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Oysters Australia IPA: Pacific Oyster Mortality Syndrome

Closing knowledge gaps to continue farming *Crassotrea gigas* in Australia

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June 2019

FRDC Project No **2014-040**

ISBN **978-1-74210-445-4**

Oysters Australia IPA: Pacific Oyster Mortality Syndrome. Closing knowledge gaps to continue farming *Crassotrea gigas* in Australia FRDC Project No 2014-040

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Foreword

An ongoing research program was initiated at the University of Sydney to address the threat of Pacific Oyster Mortality Syndrome (POMS) to oyster farming since the emergence of *Ostreid herpesvirus 1* (OsHV-1) in Australia. The present project has provided a deeper understanding of the epidemiology of this disease. This report includes the analyses of research methodologies that have been employed across five seasons of recurrent POMS outbreaks in NSW. Recurrent seasonal outbreaks of POMS have occurred consistently in estuaries where OsHV-1 has become endemic, and there is a high likelihood of spread of the virus to new locations. Therefore this disease will remain one of the greatest concerns for oyster farming into the foreseeable future.

Pacific oyster production in Australia was seriously impacted by the incursion of OsHV-1 into Tasmania during the course of this project. The devastating disease resulted in job losses and a restructure of the industry including the need to develop new hatcheries outside of the state. Movement restrictions impacted commercial farming operations and included important broodstock. A detailed outbreak investigation during the index cases in Tasmania identified risk factors for mortality that have formed the basis of important recommendations to farmers. These included new understandings about the susceptibility of different age and size classes of oysters to mortality and the importance of not handling stock during POMS risk periods. The project team then collaborated officially with further POMS research efforts in Tasmania.

Surveillance using frequent deployment and inspection of sentinel spat together with controlled field and laboratory experiments has now been continued over 5 disease seasons in NSW. Synthesised understandings from the sub-projects within this work have helped explain much of the apparent unpredictability in the incidence and severity of POMS. Transmission of OsHV-1 occurs from environmental sources in point source epidemic events. Particle attachment of the virus results in a marked spatial and temporal clustering of disease on the large and small scales of lease and bay. This refined understanding of OsHV-1 transmission reinforces the potential for long distance transmission to regions presently free from disease.

Risk factors for POMS arise in the environment, host and pathogen. The many combinations of these risks result in variation in disease expression from subclinical infection with OsHV-1 to 100% mortality. Intrinsic host oyster factors contribute most substantially to the outcome of OsHV-1 infection. The risk of disease is reduced with increasing age and size. Overlying this basic relationship is variation in susceptibility reflecting the physiologic and metabolic status of the individuals. These have the potential to be manipulated by farm management and growing infrastructure. Further, reduced occurrence of secondary diseases and survival of prior exposure to OsHV-1 can reduce the risk of mortality. Water temperature is a key environmental risk factor. The present study has established that elevation to an average daily on-lease temperature of 20°C in spring and decline below 17°C in autumn defines the start and end of the POMS risk period in NSW. Detailed monitoring of on-lease water temperature in other growing regions enabled the POMS risk period to be modelled. Knowledge of the environmental and host risk factors have been exploited by farmers to plan commercially viable production in OsHV-1 endemic estuaries using window farming and informed stock management calendars. With this study comes the first evidence that attenuated isolates of microvariant OsHV-1 might reduce the disease risk in the future.

Epidemiological research provides the key understandings needed to develop disease management strategies. These strategies are required to gain maximum performance from the best available stocks of oysters, in addition to any host resistance to disease. Disease minimisation strategies can also act at bay level to reduce OsHV-1 infection pressure, such that it does not build up to a level that overwhelms the resilience of oysters selectively bred for POMS resistance. Continued research is essential to refine the response to POMS as the impact on oyster farming continues. The specific and unique knowledge about OsHV-1 transmission and the risk factors for mortality of infected oysters described in this report requires incorporation into farm management at individual, industry and policy development level. These understandings are also critical for the design and interpretation of further POMS research and surveillance efforts.

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Acknowledgments

This project was funded by the Australian Government through the Fisheries Research and Development Corporation Industry Partnership Agreement with Oysters Australia, with additional funding from the University of Sydney and a cash contribution from NSW DPI. Support from oyster farmers was essential to provide valuable assistance and ongoing access to field sites for research. Thanks are due to Bruce Alford, Broken Bay Oysters, Hawkesbury River, Robert Hill, Endeavour Oysters in the Georges River, and Leon and Angela Reipsamen, Goodnight Oysters, Greenwell Point. Len Drake continued his energetic and knowledgeable support of the research team. The authors are grateful for the support of the technical assistance provided in the laboratory by Alison Tweedie, Vickie Patten, Anne-Michelle Whittington, Craig Kristo, Stuart Glover and Anna Waldron.

We gratefully acknowledge the support of Shellfish Culture Tasmania, Greg Kent, Southern Cross Shellfish, Port Stephens NSW for providing spat for the window of infection experiments and Seapa and BST for supply of oyster baskets. The assistance of the oyster farmers of Tasmania was invaluable in supporting the POMS outbreak investigation during 2016.

Abbreviations

OsHV-1 *Ostreid herpesvirus 1*

OsHV-1 µVar Microvariant Ostreid herpesvirus 1

POMS Pacific oyster mortality syndrome

qPCR quantitative Polymerase chain reaction

Executive Summary

Microvariant genotypes of *Ostreid herpesvirus 1* (OsHV-1) have emerged since the first detection in France in 2008 to impact Pacific oyster farming in Europe, Australia and New Zealand. This virus is the cause of a high mortality disease that has reshaped farming of Pacific oysters (*Crassostrea gigas*). The present report describes continued research at the University of Sydney that has been undertaken since OsHV-1 first impacted Australia. Several concurrent research methodologies have been employed to generate an understanding of the epidemiology of OsHV-1 and the disease it causes, Pacific Oyster Mortality Syndrome (POMS). Long-term engagement with farms in affected waterways has enabled descriptive studies of disease outbreaks to generate hypotheses about the disease that have been tested with replicated field trials and a controlled laboratory infection model. Improved understanding of the epidemiology of POMS enables rational disease control strategies to be developed for use on farms.

This project was developed based on the findings of previous research and in consultation with industry. Key knowledge gaps and translation of findings from previous research by the same group (FRDC projects 2011/053 and 2012/032) were addressed in the present project. The unpredictable nature of POMS has hampered efforts to farm Pacific oysters in the presence of OsHV-1 and to plan for an industry with an unknown distribution of the virus. There was a requirement to better understand factors which influenced the occurrence and severity of disease to enable decision making at individual farms and at industry and policy level.

The overall aim of better understanding the epidemiology of POMS was addressed through several aspects of the present research:

- Risk factors for occurrence of POMS were determined using a long-term intensive program of sentinel surveillance with deployment of spat and concurrent collection of environmental data.
- Risk factors in the setting of commercial farming were evaluated through detailed disease outbreak investigations of index cases. Descriptive studies of preliminary adaptive farming methods that incorporated window farming and stock management informed by monitoring of risk factors.
- Prediction of POMS risk in areas presently free of OsHV-1 was enabled by detailed data collection including high resolution water temperature monitoring at the location of oyster leases in all important growing areas.
- To better understand the host risk factors for mortality and determine if resilience of oysters to OsHV-1could be improved by Improving host oyster resilience to disease through manipulation of the age-size relationship and prior exposure to OsHV-1.
- Characterisation of OsHV-1 isolates to determine if differences in virulence or transmissibility of the pathogen could contribute to variable disease outcomes.

Methodology

Risk factors for temporal and spatial occurrence of POMS in endemic estuaries were investigated by continuation of a long term surveillance program. This intensive exercise required frequent deployment of Pacific oyster spat at 15 well characterised locations throughout the Hawkesbury and Georges River where OsHV-1 was endemic. The spat were assessed by regular counts of mortality and laboratory tests for OsHV-1 independent of the disease outcome. This window of infection study was continued form previous research enabling analysis of mortality and environmental data across 5 summers. Long-term patterns of seasonality and periodicity of infection were demonstrated and the site-specific and general environmental factors that impacted disease severity were evaluated. The researchers were highly engaged with field observations of experimental oysters alongside commercial production. This overview enabled synthesis of multiple datasets and revealed aspects of the unique OsHV-1 transmission mechanisms that explain the characteristic spatial and temporal clustering of disease epidemics. The unfortunate spread of OsHV-1 to Tasmania during the course of this project provided the opportunity for

a detailed outbreak investigation of the index cases. Detailed mortality counts and audits of farm records were analysed to reveal risk factors for disease expression.

With the identification of water temperature as a direct risk factor and strong predictor of mortality caused by OsHV-1, a monitoring program was established over several seasons to collect water temperature profiles at oyster leases throughout Australia growing regions. A standardised floating basket design for temperature probe deployment provided an assessment of the thermal experience of oysters. Collaborating oyster farmers deployed the probes and exchanged them via a mail-back system to provide data to a centrally curated database.

Commercial supplies of triploid Pacific oyster spat that were representative of the stock being used by industry were supplied by a collaborating hatchery (Shellfish Culture). These were managed according to commercial farming practices in regions free of OsHV-1 for later use in replicated field and controlled laboratory experiments. Some oysters were specifically managed to manipulate the age-size relationship by altering growing conditions. A combination of field and laboratory exposure to OsHV-1 was undertaken to utilise the advantages of each virus challenge method. The realistic disease outcomes provided in the complex environment of field exposure were combined with highly replicated experiment design to account for new knowledge about the clustering of OsHV-1 transmission. Laboratory experiments ensured environmental factors and the timing and dose of OsHV-1 were controlled so that individual risk factors could be evaluated independent of confounding variables. For example, the laboratory infection model was used with fine scale water temperature control to assess the susceptibility of oysters re-challenged with OsHV-1 at a temperature permissive for disease expression after survival at lower temperature. A combination of challenge by injection or cohabitation exposure balanced the advantages of greater control of exposure with reduced assessment of the complete pathophysiology of infection.

The diversity of OsHV-1 genotypes in Australia was evaluated with a 6 target multi-locus sequence analysis of 107 clinical samples using previously described well characterised amplification targets to facilitate global analyses. An evaluation of strain variation was achieved for a select number of genotypic diverse samples that were isolated and evaluated for virulence and transmissibility in a controlled *in-vivo* laboratory experiment.

The culmination of the knowledge of the epidemiology of the disease was combined as advice to farmers attempting commercial production of Pacific oysters in an endemic estuary. Three crops in the Hawkesbury River were monitored in a descriptive study describing the use of window farming and stock management calendars that were informed by all available knowledge of environmental and host risk factors for POMS.

Results

Across 5 summers of sentinel surveillance in the Georges and Hawkesbury Rivers, the earliest and latest date of disease occurrence was 28th October and 14th May. The onset date showed minmal variation across years. Mean daily water temperature in the oyster baskets rose above 20°C in mid-October and fell below 17°C after mid-May. These temperatures were taken to define the seasonal risk for POMS. It was noted that the surveillance sensitivity was not sufficient at the intra-estuary level to capture all OsHV-1 transmission events, despite the use of 15 sites. Disease investigations in commercial and research oysters detected additional incidence of POMS, consistent with the clustered nature of this disease. Mortality due to OsHV-1 in sentinel spat was diminished after 2013, although the transmission of the virus appeared to remain constant every year. Mortality due to diseases other than OsHV-1 observed in all years and was highest in the summer of 2015-16. Transmission of OsHV-1 occurred at all sites except Patonga Creek, indicating that the range of benthic communities, freshwater in-flow and oceanic influences were not important risk factors. However, disease did not occur at 1 of 2 sites at Kimmerikong which was partitioned by a tidal barrier. Taken together with the observation at Patonga Creek this suggested that local hydrodynamics are important in OsHV-1 transmission. The final season of the spat surveillance indicated more frequent incidence of POMS in spat that were unselected compared to a co-deployed POMS resistant line of spat, although the degree of mortality was similar when disease occurred in both batches.

Interstate water temperature monitoring predicted that the duration of POMS risk was longer than the Hawkesbury River in Northern NSW estuaries, and slightly shorter in Southern NSW estuaries that are not yet impacted by POMS. The window of infection in Tasmania is substantially shorter than NSW. This creates more profitable options when considering window farming and stock management calendars. In South Australia, the water temperature profile suggests a POMS risk period of similar duration to NSW.

Challenge of spat with OsHV-1 at a sub-permissive water temperature and titration of the dose of virus was able to generate a high proportion of PCR positive survivors of disease. However, spat <6 months of age that survived pre-exposure did not have a survival advantage when rechallenged with OsHV-1. Preliminary experiments using spat >6 months of age and >5 cm shell length indicated that surviving an OsHV-1 challenge at 18°C conferred a specific reduction in the hazard of mortality when the oysters were subsequently challenged at 22°C compared to oysters pre-exposed with an OsHV-1 free tissue inoculum or no pre-challenge.

Farmers are able to make stock management decisions based on the protective effect of increasing age. The hazard of death for oyster spat (8 months old) was 5.5 times that of adult oysters (17 months old) after adjusting for variation in exposure due to location in the field. However, accessing the substantial decline in susceptibility to POMS that is observed over 12 months of age requires an OsHV-1 free growing location for at least one summer season. In managing stock of any given age, the oysters which grow larger under standard growing conditions are more likely to survive POMS. The hazard of death for smaller spat and adult oysters was 1.9 times that of larger individuals from the same groups. However, no advantage was conferred by attempting to manipulate the growth of oysters to produce larger oysters at a given age. Laboratory trials using oysters prospectively differentiated for size indicated that growth restricted (smaller) spat were relatively protected (hazard ratio 0.6) compared to those grown to a larger size. Conversely, for adult oysters the hazard of death was higher (HR=2.3) for the growth restricted (smaller) oysters. Further investigation is required to understand how energetic reserves and physiological condition impact on POMS disease expression before attempting to manage disease by altering growth.

Implications and Recommendations

Improved understanding of the potential for long range transmission of OsHV-1 indicate that disease management plans should consider the potential for the majority of growing areas in Australia to be impacted in the future. When OsHV-1 is present in a region it becomes endemic and causes seasonally recurrent disease. The attenuation in disease severity observed with surveillance using sentinel spat most likely reflects reduced stocking density in NSW estuaries.

Farmers need to develop stock management calendars and window farming that incorporates known risk factor information to minimise production losses caused by POMS. Considerations include the on-lease thermal profile, local hydrodynamics, the age-size profile of oysters and the exposure history of the stock to OsHV-1. To maximise production with advantages conferred by stock selectively bred for resilience to POMS requires management of disease at bay level. Coordinated stock management will be important in reducing the OsHV-1 infection pressure to maximise the advantage conferred by genetically resilient stock.

This research has highlighted key areas for further research activity that are likely to assist Pacific oyster farming:

- There may be opportunities to increase the resilience of spat > 6 months of age through controlled pre-exposure to OsHV-1. Further research considering disease pathogenesis and immune response is necessary to underpin studies which might provide a practical disease management tool.
- Epidemiologic approaches to understanding of the risk factors for diseases other than POMS that cause of mass mortality of Pacific oysters are required. Integrated approaches to disease management that consider methods of reducing the impact of all possible disease threats concurrently will be essential to assist farmers in the future.

- Surveillance for low virulence genotyopes of OsHV-1. Attenuated strains of the virus should be isolated and investigated to determine how infection with a low virulence OsHV-1 isolate might alter or prevent infection with more virulent genotypes.
- Further evaluation of the local hydrodynamic effects on transmission of OsHV-1 should be investigated in new environments where OsHV-1 becomes endemic. Consultation with farmers to develop and evaluate growing infrastructure that minimise the impacts of known risks for high mortality disease is warranted in conjunction with this objective.

Keywords

Pacific Oyster Mortality Syndrome (POMS), Ostreid herpesvirus 1 (OsHV-1), Pacific oyster *Crassostrea gigas*

1. Introduction

The emergence of microvariant genotypes of *Ostried herpesvirus 1* (OsHV-1) has devastated Pacific oyster (*Crassostrea gigas*) production in Europe, Australia and New Zealand (EFSA, 2015). The socio-economic impacts have been substantial with OsHV-1 spreading to new locations and causing recurrent seasonal outbreaks of disease when endemic (EFSA, 2015; Pernet et al., 2016). The disease caused by OsHV-1 is referred to as Pacific Oyster Mortality Syndrome (POMS) in Australia. The present impacts of recurrent high mortality in affected regions and the potential for spread of OsHV-1 into additional regions make it one of the greatest threats to the oyster farming industry in Australia at present. In Australia, the species supports a productive industry with an established market that has a farm gate value of AUS$86million (ABARES 2016). If OsHV-1 continues to spread adaptations to limit the impact of POMS are essential to maintain this market.

Microvariant genotypes of *Ostreid herpesvirus-1* (OsHV-1) are members of the family *Malacoherpesviridae* from the order *Herpesvirale (Davison et al., 2005).* The first OsHV-1 microvariant (OsHV-1 µVar) was detected in France in 2008 as the causative agent for mass mortality of farmed *C. gigas* (Segarra et al., 2010). Despite attempts to constrain this notifiable disease, microvariant genotypes of OsHV-1 have spread rapidly across the globe where outbreaks have occurred in Europe (Roque et al., 2012), New Zealand (Keeling et al., 2014) and Australia (Paul-Pont et al., 2014b). The microvariant genotypes have predominantly replaced the reference genotype in France to become the dominant cause of mortality events in *C. gigas* (Renault et al., 2012). Microvariants of OsHV-1 are defined by a deletion in the microsatellite locus upstream of open reading frame (ORF) 4 compared to the reference OsHV-1 genotype (GenBank accession number AY509253) and several point source mutations including polymorphism in ORF 4 (C region) and ORF 42/43 encoding an inhibitor of apoptosis (OIE, 2014).

OsHV-1 was first detected in Australia in November 2010, causing >95% mortality of farmed *C. gigas* in the Georges River, NSW (Jenkins et al., 2013b). Recurrent high mortality disease occurred subsequently in the Georges River and despite biosecurity measures, in 2013 an outbreak of extensive disease occurred in the Hawkesbury River (Paul-Pont et al., 2014b). Subsequently the virus entered Tasmania in 2016 disrupting farming and causing extensive mortality in the country's most important spat producing state (de Kantzow et al., 2017). There have been significant social and economic impacts due to substantially reduced production in regions where OsHV-1 is endemic. Job losses and flow on effects impacted uninfected regions such as South Australia through a lack of hatchery spat. Pacific oysters represent a large proportion of global edible oyster production.

Disease expression is unpredictable due spatial and temporal clustering of mortality in regions where OsHV-1 has become endemic (Pernet et al., 2014; Whittington et al., 2015a). The variability in disease expression can range from 100% mortality to subclinical infection (Evans et al., 2017; Paul-Pont et al., 2013b). This is partially explained by the known risk factors that contribute to the multifactorial relationship between OsHV-1, the environment and the host (Paul-Pont et al., 2013a; Pernet et al., 2012b). Water temperature is a key environmental risk factor with mortality events observed between 16°C -24°C in Europe (Oden et al., 2011; Petton et al., 2013; Renault et al., 2014b) and 22°C - 25°C in Australia (Paul-Pont et al., 2013a). Attempts to modify the host oyster and environmental risk factors can alter the disease outcome have shown some success. For example, raising the growing heights of intertidal trays and baskets to decrease immersion time (Whittington et al., 2015b).

OsHV-1 is capable of causing mortality in all age and size classes of *C. gigas* (Azema et al., 2017; Degremont, 2013; Paul-Pont et al., 2014b; Petton et al., 2015). However, lower age and size are key host risk factors for increased mortality. In France, OsHV-1 predominantly affects *C. gigas* spat (defined as oysters <12 months old), with successively lower rates of mortality reported in juveniles (oysters 12–24 months old), followed by adults (oysters > 24 months old) (EFSA, 2015; Oden et al.,

2011). In Ireland, a successive decrease in OsHV-1 associated mortality was reported between *C. gigas* spat, and small and large oysters, with average mortalities of 50%, 34%, and 14% reported, respectively (Lynch et al., 2011; Lynch et al., 2012). Similarly in Ireland, OsHV-1 resulted in mortalities of 55% in oyster spat (defined as oysters weighing<5 g), compared with 18% mortality in juveniles (oysters weighing 5–40 g), and 25% in adults (oysters weighing>40 g) (Peeler et al., 2012). During the first OsHV-1 outbreak that occurred in the Hawkesbury River, Australia, mortalities>50% were reported for oysters up to 2 years of age or 140mm shell length, but mortality was highest in smaller or younger oysters (Paul-Pont et al., 2014b). Lower mortalities were observed in larger oysters (shell length 61–115 mm) compared to smaller oysters (shell length 0–20 mm) during the first OsHV-1 outbreak that occurred in south-eastern Tasmania, Australia (de Kantzow et al., 2017). These index cases in Ireland and Australia reveal the susceptibility of older/larger oysters to OsHV-1 associated mortality independent of the effects of prior exposure to OsHV-1, which can be exacerbated by other pathogens (Azema et al., 2016). A more complex situation occurs in endemic regions because oysters that have survived a disease event subsequently experience lower mortality when re-exposed to OsHV-1 (Evans et al., 2017), a circumstance that leads to apparent higher survival in larger/adult stock. Several additional host factors contribute to the expression of OsHV-1 disease in addition to the age and size of oysters. These include the genotype (Degremont, 2013; Dégremont et al., 2010) and energy reserves (Moreau et al., 2015; Tamayo et al., 2014). The presence of food has been shown to increase the hazard of death due to water-borne exposure at low and high doses of OsHV-1 (Evans et al., 2015b).

Considerable genotypic diversity has now been documented within the OsHV-1 virus species from throughout the world (Bai et al., 2015; Martenot et al., 2015; Mineur et al., 2015). To date there is no information about potential strain differences between genotypes and the extent to which the nature of the OsHV-1 isolate might affect disease outcomes. Since the emergence of the disease in the Georges River, Australia in 2010, there has been extreme selection pressure on OsHV-1 due to the greatly restricted host density. This may have driven genotypic diversification and possibly favoured a less virulent viral phenotype.

2. Objectives

1. To determine methods for the conditioning/husbandry of spat and juvenile oysters to obtain survival after exposure to OsHV-1 based on improved scientific understanding of exposure, pathogenesis, immunity, tolerance or latency

2. To confirm i) the consistency of seasonal patterns of POMS, ii) the periodicity of infection within season, iii) inter-estuary temperature variation, and iv) predict POMS seasonal behaviour.

3. To identify changes in OsHV-1 DNA sequence over time (2010-2016) to understand infection and disease patterns

4. To describe an integrated disease control strategy based on complementary use of genetically resistant oysters (when available) and husbandry methods throughout the production cycle: hatchery-juvenile-growout to market

5. To build capacity in aquatic animal health for Australian industry through training a post graduate student

The objectives from the original project application were revised following milestone report 4 and after the POMS outbreak in Tasmania to ensure resources were focused on relevant objective.

Project variation (1/2/2017)

Additional objectives were added subsequent to the outbreak of POMS in Tasmania.

6. Collaboration with University of Tasmania CRC-P Project. Individual items subject to opportunity and need:

6.1 Join steering committee for CRC-P project at UTAS

6.2 Collaborate on design and analysis for outbreak investigation; USyd PhD student Max de Kantzow will assist UTAS with implementation subject to inclusion of data in his thesis and the cost centre except travel in UTAS budget, with joint publication

6.3 Epidemiology advice and collaboration in window of infection and farm management practices

6.4 Virology collaboration/training in OsHV-1 PCR and aquatic PC2 lab, advice on protocols and interpretation of results, better understanding of OsHV-1

6.5 Advice on POMS data harmonisation with the Yield;

6.6 qPCR testing services for OsHV-1 from UTAS budget and professional interpretation

6.7 Collaboration and joint publications.

3. Method

The methods and results are described according to numbered sub-headings which describe the different project activities that align to each objective.

3.1. The influence of size and age on susceptibility of juvenile Pacific oysters to OsHV-1

Background

Observations during natural outbreaks of disease caused by OsHV-1 indicate that mortality of oysters is lower for older and larger oysters (Paul-Pont et al., 2013a; Paul-Pont et al., 2014a).

Objective

The purpose of this study was to determine if the age and size of Pacific oysters have independent effects on susceptibility to disease caused by OsHV-1. Differential susceptibility of oysters at ages that are important for management of this seasonal disease in endemic estuaries was measured. Further, it was determined if the size of oysters within an age class altered susceptibility to infection and disease. These data were investigated to determine practical management solutions for farming Pacific oysters in waterways where OsHV-1 is endemic.

Materials and methods

Oysters

Experiment 1. Retrospective differentiation of size by age

Triploid Pacific oysters were recruited from commercial stock at a farm in the Shoalhaven River (Goodnight Oysters, Greenwell Point, NSW, Australia). This growing region and the location of the hatchery that produced the spat were declared free of OsHV-1 (Animal Health Australia, 2011). A subsample of the oysters tested negative for OsHV-1 at the time of recruitment (n=60). The two batches used (SPL13A and SPL12FT) were 8 and 14 months of age at the start of the trial, respectively (Table 3). These oysters had been grown in plastic cylinders according to routine commercial husbandry practices with regular grading for size within each batch. Oysters were graded using a Shellquip machine set to provide 8-9% frequency distribution in the smallest size category based on shell length. A selection of the largest and smallest oysters from each batch were collected by convenience (n=700).

Experiment 2. Purposive differentiation of size by age

Triploid Pacific oysters were sourced from batches SPL14B and SPL13B that were produced at the same hatchery in Tasmania (Shellfish Culture) as the previous batches (Table 3). These oysters represented standard commercial stock available to farmers at the time and were not purposefully selected for disease resistance. A representative selection of 2500 oysters from each batch was obtained at the time of sale of the spat to growers and transferred to the OsHV-1 free growing region in the Shoalhaven River. Half of the baskets from each batch were placed on fixed longlines at standard growing height and the other half on a line 450 mm higher for decreased immersion time. This provided for differential growth of both age groups over a period of 6 months prior to field deployment and 8 months prior to laboratory challenge (Table 3). The batches were 8 and 17 months at the time of field exposure to OsHV-1.

Experiment 3. Extreme size restriction

An opportunistic experiment aimed to determine the susceptibility of oysters with extreme growth restriction to mortality from OsHV-1. This experiment was possible through use of oysters with extreme growth restriction by virtue of long-term retention in hatchery upwellers in Tasmania. The experiment was justified by the outcome of the age-size trial, where a modest increase in survival after OsHV-1 challenge was noted in 8 month old oysters with restricted growth. The age-size trial is described below (Batch effects).

A subset of batch SPL13B was recruited from Shellfish Culture, Tasmania. At the time of laboratory and field challenge with OsHV-1, these oysters were 16 months of age and approximately 12 - 30 mm shell length. A laboratory challenge by immersion was conducted and for a field challenge oysters were deployed directly to Site C in the Georges River in socks in floating baskets (4 replicates of approximately 50 oysters) in January 2015.

Field challenge

Field deployments were conducted in February-May 2014 for retrospectively recruited oysters and January-March 2015 for the prospectively recruited oysters. The sites A, B and C in Woolloware Bay, Georges River, NSW that were described in Whittington et al., (2015a) were used. POMS had recurred seasonally at these locations since 2011 (Whittington et al., 2015a). Each experiment included 48 baskets on fixed long lines, 300 mm above standard growing height. Oysters within each age-size category were mixed thoroughly and then distributed by convenience to approximate random sampling into 12 replicate 15 L plastic baskets (Seapa, Edwardstown, Australia) per site, each containing 50 oysters. Basket position was determined according to a systematic random strategy to allocate the first four positions to each age-size category such that treatments from the same batch (age group) were positioned in adjacent baskets. The order of the four treatments for replicate 1 was repeated along the lines for the remaining 5 replicates, and the pattern was repeated at the second site. The baskets were placed at Sites A and C for Experiment 1 and Sites B and C for Experiment 2 based on knowledge of disease occurrence during each season from concurrent research. Baskets were left *in-situ* for 12 weeks and inspected every 2 weeks during which time dead oysters were counted and removed. Dead oysters were categorized as recently dead or dead based on observation of residual soft tissues or only shell remaining. A selection of fresh dead oysters at each time point were tested for OsHV-1. At the completion of the trial a random selection of surviving oysters was sampled for qPCR (n=30, treatment group).

Laboratory challenge

Two laboratory challenge experiments were conducted to assess the susceptibility of the retrospectively and prospectively recruited oysters of each age-size category to a measured dose of OsHV-1 administered by injection or adsorption (immersion). Experiment 1 (April 2014) and Experiment 2 (March 2015) were conducted according to the same trial design such that the oysters were 2 months older from the time of field challenge (Table 3).

Management. Oysters from the four treatment groups were randomly allocated to 6 replicate tanks and 1 control tank (n=13) in a level 2 physical containment (PC2) aquatic animal facility. Each 15 L replicate tank contained an entirely separate volume of aerate artificial seawater (30-31 $g.L^{-1}$ salinity; Red Sea) maintained at 20°C + 1.5°C, pH 8.2 (range 8.0 – 8.8), ammonia, nitrite and nitrate levels <0.25 ppm with constant aeration. Water quality parameters were measured each day and adjustments were performed as required, with total water exchange every 48 hours. A maintenance ration of Instant Algae® Shellfish Diet 1800 (Reed Mariculture, USA) was provided once a day according to the directions of the manufacturer (http://www.reedmariculture.com/support_feeding_shellfish.php). A 2 day acclimation period preceded infection challenge.

Challenge with OsHV-1. An inoculum of freshly amplified OsHV-1 was prepared immediately prior to each experiment. Juvenile Pacific oysters were relaxed in 50g.L^{-1} $MgCl_2$ and 0.1 mL of a cryopreserved tissue homogenate containing the isolate OsHV-1_Georges River 2011 was injected into the adductor muscle (n=8). After maintenance of these oysters at 20°C for 48 – 60 hours, the mantle and gill tissue was collected and prepared as a clarified tissue homogenate according to the previously described methods (Hick et al., 2016). A negative control tissue homogenate was produced after injection of oysters with an OsHV-1 free oyster tissue.

The number of copies of OsHV-1 DNA in the homogenates was estimated by qPCR. A dilution in sterile artificial seawater was prepared immediately prior to injection to provide high and low doses of 10^6 or 10^5 genome copies per oyster. Experimental oysters were relaxed by immersion in 50g/L $MgCl_2$ for 1 – 4 hours before challenge by one of two routes of administration: (i) injection into the adductor muscle a volume of 50 µL (small oysters) or 100 µL (large oysters); or (ii) adsorption, whereby the total dose was mixed in a 0.5 mL volume of ASW and placed over the gill and mantle tissue after emptying the pallial cavity of liquid. This inoluculum was left *in-situ* for 10 min before returning to the oysters to the maintenance tanks.

Observation and sampling. Oysters were checked twice a day and dead and moribund individuals were removed and stored at -80°C. Oysters were considered to be dead when they were open, non-responsive to disturbance of the tank and did not retract the mantle following stimulation with a 25 gauge (25G) needle.

Laboratory methods

Samples for OsHV-1 detection. A combined sample of mantle and gill (0.08 – 0.12 g) from each individual was collect using aseptic technique and placed in a 2 mL laboratory tube with 1 mL water (Ultrapure) and 0.4 g of 0.1 mm silica-zirconia beads (Daintree Scientific). Tissues were disrupted by bead beating using a TissueLyser II machine (Qiagen®, Chadstone, Australia) for 2 min at frequency 30, repeated after rotating the insert 180°. Tissue homogenates were centrifuged at 900 x *g* for 10 minutes and nucleic acids were purified from a 50 µL aliquot of the supernatant using a MagMAX-96 Viral RNA Isolation Kit (Ambion®, Life Technologies). Extraction was performed according to the directions of the manufacturer using the AM-1836 deep-well standard program on a MagMAX Express-96 magnetic particle processor (Applied Biosystems, Life Technologies).

Quantitative (q)PCR. Individual oysters were tested for OsHV-1 and the number of copies of the B-region of the OsHV-1 genome were quantified as previously described (Paul-Pont et al., 2013a), with oligonucleotide primers and probe described by Martenot et al. (2010). Samples were tested in duplicate 25 µL reactions containing 5 µL of template DNA; 900 nM each of the forward primer (OsHV1BF, 5'- GTC GCA TCT TTG GAT TTA ACA A -3') and reverse primer (OsHV1B4, 5'-ACT GGG ATC CGA CTG ACA AC-3'), 250 nM probe (5'-6FAM-TGC CCC TGT CAT CTT GAG GTA TAG ACA ATC-BHQ1-3') with the AgPath-ID qPCR master mix (Life Technologies). Thermocycling parameters were: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 45 s and fluorescence data was analysed with an Mx3000p qPCR system (Stratagene).

Statistical analysis. Total cumulative mortality was calculated as the proportion of dead oysters with 95% exact binomial confidence limits and compared between groups with Chi-squared test (Epitools: http://epitools.ausvet.com.au/content.php?page=chi_sq1). The quantity of OsHV-1 DNA determined by qPCR for oysters in the laboratory trials were $\log_{10}$ transformed to satisfy the assumption of normality and compared across different treatments using a restricted maximum likelihood, linear mixed model (REML) (GenStat, 16th Edition, 2015 VSN International Ltd, Oxford, United Kingdom). Water temperature, dose of virus and age of the oyster were considered as fixed effects with tank as a random effect. Pairwise comparisons of estimated means used the least significant differences with significance accepted at $p<0.05$.

Separate survival analyses were conducted for each experiment and observational study. The survival time was defined as the number of hours from OsHV-1 exposure until mortality for oysters that tested positive for OsHV-1 by qPCR for the laboratory exposures. For the field trials, survival time was the number of days in the endemic estuary until death for oysters in groups that tested positive for OsVH-1. Oyster surviving at the end of the experiment or the observation period were right censored and considered to have a survival time greater than two times the incubation period of OsHV-1. Kaplan-Meier (KM) survival curves were plotted for each treatment group and compared using log-rank test. Further Cox proportional hazards (PH) models with shared frailty to account for clustering were fitted for each dataset using PHREG procedure in SAS statistical program (Version 9.4© 2002-2012 by SAS Institute Inc., Cary, NC, USA). Interactions were tested between age and size and between dose and method and retained, if significant. Hazard ratios and their 95% confidence intervals were presented.

3.2 Pre-exposure of Pacific oyster spat to OsHV-1

3.2.1 Spat conditioning Experiment 1: Conditioning by prior exposure to generate OsHV-1 survivor spat

Background

Field observations indicate that individual oysters which survive outbreaks of POMS have survival approaching 100% during subsequent outbreaks (Pernet et al., 2012a; Whittington et al., 2015a). In a laboratory challenge, some oysters that survived injection with a low dose OsHV-1 appeared to have become infected and the virus had replicated (Paul-Pont et al., 2015b). Survivors of OsHV-1 challenge by cohabitation and immersion were also positive for OsHV-1 DNA (Evans et al., 2015a; Hick et al., 2016). The susceptibility to infection of these survivors of a laboratory challenge to subsequent OsHV-1 has not been assessed. In addition to being an innate quality of the individual, subsequent survival might involve a form of immune priming (Green and Montagnani, 2013). The persistence of small quantities of OsHV-1 DNA might indicate a persistent infection which is protective against subsequent lethal infection.

Observations of survival after repeated exposure to OsHV-1 have not included spat. Young oysters are most relevant for disease management strategies using interventions based on deliberate exposure.

Aims

To identify an OsHV-1 exposure regime that would generate a high proportion of PCR positive surviving Pacific oyster spat. To assess the susceptibility of surviving spat to repeated OsHV-1 exposure.

Materials and Methods

Oysters

Triploid Pacific oyster spat from a commercial hatchery (Shellfish Culture, Tasmania). SPL14B were produced in May 2014 and were approximately 5mm total shell length and 7 months of age at the time of challenge. These oysters were obtained in the same condition in which they were supplied for commercial farming.

OsHV-1 Challenge

An inoculum of freshly amplified OsHV-1 was generated according to the method described by Hick et al. (2016). The source of OsHV-1 was a cryopreserved filtered tissue homogenate from oysters infected during a field outbreak of POMS in the Georges River in 2011 (V171). This virus preparation (n=12) or a negative control tissue homogenate (n=3) was injected into the adductor 8 month old

Pacific oysters. These donor oysters were also from Batch SPL13B, but had been grown to 6 cm length in the Shoalhaven River, which was declared free of OsHV-1. After 72 hours in artificial seawater (ASW) at 20°C with daily feeding, the mantle and gill tissue of all challenged oysters was prepared as a 1/10 w/v, 0.2 µm filtered tissue homogenate in ASW. The inoculum was held at 4^0C and the concentration of OsHV-1 DNA was quantified by qPCR before use.

Spat were divided into groups of approximately 250 by weight and then relaxed in 50 mg/L $MgCL_2$ for 4 hours at 20°C, after which time the majority were completely open. Immersion challenge was conducted in a plastic container with the final dilution of the OsHV-1 inoculum prepared in ASW (Table 1). The dose of OsHV-1 was titrated using 4 x 10-fold dilutions from of $1/10^2$ to $1/10^5$ final w/v OsHV-1 inoculum in ASW and $1/10^3$ final dilution of the negative control tissue homogenate (Table 1).

