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Authors	Bookelaar, Babette E.; O'Reilly, A. J.; Lynch, Sharon A.; Culloty, Sarah C.
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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

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

4
5 B. E. Bookelaar, A. J. O'Reilly, S. A. Lynch, S. C. Culloty,

6 Aquaculture and Fisheries Development Centre, School of Biological, Earth and

7 Environmental Sciences &

8 Environmental Research Institute, University College Cork, Cork, Ireland

9 Corresponding author: b.bookelaar@umail.ucc.ie

10
11 **SUMMARY**

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

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

28

29

KEY WORDS

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

32

33

INTRODUCTION

34 Diseases, parasites and pathogens are common in marine ecosystems (Lafferty et al. 2015) and
35 have a significant impact on fisheries and aquaculture (Willman et al. 2009; Lafferty et al.
36 2015), as well as the ecology of marine habitats (Harvell et al. 2002). Development of disease
37 is in general due to a complex aetiology including numerous physical, chemical, biological,
38 and ecological interactions. Hence, the environment and its constituents play a significant role
39 in disease transmission (Mydlarz et al. 2006; Degremont 2011), also known as the ‘pathogen–
40 host–environment interplay’ (Engering et al. 2013).

41

42 Virus infections in bivalve species have been associated with high mortality rates, when
43 conditions become less favorable for the host species (Rowley et al. 2014). A significant
44 pathogen-host-environment interplay has been observed for the commercially important
45 Pacific oyster *Crassostrea gigas* with ostreid herpesvirus (OsHV-1) and variants, which has
46 resulted in mass mortalities among early life stages of *C. gigas* worldwide (Burge et al. 2007;
47 Lynch et al. 2012; Prado-Alvarez et al. 2016). In particular, nowadays these mortalities have
48 been associated with the variant OsHV-1 microVar (OsHV-1 μ Var), which is considered highly
49 virulent (Segarra et al. 2010) especially when seawater temperatures reach 16°C and higher
50 (Clegg et al. 2014; Renault et al. 2014; Pernet et al. 2015). The virus has already been proven

51 to be waterborne in previous studies (Vigneron et al. 2004; Sauvage et al. 2010; Schikorski et
52 al. 2011; Evans et al. 2015). Infected adult oysters may function as carriers and infect naïve
53 spat by vertical transmission (Burge and Friedman 2012) and horizontal transmission between
54 healthy and experimental infected oysters has been observed (Schikorski et al. 2011).

55

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

68

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

76 2000; Arzul et al. 2001a; Arzul et al. 2001b; Renault 2001) and recently also in invertebrates
77 such as the oyster *Crassostrea virginica* (Burge et al. 2011), Mediterranean mussel *Mytilus*
78 *galloprovincialis* (Burge et al. 2011) and Chinese scallop *Chlamys farreri* (Ren et al. 2013).
79 More recently OsHV-1 μ Var was detected in the Sydney rock oyster *Saccostrea glomerata*,
80 Sydney cockle *Anadara trapezia*, blue mussels *Mytilus spp.*, hairy mussel *Trichomya hirsuta*,
81 whelks *Batillaria australis* and barnacles *Balanus spp.* (Evans et al. 2017). For most
82 invertebrate species other than oysters infected with herpes-like virus the pathogenic effect is
83 still unknown, however Chinese scallop *Chlamys farreri* suffered mass mortality after infection
84 (Ren et al. 2013) highlighting the potential impact of this virus on its marine environment.

85

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

101

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

107

108 Different routes of entry for diseases and pathogens seem to be possible for *C. maenas*. Firstly,
109 due to ingestion of disease infected tissue (www¹) *C. maenas* is a mobile predator feeding upon
110 Pacific oysters (McManus 1988) and preferentially targeting moribund (and thus potentially
111 infected) individuals compared to healthy individuals (Moore 2002), resulting in direct take up
112 of pathogens or diseases. Secondly, disease intake could happen by intraspecific contact of
113 diseased scavengers and also cannibalism (Moksnes et al. 1998; Moksnes 2004). In addition,
114 during respiration the gill tissue of *C. maenas* is in direct contact with infected particles in the
115 water column, and the gills of *C. maenas* are recognized as a selective interface between the
116 external environment and the internal milieu (www¹; Henry et al. 2012).

