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Unraveling concordant and varying responses of oyster species to Ostreid Herpesvirus 1 variants

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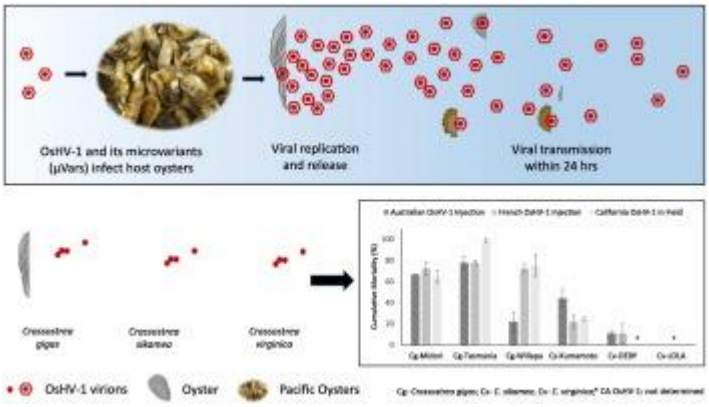
Abstract :

The Ostreid herpesvirus 1 (OsHV-1) and variants, particularly the microvariants (µVars), are virulent and economically devastating viruses impacting oysters. Since 2008 OsHV-1 µVars have emerged rapidly having particularly damaging effects on aquaculture industries in Europe, Australia and New Zealand. We conducted field trials in Tomales Bay (TB), California where a non-µVar strain of OsHV-1 is established and demonstrated differential mortality of naturally exposed seed of three stocks of Pacific oyster, Crassostrea gigas, and one stock of Kumamoto oyster, C. sikamea. Oysters exposed in the field experienced differential mortality that ranged from 64 to 99% in Pacific oysters (Tasmania>Midori = Willapa stocks), which was much higher than that of Kumamoto oysters (25%). Injection trials were done using French (FRA) and Australian (AUS) µVars with the same oyster stocks as planted in the field and, in addition, two stocks of the Eastern oyster, C. virginica. No mortality was observed in control oysters. One C. virginica stock suffered ~10% mortality when challenged with both µVars tested. Two Pacific oyster stocks suffered 75–90% mortality, while one C. gigas stock had relatively low mortality when challenged with the AUS µVar (~22%) and higher mortality when challenged with the French µVar (~72%). Conversely, C. sikamea suffered lower mortality when challenged with the French µVar (~22%) and higher mortality with the AUS µVar (~44%). All dead oysters had higher viral loads (~1000×) as measured by quantitative PCR relative to those that survived. However, some survivors had high levels of virus, including those from species with lower mortality. Field mortality in TB correlated with laboratory mortality of the FRA µVar (69% correlation) but not with that of the AUS µVar, which also lacked correlation with the FRA µVar. The variation in response to OsHV-1 variant challenges by oyster species and stocks demonstrates the need for empirical assessment of multiple OsHV-1 variants.

Graphical abstract



Highlights

► Variants of the Ostreid herpesvirus 1 are infectious pathogens of oysters. ► First comparison of lethality of Ostreid herpesvirus 1 µVars in three oyster species ► Three oyster species show differential susceptibility to the Ostreid herpesvirus 1. ► Dead oysters had 1000 times higher viral loads than survivors.

Keywords : OsHV-1, *Crassostrea gigas*, Crassostrea sikamea, *Crassostrea virginica*, Mortality, Viral load

1. Introduction

The Ostreid herpes virus (OsHV-1) and its microvariants (µVars) threaten the oyster culture industry, which comprises approximately a third of the global marine mollusc aquaculture

production (FAO 2018). Oyster farming is worth billions of dollars to global economies and is dominated by the Pacific oyster, *Crassostrea gigas*, the primary oyster species known to be affected by OsHV-1 and its µVars (Friedman et al. 2005, Burge et al. 2006, Burge et al. 2007, Segarra et al. 2010, Burge et al. 2011, Paul-Pont et al. 2015, Pernet et al. 2016, San Martin et al. 2016, Arzul et al. 2017, Bai et al. 2018). Since the first observation of mortalities in larval and seed Pacific oysters in France and New Zealand in 1991 (Hine et al. 1992, Nicolas et al. 1992, Renault et al. 1994 a,b), OsHV-1 and related herpesviruses have emerged globally and typically cause significant losses in early life history stages of Pacific oysters (Reviewed by Arzul et al. 2017, Burge et al. 2018). Concern over OsHV-1 heightened in 2008 with the emergence of the OsHV-1 µVar in France (Segarra et al. 2010), followed by the detection of multiple OsHV-1 µVars (Martenot et al. 2011, 2015). OsHV-1 µVars rapidly spread throughout Europe, Australia and New Zealand causing mortality in Pacific oysters of all life history stages including adult oysters (Segarra et al. 2010, Schikorski et al. 2011 a,b, Bingham et al. 2013, Jenkins et al. 2013, Keeling et al. 2014, Whittington et al. 2018).

Sequence analyses revealed that many OsHV-1 variants have emerged over the past decade and cause oyster disease outbreaks, which vary in observed temperature ranges, mortality kinetics and host susceptibility (reviewed by Arzul et al. 2017, Burge et al. 2018, Whittington et al. 2018). For example, in France, outbreaks of OsHV-1 µVars occur (in nature) at temperatures as low as 16^{o}C with an upper limit of ~24 ^{o}C, while in Australia, wide-spread mortalities occur when water temperatures exceed 20^{o}C (reviewed in Pernet et al. 2016, Arzul et al. 2017, Whittington et al. 2019). Studies focused on Pacific oyster survival in areas affected OsHV-1 outbreaks including areas affected by OsHV-1 µVar disease, show that size and age impact host survival; improved survival is found in larger and older oysters (Burge et al. 2007, Dégremont 2013, Azéma et al. 2017b Hick et al. 2018). However, for some species, such as *C. angulata*, higher losses may occur in adults relative to younger oysters (Batista et al. 2015). To date, OsHV-1 µVars have been detected across Europe, Australia, New Zealand, and Asia (reviewed in Burge et al. 2018, OIE 2019) but we still have a poor understanding of how virus genotype affects phenotype; that is both the ability to infect and cause disease across species and life stages. Additionally, studies comparing the relative susceptibility of oysters to OsHV-1 µVars are few (Burge et al. 2020, Divilov et al. 2019).