The spat were held with occasional mixing for 4 h without feed during which time the relaxation effect of magnesium chloride resolved in the majority of spat.

Maintenance of Oysters

A physical containment Level 2 Aquatic Facility was used for all procedures. Each replicate group was maintained in individual aquaria that did not share water and lids were used to reduce cross contamination. Artificial seawater (ASW, Red Sea Salt) was prepared at salinity of 30 $g.L^{-1}$ and the temperature was maintained at 20 $\pm$1.5°C. Aeration was continuous and a full water exchange was undertaken every 48 h. Feed was supplied once a day as a maintenance ration of a commercial algae concentrate (Shellfish Diet 1800, Reed Mariculture).

Dead oysters were counted and removed with daily examination and a sample of live oysters was taken on day 6 post-exposure. Remaining spat were kept under the same husbandry regime until 12 days after the first exposure and the number of live oysters was counted and further samples were taken. Samples of whole spat were stored at -80°C.

Detection and quantification of OsHV-1

Spat were prepared as pools of 0.1-0.2 g of tissue in 1.5 mL distilled water (Ultrapure) according to methods previously described by Evans et al. (2015a). Briefly, tissues were homogenized by bead beating with 3 mm stainless steel balls (Aussie Sapphires) and nucleic acids were purified using the MagMax-96 Viral Isolation Kit (Ambion, Life Technologies). The number of copies of the B-region of the OsHV-1 genome was estimated using a hydrolysis probe qPCR protocol adapted from Martenot et al. (2010) as previously described (Paul-Pont et al., 2013a).

3.2.2 Spat conditioning Experiment 2: Water temperature manipulation to alter survival of spat during OsHV-1 challenge

The aim of this experiment was to evaluate the impact of a reduction on water temperature below that which is permissive for OsHV-1 at different times after infection challenge on the survival of spat. Further, the susceptibility of surviving spat to subsequent challenge was compared with naïve spat and those which had undergone temperature fluctuations with OsHV-1 challenge.

Methods

Triploid Pacific oyster (*C. gigas*) spat (2mm) were recruited from a commercial hatchery and acclimated for 7 days prior to the trial. They were kept in artificial seawater (Redsea salt) at 30 $g.L^{-1}$ with biological filtration and limited water exchange to maintain water quality and fed daily with a commercial algae concentrate (Shellfish diet 1800, Reed Mariculture).

Fresh OsHV-1 inoculum was produced by injecting juvenile oysters with cryopreserved OsHV-1 produced from a gill and mantle sample taken from the initial outbreak of OsHV-1 in the Georges River as previously described (Paul-Pont et al., 2015a). A similar procedure was used to produce inoculum from the OsHV-1 negative donor oysters.

OsHV-1 infection challenge

Spat were relaxed for 1 hour in a 50gL^{-1} solution of magnesium chloride in fresh aquarium water. Residual magnesium chloride was removed by rinsing spat in ASW before immersion in a 1 in 1000 w/v dilution of the 0.2 µm filtered positive or negative control inoulum in ASW. Spat were challenged for 4 hours by placing 1800 spat in a single layer at the bottom of a vessel with per 250 mL liquid. The OsHV-1 challenge was repeated for surviving spat in two tanks for each treatment group 28 days after the first challenge as indicted in Figure 1. The remaining tank from each group was exposed to an OsHV-1 free control inoculum.

Water temperature profile

The water temperature profile for each treatment was a reduction from 22°C to 14°C at 4, 8 or 12 hours post exposure, and a control group with no reduction in temperature (Figure 1). The change in water temperature was timed from the middle of the OsHV-1 exposure period. The lower temperature was held for 14 days. The water temperature was increased to 22°C on day 14 for all treatment groups that were returned to a water temperature of 22°C for the remainder of the trial period.

Sampling

A sample of 150 oysters was randomly selected across all treatment groups prior to OsHV-1 exposure to ensure freedom from previous infection. A sample of n = 104 oysters (or 1 gram) from each treatment group was randomly selected for quantification of OsHV-1 DNA immediately prior to initial OsHV-1 exposure and at 24 hours, 13 days and 15 days post initial exposure. A selection of dead oysters with tissues present was sampled for quantification of OsHV-1. At the conclusion of the trial mortality in each tank was determined and all oysters in all treatment groups were sampled for quantification of OsHV-1.

Quantification of mortality

Total cumulative mortality was determined in each tank on Day 13, prior to the temperature change, and on Day 28, prior to the second exposure. Visual inspection for mortality was extremely slow because many dead oysters remained closed and was limited to just 1 tank per treatment group. These counts were used to validate a procedure to estimate survival based on the mass of the oysters by determining the average weight of dead shell and confirmed live oysters:

$$Survival = \frac{(Sample\ mass/n) - Avg(Empty\ shell\ mass)}{Avg(live\ oyster\ mass)}$$

Where:

Survival (%) is the estimated proportion of live oysters

Sample mass (g) of all oysters with unknown mortality and n is the number of oysters.

Avg(Empty shell mass) is the average mass of dead oysters from the group (empty shell)

Avg(live oyster mass) is determined from the surviving oysters during validation.

Data were analysed as total cumulative mortality = 1 – Survival (%)

Statistical analysis

Statistical analysis was performed using SAS version 9.3 (SAS institute, Carey, NC, USA) and Microsoft R Open (R Core Team, 2016). Data were stored, aggregated and summarized using Microsoft SQL Server 2016 (Microsoft Corporation, Redmond, WA, USA). Exploratory statistical analysis was done graphically using ggplot2 in R and numerically using SQL Server 2016 and R.

3.2.3 Spat conditioning Experiment 3: Pre-exposure of Pacific oysters > 6 months of age and re-challenge OsHV-1

Aim

To evaluate the susceptibility of 6 month old Pacific oyster spat that had survived previous exposure to OsHV-1 when re-exposed in a controlled laboratory experiment. Further, to determine if low water temperature at the time of initial exposure might increase resistance to subsequent disease at the most permissive water temperature.

Materials and Methods

Oysters and management

Pacific oysters (~6 months, 40 mm, Batch SPL16A) were originally produced at a hatchery in Tasmania and were grown under commercial conditions in a waterway in which POMS has not been recorded. They were held in tanks with 12L artificial sea water (Red Sea Salt, 30 $g.L^{-1}$) and individual biofilter and aeration units. The oysters were fed algae concentrate (Reed Mariculture 1800) and a 12 hour photoperiod was used. The water temperature profile of each tank was controlled by immersing each tank in a water bath that was controlled by a heater chiller unit.

OsHV-1 challenge

Oysters were relaxed in 50$g.L^{-1}$ magnesium chloride and challenged by injection into the adductor muscle with 100 µL of an inoculum made of a 1/1000 dilution of a cryopreserved stock of OsHV-1. The control oysters were given an injection of an inoculum made from oysters with no history of OsHV-1 and which were negative for the virus when tested by qPCR.

Experiment design

All groups were acclimated to their first challenge temperature for 5 days prior to the injection. The water temperature profile was then maintained according to the design indicated in Table 1. Changes in water temperature from 18°C to 22°C occurred over 6 hours, 14 days after OsHV-1 challenge for some treatment groups. A period of 21 days was allowed to determine subsequent mortality before the surviving oysters were challenged for a second time with OsHV-1. Final cumulative mortality was determined 14 days after the second infection challenge.

Dead and moribund oysters were removed during inspections every 12 hours. Dead oysters and those collected live at the end of the trial were stored frozen until processed for viral quantification by qPCR.

3.3 Interstate temperature monitoring

Temperature data loggers (Thermocron DS1921G, Thermodata) were deployed with cooperation from oyster growers on farm leases in Tasmania, South Australia and New South Wales progressively from 18 October 2014; this was prior to the official start of the project in August 2015. This was to maximize the chances of recording a continuous stream of data covering relevant periods over several years. Probes were configured to record temperature every 72 mins, enabling about 100 days of recording capacity. Growers were asked to deploy probes in a systematic way so as to record water temperature on leases at a depth of about 200 mm; probes were deployed attached to the base of a floating basket on a floating long line (Figure 2.1), except for one probe in Tasmania that was placed in a deep-water lease (Freycinet, TAS6). Two probes were prepared for each lease site to enable a mail back system. About every 90 days growers were requested to mail one probe back to Richard Whittington at Camden using pre-addressed Australia Post express post mailbags, and deploy the second probe. For each cycle the researchers contacted growers by letter and text message to ensure real time communication.

Probes were downloaded into proprietary software (Thermodata) in Access (Microsoft), then exported to Excel (Microsoft) and Statistica (StatSoft Inc) for manipulation. Generally the paper records submitted by the oyster farmers included the deployment and retrieval times and dates at each site. The data were edited to remove records that did not correspond to water temperatures, for example if probe deployment was delayed due to bad weather and probes were kept in an oyster shed. Average daily temperatures were plotted for each site over time.

After each probe download, each grower was sent the data for his/her site since the start of the trial, as well as the average water temperature for the state, in graphical format. Data for all sites were uploaded progressively onto the project website www.oysterhealthsydney.org. The project website showed the geographical location of each site on a map, and from there it was possible to view all of the results; data tracking confirmed the website was regularly accessed from multiple computers in Australia during the project.

The reproducibility of temperature records between probes was confirmed in separate experiments and the co-efficient of variation between probes was < 2.5% for water temperatures in the range 10-50°C, and <14% at 5°C.

Significant water temperatures for risk of mortality due to OsHV-1 were determined separately in epidemiological studies in NSW, including in the Window of Infection trial which was conducted between the 2012-13 and 2016-17 summer seasons. The risk of mortality commenced when water temperatures rose above 20°C in spring and ceased when water temperatures fell below 17 °C in autumn (this final report). The index cases in the Hawkesbury River estuary occurred at water temperatures of approximately 24° but it was not recorded in the Georges River in 2010 (Jenkins et al., 2013a; Paul-Pont et al., 2014a), suggesting that outbreaks can occur over a wide range of temperatures greater than 20°C in Australia. The index cases in Tasmania in mid-January 2016 in Upper and Lower Pitt Water occurred when water temperatures were approximately 22°C (Figure 2.4). Using these data as reference points, the risk of mortality due to OsHV-1 was assessed at each site in NSW (Figure 2.3), Tasmania (Figure 2.4) and South Australia (Figure 2.5) and was classified as low if average daily temperatures were mostly <20°C or as significant if average daily temperatures were >20°C for more than a few weeks.

3.4 Window of Infection: seasonal, spatial and temporal patterns of OsHV-1 disease between 2013 and 2018 in Pacific oysters (*Crassotrea gigas*) in the Georges River and Hawkesbury River

The spatial and temporal pattern of disease associated with OsHV-1 infection in *C. gigas* spat was monitored over 5 consecutive summers in the Georges River and Hawkesbury River estuaries. Sentinel oysters were placed in subtidal floating baskets at multiple locations in each estuary (Figure 3.1). Up to 7 sites were monitored in the Hawkesbury River (Patonga Creek, Mullet Creek, Porto Bay, Marra Marra Creek, Coba Bay, Kimmerikong Bay and Mooney Mooney) and up to 8 in the Georges River (Pelican Gut, Site A, Site B, Site C, The Shed, Sylvania Waters, Neverfail Bay and Limekiln Bar). The selection included downstream and upstream sites in both estuaries with varying the exposure to freshwater inflows and oceanic influence.

The number of sites that were monitored in each estuary each season, the number of floating baskets used at each site, the number of sentinel oysters placed in each basket and the number of separate placements of sentinel oysters each season over the 5 consecutive summers are shown in Table 3.1.

Sentinel oysters

All naïve oysters used in this study were hatchery reared, single seed *C. gigas* spat. Spat were provided by Shellfish Culture, Tasmania (for the 2012-13 to 2016-17 summer seasons) and Southern Cross Shellfish, Port Stephens (2015-16, 2016-17). Spat from Tasmania were certified negative for OsHV-1 by the competent government authority and shipped to Sydney by air, or road (Port Stephens spat). Spat batches were either sent from the hatchery every 2 weeks for immediate deployment (2012-2013, 2013-2014), or sent in larger batches and held in a recirculation tank at the University of Sydney, Camden, NSW and deployed progressively during the season (2014-2015 to 2016-2017). Spat held at the University of Sydney were divided into lots of 250 and placed into 1 mm mesh polypropylene socks. Each sock was suspended from a hanger in a communal tank (500 L) supplied with aeration and artificial seawater (Red Sea; 29 ppt ± 1 ppt salinity; prepared with unfiltered, dechlorinated water from the municipal supply) at ambient temperature. Oysters were fed daily using concentrated marine microalgae (Instant Algae® Shellfish Diet 1800 Reed Mariculture).

All batches of spat were reconfirmed negative for OsHV-1 at the University of Sydney prior to use, using real-time quantitative PCR (qPCR). The approximate number of oysters used each season and the identity of each of the 15 batches used are shown in Table 3.2. In general, several different batches were used each season, and a given batch was used in only one season.

All batches except one used in 2015-16 and some used in 2016-17 (Table 3.2) were believed to be fully susceptible to OsHV-1: those from Southern Cross Shellfish were unselected for resistance to OsHV-1 and those from Shellfish culture mostly pre-dated influences of the POMS breeding program. However, 3N oysters shown as "selected" in Table 3.2 were derived from ASI diploid lines that had been selected in the ASI POMS breeding program together with Shellfish culture tetraploid individuals. The tetraploids used to produce "selected" spat before January 2016 (i.e. batch SPL15AT) had not been exposed naturally to OsHV-1, therefore their OsHV-1 resistance was untested. The tetraploids used to produce "selected" spat after January 2016 (i.e. batches SPL16A, SPL16B) had been exposed naturally to OsHV-1 and survived and therefore may have had resistance due to genetic factors. The diploids used to produce 2N "selected" spat for batch THO15J were from ASI POMS selected lines. Estimated breeding values with respect to POMS resistance for the "selected" batches were unknown.

During the 2016-2017 season, two different batches of spat were deployed at each site – an unselected batch from Port Stephens NSW and a commercially available "selected" Shellfish culture batch. These batches were deployed together in the same baskets and sampled on the same days.

Data from other research trials and disease investigations

There were concurrent research trials and disease investigations during some seasons. Data for infection and disease caused by OsHV-1 from these trials was included in this report where relevant. This enabled some evaluation of the sensitivity of sentinel spat for detection of OsHV-1transmission and disease incidence.

Placement of oysters

Spat were divided into lots of 250 or 500 and placed into 1 mm mesh polypropylene socks. Each sock was placed into a 6 mm mesh floating basket (Seapa or BST). Each basket had a float and was attached to a floating longline. The baskets were adjacent to one another at each site in the Georges River, but 50 to 150 m apart at each site in the Hawkesbury River (except Mooney Mooney). Baskets were clearly identified with a label and were returned to the same locations after each inspection.

In general, the pattern of deployment and sampling of spat on each occasion was as depicted in Figure 3.2. Briefly, a new lot of spat was placed into each basket at each site. Samples were collected from the lot of spat that was already in the basket after checking for mortality. At the next inspection both lots of spat were checked for mortality and the first lot was removed without sampling, and the pattern was repeated. By this means, if early infection without mortality was present at the time of sampling it was possible to check for progression of the disease in that lot of spat following sample collection. The most common interval between deployment and sampling was two weeks, but this was reduced to 1 week in October-November in some seasons, and extended to 4 weeks prior to October and after May in some seasons.

All inspections were conducted by the researchers except during the 2014-15 season, when the spat at each site were checked by the oyster farmers who alerted the researchers when significant mortality was observed at a particular site. Samples were collected and no further checks were made or samples collected at the affected sites. However, a new batch of spat was placed at each site in May 2015 to check for further exposure events at the end of the season. All inspections were conducted from a boat and the baskets and spat were out of water for less than 30 mins each time.

Examination of spat and sampling

Spat were removed from the mesh bag and placed on a white tray. Mortality was assessed by visual inspection; empty shells were usually obvious but individual spat were probed with a pointed plastic tool to determine whether the valves were sealed. Spat were then thoroughly mixed on the tray and individuals were sampled opportunistically but non-selectively into sterile plastic tubes. These were transported directly to the laboratory and stored at -80°C until processing for PCR, or occasionally frozen at -20°C until transport.

Case Definition

Mortality due to OsHV-1 was defined as the onset of mortality between consecutive inspections with a cumulative total $\geq$10% in a basket which was not be explained by other obvious causes (e.g. prolonged immersion in freshwater) with confirmation of the presence of OsHV-1 by PCR from a sample of *C. gigas* from spat in the basket. The viral load was not specified because very often empty shell were tested and this type of sample can contain a very low concentration of virus, unlike the tissues of freshly dead oysters. Sub-clinical infection was defined as the detection of OsHV-1 in spat in which mortality was <10% at the time of sampling and at the second inspection.

Water Temperature

A temperature data logger (DS1921G, Thermochron) was inserted inside one basket at each site with the frequency of acquisition of data configured to between 30 and 72 minutes. Minimum, maximum

and average daily water temperatures were calculated. To determine representative water temperature ranges for each estuary by month, average daily water temperatures based on 20 readings per day from Porto and Mooney Mooney in the Hawkesbury River, and Sites A, B and C in the Georges River were used to calculate mean monthly water temperature for each estuary. Smoothed data lines were plotted.

Spat measurement and processing

Spat were pooled for homogenisation and testing. Spat were thawed at 4°C for up to 3 hours and placed onto sheets of plastic laminated 2 mm grid paper. Spat were then arranged into pools of between 5 and 12 oysters (depending on size) by opportunistic, non-selective sampling, with up to 5 pools of spat processed per basket per site. A digital photograph was taken of each pool and the length of every oyster in each pool was determined later from enlarged images. The average length of the spat in each pool was recorded and used to calculate the average length of spat in each basket. Each pool contained 300-500 mg of spat tissue (total pooled weight); spat <6 mm in length were pooled whole, spat 6-15 mm in length were dissected using sterile, disposable scalpel blades to remove excess shell, and spat >15 mm in length were dissected using sterile, disposable scalpel blades to exclude the shell, adductor muscle, gonad and digestive gland from the sample. Pooled spat tissues were then placed into a 2 mL safe lock micro test tube (VWR) containing 2 x 3 mm sterilised stainless steel beads (Aussie Sapphires) and 1 mL distilled water (Ultrapure™) and ground in the tube with a toothpick to break up the remaining shells. New scalpel blades and toothpicks were used for each pool, and a new plastic laminated sheet was used for each basket or site. All work surfaces were disinfected with sodium hypochlorite solution (500 mg L^{-1}) rinsed and dried between each sheet. Tubes were stored at -80°C until tissue homogenisation.

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

Spat tubes were homogenised by one of two functionally equivalent bead beating procedures (unpublished data) to disaggregate OsHV-1 from cells and particles within the samples. Tubes were thawed for 20 min at room temperature and placed into a bead-beating machine (Fastprep®-24 MP Biomedical) for 15 s at a speed of 6.5 m s^{-1} or a TissueLyser II machine (Qiagen®) for 2 min at frequency 30, then the insert containing the samples was rotated 180° with a further 2 min at 30. All samples were clarified by centrifugation at 1340 x *g* for 2 min in a microcentrifuge and supernatants removed and stored at -80°C until nucleic acid purification.

Nucleic acids were purified from each clarified tissue homogenate using a 5× Mag-MAX™-96 Viral RNA Isolation Kit (Ambion®, Life Technologies™) and a MagMAX™ Express 96 magnetic particle processor (Applied Biosystems™, Life Technologies™) according to the manufacturer's instructions. A 50 µL sample volume with the AM1836 deep well standard program (Ambion®, Life Technologies™). Purified nucleic acids were stored at -20°C until qPCR analysis.

Samples were analysed in duplicate in a 25 µL reaction volume using a real-time fluorescent probe assay adapted from that developed by Martenot et al. (2010) as previously described by Evans et al. (2014). An AgPath-ID One Step RT-PCR kit (Life Technologies™) was used. The reaction contained 12.5 µL of 2x real-time qPCR buffer, , 900 nM of each primer (OsHV1BF 5'- GTC GCATCT TTG GAT TTA ACA A -3' and OsHV1B4 5'- ACT GGG ATC CGA CTG ACA AC-3'), 250 nM OsHV-1 probe (5'-6FAM-TGC CCC TGT CAT CTT GAG GTA TAG ACAATC-TAMRA-3'), 1 µL of 25× real-time qPCR enzyme mix, nuclease free water and 5 µL of neat nucleic acid extract. Purified nucleic acid from known OsHV-1 infected oysters was used for the positive control. Negative controls consisted of an extract from an OsHV-1 oyster and a no template DNA reaction. Standards were created from plasmid pOSHV1-Breg (2×10^6copies/µL) (University of Sydney, Camden, Australia) with a 10-fold dilution series (10^7–10^0 copies/µL) used to create the standard curve. All controls, standards and sample DNA were tested in duplicate. A real time thermocycler (Mx3000P, Stratagene, Agilent Technologies) was used with a hot start activation phase of 10 min for 1 cycle at 95°C, then

for 45 cycles of denaturation for15 s at 95°C and annealing and extension for 45 s at 60°C . A valid PCR run was defined as with no amplification of the negative controls, amplification of both replicates of the positive control with a Ct within the range of the standard curve, a standard curve with r2> 0.95 and efficiency 90 -110%. The fluorescence threshold for each run was determined by the amplification-based threshold algorithm (Stratagene) calculated for the standard samples and applied to all samples. A positive result was defined by one or both replicates exhibiting an exponential increase in FAM fluorescence signal and a cycle threshold of <40. The quantification limit of the assay was 12 DNA copies per PCR reaction. Samples that satisfied the criteria for detection but had a Ct value below quantification limit of the assay were described as positive below the limit of quantification (bloq).

3.5 Sequence of OsHV-1 and phenotype evaluation

3.5.1 Conventional PCR and multi-locus sequence determination

Six regions of the OsHV-1 genome were targeted for sequencing according to previously described methods (Table 4.1). These regions were selected based on discriminatory value for distinguishing microvariant genotypes from other OsHV-1 variants and/or as regions with a high level of polymorphism suitable for distinguishing related genotypes. Conventional PCR assays were prepared with the Expand High Fidelity PCR System (Roche) according to directions with a reaction volume 50 µl. Each reaction contained a primer pair (Table 4.1) at a final concentration of 400 nM each and used 5 µl of neat nucleic acid template derived from mantle and gill tissue homogenates using the MagMax Viral Nucleic acid Extraction Kit (Life Technologies).

Thermocylcing was conducted with Corbett palm cycler and Biorad T100 according to the following program: 98°C for 2min followed by 35 cycles of 98°C for 30s, 62°C for 30s and 72°C for 45s with a final incubation at 72°C for 10min.The results were determined by loading 10 µl of the reaction into a 2% agarose gel stained with RedSafe (iNtRON Biotechnology) and subject. Amplification products were visualised with a GelDoc transilluminator (Biorad) after electrophoresis. Products of the expected size were excised from the gel and DNA bands of the appropriate size were purified using the QIAquick Gel Extraction Kit (Qiagen). Sanger sequencing with BigDye Terminator v3.1 Cycle Sequencing chemistry was performed at the Medical Genomics Facility services (Monash Health Translation Precinct). A reaction was primed by each of the PCR primers used to generate the amplicons. Chromatograms were analyzed and primer sequence was removed, using FinchTV (Geospiza).

Selection of clinical samples

Clinical samples were selected to represent OsHV-1 infections from the full geographical distribution and time-span accessed through University of Sydney research efforts (Table 4.2). This included the Georges and Hawkesbury River, New South Wales from 2011 or 2013, respectively. A sample from Pittwater represented the initial outbreak in Tasmania from the summer of 2016. Individual oysters from infected populations were selected according to the highest concentration of OsHV-1 genomic DNA detected by qPCR undertaken for other research objectives.

Phylogenetic analyses

Multiple sequence alignments were compiled for each region using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and compared with OSHV-1 reference genome (AY509523) and other variants identified in GenBank (Table 4.3). Unique virus sequence types were identified by analysing the polymorphisms across each region. Examples of genotypic variants identified in this study were presented as multiple sequence alignments with the OSHV-1 reference sequence (1999-FRA-001), Acute viral necrosis virus of Chinese scallops (AVNV) (2007-CHN-001),

OSHV-1 µvar from France (2008-FRA-002) and the NSW isolate from 2010 (2010-AUS-001). Phylogenetic analyses were conducted in Mega6 (Tamura et al., 2013) with representative nucleotide sequences available on Genbank (http://www.ncbi.nlm.nih.gov/genbank/).

3.5.2 Phenotypic comparison of diverse OsHV-1 genotypes

The purpose of the present study was to compare the phenotype of different OsHV-1 isolates in a standard laboratory infection model system. The experiment was conducted with a single batch of oysters to reduce the effect of the host. Triploid Pacific oysters were sourced at 6 months of age and approximately 5 cm shell length. Originally from a commercial hatchery (Shellfish Culture, Tasmania), they were grown under commercial conditions in Patonga Creek, NSW. They were certified free form OsHV-1 at shipment and a sample at recruitment to the laboratory was tested by qPCR to demonstrate freedom from OsHV-1 (n=30).

Clinical samples were selected to represent the most diverse genotypes. However, it was determined that only those stored as fresh frozen tissues could be used to amplify OsHV-1. Many samples stored at -80°C as tissue homogenate supernatants were not suitable for this study.

A preliminary amplification of fresh OsHV-1 was undertaken by preparation of a mantle and gill tissue homogenate and intramuscular injection into oysters according to previously described methods (Paul-Pont et al., 2015a). This was intended to overcome differences between stage of infection, time of storage and nature of host oyster before comparing the phenotype of 3 isolates of OsHV-1 Table 4.4.

The virulence and transmissibility was assessed for the 3 freshly amplified isolates using 24 tanks of 40 oysters acclimated for 7 days to the laboratory conditions. The 3 isolates and a negative control homogenate were evaluated with two replicate tanks per treatment each containing 20 injected oysters and 20 cohabitated oysters for each treatment. Injection was done with 3 different doses: Dose 1, $1x10^4$ OsHV-1 genome copies per injection; Dose 2, $1x10^3$ copies per injection; and Dose 3, $1x10^2$ copies per injection. Extreme care was taken to avoid cross contamination between isolates. Injected and cohabited oysters were distinguished by cutting a notch on the shell during relaxation of injected oysters. Visual inspection and removal of dead oysters was conducted twice a day and samples were stored at -80°C fore qPCR analysis. The trial was completed 14 days post injection.

The quantity of OsHV-1 was determined by qPCR in the mantle and gill tissue according to previously described methods for the first 5 oysters to die by each challenge method and for a random selection of survivors from each tank.

The quantity of OsHV-1 DNA in oysters that died was compared using a general linear model with the factors: isolate; dose; and challenge method; with tank as a random effect. Differences in the quantity of viral DNA were assessed using the least significant difference method. Survival analysis was performed with failure defined as mortality and detection of $>10^4$ copies OsHV-1 DNA per mg in oysters from the tank. Kaplan Meyer survival curves were prepared for each isolate, dose and challenge method and tested with a log rank test ($P<0.05$). A Cox proportional hazards model was prepared considering the factors: isolate; challenge method; and dose, with tank as a random effect.

3.6 Evaluation of an integrated OsHV-1 disease control strategy

Aim

The aim of the present studies was to demonstrate commercial scale production of *C. gigas* in a POMS infected estuary using a range of genetic (partially POMS resistant) and husbandry (lease location, seasonal window) approaches to minimize mortality due to OsHV-1.

The specific aims of the 3 trials conducted were:

Trial 1 (Commercial Grow-out Monitoring Trial 2013-2014)

To determine whether locations in the Hawkesbury River in which commercial stocks of Pacific oysters were not affected by epizootic POMS in Jan-Feb 2013 would remain unaffected in the summer 2013-2014.

Trial 2 (Commercial grow-out Monitoring Trial 2014-2015)

To monitor the outcome of commercial scale attempts to take advantage of the window of infection, provide data that can be used to inform biosecurity policy (translocations of large oysters) and provide more confidence in the approach of multi-estuary farming to avoid disease.

Trial 3 (POMS Commercial grow-out trial 2016-2017)

To demonstrate commercial production of Pacific oysters using temporal and geographic information about the distribution of OsHV-1 and the occurrence of POMS in the Hawkesbury River.

Background

The Window of Infection trials have provided information about the distribution and activity of of OsHV-1 in the c Hawkesbury River that can be exploited by farmers to develop commercially relevant window farming calendars. Temporal risk factors have been defined by the recurrent seasonal activity of OsHV-1 in the Hawkesbury River between October and May. The period between May and September inclusive provides a 5 month window during which oysters may be finished with minimal risk of mortality due to OsHV-1. Geographic risk factors are also identified with all major bays except Patonga have been affected since January 2013. During the initial POMS in the Hawkesbury River, although OsHV-1 was widely distributed in the estuary, clinical disease was observed mainly in the lower river at Mullet Creek, Porto Bay and Mooney Mooney Creek. Several areas remained free of clinical disease. Coba Bay, Kimmerikong Bay and Marra Marra Creek, all of which are in the upper river and are subject to influences from fresh water inflows from the main river system. In Jan 2013 POMS was expressed in trays of oysters in Coba Bay that had been relocated from Mullet Creek, the index case site. However, a propagating epizootic did not occur in Coba Bay. These observations suggested that there may be environmental risk factors for POMS may not be uniformly distributed. This led to speculation that it might be possible to identify risk-based locations for commercial culture.

Despite the considerable unpredictability in the spatio-temoral occurrence of POMS in the Hawkesbury River, farmers have attempted to use this information for commercially viable farming activities.The research team has developed methods to evaluate these first attempts to farm Pacific oysters in an estuary with recurrent seasonal POMS in Australia.

Materials and Methods

Oysters were purchased by growers from Shellfish Culture, Tasmania and stocked into an estuary at Wapengo on the south coast of NSW during the high risk period for POMS. Wapengo has remained freed from POMS for the duration of the research period, although the outbreak of POMS in Tasmania impacted this source of spat supply. Stock was transferred to the Hawkesbury River in batches commencing in May 2014. An observational study was conducted: each batch was monitored to obtain basic production statistics. For the third trial, spat were sourced directly from Tasmania to Patonga Creek.

For each batch of oysters at Wapengo a record was made of: shipment date; batch identification; number; destination lease and basic growing methods. For each translocation to the Hawkesbury, a

record was made of: date, number, owner and lease holder, size, location grown. Subsequently records were made of the date and number sold from each size category. Samples of 30 oysters were randomly collected at the time of arrival of each batch at the Hawkesbury should be collected and frozen for OsHV-1 qPCR tests and again when any mortality was noted.

Environmental monitoring was conducted using retrospective analysis of data from the main river probes and temperature probes set to 60 min acquisition frequency provided to participating farmers for each lease location. These have been named according to trial, grower and location.

The collaborating farmer Bruce Alford, Broken Bay Oysters was requested to identify each basket and tray used for this batch of Shellfish Culture spat with an orange fluorescent cable tie. This distinctive tag was to be added to any new baskets and trays used as the batch was split during grading. This enabled tracking of the cohort.

3.7 Outbreak investigation: Index cases of POMS in Tasmania

A physical survey was undertaken on affected farms in Upper and Lower Pitt Water and Blackman Bay to measure the severity of the first occurrence of POMS in Tasmania in January 2016. This study documented the wide variation in mortality and evaluated risk factors for disease. Survival within each bay ranged from 13% to 45% and there was up to 75% variation in survival between groups of oysters from the same batch. Smaller and younger oysters, handling for routine husbandry within a week of a disease outbreak and stocking density that was higher or lower than typical were risk factors for increased mortality. Factors that did not influence survival during the initial POMS outbreak in Tasmania included ploidy, hatchery of origin, use of on-growers and the immersion times obtained from the narrow range of clip heights in use for intertidal infrastructure. These findings were important to inform farmers planning for the impact of recurrent outbreaks of POMS in Tasmania. Recommendations for further research included investigation of sub-tidal growing infrastructure and further evaluation of hatchery batch and growing location as possible explanations for the highly variable mortality.

This work was communicated in an extremely timely manner by provision of a factsheet for industry, presentation at Shellfish Futures (Sorell, October 2016) and rapid publication in the international peer reviewed scientific literature (de Kantzow, et al., 2017).

Materials and Methods

The field investigation was conducted between the 21st and the 24th of March and the 11th to the 22nd of April, 2016 as part of the recommencement of farm operations following the POMS outbreak.

Farms

Farms affected by the outbreak were invited to participate according to the availability of stock, detailed farm records and their willingness to participate, which required farmers to physically assist in sampling and examining stock. In Upper and Lower Pitt Water there were 5 farms of which 3 participated. In Blackman Bay there were 6 farms of which 3 participated.

Study design

The sampling frame for each farm was determined at the time of the survey by examining farm records for batches that were present on the farm at the time of the outbreak. Batches were selected for sampling based on having a number of sub-batches in a variety of locations and growing structures across the farm, each of which was then sampled. The sample size to estimate farm mortality with a precision of 0.05 and a confidence of 95% was calculated based on an estimated mortality of 50% and adjusted for clustering within baskets. This produced a sample size of ~8000, indicating 80 baskets of

100 oysters each were required at each farm. The actual sample size that was possible depended on logistical considerations including time available and tides, number of baskets present and the time to count and measure the oysters in each basket which depended on oyster size, number per basket and over catch present on the baskets and oysters.

Sampling and field audit

Systematic random sampling was used to obtain a representative sample of baskets for mortality determination. The size and age range of oysters in the survey reflected the stage in the production cycle at the time of year. Peak sales around Christmas occurred immediately prior to the outbreak therefore the number of plate-size stock present on-farm was low. Husbandry conditions were also affected by this event; much of the stock on the top clip height had been sold before the outbreak and stock on the lower clip height had been brought up to the middle clip height to replenish these at the time of the outbreak. On farms 2 and 3 cleanup operations had commenced prior to the survey which also decreased the availability of larger stock sizes to sample as they were being preferentially targeted due to the lower mortality and higher immediate value.

The size of the oysters within each basket on all farms was determined at the time of sampling according to standard size categories used for sales, based on size grading by the farmer. In addition, on Farms 1 and 2 the length of oysters was determined using a carpenter's square.

The total cumulative mortality in each basket was determined by counting the number of apparently live oysters and the number of empty shells.

Risk factors and statistical analysis

For each basket farm management including husbandry and growing conditions and biological parameters such as length were recorded. Risk factors for investigation will include size, age, handling, on-growing, ploidy, hatchery and clip height. Statistical analysis will be performed using Microsoft R open (version 3.2.5, 2016-04-14) with the lme4, ggplot2 and ggmap. The glmer () function (lme4 package) will be used for fitting the GLMM and screening variables. Data summaries, histograms and scatter plots against mortality as a percent of total basket count will be used to assess normality of the data and screen for any graphical association with mortality. A generalized linear mixed model (GLMM) will be used to assess the effect of management practices across farms and intrinsic factors to the oyster. The count of dead oysters relative to the total number of oysters per basket will be used as the outcome variable (dead/total). The random effects farm, block and sub-batch will be included in the model to account for the variation arising from differences in geographical location and positioning due to husbandry practices. Forward stepwise multivariable logistic regression analysis will be performed between each unique combination of factors starting with two variable models and progressing with the addition of further variables to significant models. Interactions will be assessed between all variables selected for inclusion in the final model.

4. Results and Discussion

4.1 The influence of size and age on susceptibility of juvenile Pacific oysters to OsHV-1

Experiment 1. Retrospective recruitment of oysters

Two distinct size classes were identified within the batches of 8 month and 14 month old oysters (Table 1.1). The two batches were free from OsHV-1 at the time of deployment based on negative tests on pools of 5 prepared from 30 randomly selected individuals from each age-size group prior to deployment.

Field trial

POMS was confirmed in the field challenge based on positive tests for OsHV-1 with $>10^4$ OsHV-1 genome copies/mg oyster tissue in a selection of fresh dead oysters at each time point. Tests for OsHV-1 prior to deployment were negative (10 pools of 3 for each of the 4 age-size categories). The disease was much more severe at site A where the onset of mortality was later, but progressed rapidly to >60 % mortality (Figure 1.1a). Mortality of the 8 month old oysters was 10.2% higher than the 14 month oysters (Table 1.2, $p<0.05$). Within the younger batch, the mortality was 23.2% higher for the smaller oysters compared to the larger oysters ($p<0.01$). In the older batch, mortality was 21.8% higher for the group of smaller oysters ($p<0.01$). Kaplan Meier curves are shown in Figure 1.1. Log rank tests indicated that survival was significantly different between younger and older oysters ($P<0.001$), smaller and larger oysters ($P<0.001$) and both sites in the Georges river ($P<0.001$). However, only size was significant after adjusting for clustering due to site and basket in the Cox proportional hazard model ($P=0.019$). The results suggested that smaller oysters had about twice the hazard of mortality compared to the larger oysters (Table 1.4).

