The role of the intertidal mobile predator and scavenger the shore crab *Carcinus maenas* in transmission dynamics of the Pacific oyster pathogen ostreid herpesvirus-1 microVar

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SUMMARY

Ostreid herpesvirus-1 microVar (OsHV-1 μVar) has been responsible for significant mortalities globally in the Pacific oyster, *Crassostrea gigas.* While the impact of this virus on the Pacific oyster has been significant, this pathogen may have wider ecosystem consequences. It has not been definitively determined how the virus is sustaining itself in the marine environment and whether other species are susceptible. *Carcinus maenas* is a mobile predator and scavenger of *C. gigas,* commonly found at Pacific oyster culture sites. The aim of this study was to investigate the role of the crab in viral maintenance and transmission to the Pacific oyster. A field trial took place at different shore heights at two Irish Pacific oyster culture sites, over a summer, that are endemic for OsHV-1 μVar. Infection of OsHV-1 μVar in tissues of *C. maenas* at both shore heights of both sites was detected by polymerase chain reaction (PCR), quantitative PCR (qPCR), *in situ* hybridization and direct Sanger sequencing. In addition, a laboratory trial demonstrated that transmission of the virus could occur to naïve *C. gigas* within four days, from *C. maenas* previously exposed to the virus in the wild. These findings provide some insight into the possibility that the virus can be transmitted through marine food webs

and suggests viral plasticity in the hosts required by the virus and potential impacts on a range of crustacean species with wider ecosystem impacts if transmission to other species occurs.

KEY WORDS

Crassostrea gigas, *Carcinus maenas*, ostreid herpesvirus-1 microvar, pathogen–host–environment interplay, predator-prey, scavenger

INTRODUCTION

Diseases, parasites and pathogens are common in marine ecosystems (Lafferty et al. 2015) and have a significant impact on fisheries and aquaculture (Willman et al. 2009; Lafferty et al. 2015), as well as the ecology of marine habitats (Harvell et al. 2002). Development of disease is in general due to a complex aetiology including numerous physical, chemical, biological, and ecological interactions. Hence, the environment and its constituents play a significant role in disease transmission (Mydlarz et al. 2006; Degremont 2011*)*, also known as the 'pathogen–host–environment interplay' (Engering et al. 2013).

Virus infections in bivalve species have been associated with high mortality rates, when conditions become less favorable for the host species (Rowley et al. 2014). A significant pathogen-host-environment interplay has been observed for the commercially important Pacific oyster *Crassostrea gigas* with ostreid herpesvirus (OsHV-1) and variants, which has resulted in mass mortalities among early life stages of *C. gigas* worldwide (Burge et al. 2007; Lynch et al. 2012; Prado-Alvarez et al. 2016*)*. In particular, nowadays these mortalities have been associated with the variant OsHV-1 microVar (OsHV-1 μVar), which is considered highly virulent (Segarra et al. 2010) especially when seawater temperatures reach 16°C and higher (Clegg et al. 2014; Renault et al. 2014; Pernet et al. 2015). The virus has already been proven to be waterborne in previous studies (Vigneron et al. 2004; Sauvage et al. 2010; Schikorski et al. 2011; Evans et al. 2015). Infected adult oysters may function as carriers and infect naïve spat by vertical transmission (Burge and Friedman 2012) and horizontal transmission between healthy and experimental infected oysters has been observed (Schikorski et al. 2011).

Viral transmission within the marine environment provides a medium that can expose all animals within that habitat to a source of infection. Whether viral transmission occurs solely from primary host to primary host is a key point in understanding those dynamics. However, in other host:pathogen interactions in marine systems a range of species and trophic interactions may play a role in disease transmission, with other animals acting as carriers and reservoirs for pathogens (Lynch et al. 2007; Lynch et al. 2010; Small and Pagenkopp 2011). Carriers or reservoirs have been defined as species that can function as a source of infection. A carrier is seen as an incidental, asymptomatic host and a distributor of infection, while a reservoir can retain the pathogen permanently and transmit it back to the natural host (Haydon et al. 2002; Lynch et al. 2010). Furthermore, in specific scenarios, pathogens and diseases can change their host range by selecting new target species as an alternative host (Howard and Fletcher 2012; Engering et al. 2013; Schrauwen and Fouchier 2014).

Infectious disease outbreaks can occur when carrier species, mostly "non-pathogenic" for the specific pathogen, come in contact with a susceptible host species (Burek et al. 2008). It is important to note that viruses are able to jump host as they have been shown to demonstrate plasticity and rapid evolution in terms of hosts targeted, allowing them to respond to and infect a range of potential hosts in new habitats (Johnson et al. 2015; Geoghegan et al. 2017). It is uncertain if *C. gigas* functions as a single host (Arzul et al. 2001a) as herpes-like virus have been detected in multiple different marine species in the past (Renault 1998; Renault et al.

2000; Arzul et al. 2001a; Arzul et al. 2001b; Renault 2001) and recently also in invertebrates such as the oyster *Crassostrea virginica* (Burge et al. 2011), Mediterranean mussel *Mytilus galloprovincialis* (Burge et al. 2011) and Chinese scallop *Chlamys farreri* (Ren et al. 2013). More recently OsHV-1 μVar was detected in the Sydney rock oyster *Saccostrea glomerata*, Sydney cockle *Anadara trapezia*, blue mussels *Mytilus spp.*, hairy mussel *Trichomya hirsuta*, whelks *Batillaria australis* and barnacles *Balanus spp*. (Evans et al. 2017). For most invertebrate species other than oysters infected with herpes-like virus the pathogenic effect is still unknown, however Chinese scallop *Chlamys farreri* suffered mass mortality after infection (Ren et al. 2013) highlighting the potential impact of this virus on its marine environment.