117

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

124 In this study, disease dynamics involving OsHV-1 μ Var, *C. gigas* and a mobile scavenger, *C.*
125 *maenas* was studied at two Irish Pacific oyster culture sites, responsible for the majority of

126 production of Irish *C. gigas* with a history of OsHV-1 μ Var and having different ecosystem
127 characteristics. The role of *C. maenas* as a potential carrier, reservoir or alternative host of
128 OsHV-1 μ Var was investigated, taking into consideration the potential extension range of the
129 virus in crabs as they migrated up and down the intertidal zone, associated with changing
130 morphological and ecological characteristics during the crab's life cycle. The nature of the role
131 of crabs in viral transmission was determined by laboratory-based trials. The focus of the study
132 was to gain a better understanding of how the virus might sustain itself in the marine
133 environment once introduced into a particular habitat and give a better insight into the potential
134 wider ecosystem impacts of such introductions.

135

136

MATERIAL AND METHODS

137 *(1) Field trial*

138 *Study sites*

139 Invertebrate sampling took place at two main Irish oyster culture sites, with different habitat
140 structure; Dungarvan, Co. Waterford (52.0936 °N -7.6204°W) and Carlingford Lough, Co.
141 Louth (54.0733°N -6.1994°W), approximately 245 km apart (Figure 1). Both sites are the main
142 areas of production of Irish *C. gigas* and have a history of OsHV-1 μ Var (www¹) and oyster
143 trestles are held in intertidal area with a tidal cycle of approximately 7-9 hours of emersion
144 depending on neap or spring tides (Oyster farmers Pers. Comm.).

145

146 The oyster culture site in Dungarvan is sheltered, being almost closed off by the linear Cunnigar
147 spit to the east (www²). Intertidal habitats are dominated by sandflats and it has mudflats at the
148 edge of saltmarsh habitats. The water quality of Dungarvan Harbour varies from moderate to
149 good, representing unpolluted water and acceptable levels of biochemical oxygen demand
150 (EPA 2015). The oyster culture site in Carlingford Lough has a gravelly substrate covered by

151 3-5cm of muddy silt. Carlingford Lough, fed by the Newry River, has generally shallow waters
152 of 2-5 m. Water quality within the lough is good; mean salinity is 32.5 and the annual
153 temperature varies between 3 - 20°C (www³).

154

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

160

161 *Macroinvertebrate sampling*

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

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

176

177 *Morphometric characteristics of C. maenas*

178 Weight (g) and carapace width (mm) were recorded using a balance scales and vernier calipers.
179 Carapace width was divided into 4 different length classes, Class 1: 9.3-20 mm, Class 2: 20.1-
180 30 mm, Class 3: 30.1-40 mm, Class 4 > 40.1 mm. Weights were divided into 4 different weight
181 classes, Class 1: 0 – 10.0 g, Class 2: 10.1 – 20 g, Class 3: 20.1 - 30 g, Class 4 >30.1 g.
182 Classification of crab carapace colour (brown, green and red)/moult stage and sex was noted
183 by gross visual examination.

184

185 *(2) Laboratory transmission trial of OsHV-1 μ Var from Carcinus maenas to Crassostrea*
186 *gigas*

187 A laboratory transmission trial was designed to determine the nature of positive results detected
188 in the wild and to assess the possibility of viral transmission from the crabs to oysters. Naïve
189 *C. gigas* (n=180) with an average weight of 3.4 g and an average length of 31.9 mm, which
190 had never been exposed to OsHV-1 μ Var and proven to be naïve by the Marine Institute
191 (www⁴), were obtained from a hatchery at New Quay, Galway Bay (53° 09' 16.27" N, 9°04'
192 58.19" W). Crabs with an average weight of 18.5 g and an average carapace width of 40.2 mm
193 were randomly collected from Carlingford Lough in September 2015 where OsHV-1 μ Var had
194 been detected in oysters and in crabs during the field study. Prior to the start of the trial, 30
195 naïve *C. gigas* and 30 *C. maenas* were screened for OsHV-1 μ Var by polymerase chain reaction
196 (PCR), to confirm the oysters were uninfected and to determine if the virus could be detected
197 in *C. maenas*. Before placing in tanks, *C. maenas* were washed several times in ddH₂O to
198 remove any pathogens that may have been incidentally attached to their external body/shell.
199 10 l tanks were filled with 8 l of UV treated seawater. In Ireland, water temperatures often
200 remain below the threshold temperature of 16°C (www⁵) and to imitate natural water

