Vol. 122: 247–255, 2017
doi: 10.3354/dao03078

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published January 24

Detection of ostreid herpesvirus 1 microvariant DNA in aquatic invertebrate species, sediment and other samples collected from the Georges River estuary, New South Wales, Australia

Olivia Evans[1], Ika Paul-Pont[1,2], Richard J. Whittington[1,*]

[1]Faculty of Veterinary Science, School of Life and Environmental Sciences, The University of Sydney, Camden, NSW 2570, Australia

[2]Laboratoire des Sciences de l'Environnement Marin (LEMAR), UMR 6539 CNRS/UBO/ IRD/IFREMER e Institut Universitaire Europeen de la Mer, Technopole Brest-Iroise e Rue Dumont d'Urville, 29280 Plouzane, France

ABSTRACT: Ostreid herpesvirus 1 microvariants (OsHV-1) present a serious threat to the Australian *Crassostrea gigas* industry. Of great concern is the propensity for mortality due to the virus recurring each season in farmed oysters. However, the source of the virus in recurrent outbreaks remains unclear. Reference strain ostreid herpesvirus 1 (OsHV-1 ref) and other related variants have been detected in several aquatic invertebrate species other than *C. gigas* in Europe, Asia and the USA. The aim of this study was to confirm the presence or absence of OsHV-1 in a range of opportunistically sampled aquatic invertebrate species inhabiting specific locations within the Georges River estuary in New South Wales, Australia. OsHV-1 DNA was detected in samples of wild *C. gigas*, *Saccostrea glomerata*, *Anadara trapezia*, mussels (*Mytilus* spp., *Trichomya hirsuta*), whelks (*Batillaria australis* or *Pyrazus ebeninus*) and barnacles *Balanus* spp. collected from several sites between October 2012 and April 2013. Viral loads in non-ostreid species were consistently low, as was the prevalence of OsHV-1 DNA detection. Viral concentrations were highest in wild *C. gigas* and *S. glomerata*; the prevalence of detectable OsHV-1 DNA in these oysters reached approximately 68 and 43%, respectively, at least once during the study. These species may be important to the transmission and/or persistence of OsHV-1 in endemically infected Australian estuaries.

KEY WORDS: Ostreid herpesvirus 1 microvariant · Hosts · Detection · Prevalence · Estuary · Australia

INTRODUCTION

The Australian *Crassostrea gigas* industry consists of many farming sectors distributed along the coastlines of New South Wales (NSW), South Australia (SA) and Tasmania (TAS). The industry was worth an estimated AUD $53 million in 2007 and 2008 (SA $30.1 million, TAS $19.4 million, NSW $3.3 million) (Horvat 2011). Ostreid herpesvirus 1 microvariant (OsHV-1) was first detected in Australia in the Georges River estuary, NSW in November 2010 (Jenkins et al. 2013). In January 2013 OsHV-1 was later detected in diseased oysters in the Hawkesbury River estuary, located approximately 50 km north of the Georges River estuary (Paul-Pont et al. 2014). Once major sites for the commercial production of *C. gigas* in NSW (O'Connor & Dove 2009), *C. gigas* farming has now ceased entirely in both of these

*Corresponding author: richard.whittington@sydney.edu.au

estuaries as a consequence of the recurrent, seasonal mass mortality outbreaks caused by OsHV-1 (Paul-Pont et al. 2014, Whittington et al. 2015a). Larval oysters and oysters less than 1 yr of age are the most susceptible age classes, with losses of between 60 and 100% commonly reported in Australia and Europe (Garcia et al. 2011, Martenot et al. 2011, Paul-Pont et al. 2013b, Whittington et al. 2015a). Most recently, OsHV-1 caused an epizootic in the southeast of TAS (DPI 2016, de Kantzow et al. 2017).

It is understood that OsHV-1 is a waterborne virus and that seawater may act as a medium in the horizontal transmission of the virus (Schikorski et al. 2011a, Evans et al. 2015). OsHV-1 transmits during the warm summer months, with mortality events in Europe reported at seawater temperatures ranging between 16 and 24°C (Pernet et al. 2012, Petton et al. 2013). Mortality events begin in the south of France and progress north as the seawater temperature increases above the threshold of 16°C (Oden et al. 2011, Petton et al. 2013, Renault et al. 2014). OsHV-1 has been detected in clinically healthy oysters at field sites in La Mortanne and Les Sables de l'Are, France, at seawater temperatures less than 16°C, but transmission at such a low temperature is yet to be proven (Renault et al. 2014). In Australia the permissive temperature range of the virus is not defined; however, disease events have been reported at 22 to 25°C (Jenkins et al. 2013, Paul-Pont et al. 2013b), and experimental infection with mortality occurs at ≥18°C (de Kantzow et al. 2016). It is unknown where, or how, OsHV-1 persists in the estuarine environment during the colder months of the year.

