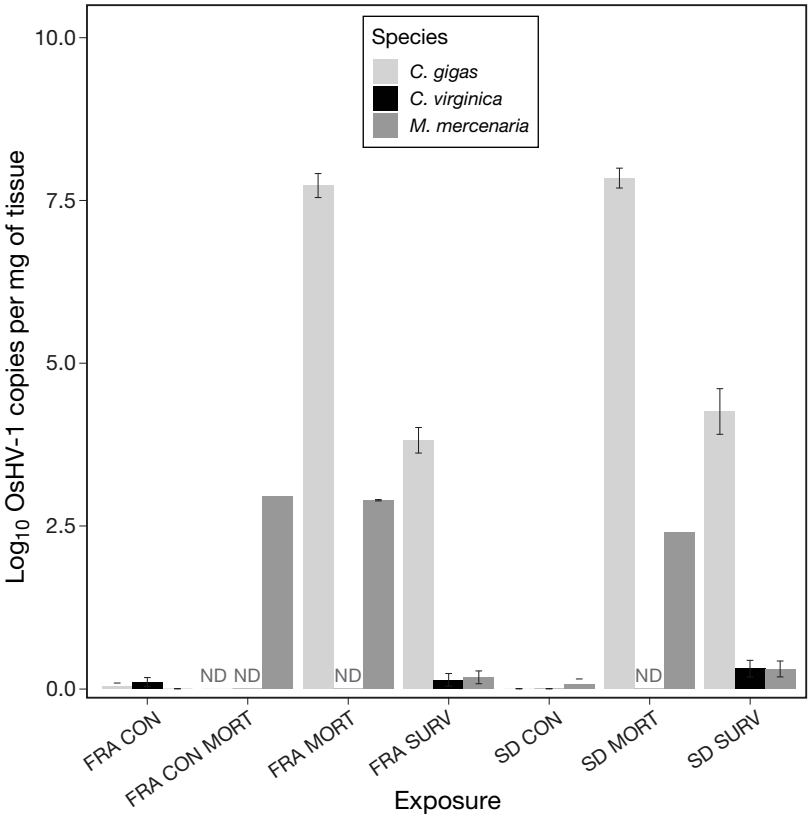

Fig. 3. Dose response experiment: OsHV-1 log-transformed quantification for exposures to the San Diego (SD) and French (FRA) microvariants. *C. gigas*: *Crassostrea gigas*; *C. virginica*: *Crassostrea virginica*; *M. mercenaria*: *Mercenaria mercenaria*; CON: control; MORT: mortalities; SURV: survivors. ND: not detected. Note: SE is absent when n < 2

594 nucleotides. *C. gigas* mortalities exposed to the SD µVar (GenBank accession ID: MW504462) had 100% identity of 636 nucleotides, and *C. gigas* mortalities exposed to the FRA µVar (GenBank accession ID: MT157287) had 100% identity of 608 nucleotides.

3.3. Vector study

3.3.1. Viral load of water treatments

Following the 24 h incubation period, water samples from injected bivalve species tanks were taken immediately before being used to expose spat for the ExpSW treatments to determine viral concentrations. Pacific oysters shed $2.9 \times 10^7 \pm 1.6 \times 10^4$ SE viral copies ml^{-1} of the SD µVar and $8.9 \times 10^6 \pm 5.5 \times 10^3$ SE viral copies ml^{-1} of the FRA µVar. Hard clams shed $4.8 \times 10^2 \pm 4.6$ SE viral copies ml^{-1} of the SD µVar and $6.9 \times 10^2 \pm 3.1$ SE viral copies ml^{-1} of the FRA µVar (Fig. 4).

All Pacific oysters died by Day 3 (FRA µVar) and Day 5 (SD µVar) following injections. Two of the 30 eastern oysters injected with the SD µVar died on Days 1 and 5 post injection. Eastern oysters injected with the FRA µVar had 1 individual die on Day 3 post injection. No hard clams experienced mortality from injection of either µVar during the vector study. We did not quantify virus in tissues of donor animals.