Laboratory challenge

The laboratory challenge resulted in higher mortality compared to the field challenge conducted 2 months earlier, due to higher mortality (67.3%) of the younger, now 10 month old oysters, compared to the 16 month old group (26.9%). There was no mortality in the control oysters challenged with OsHV-1 free inoculum and each individual tested negative for OsHV-1 DNA. Of the dead oysters, all but 2 (98.6%) were positive for OsHV-1 DNA. At the completion of the trial the proportion of live oysters that were positive for OsHV-1 was 40.0% (Table 1.3). The viral load was below the limit of quantification in all but 10.3% of these survivors, and of these, the quantifiable viral load was 42.2 times lower than the dead oysters ($p<0.01$). For the dead oysters, the quantity of OsHV-1 DNA was greater than 10^3 copies per mg tissue in 73.5% and $>10^4$ copies per mg tissue in 51.4% of oysters.

Kaplan Meier curve analyses indicated a higher survival for older compared to the younger and larger compared to the smaller oysters. There was no significant difference in survival between the two doses tested but it was significantly higher in the adsorption than in the injection method. In the Cox proportional hazard analyses, the interaction between method and dose was significant, but that between age and size was not, and hence, removed. The results suggest a greater hazard of death for smaller and younger oysters relative to larger and older oysters, respectively (Table 1.4). Oysters infected via injection had more than 7 times the hazard than those infected by adsorption if they were given low doses, but only about 3 times the hazard, if they were given high doses (Table 1.5). The effect of dose was not significant for the injection method but a lower dose resulted in a lower hazard of death in the adsorption method (Table 1.5). The smaller group within the 10 month old oysters had 19.2% higher mortality compared to the larger oysters of this age ($p<0.05$). There was a differential mortality with 7.7% higher mortality for smaller group of 16 month old oysters, but this was not

significant (p=0.28). Accounting for batch and dose, the protective effect of being larger was greater than the protective effect of being 6 months older (Figure 1.1b).

Experiment 2. Prospective recruitment of oysters

There was a substantial restriction growth indicated by the shorter total shell length and wet weight of the oysters grown at +450 mm compared to those stocked at standard growing height for 6 months in both batches (Table 1.1). The two batches were free from OsHV-1 at the time of deployment based on negative tests on pools of 5 prepared from 30 randomly selected individuals from each age-size group prior to deployment.

Field trial

The occurrence of POMS at both sites was confirmed by positive tests for OsHV-1 in a selection of fresh dead oysters from each site and time point and $>10^4$ OsHV-1 genome copies/mg in pools of the control spat in placed in baskets (SPL14B 8 months, 8 mm). At the completion of the trial there was a positive test for OsHV-1 DNA in pools of 5 oysters from 7 of the 34 baskets in which live oysters remained, but the viral load was below the limit of quantification for all of these. The mortality at both sites in the River was the same (Figure 1.1c).

In this experiment the mortality of the 8 month old batch of oysters was extremely high (94.6%), much higher than the batch of 17 month old oysters (27.7%). In the crude Kaplan-Meier analysis (Fig 1c), younger oysters had significantly lower survival than older oysters (P<0.001) but no significant difference in survival was observed between larger and smaller (growth restricted) oysters (P=0.50). Oysters at Site C had a lower rate of survival than at Site B (P =0.004). In the Cox proportional hazard analyses neither size nor interaction between age and size was significant. Age was the only variable left in the final model after adjusting for site and basket and the results suggested that younger oysters had 5.49 times the hazard compared to the older oysters (95% CI: 4.79, 6.31).

Laboratory challenge

The laboratory challenge resulted in similar outcome to the field challenge with much higher mortality in the younger (84.6%), now 10 month old oysters, compared to the 19 month old group (37.2%). There was no mortality in the control oysters challenged with OsHV-1 free inoculum and each individual tested negative for OsHV-1 DNA. Of the dead oysters, all but 3 (98.4%) were positive for OsHV-1 DNA. The proportion of live oysters at the completion of the trial that were positive for OsHV-1 was 48.4%. The viral load was below the limit of quantification in all but 23.0% of the survivors, and for these, the quantifiable viral load was 29.8 times lower than the dead oysters (p<0.01). For the dead oysters, the quantity of OsHV-1 DNA was $> 10^3$ copies per mg tissue in 84% of oysters and $>10^4$ copies per mg tissue in 45% of oysters. In this experiment, there were significant differences in survival between younger and older oysters, between two doses and between two infection methods (all P<0.001), but not between different sized oysters (P=0.41). Both the interactions, i.e. between age and size and between dose and method, were significant in the Cox proportional hazard model. The results for interactions presented in Table 1.5 indicated that younger oysters had more than 10 times the hazard of death compared to the older oysters if the oysters were *larger* but this hazard of death was only 2.7 times if the oysters were *smaller* (restricted growth). Further smaller oysters had almost twice the hazard of death than larger oysters when the oysters were *adult*, but this association reversed for younger oysters, i.e. smaller oysters (restricted growth) had less than half the hazard of death than larger oysters for adults (Table 1.5).

Experiment 3. Extreme restriction of size for age

Field

One month after deployment in the field, the cumulative mortality for this cohort was 100%. No samples of soft tissues were recovered for testing. However, OsHV-1 was detected in co-located oysters from a different trial (Age-size trial reported below). Oysters from the same batch that were retained in holding tanks at USyd remained alive, i.e. oysters that were not exposed to OsHV-1.

Laboratory

The total cumulative mortality 12 days after challenge was 59% after exposure to the highest dose of OsHV-1. This mortality was 35.4% higher than for a routine commercial batch of 7 month old, 5 mm triploid Pacific oyster spat with the same exposure ($p<0.01$). Further, a dilution of the inoculum below the minimum dose for infection of the younger batch of spat was sufficient to induce 20% mortality in this cohort of extreme growth restricted oysters. The viral loads in pools of live and dead spat exceeded 10^4 copies of the OsHV-1 genome per milligram of tissue 6 days after challenge, and remained above 10^3 copies per mg after 12 days in live and dead oysters.

This study confirmed and quantified higher survival of older oysters when challenged with OsHV-1. This will inform stock management by farmers expecting recurrent seasonal incidence of POMS. The observation that a protective effect from age requires oysters greater than 14 months of age, and that oysters up to 10 months of age can experience very high mortality confirms the need for stock that are too old to have avoided a prior POMS risk period. There was an increased risk of death after OsHV-1 challenge in the smaller oysters within batches of routinely managed oysters. This likely reflected general characteristics of individuals within the group whereby increased growth was associated with general disease resilience. Differentiation of the size of otherwise unselected cohorts by restricting or accelerating growth through immersion was demonstrated. A key management strategy for stock maintenance in POMS infected estuaries is the protective effect of restricted growth for the preceding 6 months when oysters are challenged at 10 months of age. The protective effect of restricted growth might not be useful for commercial production with the very high mortality observed in the juvenile age class in this study. However, physiological conditioning of oysters should be explored further for potential to increase the resilience of oysters to POMS.

Table 1.1. Triploid Pacific oysters recruited to test the effect of size within age category on susceptibility to OsHV-1.

Experiment	Batch ID	Spawning Date	Age-size category	Age (months) field / lab exposure	Size (mean +/- st dev)	
					Shell length (mm)	Weight (g) [a]
1 Retrospective assignment of size categories	SPL 12FT	3/12/12	Old-large	14 / 16	79.4 + 11.5	52.3 + 15.3
			Old-small	14 / 16	49.1 + 2.8	17.8 + 3.0
	SPL 13A	10/6/13	Young-large	8 / 10	73.0 + 15.6	30.0 + 5.3
			Young-small	8 / 10	36.9 + 4.1	3.6 + 0.9
2 Prospective differentiation of size categories	SPL13B	19/8/13	Old-large	17 / 19	86.0 + 12.3	79.1 + 28.7
			Old-small	17 / 19	64.4 + 19.2	41.2 + 19.4
	SPL14B	12/05/14	Young-large	8 / 10	69.0 + 11.0	
			Young-small	8 / 10	29.6 + 13.4	

[a] measured in relaxed oysters after emptying the pallial cavity of liquid

Table 1.2. Mortality in laboratory and field trials with 95% exact binomial confidence limits.

Experiment	Cohort	Description	Laboratory		Field	
			Total cumulative mortality (%)	95% CI	Total cumulative mortality (%)	95% CI
1	SPL 13A	Younger-smaller	76.9	66.0 – 85.7	59.3	54.9 – 63.6
		Younger-larger	57.7	46.0 – 57.7	36.1	31.9 – 40.5
	SPL 12FT	Older-smaller	30.8	20.8 - 42.2	48.4	44.0 – 52.9
		Older-larger	23.1	14.3 – 34.0	26.6	22.8 – 30.7
2	SPL14B	Younger-smaller	74.4	63.2 – 83.6	91.1	88.3 – 93.4
		Younger-larger	94.9	87.4 – 98.6	98.8	97.2 – 99.5
	SPL13B	Older-smaller	43.6	32.4 – 55.3	28.6	24.8 – 32.7
		Older-larger	30.8	20.8 – 42.2	26.8	23.1 – 30.7

Table 1.3. Detection and quantification of OsHV-1 DNA in laboratory trials.

Data are $\log_{10}$(OsHV-1 genome copies/mg) at the time of death or in live oysters at the completion of the trial.

Experiment	Batch	Classification	PCR				
			Positive	Negative	Quantifiable viral load		
			n	n	n	average	St dev.
1		All live oysters	66	99	17	2.34	0.90
		All dead oysters	145	2	140	3.87	1.16
	SPL 13A	Younger-smaller	67	11	61	4.60	0.76
		Younger-larger	53	25	45	2.91	0.74
	SPL 12FT	Older-smaller	33	45	25	2.89	1.02
		Older-larger	32	46	24	3.74	1.50
2		All live oysters	59	63	28	2.37	0.97
		All dead oysters	187	3	185	3.85	0.73
	SPL14B	Younger-smaller	62	16	56	3.88	0.66
		Younger-larger	78	25	74	3.97	0.69
	SPL13B	Older-smaller	48	30	38	3.32	0.84
		Older-larger	58	20	45	3.14	1.20

Table 1.4. Final Cox-proportional hazards models for all laboratory and field studies.

Study	Factor	Comparison	Parameter estimate	Standard error	Hazard ratio[a]	LCL	UCL	P-value
Field Trial 1								
	Size	Smaller versus larger	0.64	0.27	1.91	1.66	2.19	0.02
	Age	Not significant						
Lab Experiment 1								
	Size	Smaller versus larger	1.57	0.17	4.79	3.33	7.01	<0.001
	Age	Younger versus older	0.52	0.12	1.69	1.22	2.36	<0.001
	Method	Injection versus adsorption	1.09	0.19	-	-	-	<0.001
	Dose	Low versus high	-0.91	0.18	-	-	-	<0.001
	Dose*Method	Low and Injection	0.89	0.24	-	-	-	<0.001
Field Trial 2								
	Age	Younger versus older	1.70	0.18	5.49	4.79	6.31	<0.001
	Size	Not significant						
Lab Experiment 2								
	Size	Smaller versus larger	2.36	0.28	-	-	-	<0.001
	Age	Younger versus older	0.60	0.22	-	-	-	0.006
	Age*Size	Younger and Smaller	-1.34	0.32	-	-	-	<0.001
	Method	Injection versus adsorption	0.53	0.18	-	-	-	0.002
	Dose	Low versus high	-1.25	0.32	-	-	-	<0.001
	Dose*Method	Low and Injection	1.04	0.35	-	-	-	0.003

LCL: Lower 95% Confidence Limit; UCL: Lower 95% Confidence Limit;
[a]Hazard ratios for variables involved in an interaction are presented in Table xx.

Table 1.5. Hazard ratios from laboratory experiments.

Hazard ratios for the variables involved in interactions in presented in Table 1.4 based on the two laboratory experiments. No interactions were significant in the two field studies.

Factor	Description	Hazard ratio	LCL	UCL
Lab Experiment 1				
Method	Injection versus adsorption			
	For low dose	7.24	5.12	10.24
	For high dose	2.98	2.06	4.30
Dose	Low versus high			
	For injection	0.98	0.72	1.33
	For adsorption	0.40	0.28	0.57
Lab Experiment 2				
Age	Young versus adult			
	For Small size	2.75	1.83	4.15
	For Large size	10.55	6.06	18.37
Size	Small versus large			
	For young age	0.47	0.30	0.74
	For adult age	1.82	1.19	2.78
Method	Injection versus adsorption			
	For low dose	4.84	2.74	8.52
	For high dose	1.70	1.21	2.40
Dose	Low versus high			
	For injection	0.81	0.62	1.07
	For adsorption	0.29	0.15	0.53

LCL: Lower 95% Confidence Limit; UCL: Lower 95% Confidence Limit;

Figure 1.1. Kaplan-Meyer survival curves for laboratory and field trials.

A. Experiment 1, field trial. B. Experiment 1, laboratory challenge. C. Experiment 2, field trial. D. Experiment 2, laboratory challenge.

Figure 1.1a. Experiment 1, field trial. Retrospectively recruited oysters representing large and small groups from a batch at 14 months of age and large and small groups within a batch of oysters at 8 months of age. Estimated survivor function for (i) oysters of different age, (ii) oysters of different size, (iii) oysters placed at different locations in the Georges River.

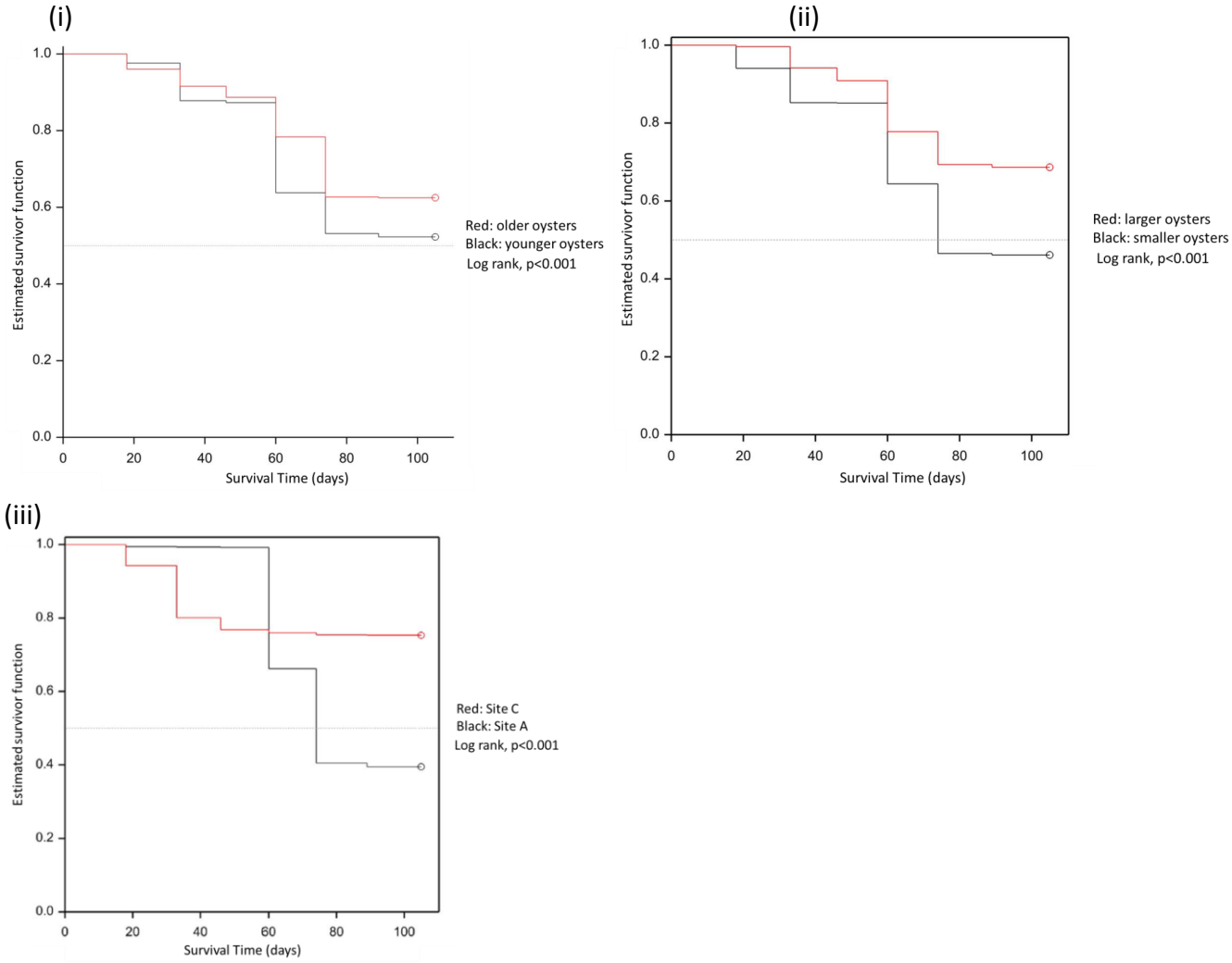
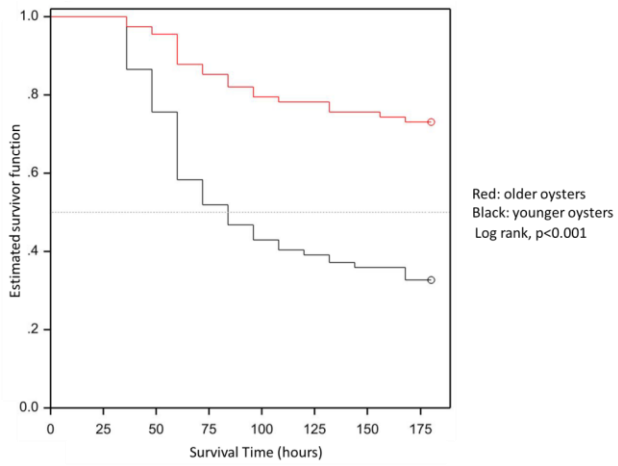


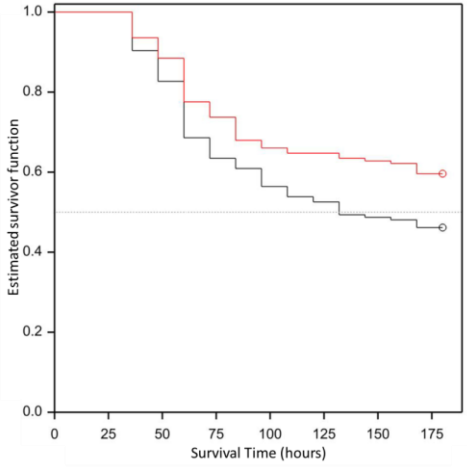
Figure 1.1b. Experiment 1, laboratory challenge. Retrospectively recruited oysters representing large and small groups from a batch at 14 months of age and large and small groups within a batch of oysters at 8 months of age. Estimated survivor function for (i) oysters of different age, (ii) oysters of different size, (iii) oysters challenged with OsHV-1 by different methods.

(i)



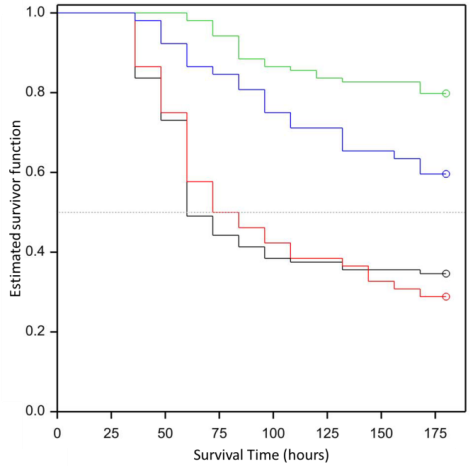
Red: older oysters
Black: younger oysters
Log rank, p<0.001

(ii)



Red: larger oysters
Black: smaller oysters
Log rank, p=0.017

(iii)



Green: low dose adsorption
Blue: high dose adsorption
Red: high dose injection
Black: low dose injection

Log rank, p<0.001

Figure 1.1c. Experiment 2, field trial. Prospectively recruited oysters representing large and small groups from a batch at 17 months of age and large and small groups within a batch of oysters at 8 months of age. Estimated survivor function for (i) oysters of different age, (ii) oysters of different size, (iii) oysters placed at different locations in the Georges River.

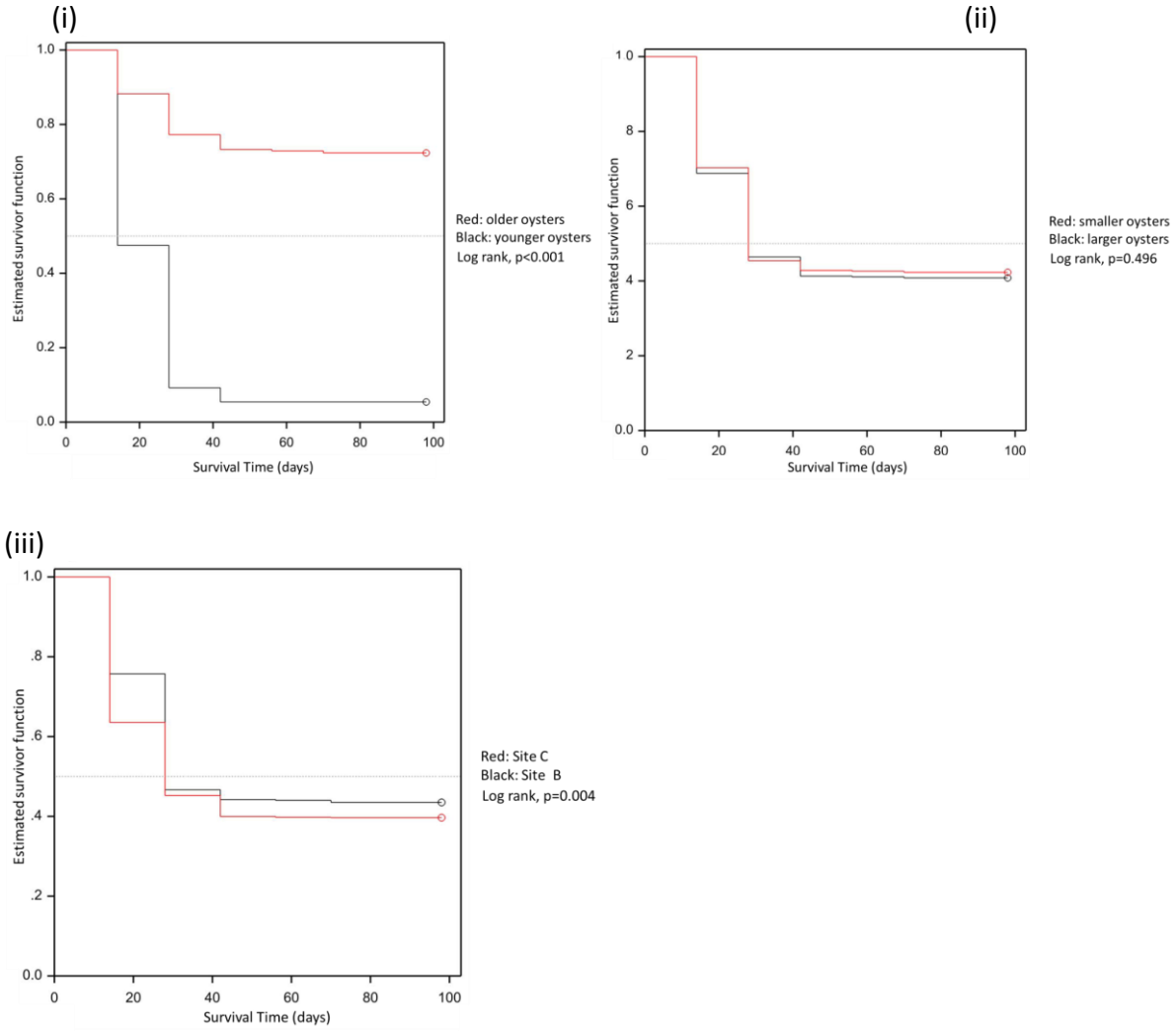
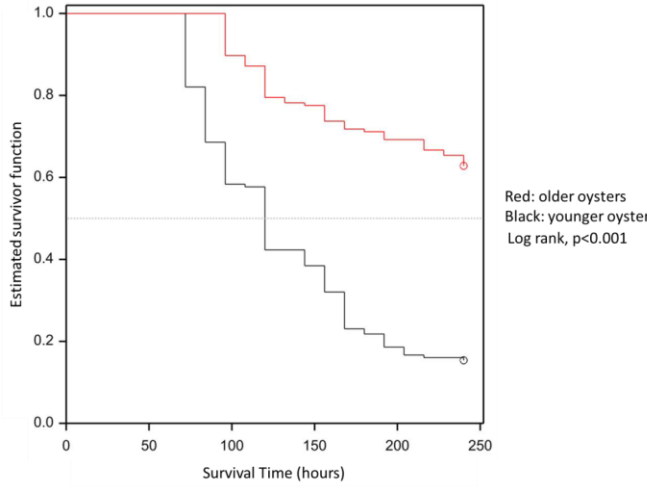
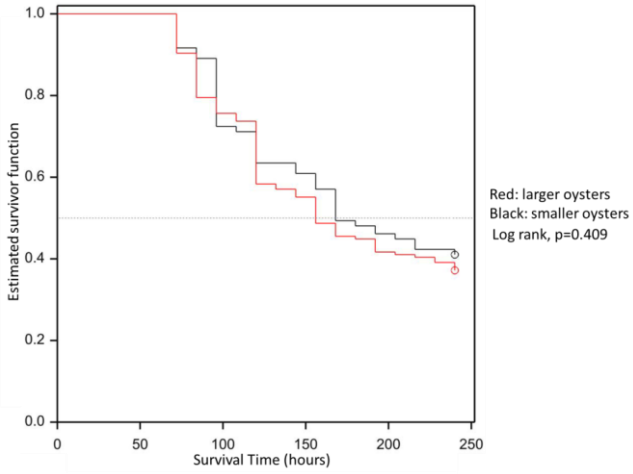


Figure 1.1d. Experiment 2, laboratory challenge. Prospectively recruited oysters representing large and small groups from a batch at 17 months of age and large and small groups within a batch of oysters at 8 months of age. Estimated survivor function for (i) oysters of different age, (ii) oysters of different size, (iii) oysters challenged with OsHV-1 by different methods.

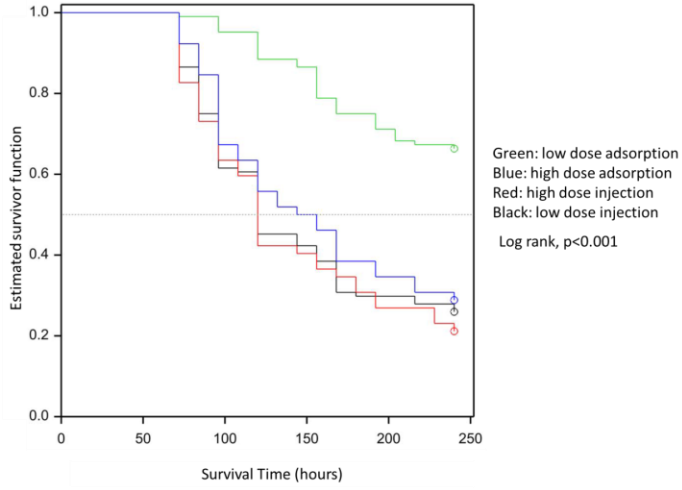
(i)



(ii)



(iii)



4.2 Pre-exposure of Pacific oyster spat to OsHV-1

4.2.1 Spat conditioning Experiment 1. Conditioning by prior exposure to generate OsHV-1 survivor spat

The initial immersion challenge of Batch SPL14B resulted in modest mortality over a 6 day period with survival > 84% of OsHV-1 challenged spat, and a dose-response according to the titration of the OsHV-1 inoculum (Table 1.6). The endpoint dose was identified as 2.4×10^3 OsHV-1 genome copies per mL in the challenge water. A very high viral load ($6.9 \times 10^3 – 6.0 \times 10^5$ OsHV-1 genome copies/mg) in pools of dead spat indicated that mortality was attributable to OsHV-1 (Table 1.7). The viral load and prevalence of infection in live oysters sampled 6 days after challenge indicated that OsHV-1 infection was induced without acute mortality in a large proportion of the spat.

There was less than 10% mortality of the spat that survived the initial challenge over the subsequent 6 days, during which they were continuously re-exposed to OsHV-1 by cohabitation. The virus load was the same for all infected treatment groups at this time ($p=0.36$), but there was a 12-fold reduction in the viral load compared to the previous time point ($p<0.001$).

Minimal mortality was detected for spat that were challenged with the negative control homogenate or were not exposed and all PCR tests on these groups were negative for OsHV-1.

The low dose immersion exposure provided an effective method to generate a high proportion of PCR positive spat that survived a virulent OsHV-1 challenge. In a short-term period of continuous re-exposure by virtue of cohabitation, there was not a substantial increase in mortality. The long-term outcome of this apparently subclinical infection remains to be determined. A more rigorous re-challenge with OsHV-1 is required to determine if a state of disease resilience was conferred in surviving the experimental infection.

Table 1.6. Prevalence and quantity of OsHV-1 detected by qPCR after first challenge by immersion.

Data are for the experiment in which temperature was manipulated to alter survival of an OsHV-1 laboratory challenge.

Time of temperature change 22°C to 14°C (hours post challenge)	Challenge	OsHV-1 qPCR (pools positive / pools tested)	Quantity OsHV-1 DNA (genome equivalents.mg^{-1})	
			Average	St. error
Control (22°C)	Negative control	0/7	Not Detected	-
	OsHV-1	5/5	9.26×10^4	1.05×10^4
4	Negative control	0/0	Not Detected	-
	OsHV-1	1/8	Not Quantified	-
8	Negative control	0/5	Not Detected	-
	OsHV-1	4/7	Not Quantified	-
12	Negative control	0/7	Not Detected	-
	OsHV-1	4/7	16.40	1.47

Table 1.7. Total cumulative mortality (%) for spat challenged with OsHV-1.

at the completion of the trial for spat challenged with OsHV-1 there was a high background mortality independent of the change in water temperature and challenge with OsHV-1 or negative control inoculum in this batch of spat in the laboratory.

Inoculum preparation		Mortality (%)			
		Time until water temperature change (hours post challenge)			
First challenge	Second challenge	No change	4	8	12
Control	Control	57	43	63	61
	OsHV-1	68	31	24	56
OsHV-1	Control	67	70	45	58
	OsHV-1	37	53	56	45

4.2.2 Experiment 2. Water temperature manipulation to alter survival of spat during OsHV-1 challenge

A successful initial immersion challenge with OsHV-1 was indicated by high prevalence of infection and load of OsHV-1 DNA (>10^4 genome equivalents per mg) in spat challenged and maintained at 22°C water temperature (Table 1.8). Interestingly, the same immersion challenge did not lead to a productive OsHV-1 infection when the water temperature was reduced rapidly to 14°C at 4, 8 or 12 hours after challenge. The arresting of OsHV-1 replication below a quantity that is consistent with disease was achieved by a change in water temperature as late as 12 hours after challenge.

There was no indication across the duration of the study that survival of spat was increased after previously surviving a challenge with OsHV-1. This is consistent with the finding of the preliminary spat conditioning trial reported in Milestone 4 (1/8/2016). In the present experiment, a rigorous re-challenge demonstrated that PCR positive survivors of a pre-exposure to OsHV-1 did not generate a cohort of more resilient spat. Further, there was no protective effect evident for spat that were exposed to an infectious OsHV-1 challenge but then protected temporarily from disease by arresting virus replication with a sudden decrease in water temperature to 14°C.

The total cumulative mortality for this batch of oysters was 52.1% for all replicates and treatment groups. This indicates high background mortality in the present laboratory husbandry system over the period of almost 2 months. The mortality was the same for spat that were only challenged by immersion with a negative control tissue homogenate inoculum on the first and second challenges (average mortality 56.0%) and were not subject to a temperature change (57.0%) compared to treatment groups in which OsHV-1 replicated (Table 1.9).

4.2.3 Experiment 3. Pre-exposure of Pacific oysters > 6 months of age and re-challenge OsHV-1

There was improved survival of 6 month old spat challenged with OsHV-1 at 22°C when they had previously survived a challenge at 18°C, when compared with naïve spat challenged at 22°C and spat that had been given a negative control inoculum at 18°C before being challenged at 22°C (Table 1.10)

This preliminary trial provides first laboratory indication of resistance to disease caused by OsHV-1 conferred specifically by survival of a previous OsHV-1 challenge. Controlled exposure to OsHV-1 at a specific water temperature presents an important potential management tool for farmers faced with seasonal recurrence of POMS.

A series of experiments have considered the survival of spat < 6 months of age and/or < 40 mm total shell length that were challenged with OsHV-1 subsequent to a controlled pre-exposure to OsHV-1. There was no evidence in these experiments that pre-exposure to OsHV-1 conferred any resistance to disease in surviving spat when challenged again with OsHV-1. In a preliminary experiment using 6 month old spat, there was evidence that surviving pre-exposure conferred some resistance to mortality when re-exposed to OsHV-1 in a controlled laboratory experiment. The possibility that an age-dependent mechanism for improved survival by virtue of previous exposure to OsHV-1 requires further investigation as it would form the basis of an important disease mitigation strategy.

Figure 1.2. Experiment design for evaluation of the effect of pre-exposure on survival of spat.

Spat were subject to repeated immersion exposure to OsHV-1 with manipualtion of water temperature to alter survival.

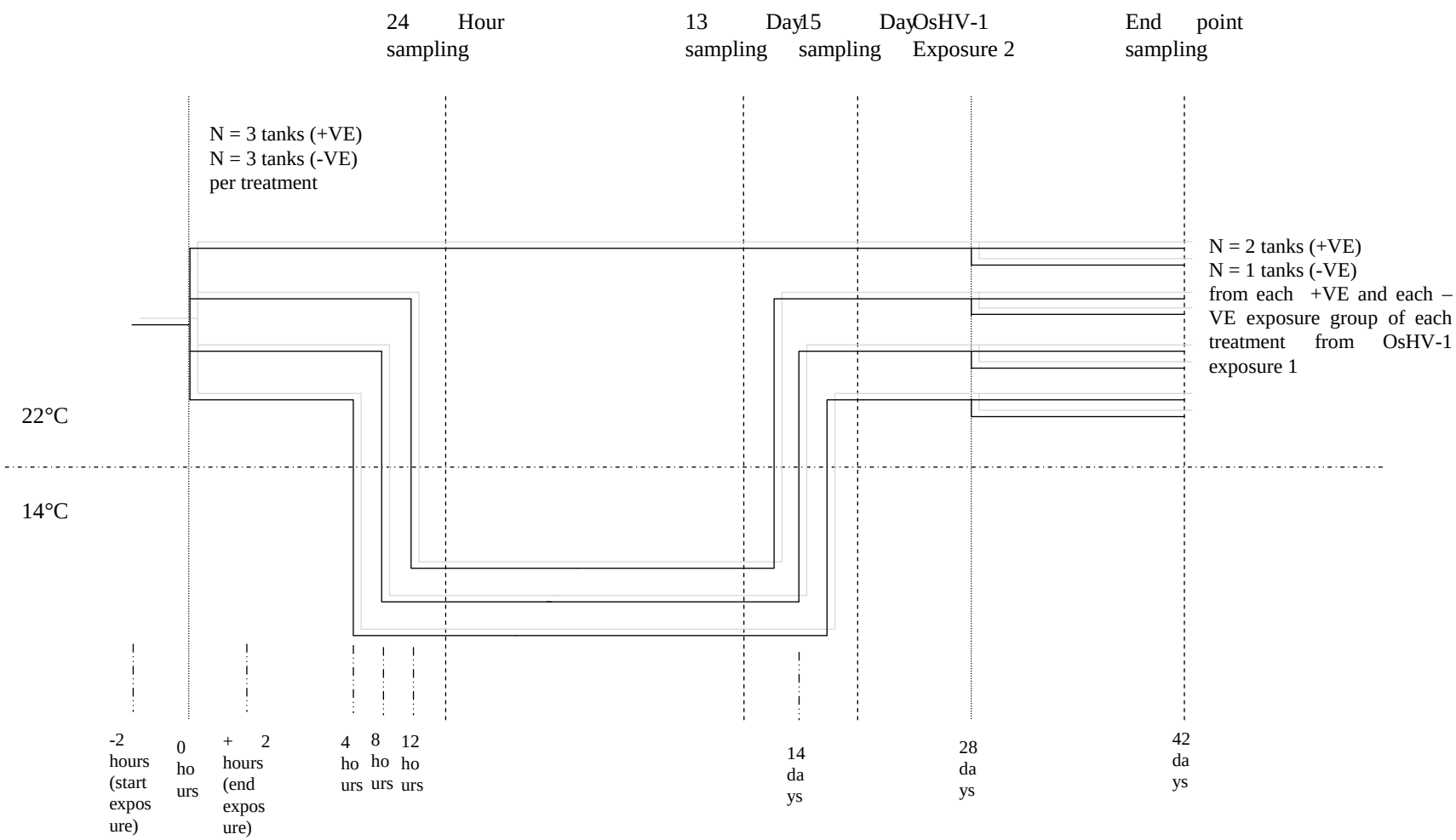


Table 1.8. Experiment design and survival of two batches of spat with repeated OsHV-1 immersion challenge.

Data are total cumulative mortality after a low dose immersion challenge with OsHV-1 and subsequent re-challenge of the survivors through cohabitation. Results for all replicate tanks were similar and were combined for analysis.