Reference strain ostreid herpesvirus 1 (OsHV-1 ref) and other related variants have been detected in several aquatic invertebrate species other than *C. gigas* in Europe, Asia and the USA (Arzul et al. 2001b, Moss et al. 2007, Burge et al. 2011). This suggests that reservoir hosts of ostreid herpesviruses may exist and that interspecies transmission of these viruses may be possible. In Europe, OsHV-1 ref and the variant OsHV-1 var were detected in association with sporadic high mortalities in larval grooved shell clams *Ruditapes decussatus*, larval Manila clams *Venerupis philippinarum*, and larval French scallops *Pecten maximus* (Arzul et al. 2001a,b, Renault et al. 2001). Arzul et al. (2001a) demonstrated that naïve larval scallops and naïve *C. gigas* larvae, which had never previously been exposed to OsHV-1 var, could be infected with the virus when exposed to an OsHV-1 var-positive, semi-purified tissue homogenate by immersion. This homogenate was created from the tissues of larval scallops that had died from OsHV-1 var infection, suggesting that both intra-species and inter-species transmission of OsHV-1 var may be possible (Arzul et al. 2001a). However, the role that these species have in the transmission, spread and/or persistence of the OsHV-1 variants in the field remains unclear. In China, the OsHV-1 variant called acute viral necrosis virus (AVNV) caused severe mass mortality in 2 yr old Zhikong scallops *Chlamys farreri*; mortalities reached 90% within 5 d of exposure to the virus (Yu et al. 1998, Ren et al. 2013, Bai et al. 2015). Another variant, known as OsHV-1-SB has been detected in association with mass mortalities in commercially produced blood clams *Scapharca broughtonii* (Xia et al. 2015), and OsHV-1 ref DNA was detected in several other *Crassostrea* spp., as well as in Asiatic hard shell clams *Meretrix meretrix* and Yesso scallops *Patinopecten yessonesis* in China, Japan and Korea (Moss et al. 2007, Bai et al. 2015).

Interspecies transmission of OsHV-1 has not been investigated in Australian estuaries. However, the detection of related OsHV-1 variants in a number of species other than *C. gigas* in Europe and Asia suggests that a survey of other species is warranted in Australia. To fully understand the pathogenesis of the virus and the host–pathogen–environment dynamic, the reservoir host(s) of the virus and/or possible vectors or carriers of the virus must be identified. Understanding which species may become infected with OsHV-1 in the estuarine environment will allow for thorough prevention and management strategies to be developed. These strategies, in combination with advances in oyster husbandry and genetics, may allow Australian farmers to continue the production of *C. gigas* in estuaries where OsHV-1 is now endemic (Degremont 2013, Paul-Pont et al. 2013b, Whittington et al. 2015a,b, Pernet et al. 2016).

The aim of this study was to perform a preliminary investigation into the role that wild hosts in the Australian seashore population may play in the transmission of OsHV-1 by (1) confirming the presence or absence of OsHV-1 in a range of opportunistically sampled aquatic invertebrate species and (2) identify which of these species may be important to the transmission cycle of OsHV-1 by measuring the viral load detected and the prevalence of OsHV-1.

MATERIALS AND METHODS

Study site

The Georges River estuary is located approximately 16 km south of the centre of Sydney, NSW. There is

Fig. 1. Location of the 8 sampling sites (stars) within Woolooware Bay and the Georges River estuary, NSW, Australia. A range of wild aquatic invertebrate species was collected from October 2012 to April 2013. For the specific sampling dates, refer to Table 1

major bay known as Woolooware Bay (34° 01′ 54.0″ S, 151° 09′ 04.0″ E) on the southern side of the larger Botany Bay (see Fig. 1). The former is a shallow estuarine area composed of large mud flats, creating a nutrient-rich area for the growth of mangroves and saltmarsh communities. Woolooware Bay was an important area for the production of native Sydney rock oysters *Saccostrea glomerata* in NSW (Nell 2001, O'Connor & Dove 2009). However, disease outbreaks of the parasite *Marteilia sydneyi* (QX disease) in the mid-1990s led to a progressive shift towards *Crassostrea gigas* production (Adlard & Ernst 1995, O'Connor & Dove 2009). After the OsHV-1 outbreak that occurred in 2010 (Jenkins et al. 2013), the farming of *C. gigas* ceased completely. The only remaining *C. gigas* populations within the bay are small, interspersed groups of wild oysters. *S. glomerata* cultivation recommenced in 2011 at small scale.

Species sampled

A range of aquatic invertebrates were opportunistically, non-selectively sampled from 8 sites in the estuary on 6 separate occasions from October 2012 to April 2013 (Fig. 1, Table 1). Sampling sites included a range of estuarine habitats to ensure that a diverse range of invertebrates were collected. Sites included commercial oyster leases, natural oyster reef, mangrove, sandy intertidal beaches and areas of urban infrastructure such as boat ramps and jetties. Species collected included wild *C. gigas* (n = 279), wild *S. glomerata* (n = 535), Sydney cockles *Anadara trapezia* (n = 46), mussels *Mytilus* spp. (n = 5), hairy mussels *Trichomya hirsuta* (n = 10), whelks (*Batillaria australis* or *Pyrazus ebeninus*; n = 95), small gastropods *Littorina* spp. (n = 10), wedge-shell pipis *Amesodesma* spp. (n = 5), crabs (*Scylla* spp. or *Portunus* spp.; n = 22), and barnacles *Balanus* spp. (n = 300), as well as a single flatworm *Pseudostylochus* spp. and a single unidentified species of conch (Family Strombidae) (Table 1). Samples of brown algae (Class Phaeophyceae) and excrement (urates and faeces) from unidentified seabirds were collected once in November 2012, and samples of mangrove sediments were collected once in December 2012 (Table 1). The date and site of collection for each sample is summarised in Table 1. A variety of age and size classes of invertebrates were sampled, and all of the organisms collected appeared apparently healthy at the time of sampling. Samples were grouped by genus (where possible), date and site of collection, and frozen at –80°C for 1 to 3 mo prior to processing.

