

# Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 $\mu$ -var) in *Crassostrea gigas* (Pacific oysters) in Australia

Cheryl Jenkins<sup>1,\*</sup>, Paul Hick<sup>1</sup>, Melinda Gabor<sup>1</sup>, Zoe Spiers<sup>1</sup>, Shayne A. Fell<sup>1</sup>, Xingnian Gu<sup>1</sup>, Andrew Read<sup>1</sup>, Jeffrey Go<sup>1,2</sup>, Michael Dove<sup>3</sup>, Wayne O'Connor<sup>3</sup>, Peter D. Kirkland<sup>1</sup>, Jane Frances<sup>3</sup>

<sup>1</sup>NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales 2568, Australia

<sup>2</sup>Faculty of Veterinary Science, University of Sydney, New South Wales 2570, Australia

<sup>3</sup>NSW Department of Primary Industries, Port Stephens Fisheries Institute, Port Stephens, New South Wales 2316, Australia

**ABSTRACT:** Between November 2010 and January 2011, triploid *Crassostrea gigas* (Pacific oysters) cultivated in the Georges River, New South Wales, experienced >95 % mortality. Mortalities also occurred in wild diploid *C. gigas* in the Georges River and shortly thereafter in the adjacent Parramatta River estuary upstream from Sydney Harbour. Neighbouring *Saccostrea glomerata* (Sydney rock oysters) did not experience mortalities in either estuary. Surviving oysters were collected to investigate the cause of mortalities. Histologically all oysters displayed significant pathology, and molecular testing revealed a high prevalence of ostreid herpesvirus-1 (OsHV-1). Quantitative PCR indicated that many *C. gigas* were carrying a high viral load at the time of sampling, while the load in *S. glomerata* was significantly lower ( $p < 0.001$ ). Subsequent *in situ* hybridisation experiments confirmed the presence of a herpesvirus in *C. gigas* but not *S. glomerata* tissues, suggesting that *S. glomerata* is not susceptible to infection with OsHV-1. Naïve sentinel triploid *C. gigas* placed in the Georges River estuary in January 2011 quickly became infected and experienced nearly 100 % mortality within 2 wk of exposure, indicating the persistence of the virus in the environment. Phylogenetic analysis of sequences derived from the C2/C6 region of the virus revealed that the Australian strain of OsHV-1 belongs to the microvariant ( $\mu$ -var) cluster, which has been associated with severe mortalities in *C. gigas* in other countries since 2008. Environmental data revealed that the Woonooware Bay outbreaks occurred during a time of considerable environmental disturbance, with increased water temperatures, heavy rainfall, a toxic phytoplankton bloom and the presence of a pathogenic *Vibrio* sp. all potentially contributing to oyster stress. This is the first confirmed report of OsHV-1  $\mu$ -var related *C. gigas* mortalities in Australia.

**KEY WORDS:** Viral disease · Triploid · Bivalve · Aquaculture · Environmental stressor · Phytoplankton · Bloom · Biotoxin · *Vibrio* · New South Wales

— Resale or republication not permitted without written consent of the publisher —

## INTRODUCTION

Herpesviruses that infect bivalve species are emerging as significant pathogens in the aquaculture industry. Herpes and herpes-like viruses have been

reported in a variety of commercially important oyster species of the genera *Crassostrea* (Farley et al. 1972, Hine et al. 1992, Burge et al. 2011, Lynch et al. 2012) and *Ostrea* (Hine & Thorne 1997, Hine et al. 1998, da Silva et al. 2008), as well as in the scallops

*Pecten maximus* and *Chlamys farreri* (Arzul et al. 2001a, Tang et al. 2010), clams *Ruditapes* spp. (Arzul et al. 2001c) and abalone *Haliotis* spp. (Tan et al. 2008, Savin et al. 2010). Despite the significant impacts of herpesviruses on molluscan aquaculture, ostreid herpesvirus (OsHV) is one of only 3 molluscan herpesviruses (family *Malacoherpesviridae*) that have been characterised at the molecular level (Davison et al. 2009, Savin et al. 2010); the others being abalone ganglioneuritis virus from *Haliotis* spp. (Savin et al. 2010) and acute viral necrobiotic virus (AVNV) from the Chinese scallop *Chlamys farreri* (Tang et al. 2010). Phylogenetic comparisons indicate that AVNV and OsHV are variants of the same virus (Renault et al. 2012). OsHV is known to cause severe mortality events in the Pacific oyster *C. gigas*; however, molecular analyses of hatchery-reared *O. edulis* and *Ruditapes* spp. (Arzul et al. 2001c), as well as samples derived from experimental transmission trials (Arzul et al. 2001b), indicate that OsHV Type 1 (OsHV-1) can infect a range of bivalve hosts. Spat and juvenile *C. gigas* infected with OsHV appear to be particularly susceptible to mortality, with adults less severely affected or asymptomatic (Arzul et al. 2002). As *C. gigas* represents over 95% of the world's cultured oyster production, estimated at 661 500 t and US\$1.3 billion in 2010 (FAO 2013), OsHV represents a significant risk globally to commercial bivalve production.

Several studies conducted in France (Le Deuff et al. 1996, Sauvage et al. 2009), Ireland (Malham et al. 2009, Cotter et al. 2010) and the United States (Friedman et al. 2005, Burge et al. 2006) strongly suggest a role for environmental stress in OsHV outbreaks. Specifically, OsHV outbreaks appear to be related to both increases in water temperature ( $>16^{\circ}\text{C}$ ) and intensive farming (Pernet et al. 2012). In some cases, OsHV mortality events have also been linked to the presence of *Vibrio* species including *V. harveyi*, *V. splendidus* and *V. aestuarianus* (Segarra et al. 2010). Particularly severe *Crassostrea gigas* mortalities reported in France, the United Kingdom and the Republic of Ireland in 2008 to 2009 were associated with the presence of an OsHV-1 variant known as the microvariant ( $\mu$ -var) (Segarra et al. 2010). However, recent studies demonstrate that OsHV-1  $\mu$ -var can also be detected in asymptomatic oysters, possibly reflecting latent infection or a low viral load (Dundon et al. 2011).

Mortality events in which OsHV has been definitively identified as the causative agent have previously been confined in the northern hemisphere, with outbreaks in Europe (da Silva et al. 2008,

Sauvage et al. 2009, Segarra et al. 2010), the United States (Friedman et al. 2005, Burge et al. 2006) and Mexico (Vásquez-Yeomans et al. 2010). Herpes-like virus particles have been observed in moribund *Crassostrea gigas* and *Ostrea chilensis* in New Zealand (Hine et al. 1992, 1998), in *Bonamia*-infected *O. angasi* (Hine & Thorne 1997) and in hatchery-reared *Saccostrea glomerata* larvae experiencing mortalities (M. Hine pers. comm.) in Australia. Due to a lack of molecular data, it is unclear whether OsHV was the viral agent in these cases; however, the herpesvirus-like particles described in *O. angasi* appeared to be ultrastructurally distinct from those described in *C. gigas* (Hine & Thorne 1997).

While *Crassostrea gigas* is not native to Australia, it has become the major commercial oyster in Tasmania and South Australia and is preferentially farmed in some New South Wales (NSW) estuaries due to the impact of QX disease (infection with *Marteilia sydneyi*) on the indigenous species *Saccostrea glomerata*. In the Georges River, NSW, triploid *C. gigas* are farmed alongside *S. glomerata*, while in the Parramatta River (Sydney Harbour), only wild populations of diploid *C. gigas* and *S. glomerata* are present; with *C. gigas* considered an invasive pest species. Triploid *C. gigas* culture commenced in the Georges River in 2004, and it generally takes approximately 7 to 18 mo to grow small spat (shell height: 4 mm) to a marketable size (shell height: 50 to 120 mm).

In the summer of 2010, mortalities of  $>95\%$  were observed in both wild (diploid) and farmed (triploid) *Crassostrea gigas* in the Georges River, and in wild populations of diploid *C. gigas* in the Parramatta River. Subsequent testing revealed that the mortalities were caused by OsHV-1  $\mu$ -var, the first confirmed case in Australian oysters.

## MATERIALS AND METHODS

### Oyster cultivation, chronology of events and study sites

Triploid *Crassostrea gigas* cultivated at Woolooware Bay, Georges River, were all from the same broodstock population and were sourced from either the Port Stephens or the Tasmanian hatchery. Prior studies indicate that there is a high genetic diversity within both Australian and NSW *C. gigas* stocks (Miller et al. 2012). Diploid *C. gigas* are considered a pest in NSW and are also known to have been present in the Georges River since 1984. *C. gigas* is not cultivated in the Parramatta River; therefore, samples

collected from this estuary constituted diploid oysters from wild populations. Records indicate that diploid *C. gigas* have been present in the Sydney area since the late 1960s (for >10 generations). Inter-estuarine movements of farmed oysters, including diploid and triploid *C. gigas*, as well as *Saccostrea glomerata*, are regulated in NSW.

Investigations of unexplained mortalities in triploid and diploid *Crassostrea gigas* were conducted at 3 oyster leases located in Woolooware and Quibray Bays in the Georges River and 4 sites within the Parramatta River (Fig. 1). Key dates and the types and number of samples collected from each estuary are summarised in Table 1. *C. gigas* and *Saccostrea glomerata* samples collected from the Georges River were cultivated in trays according to standard industry practice and at standard growing height (Smith et al. 2000); however, floating baskets and trays on the substratum were also in use at the time of the study. As poor water quality was considered a potential cause of the initial Woolooware Bay mortalities, water samples were also collected for toxicological analysis (Table 1). No prior screening for OsHV-1 had been carried out on samples from either the Georges or Parramatta Rivers.

### Sampling of oysters

Oyster samples from mortality events were collected in accordance with NSW Department of Primary Industries (DPI) fish kill policy (NSW DPI 2011). Oysters from cultivation areas were submitted directly to NSW DPI for testing, while field personnel were deployed to identify evidence of disease or mortality of wild oysters. Samples of farmed oysters were collected from trays, and wild oysters were detached from the substratum. Each sample was enclosed in an individual zip lock plastic bag and transported to the laboratory on ice. In the case of mortality events, live oysters adjacent to areas showing evidence of disease were selected preferentially to provide suitable diagnostic samples. Surviving triploid *Crassostrea gigas* from Lease 1 (Fig. 1) were sampled approximately 12 and 26 wk after the outbreak (Table 1). For the purpose of testing for continued transmission of OsHV-1, naïve, triploid *C. gigas* (sentinel oysters, 60 mm shell height) were translocated from the Hawkesbury River to sites in Woolooware Bay and Quibray Bay (Table 1). Oysters were placed in a basket at normal commercial tray growing height on 6 January 2011.