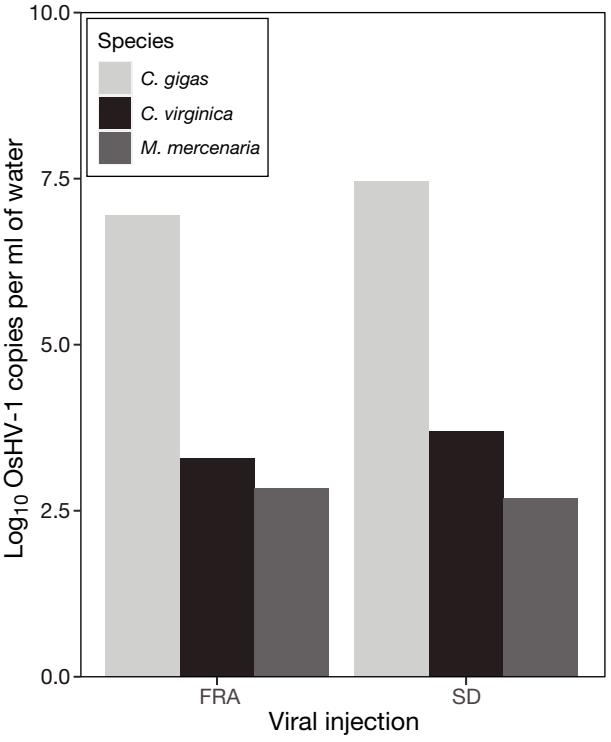

Fig. 4. Log-transformed quantification of 'exposed seawater' from Pacific oysters *C. gigas*, eastern oysters *C. virginica*, and hard clams *M. mercenaria*. FRA: French microvariant (µVar); SD: San Diego µVar

3.3.2. Mortality

Survival analysis based on mortality counts of Pacific oyster spat from Days 3 through 7 of the vector study indicated that there was a significant difference in survival across viruses and between treatments of ExpSW from Pacific oysters (25.6% survival), eastern oysters (66.3% survival), and hard clams (99.8% survival) (log-rank test, chisq = 731, df = 2, $p < 0.0001$) (Fig. 5). Survival probability was examined as a factor of treatment (ExpSW species) and viral exposure (FRA µVar or SD µVar and controls). Across all treatments, there was a significant increase in mortality of animals exposed to the FRA µVar (62.7 ± 3.0% SE) compared to the SD µVar (41.0 ± 2.1% SE) (log-rank test, chisq = 276, df = 2, $p < 0.0001$). Pacific oysters exposed to the FRA µVar (pairwise comparison using log-rank test, $p < 2 \times 10^{-16}$) and SD µVar (pairwise comparison using log-rank test, $p < 0.0001$) had significantly lower survival compared to controls. Survival probability varied significantly between treatments of ExpSW and virus and was significantly lower than controls, which did not have any mortality (log-rank test, chisq = 1991, df = 8, $p < 0.0001$). The spat treated with Pacific oyster ExpSW had significantly lower survival (SD µVars 2.2 ± 1.1 × 10^{-2}% SE, FRA µVars 0%) than spat treated with eastern oyster ExpSW (SD µVars 21.9 ± 3.1 × 10^{-2}% SE, FRA µVars 88.9 ± 2.3 × 10^{-2}% SE) (pairwise comparison using log-rank test, $p < 0.0001$). Spat treated with either the Pacific oyster or eastern oyster ExpSW had significantly lower survival than those exposed to the hard clam ExpSW (SD µVars 99.4 ± 0.5% SE, FRA µVars 100%, pairwise comparison using log-rank test, $p < 0.0001$) or the controls (pairwise comparison using log-rank test, $p < 0.0001$). Survival probability was not significantly different between Pacific oyster spat exposed to hard clam ExpSW when compared to controls for either the FRA µVar (pairwise comparison using log-rank test, $p = 0.497$) or SD µVar (pairwise comparison using log-rank test, $p = 1$).

