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Title	Role of the intertidal predatory shore crab Carcinus maenas in transmission dynamics of ostreid herpesvirus-1 microvariant
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Publication date	2018-09-27
Original Citation	Bookelaar, B. E., O'Reilly, A. J., Lynch, S. A. and Culloty, S. C. (2018) 'Role of the intertidal predatory shore crab Carcinus maenas in transmission dynamics of ostreid herpesvirus-1 microvariant', Diseases of Aquatic Organisms, 130(3), pp. 221-233. doi:10.3354/dao03264
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.int-res.com/abstracts/dao/v130/n3/p221-233/ - 10.3354/dao03264
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Download date	2026-11-16 11:54:21
Item downloaded from	https://hdl.handle.net/10468/7107



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Vol. 130: 221–233, 2018
https://doi.org/10.3354/dao03264

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 27

Role of the intertidal predatory shore crab *Carcinus maenas* in transmission dynamics of ostreid herpesvirus-1 microvariant

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ABSTRACT: 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. The shore crab *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 over 1 summer at different shore heights at 2 Irish Pacific oyster culture sites 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 4 d, 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. The results also suggest 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

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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, Dégremont 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 favourable 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, 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

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and higher (Clegg et al. 2014, Renault et al. 2014, Pernet et al. 2015). The virus has already been proven to be waterborne (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 & 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, 2010, Small & 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 adapting to new target species as an alternative host (Howard & Fletcher 2012, Engering et al. 2013, Schrauwen & 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 hosts, 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 (Kreuder 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, 2001, Arzul et al. 2001a,b) and recently also in invertebrates such as the congeneric oyster *C. 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*, whelk *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, although Chinese scallops 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 coasts of North America, South Africa, and Australia (Torchin et al. 2001, Carlton & Cohen 2003). Outside its natural range, *C. maenas* has often been seen as a pest (Lafferty & 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 & Ruiz 1995). *C. maenas* is common in estuarine intertidal habitats (Amaral & Paula 2007) and feeds upon a diverse variety of prey including commercially important species such as blue mussel *M. edulis* and Pacific oyster seed and juveniles (Lovely et al. 2015). *C. maenas* are 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, fish 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). Moreover, 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, pathogens can enter as a result of ingestion of disease-infected tissue (Bivalife 2010). *C. maenas* is a mobile predator that feeds upon Pacific oysters (McManus 1988) and preferentially targets 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 via cannibalism (Moksnes et al. 1998, Moksnes 2004). In addition,

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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 considered a selective interface between the external environment and the internal milieu (Bivalife 2010, Henry et al. 2012).

Differences in crab morphology, such as colouration, 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 behaviour for different size classes and moult stages (Hunter & Naylor 1993). It is not well known how man-made structures, such as oyster trestles, and a virus-infected culture species might influence the natural migration patterns and behaviour of *C. maenas*.

In this study, we studied disease dynamics involving OsHV-1 µVar, *C. gigas* and a mobile scavenger (*C. maenas*) at 2 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 crabs' 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.

MATERIALS AND METHODS

Field trial

Study sites

Invertebrate sampling took place at 2 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 (Fig. 1). Both sites are the main areas of production of Irish *Crassostrea gigas* and have a history of OsHV-1 µVar (Bivalife 2010). Oyster trestles are held in intertidal areas with a tidal cycle of approximately 7–9 h of emersion depending on neap or spring tides (Dungarvan oyster farmers pers. comm.).

The oyster culture site in Dungarvan is sheltered, being almost closed off by the linear Cunnigar spit to the east (NPWS 2017). Intertidal habitats are dominated by sandflats, and the site 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 3–5 cm 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 and 20°C (Agri-Food and Biosciences Institute 2015).

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 were 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.