Batch	Exposure [a]	Dilution (dose) [b]	Reps	No. of spat / rep	Survivors (%)		Total cumulative mortality (%)
					Initial challenge [c]	Re-challenge period [d]	
SPL14B	OsHV-1	$1/10^3$	2	250	84	91	23.6
		$1/10^4$	2	250	88	95	16.4
		$1/10^5$	2	250	97	98	4.9
		$1/10^6$	2	250	100	100	0.0
SPL13B	OsHV-1	$1/10^3$	2	250	64	64	59.0
		$1/10^4$	2	250	83	72	40.2
		$1/10^5$	2	250	91	84	23.6
		$1/10^6$	2	250	93	85	21.0
SPL14B	negative control	$1/10^3$	2	250	100	99	1.0
SPL13B	negative control	$1/10^3$	2	250	100	98	2.0
SPL14B	no lab exposure	n/a	1	250	100	100	0.0

[a] OsHV-1 as a fresh clarified mantle and gill tissue homogenate or a negative control tissue homogenate without OsHV-1 infection.

[b] Expressed as a final dilution of inoculum in ASW, the undiluted (1/10 w/v) inoculum had 2.4×10^7 OsHV-1 genome copies per µl

[c] A detailed examination and removal of all dead oysters at 6 days post-exposure.

[d] Proportion of live spat at 6 days that were still alive after a further 6 days in the laboratory with re-exposure to OsHV-1 by cohabitation.

Table 1.9. Quantity of OsHV-1 DNA at different time points after exposure to OsHV-1 by immersion challenge.

Data are for pools of spat exposed to OsHV-1 by immersion. Spat exposed to negative control tissue homogenate are not included in this table as OsHV-1 qPCR tests were negative, as were the samples tested prior to exposure. Replicates were pooled for PCR analysis.

Batch	**Dilution (dose)** [a]	**Selection**	**6 days after challenge**		**12 days after first challenge**	
			No. pools positive[b]	**Log_{10}(OsHV-1 genome copies/mg) (mean ± sd)**	**No. pools positive**[b]	**Log_{10}(OsHV-1 genome copies/mg) (mean ± sd)**
SPL14B	$1/10^2$	Live	5 / 5	4.28 ± 0.53	5 / 5	3.19 + 0.65
		Dead	3 / 3	3.84 ± 0.35	No sample	-
	$1/10^3$	Live	5 / 5	4.83 ± 0.91	5 / 5	3.57 + 0.35
		Dead	3 / 3	3.97 ± 0.45	No sample	-
	$1/10^4$	Live	3 / 5	4.26 ± 1.16	5 / 5	3.79 + 1.26
		Dead	No sample	-	No sample	-
	$1/10^5$	Live	0 / 5	0	0 / 5	0
		Dead	No sample	-	No sample	-
SPL13B	$1/10^2$	Live	5 / 5	4.94 ± 0.33	5 / 5	2.88 + 1.86
		Dead	3 / 3	4.90 ± 0.44	3 / 3	3.59 + 0.63
	$1/10^3$	Live	5 / 5	4.44 ± 1.01	5 / 5	3.14 + 1.39
		Dead	3 / 3	5.78 ± 0.24	3 / 3	2.50 + 0.48
	$1/10^4$	Live	5 / 5	4.17 ± 1.03	5 / 5	3.50 + 1.04
		Dead	3 / 3	5.71 ± 0.26	3 / 3	3.32 + 0.54
	$1/10^5$	Live	5 / 5	4.07 ± 0.72	5 / 5	3.76 + 1.28
		Dead	3 / 3	5.18 ± 0.46	3 / 3	3.60 + 1.34

[a] Expressed as a final dilution of inoculum in ASW, the undiluted (1/10 w/v) inoculum had 2.4×10^7 OsHV-1 genome copies per µl

[b] Pools containing quantifiable concentration of OsHV-1 DNA were considered positive.

Table 1.10. Cumulative mortality for juvenile oysters with repeated laboratory challenge with OsHV-1.

Experiment design and total cumulative mortality for 6 month old triploid Pacific oyster spat challenged with OsHV-1 and then subsequently re-challenged in a laboratory environment with water temperature at 18°C or 22°C.

1st Exposure					2nd Exposure			
OsHV-1 Challenge	Water temperature (°C)		n	Mortality % (95% CI)	OsHV-1 Challenge	Water temperature (°C)	n	Mortality % (95% CI)
	0-14 days	14–35 days						
+VE	18	22	135	3.0 (0.1 - 5.8)	+VE	22	112	8.9 (3.7 - 14.2)
					-VE		19	0
-VE	18	22	145	4.8 (1.3 - 8.3)	+VE	22	114	20.0 (15.9 - 31.5)
					-VE		24	0
+VE	18	18	46	2.2 (-2.0 - 6.4)	+VE	18	23	6.52 (0 - 26.8)
					-VE		22	0
+VE	22	22	208	73.6 (67.6 - 79.6)	+VE	22	41	27.45 (19.6 - 48.7)
					-VE		13	23.08 (0.2 - 46.0)
-VE	22	22	143	3.5 (0.5 - 6.5)	+VE	22	114	43.3 (31.3 - 49.4)
					-VE		24	100
-VE	18	18	55	3.6 (0 - 8.6)	+VE	18	27	1.9 (0 - 10.8)
					-VE		26	0

4.3 Interstate temperature monitoring

Probes were deployed in estuaries at 7 locations in NSW, 14 locations in Tasmania and 12 locations in South Australia (Figure 2). Data recording commenced at most sites on 18 October 2014; one additional site was added in March 2016 (Bruny Island, Tasmania). The rate of return of probes was excellent; several probes were lost at sea during bad weather and these were replaced each time, but left gaps in the data for variable periods. Two farms were sold or ceased trading and recording was terminated in July 2015 and August 2016, respectively. Therefore, comprehensive water temperature data for three consecutive summer seasons were obtained for the period October 2014 to June 2017 from the majority of sites. The average daily water temperatures at each site are shown in Figures 1, 2 and 3.

The significant water temperatures for risk of mortality due to OsHV-1 were determined separately in epidemiological studies in NSW, including in the Window of /Infection trial which was conducted between the 2012-13 and 2016-17 summer seasons. The risk of mortality commenced when water temperatures rose above 20°C in spring and ceased when water temperatures fell below 17 °C in autumn (this final report). The index cases in Tasmania in January 2016 in Upper and Lower Pitt Water occurred when water temperatures were >20°C (Figure 3). Using these data as reference points, the risk of mortality due to OsHV-1 was assessed at each site in NSW (Figure 1), Tasmania (Figure 2) and South Australia (Figure 3).

In NSW, the risk in the northern estuaries of Wallis Lakes and Port Stephens is of similar duration or longer than in the Hawkesbury and Georges River estuaries, while that in the southern estuaries of the Shoalhaven River, Clyde River and Wapengo appears to be slightly shorter. All estuaries are considered to be at risk based on water temperature profiles.

In Tasmania, the 2015-16 summer was warmer than 2014-15 and 2016-17. In the north coast estuaries at Smithton and Port Sorell the risk period ran from early November through to mid-March across 2014-15 and 2015-16, but in the 2016-17 risk was delayed to December but extended to late March. At St Helens on the northern part of the east coast, there was low risk in 2014-15 but in both 2015-16 and 2016-17 water temperatures exceeded 20°C for a short period in mid-summer. At Freycinet, water temperatures barely exceed 20°C in both subtidal and deep water locations in mid-summer 2015-16 therefore risk overall was low. In each of the more southern locations on the east coast average daily water temperatures exceeded 20°C in one or more seasons but particularly 2015-16 (Little Swanport, Dunnalley, Eagle Hawk Neck), but in the shallow estuaries (Pitt Water, Pipeclay Lagoon) this occurred each season for a variable period.

In South Australia the water temperature profiles at each location except Kangaroo Island (for which records were incomplete) resembled those in NSW, with prolonged periods each summer season with water temperatures above 20°C commencing in September-October and temperatures remaining above 17°C until May (Figure 3). At Kangaroo Island, the profile was more similar to Tasmania than to New South Wales, with a shorter period in mid-summer being at risk.

Figure 2.1. Schematic showing the method of deployment of a sub-tidal temperature probes.

The probes (Thermocron DS1921G, Thermodata) were enclosed within a sealed tube attached to the base of a floating oyster basket, which was suspended from a floating long-line.

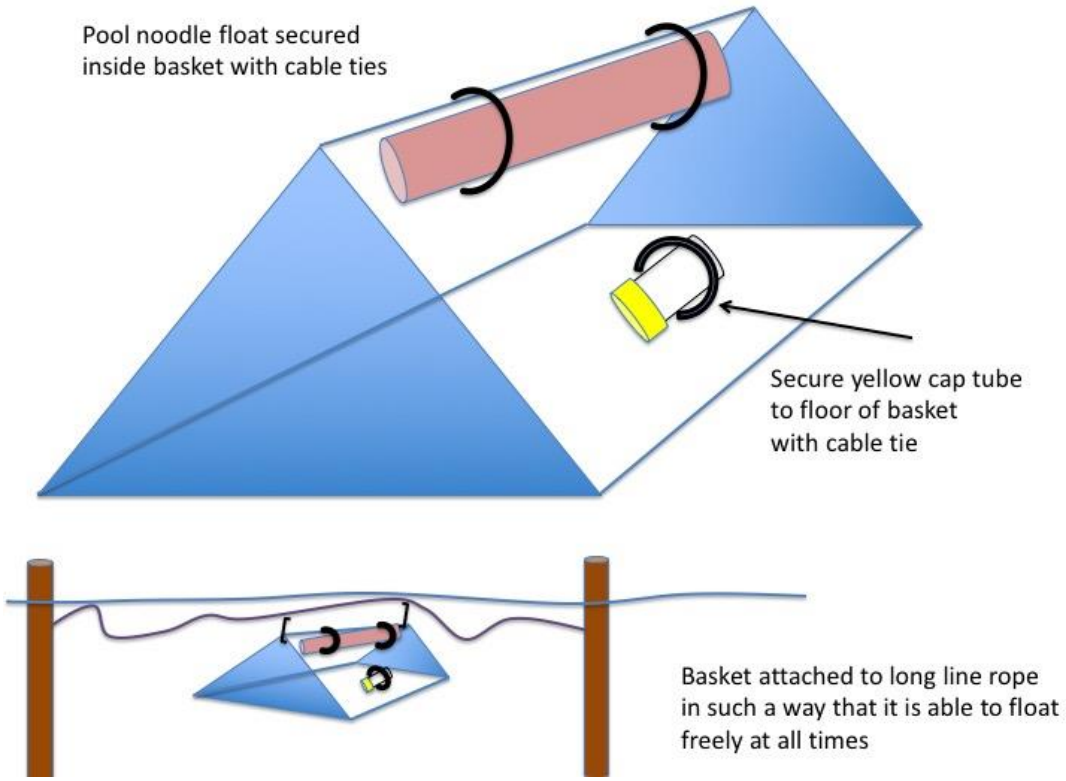
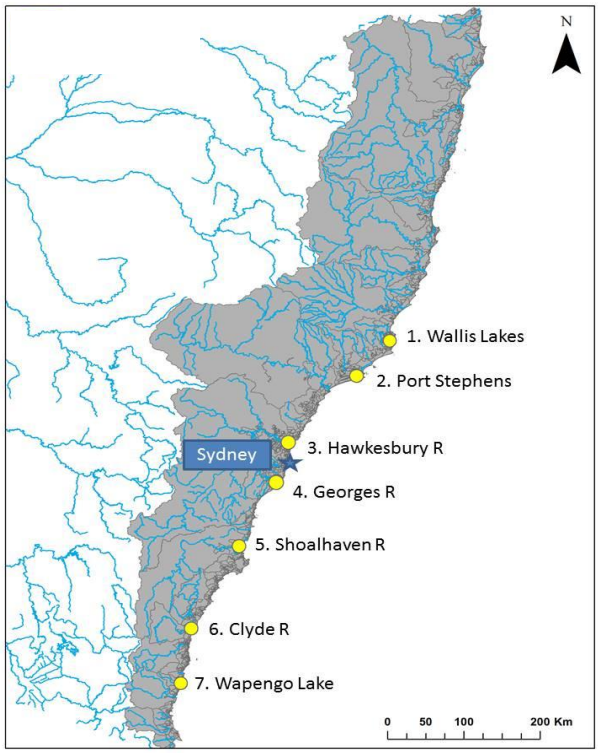


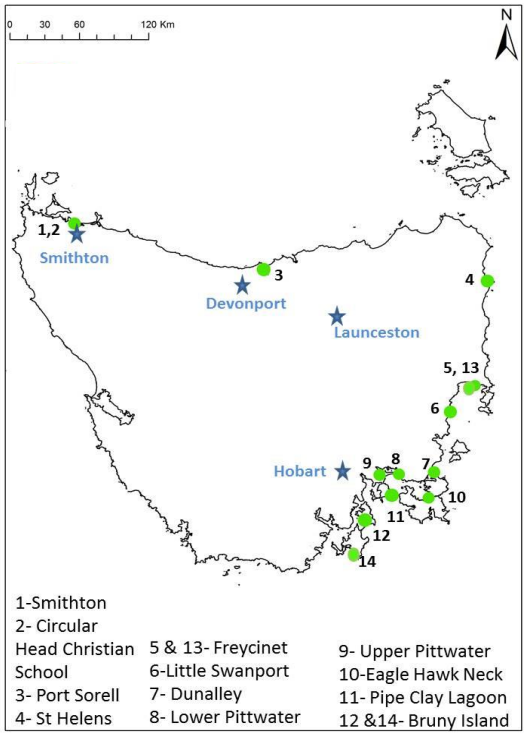
Figure 2.2. Location of estuaries where temperature loggers were deployed to monitor water temperature on oyster leases.

Panel A, New South Wales; Panel B, Tasmania; Panel C, South Australia.

A.



B.



C.

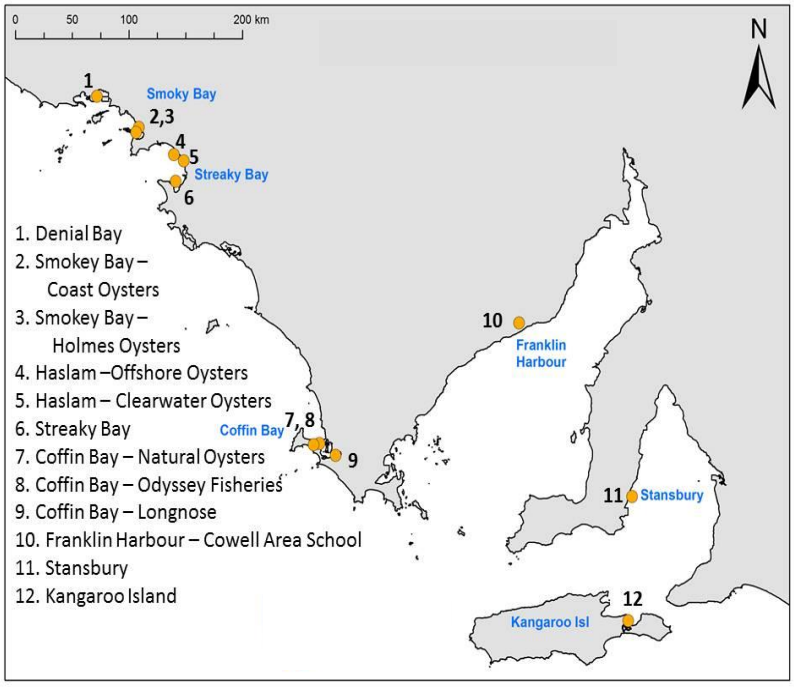
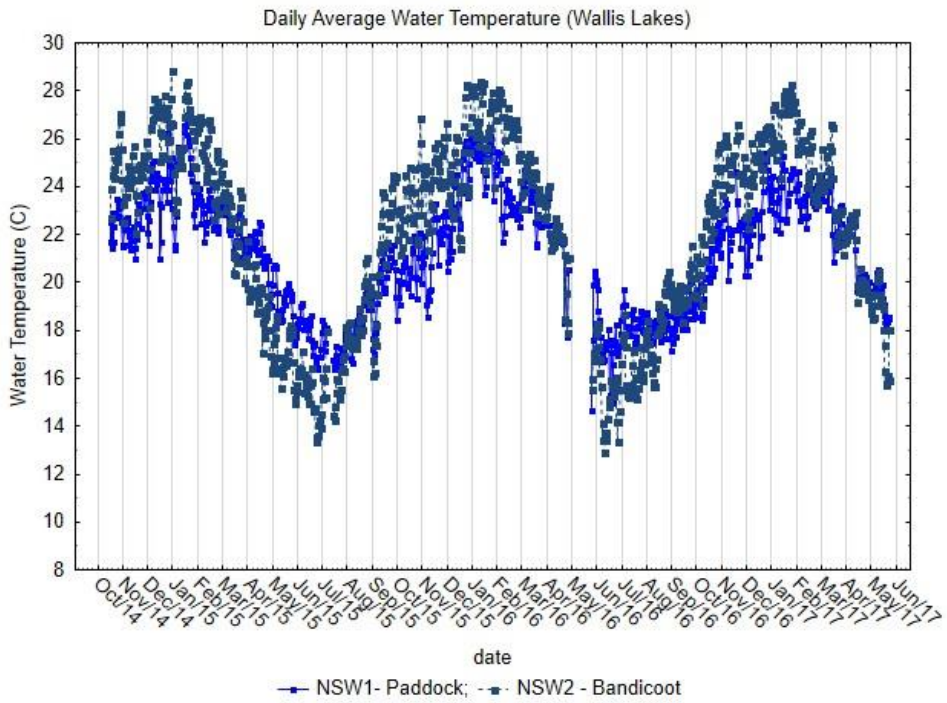
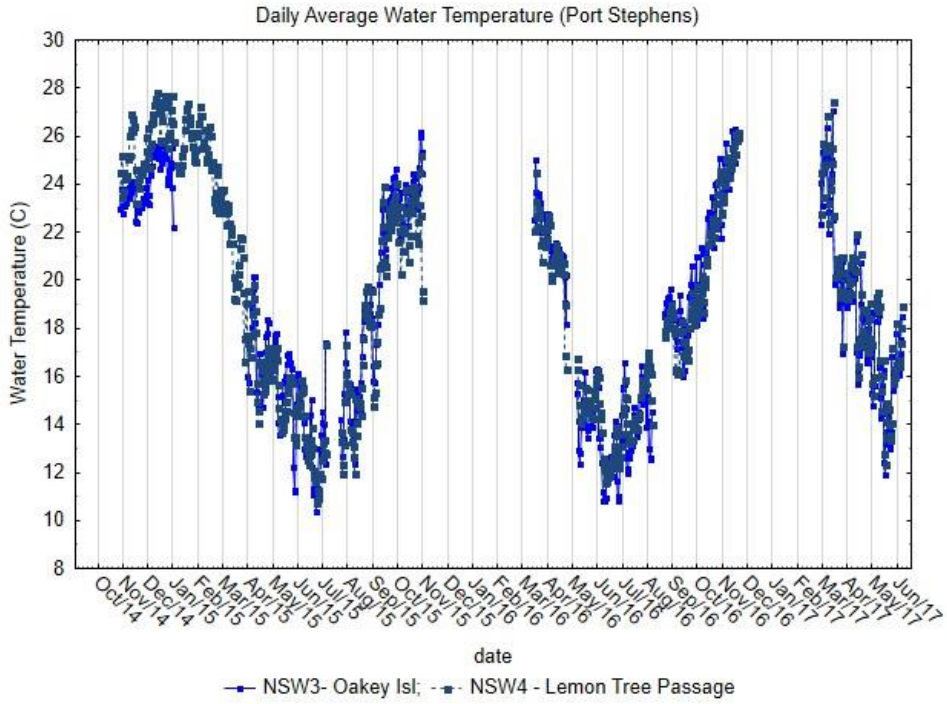
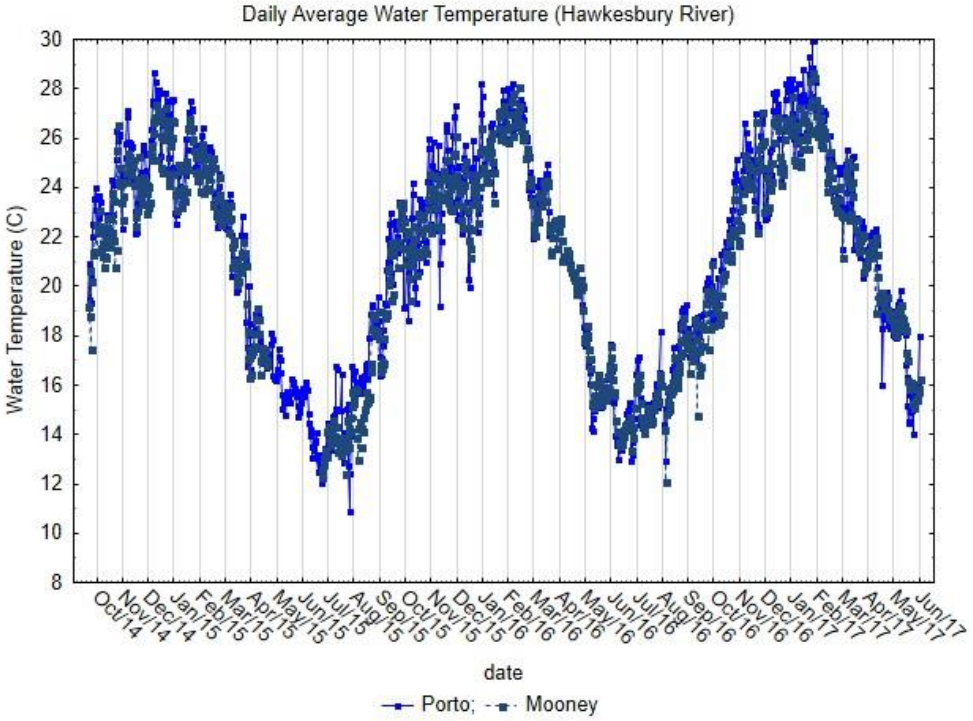


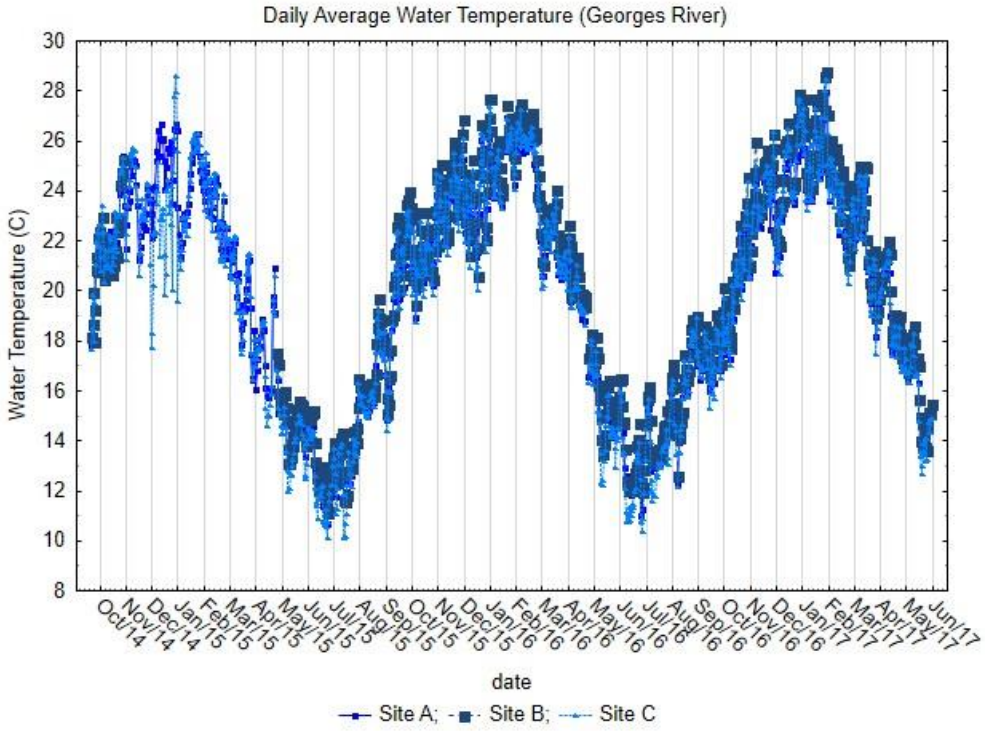
Figure 2.3. Daily average water temperature in oyster leases monitored in New South Wales.

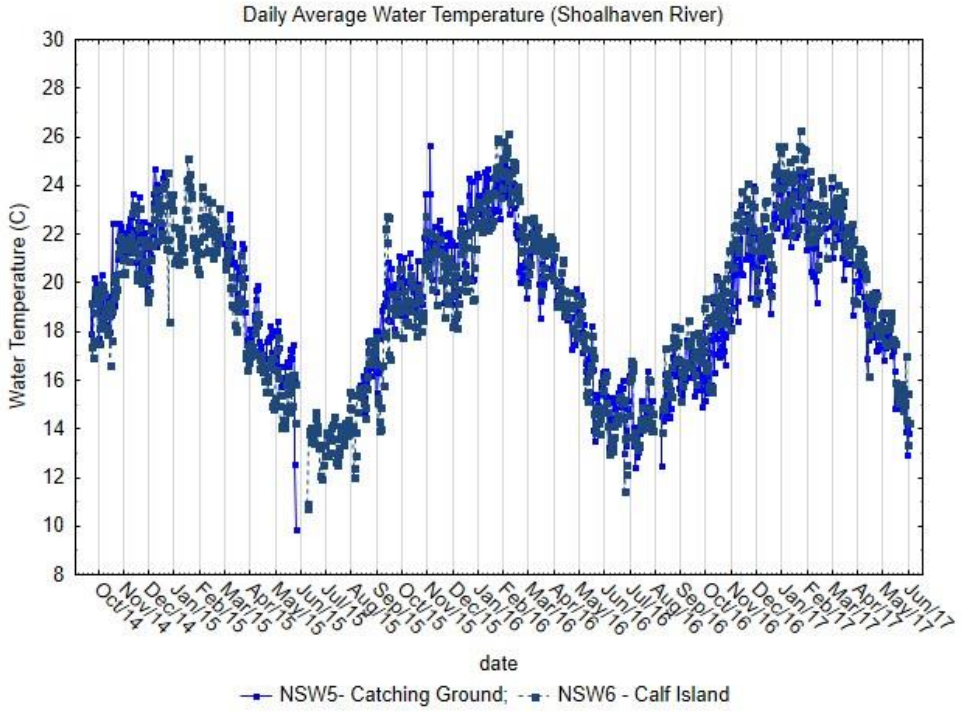
The average daily water temperature as measured in subtidal baskets on oyster leases in New South Wales between October 2014 and June 2017, arranged by site from north to south.

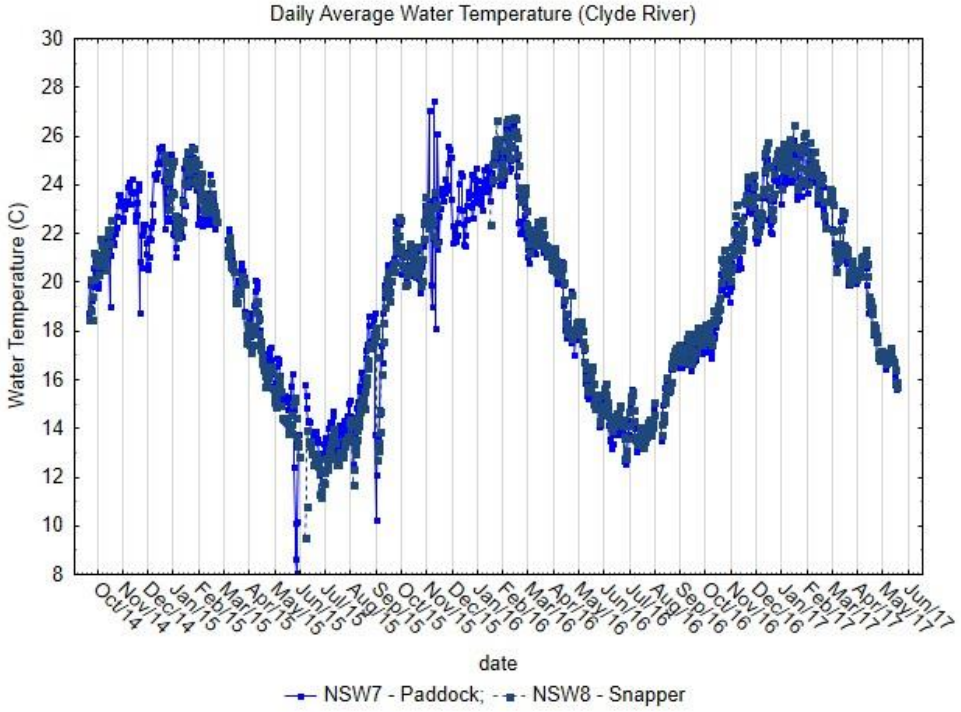


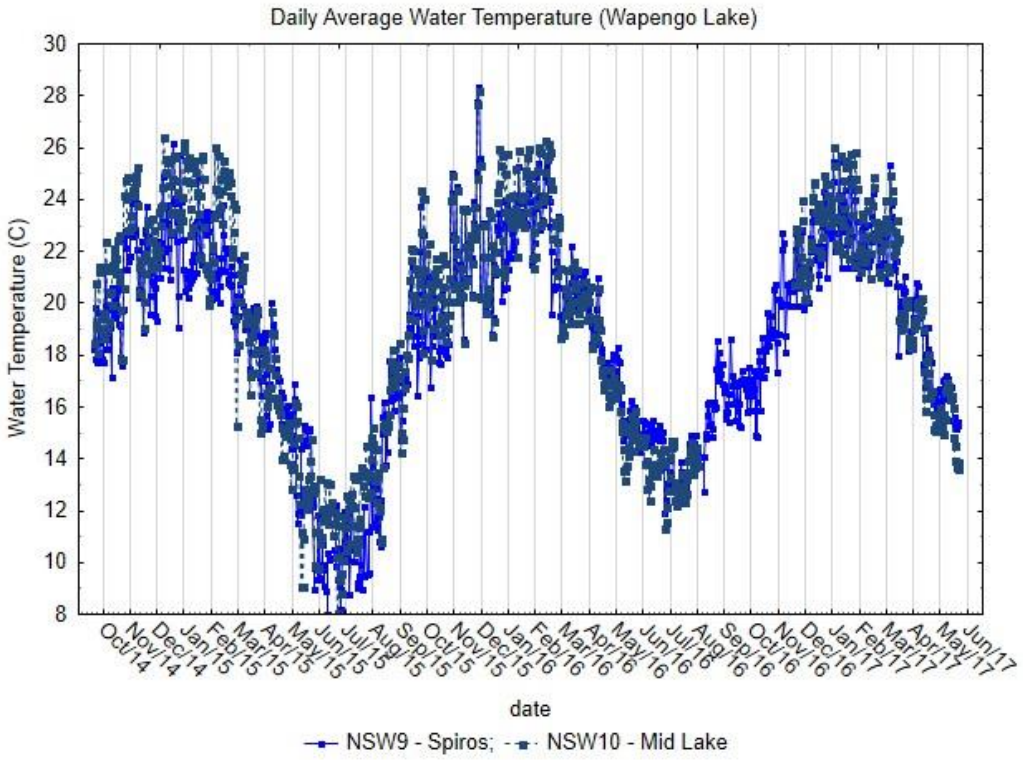



Daily Average Water Temperature (Hawkesbury River)
Water Temperature (C)
30
28
26
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
Porto; Mooney


Daily Average Water Temperature (Georges River)
Water Temperature (C)
30
28
26
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
Site A; Site B; Site C


Daily Average Water Temperature (Shoalhaven River)
Water Temperature (C)
30
28
26
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
NSW5- Catching Ground;
NSW6 - Calf Island


Daily Average Water Temperature (Clyde River)
Water Temperature (C)
30
28
26
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
NSW7 - Paddock;
NSW8 - Snapper


Daily Average Water Temperature (Wapengo Lake)
Water Temperature (C)
30
28
26
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
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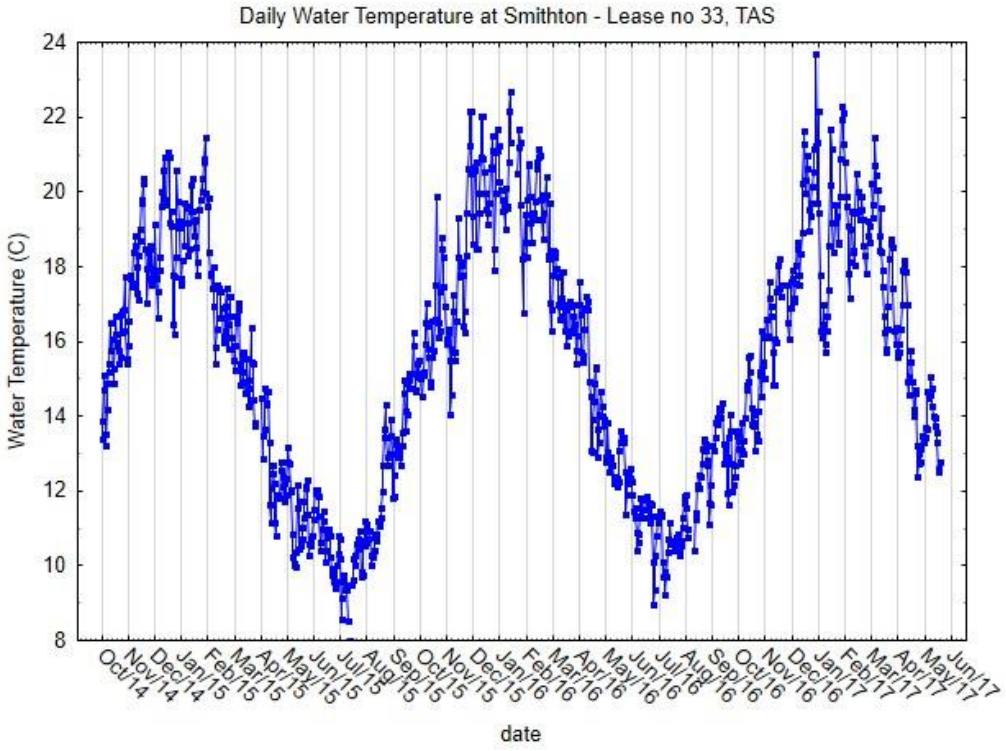
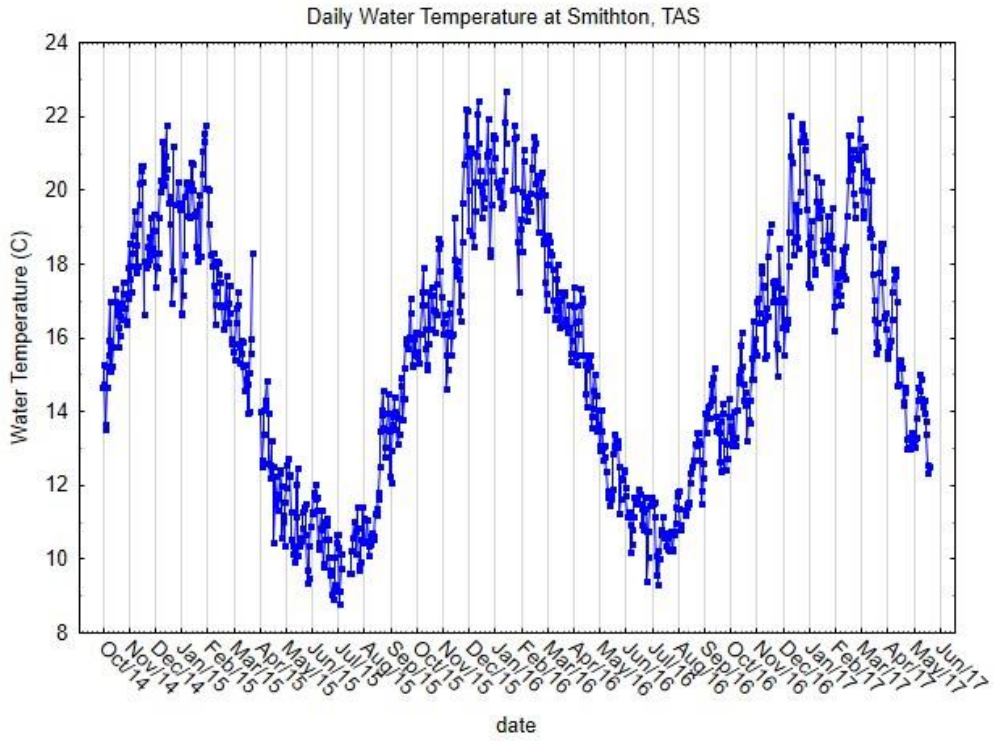
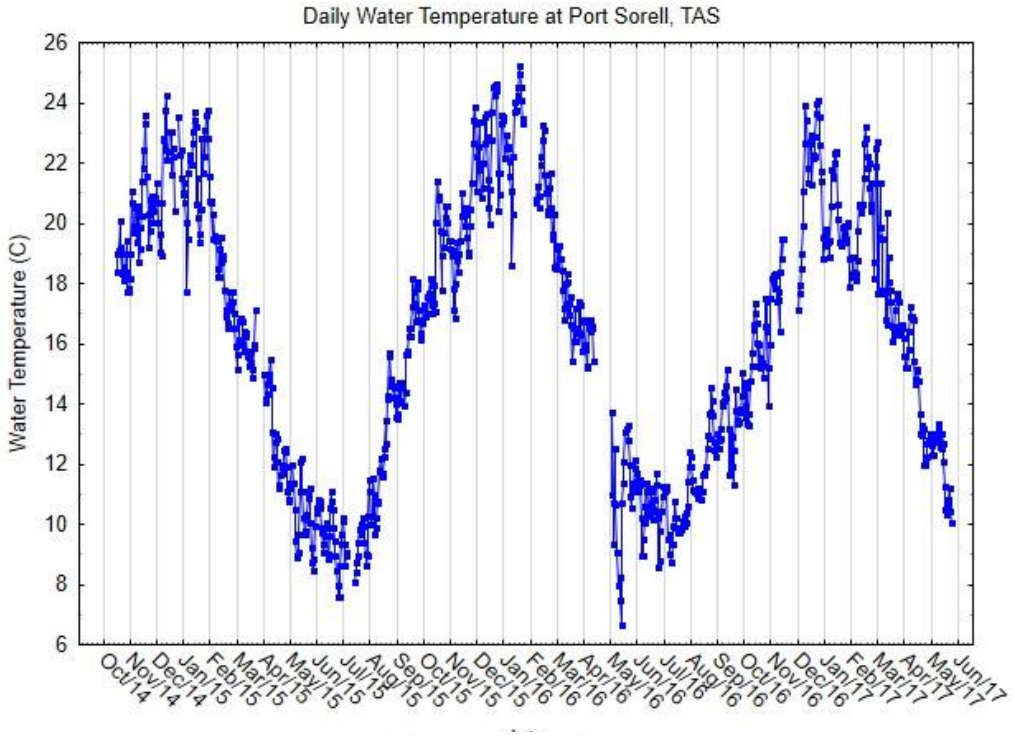
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
NSW9 - Spiros;
NSW10 - Mid Lake

Figure 2.4. Daily average water temperature in oyster leases monitored in Tasmania.