The intertidal zone where *C. gigas* are cultured on trestles contains a range of sessile and mobile filter feeders, scavengers and predators. The European shore crab *Carcinus maenas* is native to the Atlantic coasts of Europe and Northern Africa and is invasive on the west coast of North America, South Africa, Australia and Tasmania (Torchin et al. 2001; Carlton and Cohen 2003). Outside its natural range, *C. maenas* has often been seen as a pest (Lafferty and Kuris 1996) by causing significant ecological and evolutionary impacts, such as altering community structures (Torchin et al. 2002) and by reducing densities of different species of taxa including bivalves, cumaceans and amphipods (Grosholz and Ruiz 1995). *C. maenas* is common at estuarine intertidal habitats (Amaral and Paula 2007) and feeds upon a diverse variety of prey including commercially important species blue mussel *Mytilus edulis* and Pacific oyster seed and juveniles (Lovely et al. 2015). *C. maenas* are known to be attracted to oyster trestles both as a food source and for protection from predation (Lovely et al. 2015). Of significance, *C. maenas* acts as an intermediate host to a number of parasites (Torchin et al. 2001) and may function as a source of infection by transmitting pathogens to predators including birds and fish species and mammals (Bush et al. 1993; Lotz et al. 1995).

It is accepted that predator–prey interactions might affect disease transmission and alter different trophic levels in an ecosystem (Marcogliese 1995) and even affect pathogen persistence in the host species (Hall et al. 2005). It is recognized that predator inhibition or enhancement of the pathogen is ecosystem specific and needs to be explored independently for each specific situation (Moore et al. 2010).

Different routes of entry for diseases and pathogens seem to be possible for *C. maenas*. Firstly, due to ingestion of disease infected tissue (www[1]) *C. maenas* is a mobile predator feeding upon Pacific oysters (McManus 1988) and preferentially targeting moribund (and thus potentially infected) individuals compared to healthy individuals (Moore 2002), resulting in direct take up of pathogens or diseases. Secondly, disease intake could happen by intraspecific contact of diseased scavengers and also cannibalism (Moksnes et al. 1998; Moksnes 2004). In addition, during respiration the gill tissue of *C. maenas* is in direct contact with infected particles in the water column, and the gills of *C. maenas* are recognized as a selective interface between the external environment and the internal milieu (www[1]; Henry et al. 2012).

Differences in crab morphology, like coloration, sexual and life stage migrations are associated with ecosystem characteristics (Stevens et al. 2014). Within the intertidal zones shore crabs are well known to be migrants, both on a tidal and seasonal basis (Crothers 1968) with specific migratory behavior for different size classes and molt stages (Hunter and Naylor 1993). It is not well known how man-made structures, like oyster trestles and a virus infected culture species, might influence the natural migration patterns and behavior of *C. maenas*.

In this study, disease dynamics involving OsHV-1 μVar, *C. gigas* and a mobile scavenger, *C. maenas* was studied at two Irish Pacific oyster culture sites, responsible for the majority of production of Irish *C. gigas* with a history of OsHV-1 μVar and having different ecosystem characteristics. The role of *C. maenas* as a potential carrier, reservoir or alternative host of OsHV-1 μVar was investigated, taking into consideration the potential extension range of the virus in crabs as they migrated up and down the intertidal zone, associated with changing morphological and ecological characteristics during the crab's life cycle. The nature of the role of crabs in viral transmission was determined by laboratory-based trials. The focus of the study was to gain a better understanding of how the virus might sustain itself in the marine environment once introduced into a particular habitat and give a better insight into the potential wider ecosystem impacts of such introductions.

MATERIAL AND METHODS

(1) Field trial

Study sites

Invertebrate sampling took place at two main Irish oyster culture sites, with different habitat structure; Dungarvan, Co. Waterford (52.0936 °N -7.6204°W) and Carlingford Lough, Co. Louth (54.0733°N -6.1994°W), approximately 245 km apart (Figure 1). Both sites are the main areas of production of Irish *C. gigas* and have a history of OsHV-1 μVar (www[1]) and oyster trestles are held in intertidal area with a tidal cycle of approximately 7-9 hours of emersion depending on neap or spring tides (Oyster farmers Pers. Comm.).

The oyster culture site in Dungarvan is sheltered, being almost closed off by the linear Cunnigar spit to the east (www[2]). Intertidal habitats are dominated by sandflats and it has mudflats at the edge of saltmarsh habitats. The water quality of Dungarvan Harbour varies from moderate to good, representing unpolluted water and acceptable levels of biochemical oxygen demand (EPA 2015). The oyster culture site in Carlingford Lough has a gravelly substrate covered by

3-5cm of muddy silt. Carlingford Lough, fed by the Newry River, has generally shallow waters of 2-5 m. Water quality within the lough is good; mean salinity is 32.5 and the annual temperature varies between 3 - 20°C (www[3]).

Environmental (salinity, pH and temperature) data loggers (Star-Oddi) *in situ* at the oyster trestles were used to measure and record water temperature continuously every hour from the end of May until the end of August 2015 at both sites, however, due to a technical issue with the logger, data was not recorded from the end of June to the end of July at Dungarvan. Average water temperatures were calculated as average temperature per day for the time submerged.

Macroinvertebrate sampling

Up to 30 crabs were collected randomly on the mid to low shore at the oyster trestles and at the high shore approximately 500 m from the trestles, every two weeks from the end of April until the end of August 2015 to detect possible infection of the virus. At Dungarvan, *C. maenas* were sampled directly from the oyster bags on the trestles approximately 1 foot above the sediment, as no crabs were observed outside the oyster bags. At Carlingford Lough, crabs were sampled outside the oyster bags on the sediment around the trestles. At the high shore at both sites, *C. maenas* were sampled from rock pools and rocky outcrops. In addition, to detect baseline levels of virus in the natural host, at every sampling date, 30 *C. gigas*, originally imported from French hatcheries which were selectively bred for resistance to the virus (Oyster farmers Pers. Comm.), were collected at the oyster trestles at both sites.

In total, 806 crabs and 510 oysters were collected. Dungarvan was sampled nine times, with 60 crabs sampled at the high shore (as it was difficult to find crabs at this location) and 270 crabs and 270 oysters at the trestles. Carlingford Lough was sampled eight times with 238 crabs sampled at the high shore and 238 crabs and 240 oysters at the trestles.