The Cox proportional hazard model indicated that due to the significant difference in survival between the FRA and SD viruses and compared to controls, the hazard ratio was numerically infinite (coefficients = 18.82 and 18.83; likelihood ratio test = 408.3, df = 2,

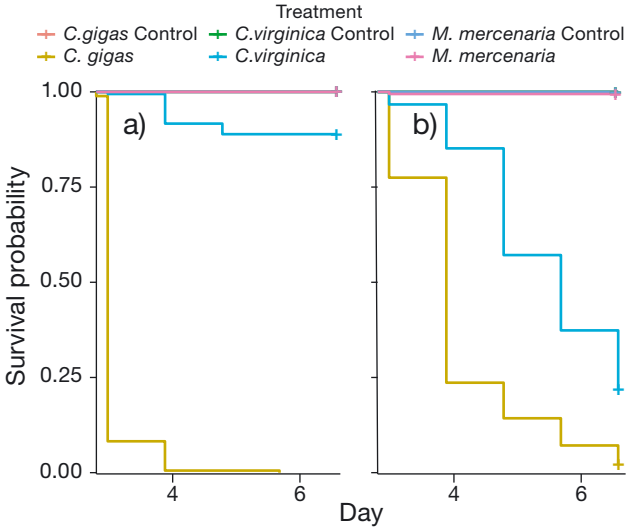

Fig. 5. Kaplan Meier survivorship curves for Pacific oyster spat exposed to (a) French microvariant (µVar) or (b) San Diego µVar 'exposed seawater' from eastern oysters *C. virginica*, hard clams *M. mercenaria*, and Pacific oysters *C. gigas*

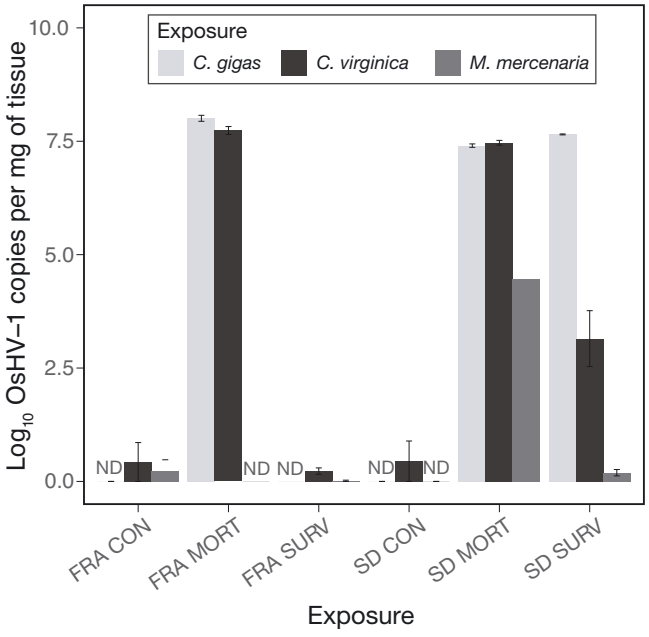

Fig. 6. Log-transformed quantification of Pacific oysters treated with 'exposed seawater' from Pacific oysters *C. gigas*, eastern oysters *C. virginica*, and hard clams *M. mercenaria*. CON: control; MORT: mortalities; SURV: survivors. ND: not detected. Note: SE is absent when n < 2

$p < 0.0001$; score (log-rank test) = 263.9, df = 2, $p < 0.0001$) (see Appendix 4). Hazard ratios indicated that spat exposed to hard clam ExpSW had a decreased risk of mortality compared to those exposed to eastern oyster and Pacific oyster ExpSW, which had a significantly increased risk of mortality compared to the controls (Fig. S3).