Susceptibility to both OsHV-1 and µVars also varies within host species as has been demonstrated in Pacific oysters (Burge, et al. 2006, 2007, Dégremont et al. 2011, 2015a,b,c, Divilov et al. 2019). For example, oysters selected for resistance to summer mortality in France experienced lower mortality when exposed to either the reference strain of OsHV-1 and a French µVar (Dégremont et al. 2011, 2015a). These resistant oysters were able to both limit infection loads and eliminate an OsHV-1 µVar from their tissues (Dégremont et al. 2011, 2015a). Other bivalve species, such as black lip pearl oyster (*Pinctada margaritifera*) from French Polynesia, were resistant to infections with the New South Wales, Australian OsHV-1 µVar variant in laboratory challenges, and *Ostrea edulis* experienced lower susceptibility to these viruses relative to Pacific oysters (Tan et al. 2015, Sanmartin et al. 2016). These observations suggest that resistance varies among taxa and oyster stocks. Characterization of the susceptibility to infection and disease dynamics among and within different species is needed.

It is also important to consider the role of life history stages and taxa that may exhibit tolerance to virus infection. OsHV-1 may persist in later life history stages (e.g. adult) of Pacific oysters that survive an outbreak, and these animals could therefore serve as reservoirs of infectious virus (Arzul et al. 2002, Dégremont et al., 2013, Evans et al. 2017a). Although the µVars can cause losses in adult oysters, the role of adult oysters as reservoirs of infection for their transmission may vary with strain, location and host (Evans et al. 2017a, Whittington et al. 2018). In order to begin addressing some of these questions, we compared the resistance of three different oyster species including Pacific, Kumamoto (*C. sikamea*) and Eastern (*C. virginica*) oysters (up to three stocks of each species) to two variants of OsHV-1 µVar by injection challenge: a French µVar (FRA) and an Australian µVar (AUS) (Burge et al. 2020). As to date no µVars have become established in the USA, we compared laboratory challenge results with those from field exposures of our experimental oyster groups in Tomales Bay, CA where a non-µVar variant of OsHV-1 is established (Friedman et al. 2005, Burge et al. 2006, 2007).

2. Methods and Materials

2.1 Field exposures

All oysters used in the field trial originated from commercial US west coast stocks as follows: Juvenile diploid Pacific oysters (Tasmania stock, Midori stock and Willapa stock) were provided by Hog Island Oyster Company (Humboldt Bay, CA), Hawaiian Shellfish (Kona, HI), and Taylor Shellfish Farms (Shelton, WA). Kumamoto oysters were provided by Taylor Shellfish Farms. Oysters were shipped directly to Hog Island Oyster Company (Humboldt Bay, CA) and held prior to field and laboratory exposures; to date, OsHV-1 has not been detected in Humboldt Bay.

Triplicate bags (n=500 seed per bag) of 3-6 mo old oysters from each stock of Pacific (mean shell length in mm ± SE: Midori=27.41 ± 0.48, Tasmania=15.85± 0.33 and Willapa=14.65± 0.36) and Kumamoto (*Cs*: 19.88 ± 0.36 mm) oysters were planted at commercial leases held by Hog Island Oyster Company (38°07'30.9"N 122°51'48.4"W) in TB, CA according to the methods of Burge et al. (2006, 2007). Seed oysters were planted on July 5, 2017 and mortality surveys were conducted using established methods (Burge et al. 2006, 2007) on the following dates: 7/11/2017, 7/25/2017, 8/9/2017, and 8/24/2017 with a final mortality survey on 11/14/2017. Cumulative mortality was calculated as the estimated number of oysters that died from 7/5/2017 to 11/14/2017. Prior to outplant, sixty oysters per stock were screened for OsHV-1 using standard protocols (see OsHV-1 quantification) except that oysters were tested for OsHV-1 using pools as allowed by the OIE manual (n=12 pools with n=5 individuals per pool, OIE 2019).

Sentinel oysters (~2 months of age, Tasmania stock, 15.00 ± 0.28) were planted on May 22, 2017 at the same location described above. Once a week, 10 oysters per bag (n=3 bags using identical methods as above) were sampled for OsHV-1 through 8/24/2017; mortality surveys were conducted every two weeks through 8/24/2017 with a final survey on 11/14/2017. For each bag, pools of five oysters were screened (n=2 per bag or n=6 total per sentinel survey) for OsHV-1 (see OsHV-1 quantification). Mean values were calculated by averaging the viral load in oyster pools in each bag.

2.2 Laboratory trials

2.2.1 Oyster holding

Six stocks from three cupped oyster species 9-12 mo in age (40-50 mm in shell height) were used in this study. Three juvenile diploid stocks of Pacific oysters (Tasmania stock, Midori stock and Willapa stock) were provided by Hog Island Oyster Company (Humboldt Bay, WA), Hawaiian Shellfish (Kona, HI), and Taylor Shellfish Farms (Shelton, WA), respectively. One stock of Kumamoto oysters was provided by Taylor Shellfish Farms, and two Eastern oyster stocks (DEBY stock and LOLA stock) were provided by the Virginia Institute of Marine Science Aquaculture Genetics and Breeding Technology Center (Gloucester Pt, VA). All animals were held in local natural environmental conditions at Humboldt Bay, CA and the York River, VA prior to shipment for the experiments. DEBY and LOLA stocks were confirmed to be free of important *C. virginica* pathogens, MSX and *Perkinsus marinus* by histopathology prior to the experiments (data not shown). Oysters were shipped overnight (on ice) to the University of Arizona, Aquaculture Pathology Laboratory (UA APL) in Tucson, Arizona in early January 2018. Shell heights for each stock are shown in Table 1. On arrival, oysters were acclimated to ~18°C at the salinity at which oysters had been reared: 30-31 ppt salinity for the Pacific and Kumamoto oyster stocks and 20-21 ppt for the Eastern oysters over 24 hrs. Temperature, salinity, ammonia levels and pH were monitored daily. Oysters were held for at least three days in quarantine prior to the beginning of the experiment. Prior to use in experiments, oysters were fed Reed Mariculture Shellfish Diet 1800 (Campbell, CA) daily according to manufacturers' recommendation. The day prior to the study, all oysters were notched with a file adjacent to the adductor muscle for subsequent viral (exposed) or sham viral injection (using saline for controls). Artificial seawater was made with Crystal Sea® MarineMix 150 gallon mixture (Marine Enterprises International, Baltimore, MD) using well-water available at the UA APL.