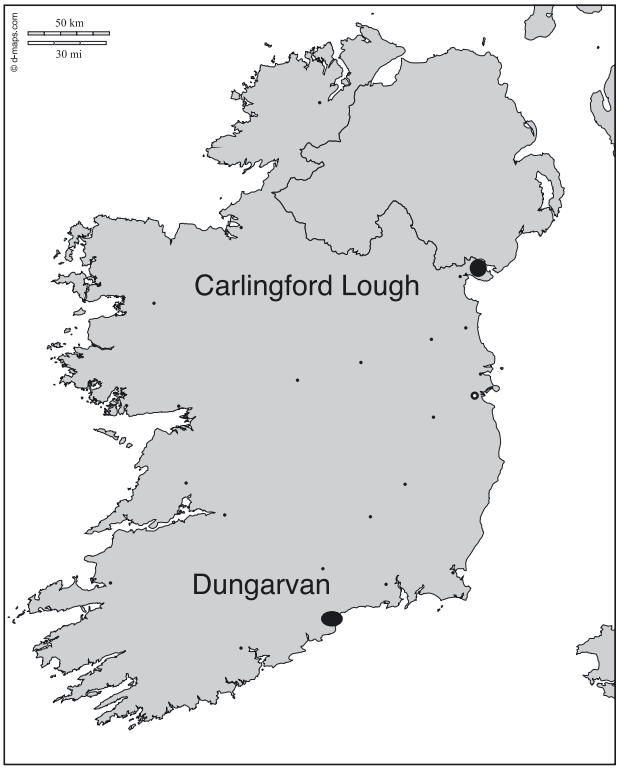


Fig. 1. *Crassostrea gigas* culture sites at Dungarvan, Co. Waterford, and Carlingford Lough, Co. Louth, Ireland

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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 2 wk 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 30.5 cm 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, *Carcinus 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 9 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 8 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 callipers. Carapace width was divided into 4 different length classes: 9.3–20, 20.1–30, 30.1–40 and >40.1 mm for Class 1 to 4, respectively. Weights were also divided into 4 different weight classes: 0–10.0, 10.1–20, 20.1–30 and >30.1 g, respectively. Classification of crab carapace colour (brown, green and red)/moult stage and sex was noted by gross visual examination.

Laboratory transmission trial

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 (Marine Institute Fish Health Unit 2015), were obtained from a hatchery at New Quay, Galway Bay (53.1545° N, 9.0828° W). Crabs with an average weight of 18.5 g and an average age 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 that the oysters were uninfected and to determine if the virus could be detected in *C. maenas*. Before being placed in tanks, *C. maenas* were washed several times in double distilled water (ddH_2O) to remove any pathogens that may have been incidentally attached to their external body/shell. Tanks (10 l capacity) were filled with 8 l of UV-treated seawater. In Ireland, water temperatures often remain below the threshold temperature of 16°C (https://www.seatemperature.org/europe/ireland/), 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 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 2 control tanks each containing 30 naïve oysters, and 3 experimental tanks, which contained 30 naïve oysters and 10 virus-exposed crabs each. The trial ran for 14 d. The tanks were checked twice a day for oyster mortality (open shells), and dead individuals were removed, but no tissues could be recovered for OsHV-1 µVar screening from these animals due to predation. After Day 2 (48 h), Day 4 (96 h), Day 7 (168 h) and Day 11 (264 h), living oysters (n = 3) were arbitrarily selected from the tanks each time to screen for OsHV-1 µVar. All individual oysters and crabs that were 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 thoroughly washed in ddH_2O and blotted dry using tissue paper. DNA extraction was performed using the Chelex-100 methodology. Tissue samples from the invertebrates (ca. 5 mm^2) were placed in a 10% chelex solution (100 µl volume; Sigma Aldrich) and then placed in a thermo Hybaid thermal cycler for 1 h and 10 min

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heated at 99°C to facilitate cell lysis (Walsh et al. 1991). To avoid false negatives, a subsample of DNA samples (n = 30) was checked for DNA quantity and quality by using a NanoDrop 1000 spectrophotometer following the manufacturer's protocol (www.nhm.ac.uk/content/dam/nhmwww/our-science/dpts-facilities-staff/Coreresearchlabs/nanodrop.pdf). 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, with 270 being screened (gill tissues only). 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 from 137 *C. gigas* (137 gill tissues screened).

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_2O, 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 ddH_2O were used for each PCR. Thermo cycling conditions were as follows: initial denaturation of 1 min at 95°C; followed by 35 cycles including a denaturation step of 20 s at 94°C, an annealing step of 30 s at 56°C and an elongation step at 72°C; and finishing with a final elongation step of 7 min 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 (10 mg l^{-1} stock) and run with an electrical charge of 110 V for 45–60 min.

qPCR

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 EURLMD (2011) protocol 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× Brilliant Sybr Green ® Q PCR Master Mix, 2.5 µl HVDP-F (5 µM) 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. qPCR 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 ddH_2O were used for each qPCR. Thermo cycling conditions were initial denaturation of 2 min at 50°C and 10 min at 95°C; followed by 40 cycles of 15 s at 95°C and 1 min at 60°C and a melt curve of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s by using a thermo Hybaid PCR express thermal cycler (EURLMD 2011).

