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Spatial distribution of mortality in Pacific oysters *Crassostrea gigas*: reflection on mechanisms of OsHV-1 transmission

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ABSTRACT: The ostreid herpesvirus OsHV-1 has the potential to devastate Pacific oyster *Crassostrea gigas* culture in Australia as it has done in many other countries, highlighting the need for a better understanding of disease expression and transmission. The aim of this study was to assess the spatial distribution of OsHV-1-associated mortalities in one of only two infected areas in Australia, Woolooware Bay (Botany Bay, New South Wales). In October 2011, healthy sentinel Pacific oysters were placed in 3 different locations at 3 different tidal levels, and OsHV-1 associated mortalities were closely monitored over 7 mo. The outbreak started in November 2011, and the disease remained active until April 2012. Three major mortality events were detected. Rather than being a propagating epizootic, it appeared that most oysters were infected from a common environmental source. The distribution of OsHV-1-associated mortalities was spatially clustered, highly variable and clearly dependent on the age of oysters and their position in the water column. Non-random distribution of mortalities at macro scale (sites several km apart) and micro scale (within rearing trays), and vertical clustering patterns in the water column are discussed in regard to factors known to influence mechanism of disease transmission in aquatic environments (hydrodynamics, physical disturbances, host density/distribution, and variations of environmental parameters). A new hypothesis proposing that OsHV-1 may be carried through water by particles, possibly plankton, is also suggested to explain the patchy distribution of mortalities in Woolooware Bay.

KEY WORDS: *Crassostrea gigas* · Ostreid herpesvirus 1 · Summer mortalities · Spatial distribution · Plankton · Disease transmission

INTRODUCTION

Summer mortalities of Pacific oysters *Crassostrea gigas* may be due to multifactorial interactions among the environment, pathogens and the physiological status of the host and were first recorded in the US and Japan in the early 1960s (Mori 1979, Perdue et al. 1981). The first identification of herpes-like virus infection associated with major summer mortality events of spat and juveniles was reported in France in 1993 (Renault et al. 1994, Renault 1998). This virus was classified as the first member of the family *Malacoherpesviridae*, and was called ostreid herpesvirus 1 (OsHV-1) after its genome was completely sequenced (Davison et al. 2005, 2009). Since then, massive mortalities of spat and juvenile Pacific oysters associated with OsHV-1 have been reported in France, the UK, Jersey, Ireland, Spain, The Netherlands and the US (Renault et al. 1994, Renault & Novoa 2004, Friedman et al. 2005, Schikorski et al. 2011a). The most severe disease events are specifically associated with a virulent variant of the virus, OsHV-1 µVar (Segarra et al. 2010). In 2010, high mortalities of farmed and wild Pacific oysters were recorded for the first time in both New Zealand and Australia in association with OsHV-1 (Cameron & Crane 2011). Renault et al. (2012) demonstrated that a variant virus infecting Pacific oyster in New

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Zealand was related to the variant µVar but showed some significant differences in terms of sequences. In Australia, detailed sequencing is still required to determine the identity of the OsHV-1 variant(s) present since 2010. Therefore, the term 'OsHV-1' as a species name including the different forms of variants is used in this study.

Despite the impact that OsHV-1 has on the Pacific oyster, the most economically important shellfish in the world (see www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en), little information is available about disease epidemiology in open marine environments (Garcia et al. 2011). Disease outbreaks occur in summer, preferentially in sheltered locations (Renault 2011). Water temperature plays a key role but does not fully explain the pattern of mortalities. Several studies have also suggested the role of some *Vibrio* species in disease expression (Burge et al. 2007, Sauvage et al. 2009, Saulnier et al. 2010, Pernet et al. 2012). The distribution of outbreaks in Europe suggested that the disease spread with the movement of animals from infected areas (Peeler et al. 2012). Some evidence indicates that boat movements, biofouling and the introduction of materials may play a role in the spread of the disease (Cameron & Crane 2011). The spatial distribution of mortalities between and within bays is still poorly understood (Peeler et al. 2012, Pernet et al. 2012). Indeed, the lack of knowledge about the role of hydrodynamics and other environmental/biological forces on OsHV-1 spread, at small and large scales, constitutes one of the main knowledge gaps to implement efficient control mechanisms to prevent the spread of the disease. Moreover, data on the existence of potential vectors for OsHV-1 transmission in open marine systems are lacking. In Australia, the presence of OsHV-1 is restricted to 2 estuaries in New South Wales (NSW) where it is now considered to be endemic: Port Jackson and Wooloware Bay/Georges River. It is unknown how the virus arose or arrived in the country. Consequently, particular efforts are required to understand how the disease transmits and spreads in a natural environment. Indeed, improving knowledge about OsHV-1 transmission could help limit the impact of the disease on the oyster industry.

The aim of the present work was to assess the spatial distribution of OsHV-1-associated mortalities in one of the infected areas in Australia, Woolooware Bay (Botany Bay, NSW), across a range of spatial scales during the Australian summer 2011/2012.