2.2.2 Viral Inoculum preparation

Virus stocks from France (FRA) and Australia (AUS) were the same as those used in Burge et al. (2020) and imported to the US with permission from the USDA-APHIS and the Arizona state veterinarian. Briefly, 0.22 µm filtered homogenate was produced by injecting stocks of susceptible naïve sub-adult Pacific oysters (~10) with 100 µl of one viral isolate (AUS or FRA). At 48 hours post injection, gill and mantle tissues were excised, homogenized and passed through 0.22 µm filters to create inoculum (Burge & Friedman 2012, Kirkland et al. 2015).

OsHV-1 copy numbers were quantified by extracting 200 µl of each inoculum using a Zymo Quick DNA Mini-Plus Kit and the Biological Fluids and Cells method. Extracted DNA was amplified using the OsHV-1 specific qPCR described below. Virus stocks (OsHV-1 µVar AUS and OsHV-1 µVar FRA) were a single passage from filtered homogenates created oysters collected during outbreaks in the George's River, Australia (a derivative of the Australian prototype strain; Kirkland et al. 2015) and Marennes-Oléron Bay, France. Genetic characterization of the 'C' and 'IA' regions for these variants can be found in GenBank under accession numbers MT157286 – MT157289.

2.2.3 Experimental challenges

For each stock, six oysters of each species/stock were placed into each of 18 3.5 L tanks for a total of 54 experimental tanks (Table 1). Triplicate tanks were used for each oyster stock and each treatment that consisted of each viral strain (independently) or no-virus control (Table 1). Oysters were challenged by intramuscular injection (IM) into the adductor muscle with 10^6 genome copies of OsHV-1 µVar per oyster in 0.22 µm filtered ASW or 0.22 µm filtered ASW for controls (Hick et al. 2018, de Kantzow et al. 2019). All tanks were aerated and held at 22^{o}C at the same salinities as experienced during the holding period throughout the study. Oysters were monitored each day for mortality (by lightly tapping the tank) and dead animals (gaping and failed to close valves) were removed and frozen at -80^{o}C for further analysis. Tank water was changed every other day. Oysters were not fed during injection trials. After 6 days, all survivors were collected and frozen at -80^{o}C.

2.2.4 OsHV-1 quantification

All samples from experimental challenges were processed in a BSL3 facility using the Zymo Quick-DNA Mini Plus kit following the manufacturer's protocol to extract total nucleic acids from ~25 mg of gill and mantle tissue (Solid Tissues method) from each oyster. All tissue samples were weighed prior to extraction and thus data is standardized per milligram of tissue. Using methods of Burge & Friedman (2012) as modified by Burge et al. (2020), we targeted the OsHV-1 ORF 100/catalytic subunit of a DNA polymerase δ using the following forward (100 F 5′-TGA TGG ATT GTT GGA CGA GA-3′) and reverse (ORF 100R 5′-ATC ACATCC CTG GAC GCT AC-3') primers and a plasmid standard curve (Burge & Friedman 2012) from 3 to 3 x

10^6 copies per reaction to quantify viral DNA, a proxy for viral load. Briefly, each 20 µl reaction contained 10 µl of the Fast SYBR™ Green Master Mix, 15 µg of BSA, 300 nM of each primer, and 2 µl of DNA. All standard curves were run in triplicate and samples were run in duplicate using the Applied Biosystems 7500 Fast Real-time PCR system or Applied Biosystems QuantStudio 3 with a limit of detection of 3 copies per reaction (Burge & Friedman 2012). Cycling conditions for each qPCR run included: 95°C for 20 s followed by forty cycles of 3 s at 95°C and 30 s at 60°C. Melt curve analysis was performed to confirm the specificity of each qPCR reaction by comparing the melting temperature peak of the positive control DNA to that of the experimental samples after each run following Burge et al. (2020).

2.3 Data analysis.

All analyses were conducted in JMP 14.0 (SAS Corporation). Survivorship was analyzed using a Kaplan-Meier with log-rank Chi-Square statistics, Cox Proportional Hazard tests and risk ratios ($p<0.05$) to test for differences in survival among and within oyster species and stocks after exposure to the AUS and FRA viruses. Exponential, Weibull and Log-normal distribution plots tested for linear failure rates; data best fit the Weibull plot and thus β (slope) was used to assess if the risk of mortality changed over time. Differences in cumulative mortality and qPCR loads (log10 (x+1) transformation) among oyster species, stocks and treatments were tested using least square regression with oyster species/stock and virus as fixed factors and replicate as a random factor. Spearman's rho tested for a linear relationship between qPCR loads (at time of death and, for those that survived, at day 6) and cumulative mortality (day 6). Pearson Product-Moment Correlation was used to test for correlations in cumulative mortality for each stock among the AUS and FRA viruses and controls (at day 6) in the laboratory and among the laboratory and field-challenged oysters. Differences in mean virus loads between survivors and mortalities for each µVar and among variants and controls were tested using least square regression. Post hoc testing to identify differences between or among stocks were identified using the Tukey HSD.

3. Results

3.1 Field exposures in TB, California

The primary period of mortality in the field occurred 3-5 weeks post outplant where fresh mortalities were noted on 7/25/2017 and 8/9/2017. In oysters (Tasmania stock) in adjacent sentinel bags (monitored weekly), peak copy numbers of $1.20x10^7$ +/- $3.39x10^6$ copies/mg were observed on 8/3/2017 (Burge unpub. data). Cumulative mortality varied among species in TB (Tasmania>Willapa=Midori>Kumamoto; $p < 0.05$) TB (DF=3, SS=0.87, MS=0.288, F Ratio=76.77, $p<0.0001$; Figure 1).