Morphometric characteristics of C. maenas

Weight (g) and carapace width (mm) were recorded using a balance scales and vernier calipers. Carapace width was divided into 4 different length classes, Class 1: 9.3-20 mm, Class 2: 20.1-30 mm, Class 3: 30.1-40 mm, Class 4 > 40.1 mm. Weights were divided into 4 different weight classes, Class 1: 0 – 10.0 g, Class 2: 10.1 – 20 g, Class 3: 20.1 - 30 g, Class 4 >30.1 g. Classification of crab carapace colour (brown, green and red)/moult stage and sex was noted by gross visual examination.

(2) Laboratory transmission trial of OsHV-1μvar from Carcinus maenas to Crassostrea gigas

A laboratory transmission trial was designed to determine the nature of positive results detected in the wild and to assess the possibility of viral transmission from the crabs to oysters. Naïve *C. gigas* (n=180) with an average weight of 3.4 g and an average length of 31.9 mm, which had never been exposed to OsHV-1 μVar and proven to be naïve by the Marine Institute (www[4]), were obtained from a hatchery at New Quay, Galway Bay (53° 09′ 16·27″ N, 9°04′ 58·19″ W). Crabs with an average weight of 18.5 g and an average carapace width of 40.2 mm were randomly collected from Carlingford Lough in September 2015 where OsHV-1 μVar had been detected in oysters and in crabs during the field study. Prior to the start of the trial, 30 naïve *C. gigas* and 30 C. *maenas* were screened for OsHV-1 μVar by polymerase chain reaction (PCR), to confirm the oysters were uninfected and to determine if the virus could be detected in *C. maenas*. Before placing in tanks, *C. maenas* were washed several times in ddH_2O to remove any pathogens that may have been incidentally attached to their external body/shell. 10 l tanks were filled with 8 l of UV treated seawater. In Ireland, water temperatures often remain below the threshold temperature of 16°C (www[5]) and to imitate natural water temperatures, a lower temperature was chosen during the laboratory trial. UV filtered natural seawater and animals were held at 14°C in a constant temperature (CT) room with a salinity of 35 ppt. At the start of the trial a water conditioner (1 ml of Aqueon) was used, to keep the water quality to an optimum. The experimental set up consisted of two control tanks each containing 30 naïve oysters and three experimental tanks, which contained 30 naïve oysters and 10 virus-exposed crabs each. The trial ran for 14 days. The tanks were checked twice a day for mortality (open shells) and dead individuals were removed and screened for OsHV-1 μVar if tissue was present and of a suitable quality, but no tissues could be recovered for screening from these animals due to predation. After day 2 (48 hours), Day 4 (96 hours), Day 7 (168 hours) and Day 11 (264 hours), living oysters (n=3) were arbitrarily selected from the tanks each time to screen for OsHV-1 μVar. All individuals, oysters and crabs, still alive at the end of the experiment were removed and screened for OsHV-1 μVar.

Molecular diagnostic screening

DNA extraction

Gill and internal tissues made up of connective, digestive and reproductive tissues of both oysters and crabs were stored in 70% ethanol for DNA extraction. Prior to extraction, tissues were washed in double deionized water (ddH_2O) thoroughly and blot dried using tissue paper. DNA extraction was performed using the Chelex-100 methodology. Tissue samples from the invertebrates (approx. 5mm^2) were placed in a 10% chelex solution (100 microlitres volume) (Sigma Aldrich) and following the samples were placed in a thermo Hybaid thermal cycler for 1 hour and 10 minutes heated at 99°C to facilitate cell lysis (Walsh et al.1991). To avoid false negatives, a subsample of DNA samples (n=30) were checked for DNA quantity and quality by using a NanoDrop 1000 spectrophotometer following protocol T042-TECHNICAL BULLETIN NanoDrop Spectrophotometers (www[6]). From the samples collected from

Dungarvan during the field trial, DNA was extracted from 330 individual *C. maenas* with (330 gill and 330 internal tissues being screened) from those crabs and 270 *C. gigas* were sampled (270 gill tissues only) being screened. DNA was extracted from 476 *C. maenas* (476 gill and 476 internal tissues screened) and 240 *C. gigas* (240 gill tissues screened) in Carlingford Lough. For the laboratory trial, DNA was extracted from 58 *C. maenas (*58 gill and 58 internal tissues screened) and for 137 C. *gigas* (137 gill tissues screened).

Polymerase chain reaction (PCR)

For all samples collected in the field and laboratory trial standard PCR to detect OsHV-1 µVar was performed following the protocol of Lynch et al. (2013) by using OHVA/OHVB primers. All PCRs used a total of 2 µL genomic DNA template per individual. Expected size of amplified PCR products for OsHV-1 µVar was 385 bp and PCR was carried out in 25 µL containing 12·9 µL ddH$_2$0, 5 µL, 5× buffer, 5 µL dNTPs (0·2 mM), 0·5 µL MgCl$_2$ (25 mM stock), 0·25 µL of each primer (100 pmol mL^{-1} stock) and 0·1 µL Taq DNA polymerase. Positive controls (duplicate) consisting of OsHV-1 µVar infected oyster tissue and negative controls (duplicate) of double distilled water (ddH$_2$O) were used for each PCR. Thermo cycling conditions were performed by initial denaturation of 1 min of 95 °C, following by 35 cycles including a denaturation step of 20 seconds at 94 °C, an annealing step of 30 seconds at 56 °C and an elongation step at 72 °C and finishing with a final elongation step of 7 minutes at 72 °C by using a thermo Hybaid PCR express thermal cycler (Lynch et al. 2013). Presence of amplified PCR products was confirmed by electrophoresis using a 2% agarose gel stained with ethidium bromide (10mg/l stock) and was run with an electrical charge of 110V for 45-60 minutes.

Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) was carried out to determine the viral load of samples deemed positive for OsHV-1 μVar by PCR, on a subsample of *C. maenas* collected in the field trial (n=43) and *C. maenas* (n=24) and *C. gigas* (n=5) in the laboratory trial, following the protocol "http://www.eurl-mollusc.eu/content/download/42545/578238/file/OsHV-" (www[7]) using primers HVDP-F and HVDP-R (Webb et al. 2007). All qPCRs used a total of 5 μL genomic DNA template per individual (duplicate). The qPCR mix was carried out in 25 μL containing 12.5 μl 2 x Brilliant Sybr Green ® Q PCR Master Mix, 2.5 μl HVDP-F (5μM) and 2.5 μl HVDP-R (μM) primers and 2.5 μl ddH_2O. Standards were used to detect the exact amount of viral copies μl^{-1} of genomic DNA in tested samples. Standard curves were prepared by diluting a viral DNA suspension of 10^8 viral copies μl^{-1} of genomic DNA of OsHV-1. Q PCR plates included 5 dilutions of 10^5, 10^4, 10^3, 10^2 and 10^1 viral copies μl^{-1} of genomic DNA. Negative controls (duplicate) of double distilled water (ddH_2O) were used for each qPCR. Thermo cycling conditions were performed by initial denaturation of 2 min of 50 °C and 10 min at 95°C, following by 40 cycles of 15 seconds at 95°C and 1 min at 60 °C and a melt curve of 95°C for 15 seconds, 60 °C for 1 minute, 95°C for 30 seconds and 60 °C for 15 seconds by using a thermo Hybaid PCR express thermal cycler (www[7]).

In situ hybridization (ISH) with DIG labelled probe

In situ hybridization (ISH) was carried out to detect the viral genome within different tissue sections of virus infected individuals. For each individual collected in this study, a section of internal tissue including gills, digestive and reproductive organs, were removed for histological analysis and immediately fixed in Davidson's solution at 4 °C for 24-48 h after which they were placed in 70% ethanol. *In situ* hybridization assays were carried out on *C. gigas* and *C. maenas* from the field trial screened negative (n=3 per species) and positive (n=3 per species) for OsHV-1 μVar by PCR. Samples were processed (Shandon Citadel 1000) and sectioned to 7 μm tissue thickness. ISH was carried out using a digoxigenin (DIG)-labelled probe (Lynch et al. 2010). Sections were viewed and viral cells were noted with a Nikon Eclipse 80i and images were captured using NIS elements software (at 100×, 200× and 400×).

Direct Sequencing

Direct Sanger sequencing of DNA of PCR products (385-bp) amplified in *C. maenas* from the field trial (n=3 gill tissues and n=3 internal tissues) was carried out to confirm OsHV-1 μVar detection. DNA was isolated from PCR products of separate tissues (pooled 4 replicates per tissue to increase the DNA concentration). Qiagen Qiaquick gel extraction kit was used to isolate and clean up the DNA, prior to direct sequencing of both forward and reverse strands of DNA by Eurofins MWG. Sequences were matched by BLASTn nucleotide database (https://blast.ncbi. nlm.nih.gov/) to confirm true infection of OsHV-1 μVar.

Statistical analyses

Statistical analyses were performed in Statistical model program R studio (R core team 2013). Normality was tested using the Shapiro-Wilks Normality test. A Mann Whitney test was used to determine if there was a significant difference between the mean weight and mean carapace width between the sites. Pearsons Chi-squared tests were used to compare sex and colour between sites and within sites at the two shore heights and to test for differences in prevalence of OsHV-1 μVar within gill and internal tissue for crab length classes, weight classes, sex and colour/moult stage. For all analyses, a critical value of 0.05 was used to confirm significant results. Data are presented as mean ± standard error.

RESULTS

(1) Field trial

Prevalence of OsHV-1 μVar in Crassostrea gigas

Herpes virus was detected in oysters at both sites during the study period. Overall prevalence of OsHV-1 μVar detected by PCR in oysters at the two sites for the duration of the field trial was low with a mean prevalence of 3.75% at both sites, with a range in prevalence of 0-27% at Dungarvan and a range of 0-23% at Carlingford Lough. However, the mean temperature over the study period was 15.0°C for Dungarvan and 14.2 °C for Carlingford Lough, with the overall water temperature during summer 2015 being low, rarely reaching temperatures of 16 °C or higher (Figure 2). At the farms, a tidal cycle of approximately 7-9 hours of emersion depending on neap or spring tides are common (Oyster farmers, pers comm) and therefore the sites were exposed to higher temperatures during low tides. Periods of air temperature above 16 °C were measured from the end of May (www[8]). Significant difference in prevalence between months were observed for both Dungarvan ($P < 0.01$) and Carlingford Lough ($P < 0.01$) with highest prevalence in June for both sites.

Crab morphometrics

C. maenas were significantly larger ($P < 0.01$) and heavier ($P < 0.01$) at Carlingford compared with Dungarvan (Table 1). All four carapace classes were present at both locations and shore heights, with crabs at Carlingford Lough having significantly larger carapace widths compared to crabs at Dungarvan ($P < 0.01$). Within Dugarvan, larger crabs were significantly more abundant at the trestles ($P < 0.01$), while no significant difference in carapace widths was observed between crabs at the trestles and high shore in Carlingford Lough ($P > 0.05$). All four crab weight classes were present in Carlingford lough while three weight classes were observed at Dungarvan, no significant differences were found for weight classes between high shore and trestles. A significantly higher ($P < 0.01$) female-male ratio of 1:0.6 in Carlingford Lough was observed relative to 1:1 in Dungarvan. Within each site, no significant difference in female-male ratio was observed between the high shore and trestles. Green, brown and red coloured *C. maenas* were observed at Dungarvan and Carlingford Lough. At Dungarvan, green, recently moulted crabs were most abundant, followed by brown and red (1:4.1:2.2 for red:green:brown crabs), while at Carlingford Lough brown crabs were most common, followed by green and red crabs (1:1.9:2.0 for red:green:brown crabs). Colour ratio did differ significantly between both culture sites ($P < 0.01$). Within sites only a significant difference within coloration was observed between high shore and trestle at Carlingford Lough ($P < 0.01$), with significantly more green crabs at the trestles and brown crabs at the high shore.