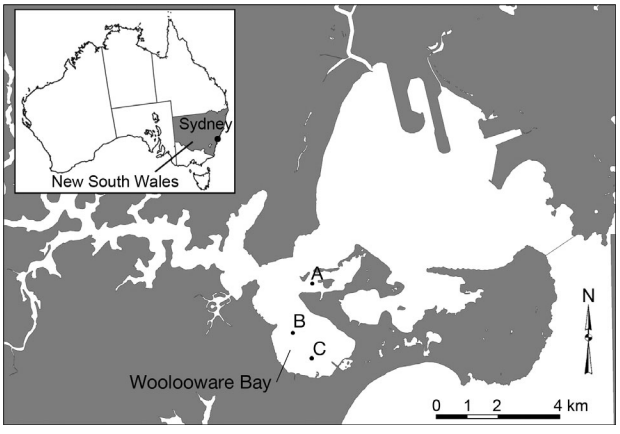

Fig. 1. Experimental sites (Site A: 34.0118° S, 151.1463° E; Site B: 34.0254° S, 151.1400° E; Site C: 34.0329° S, 151.1466° E) in Woolooware Bay (Botany Bay, New South Wales, Australia)

MATERIALS AND METHODS

Study site

Woolooware Bay is located on the southern shore of Botany Bay, approximately 16 km from the centre of Sydney, NSW (Fig. 1). This shallow estuarine area is constituted by mud flats creating an excellent nutrient-rich area for the growth of mangroves and saltmarsh communities. Woolooware Bay used to be one of the most important areas for the native Sydney rock oyster *Saccostrea glomerata* in NSW. Parasitic disease outbreaks decimated a major part of the industry in this area, and the Sydney rock oyster was progressively replaced by the Pacific oyster *Crassostrea gigas* from 1991. As a consequence of the OsHV-1 outbreak in 2010, the farming of *C. gigas* ceased almost completely, and the remaining population in the bay is almost exclusively made up of small populations of wild individuals. The experiment was set up in 3 different oyster leases located up to 1000 m apart from each other (Fig. 1).

Experimental design

Healthy oysters (n = 8500) were sampled from Hawkesbury River, and were free of any clinical signs of disease and tested negative for OsHV-1 using the TaqMan assay developed by Martenot et al. (2010). All oysters used during this study correspond to hatchery single-seed oysters and came from 2 distinct batches: SPL11D (2 mo old spat) and SPL10GSD (12 mo old adults). Both batches were harvested from the same location (Porto Bay) in

Hawkesbury River. From October 2011, oysters were transferred to Woolooware Bay and placed at Sites A, B and C (Fig. 1). Oysters comprised 4250 adults (mean ± SE length: 77.9 ± 1.0 mm) and 4250 spat (length: 28.4 ± 0.9 mm). These were divided randomly and used in 2 experiments conducted in parallel across the 3 oyster leases. The different systems used in Expts 1 and 2 are schematically presented in Fig. 2. For logistic reasons, Expt 1 started on 20 October 2011, whereas Expt 2 began on 24 November 2011.

Expt 1: Effect of height on oyster mortality using intertidal cultivation systems

This experiment was designed to compare the mortality rates as a function of the depth of intertidal trays in the water column leading to different immersion times and air/UV/heat exposures. Adult oysters and spat were placed intertidally in plastic trays (2 m × 1 m) fixed on wooden racks at a standard height commonly used by local oyster farmers, and at a height 300 mm above standard rack height (Fig. 2A). For the purpose of this study, the standard and higher heights are called 'low' and 'high', respectively. The experimental design thus led to 4 treatments site^{-1}: high adult, high spat, low adult and low spat.

The initial stocking density was 320 oysters tray^{-1}. With 2 trays treatment^{-1}, this corresponded to 640 oysters treatment^{-1} (high adult, low adult, high spat, low spat) site^{-1} (A, B, C). Regular random sampling of 25 to 50 live oysters treatment^{-1} site^{-1} was performed weekly to fortnightly from October 2011 until May 2012 for OsHV-1 detection (data not shown). In addition, dead oysters were systematically removed from trays and were not replaced. Therefore, the total number of oysters and the stocking density declined over time. This was taken into consideration when calculating the cumulative mortality (see 'Mortality data' below).

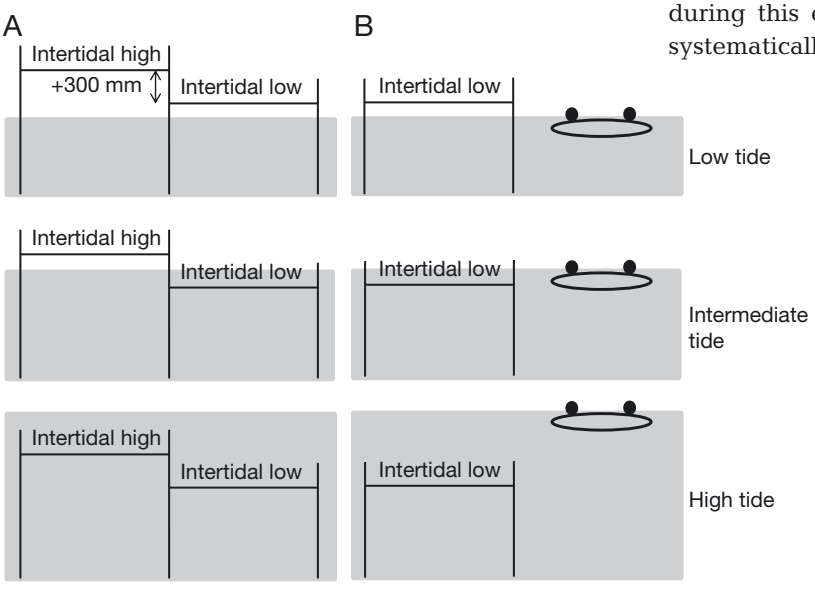

Fig. 2. Schematic representation of the 2 experiments in different tide scenarios. (A) Intertidal trays at high and low heights in Expt 1; (B) intertidal trays at low height and subtidal floating baskets in Expt 2

Expt 2: Effect of cultivation system (subtidal versus intertidal) on oyster mortality

The aim of this second experiment was to compare the mortality rates among spat and adult oysters placed in 2 contrasting cultivation systems: intertidal trays fixed on wooden racks (standard height called 'low', as in Expt 1) and subtidal floating baskets (Fig. 2B). These structures led to different immersion/emersion patterns and absolute position in the water column. Both spat (S1) and adult oysters (A1) were used for this experiment.