3.3.3. Viral load in exposed Pacific oyster spat

Differences among treatment, virus, and species for tissue concentrations were tested. OsHV-1 copy numbers detected in live and dead animals did not significantly differ between the FRA µVar or SD µVar (Kruskal-Wallis chisq = 0.419, df = 1, p = 0.516, Wilcoxon sum rank test p = 0.434). No Pacific oyster spat in the FRA µVar hard clam treatments experienced mortality. No Pacific oysters survived in the FRA µVar Pacific oyster treatments. Pacific oyster spat controls treated with eastern oyster seawater had 2 pools having a mean of 19.5 copies mg^{-1} of tissue, while viral copies in the remaining pools could not be detected. Pacific oyster controls treated with hard clam seawater had 1 pooled sample amplify with a mean of 4.2 viral copies mg^{-1} of tissue (Fig. 6, Table S3).

OsHV-1 copy numbers were significantly different between ExpSW treatments for either the FRA µVar or SD µVar (Kruskal-Wallis chisq = 149.8, df = 2, $p < 0.0001$) (Table S3). Spat exposed to Pacific oyster ExpSW accumulated significantly higher (26-fold) viral copy numbers than eastern oyster ExpSW treatments (Wilcoxon sum rank test *C. virginica* to *C. gigas*, $p < 0.0001$) and hard clam treatments (2.7×10^3-fold) (Wilcoxon sum rank test *M. mercenaria* to *C. gigas*, $p < 0.0001$) for both viruses. Spat exposed to eastern oyster ExpSW had significantly higher viral copy numbers than hard clam ExpSW treatments (Wilcoxon sum rank test *M. mercenaria* to *C. virginica*, $p < 0.0001$). Across all ExpSW treatments and viral exposures, dead animals accumulated higher viral copy numbers than surviving animals (5.1-fold) and controls (9.4×10^6-fold) (Kruskal-Wallis chisq = 217.1, df = 2, $p < 0.0001$; Wilcoxon sum rank test survivors to controls, $p < 0.0001$; Wilcoxon sum rank test survivors to mortalities, $p < 0.0001$).

4. DISCUSSION

This is the first study to expose eastern oysters and hard clams to OsHV-1 µVars by bath exposure to determine susceptibility to mortality and infection and to study the potential for horizontal viral transmission by these species. In this series of studies, the eastern oyster and hard clam stocks exposed to OsHV-1 SD or FRA µVars via bath exposure did not experience infection or mortality under conditions that can lead to

100% infection of the natural host, the Pacific oyster. However, a different stock of eastern oysters, when injected directly with OsHV-1 µVars (FRA and SD), were able to pass the virus to naïve hosts. OsHV-1 may have replicated in the *C. virginica*, leading to shedding, but further study is needed to confirm replication of the virus in the host. 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 oyster samples from the Maryland portion of the Chesapeake Bay during the 2021 summer surveys. Viral replication is heavily influenced by environmental elements such as temperature (Martenot et al. 2015, de Kantzow et al. 2016). Most OsHV-1 experiments are conducted at temperatures typically between 18 and 26°C due to estimated increased risk of mortality at these temperatures (de Kantzow et al. 2016). Infections in the field have been observed at temperatures between 16.2 and 21.9°C in France (Dégremont 2013, Petton et al. 2013, Pernet et al. 2014) and 21 to 27°C in Australia (Paul-Pont et al. 2014). During June through August of the 2021 surveys, the mean temperatures at the sampling sites were ~26°C (±2°C) (Table 2), which may begin to exceed the threshold for viral replication. Delisle et al. (2020) investigated how temperature influenced a host's (Pacific oyster) response to OsHV-1 by comparing transcriptional profiles using RNA sequencing during experimental infections acclimated to 21 and 29°C, as high survival of oysters infected with OsHV-1 at high temperatures (29°C) has previously been observed (Delisle et al. 2018). Host (*C. gigas*) susceptibility to OsHV-1 infection has been shown to be reduced at 29°C, with modulated host responses limiting entry (up-regulation of genes related to membrane composition), early viral infection (up-regulation of apoptosis genes), and viral replication (down-regulation of genes needed for cellular components essential to viral replication) (Delisle et al. 2020). Considering the findings of the 2021 survey, further investigation of the immune response of eastern oysters exposed to OsHV-1 at varying temperatures may help understand their susceptibility. Based on this knowledge, the temperature in the Chesapeake Bay at the time of sampling may have been too high to support an active replicating infection.