201 temperatures, a lower temperature was chosen during the laboratory trial. UV filtered natural
202 seawater and animals were held at 14°C in a constant temperature (CT) room with a salinity of
203 35 ppt. At the start of the trial a water conditioner (1 ml of Aqueon) was used, to keep the water
204 quality to an optimum. The experimental set up consisted of two control tanks each containing
205 30 naïve oysters and three experimental tanks, which contained 30 naïve oysters and 10 virus-
206 exposed crabs each. The trial ran for 14 days. The tanks were checked twice a day for mortality
207 (open shells) and dead individuals were removed and screened for OsHV-1 μ Var if tissue was
208 present and of a suitable quality, but no tissues could be recovered for screening from these
209 animals due to predation. After day 2 (48 hours), Day 4 (96 hours), Day 7 (168 hours) and Day
210 11 (264 hours), living oysters (n=3) were arbitrarily selected from the tanks each time to screen
211 for OsHV-1 μ Var. All individuals, oysters and crabs, still alive at the end of the experiment
212 were removed and screened for OsHV-1 μ Var.

213

214 ***Molecular diagnostic screening***

215 *DNA extraction*

216 Gill and internal tissues made up of connective, digestive and reproductive tissues of both
217 oysters and crabs were stored in 70% ethanol for DNA extraction. Prior to extraction, tissues
218 were washed in double deionized water (ddH₂O) thoroughly and blot dried using tissue paper.
219 DNA extraction was performed using the Chelex-100 methodology. Tissue samples from the
220 invertebrates (approx. 5mm²) were placed in a 10% chelex solution (100 microlitres volume)
221 (Sigma Aldrich) and following the samples were placed in a thermo Hybaid thermal cycler for
222 1 hour and 10 minutes heated at 99°C to facilitate cell lysis (Walsh et al.1991). To avoid false
223 negatives, a subsample of DNA samples (n=30) were checked for DNA quantity and quality
224 by using a NanoDrop 1000 spectrophotometer following protocol T042-TECHNICAL
225 BULLETIN NanoDrop Spectrophotometers (www⁶). From the samples collected from

226 Dungarvan during the field trial, DNA was extracted from 330 individual *C. maenas* with (330
227 gill and 330 internal tissues being screened) from those crabs and 270 *C. gigas* were sampled
228 (270 gill tissues only) being screened. DNA was extracted from 476 *C. maenas* (476 gill and
229 476 internal tissues screened) and 240 *C. gigas* (240 gill tissues screened) in Carlingford
230 Lough. For the laboratory trial, DNA was extracted from 58 *C. maenas* (58 gill and 58 internal
231 tissues screened) and for 137 *C. gigas* (137 gill tissues screened).

232

233 *Polymerase chain reaction (PCR)*

234 For all samples collected in the field and laboratory trial standard PCR to detect OsHV-1 μ Var
235 was performed following the protocol of Lynch et al. (2013) by using OHVA/OHVB primers.
236 All PCRs used a total of 2 μ L genomic DNA template per individual. Expected size of
237 amplified PCR products for OsHV-1 μ Var was 385 bp and PCR was carried out in 25 μ L
238 containing 12.9 μ L ddH₂O, 5 μ L, 5 \times buffer, 5 μ L dNTPs (0.2 mM), 0.5 μ L MgCl₂ (25 mM
239 stock), 0.25 μ L of each primer (100 pmol mL⁻¹ stock) and 0.1 μ L Taq DNA polymerase.
240 Positive controls (duplicate) consisting of OsHV-1 μ Var infected oyster tissue and negative
241 controls (duplicate) of double distilled water (ddH₂O) were used for each PCR. Thermo cycling
242 conditions were performed by initial denaturation of 1 min of 95 °C, following by 35 cycles
243 including a denaturation step of 20 seconds at 94 °C, an annealing step of 30 seconds at 56 °C
244 and an elongation step at 72 °C and finishing with a final elongation step of 7 minutes at 72 °C
245 by using a thermo Hybaid PCR express thermal cycler (Lynch et al. 2013). Presence of
246 amplified PCR products was confirmed by electrophoresis using a 2% agarose gel stained with
247 ethidium bromide (10mg/l stock) and was run with an electrical charge of 110V for 45-60
248 minutes.