The initial stocking density was lower in Expt 2 than in Expt 1, as the size of each floating basket did not allow more than 15 adults basket^{-1}. Consequently, 15 oysters were placed in each floating basket with 2 basket replicates for each age class (spat/adult) at each site (A, B, C). Additionally, 30 oysters of each age class (spat/adult) were placed in one intertidal tray at each site. Therefore, the total number of oysters at the beginning of the experiment (24 November 2011) was 360. No sampling was performed during this experiment, but dead individuals were systematically removed from trays and baskets. In contrast to Expt 1, dead batches of spat were replaced by later transfers of healthy individuals in order to keep live naive animals and determine the window of infection for OsHV-1 disease. In total, 2 additional batches of healthy spat (n = 180 × 2, called 'S2' and 'S3') were deployed in trays and baskets on 20 December 2011 and 16 March 2012.

Mortality data

Mortality was assessed by manually counting dead and live oysters in each treatment for each experiment. Additionally, in Expt 1, the number of dead/alive oysters was carefully recorded in

each segment/square of each tray (8 segments/squares tray^{-1}) in order to assess the spatial distribution of mortality. To take into account the proportion of oysters sampled for pathogen detection (Expt 1), the cumulative mortality was expressed after correction for sampling according to the following equation:

$$Cumulative\ mortality_t = [(Observed\ mortality\ rate_t \times Adjusted\ number\ of\ live\ oysters_{t-1}) + Cumulative\ mortality_{t-1}]/Initial\ population\ size_{t_0}$$

where $Observed\ mortality\ rate_t = Number\ of\ dead\ oysters_t/Actual\ population\ size_t$ and $Adjusted\ number\ of\ live\ oysters_{t-1} = Adjusted\ number\ of\ live\ oysters_{t-2} - (Observed\ mortality\ rate_{t-1} \times Adjusted\ number\ of\ live\ oysters_{t-2})$ where t is the observation time, and t–1 and t–2 refer to the first and second previous observation times, respectively.

Statistical analyses

Cumulative mortality was compared between age and tidal height groups for each observation period and site (and for each rearing system in the second experiment) by using a chi-squared test or Fisher's exact test as appropriate (Altman 1991). These analyses were conducted using the SAS statistical program (release 9.2, SAS Institute).

Spatial analysis was undertaken to test the hypothesis that the pattern of mortality was random using commercial scan statistic software (SatScanV9.1.1, Information Management Services) based on Kulldorff (1997). Unique Cartesian coordinates were allocated to each of the 8 segments in each tray at each site, with a single x-coordinate gap inserted at each site between age groups, heights and replicates within treatments, and 4 y-coordinate gaps inserted between sites as notional and not-to-scale representation of the geographical location of trays. Cases were defined as the number of dead oysters while the population was defined as the number of live plus dead oysters at each time point. A discrete Poisson scan statistic was used, with the covariates age and height. To confirm site effects, spatial analyses were conducted across all sites at selected time points: 16, 21 and 24 November 2011, and 10 and 13 February 2012. To look for within-site effects, analyses were conducted for Site C on 16 and 21 November, Site A on 21 and 24 November and Site B on 10 and 13 February 2012.

Contingency tables of the frequencies of dead and live oysters for each age–height group and replicate were prepared at selected time points: 16 November 2011 for Site C and 21 and 24 November 2011 for Site A. Chi-squared and Fisher's exact tests (as appropriate) were conducted to compare proportional mortality between the left and right sides of trays and between the 2 replicates using SAS.

RESULTS

Three summer mortality events were recorded in Woolooware Bay: November 2011, February 2012 and April 2012. All dead oysters which were tested for OsHV-1 by PCR using the TaqMan assay developed by Martenot et al. (2010) were heavily infected, confirming the role of OsHV-1 (C_t values comprised between 18 and 30, data not shown). Cumulative mortalities were determined at the end of each major mortality event (Figs. 3 to 5). Overall, for all sites and observations, spat had a significantly higher mortality (89.1%) than adults (49.4%) in Expt 1 (May 2012; $p < 0.001$ for each site and overall for all sites; Fig. 3). Similarly, in Expt 2, spat experienced a significantly higher level of mortality (48.1%) than adults (25.9%; $p < 0.001$; Fig. 4). All spat in trays died regardless of the site or height, while a substantial proportion of spat survived in floating baskets (Figs. 4 & 5). Adult oysters had final mortality rates ranging from 40 to 80% depending on the height and the site (Fig. 3).

Mortality patterns at the macro level

Expt 1

Mortality levels differed among the 3 sites ($p < 0.001$). Mortality was observed first at Site C on 16 November 2011. Within 5 d, mortality began at Site A, but there was no evidence of mortality at Site B until February 2012. By the end of December 2011, Sites A and C had similar mortality patterns (Fig. 3) except for the high-adult group at Site A that had a significantly higher mortality than that at Site C ($p < 0.05$). After this first mortality event, no further substantial mortalities were recorded in the adult population left alive at Site A as shown by the similar cumulative mortality recorded in December, February and May 2012 ($p > 0.05$; Fig. 3). Low adults at Site C exhibited a significant increase in mortality over time ($p = 0.003$). The mortality pattern displayed at Site B was different from the other sites, as no mortality was recorded in December (Fig. 3). However, at the end of February, all spat died and the cumulative mortalities for adults reached ~35%, and by May approximately half of the adult oysters had died (Fig. 3).