Table 1. Results of real-time quantitative PCR (qPCR) analysis for the detection of ostreid herpesvirus 1 microvariant (OsHV-1) DNA in a range of aquatic invertebrates collected from 8 sites in the Georges River estuary, NSW, Australia, from October 2012 to April 2013 (see Fig. 1). Samples were collected on 6 separate occasions. The number of individuals collected, the pooling rate and the number of positive pools are shown. The prevalence of OsHV-1 was calculated for each species, site and time where possible. bloq: samples positive for OsHV-1 DNA but with viral concentrations below the quantification limit of the qPCR assay (<12 DNA copies per PCR reaction); nd: not detected; n/a: not applicable; –: not determined. Viral load (geometric mean): DNA copies mg^{-1} tissue ± SD (where more than 1 pool was quantifiable)

Scientific (common) name	Date sampled	Site	No. ind. collected	Pool size	Pools positive	Viral load	Prevalence	Lower 2.5% CI	Upper 95% CI
Crassostrea gigas (Pacific oyster)	26-Oct-12	A	33	1–3	1	bloq	0.030	0.002	0.126
	15-Nov-12	A	20	5	4	bloq	0.661	0.227	0.999
	28-Nov-12	A	2	2	0	nd	0	–	–
	28-Nov-12	B	2	2	0	nd	0	–	–
	28-Nov-12	C	6	1–5	1	bloq	0.302	0.018	0.915
	20-Dec-12	A	21	1–5	6	bloq	0.140	0.036	0.331
	20-Dec-12	B	15	5	0	nd	0	–	–
	20-Dec-12	C	36	5	0	nd	0	–	–
	20-Dec-12	NE shore	30	5	0	nd	0	–	–
	14-Feb-13	Mangrove	3	3	0	nd	0	–	–
	11-Apr-13	A	7	2–5	0	nd	0	–	–
	11-Apr-13	B	10	5	2	bloq	0.602	0.152	0.980
	11-Apr-13	C	3	3	0	nd	0	–	–
	11-Apr-13	Gwawley Bay	15	5	3	bloq	0.634	0.192	0.982
	11-Apr-13	Oyster Bay	35	5	6	bloq	0.322	0.104	0.675
	11-Apr-13	Lime Kiln	41	1–5	9	$6.74 \times 10^3 \pm 1.29 \times 10^0$	0.680	0.301	0.984
Saccostrea glomerata (Sydney rock oyster)	26-Oct-12	A	24	4–5	4	bloq	0.081	0.014	0.233
	15-Nov-12	A	10	5	1	bloq	0.078	0.002	0.376
	28-Nov-12	A	25	5	3	bloq	0.056	0.011	0.156
	28-Nov-12	B	66	3–5	3	6.18×10^1	0.067	0.017	0.164
	28-Nov-12	C	35	2–5	4	1.77×10^1	0.122	0.039	0.266
	20-Dec-12	A	135	5	4	bloq	0.032	0.010	0.072
	20-Dec-12	B	35	5	0	nd	0	–	–
	20-Dec-12	C	44	2–5	0	nd	0	–	–
	20-Dec-12	NE shore	41	2–5	0	nd	0	–	–
	14-Feb-13	Mangrove	3	3	0	nd	0	–	–
	11-Apr-13	A	42	2–5	2	bloq	0.053	0.009	0.155
	11-Apr-13	B	14	4–5	2	bloq	0.222	0.039	0.591
	11-Apr-13	C	1	1	0	nd	0	–	–
	11-Apr-13	Gwawley Bay	19	4–5	3	bloq	0.268	0.070	0.618
	11-Apr-13	Oyster Bay	7	2–5	2	bloq	0.322	0.104	0.675
	11-Apr-13	Lime Kiln	9	4–5	1	bloq	0.436	0.050	0.962
Anadara trapezia (Sydney cockle)	28-Nov-12	A	30	5	1	bloq	0.036	0.001	0.185
	28-Nov-12	B	1	1	0	nd	0	–	–
	11-Apr-13	A	15	5	0	nd	0	–	–
Mytilus spp. (mussels)	28-Nov-12	A	1	1	1	bloq	n/a	–	–
	28-Nov-12	C	4	4	0	nd	0	–	–
Trichomya hirsuta (hairy mussel)	20-Dec-12	A	10	5	1	bloq	0.129	0.003	0.583
Amesodesma spp. (pipis)	28-Nov-12	A	2	2	0	nd	0	–	–
	11-Apr-13	A	1	1	0	nd	0	–	–
	11-Apr-13	B	2	2	0	nd	0	–	–
Batillaria australis/ *Pyrazus ebeninus* (whelks)	28-Nov-12	A	10	5	0	nd	0	–	–
	20-Dec-12	A	50	5	1	bloq	0.021	0.001	0.111
	20-Dec-12	B	35	5	0	nd	0	–	–
Littorina spp. (gastropods)	28-Nov-12	A	10	5	0	nd	0	–	–
Family Strombidae (conch)	11-Apr-13	A	1	1	0	nd	0	–	–