The average daily water temperature as measured in subtidal baskets on oyster leases in Tasmania between October 2014 and June 2017 arranged by site from north-west to south east. The time of the first reported outbreak at upper and lower Pittwater is indicated






Daily Water Temperature at Port Sorell, TAS
Water Temperature (C)
26
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
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Sep/16
Oct/16
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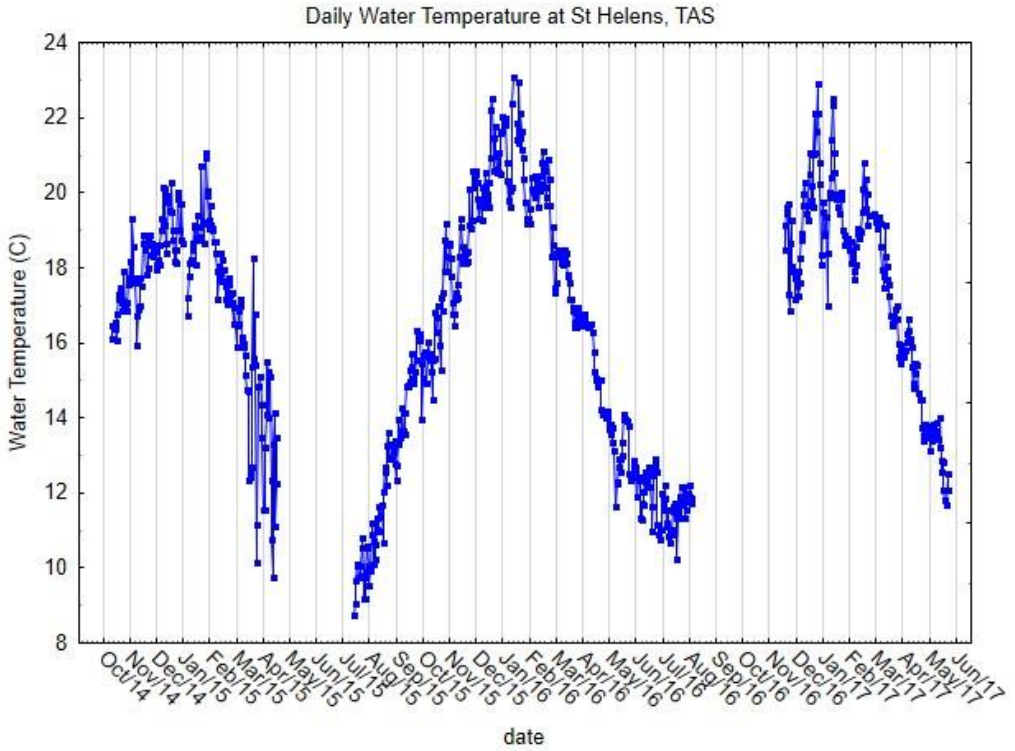
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17


Daily Water Temperature at St Helens, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
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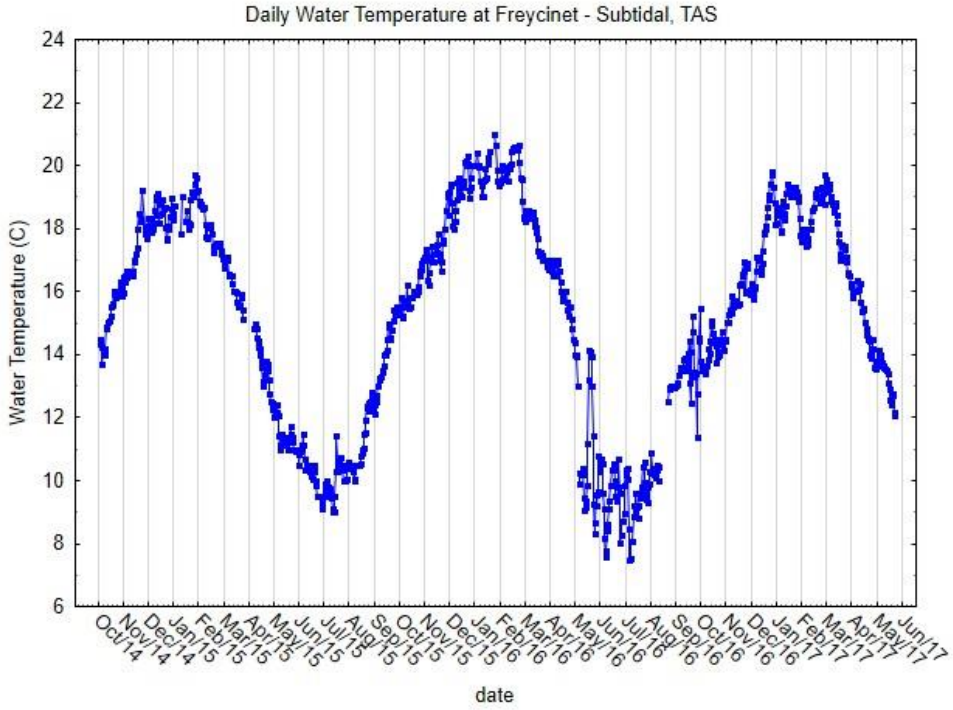
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date


Daily Water Temperature at Freycinet - Subtidal, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
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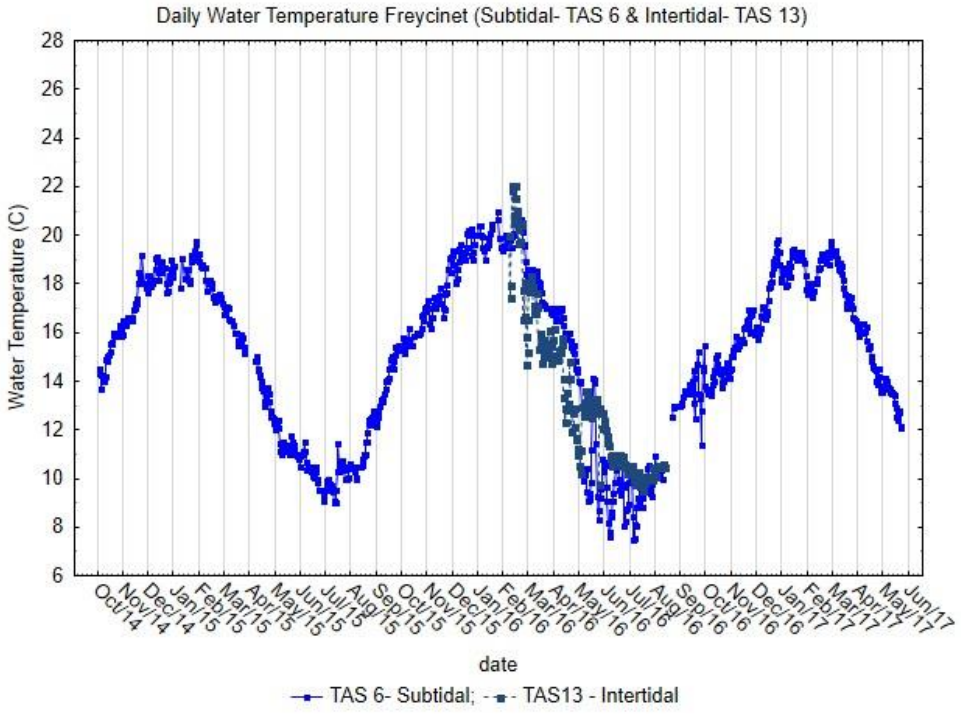
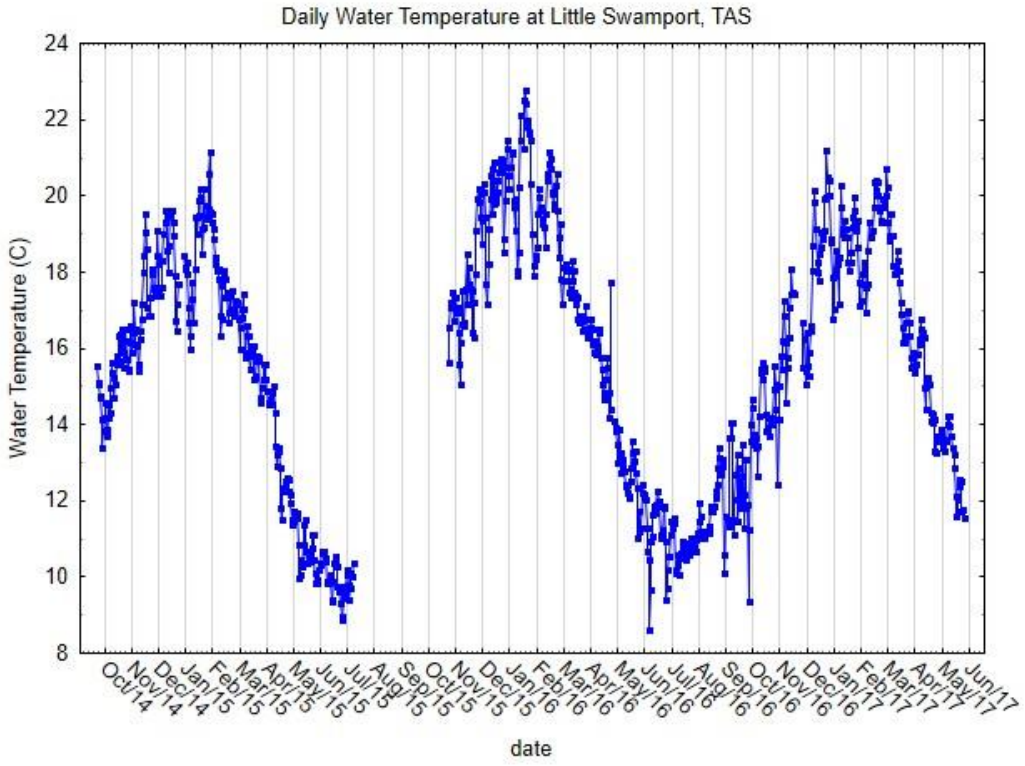
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date


Daily Water Temperature Freycinet (Subtidal- TAS 6 & Intertidal- TAS 13)
Water Temperature (C)
28
26
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
TAS 6- Subtidal;
TAS13 - Intertidal


Daily Water Temperature at Little Swamport, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
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May/16
Jun/16
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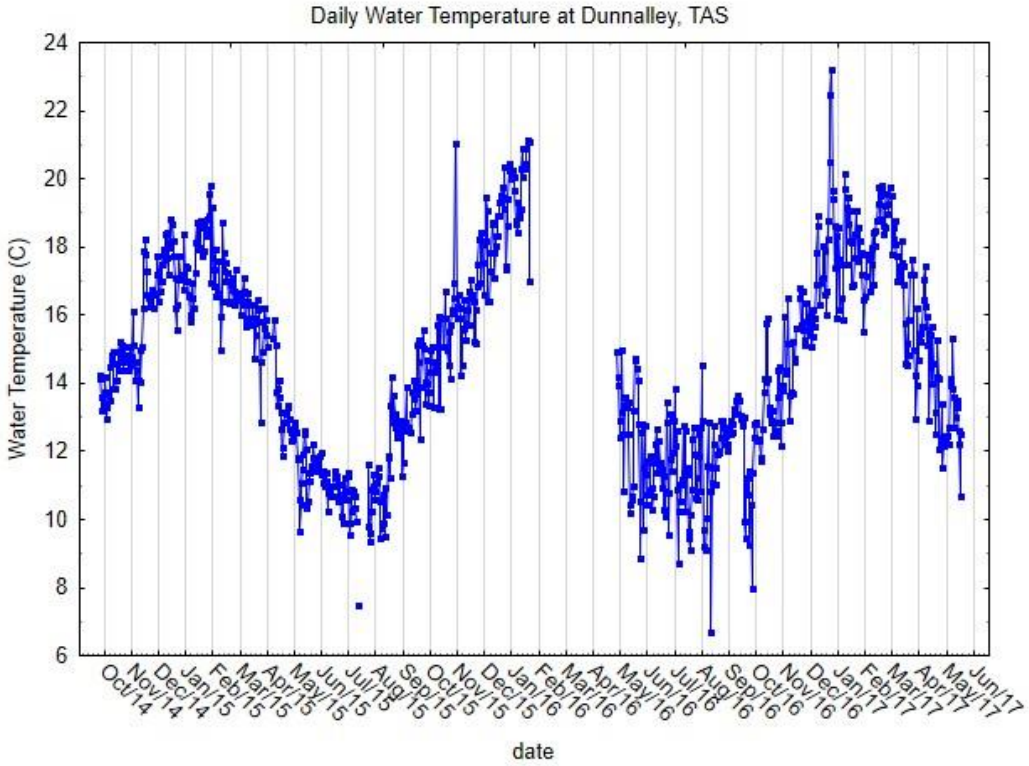
May/17
Jun/17
date


Daily Water Temperature at Dunnalley, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
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May/16
Jun/16
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Feb/17
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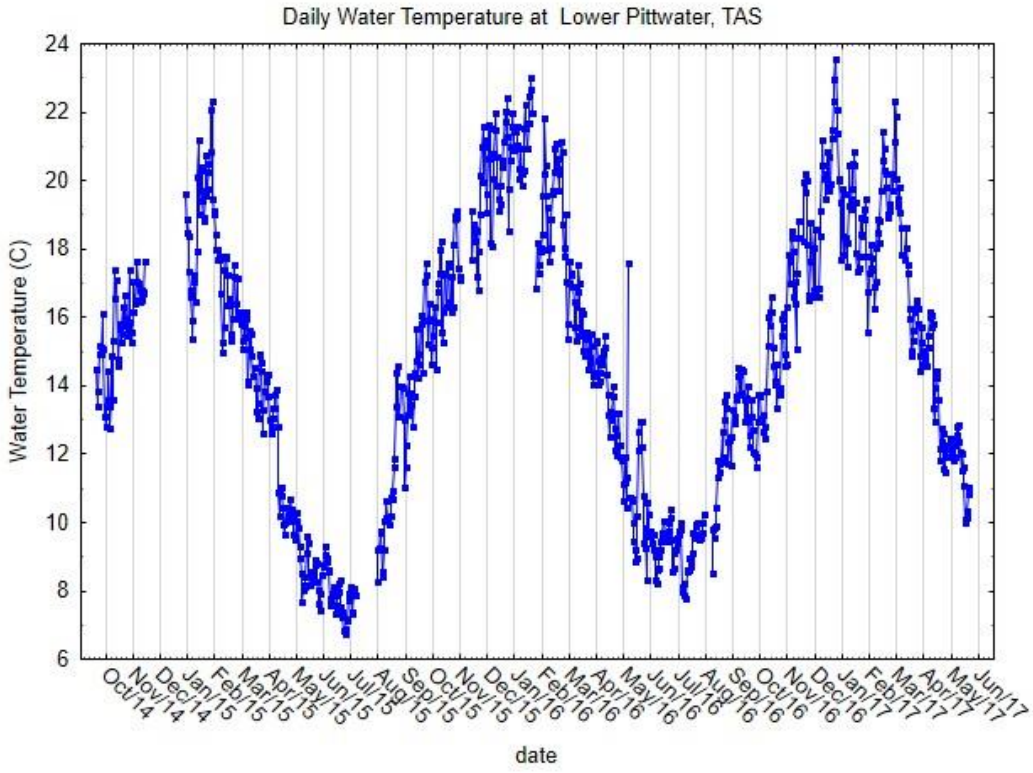
Apr/17
May/17
Jun/17
date


Daily Water Temperature at Lower Pittwater, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
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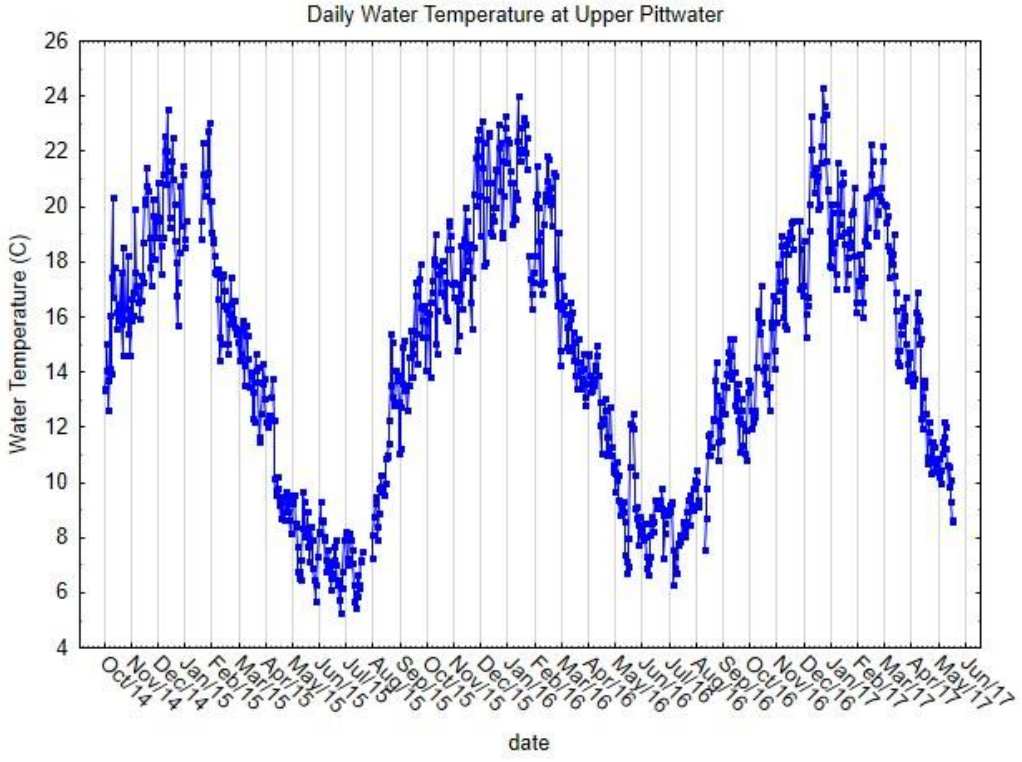
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date


Daily Water Temperature at Upper Pittwater
Water Temperature (C)
26
24
22
20
18
16
14
12
10
8
6
4
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
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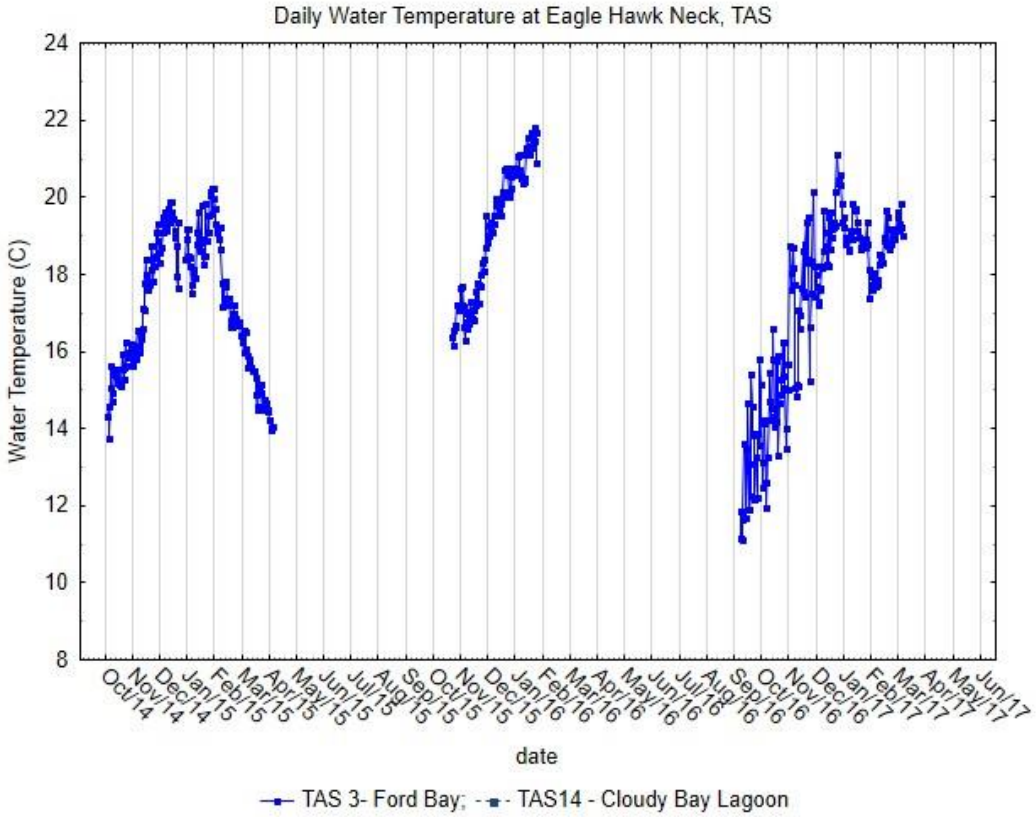
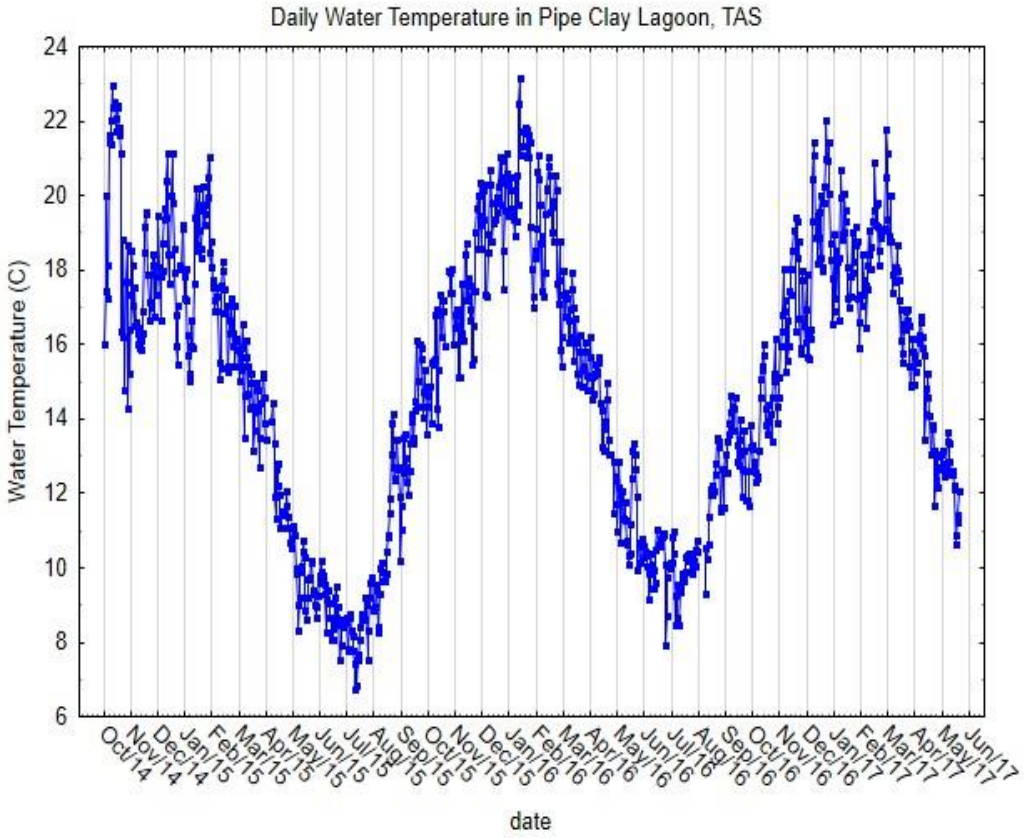
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date


Daily Water Temperature at Eagle Hawk Neck, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
Feb/16
Mar/16
Apr/16
May/16
Jun/16
Jul/16
Aug/16
Sep/16
Oct/16
Nov/16
Dec/16
Jan/17
Feb/17
Mar/17
Apr/17
May/17
Jun/17
date
TAS 3- Ford Bay; TAS14 - Cloudy Bay Lagoon


Daily Water Temperature in Pipe Clay Lagoon, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
Sep/15
Oct/15
Nov/15
Dec/15
Jan/16
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Mar/16
Apr/16
May/16
Jun/16
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Jan/17
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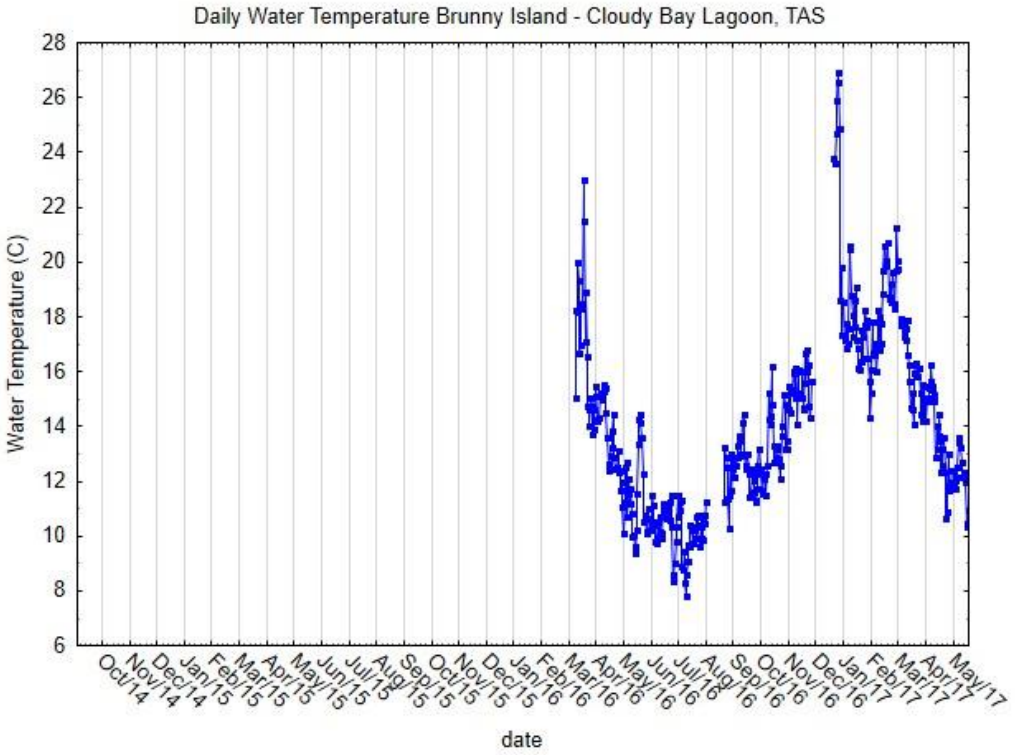
Apr/17
May/17
Jun/17
date


Daily Water Temperature Brunny Island - Cloudy Bay Lagoon, TAS
Water Temperature (C)
28
26
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
Jul/15
Aug/15
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Jan/16
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Apr/16
May/16
Jun/16
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Feb/17
Mar/17
Apr/17
May/17
date

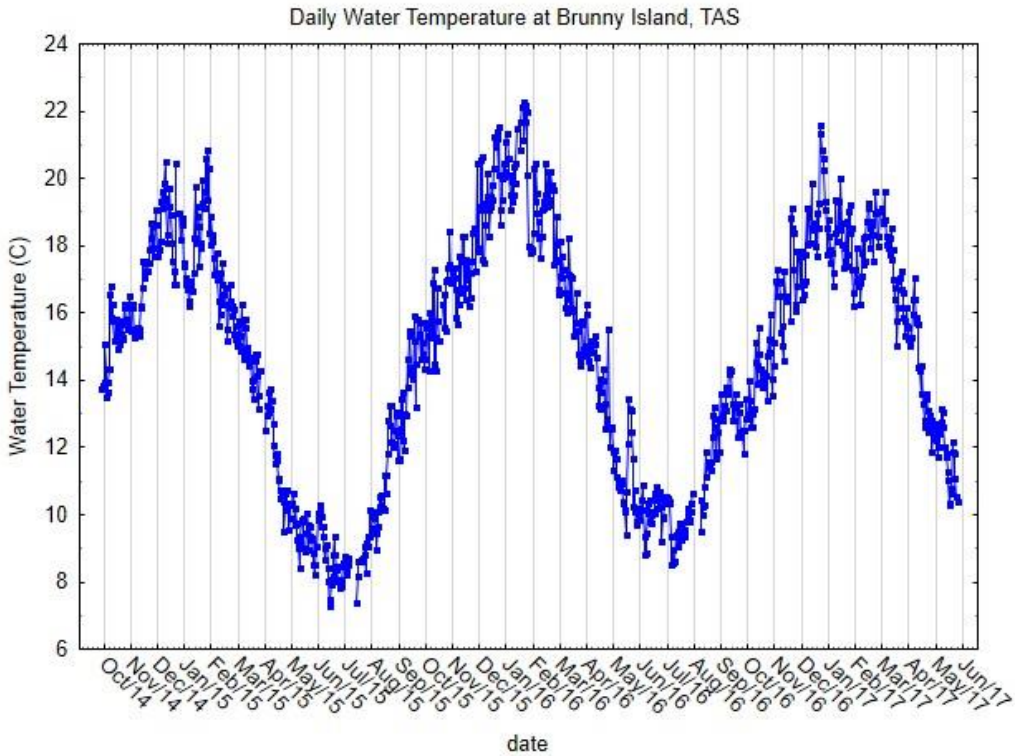
Daily Water Temperature at Brunny Island, TAS
Water Temperature (C)
24
22
20
18
16
14
12
10
8
6
Oct/14
Nov/14
Dec/14
Jan/15
Feb/15
Mar/15
Apr/15
May/15
Jun/15
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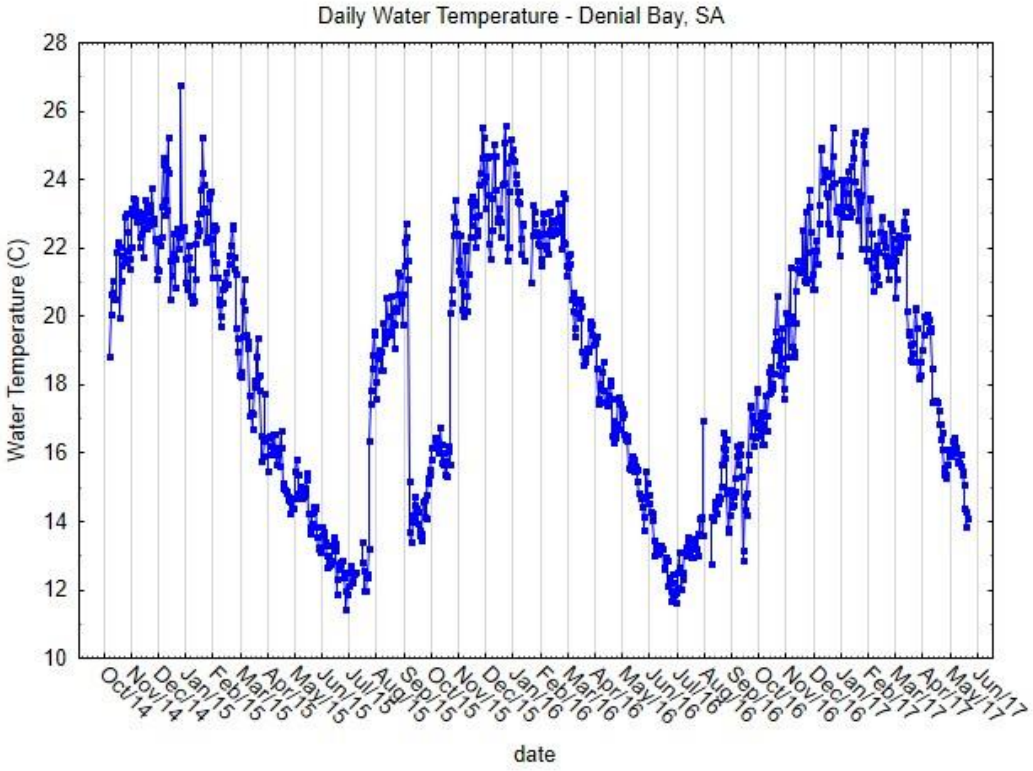
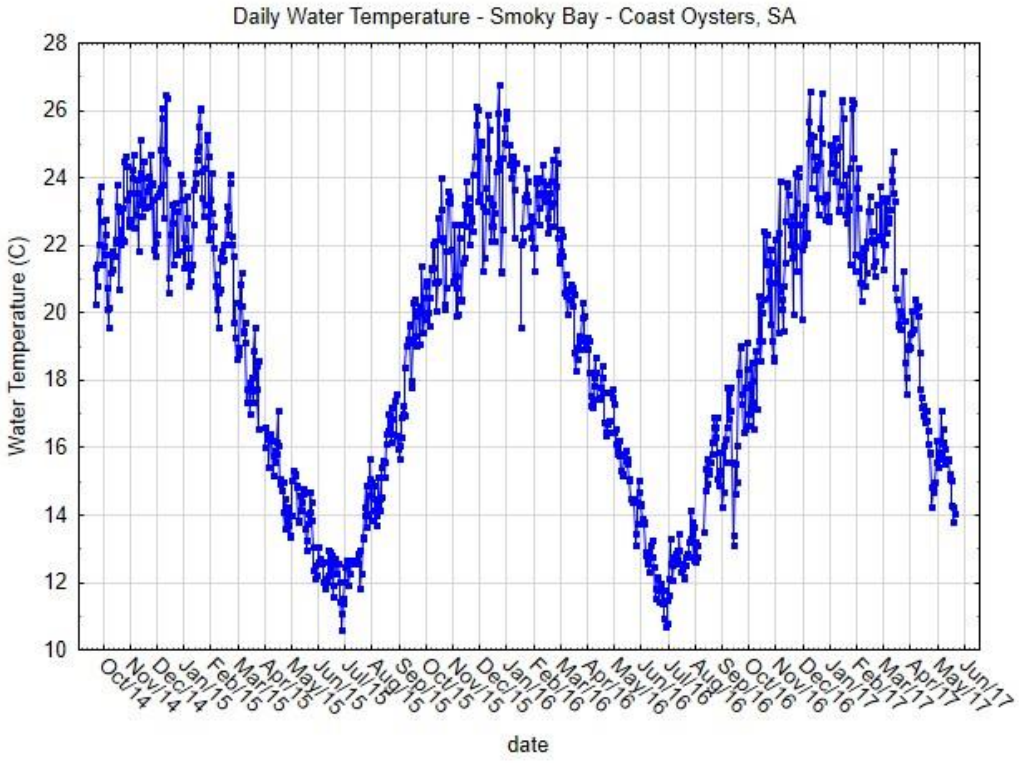
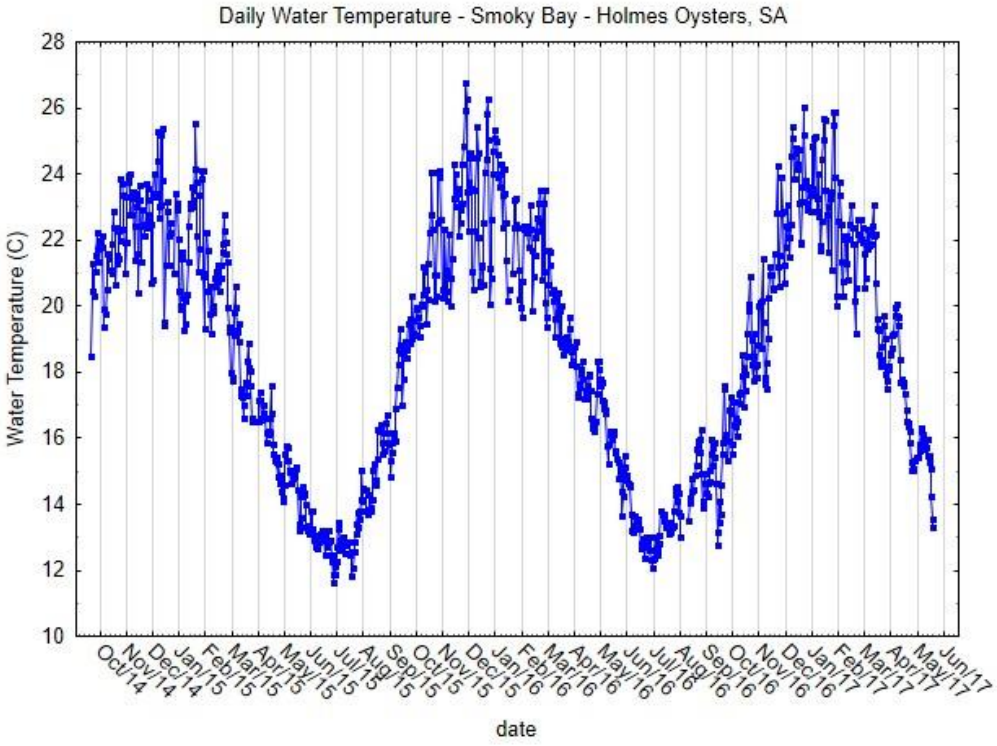
Figure 2.5. Daily average water temperature in oyster leases monitored in South Australia.