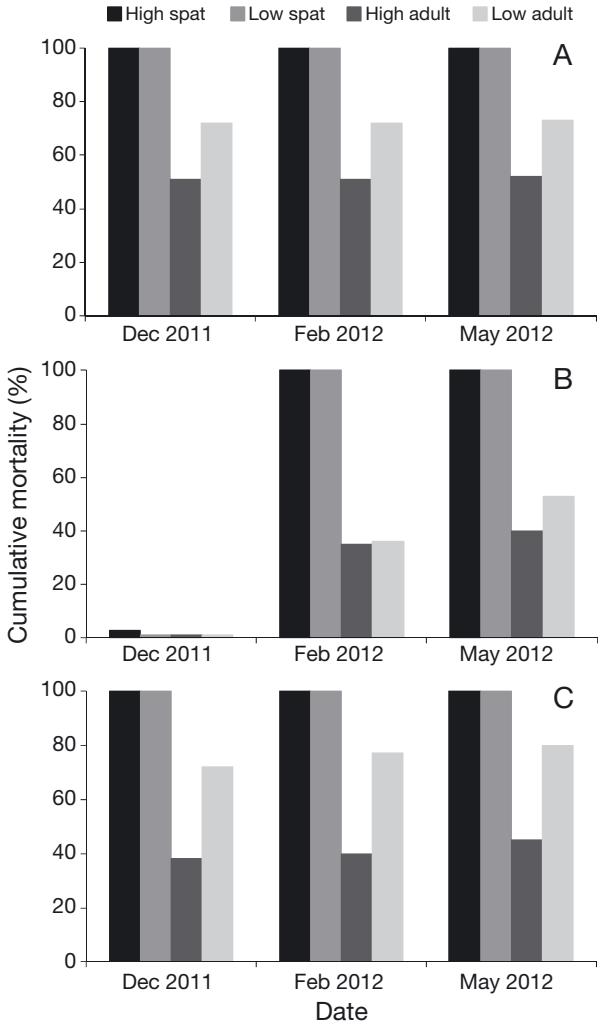

Fig. 3. Cumulative mortality of oysters at Sites A, B and C in Woolooware Bay in 2011/2012 as a function of age and height of trays (Expt 1). The initial stocking density was 640 ind. treatment^{-1}

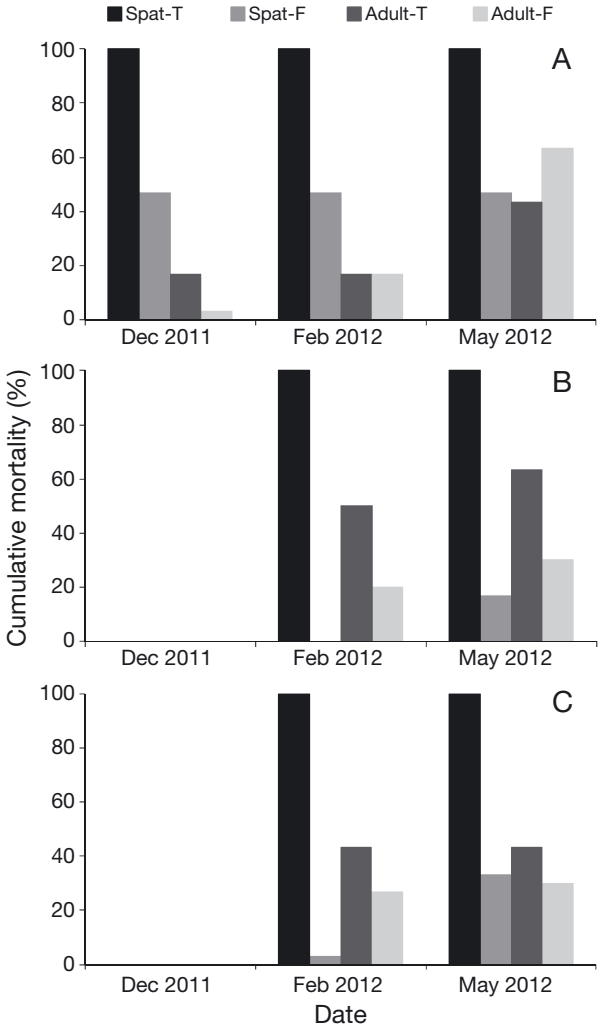

Fig. 4. Cumulative mortality of oysters at Sites A, B and C in Woolooware Bay according to age and cultivation system (Expt 2: adults and initial batch S1 spat deployed on 24 November 2011). Spat-T: spat in intertidal trays; Spat-F: spat in floating baskets; Adult-T: adult oysters in intertidal trays; Adult-F: adult oysters in floating baskets. The initial stocking density was 30 ind. treatment^{-1} batch^{-1}

Retrospective spatial analysis across the 3 sites revealed highly significant clustering of mortality at Site C on 16 November (relative risk, RR: 87, $p < 0.001$) which persisted until 21 November (RR: 6.3, $p < 0.001$), by which time there was a secondary cluster at Site A (RR: 2.9, $p < 0.001$). By 24 November 2011, mortality was clustered primarily at Site A (RR: 9.9, $p < 0.001$) with a secondary cluster at Site C (RR: 2.6, $p < 0.001$). Observed mortalities were about 2.3 to 3.5 times greater than expected within these clusters. Note that in spatial analysis, the mortality inside a significant cluster differs from that outside the cluster; the degree of difference between the cluster and the study region as a whole is specified by a fold difference (observed/expected). On 10 and 13 February 2012, mortality was clustered at Site B (RR: 2.4–5.1, $p < 0.001$), with observed mortality 2.1 to 4.8 times greater than expected.