Table 1 (continued)

Scientific (common) name	Date sampled	Site	No. ind. collected	Pool size	Pools positive	Viral load	Prevalence	Lower 2.5% CI	Upper 95% CI
Balanus spp. (barnacles)	15-Nov-12	A	150	30	2	bloq	0.017	0.002	0.062
	28-Nov-12	A	30	30	0	nd	0	–	–
	28-Nov-12	B	120	30	0	nd	0	–	–
Scylla spp./*Portunus* spp. (crab)	21-Feb-13	A	22	1	0	nd	0	–	–
Pseudostylochus spp. (flatworm)	20-Dec-12	A	1	1	0	nd	0	–	–
Class Phaeophyceae (brown algae)	28-Nov-12	C	1	1	1	bloq	n/a	–	–
Unidentified sediments	20-Dec-12	A	6	1	2	bloq	n/a	–	–
	20-Dec-12	B	5	1	0	nd	n/a	–	–
	20-Dec-12	NE shore	2	1	0	nd	n/a	–	–
Unidentified bird excrement	28-Nov-12	A	3	1	0	nd	0	–	–

Sample processing

Each species was processed separately to minimise the potential for cross contamination. Bivalve species (oysters, cockles, mussels and pipis) were processed by stomaching. Depending on the size of the bivalve, samples were stomached either as individuals (if >90 mm in length) or in pools of 2 to 5 (if ≤90 mm in length) (Table 1). All soft tissues of the bivalve were removed from the shell using sterile, disposable scalpel blades (Livingstone) and placed whole into a 190 × 300 mm stomaching bag (Interscience). Bags were placed on wet ice until stomaching. To lubricate the tissues, 2 to 4 ml of saline (0.85% w/v) was added to each stomaching bag. Bags were stomached individually in a MiniMix® machine (Crown Scientific) at maximum speed for 1 min. A 1 ml aliquot of the tissue homogenate was placed into a 2 ml tube (Interpath Services) containing 0.4 g of 0.1 mm silica-zirconia beads (Daintree Scientific). Tubes were stored at −80°C until further tissue homogenisation.

Barnacles, gastropods, and the conch were similarly processed using the stomaching method described above. However, the soft tissues of these species were removed from the shell by crushing with a hammer, and the soft tissues were sorted from the shell using sterile, disposable scalpel blades. Where the shell was not easily or sufficiently crushed, the entire or remaining soft tissues were excised from the shell using sterile scalpel blades and laboratory tweezers. Whelks and small gastropods were pooled (n = 5) for stomaching, and barnacles were pooled (n = 30) (Table 1).

Crabs were dissected individually by first removing the legs and carapace with a sterile, disposable scalpel blade. The gill and digestive gland were excised and placed into separate 2 ml tubes containing 9 volumes (w/v) of nuclease-free water (Ultrapure™) and 0.4 g of silica-zirconia beads. Crabs <10 mm in length were placed whole into the tube after the removal of the legs and carapace. The flatworm was placed whole into a 2 ml tube containing 1 ml of nuclease-free water and 0.4 g of silica-zirconia beads. Tubes were stored at −80°C until tissue homogenisation.

The brown algae sample was processed by finely mincing with sterile, disposable scalpel blades, and placing a weighed portion of the minced material into a 2 ml tube with 9 volumes of nuclease-free water and 0.4 g of silica-zirconia beads. To process the sediment samples and bird excrement, samples were diluted in 2 to 10 ml of nuclease-free water and vortexed for 30 to 60 s to homogenise the suspension. A 1 ml sample of the suspension was placed into a 2 ml tube containing 0.4 g of silica-zirconia beads, and samples were stored at −80°C until tissue homogenisation.

Detection of OsHV-1 DNA using real-time quantitative PCR

All samples were homogenised by bead beating in a Fastprep®-24 machine (Fastprep®-24 System; MP Biomedical). Tubes were thawed for 20 min at room temperature and placed into the machine for 15 s at a speed of 6.5 m s^{-1}. Samples were clarified by cen-

trifugation at 900 × *g* for 10 min in a microcentrifuge (Heraeus® Biofuge® Pico; Thermo Electron Corporation). A total of 200 µl of supernatant was removed and stored at −80°C until nucleic acid purification.