Salinity can also play a role in the ability for OsHV-1 to infect a host. 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) determined that survival of oysters acclimated to higher salinities and exposed to OsHV-1 at high salinities (15, 25, and 35‰) was between 43 and 73% compared to oysters in lower salinities (10‰) having a survival rate of ~95%. Therefore, low salinity may also reduce infectivity of OsHV-1. At the survey sites, salinity had a maximum of 17.1 ppt and a minimum of 10 ppt, with most sites ranging closer to the minimum (11–12 ppt) (Table 2). OsHV-1 may not be able to infect in lower salinity environments where eastern oysters reside in Maryland. Follow-up studies exposing eastern oysters at salinities found in the Chesapeake Bay would be useful to determine their risk of infection.

The dose response experiment provided an additional explanation to why OsHV-1 may not thrive in the Chesapeake Bay or other regions in the mid-Atlantic. It should be noted that the eastern oyster line used in the experiment was not bred in the Chesapeake Bay and therefore is not representative of stocks bred in the region. The eastern oyster and hard clam stocks tested in this study experienced minimal to no mortality and low copy numbers when exposed to OsHV-1, indicating that infection did not occur in this experiment. Any amplified OsHV-1 DNA in dead or surviving individuals of eastern oysters or hard clams is not typical or consistent with infection and mortality caused by OsHV-1 (Agnew et al. 2020, Burge et al. 2020, Friedman et al. 2020), suggesting 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 µVars; however, only 1 stock was used for each species and therefore cannot be representative of the entire species. Additionally, only high salinity adapted animals were used in these trials. Friedman et al. (2020) showed that the DEBY (high to moderate salinity performance) *C. virginica* line used in Virginia experienced ~11% mortality when injected with either the FRA µVar or an Australian (AUS) µVar (New South Wales, Georges River; Burge et al. 2020) at 20 ppt. Mortalities also yielded a high viral load in gill and mantle tissue (AUS µVar $1.8 \times 10^6 \pm 1.5 \times 10^6$ copies mg^{-1} of tissue, FRA µVar 6.5×10^6 copies mg^{-1} of tissue), suggesting that differing stocks may not be as resistant to OsHV-1 µVars as demonstrated in this study. Further experiments using other oyster and clam stocks are necessary to fully understand the susceptibility or resistance of eastern oysters and hard clams in the USA. No eastern oyster controls had viral copy numbers or mortality. However, 1 hard clam control yielding low viral copy numbers (~900 viral copies mg^{-1} of tissue) had died, which may have been a result of aerosolization of viral DNA in the wet lab and stress.

Eastern oysters injected with OsHV-1 were able to pass the virus to naïve susceptible Pacific oysters, and while viral replication was not confirmed in injected individuals, this could indicate they have the potential to be vectors or non-competent hosts of the virus when exposed via injection. The small quantities of released virus by eastern oysters were enough to induce infection and mortality (67% FRA µVar and 11% SD µVar) in directly exposed susceptible and naïve Pacific oyster spat. Biosecurity practices such as limiting transport of non-native species and performing disease screenings that include OsHV-1 detection are important to limit spread and emergence of disease to new locations. It should be noted that animals in the vector study were injected with OsHV-1, which is not characteristic of natural infection pathways. Animals naturally exposed may not shed the same amount of virus, giving opportunity for further experimentation on shedding rates of naturally exposed eastern oysters or other bivalve species over time.