The average daily water temperature as measured in subtidal baskets on oyster leases in South Australia between October 2014 and June 2017 arranged by site from north-west to south-east.






Daily Water Temperature - Smoky Bay - Holmes Oysters, SA
Water Temperature (C)
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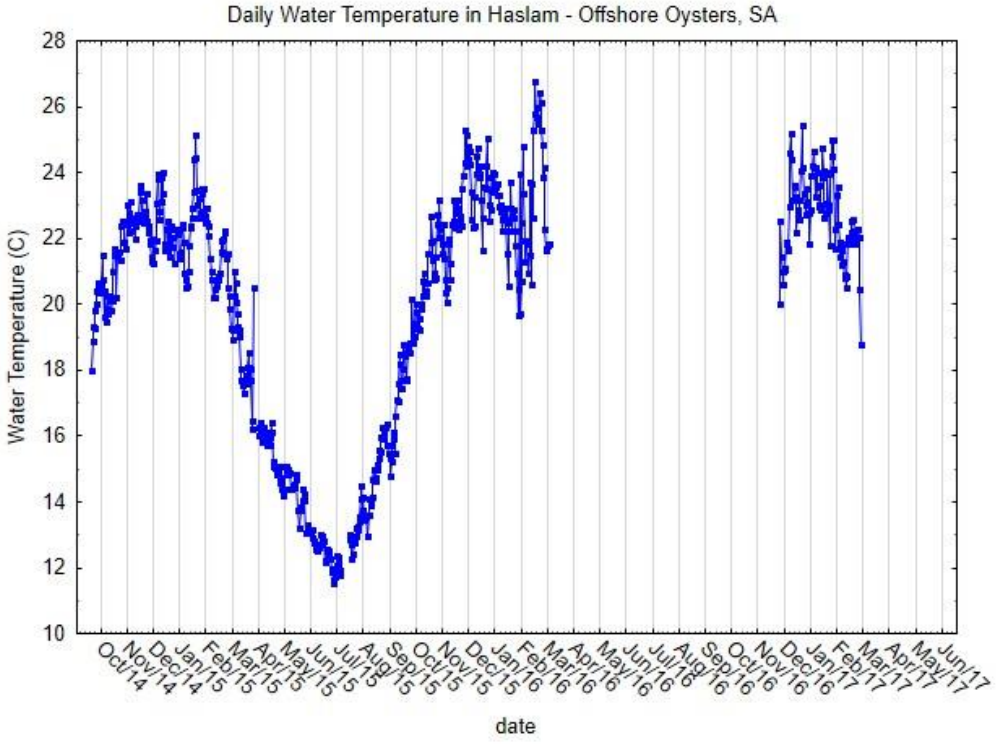
Daily Water Temperature in Haslam - Offshore Oysters, SA
Water Temperature (C)
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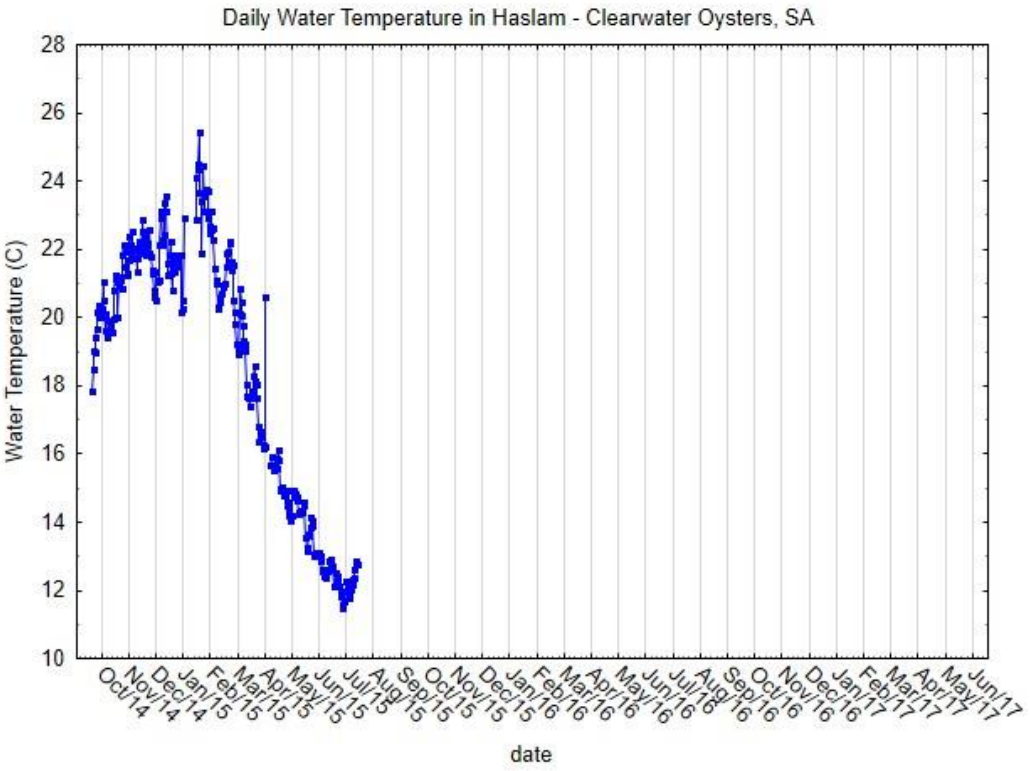
Daily Water Temperature in Haslam - Clearwater Oysters, SA
Water Temperature (C)
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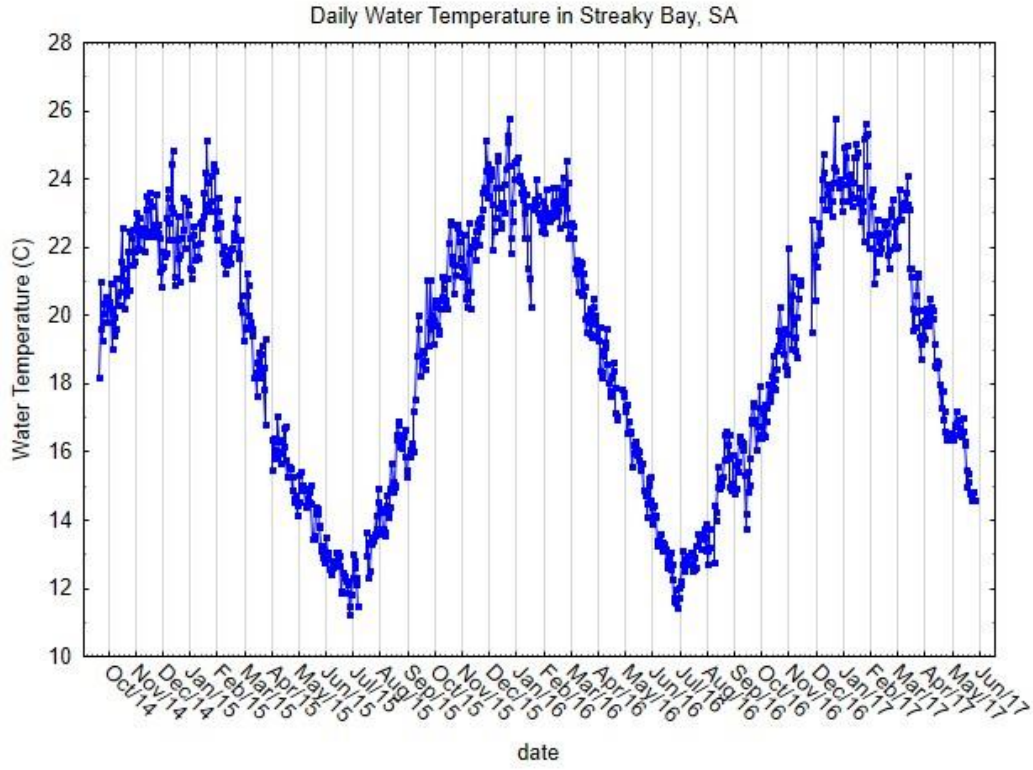
Daily Water Temperature in Streaky Bay, SA
Water Temperature (C)
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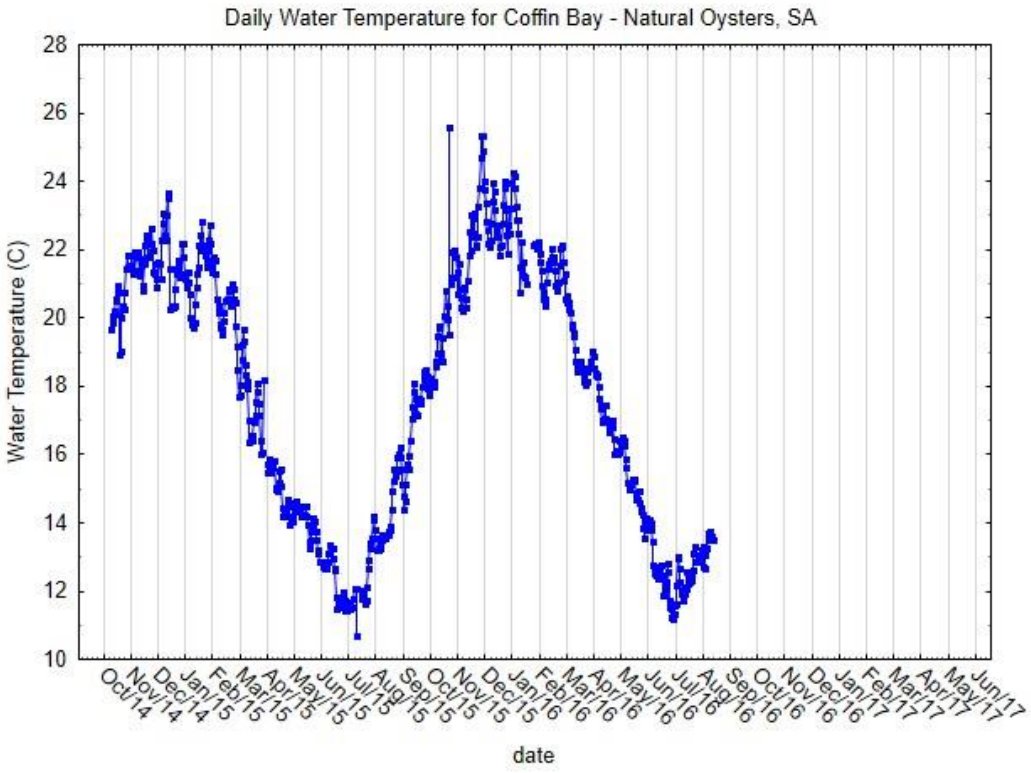
Daily Water Temperature for Coffin Bay - Natural Oysters, SA
Water Temperature (C)
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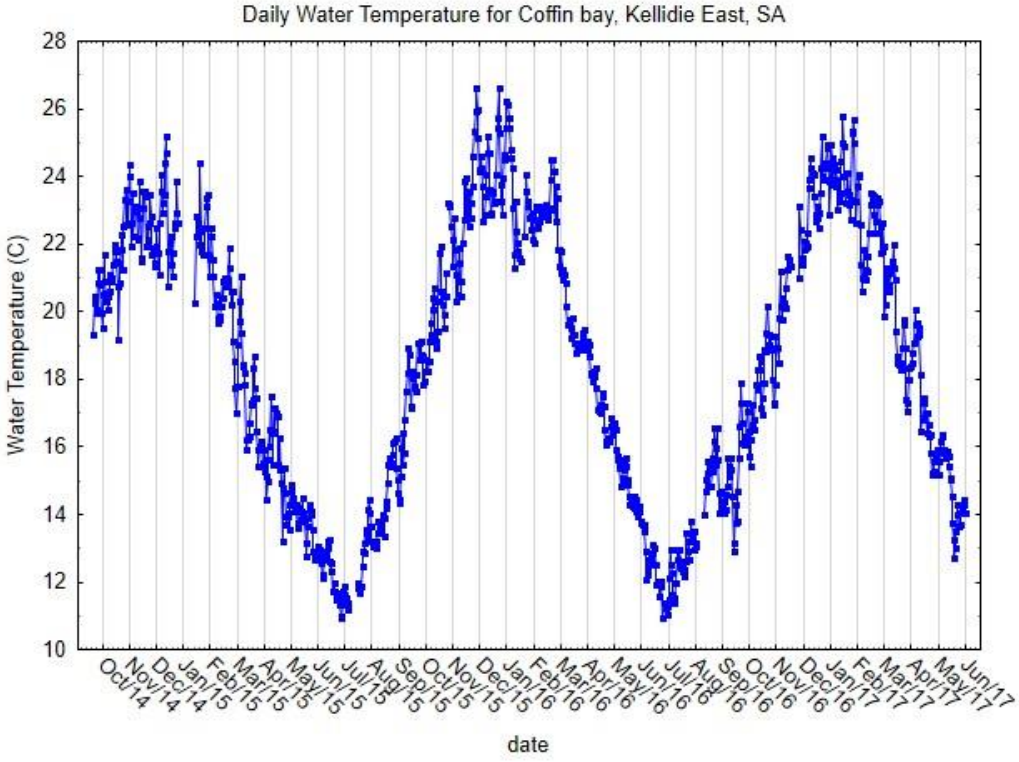
Daily Water Temperature for Coffin bay, Kellidie East, SA
Water Temperature (C)
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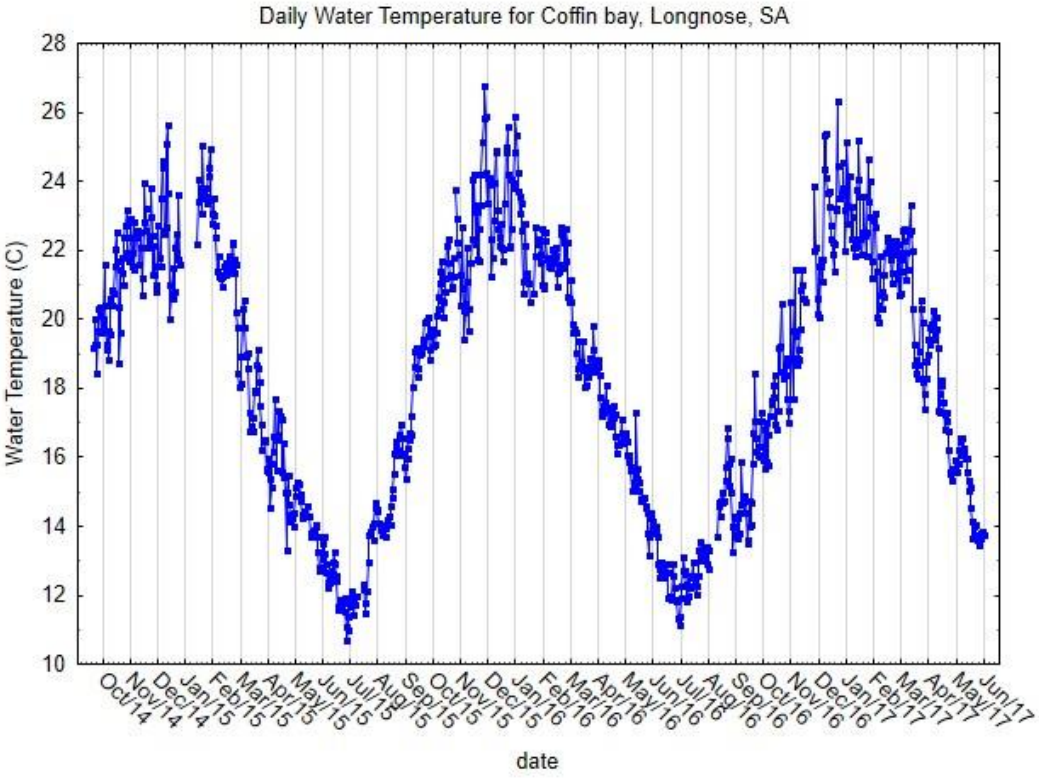
Daily Water Temperature for Coffin bay, Longnose, SA
Water Temperature (C)
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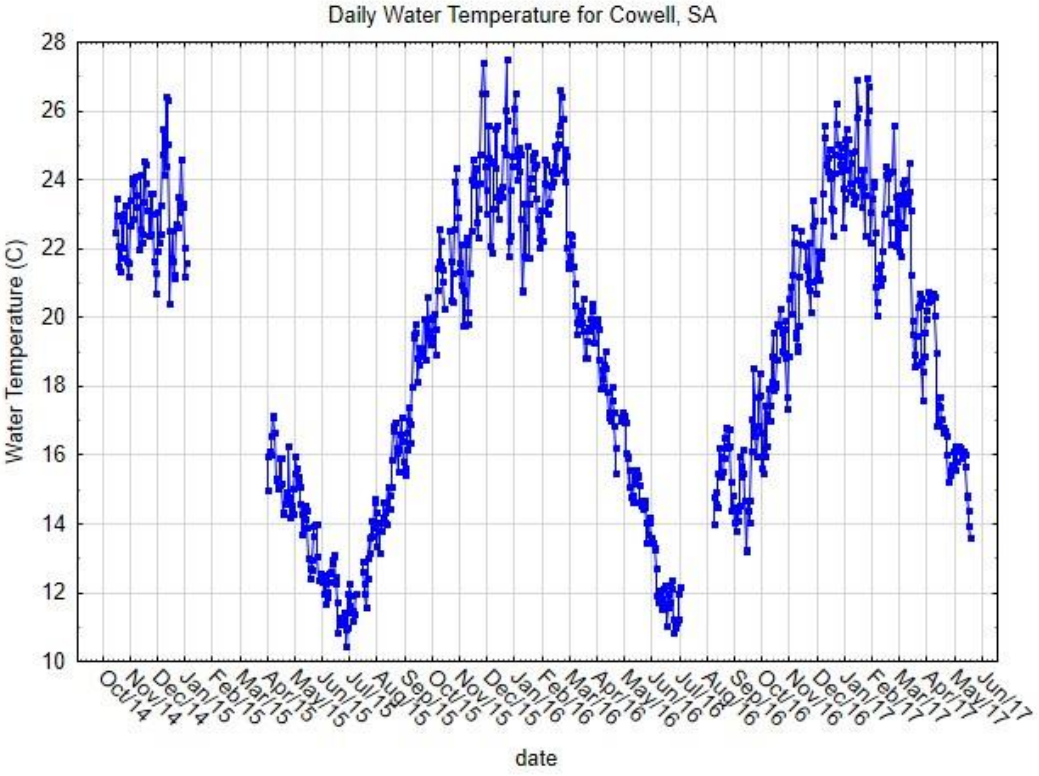
Daily Water Temperature for Cowell, SA
Water Temperature (C)
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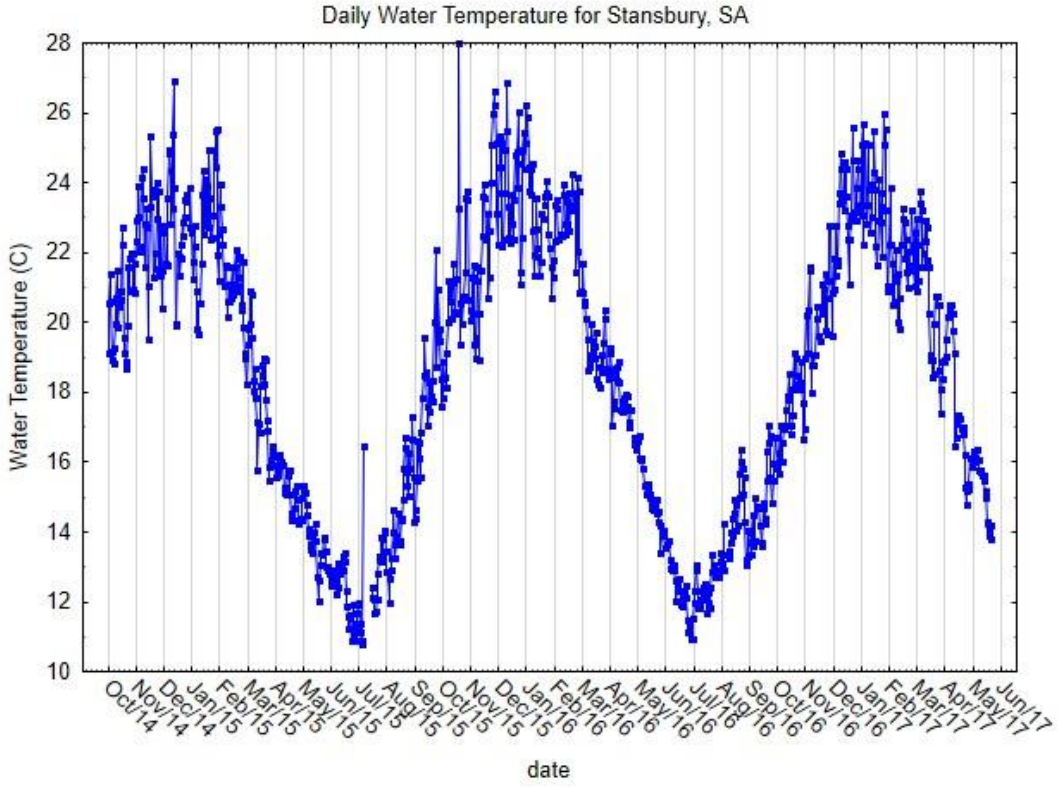
Daily Water Temperature for Stansbury, SA
Water Temperature (C)
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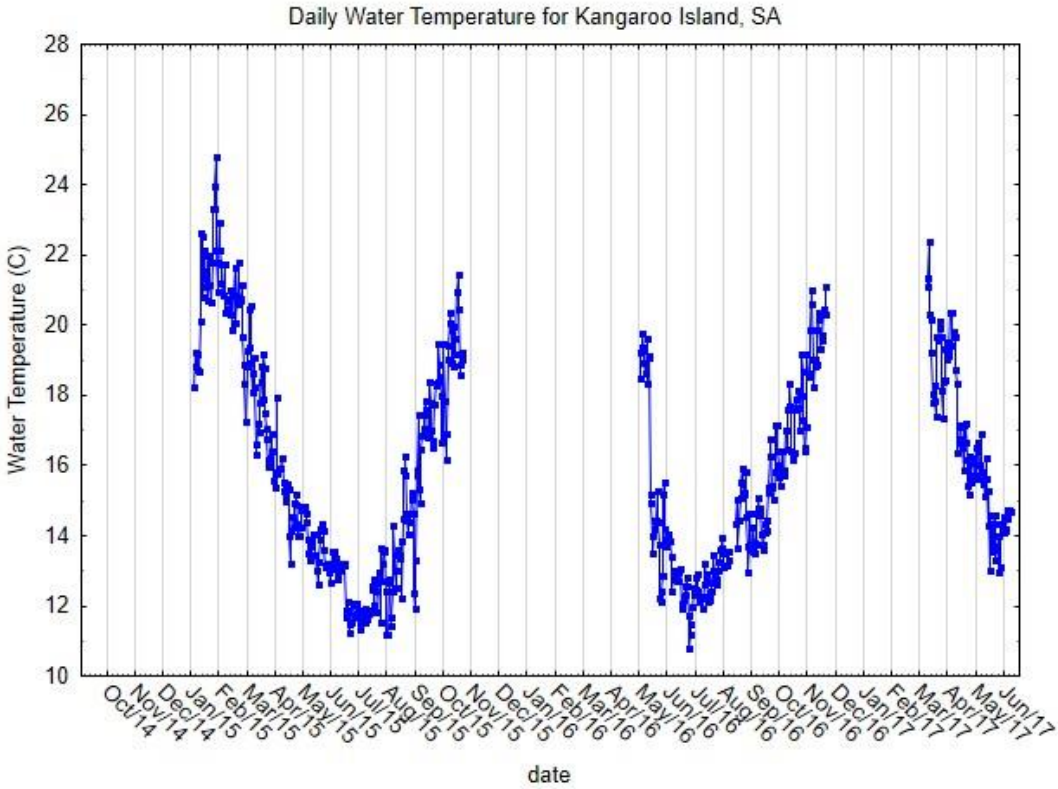
Daily Water Temperature for Kangaroo Island, SA
Water Temperature (C)
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The data in this study provide the first comprehensive assessment of on-farm water temperatures for most locations where Pacific oysters are commercially farmed in Australia. The data are unique because they represent water temperatures from oyster leases in the actual locations and in cultivation structures in which oysters are grown.

Oyster leases in all locations studied in New South Wales and South Australia with the possible exception of Kangaroo Island were considered to be at risk of mortalities due to OsHV-1 for a prolonged period from October through to May each summer season. Leases on the north coast of Tasmania had an intermediate risk, with temperatures >20°C each season. However, locations on the east coast except for the shallow estuaries tended to have short periods of risk, and may not be at risk each summer season. The shallow estuaries of Pitt Water and Pipeclay lagoon had a different temperature profile, with warmer water and therefore longer periods of risk each season. The summer season 2015-16 was of particular significance in Tasmania because it brought unusually warm water due to an East Australia current transfer across Bass Strait.

Mortality due to OsHV-1 will be accentuated by climate change and warming ocean waters in Tasmania due to extension of the risk period for OsHV-1 transmission and expression of disease.

4.4 The Window of Infection: seasonal, spatial and temporal patterns of OsHV-1 disease between 2013 and 2018 in Pacific oysters (*Crassotrea gigas*) in the Georges River and Hawkesbury River

Seasonality and periodicity of OsHV-1 associated mortality events

Spat were deployed and examined regularly throughout each summer season commencing in late August to early October, and continuing through to June-July the following year. The cumulative number of basket examinations conducted each season is shown in Table 3.1.

Earliest and latest calendar date of detection of mortality and sub-clinical infection. Across the five summer seasons, the earliest date of mortality due to OsHV-1 in sentinel spat was 28th October while the latest date of detection of mortality due to OsHV-1 in sentinel oysters was 14th May (Table 3.4). The earliest date of detection of OsHV-1 in sentinel oysters without significant mortality was 2nd October. The latest date of detection of the virus in sentinel spat was 28th June.

Pattern of mortality and sub-clinical infection according to the month. The monthly pattern of mortality due to OsHV-1 in sentinel spat across five summer seasons is shown in Figure 3.3. No mortalities due to OsHV-1 were observed in September, June or July although exposure to the virus was seen in June. The occurrence of mortality due to OsHV-1 and sub-clinical infection each month was greater in the Georges River estuary compared to the Hawkesbury River estuary.

Pattern of mortality and sub-clinical infection across consecutive summer seasons. The pattern of mortality due to OsHV-1 in sentinel oysters in successive summer seasons between 2012-13 and 2016-17 is shown in Figure 3.4. Mortalities due to OsHV-1 were observed each season but appeared to be diminishing after 2012-2013, although exposure to the virus was observed at a similar rate (about 5 to 15% of baskets sampled) each season. Note that in 2014-2015 samples were collected from a site only when mortality was observed in at least one basket so the percentage of affected baskets is not comparable to the other seasons. The occurrence of mortality due to OsHV-1 and subclinical infection was greater in sentinel oysters in the Georges River compared to the Hawkesbury River each summer season (ignoring the index case in the Hawkesbury River in January 2013).

Patterns of mortality and sub-clinical infection at different sites within each estuary. The pattern of mortality due to OsHV-1 in sentinel spat at each site in successive summer seasons between 2012-13 and 2016-17 is shown in Figure 3.5. Mortalities due to OsHV-1 were observed at each site except Patonga, but not at every site in every summer season. Subclinical infection was observed at each site including Patonga, but again not at every site in every season. Mortality due to causes other than OsHV-1 was observed at all sites where spat were deployed in 2015-16 and 2016-17.

Patterns of mortality and sub-clinical infection in paired baskets within sites. In the Georges River estuary the baskets were usually placed adjacent to one another on the long line, whereas in the Hawkesbury River they were placed 50 to 150 m apart, except at Mooney Mooney where they were about 1 m apart. In general, there was a similar pattern of occurrence of mortality in each of the baskets at any given site and time, that is both baskets were usually either affected or unaffected by mortality. However, at many sites on one or two occasions one basket had mortality due to OsHV-1 whereas the other did not (Pelican, Limekiln, Neverfail, Site A, Site B, Site C, Patonga, Porto, Mullet, Marra, Coba and Mooney). Greater differences between the paired baskets were seen at The Shed (8 and 11 occasions in Basket 1 and 2, respectively out of 26 sampling occasions when mortality >10% was observed), Sylvania (6 and 2 occasions in Basket 1 and 2, respectively out of 62 sampling occasions) and Kimmerikong (0 and 8 occasions in Basket 1 and 2, respectively, out of 59 sampling occasions). This striking pattern at Kimmerikong was seen in both 2014-15 and 2015-16. There were no occasions where both baskets were affected at Kimmerikong as Basket 1 was never affected. At

this site one basket was within a partially enclosure lease area demarcated by vertically mounted rubber conveyor belting placed to reduce wave action.

Subclinical infection was less often observed than mortality due to OsHV-1, but a similar pattern of differences between paired baskets was observed, except at Kimmerikong where OsHV-1 was detected in spat in both baskets in the absence of mortality on 4 occasions.

Suitability of spat as sentinels for OsHV-1

Different batches of spat were used over time in this study and it is possible that their susceptibility to OsHV-1 varied. However, mortality due to OsHV-1 occurred in some baskets of all batches except SPL14T, SPL16A and SPL16B. Of these batches, sub-clinical infection with OsHV-1 was observed in SPL16B (Table 5). Mortality due to OsHV-1 occurred in both triploid and diploid sentinel oysters.

Non-specific mortality (i.e. mortality >10% not associated with OsHV-1 infection) was observed in many batches over time. However, it occurred most commonly in 2015-16 and 2016-17 (Table 5). The batches affected were mainly ungraded lots of non-commercial 2N and 3N spat from Port Stephens (batches GK3N, 3NPO, GK2N). These were supplied following urgent request due to detection of OsHV-1 in Tasmania when translocation of 3N hatchery spat into NSW from Tasmania was prohibited for biosecurity reasons. Subsequently one batch from Tasmania (THO15J) performed similarly in 2016-17. Non-specific mortality was not investigated further but was attributed to batch effects combined with environmental disturbance such as low salinity, very high temperature, severe biofouling of baskets and unknown factors.

In the 2016-2017 season two batches of spat were included in each basket, one that was unselected for resistance to OsHV-1 and the other with potential resistance to OsHV-1 based on use of selected ASI diploid broodstock and previously exposed tetraploids at the Shellfish Culture hatchery. In November, December, February and March there appeared to be higher rates of mortality associated with OsHV-1, and higher rates of OsHV-1 infection without significant mortality in the unselected batches than in the selected batches. However, in January the rates were similar in selected and unselected batches (Table 3.6).

Reliability of sentinel spat to indicate OsHV-1 transmission and mortality in a bay

Commercial and research trials and diagnostic investigations were sometimes undertaken in some bays in both rivers while sentinel spat were deployed. On some occasions infection and mortality due to OsHV-1 was detected in oysters that were not evident in sentinel spat. These occasions are shown in Table 3.7. Further analysis was not warranted as this comparison is opportunistic, and it is biased in favour of detection of OsHV-1 in trials in which there was frequent observation and sampling; such trials were not conducted in all bays. Nevertheless the observations confirm that deployment and sampling of sentinel spat did not reliably indicate the presence of OsHV-1 at bay level in those bays were *C. gigas* were present on other leases. Therefore, using the design in this trial, sentinel spat may underestimate the presence of OsHV-1 at bay level.

Growth of spat

In general, the spat in each basket grew in the interval between deployment and sampling (Table 3.8). Growth appeared to be similar between spat in baskets in which mortality due to OsHV-1 occurred and spat in baskets that were unaffected.

Water temperature and association with mortality due to OsHV-1

Mean monthly water temperatures for the Georges River and the Hawkesbury River estuaries for the period October 2014 to June 2017 are shown in Figure 3.6. Temperatures in the Georges River were on average ~ 0.5°C cooler than in the Hawkesbury River. All cases of mortality due to OsHV-1 in sentinel spat as well as in other classes of oysters in other research trials and in disease investigations occurred after mid-October and before the end of May (Table 3.4), when mean daily water temperature in sentinel baskets rose above ~20°C and fell below ~17°C, respectively (Figure 3.6).

The seasonal window of risk of mortality due to OsHV-1 in the Georges River and Hawkesbury River estuaries was mid-October to the end May. There are examples of detecting OsHV-1 in oysters outside of this time, for example on 28th August in another trial. In this case spat were almost certainly exposed in May of the previous season and the detection did not represent transmission of virus outside of the risk period (Table 3.4). Evans et al. (2017) described in detail the detection of OsHV-1 in Pacific oysters that survivor mortality events and their potential role as a reservoir for further infection. Mean daily water temperature in the oyster baskets rose above 20°C in mid-October and fell below 17°C after mid-May. These temperatures can be considered to define the risk period for transmission of OsHV-1 and expression of disease. Water temperature and dose of exposure were the key risk factors identified in a laboratory infection model developed at the University of Sydney (de Kantzow et al., 2016; Paul-Pont et al., 2015b). The temperatures identified during the Window of Infection trials between 2012-13 and 2016-17 agree with those from the laboratory model in which mortality was not significant below 18°C, was very mild at 18°C, requiring a high exposure dose and very severe >22°C even at a lower exposure dose. These water temperatures are about 4-5°C warmer than the threshold reported from France for onset of seasonal mortality due to OsHV-1 (Pernet et al., 2012a; Renault et al., 2014a). The reasons for the differences observed between France and Australia are unknown, but may reflect an adaptation of *C. gigas* or the virus or both to Australian estuarine conditions.

There was an apparent decrease in the incidence of mortality due to OsHV-1 in both estuaries after the 2012-13 season. This may reflect a change in the environment, for example exhaustion of putative reservoir hosts which are potentially involved in transmission (Whittington et al., 2018) or evolution of the virus to a less virulent form. Use of potentially resistant spat in the last two years of the study may also be a factor, as may be the phenotypic variation in OsHV-1 observed in this project.

The occurrence of mortality due to OsHV-1 was not consistent over time at the sites studied. Some sites were more likely to be affected (Porto, Mooney Mooney, Site C than others (Patonga, Neverfail, Limekiln). Hydrodynamics is likely to be a major determinant of exposure and mortality and some hydrodynamic patterns may lead to consistent exposure sufficient to induce mortality at some sites and insufficient exposure of others – the best example of this was observed at Kimmerikong in 2014-15 and 2015-16. In this case spat in one basket of the pair were consistently affected by mortality whereas those in the other basket were not; the baskets were placed on either side of a barrier that had been installed to reduce wave action. Patonga was the only site with restricted tidal action; this feature was due to a sand bar at the entrance to the estuary, and spat in this location were never affected by mortality due to OsHV-1.

Mortality due to OsHV-1 can occur in a wide range of estuarine environments including shallow, muddy mangrove rich tidal bays and areas with sea grass and relatively clear water based on index case locations (Paul-Pont et al., 2014a; Whittington et al., 2013). Most of the sites studied here using sentinel oysters were shallow bays, with turbid water, deep sediment, and adjacent mangroves. The exceptions were Patonga creek (sea grass, relatively clear water), Sylvania Waters (a concrete lined canal in an urban residential estate with relatively clear water) and Neverfail (high flow channel adjacent to the main channel of the Georges River estuary). The sites represented both upstream and downstream locations. Mortality due to OsHV-1 was observed at all locations except Patonga,

suggesting that local benthic environment, flow, proximity to freshwater inflow and oceanic influence, and turbidity were not important risk factors.