Nucleic acids were purified using a 5× MagMAX™-96 Viral RNA Isolation Kit (Ambion®; Life Technologies™) and MagMAX™ Express 96 magnetic particle processor (Applied Biosystems™; Life Technologies™) according to the manufacturer's instructions for a 50 µl sample volume protocol using the AM1836 deep-well standard program. Nucleic acid preparations were stored at −20°C until real-time quantitative PCR (qPCR) analysis.

Samples were analysed in duplicate in a 25 µl total reaction volume using the TaqMan® qPCR assay previously described by Evans et al. (2014). This assay was adapted from the fluorescent probe assay first developed by Martenot et al. (2010). The quantification limit of the assay was 12 viral copies per PCR reaction. Samples that satisfied the criteria for detection but had a cycle threshold (C_T) value below the quantification limit of the assay were described as positive below the limit of quantification (bloq).

Prevalence

The prevalence of OsHV-1 was calculated for each species, at each site and time of collection. The Ausvet Animal Health service calculators for 'estimating individual prevalence from pooled samples', available at http://epitools.ausvet.com.au (Sergeant 2016), were used to estimate prevalence from the pooled samples. The 'variable pool sizes and a perfect test' calculator and a Bayesian calculator (Cowling et al. 1999) were employed. The Bayesian method required specification of priors for prevalence, sensitivity and specificity. The methods of Paul-Pont et al. (2013b) were used: a uniform beta prior for prevalence (alpha = 1 and beta = 1), an informative prior for sensitivity assuming a median of 0.90 with lower 5% interval of 0.70 (alpha = 15.03 and beta = 2.56), and an informative prior for specificity assuming a median of 0.995 with a lower 5% interval of 0.95 (alpha = 1137.5 and beta = 6.7) were specified.

RESULTS

OsHV-1 DNA was detected in wild *Crassostrea gigas*, *Saccostrea glomerata*, *Anadara trapezia*, mussels *Mytilus* spp., *Trichomya hirsuta*, whelks (*Batillaria australis* or *Pyrazus ebeninus*) and barnacles *Balanus* spp. at several sites in the Georges River estuary on separate occasions from Oct 2012 to April 2013. OsHV-1 was also detected in the single brown algae (Class Phaeophyceae) sample and in 2 of the 13 mangrove sediment samples collected in November and December 2012, respectively. OsHV-1 was not detected in any of the *Amesodesma* spp., *Littorina* spp., conch, flatworm, crab (*Scylla* spp. or *Portunus* spp.) or excrement samples analysed (Table 1).

Viral quantities in *A. trapezia*, mussels (*Mytilus* spp., *T. hirsuta*), whelks (*B. australis* or *P. ebeninus*) and barnacles (*Balanus* spp.) were consistently very low (below the quantification limit of the assay; <12 DNA copies per PCR reaction). Viral loads in oysters varied according to the species, site and time of collection (Table 1). The highest viral concentrations detected were in wild *C. gigas* collected from Lime Kiln in April 2013, at $6.74 \times 10^3 \pm 1.29 \times 10^0$ DNA copies mg^{-1} tissue. The highest viral concentration detected in wild *S. glomerata* was 6.18×10^1 DNA copies mg^{-1} tissue, at Site B in November 2012 (Table 1).

Prevalence of OsHV-1 DNA detection in *A. trapezia*, mussels *Mytilus* spp., whelks and barnacles ranged between 1.7 and 3.6%, while in *T. hirsuta* the prevalence of OsHV-1 DNA was 12.9% (Table 1). The prevalence of OsHV-1 DNA detection in wild *C. gigas* ranged between 3.0 and 68.0%, and in wild *S. glomerata* it ranged between 3.2 and 43.7% (Table 1).

DISCUSSION

This study is the first to investigate the detection and prevalence of OsHV-1 DNA in wild aquatic invertebrate species and sediments in an OsHV-1 affected estuary in Australia. Similar to what has been previously reported for OsHV-1 ref (and other related variants) in Europe, Asia and the USA, OsHV-1 DNA was detected in several bivalve species other than *Crassostrea gigas*, including a native oyster, several species of mussel and a species of native cockle (Arzul et al. 2001b, Moss et al. 2007, Burge et al. 2011, Cochennec-Laureau et al. 2011, Guichard et al. 2011, Bai et al. 2015, Batista et al. 2015, Xia et al. 2015). OsHV-1 DNA was previously detected in farmed *Saccostrea glomerata* during investigations of the initial OsHV-1 outbreak in the Georges River estuary in 2010 (Jenkins et al. 2013). However, it is unclear whether wild or farmed *S. glomerata* are susceptible to infection with OsHV-1, or whether transmission between *S.*

glomerata and *C. gigas* is possible. *In situ* hybridisation (ISH) experiments suggest that *S. glomerata* are not susceptible to infection with OsHV-1 (Jenkins et al. 2013).

This study is the first report of OsHV-1 DNA detection in non-bivalve invertebrates; OsHV-1 DNA was detected in whelks (*Batillaria australis* or *Pyrazus ebeninus*) and barnacles *Balanus* spp. at very low viral concentrations (below the quantification limit of the assay).