249 *Quantitative polymerase chain reaction (qPCR)*

250 Quantitative PCR (qPCR) was carried out to determine the viral load of samples deemed

251 positive for OsHV-1 μ Var by PCR, on a subsample of *C. maenas* collected in the field trial
252 (n=43) and *C. maenas* (n=24) and *C. gigas* (n=5) in the laboratory trial, following the protocol
253 “<http://www.eurl-mollusc.eu/content/download/42545/578238/file/OsHV->” (www⁷) using
254 primers HVDP-F and HVDP-R (Webb et al. 2007). All qPCRs used a total of 5 μ L genomic
255 DNA template per individual (duplicate). The qPCR mix was carried out in 25 μ L containing
256 12.5 μ L 2 x Brilliant Sybr Green [®] Q PCR Master Mix, 2.5 μ L HVDP-F (5 μ M) and 2.5 μ L
257 HVDP-R (μ M) primers and 2.5 μ L ddH₂O. Standards were used to detect the exact amount of
258 viral copies μ l⁻¹ of genomic DNA in tested samples. Standard curves were prepared by diluting
259 a viral DNA suspension of 10⁸ viral copies μ l⁻¹ of genomic DNA of OsHV-1. Q PCR plates
260 included 5 dilutions of 10⁵, 10⁴, 10³, 10² and 10¹ viral copies μ l⁻¹ of genomic DNA. Negative
261 controls (duplicate) of double distilled water (ddH₂O) were used for each qPCR. Thermo
262 cycling conditions were performed by initial denaturation of 2 min of 50 °C and 10 min at
263 95°C, following by 40 cycles of 15 seconds at 95°C and 1 min at 60 °C and a melt curve of
264 95°C for 15 seconds, 60 °C for 1 minute, 95°C for 30 seconds and 60 °C for 15 seconds by
265 using a thermo Hybaid PCR express thermal cycler (www⁷).

266

267 *In situ hybridization (ISH) with DIG labelled probe*

268 *In situ hybridization (ISH)* was carried out to detect the viral genome within different tissue
269 sections of virus infected individuals. For each individual collected in this study, a section of
270 internal tissue including gills, digestive and reproductive organs, were removed for histological
271 analysis and immediately fixed in Davidson’s solution at 4 °C for 24-48 h after which they
272 were placed in 70% ethanol. *In situ hybridization* assays were carried out on *C. gigas* and *C.*
273 *maenas* from the field trial screened negative (n=3 per species) and positive (n=3 per species)
274 for OsHV-1 μ Var by PCR. Samples were processed (Shandon Citadel 1000) and sectioned to
275 7 μ m tissue thickness. ISH was carried out using a digoxigenin (DIG)-labelled probe (Lynch

276 et al. 2010). Sections were viewed and viral cells were noted with a Nikon Eclipse 80i and
277 images were captured using NIS elements software (at 100×, 200× and 400×).

278

279 *Direct Sequencing*

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

287

288 **Statistical analyses**

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

297

298

299

RESULTS

300 **(1) Field trial**

301 *Prevalence of OsHV-1 μ Var in Crassostrea gigas*

302 Herpes virus was detected in oysters at both sites during the study period. Overall prevalence
303 of OsHV-1 μ Var detected by PCR in oysters at the two sites for the duration of the field trial
304 was low with a mean prevalence of 3.75% at both sites, with a range in prevalence of 0-27%
305 at Dungarvan and a range of 0-23% at Carlingford Lough. However, the mean temperature
306 over the study period was 15.0°C for Dungarvan and 14.2 °C for Carlingford Lough, with the
307 overall water temperature during summer 2015 being low, rarely reaching temperatures of 16
308 °C or higher (Figure 2). At the farms, a tidal cycle of approximately 7-9 hours of emersion
309 depending on neap or spring tides are common (Oyster farmers, pers comm) and therefore the
310 sites were exposed to higher temperatures during low tides. Periods of air temperature above
311 16 °C were measured from the end of May (www⁸). Significant difference in prevalence
312 between months were observed for both Dungarvan ($P < 0.01$) and Carlingford Lough ($P <$
313 0.01) with highest prevalence in June for both sites.