Expt 2

In the second experiment, oysters were deployed in both intertidal trays and floating baskets on 24 November 2012. Overall, the mortality levels were different at the 3 sites ($p = 0.03$). At the end of December, only Site A exhibited significant mortalities, while oysters deployed at Sites B and C remained free of any sign of disease until February 2012 (Fig. 4). As the mortality rate for intertidal spat at Site A reached 100% in December, a new batch (S2) was introduced at the end of the month at this site only. No mortality was observed in this second batch at Site A in February (Fig. 5). However, the first batch deployed at Sites B and C then demonstrated

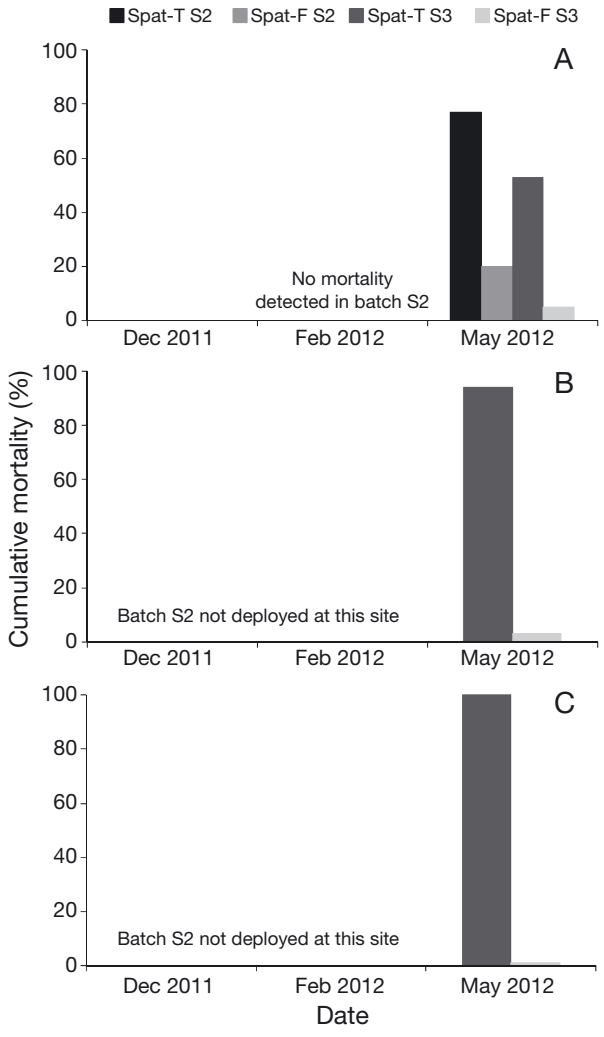

Fig. 5. Cumulative mortality of oysters at Sites A, B and C in Woolooware Bay according to placement date and cultivation system (Expt 2: additional batch S2 spat deployed on 20 December 2011 at Site A only; additional batch S3 deployed on 16 March 2012 at all sites). Spat-T: spat in intertidal trays; Spat-F: spat in floating baskets. The initial stocking density was 30 ind. treatment^{-1} and batch^{-1}

mortality rates ranging from 3 to 100% in February 2012 (Fig. 4). Consequently, a new batch of spat (S3) was deployed at Sites A, B and C in March. In May 2012, oyster mortality occurred at all sites (Fig. 5).

Mortality rates at the micro level in Woolooware Bay

Variation of mortality between and within trays

During November 2011, the mortality distribution within every segment (n = 8 segments tray^{-1}) of each tray (n = 8 trays site^{-1}) was closely observed at Sites A and C. Spatial analysis revealed non-random distribution of mortality with important clusters between and within trays (Fig. 6). Within Site C on 16 November, mortality differed between replicates and was clustered in parts of trays, with clusters identified among both spat and adults (RR: 2.1, $p < 0.001$), and observed mortalities were 2.0 to 2.4 times higher than those expected. By 21 November, the mortality was uniform at Site C. Within Site A on 24 November, mortality was clustered between and in parts of trays (RR: 1.6 to 2.5, $p < 0.001$), and observed mortalities in the clusters were 1.5 to 2.4 times those expected. At Site B on 10 and 13 February 2012, mortalities were uniform. Chi-squared analyses revealed that mortalities were significantly different between left and right sides of trays at Sites A and C (Fig. 6).

Variation of mortality among oysters exposed to different strata of the water column

The position of trays and floating baskets relative to strata of the water column at 3 tide levels is illustrated in Fig. 2. Adult oysters kept at lower height had a significantly higher mortality than those kept at higher levels at Sites A and C at all 3 time points (all $p < 0.001$; Fig. 3). As the outbreak started later at Site B, a significant difference in mortality between low and high trays was observed only in May 2012 ($p < 0.001$). In the second experiment, significantly lower mortality occurred in oysters kept in baskets (18%) than those kept in trays (60.9%; $p < 0.05$). All spat from the initial batch (S1) died in trays at all sites, while the mortality rate remained relatively low in floating baskets (17–47%, depending on the site; Fig. 4). This observation was consistent for all additional batches of spat (S2 and S3) at all sites (Fig. 5). As the absolute mortality rate was lower for adult oysters than for spat, the difference between subtidal and intertidal systems for this age class was less outstanding and only significant at Site B in February and May 2012 ($p < 0.05$; Fig. 4).