OsHV-1 DNA was not detected in the *Amesodesma* spp., *Littorina* spp., or crab (*Scylla* spp. or *Portunus* spp.), conch, flatworm or excrement samples analysed. As only small numbers of these organisms/samples were collected during the course of this study it is uncertain whether OsHV-1 DNA was indeed absent from these populations. Observations of repeated natural transmission events in the Georges River estuary have demonstrated a non-random, spatially clustered pattern of OsHV-1 disease events (Paul-Pont et al. 2013a). Thus, it is possible that the sampling strategy used may have missed detecting the virus in some samples, at some sites and on particular dates. The sample sizes collected at each site and time were variable, the consequence of which is variable power of the study and precision of prevalence estimates. This requires resolution in future investigations. More regular sampling and consistent, larger sample sizes will provide a clearer indication of the detection pattern and prevalence of OsHV-1 in these species over time.

Viral loads in cockles, mussels, whelks and barnacles were consistently very low (below the quantification limit of the assay). In contrast, the viral load in wild oysters was quantifiable on several occasions (Table 1). The highest viral concentrations were detected in wild *C. gigas* collected from Lime Kiln. Viral quantities greater than 1×10^4 OsHV-1 DNA copies mg^{-1} tissue have been shown to be systematically associated with clinically infected, moribund and dead *C. gigas*, while oysters with concentrations less than this are typically healthy (Oden et al. 2011, Schikorski et al. 2011a, Paul-Pont et al. 2015). This threshold, which was not exceeded in this study, may explain why all of the wild oysters sampled in this study appeared to be healthy at the time of collection.

It is unclear whether OsHV-1 was actively replicating within the tissues of each species which tested positive for OsHV-1. As seawater may act as a medium in the transmission of OsHV-1 (Vigneron et al. 2004, Schikorski et al. 2011a, Evans et al. 2015), it is possible that organisms were contaminated by OsHV-1 filtered from the seawater during respiration or feeding activities, rather than being actively infected by the virus. The virus detected may have instead been present within the mucus layers of the gill epithelium or within the digestive tract of the invertebrates. It is similarly unknown whether the OsHV-1 DNA detected in the mangrove sediments and brown algae sample was free virus, free viral DNA, or virus or viral DNA associated with particles in the sample. Further studies are required to ascertain whether these species are susceptible to OsHV-1 infection, and to determine whether transmission between these species is possible.

Several ISH methods are available for the detection of OsHV-1 in infected *C. gigas* tissues (Arzul et al. 2002, Lipart & Renault 2002, Corbeil et al. 2015). ISH techniques require clinically infected oysters with high viral loads of $\geq 10^4$ DNA copies mg^{-1} tissue (Oden et al. 2011, Corbeil et al. 2015). These techniques could be used to confirm the presence of OsHV-1 in infected individuals and to localise viral mRNA or DNA in different tissues, or to identify viral particles within the mucous or lumen of the digestive tract or on the outer surface of samples such as sediment or algae. ISH techniques could not be employed in the current study due to the low viral quantities detected ($<10^4$ DNA copies mg^{-1} tissue) (Table 1).

Structured, longitudinal field studies and laboratory-based studies will be required in combination to fully understand the potential for host reservoirs of OsHV-1, interspecies transmission, and the host–pathogen–environment dynamic (Burge et al. 2011). Experimental infection models and cohabitation based investigations are required to demonstrate that natural transmission of OsHV-1 between *C. gigas* and other species (or vice versa) is possible. The mechanism of infection in cohabitation models is more natural than methods involving intramuscular injection of, or immersion in, a concentrated tissue homogenate and would demonstrate more reliably what organisms are truly susceptible and at risk of infection from OsHV-1, or are resistant, or possible carriers, vectors or reservoirs of the virus in the field (Schikorski et al. 2011a,b, Paul-Pont et al. 2015). Demonstrated viral replication within the tissues of a species, or confirmation of the presence of OsHV-1 within host tissues by ISH or transmission electron microscopy would demonstrate that OsHV-1 is not simply being filtered out of the seawater during respiration or feeding activities (Segarra et al. 2014, Corbeil et al. 2015, Tan et al. 2015).

CONCLUSIONS

OsHV-1 DNA was detected in the samples of wild *Crassostrea gigas*, *Saccostrea glomerata*, *Anadara trapezia*, mussels (*Mytilus* spp., *Trichomya hirsuta*), whelks (*Batillaria australis* or *Pyrazus ebeninus*) and barnacles (*Balanus* spp.) collected from several sites within the Georges River estuary, NSW, Australia, from October 2012 to April 2013. Viral loads in non-ostreid species were consistently low, as was the prevalence of infection. Prevalence of OsHV-1 and viral loads in wild *C. gigas* and *S. glomerata* varied according to the site and time of collection; however, prevalence in these species reached approximately 68 and 43%, respectively, at least once during the 2012/2013 period. Viral loads in wild *C. gigas* and wild *S. glomerata* were the highest detected, with approximately 10^3 and 10^1 DNA copies mg^{-1} tissue, respectively. Further investigation is required to understand the role that these species play in the transmission and persistence of OsHV-1 in Australian estuaries.