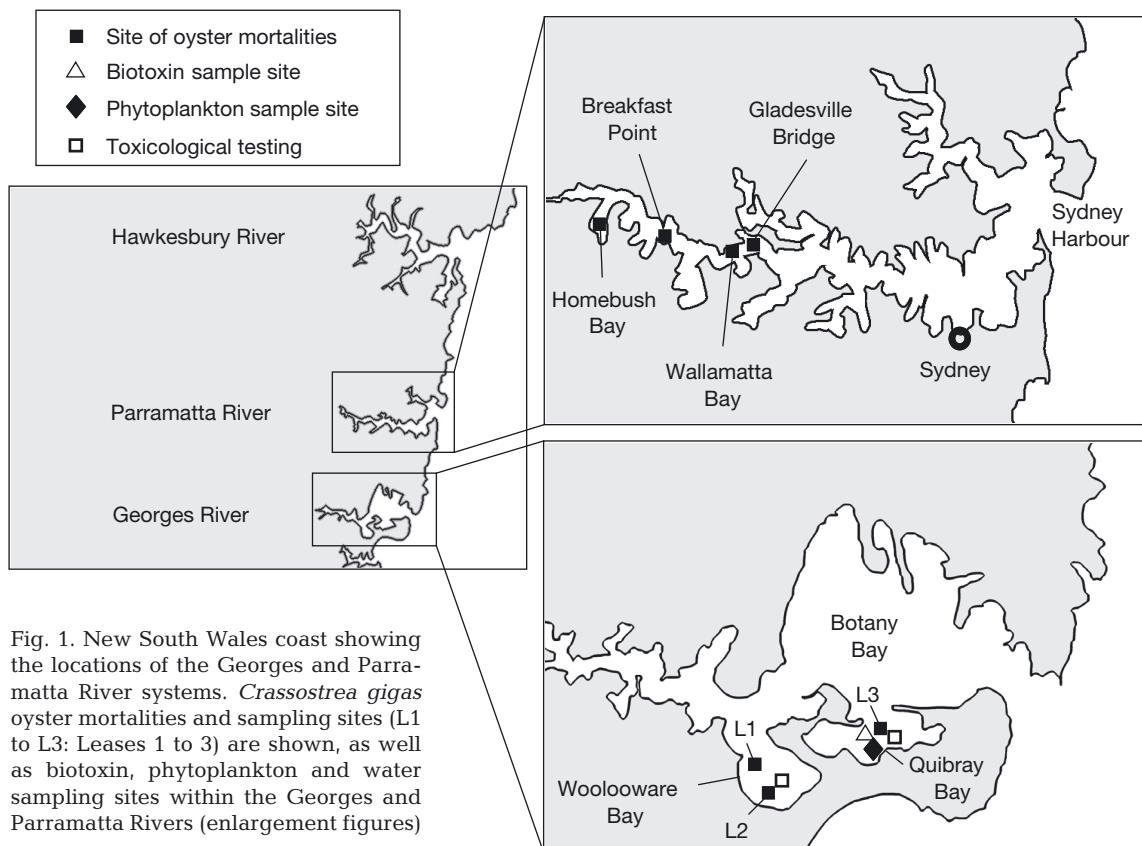


Fig. 1. New South Wales coast showing the locations of the Georges and Parramatta River systems. *Crassostrea gigas* oyster mortalities and sampling sites (L1 to L3: Leases 1 to 3) are shown, as well as biotoxin, phytoplankton and water sampling sites within the Georges and Parramatta Rivers (enlargement figures)

Table 1. Summary of chronology of disease and sample collection (*Crassostrea gigas*, *Saccostrea glomerata* and water)

Event	Sampling date	Location	Site	Samples collected	Tests performed
Mortalities (est. onset 12 Nov 2010)	24 Nov 2010	Woolooware Bay	Lease 1	12 × <i>C. gigas</i> triploid (adult, size 100 mm) 2 × <i>C. gigas</i> triploid (pooled spat <5 mm) <sup>a</sup> 12 × <i>S. glomerata</i> Water sample	OshHV-1 conventional and qPCR, histopathology, ISH, toxicology
Mortalities (est. onset 2 Dec 2010)	6 Dec 2010	Woolooware Bay	Lease 2	23 × <i>C. gigas</i> triploid (adult, size 100 mm) Water sample	OshHV-1 conventional and qPCR, histopathology, ISH, toxicology
Mortalities	22 Feb 2011	Parramatta River	First reported location	11 × <i>C. gigas</i> diploid, (adult, mixed sizes from small/medium to large, range 45–140 mm)	OshHV-1 qPCR <sup>a</sup>
	1 Mar 2011	Parramatta River	Four locations	20 <i>C. gigas</i> diploid (adult, size as above)	OshHV-1 qPCR <sup>a</sup>
Placement of sentinel <i>C. gigas</i> (triploid)	13 Jan 2011	Woolooware Bay Quibray Bay	Lease 2 Lease 3	7 <i>C. gigas</i> triploid (juvenile, size range 50–65 mm) 10 <i>C. gigas</i> triploid (juvenile, size as above)	OshHV-1 qPCR
6 Jan 2011	21 Jan 2011	Woolooware Bay Quibray Bay	Lease 2 Lease 3	12 <i>C. gigas</i> triploid (juvenile, size as above) 13 <i>C. gigas</i> triploid (juvenile, size as above)	OshHV-1 qPCR OshHV-1 qPCR
High level mortality 21 Jan 2011	No samples collected	Quibray Bay	Lease 3	No suitable tissue available	No testing conducted
Sampling of outbreak survivors	4 Feb 2011 1 Jun 2011	Woolooware Bay Woolooware Bay	Lease 1 Lease 1	10 <i>C. gigas</i> triploid (juvenile, size range 70–85 mm) 37 <i>C. gigas</i> triploid (adult, size range 90–110 mm)	OshHV-1 qPCR OshHV-1 qPCR

<sup>a</sup>Samples were of poor quality and were unsuitable for histopathology/ISH

### Collection of environmental data

Water samples were collected from Woolooware Bay and Quibray Bay on 2 separate occasions (Table 1) and were submitted to the Department of Environment, Climate Change and Water (DECCW) Environmental Forensics and Analytical Science Laboratories (EFAS) (Lidcombe, NSW) to test for potential pollutants, including organochlorine pesticides, organophosphate pesticides and metals. Water samples for the assessment of harmful phytoplankton species and oyster samples for shellfish biotoxin analyses were collected at regular intervals by a representative of the NSW Food Authority (Newington, NSW) during routine food safety testing in Quibray Bay, adjacent to Woolooware Bay. Shellfish biotoxins were analysed in presence/ absence assays and included domoic acid, saxitoxin and okadaic acid (amnesic, paralytic and diarrhetic shellfish toxin, respectively). Only limited water temperature data were available for Woolooware Bay in 2010; however, historical data collected from Woolooware Bay and the adjacent Quibray Bay are highly congruent, and therefore, where necessary, data from Quibray Bay was used. Daily rainfall data were obtained from the Australian Bureau of Meteorology (BOM) website (BOM 2010) for a station approximately 5 km distant from Woolooware Bay. Intermittent (approximately twice monthly) rainfall data were also collected from rain gauges in Quibray Bay during visits from food safety officers and were used to confirm the local accuracy of the BOM data. Salinity data were also collected at these times from Quibray Bay, and occasional readings (approximately monthly) were also taken from Woolooware Bay intake water.

### Sample processing

Upon receipt, the external surfaces of the oysters were scrubbed to remove excess mud and debris and the oysters were opened using a sterile shucking knife. Individual disposable scalpel blades were used

to sever the adductor muscles and to transfer the oysters to sterile petri dishes for dissection. Oysters were examined for any gross pathology, and transverse sections containing gill, mantle, gonad and digestive gland were taken for preservation in 100% ethanol (for molecular testing) and neutral-buffered formalin (for histological analysis). For histology, formalin-fixed tissues (24 to 48 h fixation) were embedded in paraffin, sectioned (5  $\mu$ m) and stained with haematoxylin and eosin. For molecular testing, approximately 1  $\times$  5 mg mantle and 1  $\times$  5 mg gill tissue were dissected from each oyster and pooled in a sterile microcentrifuge tube. Initial molecular diagnostic testing was performed using DNA extracted with the Qiagen DNeasy Blood and Tissue kit, according to the manufacturer's instructions. For subsequent high-throughput testing, tissue pools were digested by the addition of 180  $\mu$ l ATL buffer and 20  $\mu$ l of Proteinase K (Qiagen) with incubation at 56°C for 3 to 14 h. After digestion, the samples were mixed vigorously with a vortex mixer for 30 s and then centrifuged at 3000  $\times$  *g* for 5 min. Nucleic acids were purified from 25  $\mu$ l of the supernatant using the MagMax-96™ Viral RNA Isolation Kit (Applied Biosystems), according to the manufacturer's directions, with a Kingfisher-96 magnetic particle handling system (Thermo) using the AM1836 DW-standard program supplied for the MagMax-96™ kit (Applied Biosystems). Nucleic acids were eluted in 50  $\mu$ l of elution buffer.

### OsHV-1 conventional PCR

Assays for OsHV-1 were undertaken using 2 separate primer sets (Arzul et al. 2002). The first primer set (B3 and B2) targets an open reading frame of unknown function, while the second primer set (C2 and C6) targets 2 genes of unknown function separated by a microsatellite region (Arzul et al. 2001c, 2002). Reactions were prepared in a total volume of 50  $\mu$ l containing 1  $\times$  BioTaq buffer, 200  $\mu$ M dNTPs, 2.5 mM MgCl<sub>2</sub>, 400 nM of each primer, 1 U of BioTaq DNA polymerase (Bioline) and 4  $\mu$ l of DNA extract. Cycling parameters for both PCR assays involved a 2 min initial denaturation step at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. Reaction products were visualised after electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Products of the appropriate size (approximately 700 base pairs [bp]) were excised from the gel, and DNA

was recovered using the QIAquick Gel Extraction Kit (Qiagen). Nucleotide sequencing reactions were prepared in duplicate using the C2 and C6 primers and performed at the Australian Genome Research Facility using BigDye terminator Version 3.1 (Applied Biosystems).

### Sequence analysis

OsHV-1 sequences (C2/C6 region) derived from this study were aligned with representative strains from other outbreaks using ClustalW (Larkin et al. 2007). Genbank accession numbers of the sequences used in the alignment were as follows: New Zealand 2010 (JN800131), Japan 2010 (JN800133), USA 2007 (JN800128), Ireland 2009 (JN800129), China 2002 (JN800132), France 2010 Isolate 2 (JN800131), France 2010 Isolate 12 (JN800122), France 2010 Isolate 13 (JN800123), France 2008  $\mu$ -var (HQ842610), France 2005 Isolate 1 (JN800081), France 2003 Isolate 1 (JN800075), France 2003 Isolate 3 (JN800076), France 1995 Isolate 20 (JN800072), France OsHV-1 reference type (AY509253) and acute viral necrobiotic virus (AVNV; GQ153938). GeneDoc was used for alignment display (Nicholas et al. 1997). To establish the phylogeny of the Australian OsHV-1 strain, a maximum likelihood approach with 1000 bootstrap replications was employed within the Molecular Evolutionary Genetics Analysis package (MEGA V5.1) (Tamura et al. 2011).