314

315 *Crab morphometrics*

316 *C. maenas* were significantly larger ($P < 0.01$) and heavier ($P < 0.01$) at Carlingford compared
317 with Dungarvan (Table 1). All four carapace classes were present at both locations and shore
318 heights, with crabs at Carlingford Lough having significantly larger carapace widths compared
319 to crabs at Dungarvan ($P < 0.01$). Within Dugarvan, larger crabs were significantly more
320 abundant at the trestles ($P < 0.01$), while no significant difference in carapace widths was
321 observed between crabs at the trestles and high shore in Carlingford Lough ($P > 0.05$). All four
322 crab weight classes were present in Carlingford lough while three weight classes were observed
323 at Dungarvan, no significant differences were found for weight classes between high shore and
324 trestles. A significantly higher ($P < 0.01$) female-male ratio of 1:0.6 in Carlingford Lough was
325 observed relative to 1:1 in Dungarvan. Within each site, no significant difference in female-

326 male ratio was observed between the high shore and trestles. Green, brown and red coloured
327 *C. maenas* were observed at Dungarvan and Carlingford Lough. At Dungarvan, green, recently
328 moulted crabs were most abundant, followed by brown and red (1:4.1:2.2 for red:green:brown
329 crabs), while at Carlingford Lough brown crabs were most common, followed by green and
330 red crabs (1:1.9:2.0 for red:green:brown crabs). Colour ratio did differ significantly between
331 both culture sites ($P < 0.01$). Within sites only a significant difference within coloration was
332 observed between high shore and trestle at Carlingford Lough ($P < 0.01$), with significantly
333 more green crabs at the trestles and brown crabs at the high shore.

334

335 *Viral detection in C. maenas*

336 OsHV-1 μ Var was detected in *C. maenas* during the entire five-month field trial at both culture
337 sites (Figure 3). The mean prevalence of infection in both tissues of *C. maenas* was higher at
338 Dungarvan at 18.3% (n=121/660) compared to Carlingford Lough with 16.3% (n=155/952),
339 but not significantly different ($P > 0.05$). The overall prevalence of OsHV-1 μ Var in the
340 screened tissues of *C. maenas* for both sites combined was 17.1% (n=276/1612). For those
341 infected tissues, the virus was detected only in gill tissue in 89.9% (n=241/268) of the crabs,
342 in only the internal tissue of the crabs in 7.1% (n=19/268) of animals and was observed in both
343 gill and internal tissues in 3.0% (n=8/276) of crabs. This pattern was present at both shore
344 heights in Dungarvan and Carlingford Lough (Table 2). qPCR analyses indicated different viral
345 loads for a subsample (n= 43) of the crabs' gill tissue and internal tissue, screened positive
346 initially with PCR. Overall the viral load was low, with up to 100 viral copies μl^{-1} of genomic
347 DNA in most crabs (n=36) screened by qPCR, while some individuals (n=7) had higher viral
348 DNA load, with the highest load detected being $>10^4$ viral copies μl^{-1} of genomic DNA (Table
349 3). One forward and one reverse DNA sequence was generated from one sample of *C. maenas*
350 in the Direct sequencing. After sequencing of the PCR products, BLASTn analysis showed a

351 match with an average of 96% (94-98%) similarity and 99% identity with OsHV-1 μ Var
352 (KU861511.1) for the sequence of the PCR-amplified products for *C. maenas*. *In situ*
353 hybridization staining of crab (digestive and connective internal tissues) and oyster tissue
354 sections resulted in a positive signal for OsHV-1 μ Var in PCR-positive crabs (Figure 4A, 4B,
355 4C) and oysters, while PCR-negative crabs (Figure 4D) and oysters indicated no staining (i.e.
356 no infection) in any tissue.

357

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

364

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

376 and OsHV-1 μ Var prevalence ($P > 0.05$). This pattern was mainly observed at the trestles and
377 not at the high shore.

378

379 **(2) Laboratory transmission trial of OsHV-1 μ Var from *Carcinus maenas* to *Crassostrea***
380 ***gigas***