DISCUSSION

OsHV-1 causes a disease of international significance which has halved production of Pacific oysters in major producing countries such as France and New Zealand (Cameron & Crane 2011). Observations about the origins of epizootics and the mode of transmission of OsHV-1 may be important in devis-

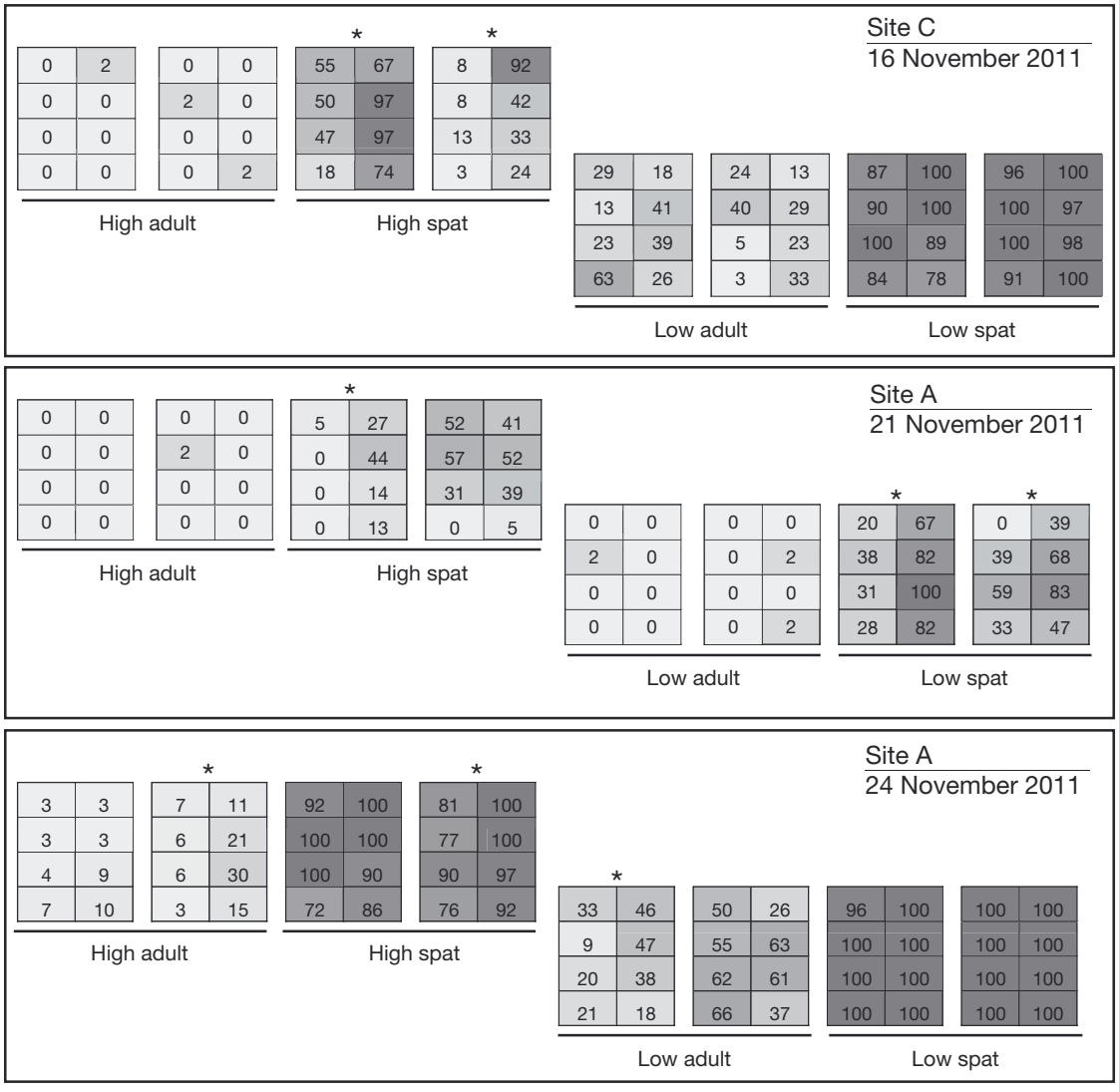

Fig. 6. Spatial distribution of oyster mortality in trays at Sites A and C for selected time points (Expt 1). Each number represents the observed mortality (%) in each segment of the tray. Shading illustrates levels of mortality. * Trays in which the mortalities were significantly different between left and right hand sides

ing future strategies for disease control. The present work demonstrated that the distribution of OsHV-1-associated mortalities in Woolooware Bay, 1 yr after the first occurrence of the disease at this location, was clustered (i.e. not uniform or random), highly variable in time and space and clearly dependent on the age of oysters. Indeed, a greater sensitivity of young age classes of oysters to OsHV-1 (micro-variant and classical strain) has already been demonstrated in several studies (Renault et al. 1994, Burge et al. 2006, 2007, Miossec et al. 2009, Dégremont et al. 2010, Dégremont 2011, Garcia et al. 2011, Schikorski et al. 2011a) and was confirmed by the present work. It was consistent among cultivation systems and sites and over time. Substantial differences in mortality were observed at the macro scale among 3 sites which were <2 km apart from each other. Additionally, we observed non-random distribution at the micro scale, i.e. within oyster trays, and vertical clustering patterns in the water column.

A great deal of useful information can be derived from an understanding of the mechanisms of disease transmission (Thrusfield 2007). Did the outbreak of OsHV-1-associated mortality in Woolooware Bay arise from a common point source (i.e. infection derived from a source which is common to all individuals) or was it propagating (i.e. the first cases in oysters are the source of infection for subsequent cases

in oysters; Thrusfield 2007)? To help answer this question, examples were sought from the literature, but few published studies of natural transmission mechanisms in marine animal virology are available. In 1995 and 1998, a herpesvirus caused massive propagating epizootics of mortality in pilchards *Sardinops sagax neopilchardus*, a schooling pelagic fish, in Australia (Whittington et al. 1997). The epizootic wave was successfully modelled using parameters of relatively close contact between individual fish, and suggested that a diseased individual infected 3 to 5 others (Murray et al. 2001a,b). Similarly, propagating epizootics of viral haemorrhagic septicaemia virus (a rhabdovirus) in schooling herring *Clupea pallasii* followed fish-to-fish transmission (Hershberger et al. 2011). In our study, while mortality in Woolooware Bay started almost concomitantly (within 5 d) at Sites A and C in Expt 1, suggesting either a simultaneous point source for the virus or a rapid dispersion of infective viral particles between sites, there was no sign of mortality at Site B during the same period (November 2011). The clustered mortality pattern was also observed during the second mortality event in February 2012 (Fig. 4). The effects of hydrodynamics and wind play an important part in disease spread in fish and shellfish in open aquatic systems (Kristoffersen et al. 2009, Viljugrein et al. 2009, Pernet et al. 2012). In Woolooware Bay, tidal flows from Sites A and C pass through Site B. As there was no progressive wave of mortality from Site A to B to C or from Site C to B to A during 3 OsHV-1 events in the summer of 2011/2012, spread of the disease due to tidal flow in and out of the bay is insufficient to explain the distribution of mortality.