### Quantitative PCR (qPCR) assays

Two assays with hydrolysis probe detection chemistry were used to detect targets in the B (Martenot et al. 2010) and C regions of the OsHV-1 genome (Table 2). Reactions were prepared in a 25  $\mu$ l volume using the AgPath-ID™ One-Step RT-PCR Kit (Life Technologies) with 200 nM primers, 100 nM probe and 5  $\mu$ l of purified nucleic acids. Thermocycling and fluorescent data acquisition were performed using an ABI 7500 Real-Time PCR machine (Applied Biosystems), with the following parameters: 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 45 s. The 6-carboxyfluorescein (FAM) fluorescence signal acquired at the end of the annealing/extension phase of each cycle was normalised to the signal from the ROX (6-Carboxyl-X-Rhodamine) passive reference dye and corrected for baseline fluorescence calculated according to a proprietary algorithm (ABI 7500 software, Applied Biosystems).

Table 2. PCR primers and probes used in this study

Assay name	Primer/ probe name	Sequence (5'-3')	Genome region	Source
<b>Conventional PCR</b>				
C2/C6	C2	CTC TTT ACC ATG AAG ATA CCC ACC	C region	Arzul et al. (2002)
	C6	GTC CAC GGC TTA CCA TTT TT		
B3/B2	B3	GTG GAG GTG GCT GTT GAA AT	B region	Arzul et al. (2002)
	B2	CAA CAG CTT TGG AGG TTG GT		
<b>Quantitative PCR</b>				
OsHV-1CR	OsHV-1CRF	CGT TTT ATC CAC CAC GAT TTT TAT T	C region	X. Gu et al. (unpubl.)
	OsHV-1CRR	TAC ATC AAA CCC ACT TTT CCT ATG AT		
	OsHV-1CR probe	FAM-CAC TCA TGA AAA CAC CGC TAA GAT CAC TGC-BHQ-1		
OsHV-1B	OsHV1BF	GTC GCA TCT TTG GAT TTA ACA A	B region	Martenot et al. (2010)
	B4	ACT GGG ATC CGA CTG ACA AC		
	B probe	FAM-TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC-BHQ-1		

Samples were considered negative if the ROX-normalised, baseline-corrected FAM fluorescence ( $\Delta R_n$ ) remained  $<0.05$ . A cycle-threshold (Ct) value was assigned for samples in which there was a logarithmic increase in fluorescence, defined as the cycle at which  $\Delta R_n$  first exceeded the fluorescence threshold of 0.05. Each test plate included the following control reactions: no template (nuclease-free water), extraction control (20 ng tRNA [transfer ribonucleic acid, from baker's yeast; Sigma] in nuclease-free water, included during nucleic acid purification) and positive control preparations. The latter were prepared from gill and mantle tissue digests, diluted 1:10 000 (v/v) in phosphate-buffered gelatin saline to give Ct values of approximately 29 and 32, and stored at  $-80^{\circ}\text{C}$  as single-use aliquots.

### Statistical analyses

DNA samples that were negative (undetermined Ct values) were excluded from the analysis and a D'Agostino and Pearson normality test was conducted on the Ct data prior to analysis. Mean Ct values derived from *Crassostrea gigas* and *Saccostrea glomerata* samples collected from Lease 1 were analysed using a 2-tailed *t*-test. One-way analysis of variation (ANOVA) employing Tukey's multiple comparison test was used to compare mean Ct values from triploid *C. gigas* collected during the Woolooware Bay outbreak, from surviving oysters collected 10 and 26 wk post-outbreak, from diploid *C. gigas* collected from the Parramatta River and from sentinel oysters collected 1 and 2 wk post-placement in Woolooware and Quibray Bays.

### *Vibrio harveyi* PCR

As some prior studies on OsHV-1 have suggested that there may be a relationship between severe mortality events associated with OsHV-1 and the presence of *Vibrio* spp., including *V. harveyi* (Segarra et al. 2010), we tested *Crassostrea gigas* and *Saccostrea glomerata* samples collected during the outbreak in Woolooware Bay together with *S. glomerata* samples that had been collected some weeks prior from the neighbouring Quibray Bay. A modification of a previously published assay targeting the ToxR gene (Pang et al. 2006) was used for the detection of *V. harveyi* in the Woolooware Bay oyster samples. Briefly, reactions contained 1× BioTaq buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.4 μM primers (ToxRF1: 5'-GAA GCA GCA CTC ACC GAT-3'; ToxRR1: 5'-GGT GAA GAC TCA TCA GCA-3'), 1 U of BioTaq polymerase (Bioline) and 2 μl of DNA template in a total volume of 25 μl. Cycling parameters were optimised for use on a BioRad Optimus thermal cycler and were as follows: 1 cycle of denaturation at  $94^{\circ}\text{C}$  for 3 min; 35 cycles of denaturation ( $94^{\circ}\text{C}$ , 1 min), annealing ( $63^{\circ}\text{C}$ , 1 min) and extension ( $72^{\circ}\text{C}$ , 1 min); and final extension at  $72^{\circ}\text{C}$  for 5 min. Total DNA extracted from an oyster sample positive for *V. harveyi* was used as a positive control template. PCR products (382 bp) were visualised after electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

### OsHV-1 probe generation and specificity

For *in situ* hybridisation (ISH) experiments, a DIG-labelled PCR probe was generated using the

B3 and B2 primers. PCR was performed as described above, except that dNTPs were replaced with DIG-labelling mix (Roche). Dot blot hybridisation was used to test the probe for cross-reactivity against purified *Crassostrea gigas* and *Saccostrea glomerata* DNA. Purified OsHV-1 PCR product generated using the B3/B2 primer set was used as a positive control. Of 2-fold serial dilutions of the DNA samples (0.15 to 20 pg  $\mu$ l $^{-1}$  for purified PCR product and 0.15 to 20 ng  $\mu$ l $^{-1}$  of purified oyster DNA) 20  $\mu$ l was boiled, chilled and then spotted onto a nylon membrane using a dot blot manifold. DNA was ultraviolet cross-linked to the membrane, prehybridised in DIG EasyHyb buffer (Roche) for 1 h at 42°C and hybridised at 42°C overnight in a roll tube. The hybridisation solution consisted of 12.5 ng ml $^{-1}$  pre-boiled probe diluted in DIG Easy-Hyb buffer. After hybridisation, the membrane was washed 2  $\times$  15 min with 2 $\times$  sodium citrate buffer (SSC)-0.1% sodium dodecyl sulphate (SDS), followed by 2  $\times$  15 min washes in 0.5 $\times$  SSC-0.1% SDS at 42°C. The membrane was then blocked for 30 min and reacted with alkaline phosphatase-conjugated anti-DIG diluted to 1:500 in blocking solution (Roche). The membrane was washed 2  $\times$  15 min with washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20) and detected with NBT-BCIP solution (Sigma Aldrich).

#### *In situ* hybridisation

Sections of oyster tissue approximately 5  $\mu$ m thick were cut from paraffin blocks, placed on Superfrost Plus slides (Menzel Gläser) and air dried. Slides were dewaxed in xylene and rehydrated in an ethanol series and then treated with 100  $\mu$ l of 10  $\mu$ g ml $^{-1}$  Proteinase K for 30 min at 37°C in a humid chamber. Sections were washed with Tris buffer (0.1 M, pH 8.0) for 3 min and prehybridised for 1 h at 37°C in 100  $\mu$ l prehybridisation buffer (50% formamide, 4 $\times$  SSC, 1 $\times$  Denhardt's solution, 0.25 mg yeast tRNA ml $^{-1}$ , 10% dextran sulfate). Prehybridisation buffer was exchanged for 100  $\mu$ l hybridisation solution (same as prehybridisation solution but with 5 ng  $\mu$ l $^{-1}$  of probe added), and slides were covered with hybridisation coverslips (ProSiTech) and heated to 95°C for 5 min on a dry heating block. Slides were cooled on ice for 5 min and then incubated overnight in a humid chamber in a Hoefer Red Roller hybridisation oven (Hoefer) at 42°C. The following day, slides were washed in wash buffer (Roche) at 40°C for 10 min.

Sections were then blocked with 500  $\mu$ l of DIG blocking solution (Roche) at room temperature (RT) for 30 min. Blocking solution was exchanged for a 1:200 solution of anti-DIG antibody (Roche) in blocking buffer, and sections were incubated at RT for 1 h. Excess antibody was removed with a 30 min wash in 1  $\times$  wash buffer (Roche) at RT. Sections were equilibrated for 5 min in detection buffer (Roche) and then incubated with 500  $\mu$ l BCIP/NBT in the dark at RT for 4 h. Following development, slides were rinsed twice in tap water and air dried.

## RESULTS

#### Field observations

Stocks of triploid *Crassostrea gigas* cultivated at 2 separate leases (Fig. 1) in Woolooware Bay, Georges River (NSW, Australia), as well as wild diploid *C. gigas* in the same estuary, experienced >95% mortality during the early summer (November and December) of 2010. Aside from the affected stock, there had been no introductions of triploid *C. gigas* to the Georges River from other sources for a period of 12 mo prior to the outbreak. The stock introduced to the estuary in November of 2010 was found to be negative for OsHV-1 on subsequent testing (authors' unpubl. data). Sentinel oysters did not experience any mortalities at their site of origin (Hawkesbury River) prior to translocation to the Georges River and were found to be negative for OsHV-1 during subsequent surveillance studies (B. Moloney et al. unpubl. data). Mortalities in the Georges River were observed in oysters with shell heights of 4 to 120 mm, comprising spat, juvenile and adult triploid *C. gigas*, while neighbouring *Saccostrea glomerata* appeared unaffected. Oysters at both leases had been in good health previously; however, just prior to the first observed mortalities (in November) at Lease 1, a plume of discoloured water and an algal bloom were observed in the vicinity of the affected oysters. Within 1 wk of the environmental disturbances, deaths were observed in *C. gigas* oyster stocks at Lease 1. *C. gigas* mortalities were also recorded in December at Lease 2 (Fig. 1) in Woolooware Bay. At this time, oyster movements were restricted to within the estuary. Macroscopic examination of these oysters revealed that the oyster shells and tissues were visibly coated in algae. The oysters were found to be moribund, with stiff and unresponsive tissues. The small proportion of *C. gigas* which survived the out-