3.2 Laboratory Challenge

3.2.1 Survivorship analysis among oyster stocks and species

Table 1 summarizes mean measured parameters for each oyster stock tested in the laboratory. No oysters died that were injected with saline (control; Table 1).

Overall, both the AUS and FRA µVars caused similar losses when mortality from all oyster stocks was pooled (Log-Rank $\chi^2=0.54$, df=1, $p>0.05$). Survival of oysters stocks varied among stocks exposed to each µVars (AUS or FRA, Figure 2, Table 1, AUS Log-Rank $\chi^2=52.83$, df=5, $p<0.0001$ and Cox Proportional Hazard: $\chi^2=43.50$, df=5, $p<0.0001$; FRA Log-Rank $\chi^2=52.50$, df=5, $p<0.0001$ and Cox Proportional Hazard: $\chi^2=49.85$, df=5, $p<0.0001$). Risk (hazard) ratios revealed that when exposed to the AUS virus more Midori and Tasmania Pacific oysters died than did those from Willapa (Hazard ratios = 0.20-0.17, $p<0.001$-0.01). Midori and Tasmania oysters shared similar risk of dying ($p>0.05$). No LOLA Eastern oysters died when exposed the AUS virus and this stock had significantly lower risk of dying than all other stocks (Hazard ratios = $1.2\text{-}7.3x10^{-10}$, $p<0.0001$-0.05) except for the DEBYs whose risk was similar to the LOLAs ($p>0.05$). Kumamoto oysters had a lower risk of dying when exposed to the AUS virus than did Midori and Tasmania stocks (Hazard ratios = 0.32-0.38, $p<0.01$-0.05) stocks, but not relative to the Willapa ($p >0.05$), DEBY ($p>0.05$) or LOLA ($p >0.05$) stocks. Risk (hazard) ratios revealed that when exposed to the FRA µVar all Pacific oyster stocks shared a similar risk of dying ($p>0.05$). No LOLA Eastern oysters died during this study when exposed to the FRA µVar and this stock had significantly lower risk of dying than all other stocks (Hazard ratios = $3.823x10^{-10}\text{-}8.56x10^{-11}$, $p<0.0001$-0.05) except for DEBYs, whose risk was similar to the LOLAs ($p >0.05$). Kumamoto oysters had a lower risk of dying when exposed to the FRA virus than did

all Pacific oysters (Hazard ratios = 0.18-0.22, p<0.01-0.001) but not a lower risk than either Eastern oyster stock: DEBY or LOLA (p>0.05).

When survival of each host stock was compared after exposure to the AUS and FRA µVars, survival was similar for all stocks (Log-Rank χ^2=0-0.1.68, df=1, p>0.05) except for Willapa Pacific oysters for which more oysters died when injected with the AUS µVar (22.2 $\pm$ 38.5%) virus versus the FRA µVar (72.0 $\pm$ 19.2%) virus (Log-Rank χ^2=8.23, df=1, p>0.01). No correlation was observed between mortality to the AUS and FRA µVars when all oyster species were examined (p>0.05).

Weibull β values were greater than 1.0 for all Pacific (β = 1.28-1.61) and Kumamoto (β = 4.12) oysters when exposed to the AUS µVar. Similarly, when exposed to FRA β exceeded 1 for all Pacific (β = 1.73-2.03) and Kumamoto (β = 2.13) stocks but β < 1.0 for DEBY Eastern oysters (β = 0.72). Too few Eastern oysters died to calculate β when exposed to the AUS µVar (LOLA and DEBY) and the FRA µVar (LOLA).

Least square regression identified that cumulative oyster mortality (%) was influenced by stock (species and stock; F-ratio = 12.51, df=5, p<0.0001) but not by virus (F-ratio = 0.6, df=1, p>0.05) or species x virus interaction (F-ratio = 1.84, df = 5, p>0.05). Pacific oysters experienced higher mortality when injected with the µVars than were Eastern oysters, while Kumamoto oysters suffered intermediate mortality levels. Overall, more Midori Pacific oysters died than did Kumamoto or Eastern oysters (DEBY, LOLA). Oysters of the same species suffered similar losses (Tables 1, Figure 1).

3.2.2 OsHV-1 loads among oyster stocks and species

All Midori and Tasmanian Pacific oysters became infected following injection with either µVar, while Willapa Pacific oysters were differentially infected with the two µVars; viral DNA was amplified in 44.4% of the Willapa oysters for the AUS µVar and 100% for the FRA µVar. Infection prevalence was high in Kumamoto oysters with100% infection with the AUS µVar and 77.8% with the FRA µVar. Both Eastern oyster stocks had low to moderate infection levels; 44.4% with the AUS µVar and 38.9% with the FRA µVar in the DEBYs, and infection levels of

22.2% with the AUS µVar and 66.7% with the FRA µVar for LOLAs. Viral loads did not vary among oyster stocks/species or virus, with no interaction among oyster stocks or between virus variant when all data from all oysters (those that died and those that survived) was examined (DF=11, SS = $1.38x10^{21}$, MS = $1.25x10^{20}$, F Ratio=0.84; $p > 0.05$, Figure 3). However, animals that died contained more OsHV-1 DNA copies (~1000x) than survivors at day 6 when the study ended (DF=1, SS=$2.0x10^{21}$, MS=$2.0x10^{21}$, F Ratio=21.4; $p < 0.0001$, Figure 3). Analysis of percent mortality and qPCR copy numbers revealed correlations for both viruses using a pool of all data (0.80 correlation, $p<0.0001$ for AUS and 0.675 correlation, $p<0.0001$ for FRA).