Substantial spatial differences were also observed at a micro level with non-uniform and non-random distribution of mortality among and within the cultivation systems at each site. The significant clusters of mortality between 2 tray replicates and among square divisions within a single tray (Fig. 6) suggest that OsHV-1 is not uniformly distributed in the seawater even at a local scale, leading to different exposures of individuals which lie quite close together.

Based on these observations, it is reasonable to conclude that oyster to oyster transmission within a tray of oysters was not important, and neither was direct spread of the virus due to water currents/tidal flows carrying infective material from infected oysters on one lease to naive oysters on another lease. The outbreak was most likely to be a point source epizootic, where infections of most animals were derived from exposure to a common environmental source, where there were different degrees of exposure at different locations (macro scale, between sites; and micro scale, between/within trays), with limited local transmission from animal to animal. Natural reservoir hosts, as well as moribund and dead infected oysters, are likely to release OsHV-1 virions into the seawater leading to horizontal transmission (Renault 2011). While we conducted our study, no commercial *Crassostrea gigas* cultivation was operating in Woolooware Bay. There is a wild population of Pacific oysters but it has not been assessed since the outbreak of OsHV-1 mortality in Woolooware Bay which began in November 2010 and affected both wild and cultivated *C. gigas* (Cameron & Crane 2011). Therefore, the contribution of this remnant population of wild *C. gigas* as a reservoir of OsHV-1 to explain spatial differences at the macro scale remains uncertain.

The effect of oyster stock and genetics on OsHV-1 (micro-variant and classical strain)-associated mortalities has been demonstrated (Burge et al. 2006, 2007, Dégremont 2011). This is particularly significant when comparing mortality rates of wild oysters from multiple parentages and hatchery-reared oysters which have less genetic variation. In the present study, single-seed oysters from defined broodstock were obtained from a hatchery and possessed clear parentage, but genetic variations between individuals must have existed and could have played a role in the difference in mortality observed between adults and spat, as these were from different spawning batches. However, the spatial effects observed in this study cannot be explained by genetic differences, as oysters used in the trial were randomly allocated among replicates, treatments and sites, and we deployed more than 600 oysters treatment^{-1} site^{-1}.

Similarly, differences in environmental parameters such as temperature, salinity, food quality/quantity or water quality may have influenced the spatial patterns of OsHV-1 disease expression among the 3 sites in Woolooware Bay (Berthelin et al. 2000, Peeler et al. 2012). However, these factors were unlikely to have operated within sites due to their small scale. On the other hand, micro-hydrodynamics could have played a role in the mortality patterns at the micro scale, i.e. in oyster trays. Physical disturbances due to the presence of rearing structures are obvious, well documented and lead to complex interactions with the water column (Forrest et al. 2009). Several studies revealed that oyster farms influence the quantity of suspended particle matter (organic matter, phytoplankton, zooplankton, larvae, bacteria, detritus) as it flows through the structures due to the intense filtration activity of the oysters (Hawkins et al. 1998, For-

rest et al. 2009). Therefore, it seems reasonable to expect that the attributes of the cultivation structure (the number, density and design of trays, their orientation in relation to currents and water flows and their depth) associated with the filtration activity and the qualitative selection capacity of oysters may have influenced the circulation of water and therefore the accessibility to viral particles in the water (Dumbauld et al. 2009).

At the micro scale (within sites), the vertical distribution of mortality also showed an interesting pattern, as oysters exposed to different strata of the water column presented contrasting mortality rates. A significant decrease in cumulative mortality was demonstrated for adults placed in intertidal trays at high height in comparison with low height. This observation was consistent among sites (Fig. 3) and may be explained by a shorter immersion time leading to a lower exposure to viral particles in the water. Similar conclusions were reached in France and Ireland after observation of reduced mortality when oysters were placed higher on the shore (Peeler et al. 2012).

The low mortality rate of spat placed in floating baskets, in comparison with spat placed in adjacent intertidal cultivation systems, cannot be explained by a reduced immersion time, as these oysters were constantly immersed. However, this could be related to the viability/infectivity of the virions in the water. As OsHV-1 is an enveloped virus, it is also reasonable to imagine that higher exposure to high temperature and sunlight (UV) in the upper centimetres of the water column than in the subjacent water layers may have significantly damaged the virus by destroying its lipid-containing envelope (Renault 2011). Indeed, as water density decreases as temperature increases, warm water overlies colder water and creates stable horizontal gradients that resist vertical mixing (Suthers & Rissik 2009). Alternatively, it is possible that oysters present in the superficial layer of water might not have been exposed to a quantity of virions sufficient to induce disease. This could be due to valve opening and feeding activities (i.e. oyster physiology) being reduced due to the cultivation structure. Côté et al. (1994) explained that the low energetic reserves in oysters placed in Australian baskets might be due to the fact that they were more exposed to shocks caused by wave and wind action thus causing reduced valve opening and feeding activities. Although the floating baskets used in the present study were quite different from the Australian baskets, intensive observations from the field clearly demonstrated that the floating systems were subjected to shocks and heavy movements caused by waves and wind at all sites in Woolooware Bay.