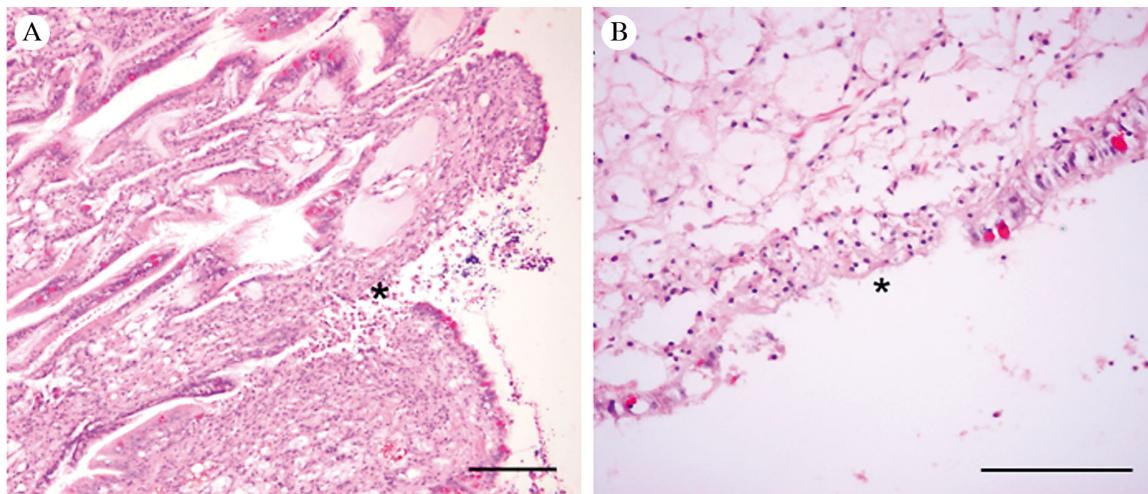


Fig. 2. *Crassostrea gigas*. Photomicrographs of haematoxylin and eosin-stained sections of surviving *C. gigas* specimens collected during the mortality events at (A) Lease 1 and (B) Lease 2, showing multifocal ulceration (stars) with focal underlying accumulations of haemocytes on palp (A) and gill (B) epithelium. Scale bars = 100 µm

break at Lease 2 had grown according to expectations when observed in June of 2011, approximately 6 mo after the start of the outbreak.

Approximately 50 naïve sentinel triploid *Crassostrea gigas*, placed at the site of the first outbreak (Lease 1) in Woolooware Bay and at Lease 3 in Quibray Bay on 6 January of 2011 experienced >50% mortality within 1 wk of translocation (Table 1). Approximately 2 wk post-placement, all of the sentinels had died. Subsequently, on 25 January 2011, a mortality approaching 100% was reported in the farmed *C. gigas* population in Quibray Bay. Due to advanced tissue degradation, no samples were collected for testing.

Mortalities in wild diploid *Crassostrea gigas* in the Parramatta River were first detected on 22 February 2011, but the time of onset of the outbreak was not known because of the absence of commercial production in this waterway. An anecdotal report from the public suggested a mortality event might have commenced up to 4 mo prior. Consequently, it was not possible to access archival environmental data relevant to the initial outbreak. Inspections at 4 sites, Gladesville Bridge, Wallamatta Bay, Breakfast Point and Homebush Bay, (Fig. 1) on 22 February 2011 indicated a mortality rate in *C. gigas* of between 50 and 100%. Surviving *C. gigas* were in poor condition. The presence of many empty shells and the previously mentioned anecdotal report suggest that the mortalities may have begun many weeks before samples were taken. There were no apparent mortalities in wild *Saccostrea glomerata* in these locations.

### Histopathology

In all oysters that were examined, there was nodular to diffuse infiltration of the leydig tissue with haemocytes, often concentrated around epithelia (gut, digestive gland and mantle), with diapedesis of haemocytes evident across digestive gland epithelia. Leydig tissue was granular, hypereosinophilic or collapsed in all oysters examined, with the severity varying from mild (*Saccostrea glomerata*) to moderate or severe (*Crassostrea gigas*). Examination of mantle, palp and gill epithelium (Fig. 2A) demonstrated multifocal erosive and focally extensive ulcerative lesions in *C. gigas* only. Remnant contiguous epithelium was often fragmented or degenerate, with pyknotic nuclei (Fig. 2B). Underlying haemocytic infiltrates were mild to moderate and consisted of scattered haemocytes with small round nuclei and minimal eosinophilic cytoplasm. These changes were not observed in examined specimens of *S. glomerata*. Cowdry Type A intranuclear inclusion bodies (marginated chromatin and central eosinophilic nuclear inclusions), sometimes associated with OsHV-1 infection (da Silva et al. 2008), were not observed in any oysters examined.

### Molecular detection of OsHV-1

All *Crassostrea gigas* oyster samples (up to 18 mo of age) collected from Woolooware Bay during the initial outbreak yielded amplicons in the 2 conventional PCR assays for OsHV-1 (Fig. 3A,B). Similarly, *Saccostrea glomerata* (12 samples) collected adjacent

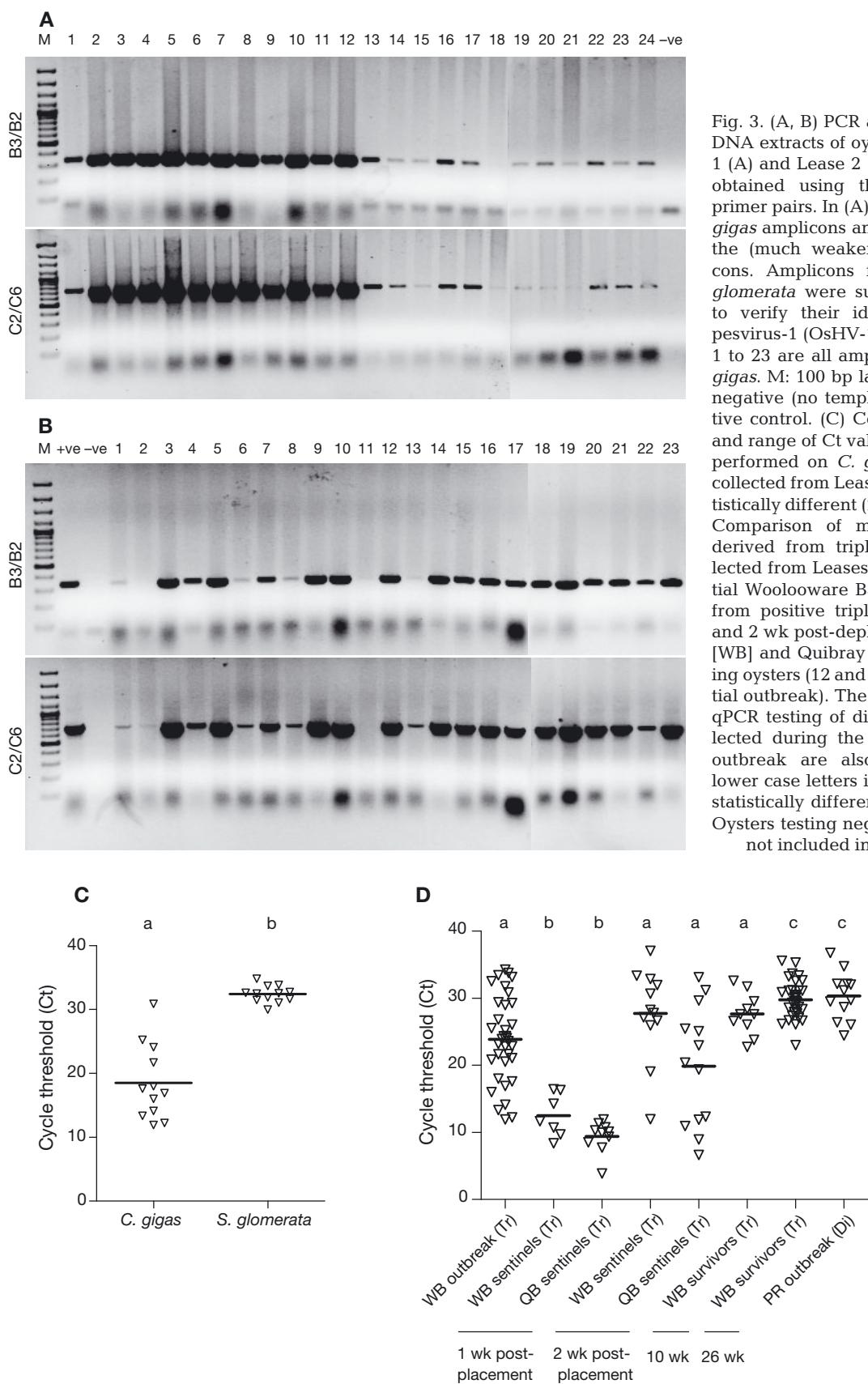


Fig. 3. (A, B) PCR amplicons derived from DNA extracts of oysters collected at Lease 1 (A) and Lease 2 (B) in Woolooware Bay, obtained using the B3/B2 and C2/C6 primer pairs. In (A), Samples 1 to 12 are *C. gigas* amplicons and Samples 13 to 24 are the (much weaker) *S. glomerata* amplicons. Amplicons from *C. gigas* and *S. glomerata* were subsequently sequenced to verify their identity as ostreid herpesvirus-1 (OsHV-1) DNA. In (B), Samples 1 to 23 are all amplicons derived from *C. gigas*. M: 100 bp ladder (Fermentas); -ve: negative (no template) control; +ve: positive control. (C) Comparison of the mean and range of Ct values derived from qPCR performed on *C. gigas* and *S. glomerata* collected from Lease 1. The means are statistically different (2-tailed,  $p < 0.0001$ ). (D) Comparison of mean qPCR Ct values derived from triploid (Tr) *C. gigas* collected from Leases 1 and 2 during the initial Woolooware Bay outbreak with those from positive triploid sentinel oysters (1 and 2 wk post-deployment in Woolooware [WB] and Quibray Bays [QB]) and surviving oysters (12 and 26 wk following the initial outbreak). The Ct values derived from qPCR testing of diploid (Di) *C. gigas* collected during the Parramatta River (PR) outbreak are also compared. Different lower case letters indicate groups that are statistically different at the  $p < 0.01$  level. Oysters testing negative for OsHV-1 were not included in statistical analyses

to the *C. gigas* were positive in both PCR assays, but discernibly less DNA was amplified from these samples (Fig. 3A, Lanes 13 to 24).