3.3 Comparison of field and laboratory challenges

A linear relationship was observed in cumulative mortalities of all species tested between FRA µVar lab challenges and those observed in TB (correlation = 0.69, $p<0.05$). In contrast, mortality associated with the injection with the AUS virus did not correlate with any observed TB field losses ($p>0.05$)

Discussion

This is the first study to show differential survival among oyster species and important US oyster stocks when exposed to two µVars from Australia and France, which are associated with population-level losses in farmed oysters (Segarra et al. 2010, Jenkins et al. 2013, ESFA 2015). Survival varied among host species and stocks within and between each µVar tested, which has important implications for selective breeding programs designed for OsHV-1 resistance, especially for those in locations where OsHV-1 µVars have not yet emerged (Dégremont 2011, Dégremont et al. 2015a, Kube et al. 2017, Divilov et al. 2019). In a recent study, Divilov and colleagues (2019) demonstrated differential mortality among families of Pacific oysters produced in the USA and exposed to a FRA µVar by bath exposure as young spat in France. Their results were compared to those from a field exposure of the same families to OsHV-1 in TB, California (Friedman et al. 2005, Burge et al. 2006, Burge & Friedman 2012,), which is not a µVar but also shows genetic differences as compared to the OsHV-1 reference strain (Burge unpubl. data, Renault et al. 2012). No correlation in mortality was observed between the results from the field

exposure in TB and those exposed in the laboratory challenges using a French µVar. In contrast, we observed a 70% correlation in mortality of the oysters from the present study when using these same two viruses (FRA µVar and TB OsHV-1). However, we observed no correlation between losses in the TB field study and those resulting from injection of the AUS variant in the laboratory. Studies in New Zealand found a high (genetic) correlation in mortality when using the same AUS µVar in laboratory and field studies (Camara et al. 2017). Lack of concordance between the Divilov et al. (2019) field and laboratory study may have been due to differences in the methods employed in the field (including planting of larger cohorts) versus the laboratory, the viruses used (French µVar vs non-µVar OsHV-1 variant), and/or experimental conditions (i.e. temperature or other important environmental factors). Bath exposure using small seed in multiwell plates was used by Divilov et al. (2019), while the present study injected the viruses into the adductor muscle of larger juvenile oysters. Thus, variation in oyster size, challenge methods and other uncharacterized parameters may be important factors to consider when using field and laboratory challenge trails to inform disease resistance selection programs.

We demonstrated high lethality of both µVars upon injection into Pacific oysters, moderate mortality in Kumamoto oysters, and low mortality for Eastern oysters over the six-day study. We challenged each host species using the salinity of their source waters. The Eastern oysters were thus challenged at 21ppt, a salinity within the range of virulence for a FRA µVar to Pacific oysters (Fuhrmann et al. 2016), where survival over six days was 63.5% at 15 ppt, 42.2% at 25 ppt and 48.5% at 35 ppt with high viral copy number, ~10^8 copies/mg in oysters exposed at 25 ppt (Fuhrmann et al. 2016). However, the salinity tolerance of the AUS µVar has not yet been characterized and needs to be considered when interpreting our results with Eastern oysters. Although intramuscular injection (IM) is not a natural means of infection as it by-passes the mantle as the first line of defense to viral entry, we chose this method to allow for direct addition of equal concentrations of virus to each animal (Hick et al. 2018, de Kantzow et al. 2019). Studies with OsHV-1 µVars have often used injection as the exposure method (e.g. Kirkland et al. 2015, Hick et al. 2018, de Kantzow et al. 2019) in addition to other methods including cohabitation (Azéma et al. 2016) and immersion (in either tissue homogenates or contaminated water) (Kirkland et al. 2015, Divilov et al. 2019); very few studies have compared techniques (Dégremont et al. 2015b, Hick et al. 2018). Two recent studies in Australia and France compared

field exposure to IM injection using the same oyster stocks and found similar mortality trends in the field and laboratory when these stocks were challenged with the same virus variant (from the Georges River estuary, NSW, Australia and Marennes-Oléron Bay, Charente Maritime, France, Dégremont et al. 2015b, Hick et al. 2018). This suggests that laboratory injection trials may prove effective in estimating field performance when oyster stocks are challenged with the local µVar variant. Future studies comparing cohabitation, immersion, and injection would allow us to understand the role of physical barriers (i.e. shell or mucus) to infection as injection bypasses these important barriers (Allam & Espinosa 2016, Ben-Horin et al 2018). For example, Ben-Horin et al. (2018) recently demonstrated that genetically resistant *Crassostrea virginica* respond to the parasite *Perkinsus marinus* by both increasing the rate of shell closure and decreasing the clearance of suspended particles as compared to susceptible oyster stocks.

Longer challenge trials are needed to better estimate long-term survival upon exposure to OsHV-1 µVars, especially when testing potentially more resistant stocks or new species. Weibull β values demonstrated that for all Pacific and Kumamoto oyster stocks tested, mortality increased with time, especially for Kumamoto oysters. Eastern oysters suffered little to no mortality rendering us unable to calculate Weibull β values, except for the DEBY stock exposed to the FRA µVar for which $\beta<1.0$, which suggests that the mortality rate decreased with time. Higher qPCR viral loads were measured in oysters that died (means of 10^6-10^7 copies/mg) than in the survivors (means of 10^1- ~10^5 copies/mg). This observation is consistent with previous studies that document higher virus copy numbers in tissues from oysters that died relative to those that survived (Burge et al. 2020, Hick et al. 2018, Dégremont 2011). Exposure of Pacific oysters to µVars typically leads to high losses within 4-8 and up to 10 days (Marion et al. 2017, Hick et al. 2018, de Kantzow et al. 2019). Our data suggests that longer studies that quantify the effects of OsHV-1 µVars on different host taxa are needed. High qPCR copy numbers were observed in some of our surviving oysters. These higher viral loads, especially in the Kumamoto (mean of ~10^4 copies/mg) and even in Eastern (mean of 10^2 copies/mg for DEBY and nearly 10^5 copies/mg for LOLA) oysters, suggest that subsequent mortality was likely. In the present study and several other studies, oysters that died contained means of 10^4-10^7 copies/mg, similar to those observed in some of our surviving oysters (Oden et al. 2011, Dégremont 2011, Hick et al. 2018, Divilov et al. 2019, Burge et al. 2020). In a study using the French variant, a mortality

threshold of $8.8x10^3$ copies/mg tissue was quantified (Oden et al. 2011) further suggesting potentially lethal loads in many of our surviving oysters. In addition to concern over mortalities, the presence of amplifiable virus DNA in many surviving oysters suggests that they may serve as reservoirs of virus for susceptible hosts. When combined with our observation of correlations between percent cumulative mortality and virus load (C=0.68-0.80), which were similar to those previously observed (C=0.81 with the French variant; Dégremont 2011), these data further suggest a need for longer trials to better assess survival potential of different oyster species and families. Thus, understanding potential impacts of µVar variants to both commercially and ecologically important species is crucial.