Overall, our findings lead to a very significant question about the mode of transmission of OsHV-1 in water. Experimental studies in aquaria have demonstrated that OsHV-1 can be a waterborne infection transmitted between infected and healthy individuals through cohabitation (Schikorski et al. 2011b). Sauvage et al. (2009) analysed seawater samples from an artificial pond during a mortality outbreak and were able to detect a significant quantity of viral DNA (10^3 viral DNA copies l^{-1}). However, whether OsHV-1 transmission mainly involves waterborne free virus or virus attached to or engulfed by particles is unknown, and data on the existence of potential vectors for OsHV-1 transmission in natural environments are lacking. Herpes-like particles have been detected previously in unicellular organisms (thraustochytrid-like organism, *Schizochytrium* sp.) from the York River estuary (Virginia, USA; Kazama & Schornstein 1972, 1973). More recently, enveloped OsHV-1-like virions were detected in a marine fungoid protist which was present in experimental rearing tanks containing Pacific oyster larvae. The protist was observed free in the water and also phagocytised inside cells of larval oysters (Renault et al. 2003). These findings suggest that marine unicellular organisms may play a role as vectors for transmission of OsHV-1 in natural environments (Renault 2011). However, the argument should not be restricted to unicellular life forms, as inanimate suspended particles and metazoans in the plankton may also be vectors. Viruses, like other microbes, attach to particles in the environment through complex reversible and irreversible interactions, and so their fate and transport is associated with that of the particles (Tufenkji 2007, Dhand et al. 2009). Previous studies also revealed that plankton affects viral ecology in the natural environment and may play a role in the transmission of finfish viruses (Faisal & Winters 2011, Minamoto et al. 2011). These published studies and field observations suggest that OsHV-1 could be associated with particles. The epidemiological observations from the present study are consistent with this idea.

The hypothesis that the dispersion and transmission of OsHV-1 could involve particles including plankton acting as a vector could explain the patchy distribution of mortality. Note that in this paper, the term 'plankton' is used in its broad sense, i.e. ranging from minute bacteria to microscopically visible phytoplankton, larger zooplankton and small invertebrate larvae. Plankton organisms are not distrib-

uted uniformly throughout the water but have a non-random and patchy distribution in both time (day/night, summer/winter) and space (horizontal and vertical) across a wide range of spatial scales (from <10 cm to >1 km; Cassie 1959, Steele 1976, Malone & McQueen 1983, Boltovskoy & Mazzoni 1988, Breitburg et al. 1995, Suthers & Rissik 2009, Benoit-Bird & McManus 2012). Malone & McQueen (1983) investigated zooplankton communities in lakes and demonstrated that they were all patchy in terms of both vertical and horizontal distributions, and the patches tended to be comprised of unique groups of species. If OsHV-1 was carried by specific plankton cells, this could reasonably explain the clusters of mortality at the macro scale, the non-progression of mortality from Site A to B to C and the clusters within sites over time. Additionally, this hypothesis could also explain the vertical pattern of mortality observed in this study. Vertical clustering of planktonic organisms is well known and is due to biological (competition, predation, gregariousness, rheotactism) as well as physical factors (thermal stratification, salinity, light exposure, O_2:CO_2 ratio) and is highly dependent on species, sex, age, habitat type and season (Cassie 1958, Malone & McQueen 1983, Van Gool & Ringelberg 1998, Reinfelds & Williams 2012). Considering the hypothesis of a planktonic vector for OsHV-1, a deeper position of this vector in the water column for biological or physical reasons would lead to less exposure of the oysters in floating baskets near the surface in comparison with oysters placed at a fixed intertidal position.

The potential role of oyster physiology and feeding behaviour on OsHV-1-associated mortality is also interesting to contemplate. If OsHV-1 is associated with a particulate vector, it is possible that size preferences for feed may influence the exposure of oysters of different sizes/ages, and hence influence mortality patterns.

There are a number of limitations in the present study. No plankton or water samples were collected to validate the plankton hypothesis. Thus, the present study aims to stimulate research about the mode of transmission of OsHV-1 in natural environments. While we observed strong vertical clustering of mortality consistent with a hypothesis of a planktonic vector, this might be disrupted by strong wind or wave action causing mixing of strata, in which case the mortality pattern may not show such vertical patterns. For this reason, our study will be repeated to observe mortality patterns associated with different seasonal weather patterns. In addition, more detailed investigations are required to clarify the roles of potential mechanisms (micro-hydrodynamics/plankton patchiness/oyster physiology) on the strong clustering pattern of OsHV-1-associated mortalities at macro and micro scales.

CONCLUSION

OsHV-1-associated mortality in *Crassostrea gigas* in Woolooware Bay in 2011/2012 followed the pattern of a point source epizootic. Beyond the influence of oyster age on mortality rates, the clustering of mortality was clearly demonstrated in time and space at a large scale (bay level) as well as a very small scale (site level, tray level, vertically). Can a single hypothesis explain these observations? A planktonic vector that enhances transmission and spread of OsHV-1 could explain the observed patterns. However, as no plankton sampling was performed to validate this hypothesis, it is evident that further work is required to clarify the possible involvement of a vector for OsHV-1 transmission. High-frequency water samples using appropriate plankton nets and water samplers before, during and after an outbreak at different tide cycles and times (day/night) will be required to investigate this question. PCR and *in situ* hybridization will constitute relevant tools to detect and observe OsHV-1 DNA in the water samples and, if found, its potential carrier. Further work on viral RNA activity will also be required to determine the infective status of the virus. The importance of such a study is that it may be possible to ameliorate the impact of OsHV-1 by disturbing the spatial distribution of a vector around an oyster farm, or by placing oysters in a husbandry system designed to avoid the vector.

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