Ct values derived from the qPCR assays were analysed statistically. Data within most groups passed the normality test ( $p > 0.05$ ), indicating that the data did not deviate significantly from a normal distribution. The only exception was the data from the 1 wk post-placement sentinels from Quibray Bay; however, inclusion of this group in subsequent ANOVA analysis was deemed valid given that the group had a low sample number (potentially explaining the lack of normality) and given that ANOVA is considered robust regardless of normality (Schmider et al. 2010). A 2-tailed *t*-test indicated that the Ct values were statistically much lower (quantity of OsHV-1 DNA was higher) in *Crassostrea gigas* compared to the *Saccostrea glomerata* collected from Woolooware Bay ( $p < 0.0001$ ; Fig. 3C). Although there was no tissue suitable for testing when 100% mortality was detected in the farmed *C. gigas*, the presence of OsHV-1 in Quibray Bay was confirmed in sentinel oysters tested in the preceding weeks. The qPCR results showed high levels of OsHV-1 in sentinel oysters within 1 wk of placement in both Quibray and Woolooware Bays. The viral load in sentinel oysters from both locations was significantly higher after 1 wk of exposure than after 2 wk of exposure ( $p < 0.01$ ) and was also significantly higher than in oysters sampled during the initial outbreak in Woolooware Bay ( $p < 0.01$ ; Fig. 3D). Conversely, the mean Ct of sentinel oysters sampled after 2 wk of exposure was not statistically different from oysters sampled during the initial outbreak ( $p > 0.05$ ; Fig. 3D). Triploid *C. gigas* survivors from Woolooware Bay had a high prevalence of infection 12 wk (10/10 positive) and 26 wk (35/37 positive) after the mortality event, but the viral load was significantly lower than in samples collected during the outbreak (Fig. 3D;  $p < 0.01$ ). OsHV-1 was detected in only 39% of adult diploid *C. gigas* sampled during the Parramatta River outbreak and, therefore, was much less prevalent in these oysters than in triploid *C. gigas* sampled from the Georges River. Indeed, only 2 oysters from a total of 124 *C. gigas* tested from the Georges River (1.6%) were negative for OsHV-1. A comparison of the mean Ct values of samples from the Parramatta River outbreak indicated that statistically lower quantities of OsHV-1 were present relative to oysters from the Woolooware Bay outbreak ( $p < 0.01$ ). The mean viral load in the Parramatta River samples was statistically indistinguishable from that found in the 26 wk survivors from Woolooware Bay ( $p > 0.05$ ; Fig. 3D).

## Sequence analysis

OsHV-1 sequences (C2/C6 region) derived from infected *Crassostrea gigas* collected from Woolooware Bay (Leases 1 and 2) and the Parramatta River, as well as a further sequence derived from OsHV-1 PCR positive *Saccostrea glomerata* (collected at Lease 1), were identical. When aligned with sequences of OsHV-1 strains derived from different temporal and geographical origins, the Australian sequence (GenBank Accession Number KC685525) was found to contain a deletion in the microsatellite region similar to the microvariant of OsHV-1 (Segarra et al. 2010) (Fig. 4A). However, there were also some nucleotide differences between the 2 sequences (data not shown). A maximum-likelihood analysis of the OsHV-1 C2/C6 region revealed 2 major phylogenetic clusters defined by the reference strain of OsHV-1 (GenBank Accession Number AY509253) and the OsHV-1 microvariant (GenBank Accession Number HQ842610) (Fig. 4B). The Australian OsHV-1 clustered within the microvariant clade and was most similar to samples sequenced from outbreaks in 2010 in New Zealand, Japan and France. As noted previously (Renault et al. 2012), the majority of sequences within the microvariant cluster are derived from samples collected from 2008 onwards. One possible exception to this is an Irish variant (GenBank Accession Number JQ963169) which was identified in *C. gigas* adults experiencing mortalities in 2003 and 2004. This sequence is not shown in Fig. 4B due to the fact that only a short sequence was available for phylogenetic analysis; however, alignment of the existing sequence indicated that this variant contained only one nucleotide substitution relative to the microvariant (GenBank Accession Number HQ842610). A further exception is the sequence derived from an OsHV-1 outbreak in China in 2002, which also clusters within the microvariant group and, according to Fig. 4B, most closely resembles the ancestral sequence for this cluster. This finding is in contrast to previous results (Renault et al. 2012) which suggested that the 2002 sample from China falls within the OsHV-1 clade containing the reference strain. The sequence of the microsatellite region of this sample also suggests that it belongs to the microvariant cluster (Fig. 4A).

## In situ hybridization

A dot blot performed on DNA extracts from uninfected *Crassostrea gigas* and *Saccostrea glomerata* samples and purified OsHV-1 PCR product demon-

strated that the OsHV-1 probe did not cross-react with oyster tissue, but was able to detect target DNA down to approximately 6 pg (Fig. 5A). ISH was performed on selected *C. gigas* and *S. glomerata* samples to determine whether OsHV-1 was present within the oyster samples collected from Woolooware Bay. No staining was detectable in *S. glomerata* (Fig. 5B); however, in *C. gigas* samples, the OsHV-1 probe stained regions within a variety of different tissues, indicating that the oysters were actively infected with OsHV-1. Staining within the gonadal tissue was noted in a number of infected *C. gigas* samples (Fig. 5C). In some samples, staining was most prominent in what appeared to be haemocytes within the connective tissue surrounding the ova (Fig. 5D—no probe added; Fig. 5E—probe added), but not within the ova themselves, while in other samples staining was confined to the ova (Fig. 5F—no probe added; Fig. 5G—probe added).

#### Detection of *Vibrio harveyi* in OsHV-1 infected oysters

Results of the *Vibrio harveyi* PCR assay indicate that *V. harveyi* was present in 10/12 and 23/23 of the *Crassostrea gigas* and 12/12 of the *Saccostrea glomerata* sampled in late November and early December during the Woolooware Bay outbreak. In contrast, *V. harveyi* was not detectable in *S. glomerata* samples collected from Quibray Bay in either October (0/30) or early November (0/30) of 2010 prior to the outbreak (Table 3).

#### Environmental data

Around the time of the initial *Crassostrea gigas* mortalities in Woolooware Bay, a plume of discoloured water and an algal bloom had been observed in the vicinity of the affected leases. Rainfall data confirmed that there had been heavy rainfall approximately 3 d prior to the onset of both mortality events (Fig. 6A). Water collected for toxicological analyses revealed that organic compounds and metals were below the level of detection except for calcium, iron and magnesium which were typical of that environment and unlikely to be acutely toxic. Historical data from Woolooware and Quibray Bays indicate that these sites are of similar salinity and that the salinity is generally high (33 to 35 ppt) decreasing to 30–32 ppt following rainfall (data not shown). The influx of freshwater from rainfall in early November 2010, just prior to the mortalities, resulted in a modest decrease in the salinity of the water at Quibray Bay to 30.9 ppt (Fig. 6B). A salinity reading from the intake water at Woolooware Bay at this same time point indicated that salinity in this bay was

logical analyses revealed that organic compounds and metals were below the level of detection except for calcium, iron and magnesium which were typical of that environment and unlikely to be acutely toxic. Historical data from Woolooware and Quibray Bays indicate that these sites are of similar salinity and that the salinity is generally high (33 to 35 ppt) decreasing to 30–32 ppt following rainfall (data not shown). The influx of freshwater from rainfall in early November 2010, just prior to the mortalities, resulted in a modest decrease in the salinity of the water at Quibray Bay to 30.9 ppt (Fig. 6B). A salinity reading from the intake water at Woolooware Bay at this same time point indicated that salinity in this bay was

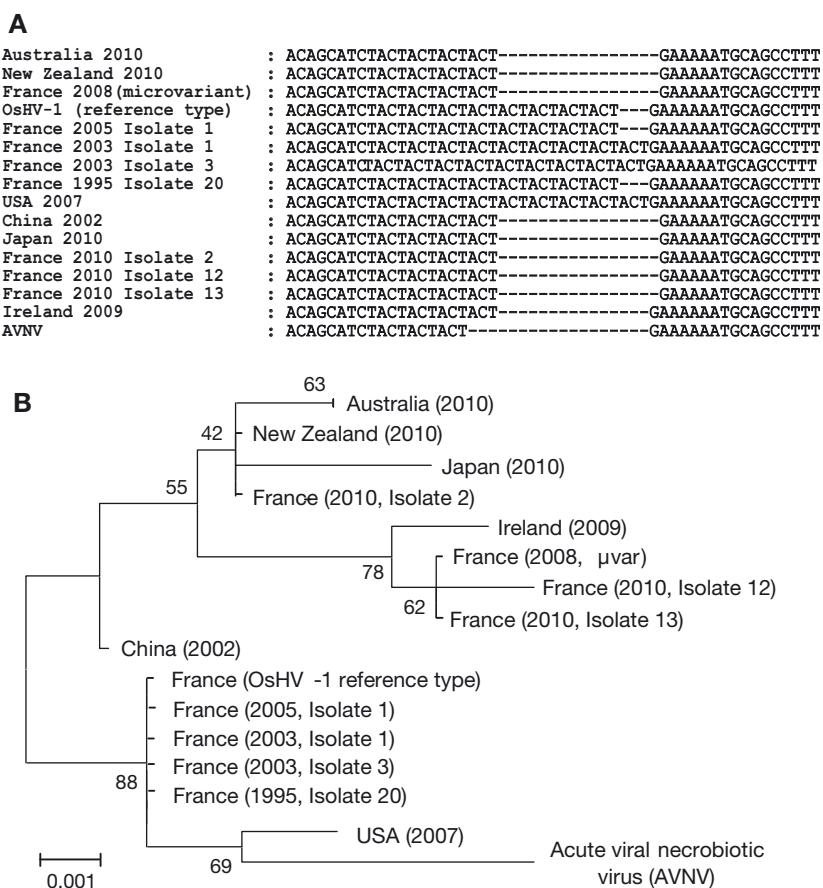
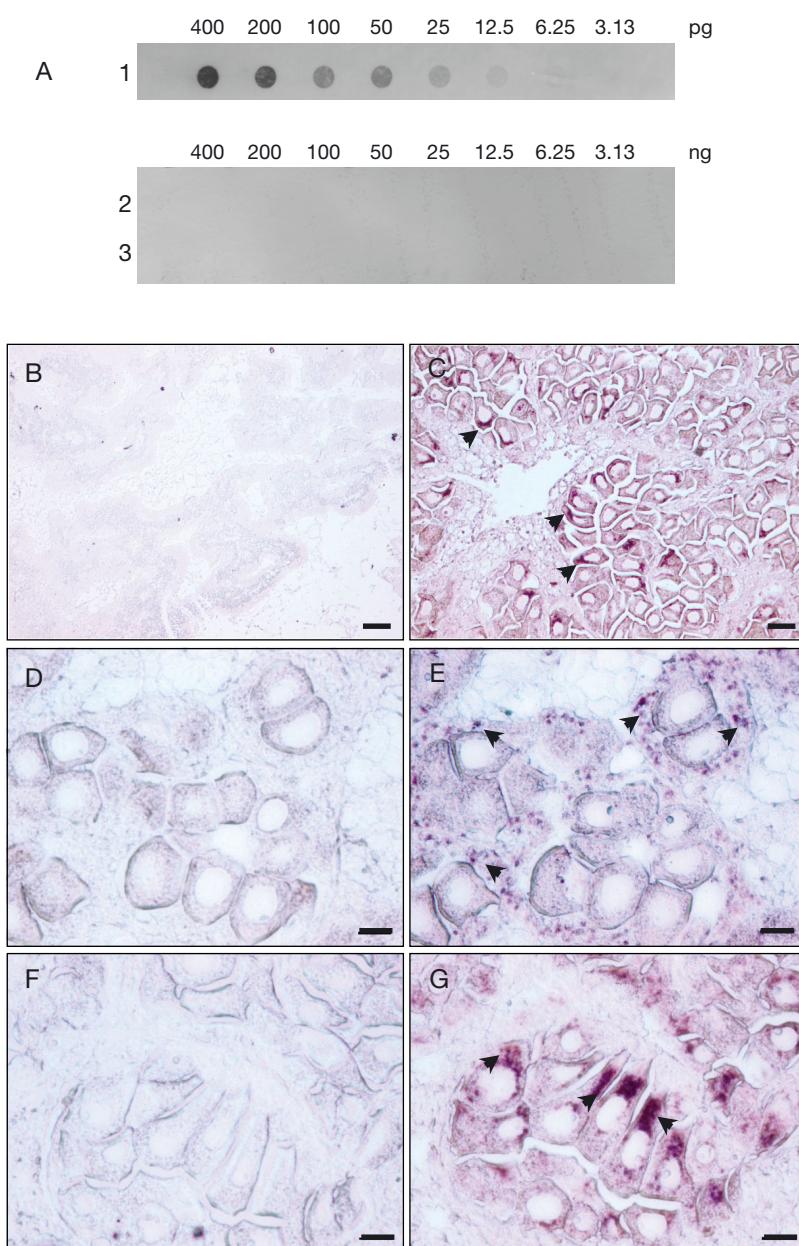