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

384

385 All oysters in the two control tanks were still alive at the end of the trial. Oysters of one of the
386 two control tanks ($n=30$) were screened for prevalence of OsHV-1 μ Var by PCR on the last
387 day of the trial. All control individuals were negative for OsHV-1 μ Var. In experimental tanks,
388 total mortality observed in *C. gigas* was 14.4% ($n=13$ out of 90 / $n=8$ in tank 1, $n=1$ in tank 2,
389 $n=4$ in tank 3) exposed to *C. maenas*, while *C. maenas* itself had very low mortalities with
390 $<10\%$ ($n=2$ out of 30 / $n=1$ in tank 1, $n=1$ in tank 2, $n=0$ in tank 3). Cumulative mortality of *C.*
391 *gigas* taking into account removal of 3 oysters per tank at day 2 (48 hours), Day 4 (96 hours),
392 Day 7 (168 hours) and Day 11 (264 hours) ($n=36$) was $<25\%$ ($n=13/54$) (Figure 5). Despite
393 daily screening of the tanks, open shells were counted and removed to assess mortality but the
394 tissues in these shells were either too degraded for screening or had been removed by crab
395 predation. As a result, infection levels in these 14 dead oysters could not be assessed and only
396 live *C. gigas* were screened. In addition, no tissue of the two dead crabs was left, possibly due
397 to cannibalism. In the *C. gigas* experimental tanks, the first positive signal of OsHV-1 μ Var
398 occurred within 96 hours. After screening all experimental oysters, *C. gigas* showed a OsHV-
399 1 μ Var prevalence of 6.5% ($n=5$ out of 77) with up to 1.2×10^2 viral copies μl^{-1} of genomic
400 DNA. The viral prevalence in *C. maenas* gill tissue was 75% ($n=21$ out of 28) with greater

401 than 1.0×10^4 viral copies μl^{-1} of genomic DNA, no screened internal tissue showed infection
402 (Table 4).

403

DISCUSSION

404 The study demonstrated that *C. maenas* can become infected with OsHV-1 μVar by using a
405 range of protocols recommended by OIE including PCR, qPCR and *In Situ* hybridization
406 ([www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_ostreid_herpesvir
407 us_1.pdf](http://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
408 using primer pairs that we or colleagues have successfully developed and have previously had
409 published,; PCR (Lynch et al. 2013) qPCR (Webb et al. 2007), ISH (Lynch et al. 2010).

410

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

426 build up the virus while the abundance of infected *C. gigas* would decrease.

427

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

443

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

449 The presence of the trestles, providing protection from predators, a readily available food
450 supply in the form of diseased and dying oysters and acting as a nursery site for *C. maenas*

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

473

474 In our transmission trial, first infection of OsHV-1 μ Var in naïve *C. gigas* was detected after 4
475 days. Even though the temperature was held below the associated activation threshold

476 temperature of 16 °C, a total prevalence of 6.5% OsHV-1 μ Var was detected in *C. gigas* after
477 14 days. This suggests that the virus, at nonfavorable temperatures, could be maintained in the
478 system by other marine species, like *C. maenas*, acting as a carrier and transmitting it to host
479 species *C. gigas*. Transmission of OsHV-1 μ Var to naïve *C. gigas* might have been a result of
480 direct contact between *C. maenas* and *C. gigas* or through filtration of virus particles in the
481 water or faeces excreted by *C. maenas*. The higher prevalence of OsHV-1 μ Var in gills of
482 experimental crabs (75%) after 14 days compared with the initial sample (10%) might be the
483 result of reactivation of the virus due to stress of transport and artificial settings. *C. gigas*
484 showed a cumulative mortality rate up to 25%, however it was not possible to screen dead *C.*
485 *gigas* as though tanks were checked twice daily there was no tissue left in those dead animals.
486 Therefore, it cannot be determined if *C. maenas* had predated on live animals or scavenged
487 tissues when the oysters were moribund. Due to this, infection of *C. gigas* might have been
488 underestimated as it could not be determined if those dead animals were infected or not. No
489 virus was detected within internal tissues of *C. maenas*, suggesting that migration of virus from
490 gills to internal tissues needs longer, only occurs through other transmission routes (e.g.
491 ingestion) or that infection in the crab shows different patterns of viral presence in the tissues.
492 Abnormal mortalities of *C. gigas* have been associated with viral loads of OsHV-1 μ Var higher
493 than 10^4 DNA copies mg^{-1} (Schikorski et al. 2011; Pernet et al. 2012). These high viral loads
494 were detected in a small percentage of living *C. maenas* in our experimental laboratory study,
495 however mortalities in *C. maenas* remained low (<10%). The transmission trial was performed
496 under threshold temperature of 16 °C, to imitate natural summers in Ireland. Keeping in mind
497 climate change, for future transmission experiments between crabs and oysters, it would be of
498 interest to choose higher temperatures and investigate the difference in transmission dynamics.
499 In addition, to gain better understanding of the viral dynamics between the species and
500 migration of the virus within crabs it would be of interest to perform new experiments in the

501 future by exposing highly infected oysters with naïve crabs.