The role of vectors in spreading OsHV-1 µVars is speculated but not well understood (Evans et al. 2017b, O'Reilly et al. 2018, Bookelaar at al. 2018, Whittington et al. 2018). Oyster species other than Pacific oysters are susceptible to infection by µVars and their potential role in the transmission of these viruses to susceptible hosts has not been quantified (e.g. Eastern and Kumamoto stocks tested in the present study, and *O. edulis*, Sanmartin et al. 2016). In addition to oysters, both bivalve (*Mytilus* spp; O'Reilly et al. 2018) and non-bivalve hosts (*Carcinus marinus;* Bookelaar et al. 2018) have been shown to be vectors of OsHV-1 µVars. Collectively, these observations suggest that a more comprehensive assessment of host susceptibility to infection by multiple OsHV-1 and its µVars is needed.

The ability of two µVars to infect, replicate and cause mortalities (albeit low-level losses) in native Eastern oysters has important implications for this native species along the US East and Gulf coasts, as well as for the rapidly growing oyster aquaculture industry in those regions. *C. virginica* has undergone significant losses due to disease and other stressors in recent decades. To our knowledge, no regular surveillance for OsHV-1 is occurring on the US East or Gulf coasts. As both OsHV-1 and its µVars cause higher levels of mortality in younger life stages, testing of younger life stages and additional *C. virginica* and *C. sikamea* stocks, as well as commercially grown species, is an important next step. In addition, other viral variants may be more virulent for the Eastern oyster or other species on the East and Gulf coasts. The hard clam, *Mercenaria mercenaria*, for example, is the target species of a lucrative shellfish aquaculture industry on the East coast, but the relative susceptibility or tolerance of *M. mercenaria* to OsHV-

1 and the µVars is unknown. Additionally, the bay scallop *Argopecten irradians* is a species used in both restoration (Tettlebach et al. 2015) and aquaculture (Leavitt and Karney 2001) in the US East Coast and has sustained a successful industry in China and Korea since 1982 for the former and 2002 for the later (Zheng et al. 2004, Kim et al. 2019). A recent study indicated that *A. irradians* larvae are susceptible to an OsHV-1 µVar in Korea (Kim et al. 2019), although no data is available for larger life stages.

Conclusions

Given the emergence of OsHV-1 µVars globally in the last decade, *its likely not a matter of if but when* additional shellfish growing areas will be affected. While it is difficult to predict potential impacts, this study and recent studies (Divilov et al. 2019, Burge et al. 2020) provide crucial data for US and global shellfish industries on disease expression in important oyster species to µVars; significantly this study indicates that species matters. Additionally, the present study suggests that longer trials are needed in species whose survivors contain high loads of virus DNA (i.e. Eastern oysters) to better estimate their long-term survival upon exposure to these viruses. More research is necessary to understand the impacts of µVars on multiple life stages of oysters (i.e. larval, spat, juvenile, and adult stages) as well as other ecologically and commercially important bivalve species (i.e. hard clams, blue mussels and bay scallops) and invertebrate species that co-habitat either in natural shellfish beds or shellfish farms (i.e. green crabs). Both abiotic and biotic stressors (i.e. temperature, salinity, pollutants, food availability, and others) may also be important in understanding the risk that OsHV-1 µVars pose to a shellfish industry or specific species (Fuhrmann et al. 2016, Pernet et al. 2018). Lessons can be learned from the emergence of disease caused by OsHV-1 and its µVars, which has been linked by movement of infected stocks and potentially commerce in large port areas (Pernet et al. 2016, Burge et al. 2018). In order to maintain a robust global shellfish industry additional surveillance prior to stock movements and through passive sentinel studies may be useful and necessary. In an era of rapid change, its necessary to be proactive and prepared for future disease emergence.

Author contributions

Carolyn Friedman (Conceptualization, Funding Acquisition, Investigation, Methodology, Formal Analysis, Visualization, Project Administration, Writing- Original Draft, Reviewing and

Editing). Kimberly Reece (Conceptualization, Funding Acquisition, Investigation, Methodology, Writing- Reviewing and Editing). Bryanda Wippel (Methodology, Editing). Victoria Agnew (Methodology, Editing). Lionel Degremont (Resources, Writing- Reviewing and Editing). Arun Dhar (Resources, Writing- Reviewing and Editing). Peter Kirkland (Resources, Writing- Reviewing and Editing). Alanna MacIntyre (Resources). Benjamin Morga (Resources, Writing- Reviewing and Editing). Clara Robison (Resources, Writing- Reviewing and Editing). Colleen Burge (Conceptualization, Funding Acquisition, Investigation, Methodology, Data Curation, Co-Project Administration, Writing- Reviewing and Editing).

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Table 1. Mean measured parameters for each stock and each oyster species for the challenged conditions using the French µVar (FRA) and an Australian µVar (AUS), and the control. All oysters ranged in age from 9-12 mo. For each species in each treatment all 18 oysters (dead and survivors) OsHV-1 DNA was quantified by qPCR.