The hard clam stock in the vector study did not become infected, experience high mortality, or transmit the virus. This may indicate that hard clams are not susceptible to OsHV-1, although further studies are necessary using additional families or stocks of hard clams. In the vector study, hard clams released a viral load approximately 10 times lower than the viral load released by the eastern oysters for both µVars. One individual Pacific oyster died in the SD µVar hard clam ExpSW treatment and had a viral load (copies mg^{-1} of tissue) high enough to be associated with mortality in the field (Dégremont 2011, Oden et al. 2011, Burge et al. 2021). According to an epidemiology study in France, the mortality threshold for Pacific oysters is 8.8×10^3 copies mg^{-1} of tissue (Oden et al. 2011). Although this low viral load released by the hard clams was not enough to induce high mortality rates in this experiment, this may not be the case on a larger scale on a farm where animals may be grown at higher densities. Further experiments are needed to determine whether most hard clams are not susceptible to or vectors of OsHV-1. Low amounts of OsHV-1 were amplified in the control samples, which could be due to aerosolization of viral DNA in the wet lab. Additionally, due to the control samples being pooled for analysis, any accumulation or amplification of DNA could be less than 3 copies of viral DNA per individual animal sample.

Our experiments augment our knowledge of the more recently detected OsHV-1 SD µVar (Burge et al. 2021). Both the vector study and dose response experiments indicated that the SD µVar had high virulence and infectivity like the historically potent FRA µVar. In the dose response experiment, survival probabilities (Table S1) of Pacific oysters were similar among µVars. Importantly, both µVars caused high mortality rates in the exposed spat and had similar viral load within tissues. In the vector study, naïve Pacific oyster spat experienced higher mortality when exposed to eastern oyster ExpSW from the SD µVar compared to the FRA µVar, which largely occurred in 1 specific replicate or petri dish, experiencing 100% mortality. While this represents the high virulence of the SD µVar, the FRA µVar results are not consistent with previous studies showing the high virulence (Agnew et al. 2020, Friedman et al. 2020, Burge et al. 2021) or what was observed in the Pacific oyster water exposures. We hypothesize that at the low exposure dose in the FRA µVar exposure, 1 animal became infected and shed virus, leading to transmission and mortality in nearby animals due to close proximity and the small volume of water. The Pacific oyster survivors exposed to the eastern oyster ExpSW had no detectable viral copies mg^{-1} of tissue, indicating that infection did not become established in the animals that survived.

Though data from this study suggests lower susceptibility of hard clams and eastern oysters, care should be taken in interpretation of the risks of these species to OsHV-1. In this study, we were logistically unable to source the same eastern oyster and hard clam stocks for both the dose response and vector study. Additionally, eastern oysters, Pacific oysters, and hard clams were sourced from different locations; due to the broad geography of the species tested and regulations regarding movement of animals, it was not possible to source these species from 1 location. Therefore, animals from all locations would be exposed to distinct environmental conditions prior to experiments, which can be related to susceptibility and vectoring of pathogens. All the animals used in the dose response and vector study that were exposed to OsHV-1 were actively filtering, particularly the hard clams (authors' pers. obs.); no data was collected on filtering rates. This indicates that all species were exposed to OsHV-1 during bath studies. Host genetics play a strong role in tolerance and resistance to OsHV-1 infection in Pacific oysters where even in an extremely susceptible species, infection (i.e. viral load as determined by qPCR) and mortalities can be variable in both previously naïve (Divilov et al. 2019, Agnew et al. 2020) and selected individuals (Dégremont et al. 2015). Taken together, this study provides a first perspective on susceptibility of eastern oysters and hard clams.