Acknowledgements. This work was funded by the Australian Government through the Fisheries Research and Development Corporation and by the University of Sydney. Len, Robert and Ted Drake are thanked for their assistance in the field. The technical teams of the Virology and Infectious Diseases Laboratories, University of Sydney, Camden Campus are thanked for their guidance and support in processing the samples (Anna Waldron, Alison Tweedie, Ann-Michele Whittington, Slavicka Patten, and Natalie Schiller). Dr. Paul Hick is thanked for his discussions and comments on the manuscript.

LITERATURE CITED

Adlard RD, Ernst I (1995) Extended range of the oyster pathogen *Marteilia sydneyi.* Bull Eur Assoc Fish Pathol 15:119–121

Arzul I, Nicolas JL, Davison AJ, Renault T (2001a) French scallops: a new host for ostreid herpesvirus-1. Virology 290:342–349

Arzul I, Renault T, Lipart C, Davison AJ (2001b) Evidence for interspecies transmission of oyster herpesvirus in marine bivalves. J Gen Virol 82:865–870

Arzul I, Renault T, Thebault A, Gerard A (2002) Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. Virus Res 84:151–160

Bai C, Wang C, Xia J, Sun H, Zhang S, Huang J (2015) Emerging and endemic types of *Ostreid herpesvirus 1* were detected in bivalves in China. J Invertebr Pathol 124:98–106

Batista FM, Lopez-Sanmartin M, Grade A, Morgado I and others (2015) Sequence variation in ostreid herpesvirus 1 microvar isolates detected in dying and asymptomatic *Crassostrea angulata* adults in the Iberian Peninsula: insights into viral origin and spread. Aquaculture 435: 43–51

Burge CA, Strenge RE, Friedman CS (2011) Detection of the oyster herpesvirus in commercial bivalves in northern California, USA: conventional and quantitative PCR. Dis Aquat Org 94:107–116

Cochennec-Laureau N, Baud JP, Pepin JF, Benabdelmouna A and others (2011) Les surmortalités des naissains d'huîtres creuses, *Crassostrea gigas*: acquis des recherches en 2010. IFREMER

Corbeil S, Faury N, Segarra A, Renault T (2015) Development of an *in situ* hybridisation assay for the detection of ostreid herpesvirus 1 mRNAs in the Pacific oyster, *Crassostrea gigas.* J Virol Methods 211:43–50

Cowling DW, Gardner IA, Johnson WO (1999) Comparison of methods for estimation of individual-level prevalence based on pooled samples. Prev Vet Med 39:211–225

de Kantzow M, Hick P, Becker JA, Whittington RJ (2016) Effect of water temperature on mortality of Pacific oysters *Crassostrea gigas* associated with microvariant ostreid herpesvirus 1 (OsHV-1 µVar). Aquacult Environ Interact 8:419–428

de Kantzow MC, Hick PM, Dhand NK, Whittington RJ (2017) Risk factors for mortality during the first occurrence of Pacific Oyster Mortality Syndrome due to *Ostreid herpesvirus-1* in Tasmania, 2016. Aquaculture 468:328–336

Degremont L (2013) Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas.* Aquaculture 416-417:129–134

DPI (Department of Primary Industries) (2016) NSW oyster industry – update: POMS outbreak in Tasmania. NSW Oyster Industry – Issues. NSW Government, Department of Primary Industries, Port Stephens

Evans O, Paul-Pont I, Hick P, Whittington R (2014) A simple centrifugation method for improving the detection of Ostreid herpesvirus-1 (OsHV-1) in natural seawater samples with an assessment of the potential for particulate attachment. J Virol Methods 210:59–66

Evans O, Hick P, Dhand N, Whittington RJ (2015) Transmission of *Ostreid herpesvirus-1* in *Crassostrea gigas* by cohabitation: effects of food and number of infected donor oysters. Aquacult Environ Interact 7:281–295

Garcia C, Thebault A, Degremont L, Arzul I and others (2011) Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. Vet Res 42:73

Guichard B, Francois C, Joly JP, Garcia C and others (2011) Bilan 2010 du réseau repamo – réseau national de surveillance de la santé des mollusques marins. IFREMER

Horvat P (2011) New threat to Pacific oysters. In: FISH. Fisheries Research and Development Corporation, Canberra, p 14–15

Jenkins C, Hick P, Gabor M Spiers Z and others (2013) Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 µ-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis Aquat Org 105:109–126

Lipart C, Renault T (2002) Herpes-like virus detection in infected *Crassostrea gigas* spat using DIG-labelled probes. J Virol Methods 101:1–10

Martenot C, Oden E, Travaille E, Malas JP, Houssin M (2010) Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas.* J Virol Methods 170:86–89

Martenot C, Oden E, Travaille E, Malas JP, Houssin M (2011) Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas*

between 2008 and 2010. Virus Res 160:25–31

Moss JA, Burreson EM, Cordes JF, Dungan CF and others (2007) Pathogens in *Crassostrea ariakensis* and other Asian oyster species: implications for non-native oyster introduction to Chesapeake bay. Dis Aquat Org 77: 207–223