502

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

513

514 **AUTHORS CONTRIBUTIONS**

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

519

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527

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TABLES AND FIGURES

793 Table 1. Weight and carapace width data for *Carcinus maenas* at the high shore and at oyster
 794 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

795

796 Table 2. Prevalence of OsHV-1 μ Var by PCR in *Carcinus maenas* gill and internal tissues at
 797 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)

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799

800 Table 3. Mean viral copies μl^{-1} of genomic DNA in samples of *Carcinus maenas* collected
 801 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%

802

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

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

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810 *Figure 1. Crassostrea gigas culture site at Dungarvan, Co. Waterford and Carlingford*

811 *Lough, Co. Louth, Ireland.*

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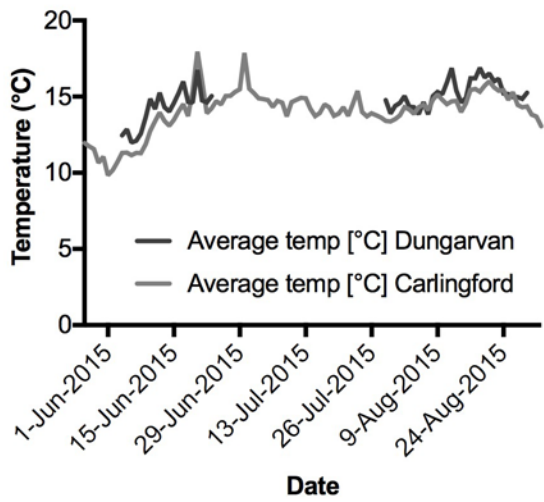
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820 *Figure 2: Average water temperature for Dungarvan and Carlingford Lough*

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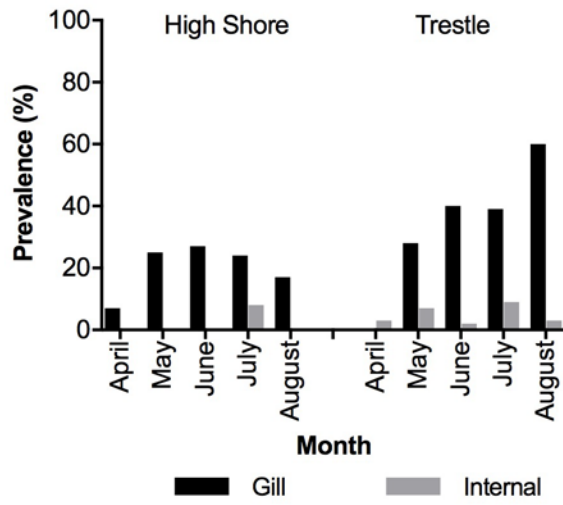
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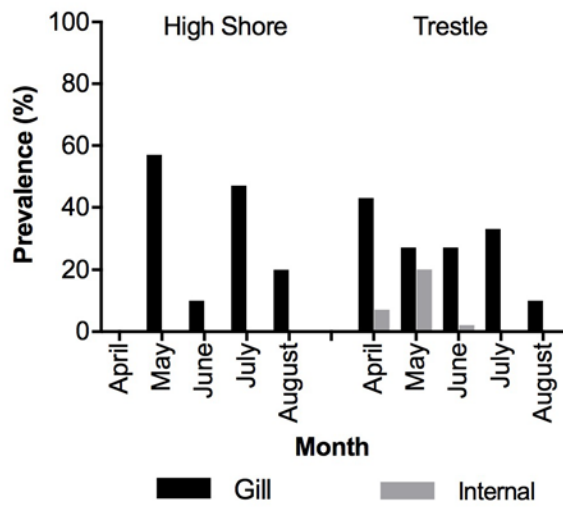
837 A



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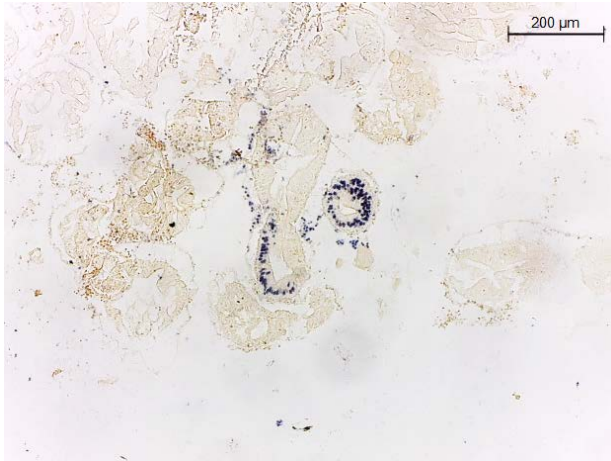
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842 *Figure 3: Prevalence of OsHV-1 μ Var in Dungarvan (A) and Carlingford Lough (B) for gill*