Fig. 4. (A) Alignment of the microsatellite region (within the C2/C6 sequence) of the Australian OsHV-1 sequence with the same sequence region from other strains of OsHV-1. The Australian strain contains a large deletion in the microsatellite region that is identical to the one seen in the  $\mu$ -var strain and other OsHV-1 strains responsible for outbreaks in 2010. (B) Phylogenetic tree constructed using the Tamura-Nei maximum-likelihood method and the C2/C6 sequences of various representatives of OsHV-1, including the Australian strain. A total of 622 nucleotide positions were used in the analysis. The Australian OsHV-1 sequence was identical in 3 individual specimens examined; therefore the branches were collapsed into a single node. The Australian strain clusters within the  $\mu$ -var group rather than the clade containing the OsHV-1 reference type



**Fig. 5.** *Crassostrea gigas*, *Saccostrea glomerata*. (A) Specificity and sensitivity of the B3/B2 OsHV-1 DIG-labelled probe tested in a dot blot against OsHV-1 PCR product (Row 1) and total DNA extracted from uninfected *C. gigas* and *S. glomerata* (Rows 2 and 3). The probe detected ~6 pg of viral DNA, but did not cross-react with either *C. gigas* or *S. glomerata* DNA. (B–G) ISH using the OsHV-1 DIG-labelled probe did not detect any virus within *S. glomerata* tissues (B), but *C. gigas* samples that were PCR positive for OsHV-1 stained strongly (C, arrowheads). No staining was observed in infected *C. gigas* oysters that received no probe (D,F), while strong staining was observed in serial sections from the same oysters that were reacted with the OsHV-1 probe (E,G). Staining (arrowheads) was largely observed within the gonadal tissue either within haemocytes (E) or within ova (G). Scale bars = (B–C) 50 µm, (D–G) 20 µm

identical to that of Quibray Bay (30.9 ppt, data not shown).

Water temperature data collected from the neighbouring Quibray Bay at the shellfish biotoxin sampling sites (Fig. 1) indicated that the water temperature was 22 to 23°C at the time of the outbreaks (Fig. 6C). The increase in water temperature from 18°C in early November to 22°C in mid-November also coincided with an increase in the detection of *Vibrio harveyi* in oyster samples collected from the Georges River (Fig. 6C, Table 3).

Counts of harmful phytoplankton species present in the area indicated that toxic diatoms and dinoflagellates bloomed during the early November to early December period. These blooms likely occurred due to a combination of nutrient run-off following the heavy rain in early November and warmer water temperatures. Diatoms belonging to various domoic acid (amnesic shellfish toxin)-producing *Pseudo-nitzschia* spp. and dinoflagellates of the saxitoxin (paralytic shellfish toxin, PST)-producing *Alexandrium fundyense/tamarensis/catanella* species complex were present in significant numbers at this time (Fig. 6D). Dinoflagellates of the *Prorocentrum* genus, which produce diarrhetic shellfish toxin (okadaic acid), were also detected during the mid-November to early December period, but were not present in high numbers (data not shown). Shellfish sampled on 3 separate occasions during the early November to early December period (but not at other times during 2010) returned positive results for the presence PST (Fig. 6D). Despite the presence of a *Pseudo-nitzschia* bloom and the detection of *Prorocentrum* spp. neither domoic acid nor okadaic acid were detected within shellfish during this period.

In early January 2011, at the time of placement of the first sentinels and the outbreak in Quibray Bay, rainfall

Table 3. *Crassostrea gigas*, *Saccostrea glomerata*. Percentage of Georges River oysters testing positive for *Vibrio harveyi*. NS: not sampled

Date sampled	<i>C. gigas</i> (% positive)	<i>S. glomerata</i> (% positive)
12 Oct 2010	NS	0
9 Nov 2010	NS	0
24 Nov 2010	83	100
6 Dec 10	100	NS

was recorded, but water quality was not greatly affected and the levels of toxic diatoms and dinoflagellates were low during this period. Biotoxin analyses were not performed at this time, but no biotoxin was detected in the preceding 3 shellfish samplings (Fig. 6D).

## DISCUSSION

Studies conducted during the 1990s demonstrated the presence of herpesvirus-like particles in *Ostrea angasi* (flat oyster) (Hine & Thorne 1997) in Australia and in *Crassostrea gigas* (Hine et al. 1992) and *Ostrea chilensis* (Hine et al. 1998) in New Zealand; however, molecular methods for the detection of OsHV-1 were not used and the identity of these herpesvirus-like particles was not established. In this study we have reported on the first confirmed outbreak of disease due to OsHV-1 infection in *C. gigas* in Australia. This strain of OsHV-1 caused mass mortality of *C. gigas* spat, juvenile and adult oysters (>95%) in Woolooware Bay, Georges River in November and December of 2010 and then in the neighbouring Quibray Bay in January 2011. Sentinel *C. gigas* oysters placed in Woolooware Bay about 7 wk after the start of the outbreak became infected and experienced approximately 50% mortality within 1 wk of exposure, indicating the persistence of the virus in the environment.

The high mortality rate observed in the Georges River oysters corresponds with the high viral load detected by PCR and is consistent with prior reports of OsHV-1 outbreaks in *Crassostrea gigas* juveniles in France (Renault et al. 1994, Segarra et al. 2010), California (Friedman et al. 2005) and Mexico (Vásquez-Yeomans et al. 2010). The Woolooware Bay mortalities coincided with reports of significant losses of *C. gigas* oyster spat in New Zealand, reported in November and December of 2010 (but perhaps commencing as early as March; Interna-

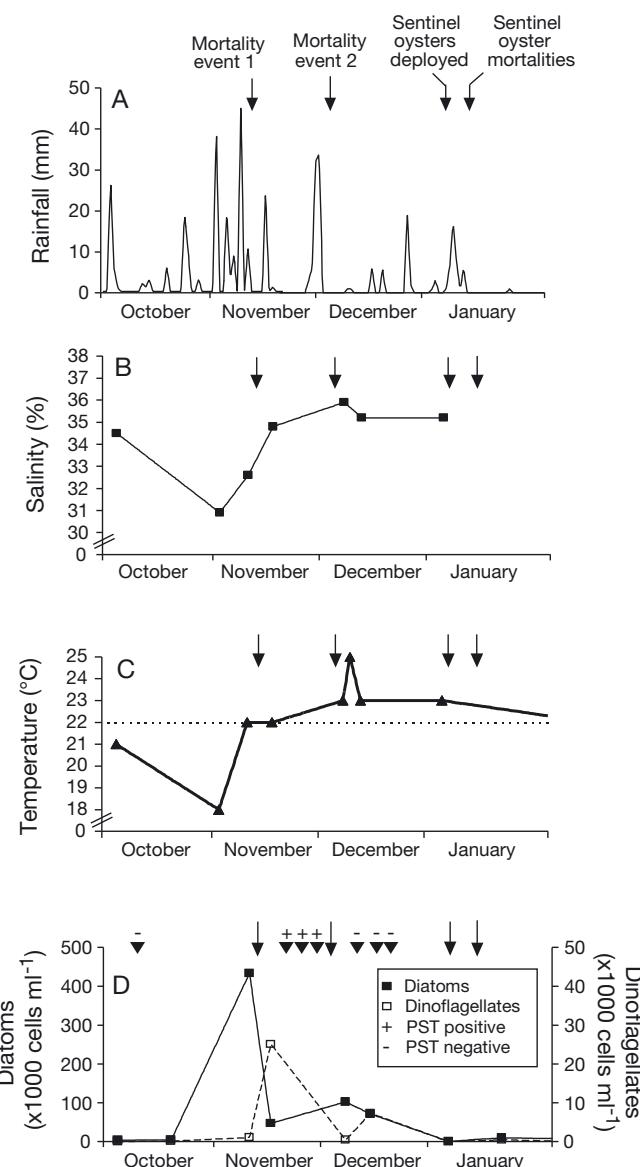


Fig. 6. Summary of environmental data collected during the OsHV-1 outbreaks and the deployment of naïve sentinel oysters in Woolooware Bay. (A) Rainfall recorded in the area from October 2010 to January 2011 indicates that both OsHV-1 outbreaks in Woolooware Bay were preceded by heavy rainfall events. (B) There was sufficient rainfall in early November to have a small impact on the salinity of both Quibray Bay and the intake water at Woolooware Bay (not shown but salinity readings at both sites were identical). (C) Water temperatures during the 2 outbreaks and during the deployment of sentinels were above the optimal temperature for OsHV-1 replication ( $22^\circ\text{C}$ ). (D) Toxic phytoplankton consisting of *Pseudo-nitzschia* diatoms and *Alexandrium* dinoflagellates bloomed during the period immediately preceding the OsHV-1 outbreaks. Oysters collected from the neighbouring Quibray Bay that were tested for the presence of biotoxin (arrowheads) returned positive results for paralytic shellfish toxin (PST) on 3 separate occasions during late November and early December 2010, coinciding with the OsHV-1 related oyster mortalities

tional OsHV-1  $\mu$ -var Workshop 2011) where elevated water temperatures were recorded and subsequent molecular testing also detected the presence of OsHV-1 (Renault et al. 2012).