Oyster Species	Virus	Shell Height (±SE)	% Mortality (±SE)	Number qPCR+	Mean Viral Load - Dead* (±SE)	Range Viral load-Dead	Mean Viral Load - Survived (±SE)	Range Viral load-Survivors
C. gigas-Midori	Control	53.9 ± 1.5	0	0	0	NA	0	NA
C. gigas -Tasmania	Control	51.2 ± 1.8	0	0	0	NA	0	NA
C. gigas - Willapa	Control	44.5 ± 1.0	0	0	0	NA	0	NA
C. sikamea	Control	41.3 ± 0.7	0	0	0	NA	0	NA
C. virginica -DEBY	Control	41.2 ± 0.8	0	0	0	NA	0	NA
C virginica -LOLA	Control	39.2 ± 1.4	0	0	0	NA	0	NA
C. gigas-Midori	AUS	51.7 ± 0.9	66.7 ± 0	18	$6.27x10^6 \pm 4.11x10^6$	$2.06x10^5$ - $4.89x10^7$	$4.61x10^2 \pm 2.64X10^2$	$4.54x10^1$ - $1.76x10^3$
C. gigas -Tasmania	AUS	54.4 ± 1.3	77.8 ± 6.01	18	$1.95x10^7 \pm 8.41x10^6$	$1.69x10^5$ - $1.14x10^8$	$4.90x10^2 \pm 3.09X10^2$	$4.27x10^1$-$1.39x10^3$
C. gigas - Willapa	AUS	44.0 ± 1.1	22.2 ± 9.07	8	$4.32x10^7 \pm 2.44x10^7$	$1.23x10^6$ - $9.40x10^7$	$2.29x10^1 \pm 1.28X10^1$	0 - $1.77x10^2$
C. sikamea	AUS	41.8 ± 0.7	44.4 ± 8.18	18	$3.16x10^6 \pm 1.43x10^6$	$3.08x10^5$ - $9.38x10^6$	$3.07x10^3 \pm 2.69x10^3$	$4.76x10^1$-$2.74x10^4$
C. virginica -DEBY	AUS	40.6 ± 0.8	11.1 ± 2.36	8	$1.83x10^6 \pm 1.56x10^6$	$2.74x10^5$ - $3.39x10^6$	$2.85x10^2 \pm 1.57x10^2$	0-$2.31x10^3$
C virginica -LOLA	AUS	38.5 ± 0.8	0	4	0	0	$5.61 \times 10^1 \pm 3.46 \times 10^1$	0-$6.06x10^2$
C. gigas-Midori	FRA	53.2 ± 0.3	72.2 ± 6.01	18	$7.48x10^6 \pm 6.87x10^6$	$1.13x10^4$ - $8.30x10^7$	$5.37x10^2 \pm 2.51x10^2$	$4.12x10^1$ - $1.54x10^3$
C. gigas -Tasmania	FRA	53.5 ± 1.3	77.8 ± 2.38	18	$1.18x10^6 \pm 3.58x10^5$	$8.25x10^3$ - $5.76x10^6$	$1.99x10^2 \pm 9.04x10^1$	$2.67x10^1$ - $3.33x10^2$
C. gigas - Willapa	FRA	46.0 ± 1.1	72.2 ± 4.53	18	$1.14x10_7 \pm 1.02x10^7$	$6.02x10^3$ - $1.43x10^8$	$2.85x10^3 \pm 1.84x10^3$	$3.78x10^1$ - $9.88x10^3$
C. sikamea	FRA	41.3 ± 0.8	22.2 ± 5.99	14	$1.89x10^6 \pm 8.98x10^5$	$2.41x10^5$ - $6.21x10^6$	$1.76x10^4 \pm 1.75x10^4$	0 - $2.61x10^5$
C. virginica -DEBY	FRA	40.5 ± 1.0	11.1 ± 9.30	7	$6.52x10^6$	NA	$1.53x10^2 \pm 9.73x10^1$	0 - $1.52x10^3$
C virginica -LOLA	FRA	39.4 ± 0.8	0	7	0	NA	$8.82x10^4 \pm 1.98x10^4$	0 - $1.51x10^6$

Figure 1. Differences in cumulative mortality (%) among oysters exposed to the AUS µVar, FRA µVar and California TB OsHV-1 viruses are illustrated below. Shared letters are statistically similar α= 0.05; lower case letters indicate significance among oyster stocks in laboratory trials while upper case letters represent those in the field trial. *Stocks that were not tested in field trials in California.

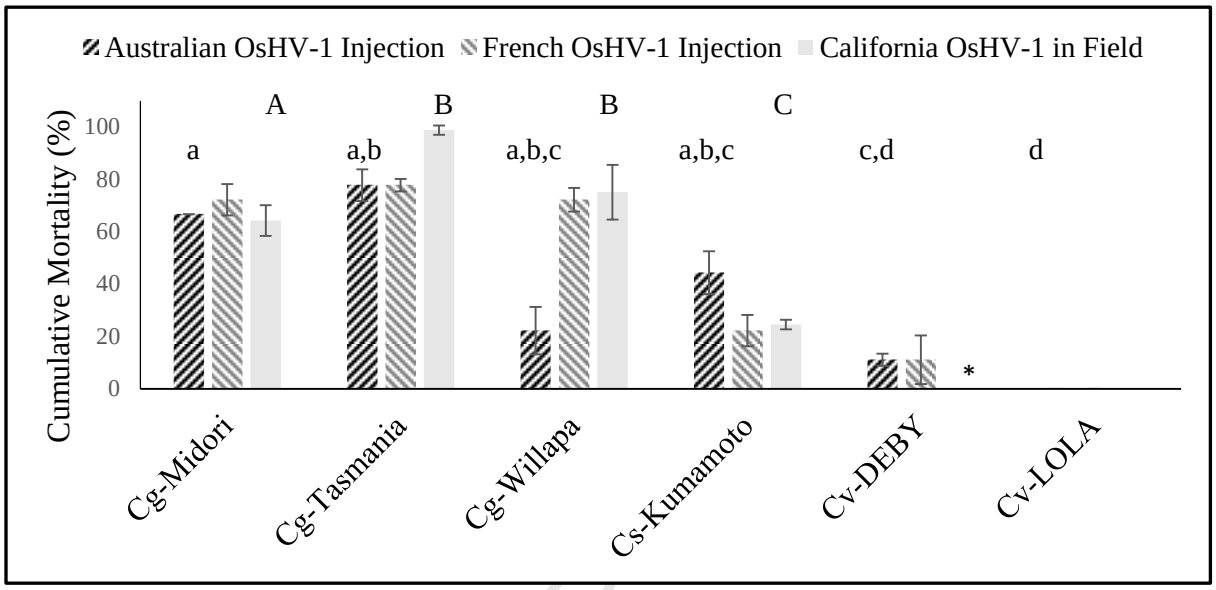


Figure 2. Kaplan-Meier survival curves for oysters injected with the Australian OsHV-µVar (A) and the French OsHV-µVar (B). Black stocks denote Pacific oyster stocks: solid line (Midori), dashed (Tasmania), and dotted (Willapa). Kumamoto oyster survival is shown by the dash/dotted dark gray line, while Eastern oyster survival is shown by the light gray lines: solid (DEBY) and dashed (LOLA).