Nell JA (2001) The history of oyster farming in Australia. Mar Fish Rev 63:14–25

O'Connor WA, Dove MC (2009) The changing face of oyster culture in New South Wales, Australia. J Shellfish Res 28: 803–811

Oden E, Martenot C, Berthaux M, Travaille E, Malas JP, Houssin M (2011) Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: determination of a viral load threshold to prevent summer mortalities. Aquaculture 317:27–31

Paul-Pont I, Dhand NK, Whittington RJ (2013a) Spatial distribution of mortality in Pacific oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission. Dis Aquat Org 105:127–138

Paul-Pont I, Dhand NK, Whittington RJ (2013b) Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters *Crassostrea gigas.* Aquaculture 412–413: 202–214

Paul-Pont I, Evans O, Dhand N, Rubio A, Coad P, Whittington R (2014) Descriptive epidemiology of mass mortality due to *Ostreid herpesvirus-1* (OsHV-1) in commercially farmed Pacific oysters (*Crassostrea gigas*) in the Hawkesbury River estuary, Australia. Aust Aquacult 422-423: 146–159

Paul-Pont I, Evans O, Dhand NK, Whittington RJ (2015) Experimental infections of Pacific oyster *Crassostrea gigas* using the Australian ostreid herpesvirus-1 (OsHV-1) µVar strain. Dis Aquat Org 113:137–147

Pernet F, Barret J, Le Gall P, Corporeau C and others (2012) Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. Aquacult Environ Interact 2:215–237

Pernet F, Lupo C, Bacher C, Whittington RJ (2016) Infectious diseases in oyster aquaculture require a new integrated approach. Philos Trans R Soc Lond B Biol Sci 371: 20150213

Petton B, Pernet F, Robert R, Boudry P (2013) Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas.* Aquacult Environ Interact 3:257–273

Ren W, Chen H, Renault T, Cai Y, Bai C, Wang C, Huang J (2013) Complete genome sequence of acute viral necrosis virus associated with massive mortality outbreaks in the Chinese scallop, *Chlamys farreri.* Virol J 10:110–117

Renault T, Lipart C, Arzul I (2001) A herpes-like virus infects a non-ostreid bivalve species: virus replication in *Ruditapes philippinarum* larvae. Dis Aquat Org 45:1–7

Renault T, Bouquet al. Maurice JT, Lupo C, Blachier P (2014) Ostreid herpesvirus 1 infection among Pacific oyster (*Crassostrea gigas*) spat: relevance of water temperature to virus replication and circulation prior to the onset of mortality. Appl Environ Microbiol 80:5419–5426

Schikorski D, Faury N, Pepin JF, Saulnier D, Tourbiez D, Renault T (2011a) Experimental ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. Virus Res 155:28–34

Schikorski D, Renault T, Saulnier D, Faury N, Moreau P, Pepin JF (2011b) Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. Vet Res 42:27

Segarra A, Baillon L, Tourbiez D, Benabdelmouna A, Faury N, Bourgougnon N, Renault T (2014) Ostreid herpesvirus type 1 replication and host response in adult Pacific oysters, *Crassostrea gigas.* Vet Res 45:103

Sergeant ESG (2016) EpiTools epidemiological calculators. Ausvet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. http://epitools.ausvet.com.au

Tan TLS, Paul-Pont I, Evans OM, Watterson D and others (2015) Resistance of black-lip pearl oyster, *Pinctada margaritifera*, to infection by *Ostreid herpes virus* 1 µvar under experimental challenge may be mediated by humoral antiviral activity. Fish Shellfish Immunol 44: 232–240

Vigneron V, Solliec G, Montanié H, Renault T (2004) Detection of Ostreid Herpesvirus 1 (OsHV-1) DNA in seawater by PCR: influence of water parameters in bioassays. Dis Aquat Org 62:35–44

Whittington RJ, Dhand NK, Evans O, Paul-Pont I (2015a) Further observations on the influence of husbandry practices on OsHV-1 µVar mortality in Pacific oysters *Crassostrea gigas*: age, cultivation structures and growing height. Aquaculture 438:82–97

Whittington RJ, Hick P, Evans O, Rubio A, Alford B, Dhand N, Paul-Pont I (2015b) Protection of Pacific oyster (*Crassostrea gigas*) spat from mortality due to ostreid herpesvirus 1 (OsHV-1 µVar) using simple treatments of incoming seawater in land-based upwellers. Aquaculture 437:10–20

Xia J, Bai C, Wang C, Song X, Huang J (2015) Complete genome sequence of *Ostreid herpesvirus-1* associated with mortalities of *Scapharca broughtonii* broodstocks. Virol J 12:110

Yu RH, Wang RC, Tian CY, Wang ZP (1998) Discussion on the high mortality and its prevention in scallop *Chlamys farreri*. Trans Oceanol Limnol 3:69–72

Editorial responsibility: Stephen Feist, Weymouth, UK

Submitted: August 7, 2016; Accepted: November 2, 2016
Proofs received from author(s): December 8, 2016