In situ hybridization

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, sections 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 that had 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-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 3 internal tissues) was carried out to confirm OsHV-1 µVar detection. DNA was iso-

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lated from PCR products of separate tissues (pooled 4 replicates per tissue to increase the DNA concentration). A 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 a BLASTn nucleotide database search (https://blast.ncbi.nlm.nih.gov/) to confirm true infection of OsHV-1 µVar.

Statistical analyses

Statistical analyses were performed in the 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. Pearson's chi-squared tests were used to compare sex and colour between sites and within sites at the 2 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, an α-value of 0.05 was used to confirm significant results. Data are presented as mean ± SE.

RESULTS

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 2 sites for the duration of the field trial was low, with a mean prevalence of 3.75% at both sites, and with a range in prevalence of 0–27% at Dungarvan and 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 (Fig. 2). At the farms, a tidal cycle of approximately 7–9 h of emersion depending on neap or spring tides are common (Dungarvan 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.met.ie/climate/irish-climate-monthly-summary.asp). Significant differences in prevalence between months were observed for both Dungarvan ($p < 0.01$) and Carlingford Lough ($p < 0.01$), with the highest prevalence in June for both sites.

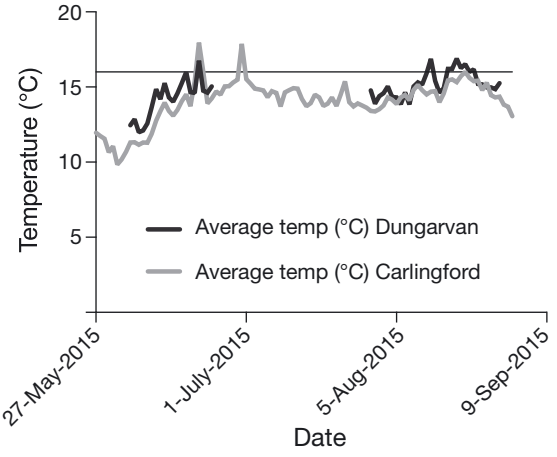


Fig. 2. Average water temperature for Dungarvan and Carlingford Lough. Horizontal line: threshold activation temperature of 16°C, above which infections with OsHV-1 are observed

Crab morphometrics

Carcinus maenas were significantly larger ($p < 0.01$) and heavier ($p < 0.01$) at Carlingford compared with Dungarvan (Table 1). All 4 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 4 crab weight classes were present in Carlingford lough, while only 3 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

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

	Weight (g)		Carapace width (mm)	
	Mean ± SD	Range	Mean ± SD	Range
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

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, a significant difference within colouration 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 5 mo field trial at both culture sites (Fig. 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% (155/952), albeit not significantly ($p > 0.05$). The overall prevalence of OsHV-1 µVar in the screened tissues of *C. maenas* for both sites combined was 17.1% (276/1612). For those infected tissues, the virus was detected only in gill tissue in 89.9% (241/268) of the crabs, in only the internal tissue in 7.1% of the crabs (19/268) and in both gill and internal tissues in 3.0% of the crabs (8/276). 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 1 reverse DNA sequence was generated from 1 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 (GenBank no. KU861511.1) for the sequence of the PCR-amplified products for *C. maenas*. ISH 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 (Fig. 4a–c) and oysters, while PCR-negative crabs (Fig. 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, although 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.

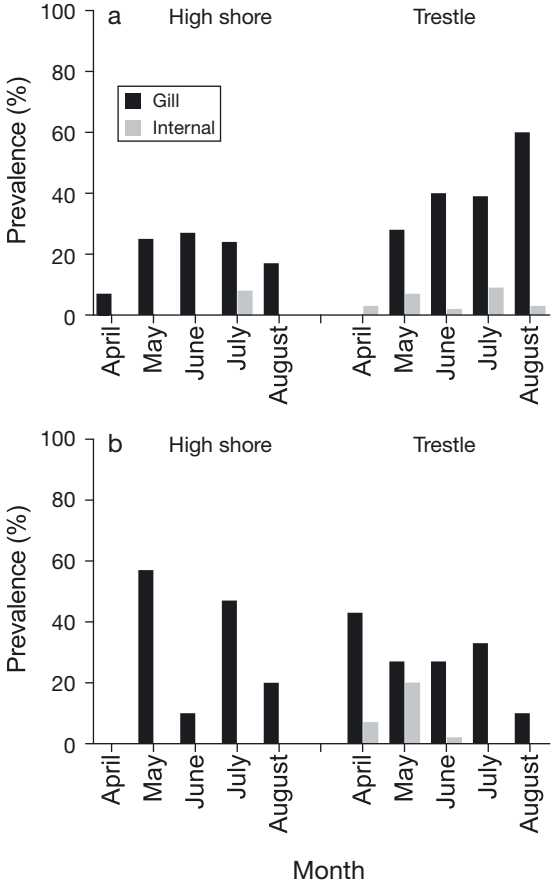


Fig. 3. Prevalence of OsHV-1 µVar in (a) Dungarvan and (b) Carlingford Lough for gill and internal tissues of *Carcinus maenas* at high shore and trestles per month