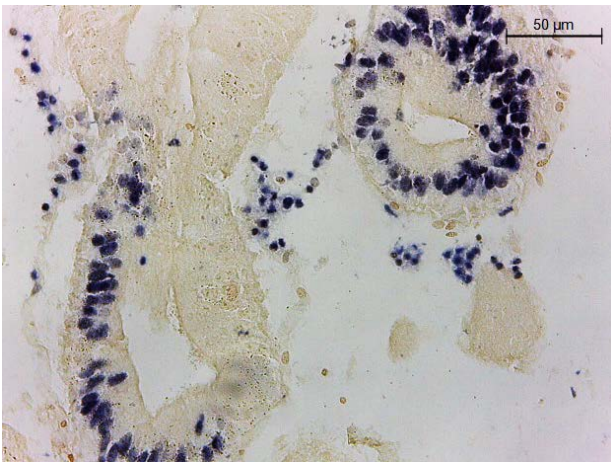
843 *and internal tissues of C. maenas at high shore and trestles per month*

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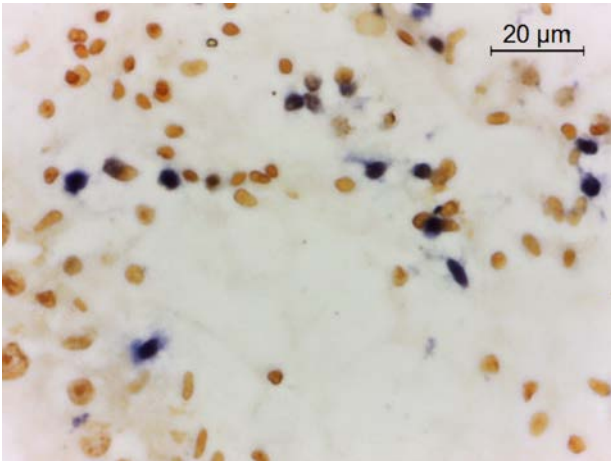
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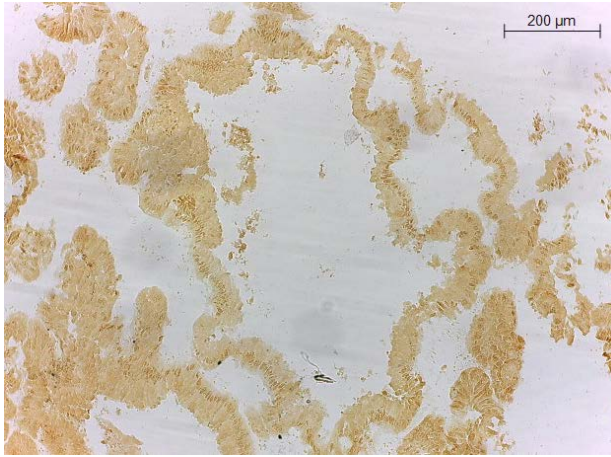
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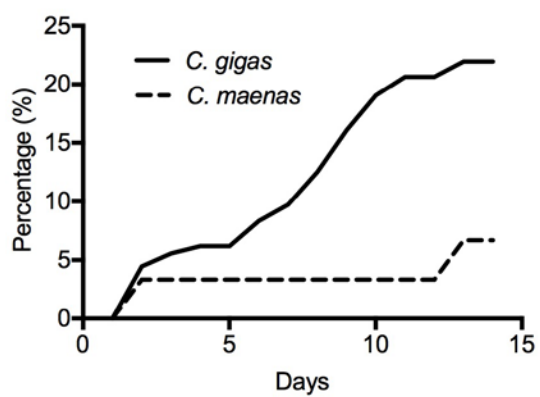


848 C



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

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855 *Figure 5: Overall cumulative mortality rates of experimental tanks with C. gigas and C.*

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maenas (derived from grouping observa