The study of the occurrence of mortality due to OsHV-1 in sentinel spat at about 7 locations provided a good indication of the presence of the virus at estuary level. However, it did not always provide a good indication at bay level. There were several occasions when sentinel spat remained unaffected while substantial mortality due to OsHV-1 occurred quite closely nearby in other oysters. These observations are consistent with the conclusions reached in independent studies of the origins and transmission of the virus, that is, that OsV-1 is a waterborne infection acquired by indirect transmission from an environmental source, is transmitted associated with animate or inanimate particles in the plankton and water column and therefore is subject to hydrodynamic as well as biological influences (Paul-Pont et al., 2013b; Whittington et al., 2018). This presents particular difficulties for surveillance and it is unlikely that conventional surveillance designs for sampling sedentary oysters or targeted surveillance through plankton sampling will be sensitive enough to obtain required levels of confidence to prove absence of the virus from a particular bay at any given time. Negative results from these sampling approaches may be highly misleading.

The sentinel spat that succumbed to OsHV-1disease in this study were feeding and growing, as shown by the consistent increase in shell length between deployment and sampling (interval generally 2 weeks). There was no evidence that oysters that succumbed to OsHV-1 were growing any more rapidly than those that did not succumb to the virus.

Table 3.1. Sentinel surveillance for OsHV-1 using *C.gigas*.

Data are the number of sites, baskets and sentinel oysters used in the Georges River and Hawkesbury River estuaries over 5 consecutive summer seasons to monitor the spatial and temporal pattern of disease associated with OsHV-1 in *C. gigas*

Season	Start[1]	End[2]	No. sites GR	No. sites HWY	No. baskets per site	No. oysters per deployment per basket	No. of separate deployments
2012-2013	20/02/13	11/06/13	3	5	3	500	8
2013-2014	28/08/13	02/07/14	8	5	2	250	21
2014-2015	17/09/14	06/07/15	8	7	2	250	2[3]
2015-2016	02/10/15	05/07/16	7	7	2	250	15
2016-2017	05/10/16	14/06/17	7	7	2	250	10

[1] Date of first deployment
[2] Date of last sampling
[3] There were additional deployments at Mooney Mooney in 2014-15; farmer monitoring of spat was conducted during this season.

Table 3.2. Batches of spat used as sentinel oysters between 2013 and 2017.

Data are the number of lots of oysters that were deployed (corresponding to basket sampling events except in 2014-2015). Spat were deployed at a rate of 500 per basket in 2012-13 and 250 per basket in other seasons.

Batch	Source	Ploidy	Resistance to OsHV-1	Season						No. spat approx
				2012-13	2013-14	2014-15	2015-16	2016-17	Total	
SPL12FT	Shellfish Culture, Tasmania	3N	Unselected	192					192	96000
SPL13A	Shellfish Culture, Tasmania	3N	Unselected		156				156	39000
SPL13B	Shellfish Culture, Tasmania	3N	Unselected		182				182	45500
SPL13C	Shellfish Culture, Tasmania	3N	Unselected		260				260	65000
SPL14B	Shellfish Culture, Tasmania	3N	Unselected			31			31	7750
SPL14FT	Shellfish Culture, Tasmania	3N	Unselected			30			30	7500
SPL15AT	Shellfish Culture, Tasmania	3N	Selected				252		252	63000
GK 2N	Southern Cross, NSW	2N	Unselected				104		104	26000
GK 3N	Southern Cross, NSW	3N	Unselected				120		120	30000
2NPO	Southern Cross, NSW	2N	Unselected					52	52	13000
3NPO	Southern Cross, NSW	3N	Unselected					192	192	48000
n/a	Shellfish Culture, Tasmania	n/a	n/a					6	6	1500
SPL16A	Shellfish Culture, Tasmania	3N	Selected					2	2	500
SPL16B	Shellfish Culture, Tasmania	3N	Selected					56	56	14000
THO15J	Shellfish Culture, Tasmania	2N	Selected					203	203	50750
No. lots deployed				192	598	61	476	511	1838*	
No. spat approx.				96000	149500	15250	119000	127750		507500

* some baskets were inadvertently sampled at too high frequency (<7 days) in GR in 2015-16 and results from dates in between the normal sampling dates were therefore excluded, and there were some missing samples. Therefore, in subsequent analyses data are shown for 1822 basket sampling events (see Table 3.3).

Table 3.3. Number of basket sampling events at each site during each summer season.

Site	Summer season					Total
	2012-13	2013-14	2014-15[1]	2015-16	2016-17	
Georges River						
Site 1 Shed		46[2]	6			52
Site 2 Pelican		46	3	38	41	128
Site 3 Sylvania		46	3	39	37	125
Site 4 Neverfail		46	4	36	36	122
Site 5 LimeKiln		46	4	36	36	122
Site A	23	46	3	37	36	145
Site B	24	44	5	33	36	142
Site C	24	46	5	34	37	146
Total	71	366	33	253	259	982
Hawkesbury River						
1 Patonga	24	44	3	16	27	114
2 Porto	24	46	4	32	35	141
3 Mullet	24	46	4	33	42	149
4 Marra	24	46	4	34	36	144
5 Kimmerikong	24	46	3	32	38	143
6 Coba			4	33	36	73
7 Mooney			6	32	38	76
Total	120	228	28	212	252	840
Grand Total	191	594	61	465	511	1822

[1] In 2014-15 the baskets were sampled only when mortality >10% was first observed.

[2] The sum of the number of baskets examined each time, for example 2 baskets at this site were sampled on 23 occasions. As one basket of a pair may have contained no oysters due to prior mortality, been missing or was unable to be recovered due to bad weather, an odd number is possible.

Table 3.4. Earliest and latest calendar dates of detection of mortality due to OsHV-1 in the Georges River and Hawkesbury River estuaries.

Data are for sentinel spat sampled between 2013 and 2017 and for all oysters in all trials or disease investigations (including index cases) sampled between 2010 and 2018, based on date of sampling.

Clinical category	Trial or investigation	Earliest date	Latest date
Virus detected with >10% mortality	Sentinel spat 2013-2017	28-Oct	14-May
	All trials and disease investigations 2010-2018	25-Oct	29-May
Virus detected but mortality <10%	Sentinel spat 2013-2017	2-Oct	28-Jun
	All trials and disease investigations 2010-2018	28-Aug [1]	28-Jun

[1] This result reflects tests on oysters that had been present in the system at before the close of the transmission risk period from the previous warm seasons and is unlikely to reflect a new OsHV-1 transmission event, rather persistent infection.

Table 3.5. Mortality and detection of OsHV-1 in batches of spat between 2013-2017 among 1822 basket sampling events.

Batch	No. of baskets		
	Virus detected and mortality at sampling or removal >=10%)	Virus detected but mortality <10% at sampling or removal	Mortality >10% at sampling but virus not detected
SPL12FT	46	17	5
SPL13A	10	14	0
SPL13B	30	9	5
SPL13C	20	16	2
SPL14B	20	1	0
SPL14FT	0	0	0
SPL15AT	36	11	7
GK 2N	3	11	12
GK 3N	20	0	96
2NPO	1	8	1
3NPO	8	10	43
n/a	0	0	0
SPL16A	0	0	1
SPL16B	0	1	0
THO15J	4	11	9
Total	198	109	181

Table 3.6. Comparison of sentinel spat with or without genetic selection for POMS resistance.

Data are the percentage of basket sampling events with each outcome in the 2016-2017 summer season.

Month	Batch	Resistance to OsHV-1	Virus and mortality	Virus but no mortality	Mortality but no virus
Oct	2NPO	unselected	0.0	19.2	0.0
Nov	2NPO	unselected	3.8	11.5	3.8
	THO15J	selected	0.0	8.1	2.3
Dec	3NPO	unselected	3.8	0.0	3.8
	THO15J	selected	0.0	0.0	6.5
Jan	3NPO	unselected	5.6	5.6	3.7
	THO15J	selected	6.9	6.9	3.4
Feb	3NPO	unselected	3.7	3.7	3.7
	THO15J	selected	0.0	0.0	10.7
Mar	3NPO	unselected	3.1	9.4	46.9
	SPL16A	selected	0.0	0.0	50.0
	3NPO	unselected	6.3	0.0	25.0
Apr	n/a		0.0	0.0	0.0
May	3NPO	unselected	0.0	0.0	100.0
	n/a		0.0	0.0	0.0
	SPL16B	selected	0.0	3.6	0.0
Jun	n/a		0.0	0.0	0.0
	SPL16B	selected	0.0	0.0	0.0
Jul	n/a		0.0	0.0	0.0
Total			2.5	5.9	10.6

Table 3.7. Occasions when mortality due to OsHV-1 was not detected in sentinel spat but instead was detected in oysters in other trials or investigations at the same site.

Site	Trial	2013-14				2014-15	2015-16	
		Oct	Jan	Feb	Mar	Jan	Oct	Nov
Porto	(Outbreak investigation)							
Marra	(Commercial Growout Trial)							
Coba	(Commercial Growout Trial)							
	(PCT1 2015-2016)							
Woolooware Site B	(Age-Size Trial 2)							
	(Spat conditioning)							
Woolooware Site C	(Age-Size Trial 1)							
	(Age-Size Trial 2)							
	(Risk Based Spat Growout Trial)							
	(Spat conditioning trial)							

Table 3.8. Growth of spat according to the month of sampling.

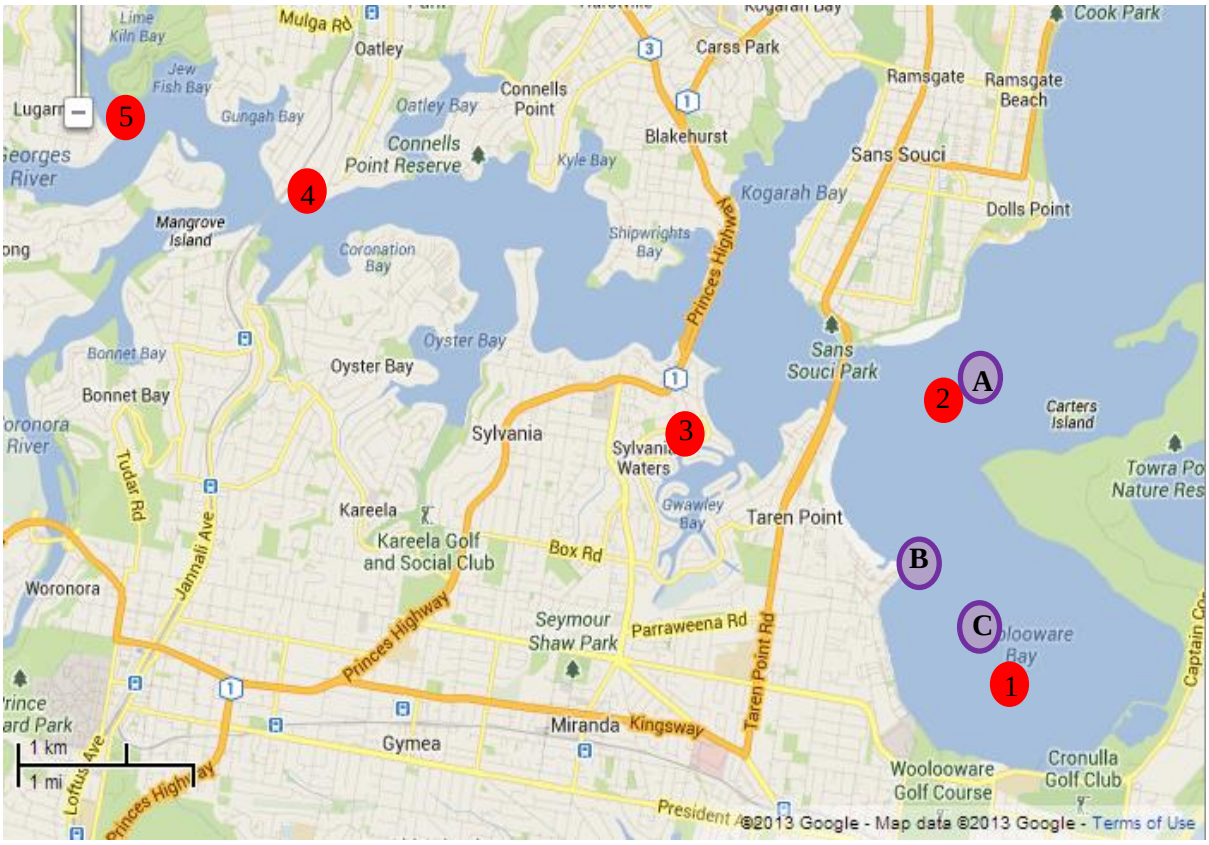
Data are the growth ratio, expressed as the average length at sampling compared to the average length at deployment after data for each basket were stratified according to whether (Yes) or not (No) mortality due to OsHV-1 was observed in each basket. Length data were not available for 2012-13.

Summer season	Mortality due to OsHV-1	Month										
		Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Georges River												
2013-14	No	1.58	1.66	1.60	1.42	1.58	1.60	1.33	1.19	1.20	1.49	1.27
	Yes			1.89	1.48	1.55	1.53	1.25	1.16	0.91		
2014-15*	No				8.65					15.88		
	Yes		4.48	4.61	8.25		12.57	16.02				
2015-16	No		2.62	2.02	1.86	3.78	3.87	1.51	1.07	1.18	1.07	
	Yes		2.35	2.26	1.62		3.75	1.35	1.00	1.40		
2016-17	No		1.80	2.50	2.72	1.82	2.30	1.08	1.44	1.30	2.37	
	Yes			1.99	2.03	2.11	1.32		1.19			
Hawkesbury River												
2013-14	No	1.43	1.76	1.64	1.60	1.92	1.66	1.25	1.39	1.47	1.69	1.39
	Yes		1.74	1.10	1.23							
2014-15*	No						9.24		17.09			
	Yes			6.40	1.15							
2015-16	No		2.49	2.31	2.42	3.27		1.36	1.11	1.73	1.29	1.08
	Yes		3.95	1.78				1.29				
2016-17	No		2.01	2.59	1.98	1.40	2.27	1.41	1.06	2.31	3.69	
	Yes							1.10				

***** As only two batches of spat were used in 2014-15, spat were left in the estuary for longer, and so spat grew to a larger size before they were sampled.

Figure 3.1. Locations where sentinel oysters were placed in the Georges (A) and Hawkesbury Rivers (B).

Panel A. Georges River estuary: A, Site A; B, Site B; C, Site C; 3, Sylvania Waters; 4, Neverfail; 5, Limekiln Bar.



Panel B, Hawkesbury River estuary. 1, Patonga Ceek; 2, Porto Bay; 3, Mullet Creek; 4, Marra Marra Creek; 5, Kimmerikong Bay; 6, Coba Bay, 7, Mooney Mooney

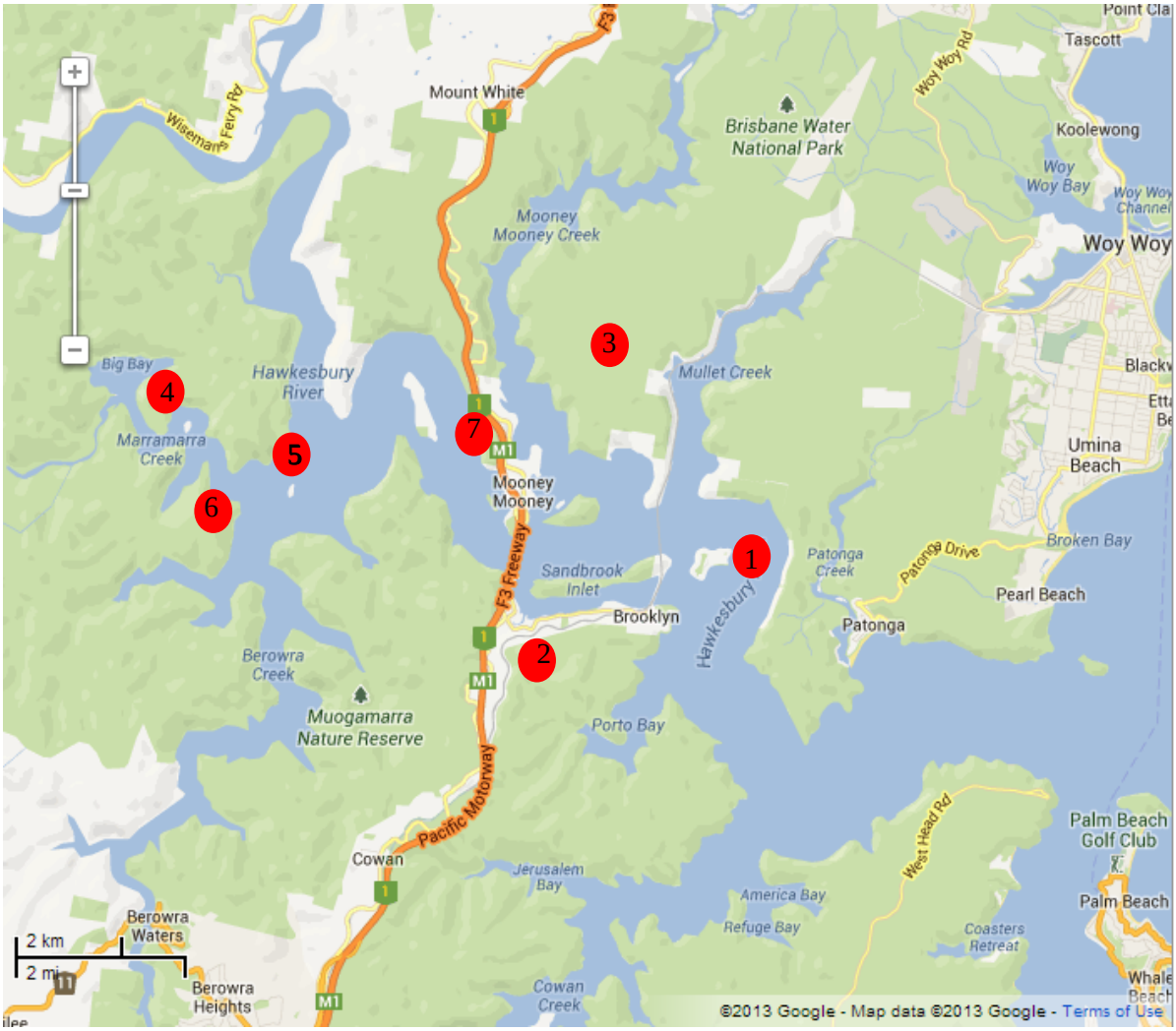
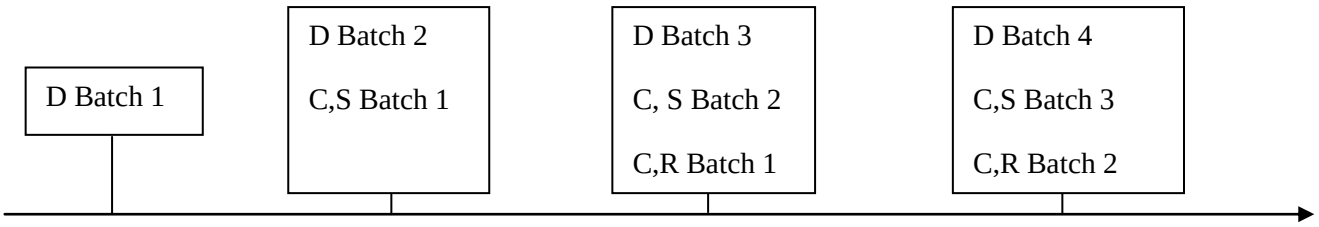


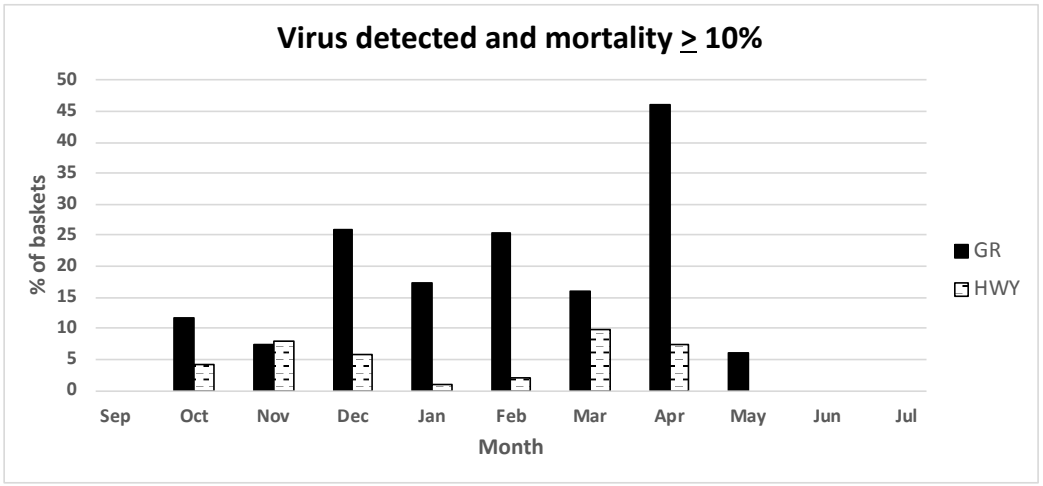
Figure 3.2. Pattern of deployment, mortality checks and sampling of sentinel spat.

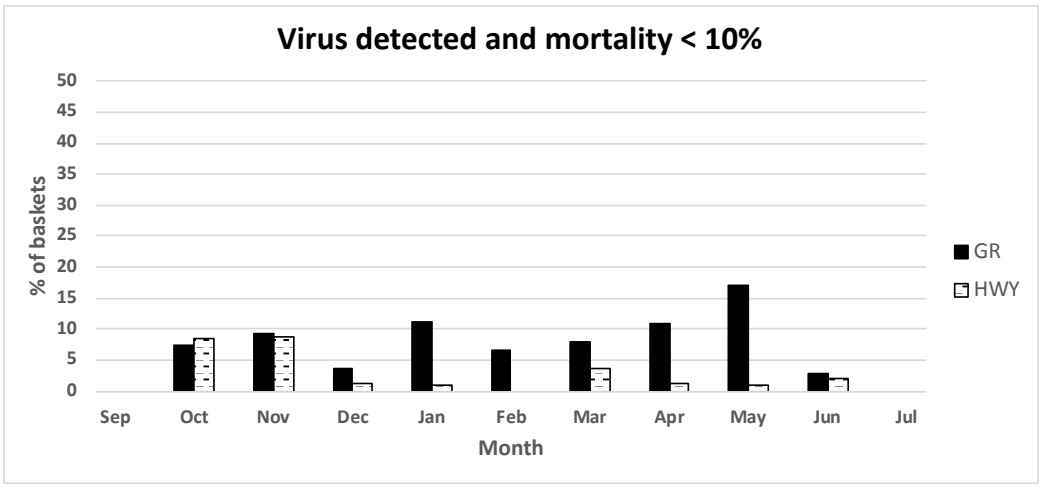


D = deployment
C = check mortality
S = sample collection
R = remove
Typically the intervals were fortnightly unless stated otherwise.

Figure 3.3. Mortality due to OsHV-1, exposure to OsHV-1 without mortality and mortality due to other causes, by month.

Data are % of baskets sampled indicating sub-clinical infection OsHV-1, mortality (POMS disease events) and mortality due to other causes in the Georges and Hawkesbury Rivers between 2013 and 2017.





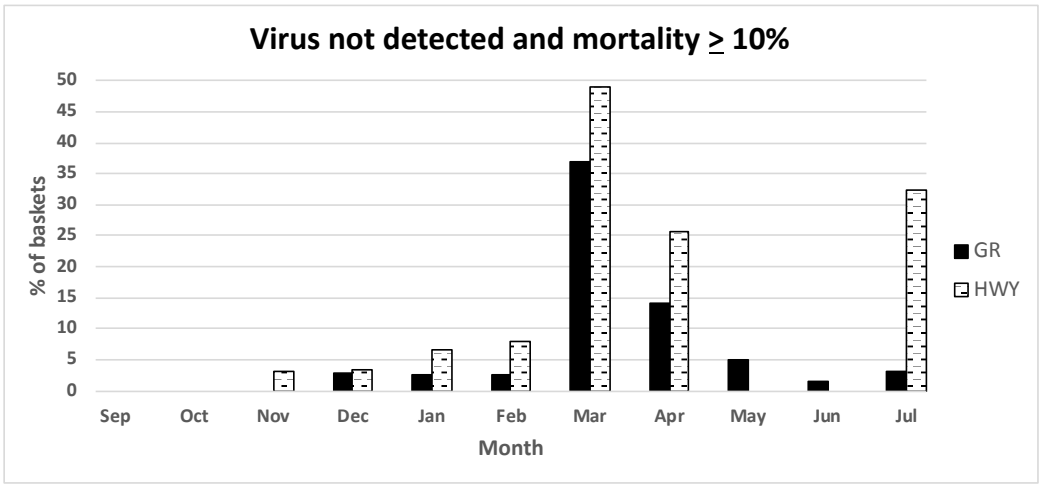


Figure 3.4. Mortality due to OsHV-1, exposure to OsHV-1 without mortality and mortality due to other causes, by year**.**

Data are % of baskets sampled indicating sub-clinical infection OsHV-1, mortality (POMS disease events) and mortality due to other causes in the Georges and Hawkesbury Rivers between 2013 and 2017.

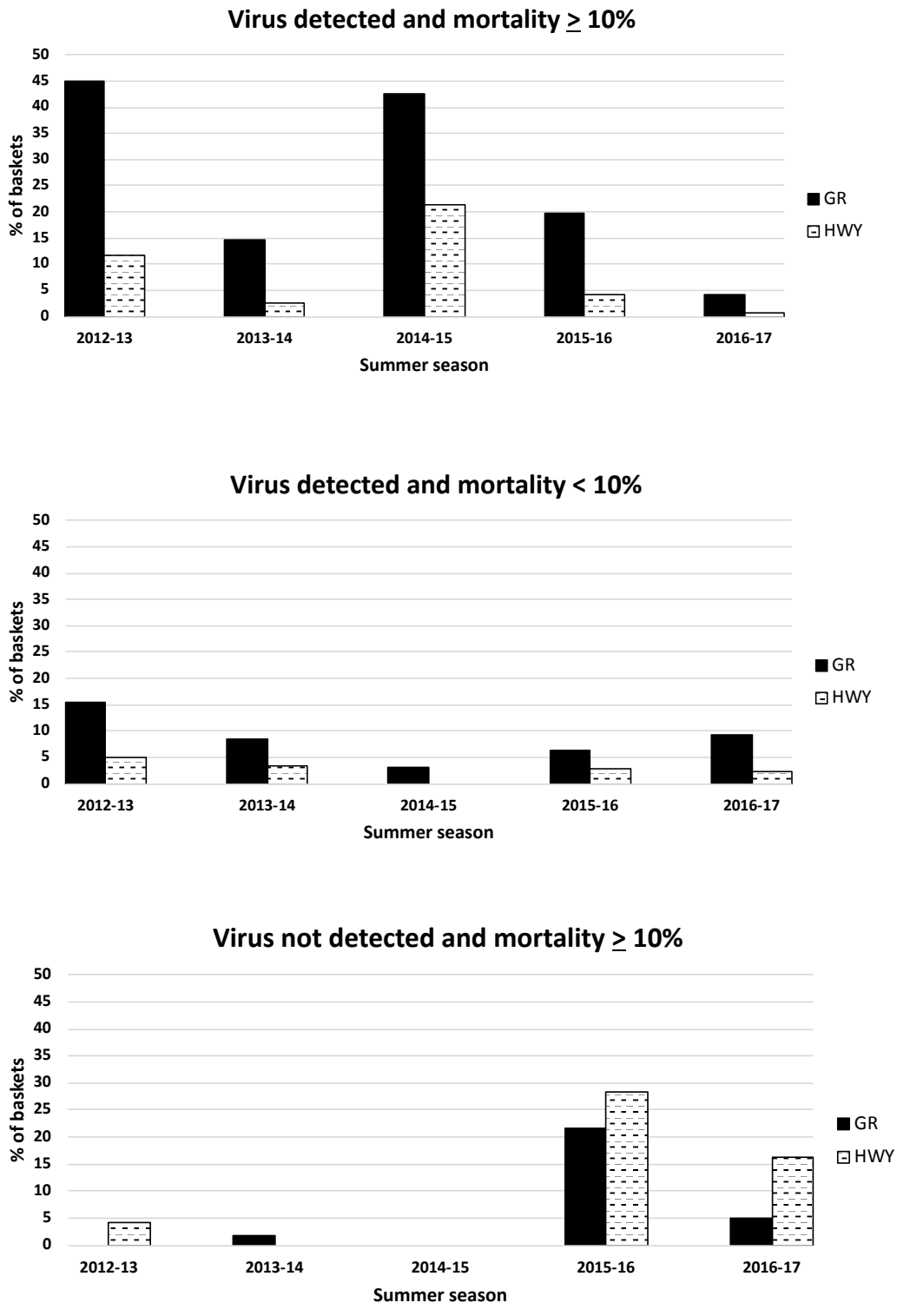
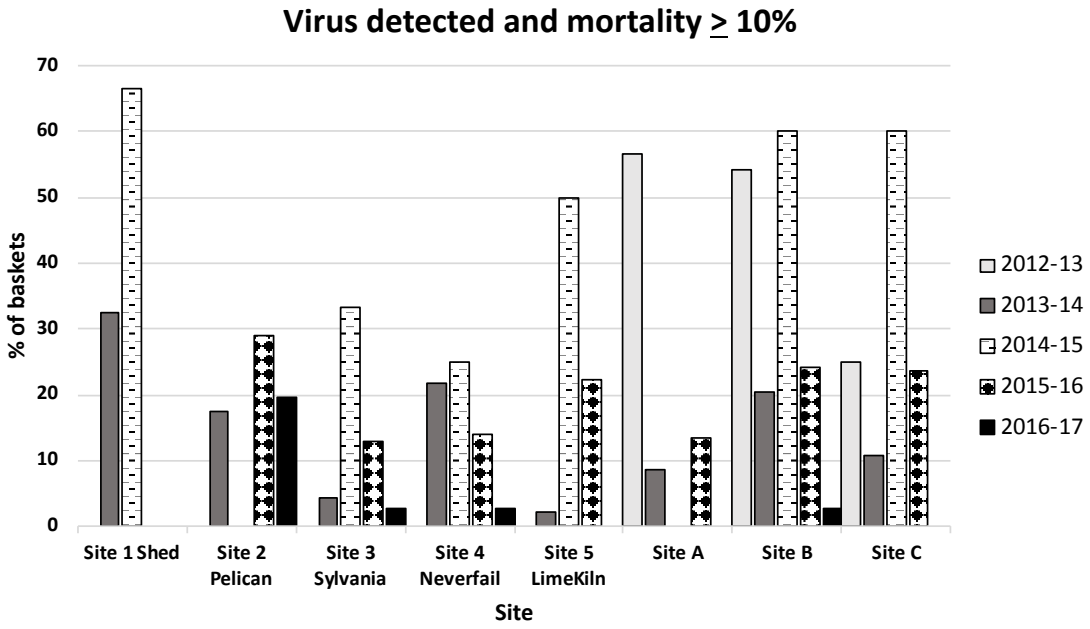


Figure 3.5. Mortality due to OsHV-1, exposure to OsHV-1 without mortality and mortality due to other causes, by Site.

Data are % of baskets sampled indicating sub-clinical infection OsHV-1, mortality (POMS disease events) and mortality due to other causes in the Georges and Hawkesbury Rivers between 2013 and 2017.

Note: The Shed, Coba and Mooney were not included each year. Data are % of baskets sampled.