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Table 2. Prevalence (Prev., %) 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			
	Gill		Internal		Gill		Internal	
	Prev.	n	Prev.	n	Prev.	n	Prev.	n
Dungarvan	35.6	96/270	5.6	15/270	21.6	13/60	1.7	1/60
Carlingford Lough	27.7	66/238	6.3	15/238	29.4	70/238	0	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		$>10^4$	
	%	n	%	n	%	n
Gill tissue						
Dungarvan high shore	100	2	0		0	
Dungarvan trestle	94.7	18	5.3	1	0	
Carlingford Lough high shore	66.6	4	33.3	2	0	
Carlingford Lough trestle	75.0	6	25.0	2	0	
Internal tissue						
Dungarvan high shore	–		–		–	
Dungarvan trestle	66.6	4	16.7	1	16.7	1
Carlingford Lough high shore	–		–		–	
Carlingford Lough trestle	100	2	0		0	

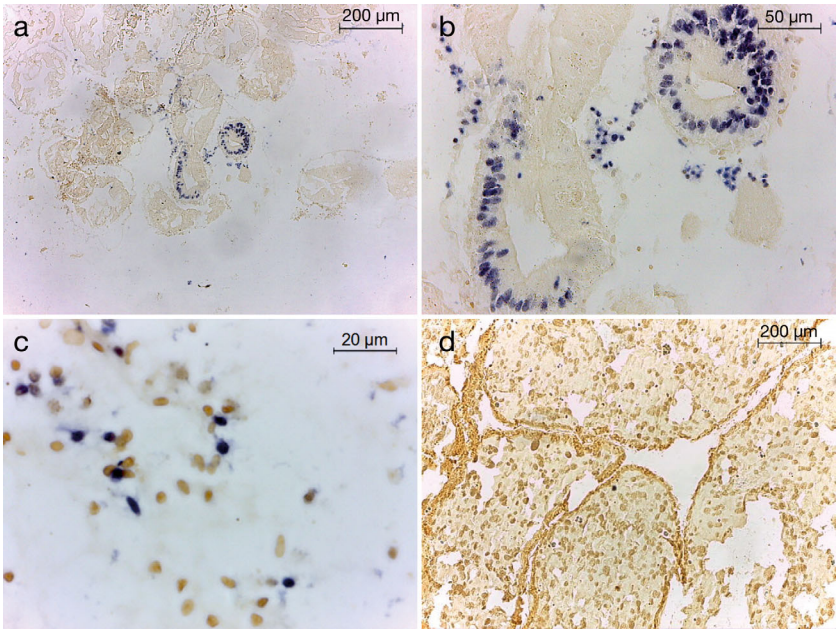


Fig. 4. *In situ* hybridization staining of OsHV-1 µVar infected blood cells (dark blue) in connective tissue (digestive tract) of *Carcinus maenas* (a–c) naturally exposed to an OsHV-1 µVar endemic area and (d) uninfected tissue

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 and weight classes. No significant difference was observed for different carapace width classes, different weight classes and crab tissues screened. Females showed a higher prevalence in gill (33.7%) and internal (4.9%) tissues compared with males (28.4 and 2.6%, respectively), but these results were not significant ($p > 0.05$ for both tissue types). Significant differences in the colouration/moult stage of *C. maenas* and the prevalence of OsHV-1 µVar were found for gill tissue ($p < 0.05$), 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.

Laboratory transmission trial

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 2 control tanks were still alive at the end of the trial. Oysters of 1 of the 2 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 = 13/90: 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

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

	Gill		Internal	
	Prev.	n	Prev.	n
Initial *C. gigas*	0	0/30	–	
Initial *C. maenas*	10	3/30	0	0/30
Experimental *C. gigas*	6.5	5/77	–	
Experimental *C. maenas*	75.0	21/28	0	0/28

low mortalities with <10% (2/30: n = 1 each in Tanks 1 and 2). Cumulative mortality of *C. gigas* taking into account removal of 3 oysters tank^{-1} on Day 2 (48 h), Day 4 (96 h), Day 7 (168 h) and Day 11 (264 h) (n = 36) was <25% (13/54) (Fig. 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 2 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 h. After screening all experimental oysters, *C. gigas* showed an OsHV-1 µVar prevalence of 6.5% (n = 5/77) with up to 1.2×10^2 viral copies µl^{-1} of genomic DNA. The viral prevalence in *C. maenas* gill tissue was 75% (21/28), with greater than 1.0×10^4 viral copies µl^{-1} of genomic DNA. No screened internal tissue showed infection (Table 4).