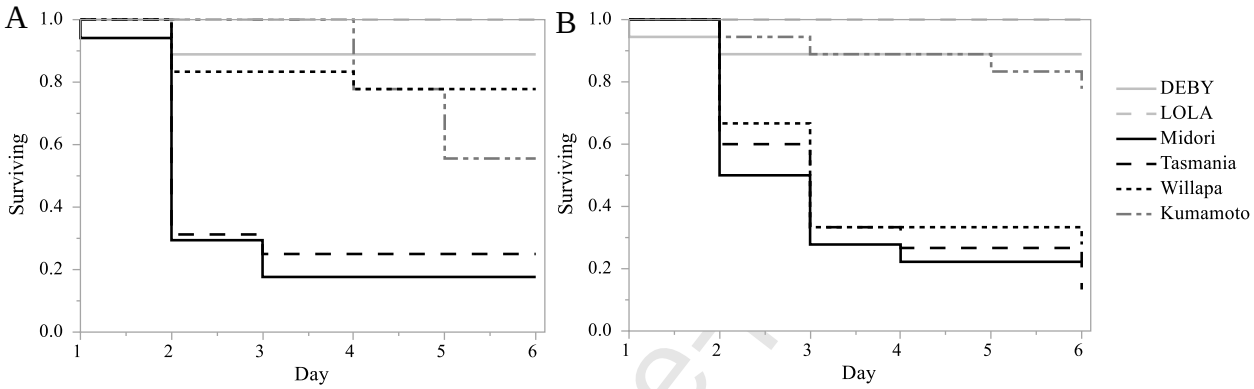
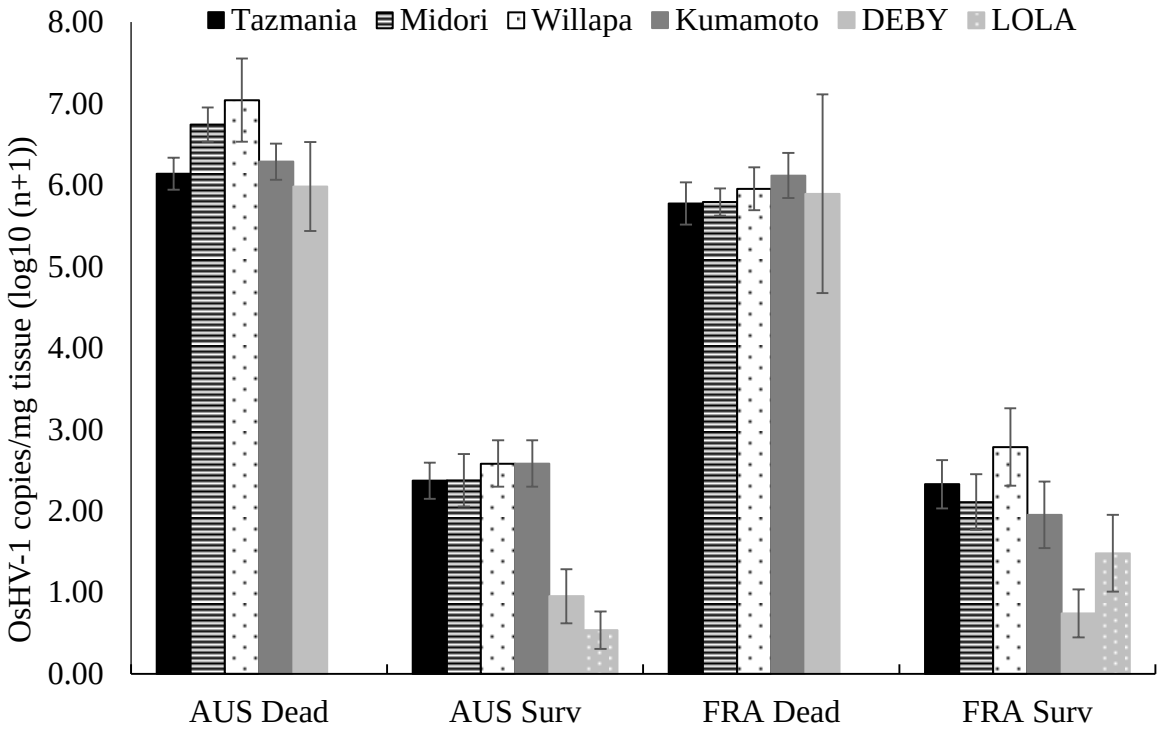


Figure 3. Number of copies of OsHV-1 (±SE) as assessed by qPCR for oysters injected with the Australian or French OsHV-µVar. Infection in Pacific oysters is represented with black bars with the solid bar (Tasmania), dashed (Midori), and dotted (Willapa). Infection in Kumamoto oysters is shown by the gray bar, while denoted in Eastern oysters by the light gray bars: solid (DEBY) and dotted (LOLA). 1000X more OsHV-1 DNA was detected in oysters that died, i.e. 'AUS Dead' and "FRA Dead' as compared to those that survived 'AUS Surv' and 'FRA Surv' ($p<0.0001$).



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract

Highlights

- Variants of the Ostreid herpesvirus 1 are infectious pathogens of oysters
- First comparison of lethality of Ostreid herpesvirus 1 µVars in three oyster species
- Three oyster species show differential susceptibility to the Ostreid herpesvirus 1
- Dead oysters had 1000 times higher viral loads than survivors