5. CONCLUSION

The susceptibility of eastern oysters and hard clams to the OsHV-1 µVars and the possibility of horizontal transmission of the virus were examined in this study. Importantly, it was shown that OsHV-1 did not cause mortality in the eastern oyster and hard clam stocks tested through these experimental challenges. OsHV-1 infection was not detected in eastern oysters surveyed in the Maryland portion of the Chesapeake Bay. However, further studies using different eastern oyster stocks and family lines under varying environmental conditions are warranted. With that, there is a need to establish a surveillance program to confirm absence and 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 its µVars. This study also demonstrated how conducting testing with multiple OsHV-1 µVars can help us understand varying virulence and the potential spread of viral variants.

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Appendix 1. Animal sources

For the dose response experiment (see Section 2.2.3), Pacific oyster donors (~30 mm) were produced and provided by an oyster farm in Humboldt Bay, California, where OsHV-1 has never been detected (C. A. Burge unpubl. data, R. A. Elston unpubl. data). Spat Pacific oysters (~5–8 mm) were produced by the University of California, Davis (UC Davis), Bodega Marine Laboratory (BML), from a spawn of Molluscan Broodstock Program broodstock. Eastern oysters (5–8 mm) were produced by the New Jersey Aquaculture Innovation Center at Rutgers University; animals were first shipped to the UC Davis BML, where they were housed for 2 wk in ambient seawater (~35 ppt, 15°C) and fed *Isochrysis galbana* daily. Hard clams (5–8 mm) were produced by the Virginia Institute of Marine Science, Eastern Shore Laboratory. For the vector study, Pacific oysters (~20 mm and spat 5–8 mm) were produced by a farm in Humboldt Bay, California. Eastern oysters (40–50 mm) were provided by the New Jersey Aquaculture Innovation Center at Rutgers University. Hard clams (~40 mm) were provided by an aquaculture farm that wishes to remain anonymous.

Appendix 2. Quantitative PCR (qPCR) protocol

OsHV-1 open reading frame (ORF) 100 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 was used to quantify viral DNA from 3 to 3×10^7 copies per reaction, 3 copies per reaction being the detection limit. No-template controls using UltraPure DNase/RNase-free distilled water in place of DNA were added to each plate in replicates of 3. Each reaction contained 10 µl of Fast SYBR Green Master Mix, 15 µg of BSA 20 mg l^{-1}, 0.4 pmol of each primer, and 2 µl of sample in a 20 µl volume. 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 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

Appendix 3. Sequence for gBlocks™ used in the TaqMan quantitative PCR assay

TGC TGG TGC CAC ATC AAC TGC TGG TGC TGA TGT TGT TGT TGG TGG AGG TGG CTG TTG AAA TAA CGG TGG TGG AGC AGT CAA TGG TAT TAC TGC TTG CTG ATT ATT TGC GCC TGT AAA TGG GTT ACT GGT TGA TGG ATT AGT TGT TGT CGC ATC TTT GGA TTT AAC AAT TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC GCC AGA AAA TTT CCC ACT CTC TTG TTC AGT CCA TGG GTT GTC AGT CGG ATC CCA GTC GAT GAC TTC TAA TCC ACA GGC GAA ACA TTT CAC CTT GTC GCC CAT TCC AGT GTA GAA GAA TCC GGC AGG TGC CAA TGT ATC TTT GTT TGG TCT CAG TTG TTT AGA CCA ACC TCC AAA GCT GTT GAC TCT ATC TTC ATA GAG TAT CAT TTC TG

Appendix 4. Hazard ratio coefficient explanation

For the vector study, Cox proportional hazard ratios were infinite between viruses due to very 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 one or more of the following: (1) ~92% mortality was observed in the FRA µVar Pacific oyster ExpSW treatment by Day 3 of the experiment, significantly decreasing or stopping the number of events compared to the SD µVar Pacific oyster ExpSW treatment, where mortality events happened on Days 3 through 7 of the experiment; (2) only 11% of the FRA µVar eastern oyster ExpSW treatment animals died compared to 66% in the SD µVar eastern oyster ExpSW treatment; and (3) few to no mortality events occurred for hard clams in either viral exposure. Therefore, many extreme events were being compared. This is also the case when comparing each viral exposure treatment.

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