Viral detection in C. maenas

OsHV-1 μVar was detected in *C. maenas* during the entire five-month field trial at both culture sites (Figure 3). The mean prevalence of infection in both tissues of *C. maenas* was higher at Dungarvan at 18.3% (n=121/660) compared to Carlingford Lough with 16.3% (n=155/952), but not significantly different ($P > 0.05$). The overall prevalence of OsHV-1 μVar in the screened tissues of *C. maenas* for both sites combined was 17.1% (n=276/1612). For those infected tissues, the virus was detected only in gill tissue in 89.9% (n=241/268) of the crabs, in only the internal tissue of the crabs in 7.1% (n=19/268) of animals and was observed in both gill and internal tissues in 3.0% (n=8/276) of crabs. This pattern was present at both shore heights in Dungarvan and Carlingford Lough (Table 2). qPCR analyses indicated different viral loads for a subsample (n= 43) of the crabs' gill tissue and internal tissue, screened positive initially with PCR. Overall the viral load was low, with up to 100 viral copies μl^{-1} of genomic DNA in most crabs (n=36) screened by qPCR, while some individuals (n=7) had higher viral DNA load, with the highest load detected being $>10^4$ viral copies μl^{-1} of genomic DNA (Table 3). One forward and one reverse DNA sequence was generated from one sample of *C. maenas* in the Direct sequencing. After sequencing of the PCR products, BLASTn analysis showed a match with an average of 96% (94-98%) similarity and 99% identity with OsHV-1 μVar (KU861511.1) for the sequence of the PCR-amplified products for *C. maenas*. *In situ* hybridization staining of crab (digestive and connective internal tissues) and oyster tissue sections resulted in a positive signal for OsHV-1 μVar in PCR-positive crabs (Figure 4A, 4B, 4C) and oysters, while PCR-negative crabs (Figure 4D) and oysters indicated no staining (i.e. no infection) in any tissue.

No temporal pattern was observed for OsHV-1 μVar prevalence in both tissue groups, however prevalence in gill tissue was significantly lower in April compared with all other sampling months (May, June, July and August ($P < 0.05$)) for both sites. Patterns in prevalence between sites at high shore and lower shore for different tissue groups at Carlingford and Dungarvan only showed significantly higher prevalence of OsHV-1 μVar in the internal tissues of crabs at the trestles ($P < 0.01$) compared to higher shore.

OsHV-1 μVar was detected in all length and weight classes sampled at both sites and shore heights. No clear trend was found for the prevalence of OsHV-1 μVar in the crab gill and internal tissues for the different length classes and weight classes. No significant difference was observed for different carapace width classes, different weight classes and crab tissue screened. Females showed a higher prevalence in gill (33.7%) and internal (4.9%) tissues compared with males (gill (28.4%) and internal of (2.6%)) however these results were not significant (gill; $P > 0.05$ and internal; $P > 0.05$). Significant differences in the colouration/moult stage of *C. maenas* and the prevalence of OsHV-1 μVar were found for gill tissue detection ($P < 0.05$) with the highest prevalence being observed in recently moulted green crabs (37.5%), followed by brown crabs (30%) and red crabs (26.6%), however for internal tissue no significant differences were observed for the different coloured individuals and OsHV-1 μVar prevalence ($P > 0.05$). This pattern was mainly observed at the trestles and not at the high shore.

(2) Laboratory transmission trial of OsHV-1 μVar from *Carcinus maenas* to *Crassostrea gigas*

In the initial sample screening, oysters were uninfected with OsHV-1 μVar as expected, while *C. maenas* (only gill tissues) showed a low prevalence of OsHV-1 μVar (<10%) (Table 4), with an average of 1.1 10^1 viral copies μl^{-1} of genomic DNA.

All oysters in the two control tanks were still alive at the end of the trial. Oysters of one of the two control tanks (n=30) were screened for prevalence of OsHV-1 μVar by PCR on the last day of the trial. All control individuals were negative for OsHV-1 μVar. In experimental tanks, total mortality observed in *C. gigas* was 14.4% (n=13 out of 90 / n=8 in tank 1, n=1 in tank 2, n=4 in tank 3) exposed to *C. maenas*, while *C. maenas* itself had very low mortalities with <10% (n=2 out of 30 / n=1 in tank 1, n=1 in tank 2, n=0 in tank 3). Cumulative mortality of *C. gigas* taking into account removal of 3 oysters per tank at day 2 (48 hours), Day 4 (96 hours), Day 7 (168 hours) and Day 11 (264 hours) (n=36) was <25% (n=13/54) (Figure 5). Despite daily screening of the tanks, open shells were counted and removed to assess mortality but the tissues in these shells were either too degraded for screening or had been removed by crab predation. As a result, infection levels in these 14 dead oysters could not be assessed and only live *C. gigas* were screened. In addition, no tissue of the two dead crabs was left, possibly due to cannibalism. In the *C. gigas* experimental tanks, the first positive signal of OsHV-1 μVar occurred within 96 hours. After screening all experimental oysters, *C. gigas* showed a OsHV-1 μVar prevalence of 6.5% (n=5 out of 77) with up to 1.2×10^2 viral copies μl^{-1} of genomic DNA. The viral prevalence in *C. maenas* gill tissue was 75% (n=21 out of 28) with greater than 1.0 x 10^4 viral copies μl^{-1} of genomic DNA, no screened internal tissue showed infection (Table 4).

DISCUSSION

The study demonstrated that *C. maenas* can become infected with OsHV-1 μVar by using a range of protocols recommended by OIE including PCR, qPCR and *In Situ* hybridization (www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_ostreid_herpesvirus_1.pdf). Although, we did not use the primer pairs as described in the OIE protocol, we were using primer pairs that we or colleagues have successfully developed and have previously had published,; PCR (Lynch et al. 2013) qPCR (Webb et al. 2007), ISH (Lynch et al. 2010).

This study indicates that the green shore crab *C. maenas*, an important mobile scavenger and predator in the intertidal area, can act as a carrier, reservoir and alternative host of oyster herpes virus, demonstrating that introduction of a virus through anthropogenic input, can have long-term and widespread ecosystem impacts, as the virus spreads amongst other cohabiting species. OsHV-1 μVar was detected in *C. maenas* at both culture sites and both shore heights, in all moult stages, crab sizes and in both crab sexes. While a seasonal effect could not be determined as the study concentrated on the summer months when viral impact is most pronounced, the virus was detected in *C. maenas* throughout the five-month study period. Highest prevalence of OsHV-1 μVar in the primary host, *C. gigas,* was detected in June at both sites. The low herpesvirus (<5%) prevalence observed in *C. gigas,* might be due to the unfavorable ambient temperatures with temperatures generally below 16 ºC during the study (Petton et al. 2013; Renault et al. 2014). Additionally, oysters selectively bred for resistance to the virus were used at the field trial in this study (Dégremont 2011) as this was what the farmers were culturing. As a scavenger, it is likely that *C. maenas* would preferentially target moribund (and thus potentially infected) *C. gigas* compared to healthy oysters (Moore 2002) and therefore possibly build up the virus while the abundance of infected *C. gigas* would decrease.