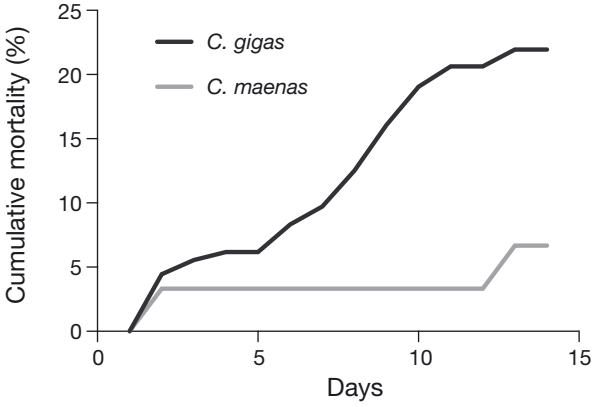


Fig. 5. Overall cumulative mortality rates of experimental tanks with *Crassostrea gigas* and *Carcinus maenas* (derived from grouping observations of 3 tanks)

DISCUSSION

Using a range of protocols recommended by OIE (2018), including PCR, qPCR and ISH, our study demonstrated that *Carcinus maenas* can become infected with OsHV-1 µVar. 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 published: PCR (Lynch et al. 2013), qPCR (Webb et al. 2007) and ISH (Lynch et al. 2010).

Our results indicate 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 herpesvirus, 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 5 mo study period. Highest prevalence of OsHV-1 µVar in the primary host, *Crassostrea gigas*, was detected in June at both sites. The low herpesvirus (<5%) prevalence observed in *C. gigas* might be due to the unfavourable ambient temperatures, which were generally below 16°C during the study (Petton et al. 2013, Renault et al. 2014). Additionally, oysters selectively bred for resistance to the virus were used during the field trial in this study (Degremont 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-1m 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 confirmed the positive detection of 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

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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 is less likely to migrate throughout internal tissues. The nature of the infection in crabs may differ from 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, colouration/moult stage did, with green, recently moulted crabs having 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 (B. E. Bookelaar pers. obs.), might result in abnormal behaviour 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 & 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* were observed between shore heights, with larger and heavier individuals at lower shore sites (trestles). Also, in agreement with a previous natural behavioural study of *C. maenas* (Hunter & Naylor 1993), a significantly higher abundance of males was observed on the high shore in Dungarvan. Those normal behavioural 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 behavioural 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), an OsHV-1 µVar free site, where *C. maenas* is a non-native species, showed significantly higher numbers of juvenile *C. maenas* (1–15 mm carapace width) 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* moult all year round (Naylor 1962), and previous studies found green, brown and red crabs at all sites and both shore heights (Lovely et al. 2015). This supports the findings of our study, i.e. all crab moult stages and corresponding carapace colouration during the sampling period at both shore heights.

In our transmission trial, the first infection of OsHV-1 µVar in naïve *C. gigas* was detected after 4 d. 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 d. This suggests that the virus, at nonfavourable temperatures, could be maintained in the system by other marine species, such as *C. maenas*, acting as a carrier and transmitting it to the 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 faeces excreted by *C. maenas*. The higher prevalence of OsHV-1 µVar in gills of experimental crabs (75%) after 14 d 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%, but it was not possible to screen dead *C. gigas* because even 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. As a result, infection of *C. gigas* might have been underestimated, as we were unable to determine whether 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 requires a longer time, 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 a threshold temperature of 16°C, to imitate natural summers in Ireland. Keeping in mind climate change, for future

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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 naïve crabs to highly infected oysters.

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 are required to elucidate the impact of the virus on *C. maenas* in the intertidal zone. However, due to the mobility of *C. maenas*, a greater geographic range extension of OsHV-1 µVar is likely. Our results suggest that man-made structures such as oyster trestles might have an effect on the ecology of *C. maenas* by facilitating the trophic transfer of OsHV-1 µVar within marine ecosystems, in particular to cohabiting top predator species of crabs such as fish and birds.

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 the Department of Agriculture, Food and the Marine (DAFM) 14 SF 820. We 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 seawater temperature raw data.

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Editorial responsibility: Stephen Feist, Weymouth, UK

Submitted: November 7, 2017; Accepted: June 24, 2018
Proofs received from author(s): September 10, 2018