Phylogenetic analysis indicated that the Australian OsHV-1 is closely related to the strain derived from the New Zealand oysters, as well as 2 other strains causing outbreaks in France and Japan during 2010 (Renault et al. 2012, Shimahara et al. 2012). All of these strains belong to the same cluster as the microvariants of OsHV-1, which have been associated with severe mortalities in *Crassostrea gigas* since the first description in France in 2008 (Segarra et al. 2010). The origin of the Australian virus remains unknown, though it is strongly suspected that it was unintentionally introduced to Australia. It is perhaps of significance that the site of the first recognised cases in Woolooware Bay is approximately 5 km upstream from one of Australia's largest shipping container terminals/ports. However, it should be noted that the farmed *C. gigas* population in Quibray Bay did not succumb to disease until about 8 wk after the outbreak was first identified in Woolooware Bay, even though it was nearby and downstream of the affected populations. This may be partially explained by sand shoaling (evident in aerial photographs), which occurs at the entrance to Quibray Bay, thus reducing tidal exchange between the bay and the rest of the estuary.

Close examination of available environmental data collected prior to, during and after the Georges River outbreaks revealed that mortalities at all leases occurred while the water temperature was at or above 22°C. This temperature is above the minimum shown to be suitable for OsHV-1 replication (16°C), or required for oyster herpesvirus transmission, in studies performed in other countries (Hine et al. 1998, Arzul et al. 2001b, Schikorski et al. 2011a,b, Pernet et al. 2012). Indeed, OsHV-1 outbreaks have now been observed in the Georges River for 3 seasons, on each occasion once the water temperature rose above 22°C (authors' unpubl. observations). This may represent an optimum temperature for rapid transmission and widespread disease occurrence, or may be related to other factors associated with disease expression. Alternatively, disease expression above 22°C may reflect local adaptation of *Crassostrea gigas* to higher water temperatures. The effect of this temperature tolerance range on the oyster immune system may be a key driver of disease susceptibility in *C. gigas*. Elevated temperatures have also been associated with increased populations of pathogenic *Vibrio* spp. (Motes et al. 1998,

Zimmerman et al. 2007, Pernet et al. 2012, Zhou et al. 2012), which is consistent with findings from this study demonstrating an increased incidence of *V. harveyi* in oysters collected from Quibray Bay when the water temperature was 22 to 23°C, compared to oysters collected in early November when the water was at 18°C.

The initial outbreaks at Leases 1 and 2 in Woolooware Bay were both preceded by heavy rainfall. The effect of rainfall on OsHV-1 transmission has not been investigated; however, rainwater run-off is a known stressor for oysters due to the influx of freshwater and a corresponding decrease in salinity (Levinton et al. 2011). Historical records indicate that both Woolooware and Quibray Bays are high-salinity areas. Due to their proximity to the confluence of Botany Bay and the Tasman Sea, the salinity at these sites rarely drops below 30 ppt. Indeed, the salinity in Woolooware Bay decreased slightly during the rainfall event in early November, just prior to the first outbreak, but nonetheless remained above 30 ppt. Furthermore, salinity did not appear to fluctuate significantly prior to subsequent mortalities; therefore, it is unclear whether salinity has a direct effect on oyster susceptibility to OsHV-1 or whether other parameters associated with rainfall, such as silting or increased nutrient loads, are more likely related to OsHV-1 outbreaks.

Both higher water temperatures and heavy rainfall are known triggers for the bloom of toxic diatoms and dinoflagellates (Hallegraeff 1992), with rainfall causing the discharge of organic and inorganic nitrogenous and phosphorus-containing compounds into the water column (Anderson et al. 2012). Indeed, during late November and early December of 2010, direct cell counts indicated that blooms of toxic phytoplankton were occurring in Quibray Bay, and biotoxin analyses confirmed that PST was present in oysters collected from this location. Observations of a plume of discoloured water and an algal bloom suggest that increased phytoplankton levels arising from nutrient run-off were also present in the neighbouring Woolooware Bay at the time of the OsHV-1 outbreak. Significant rainfall also occurred between sentinel oyster placement in Woolooware and Quibray Bays and subsequent mortalities associated with OsHV-1 (Fig. 6A). The water temperature at this time was above 22°C; however, toxic phytoplankton were not detected during this period. Interestingly, in addition to the potential to produce biotoxins, phytoplankton are also known to harbour AVNV, a variant of OsHV-1, and it has been proposed that they could act as physical carriers of the virus through the environ-

ment (Wang et al. 2011). It is possible, therefore, that phytoplankton blooms in the Georges River exacerbated virus spread.

While field observations and experimental transmission studies have demonstrated that the presence of herpesvirus and a water temperature  $>16^{\circ}\text{C}$  are sufficient for viral transmission and disease induction (Hine et al. 1998, Pernet et al. 2012), other stress factors are believed to play a role during severe mortality events in *Crassostrea gigas*, often referred to as 'summer mortality' (Gagnaire et al. 2006, Sauvage et al. 2009). These stressors include toxins, biotoxins from certain species of phytoplankton (Sauvage et al. 2009, Botner et al. 2010), pathogenic *Vibrio* spp. (Lacoste et al. 2001, Le Roux et al. 2002, Li et al. 2009, Renault et al. 2009, Botner et al. 2010, Pernet et al. 2012) and host metabolic factors related to spawning (Gagnaire et al. 2006, Li et al. 2009). Given that both a bloom of toxic phytoplankton and *V. harveyi* were demonstrated as being present during the OsHV-1 outbreak in Woolooware Bay, it is possible that they contributed to disease severity by compromising the host. The detection of *V. harveyi* in OsHV-1-infected oysters may also reflect opportunistic infection by this pathogen. These factors may also explain the pathology observed in the *Saccostrea glomerata* specimens. Indeed, the histological changes associated with the presence of PST-producing *Alexandrium* spp., which include haemocyte accumulation in the connective tissue surrounding the gut and digestive tubules (Haberkorn et al. 2010b), were also observed in the Woolooware Bay oysters.

ISH revealed that in triploid oysters from Woolooware Bay most of the viral DNA was located either within circulating haemocytes (particularly within the gonadal tissue) or within the gametes themselves. The presence of OsHV-1 DNA and proteins within the gonadal tissue of asymptomatic oysters has been reported previously (Arzul et al. 2002). When combined with the detection of viral DNA in 2 d old larvae (Barbosa-Solomieu et al. 2005), these data suggest that vertical transmission of this virus may occur. While triploid *Crassostrea gigas* are considered functionally sterile, diploid *C. gigas* were also infected by OsHV-1 in this study. Unfortunately, the tissues collected from diploid *C. gigas* in this study were of poor quality and not suitable for histological analysis; however, it would be of interest to determine whether OsHV-1 localises within the gametes in diploids as it does in triploids, as spawning could represent an important virus dispersal mechanism. Indeed, OsHV-1 disease dynamics in diploid versus triploid oysters warrants further investi-

tigation given that gonadal maturation in diploids is known to result in reduced immune parameters relative to triploids (Gagnaire et al. 2006). Conversely, higher metabolic and haemocyte (phagocytic) activity in triploids could theoretically assist viral trafficking within host tissues. Triploidy in *C. gigas* has also been shown to result in increased PST accumulation (Haberkorn et al. 2010a), which may be relevant in cases where toxic phytoplankton blooms coincide with OsHV-1 mortalities.

The mechanisms for persistence of OsHV-1 in a population are not well known. However, even though  $>99\%$  of the farmed *Crassostrea gigas* population had died, it is notable that, of the small proportion of surviving oysters, viral DNA was detected in almost all that were tested 12 and 26 wk after the onset of the outbreak. It is not known whether this DNA represents infectious virus, but it might indicate a source of infection for other oysters.

While molluscan herpesviruses are known to have a broad host range, affecting a number of oyster species (Arzul et al. 2001c, da Silva et al. 2008), as well as clams (Arzul et al. 2001c) and scallops (Arzul et al. 2001b), we noted that *Saccostrea glomerata* cultivated adjacent to *Crassostrea gigas* in Woolooware Bay, as well as wild populations of this species in the Parramatta River, did not experience mortalities during OsHV-1 outbreaks and subsequently grew normally. While OsHV-1 DNA was initially detected in samples collected from *S. glomerata* by gel-based PCR, qPCR analysis indicated that the viral load was significantly lower than that in the *C. gigas* samples ( $p < 0.0001$ ). Furthermore, ISH failed to detect any viral DNA within *S. glomerata* tissues, suggesting that *S. glomerata* is not susceptible to infection by OsHV-1. The PCR amplicons derived from *S. glomerata* samples collected during the outbreak at Lease 1 were likely to have originated from viral DNA in the water that surrounded (or had been filtered through) the oyster. Examination of further *S. glomerata* specimens by qPCR in situations where there are high viral loads in *C. gigas* have failed to detect any OsHV-1 DNA (authors' unpubl. data).

Despite the fact that *Saccostrea glomerata* appears to be unaffected by OsHV-1, histological lesions and haemocyte infiltrates were observed in both *Crassostrea gigas* and *S. glomerata* from the Georges River. It is known that *S. glomerata* farmed in the Georges River are affected by both QX disease and the winter mortality syndrome (Roughley 1926, Wolf 1972). However, neither QX sporonts nor microcell parasites were observed. Lesions in *C. gigas* were generally more severe than those observed in *S.*

*glomerata*, and, indeed, focal ulcerations were only observed in the *C. gigas* samples and may therefore be attributable specifically to OsHV-1. Histologic changes consistent in both oyster species were non-specific and indicate underlying inanition and poor health, suggesting that factors other than infection with OsHV-1 contributed to this pathology.

At the time of the disease outbreaks, freedom of the rest of the Australian *Crassostrea gigas* and the *Sacostrea glomerata* populations was supported by a structured virological survey to be described elsewhere (B. Moloney et al. unpubl. data). There was continuing freedom from OsHV-1 disease outside of the Georges and Parramatta River systems for a further 2 yr. However, just prior to submission of this manuscript, on 21 January 2013, a large-scale mortality event due to OsHV-1 was detected in farmed *C. gigas* in the Hawkesbury River. This estuary is approximately 35 km north of the Parramatta River and is the nearest population of farmed oysters to the original outbreaks.

**Acknowledgements.** We thank Dave Barker and Bob Drake for their assistance in providing samples and allowing access to their oyster leases. Anthony Zammit from the Food Authority is gratefully acknowledged for making the phytoplankton, biotoxin and water temperature data available. We are also grateful to Tony Roach and Moreno Julli for conducting the toxicological analyses, and to Damian Collins for assistance in interpreting the statistical analysis. The expertise of technical staff at EMAI in preparing specimens for histopathology and in undertaking the qPCR assays, and of NSW DPI field staff in assisting with sample collection is also gratefully acknowledged.