Although precautionary measures were taken in this study to wash and remove any incidental occurrence of OsHV-1 μVar on crab gill tissue, more detection of virus occurred in the gills compared to internal tissues, which suggests that the virus is not incidental on the gills and that the virus is being internalized in the tissue. In addition, ISH analyses in this study confirmed the positive detection OsHV-1 μVar internally in *C. maenas* digestive tissues, whereas Direct Sequencing confirmed OsHV-1 μVar within gill and connective *C. maenas* tissues. Higher prevalence in gills may indicate that crabs are being exposed via respiration rather than through feeding routes when initial exposure is occurring. With a widespread distribution of crabs around oyster trestles, with associated viral dispersion in the seawater (Schikorski et al. 2011), exposure in this way might be a likely first mode of uptake for crabs. Moreover, lower internal infection of *C. maenas* might be the result of low infection of *C. gigas,* in this case the virus is not ingested by predation by crabs and less likely to migrate throughout internal tissues. The nature of the infection in crabs may differ to that observed in oysters with localization of the virus in crabs more likely in gills than dispersed throughout the connective tissues as observed in oysters.

While crab size and sex did not have any significant effect on the prevalence of the virus in the crabs, coloration/moult stage did, with green recently moulted crabs have a slightly higher level of virus. This may suggest that this phase of the life cycle makes the animals more susceptible to infection, possibly due to easier access to tissues, or crabs being more immunocompromised during this phase.

The presence of the trestles, providing protection from predators, a readily available food supply in the form of diseased and dying oysters and acting as a nursery site for *C. maenas* replacing the high shore intertidal pools (Pers. Obs), might result in abnormal behavior in *C. maenas,* which would have an impact on ecosystem dynamics. Previous studies observed *C. maenas* varying from 25 to 55 mm in carapace width in intertidal areas in the UK (Dare et al.1983), with smaller individuals found at high shore sites, and older *C. maenas* found lower down the shore (Hunter and Naylor 1993) and actively feeding upon *C. gigas* when they were present (Dare et al. 1983). Indeed, in Dungarvan, differences in size and weight of *C. maenas* was observed between shore heights, with larger and heavier individuals at lower shore (trestles). Also, in agreement with a previous natural behavioral study of *C. maenas* (Hunter and Naylor 1993), a significantly higher abundance of males was observed at the high shore in Dungarvan. Those normal behavioral and migration patterns were missing at Carlingford Lough, with juvenile *C. maenas* being observed in and around oyster trestles at high shore. It is important to note that at Carlingford Lough, random oyster bags were found at high shore and therefore highly likely to have altered normal behavioral and migration patterns of *C. maenas*. Other studies have noted the attraction of juvenile *C. maenas* to Pacific oyster trestles. A recent study that took place at Kingston Bay, Massachusetts (USA), a OsHV-1 μVar free site, where *C. maenas* is a non-native species, showed a significantly higher numbers of juvenile *C. maenas* (1-15 mm CW) within mesh grow-out bags with oyster shells or living oysters compared to mesh grow-out bags without oyster shells at the high intertidal area (Lovely et al. 2015). *C. maenas* are known to moult all year around (Naylor 1962) and previous studies found green, brown and red coloured crabs at all sites and both shore heights (Lovely et al. 2015). This supports the findings of our study, all crab moult stages and corresponding carapace coloration were found during the sampling period at both shore heights.

In our transmission trial, first infection of OsHV-1 μVar in naïve *C. gigas* was detected after 4 days. Even though the temperature was held below the associated activation threshold temperature of 16 °C, a total prevalence of 6.5% OsHV-1 µVar was detected in *C. gigas* after 14 days. This suggests that the virus, at nonfavorable temperatures, could be maintained in the system by other marine species, like *C. maenas*, acting as a carrier and transmitting it to host species *C. gigas*. Transmission of OsHV-1 µVar to naïve *C. gigas* might have been a result of direct contact between *C. maenas* and *C. gigas* or through filtration of virus particles in the water or feaces excreted by *C. maenas*. The higher prevalence of OsHV-1 µVar in gills of experimental crabs (75%) after 14 days compared with the initial sample (10%) might be the result of reactivation of the virus due to stress of transport and artificial settings. *C. gigas* showed a cumulative mortality rate up to 25%, however it was not possible to screen dead *C. gigas* as though tanks were checked twice daily there was no tissue left in those dead animals. Therefore, it cannot be determined if *C. maenas* had predated on live animals or scavenged tissues when the oysters were moribund. Due to this, infection of *C. gigas* might have been underestimated as it could not be determined if those dead animals were infected or not. No virus was detected within internal tissues of *C. maenas*, suggesting that migration of virus from gills to internal tissues needs longer, only occurs through other transmission routes (e.g. ingestion) or that infection in the crab shows different patterns of viral presence in the tissues. Abnormal mortalities of *C. gigas* have been associated with viral loads of OsHV-1 µVar higher than 10^4 DNA copies mg^{-1} (Schikorski et al. 2011; Pernet et al. 2012). These high viral loads were detected in a small percentage of living *C. maenas* in our experimental laboratory study, however mortalities in *C. maenas* remained low (<10%). The transmission trial was performed under threshold temperature of 16 °C, to imitate natural summers in Ireland. Keeping in mind climate change, for future transmission experiments between crabs and oysters, it would be of interest to choose higher temperatures and investigate the difference in transmission dynamics**.** In addition, to gain better understanding of the viral dynamics between the species and migration of the virus within crabs it would be of interest to perform new experiments in the future by exposing highly infected oysters with naïve crabs.

The results of this study suggest that OsHV-1 μVar is highly adaptable and when the odds are in favour of the host i.e. when seawater temperatures are cooler and when disease resistant oysters are present, OsHV-1 μVar will sustain itself in the ecosystem outside the host species for a long period of time and can "species jump" to *C. maenas*. The pathogenicity of OsHV-1 μVar to *C. maenas* is not known and further studies would be required to elucidate the impact of the virus on *C. maenas* in the intertidal zone, however, due to *C. maenas's* mobility a greater geographic range extension of OsHV-1 μVar is likely. Our results suggest that man-made structures like oyster trestles might have an effect on the ecology of *C. maenas* facilitating the trophic transfer of OsHV-1 μVar within marine ecosystems, in particular, to cohabiting top predator species of crabs such as fish and bird species.

AUTHORS CONTRIBUTIONS

BB, SL and SC conceived the ideas and designed methodology; BB and AO collected the data; BB analyzed the data; BB led the writing of the manuscript with contributions and corrections from SL and SC. All authors contributed critically to the drafts and gave final approval for publication.

ACKNOWLEDGEMENTS

This study was carried out with financial assistance from the REPOSUS Project "Reducing the impact of Pathogens and disease in the Irish Oyster industry to support the sustainability and growth of the sector" funded under FIRM by Department of Agriculture, Food and the Marine (DAFM) 14 SF 820. The authors would like to thank the shellfish growers for providing material and their time for the study and for allowing access to their culture sites. The Marine Institute and Bord Iascaigh Mhara (BMI) provided sea water temperature raw data.

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TABLES AND FIGURES

Table 1. Weight and carapace width data for *Carcinus maenas* at the high shore and at oyster trestles in Dungarvan and Carlingford Lough.

	Average weight (gram)	Weight range (gram)	Average carapace width (mm)	Carapace width range (mm)
Dungarvan	4.2 ± 0.2	0.21 - 26.4	24.8 ± 0.4	9.8 - 50.5
High Shore	2.2 ± 0.4	0.21 - 23.7	20.1 ± 0.9	9.8 - 50.5
Trestle	4.6 ± 0.2	0.34 - 26.4	25.8 ± 0.4	11.9 - 49.4
Carlingford Lough	11.2 ± 0.5	0.23 - 52.0	33.5 ± 0.5	9.4 - 64.1
High Shore	11.3 ± 0.7	0.29 - 52.0	33.7 ± 0.7	10.5 - 64.1
Trestle	11.2 ± 0.7	0.23 - 47.5	33.4 ± 0.7	9.4 - 63.2

Table 2. Prevalence of OsHV-1 µVar by PCR in *Carcinus maenas* gill and internal tissues at the oyster trestles and high shore at Dungarvan and Carlingford Lough.

	Trestle		High Shore	
	Prevalence gill	Prevalence internal	Prevalence gill	Prevalence internal
Dungarvan	35.6% (n=96/270)	5.6% (n=15/270)	21.6% (n=13/60)	1.7% (n=1/60)
Carlingford Lough	27.7% (n=66/238)	6.3% (n=15/238)	29.4% (n=70/238)	0% (n=0/238)

Table 3. Mean viral copies μl^{-1} of genomic DNA in samples of *Carcinus maenas* collected from the culture sites and deemed positive for OsHV-1 μVar by PCR - =no samples screened.

	< 10^2	**10^2-10^4**	**>10^4**
Gill tissue			
Dungarvan High Shore	100% (n=2)	0%	0%
Dungarvan Trestle	94.7% (n=18)	5.3% (n=1)	0%
Carlingford Lough High Shore	66.6% (n=4)	33.3% (n=2)	0%
Carlingford Lough Trestle	75.0% (n=6)	25.0% (n=2)	0%
Internal tissue			
Dungarvan High Shore	-	-	-
Dungarvan Trestle	66.6%(n=4)	16.7% (n=1)	16.7% (n=1)
Carlingford Lough High Shore	-	-	-
Carlingford Lough Trestle	100% (n=2)	0%	0%

Table 4. Prevalence of OsHV-1 μVar in *Crassostrea gigas* and *Carcinus maenas* by PCR in the initial sample and experimental sample of laboratory transmission trial.

	Prevalence gill by PCR	Prevalence internal by PCR
Initial *C. gigas*	0% (n=0/30)	-
Initial *C. maenas*	10% (n=3/30)	0% (n=0/30)
Experimental *C. gigas*	6.5 % (n=5/77)	-
Experimental *C. maenas*	75.0% (n=21/28)	0 % (n=0/28)

Figure 1. Crassostrea gigas culture site at Dungarvan, Co. Waterford and Carlingford Lough, Co. Louth, Ireland.

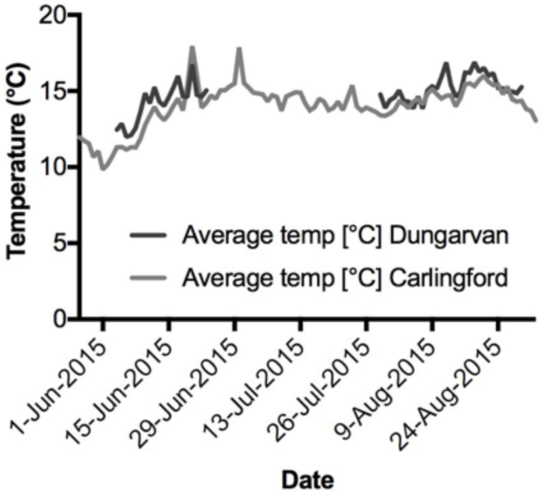
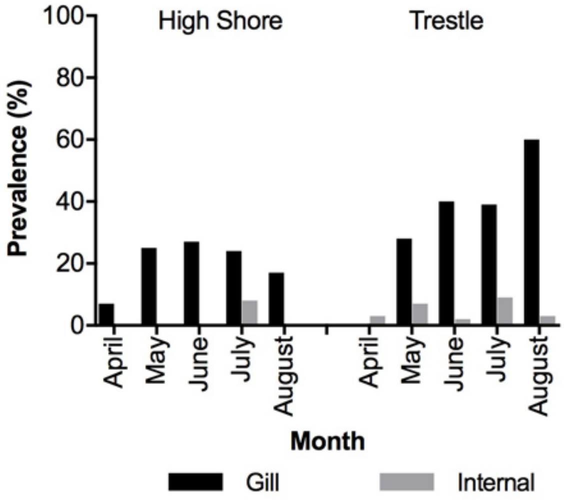