#### LITERATURE CITED

- Anderson DM, Cembella AD, Hallegraeff GM (2012) Progress in understanding harmful algal blooms: paradigm shifts and new technologies for research, monitoring, and management. *Ann Rev Mar Sci* 4:143–176
- 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 (2001b) Experimental herpes-like viral infections in marine bivalves: demonstration of interspecies transmission. *Dis Aquat Org* 46:1–6
- Arzul I, Renault T, Lipart C, Davison AJ (2001c) 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
- Barbosa-Solomieu V, Degremont L, Vazquez-Juarez R, Ascencio-Valle F, Boudry P, Renault T (2005) Ostreid herpesvirus 1 (OsHV-1) detection among three successive generations of Pacific oysters (*Crassostrea gigas*). *Virus Res* 107:47–56
- BOM (Australian Bureau of Meteorology) (2010) Weather and climate. Available at [www.bom.gov.au/climate/data/](http://www.bom.gov.au/climate/data/) (accessed 5 March 2013)
- Botner A, Broom D, Doherr MG, Domingo M and others (2010) EFSA panel on animal health and welfare: scientific opinion on the increased mortality events in Pacific oysters, *Crassostrea gigas*. *EFSA Journal* 8:1894
- Burge CA, Griffin FJ, Friedman CS (2006) Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. *Dis Aquat Org* 72: 31–43
- Burge CA, Strenge RE, Friedman CS (2011) Detection of the oyster herpesvirus in commercial bivalve in northern California, USA: conventional and quantitative PCR. *Dis Aquat Org* 94:107–116
- Cotter E, Malham SK, O'Keefe S, Lynch SA and others (2010) Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: the influence of growth, biochemistry and gametogenesis. *Aquaculture* 303:8–21
- da Silva PM, Renault T, Fuentes J, Villalba A (2008) Herpesvirus infection in European flat oysters *Ostrea edulis* obtained from brood stocks of various geographic origins and grown in Galicia (NW Spain). *Dis Aquat Org* 78: 181–188
- Davison AJ, Eberle R, Ehlers B, Hayward GS and others (2009) The order Herpesvirales. *Arch Virol* 154:171–177
- Dundon WG, Arzul I, Omnes E, Robert M and others (2011) Detection of type 1 ostreid herpes variant (OsHV-1 microvar) with no associated mortality in French-origin Pacific cupped oyster *Crassostrea gigas* farmed in Italy. *Aquaculture* 314:49–52
- FAO (Food and Agriculture Organization of the United Nations) (2013) Species fact sheet: *Crassostrea gigas* (Thunberg, 1793). FAO, Rome. Available at [www.fao.org/fishery/species/3514/en](http://www.fao.org/fishery/species/3514/en) (accessed 17 May 2013)
- Farley CA, Banfield WG, Kassnic G Jr, Foster WS (1972) Oyster herpes-type virus. *Science* 178:759–760
- Friedman CS, Estes RM, Stokes NA, Burge CA and others (2005) Herpes virus in juvenile Pacific oysters *Crassostrea gigas* from Tomales Bay, California, coincides with summer mortality episodes. *Dis Aquat Org* 63:33–41
- Gagnaire B, Soletchnik P, Madec P, Geairon P, Le Moine O, Renault T (2006) Diploid and triploid Pacific oysters *Crassostrea gigas* (Thunberg), reared at two heights above sediment in Marennes-Oleron Basin, France: difference in mortality, sexual maturation and hemocyte parameters. *Aquaculture* 254:606–616
- Haberkorn H, Lambert C, Le Goic N, Gueguen M and others (2010a) Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. *Aquat Toxicol* 97: 96–108
- Haberkorn H, Lambert C, Le Goic N, Moal J and others (2010b) Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae* 9:427–439
- Hallegraeff GM (1992) Harmful algal blooms in the Australian region. *Mar Pollut Bull* 25:186–190
- Hine PM, Thorne T (1997) Replication of herpes-like viruses in haemocytes of adult flat oysters *Ostrea angasi*: an ultrastructural study. *Dis Aquat Org* 29:189–196
- Hine PM, Wesney B, Hay BE (1992) Herpesviruses associated with mortalities among hatchery-reared larval Pacific oysters *Crassostrea gigas*. *Dis Aquat Org* 12: 135–142

- Hine PM, Wesney B, Besant P (1998) Replication of a herpes-like virus in larvae of the flat oyster *Tiosstrea chilensis* at ambient temperatures. *Dis Aquat Org* 32:161–171
- International OsHV-1  $\mu$ -var workshop (2011) Final report OsHV-1  $\mu$ -var. Available at [www.oysterstasmania.org/downloads/Oyster-Herpes-Virus-Workshop-Final-Report-111107.pdf](http://www.oysterstasmania.org/downloads/Oyster-Herpes-Virus-Workshop-Final-Report-111107.pdf) (accessed 5 March 2013)
- Lacoste A, Jalabert F, Malham S, Cueff A and others (2001) A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis Aquat Org* 46: 139–145
- Larkin MA, Blackshields G, Brown NP, Chenna R and others (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948
- Le Deuff RM, Renault T, Gerard A (1996) Effects of temperature on herpes-like virus detection among hatchery-reared larval Pacific oyster *Crassostrea gigas*. *Dis Aquat Org* 24:149–157
- Le Roux F, Gay M, Lambert C, Waechter M and others (2002) Comparative analysis of *Vibrio splendidus*-related strains isolated during *Crassostrea gigas* mortality events. *Aquat Living Resour* 15:251–258
- Levinton J, Doall M, Ralston D, Starke A, Allam B (2011) Climate change, precipitation and impacts on an estuarine refuge from disease. *PLoS ONE* 6:e18849
- Li Y, Qin JG, Li X, Benkendorff K (2009) Spawning-dependent stress responses in Pacific oysters *Crassostrea gigas*: a simulated bacterial challenge in oysters. *Aquaculture* 293:164–171
- Lynch SA, Carlsson J, O'Reilly AO, Culloty SC (2012) A previously undescribed ostreid herpes virus (OsHV-1) genotype detected in the Pacific oyster, *Crassostrea gigas*, in Ireland. *Parasitology* 139:1526–1532
- Malham SK, Cotter E, O'Keeffe S, Lynch S and others (2009) Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: the influence of temperature and nutrients on health and survival. *Aquaculture* 287:128–138
- 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
- Miller PA, Elliot NG, Koutoulis A, Kube PD, Vaillancourt RE (2012) Genetic diversity of cultured, naturalized and native Pacific oysters, *Crassostrea gigas*, determined from multiplexed microsatellite markers. *J Shellfish Res* 31:611–617
- Motes ML, DePaola A, Cook DW, Veazey JE and others (1998) Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). *Appl Environ Microbiol* 64:1459–1465
- Nicholas KB, Nicholas HBJ, Deerfield DWI (1997) GeneDoc: analysis and visualization of genetic variation. *EMBNET.NEWS* 4:14
- NSW DPI (Department of Primary Industries) (2011) Protocol for investigating and reporting fish kills. Available at [www.dpi.nsw.gov.au/\\_data/assets/pdf\\_file/0007/378385/fish-kill-protocol.pdf](http://www.dpi.nsw.gov.au/_data/assets/pdf_file/0007/378385/fish-kill-protocol.pdf) (accessed 5 March 2013)
- Pang L, Zhang XH, Zhong Y, Chen J, Li Y, Austin B (2006) Identification of *Vibrio harveyi* using PCR amplification of the toxR gene. *Lett Appl Microbiol* 43:249–255
- 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
- Renault T, Cochennec N, Le Deuff RM, Chollet R (1994) Herpes-like virus infecting Japanese oyster (*Crassostrea gigas*) spat. *Bull Eur Assoc Fish Pathol* 14:64–66
- Renault T, Allain G, Arzul I, Chollet B and others (2009) Summer mortality outbreaks of French Pacific oysters *Crassostrea gigas* in 2008 and 2009. In: Proc 14th European Association of Fish Pathologists Int Conf, 14–17 Sep 2009, Prague. Available at <http://archimer.ifremer.fr/doc/00014/12554/9420.pdf>
- Renault T, Moreau P, Faury N, Pepin JF, Segarra A, Webb S (2012) Analysis of clinical ostreid herpesvirus 1 (Malacoherpesviridae) specimens by sequencing amplified fragments from three virus genome areas. *J Virol* 86: 5942–5947
- Roughley TC (1926) An investigation of the cause of an oyster mortality on the Georges River, New South Wales, 1924–5. *Proc Linn Soc NSW* 51:446–491
- Sauvage C, Pepin JF, Lapegue S, Boudry P, Renault T (2009) Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: differences in viral DNA detection and quantification using real-time PCR. *Virus Res* 142:181–187
- Savin KW, Cocks BG, Wong F, Sawbridge T, Cogan N, Savage D, Warner S (2010) A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. *Virol J* 7:308
- 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
- Schmid E, Ziegler M, Danay E, Beyer L, Buhner M (2010) Is it really robust? Reinvestigating the robustness of ANOVA against violations of the normal distribution assumption. *Methodol Eur J Res Methods Behav Soc Sci* 6:147–151
- Segarra A, Pepin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and description of a particular ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res* 153:92–99
- Shimahara Y, Kurita J, Kiryu I, Nishioka T and others (2012) Surveillance of type 1 ostreid herpesvirus (OsHV-1) variants in Japan. *Fish Pathol* 47:129–136
- Smith IR, Nell JA, Adlard RD (2000) The effect of growing height and growing method on winter mortality in diploid and triploid Sydney rock oysters, *Saccostrea glomerata* (Gould, 1850). *Aquaculture* 185:197–205
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739
- Tan J, Lancaster M, Hyatt A, van Driel R, Wong F, Warner S (2008) Purification of a herpes-like virus from abalone (*Halopis spp.*) with ganglionitis and detection by transmission electron microscopy. *J Virol Methods* 149: 338–341

- Tang B, Liu B, Wang X, Yue X, Xiang J (2010) Physiological and immune responses of Zhikong scallop *Chlamys farreri* to the acute viral necrobiotic virus infection. Fish Shellfish Immunol 29:42–48
- Vásquez-Yeomans R, García-Ortega M, Caceres-Martinez J (2010) Gill erosion and herpesvirus in *Crassostrea gigas* cultured in Baja California, Mexico. Dis Aquat Org 89: 137–144
- Wang N, Ren Wc, Cai Yy, Wang Cm (2011) Detection of acute viral necrosis virus (AVNV) in plankton using FQ-PCR during a molecular epidemiology survey. J Fish China 34:1566–1571
- Wolf PH (1972) Occurrence of a haplosporidian in Sydney rock oysters (*Crassostrea commercialis*) from Moreton Bay Queensland, Australia. J Invertebr Pathol 19:416–417
- Zhou J, Fang W, Yang X, Zhou S and others (2012) A non-luminescent and highly virulent *Vibrio harveyi* strain is associated with 'bacterial white tail disease' of *Litopenaeus vannamei* shrimp. PLoS ONE 7:e29961
- Zimmerman AM, DePaola A, Bowers JC, Krantz JA, Nordstrom JL, Johnson CN, Grimes DJ (2007) Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Mexico water and oysters. Appl Environ Microbiol 73:7589–7596

*Editorial responsibility:* Mike Hine,  
Fouras, France

*Submitted:* March 14, 2013; *Accepted:* May 23, 2013  
*Proofs received from author(s):* July 3, 2013