Figure 2: Average water temperature for Dungarvan and Carlingford Lough

A



B

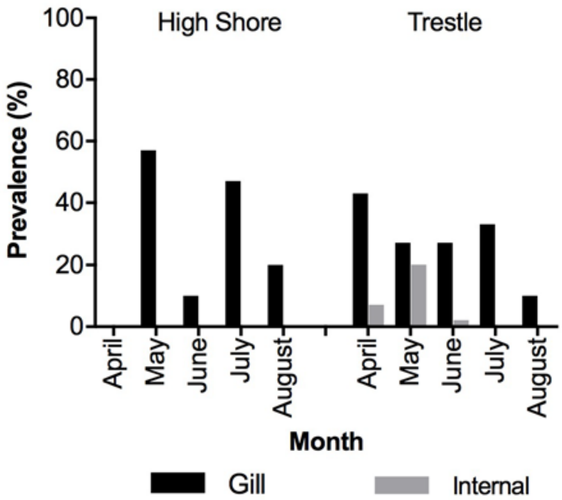
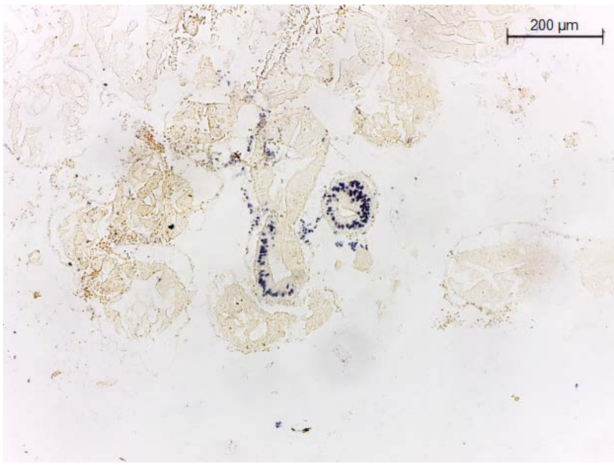
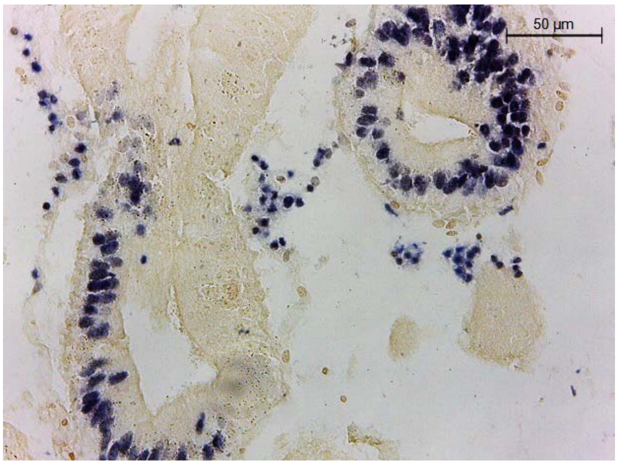


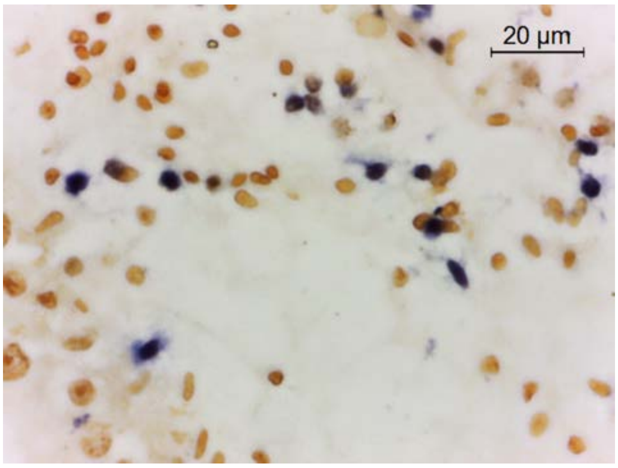
Figure 3: Prevalence of OsHV-1 µVar in Dungarvan (A) and Carlingford Lough (B) for gill and internal tissues of C. maenas at high shore and trestles per month



A



B



C

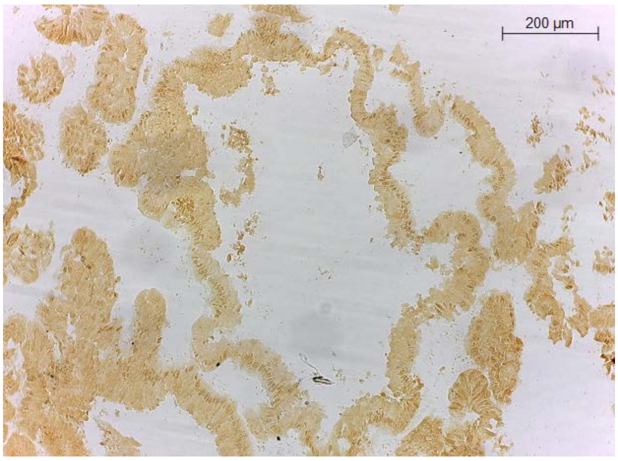
D 

Figure 4: ISH staining of OsHV-1 μVar infected blood cells (dark blue) in connective tissue (Digestive Tract) of C. maenas naturally exposed to an OsHV-1 μVar endemic area (A + B +C) and uninfected tissue (D).

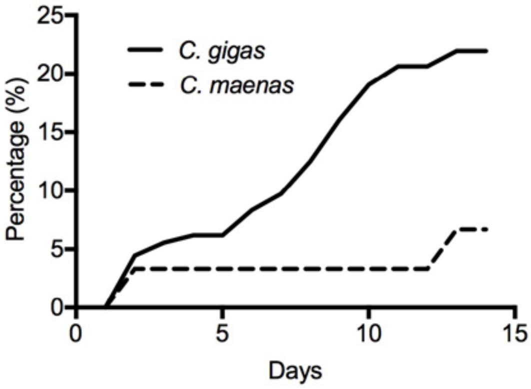


Figure 5: Overall cumulative mortality rates of experimental tanks with C. gigas and C. maenas (derived from grouping observa