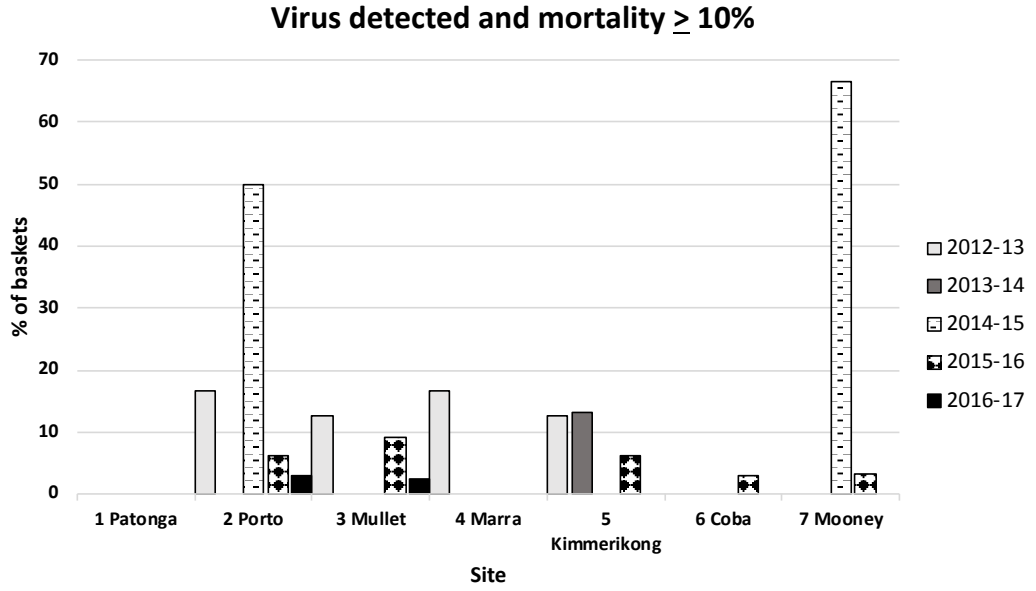
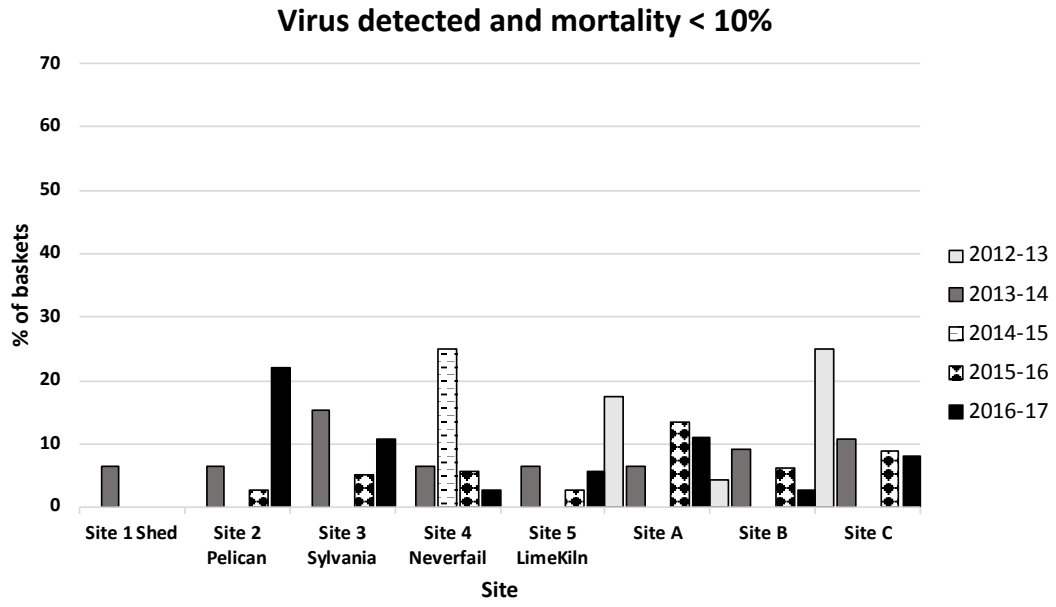


Figure 3.5 continued



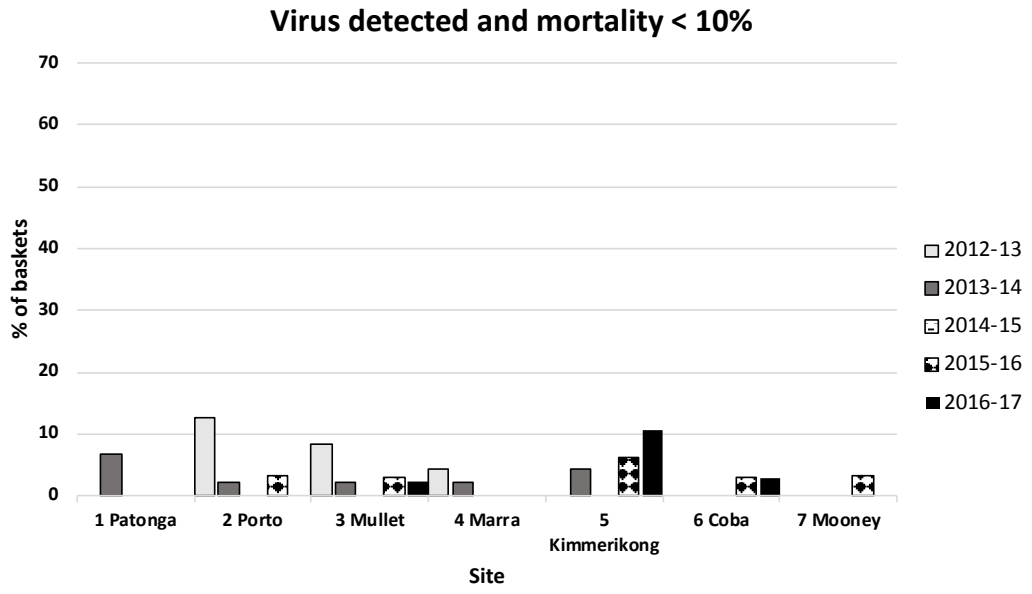
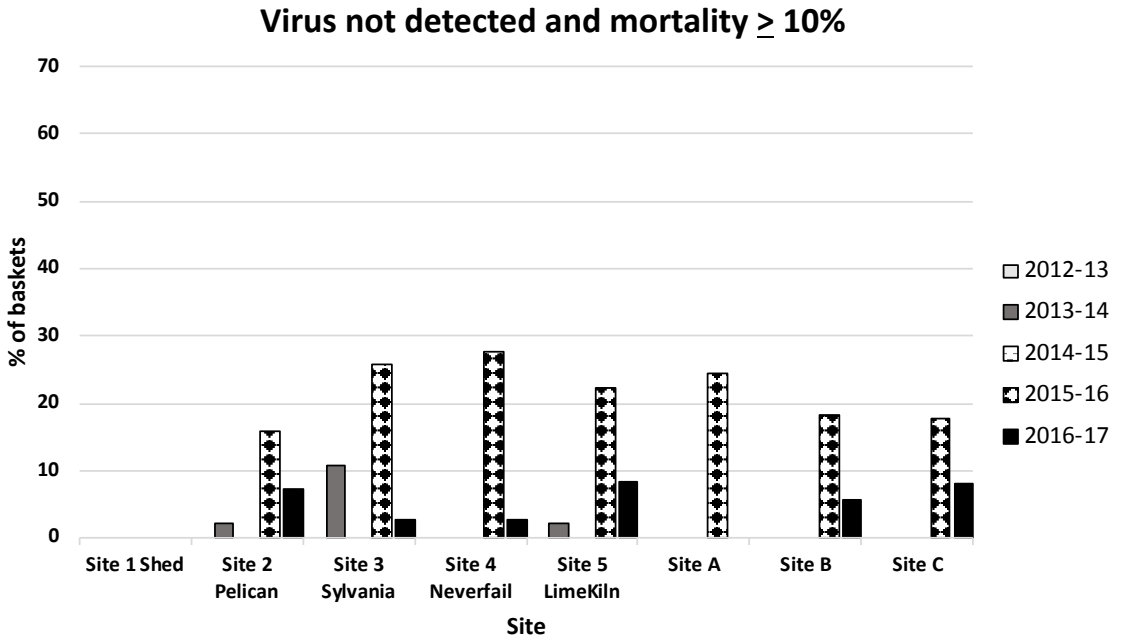


Figure 3.5 continued



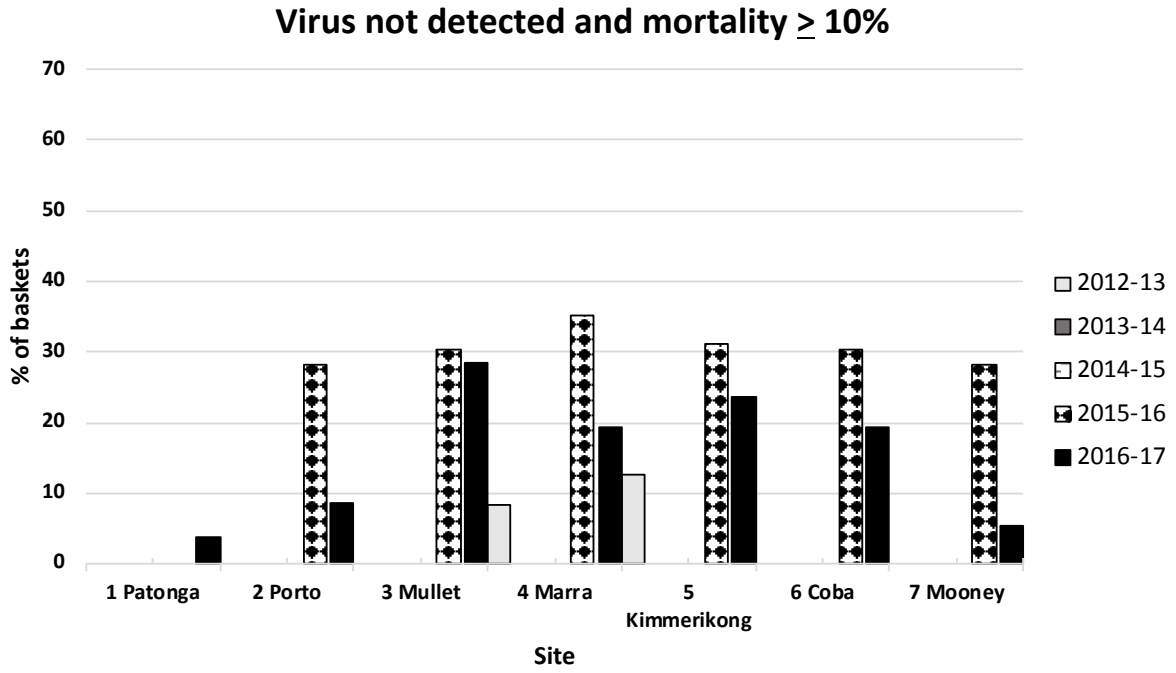
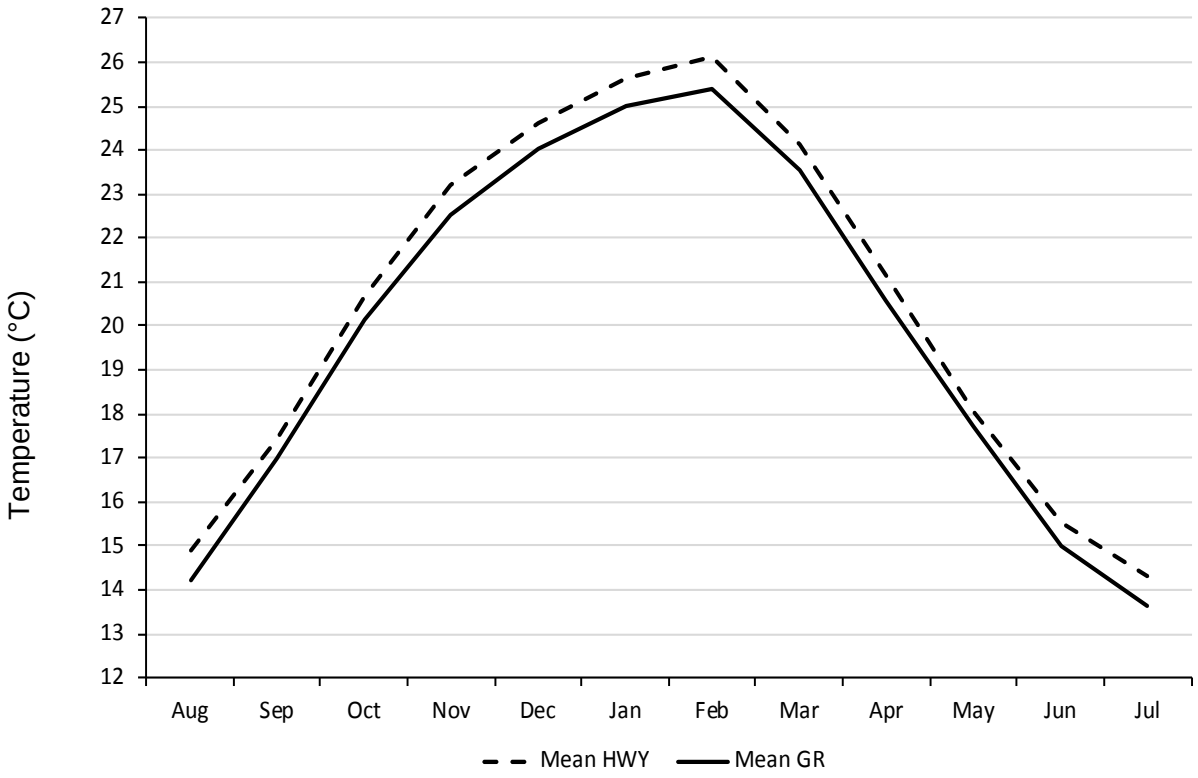


Figure 3.6. Mean monthly water temperature for the period 2014-2017 in the Georges River and Hawkesbury River.



4.5 Sequence of OsHV-1 and phenotype evaluation

4.5.1 OsHV-1 multi-locus genotyping

There was variable success for amplification of the targets different regions for each of the 107 clinical samples tested (Table 1). All of the isolates met the definition of microvariant OsHV-1 provided by the World Organisation of Animal Health (OIE, 2014).

There were 8 unique sequence types were identified from the 91 isolates sequenced in Region 1 (ORF4) (Figure 4.1a). Features in this region that defined the isolate from the original outbreak in the Georges River 2010 were seen consistently in all isolates. Additional SNPs and variation in the number of deletions in the repeat region were identified.

There was very little variation in OsHV-1 sequence in Region 2, (ORF 42-43). Five unique sequence types were identified from the 77 isolates sequenced in this study based on deletions of single nucleotides (Figure 4.1b).

Almost all samples produced an amplicon with identical sequence for Sequencing Region 3 (ORF35-36-37and-38). The sequence was consistent with microvariants of OsHV-1 including the large deletion in the region.

There was considerable variability in the non-coding region between ORF49 and 50 (Sequencing Region 4), with 19 unique sequence types were identified from the 87 isolates sequenced (Figure 4.1c). SNPS that distinguished microvariant genotypes from reference OsHV-1 genome that were sourced in France were fixed in the NSW and Tasmanian OsHV-1 sequences. There were multiple variants in this region that spanned the duration of the study and both the Hawkesbury and Georges River.

Interestingly the deletion characteristic of microvariant genotypes that is described for Sequencing Region 5 (ORF -11) was not identified. The sequence of the New South Wales and Tasmanian isolates corresponded to the OsHV-1 reference genome in this region. Five unique sequence types based on SNPs were identified in various portions of Region 5, and again the SNPs that differentiate French microvariants from the reference strain were absent in the Australian samples (Figure 4.1d).

The sequence of all samples in ORF-88 (Sequencing Region 6) was almost 100% homologous, with only a single G/C substitution at position 333 in 36/92 isolates and a second A/G substitution at position 703 in a single isolate (Figure 4.1e).

Sequencing efforts from previous studies revealed limited variation in the sequence of OsHV-1 over time and in different locations (FRDC 2012-032, Final Report) based on sequence of 3 regions of the genome. The increased number of regions of coverage in this multi-locus approach provided the increased discrimination required to distinguish the large number of isolates collected from Australia. There was no phylogenetic clustering according to time or location in this study of 107 clinical samples.

Considerable gentoypic variation in OsHV-1 has recently been appreciated in international sequencing studies where access to this technology and a broad range of samples is revealing that the virus species OsHV-1 is a polythetic group of genotypes. Despite a probable recent point source introduction to Australia, there is already considerable genotypic diversity amongst OsHV-1 detected in disease events. The extreme impact of this pathogen on host population imparts strong selective pressure on the virus.

The genotypic variation warrants phenotypic studies of diverse OsHV-1 isolates to determine the extent to which variation in the pathogen contributes to variable disease outcomes seen on farms now and in the future.

Table 4.1. Selection of samples for OsHV-1 multi-locus sequence determination.

The distribution of the samples included up to 6 different seasonal recurrences in the Georges River and between 1 and 6 different sites within each river for a total of 107.

Location	Year of collection *						Total
	2011	2012	2013	2014	2015	2016	
Georges River (NSW)	15 (2)	10 (3)	11 (6)	15 (5)	13 (2)	3 (2)	67
Hawkesbury River (NSW)	n/a	n/a	16 (4)	19 (4)	3 (1)	0	38
Pittwater (Hobart, Tasmania)	n/a	n/a	n/a	n/a	n/a	2 (1)	2

* Data are the number of OsHV-1 positive samples and the number of different sites within the river in parentheses.

Table 4.2. Regions of the OsHV-1 genome targeted for PCR sequencing regions.

Sequencing region ID	Amplification target					Comment	Reference	Result	
	Position [a]	Gene	Primer name	Primer sequence (5'→3')	Amplicon size (base pairs)			Amplicon (%)[b]	No. unique[c]
1	178211	ORF 4	C2	CTCTTTACCATGAAGATACCCACC	709	Includes microsatellite locus H10	(Renault and Arzul, 2001)	85	8
	178919		C6	GTGCACGGCTTACCATTTTT					
2	59950	ORF 42-43	IA1	CGCGGTTCATATCCAAAGTT	607		(Segarra et al., 2010)	72	5
	60557		IA2	AATCCCCATGTTTCTTGCTG					
3	51979	ORF 35 - 36 -37 and 38	Del 36-37F2	ATACGATGCGTCGGTAGAGC	989, 384 or no amplification	989 for reference genotype; 384 bp (*µvar*) OR no amplification depending on variant	(Renault et al., 2012)	97	1
	52968		Del 36-37R	CGAGAACCCCATTCCTGTAA					
4	72414	Between ORF49 and 50	NC1	ACACCTAATGACCCCAAAGG	506	Non-coding region of relatively high sequence variation	(Batista et al., 2015)	98	19
	72919		NC2	GACCAATCACCAGCTCAACA					
5	17402	ORF 11	ORF11For	ACCACCGCGCCAAAATCTG	2116 or 731	Expected Product size 2116 OR 731bp (*µvar*) depending on variant.	(Martenot et al., 2013)	90	5
	19518		ORF11Rev	CGCTTCCTATCACCTTGTGG					
6	133088	ORF 88a	ORF88aFor	CCCAGTCTATTATCCAGGTAC	1020	Polymorphic region of OsHV-1 genome coding a surface glycoprotein	(Martenot et al., 2013)	82	3
	134107		ORF88aRev	ACCGTTCCTCAATCAGTCCC					

[a] Positon on the OsHV-1 genome relative to the OsHV-1 reference genome, GenBank: AY509253
[b] The proportion of the 107 samples in the present study for which an amplicon was generated and sequenced [c] Number of unique genotypes identified in amplicon.

Table 4.3. List of OsHV-1 isolates used for comparison.

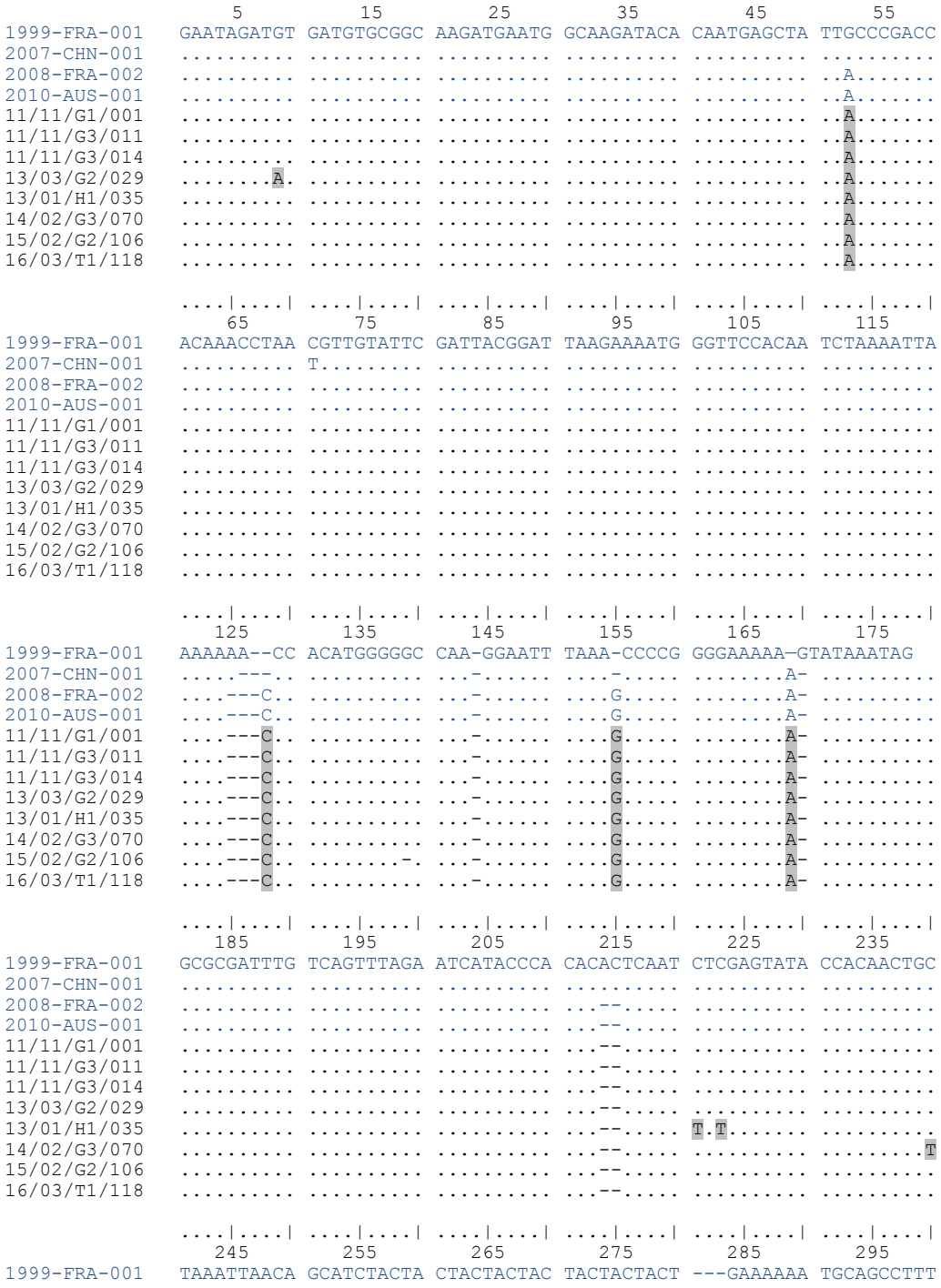
GenBank accession numbers with country of origin, year of collection and corresponding PCR sequencing region covered.

Isolate ID	Country	Year	Sequence Region						
			1	2	3	4	5	6	Whole genome
1999-FRA-	France	1999	Y	Y	Y	Y	Y	Y	AY50925
2007-CHN-	China	2007	Y	Y	Y	Y	Y	Y	GQ1539
2008-FRA-	France	2008	HQ842	N	N	N	N	N	N
2010-NZD-	New	2010-	JN6398	N	N	N	N	N	N
2010-AUS-	Australia	2010	KC6855	N	N	N	N	N	N
2010-JPN-	Japan	2010	JN8001	N	JN8002	N	N	N	N
2010-USA-	USA	2007	JN8001	JN8001	JN8002	N	N	N	N
2003-FRA-	France	2003	JN8000	JN8001	JN8002	N	N	N	N
2010-FRA-	France	2010	JN8001	JN8001	JN8002	N	N	N	N
2010-FRA-	France	2010	JN8001	JN8001	JN8002	N	N	N	N
2008-FRA-	France	2008	JN8001	JN8001	JN8002	N	N	N	N
2008-FRA-	France	2008	JN8001	JN8001	JN8002	N	N	N	N
2009-IRL-	Ireland	2009	JN8001	JN8001	JN8002	N	N	N	N
2010-NZD-	New	2010	JN8001	JN8002	JN8002	N	N	N	N
2011-POR-	Portugal	2011	KM593	KM593	N	KM593	N	N	N
2013-POR-	Portugal	2013	KM593	KM593	N	KM593	N	N	N
2013-ESP-	Spain	2013	KM593	KM593	N	KM593	N	N	N
2011-FRA-	France	2011	KF1850	KF1850	N	N	731bp	KF5172	N
2010-FRA-	France	2010	KF1850	KF1850	N	N	731bp	KF5172	N
2011-FRA-	France	2010	KF1850	KF1850	N	N	731bp	KF5172	N
2009-FRA-	France	2009	KF1850	KF1850	N	N	731bp	KF5172	N
2009-FRA-	France	2009	KF1850	KF1850	N	N	no	KF5172	N
2010-FRA-	France	2010	KF1850	KF1850	N	N	731bp	KF5172	N
2012-CHN-	China	2012	Y	Y	Y	Y	Y	Y	KP41253
2010-FRA-	France	2010	Y	Y	Y	Y	Y	Y	KY2427
2011-IRL-	Ireland	2011	Y	Y	Y	Y	Y	Y	KY2716

Figure 4.1. Multiple sequence alignment for OsHV-1 sequence regions

Figure 4.1a. Sequence Region 1, ORF4.

Eight unique sequence types were identified from the 91 isolates sequenced. Identical sequences are indicated by a dot. Deletions are indicated by a dash. Sequence base changes are highlighted grey as shown



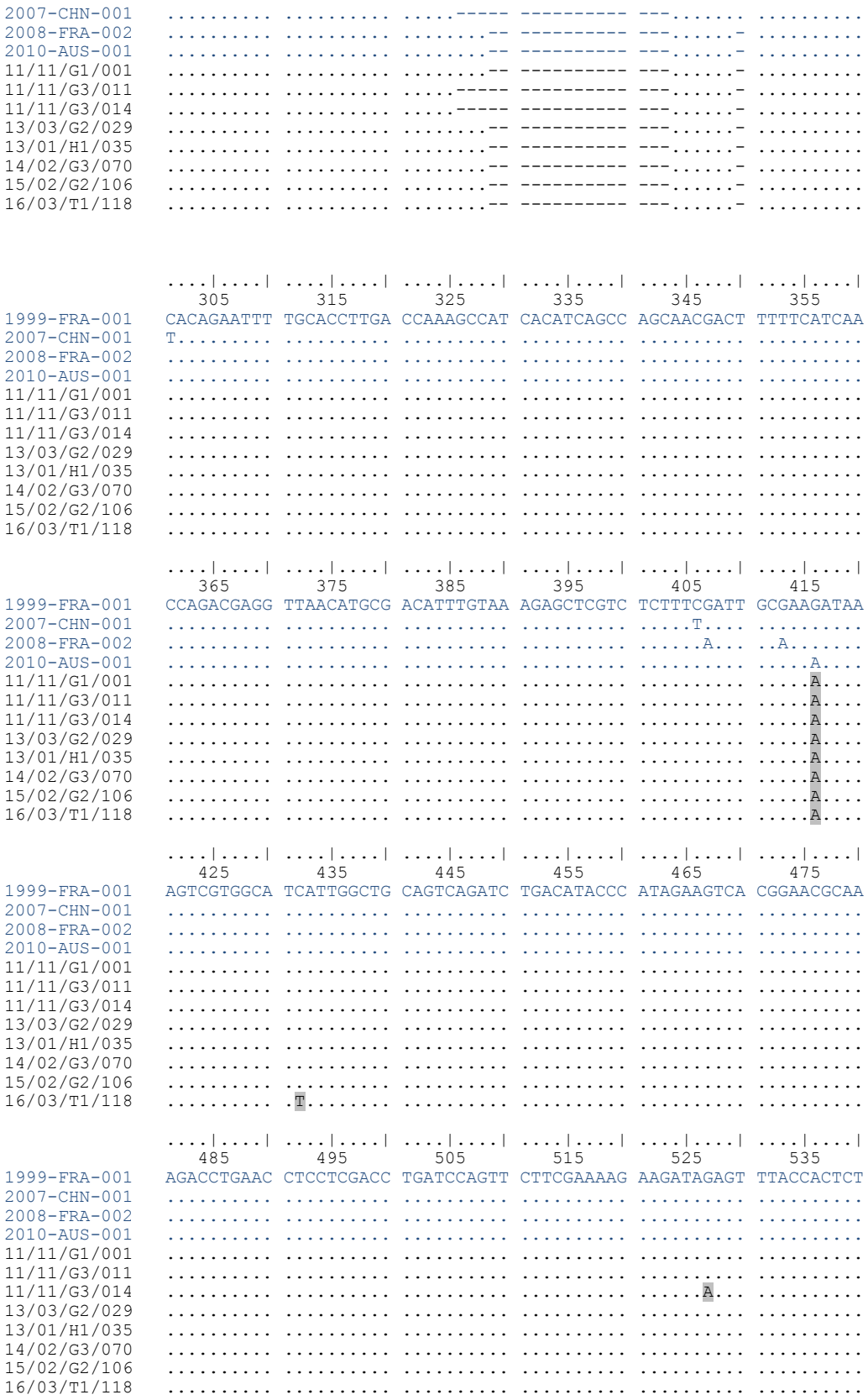

2007-CHN-001
2008-FRA-002
2010-AUS-001
11/11/G1/001
11/11/G3/011
11/11/G3/014
13/03/G2/029
13/01/H1/035
14/02/G3/070
15/02/G2/106
16/03/T1/118
305 315 325 335 345 355
1999-FRA-001 CACAGAATTT TGCACCTTGA CCAAAGCCAT CACATCAGCC AGCAACGACT TTTTCATCAA
365 375 385 395 405 415
1999-FRA-001 CCAGACGAGG TTAACATGCG ACATTTGTAA AGAGCTCGTC TCTTTCGATT GCGAAGATAA
425 435 445 455 465 475
1999-FRA-001 AGTCGTGGCA TCATTGGCTG CAGTCAGATC TGACATACCC ATAGAAGTCA CGGAACGCAA
485 495 505 515 525 535
1999-FRA-001 AGACCTGAAC CTCCTCGACC TGATCCAGTT CTTCGAAAAG AAGATAGAGT TTACCACTCT

Figure 4.1b. Sequence Region 2, ORF 42-43.

Unique sequence types from NSW isolates are aligned against OSHV-1 reference sequence (1999-FRA-001), AVNV (2007-CHN-001) and OSHV-1 µvar sequences from France (2010-FRA-0014) and New Zealand (2010-NZD-002). Identical sequences are indicated by a dot. Deletions are indicated by a dash. Sequence base changes are highlighted grey.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
1999-FRA-001    TTTTTTGTAA AGCTTTTATA TATCTTCAAA TCCGGAAGTG TTTTAACAAC AAGATTACAA
2007-CHN-001    .......... .......... .......... .......... .......... ..........
2010-FRA-014    .......... .......... .......... .......... .......... ..........
2010-NZD-002    .......... .......... .......... .......... .......... ..........
11/11/G1/004    .......... .......... .......... .......... .......... ..........
13/01/H1/031    .......... .......... .......... .......... .......... ..........
12/02/G2/009    .......... .......... .......... .......... .......... ..........
12/11/G2/026    .......... -......... .......... .......... .......... ..........
13/02/H2/040    .......... -......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
1999-FRA-001    AAAAATATCA ACGGCAATGT CTAATTTGTT CATTCCCCGA TCTACCAAAC GTGCAGTCTA
2007-CHN-001    .......... c......... .......... .......... .......... ..........
2010-FRA-014    ....-..... .......... .......... .......... .......... ..........
2010-NZD-002    ....-..... .......... .......... .......... .......... ..........
11/11/G1/004    ....-..... .......... .......... .......... .......... ..........
13/01/H1/031    ...--..... .......... .......... .......... .......... ..........
12/02/G2/009    ....-..... .......... .......... .......... .......... ..........
12/11/G2/026    ....-..... .......... .......... .......... .......... ..........
13/02/H2/040    ...--..... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
1999-FRA-001    CGACGGCCCT TTGCCAATGG TAGGCTCTTC CCTGCCGCCA ATAGAAATAA ACAGCAAAGG
2007-CHN-001    .......... .......... .......... .......... .......... ..........
2010-FRA-014    .......... .......... .......... .......... .......... ..........
2010-NZD-002    .......... .......... .......... .......... .......... ..........
11/11/G1/004    .......... .......... .......... .......... .......... ..........
13/01/H1/031    .......... .......... .......... .......... .......... ..........
12/02/G2/009    .......... .......... .......... .......... .......... ..........
12/11/G2/026    .......... .......... .......... .......... .......... ..........
13/02/H2/040    .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   185        195        205        215        225        235
1999-FRA-001    TGATAAATCG GTAGTTTATC TCAGGGGTGA TGATCAACCA ATTGATGTTA ACAGGGAACA
2007-CHN-001    .......... .......... .......... .......... .......... ..........
2010-FRA-014    .......... .......... .......... .......... .......... ..........
2010-NZD-002    .......... .......... .......... .......... .......... ..........
11/11/G1/004    .......... .......... .......... .......... .......... ..........
13/01/H1/031    .......... .......... .......... .......... .......... ..........
12/02/G2/009    .......... .......... .......... .......... .......... ..........
12/11/G2/026    .......... .......... .......... .......... .......... ..........
13/02/H2/040    .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   245        255        265        275        285        295
1999-FRA-001    TAGAATGGTA AAAGTTACGT ATAATGAATA CGATGAGCAA GAAACGATCA AGGTTATTTT
2007-CHN-001    .......... .......... .......... .......... .......... ..........
2010-FRA-014    .......... .......... .......... .......... .......... ..........
2010-NZD-002    .......... .......... .......... .......... .......... ..........
11/11/G1/004    .......... .......... .......... .......... .......... ..........
13/01/H1/031    .......... .......... .......... .......... .......... ..........
12/02/G2/009    .......... .......... .......... .......... .......... ..........
12/11/G2/026    .......... .......... .......... .......... .......... ..........
13/02/H2/040    .......... .......... .......... .......... .......... ..........


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
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                  305        315        325        335        345        355
1999-FRA-001   CCTCGACAAG AAAGCAACAA TAAAAGATCT ACATAACCTA ATGAGTGTTG GTAGGGATCT
2007-CHN-001   .......... .......... .......... .......... .......... ..........
2010-FRA-014   .......... .......... .......... .......... .......... ..........
2010-NZD-002   .......... .......... .......... .......... .......... ..........
11/11/G1/004   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... .......... .......... ..........
12/02/G2/009   .......... .......... .......... .......... .......... ..........
12/11/G2/026   .......... .......... .......... .......... .......... ..........
13/02/H2/040   .......... .......... .......... .......... .......... ..........


          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  365        375        385        395        405        415
1999-FRA-001   TACAACGGGT GTCTGCAATA TAGAAGTACA ACCGGAATAT GGATTCACAC TGAGGATACC
2007-CHN-001   .......... .......... .......... .......... .......... ..........
2010-FRA-014   .......... .......... .......... .......... .......... ..........
2010-NZD-002   .......... .......... .......... .......... .......... ..........
11/11/G1/004   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... .......... .......... ..........
12/02/G2/009   .......... .......... .......... .......... .......... ..........
12/11/G2/026   .......... .......... .......... .......... .......... ..........
13/02/H2/040   .......... .......... .......... .......... .......... ..........

                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     425        435        445        455        465        475
1999-FRA-001   AGACCCAGAC AAGTTGAAAT ATAAAAGTGA TATAGATGCA GTCTATAGAC TCTTCGCTTC
2007-CHN-001   .......... .......... .......... .......... .......... ..........
2010-FRA-014   .......... .......... .......... .......... .......... ....T.....
2010-NZD-002   .......... .......... .......... .......... .......... ..........
11/11/G1/004   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... .......... .......... ..........
12/02/G2/009   .......... .......... .......... .......... .......... ..........
12/11/G2/026   .......... .......... .......... .......... .......... ..........
13/02/H2/040   .......... .......... .......... .......... .......... ..........

                  ....|....| ....|....| ....|....| .
                     485        495        505
1999-FRA-001   AAAATACGAC AATAGCGATC TATTCGAAAG G
2007-CHN-001   .......... .......... .......... .
2010-FRA-014   .......... .......... .......... .
2010-NZD-002   .......... .......... .......... .
11/11/G1/004   .......... .......... .......... .
13/01/H1/031   .......... .......... .......... .
12/02/G2/009   .......... .......... ...-...... .
12/11/G2/026   .......... .......... .......... .
13/02/H2/040   .......... .......... .......... .
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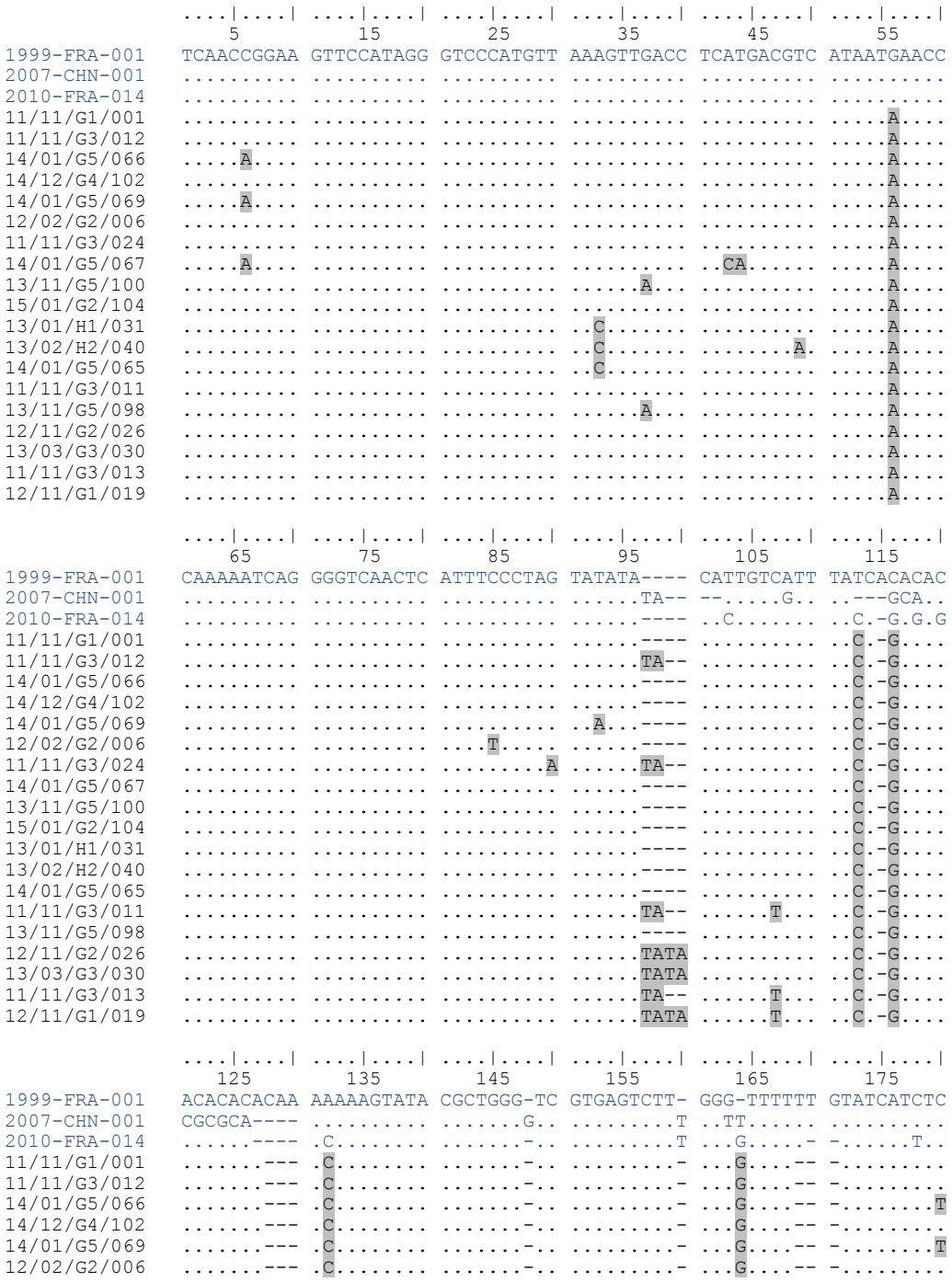
Figure 4.1c. Sequence Region 4, non-coding region between ORF49 and 50

Unique sequence types from NSW isolates are aligned against OSHV-1 reference sequence (1999-FRA-001), OSHV-1 µvar sequences from France (2010-FRA-0014) and Acute viral necrosis virus of Chinese scallops (2007-CHN-001). Identical sequences are indicated by a dot. Deletions are indicated by a dash. Sequence base changes are highlighted grey.



```
11/11/G3/024   .......--- .C........ .......-.. ..........- ...G....-- -.........
14/01/G5/067   .......--- .C........ .......-.. ..........- ...G....-- -.........T
13/11/G5/100   .......--- .C........ .......-.. ..........- ...G....-- -.........T
15/01/G2/104   .......--- .C........ .......-.. ..........- ...G....-- -.........
13/01/H1/031   .......--- .C........ .......-.. ..........- ...G....-- -.........
13/02/H2/040   .......--- .C........ .......-.. ..........- ...G....-- -.........
14/01/G5/065   .......--- .C........ .......-.. ..........- ...G....-- -.........T
11/11/G3/011   .......--- .C........ .......-.. ..........- ...G....-- -.........
13/11/G5/098   .....----- .C........ .......-.. ..........- ...G....-- -.........T
12/11/G2/026   .......--- .C........ .......-.. ..........- ...G....-- -.........
13/03/G3/030   .....----- .C........ .......-.. ..........- ...G....-- -.........
11/11/G3/013   .......--- .C........ .......-.. ..........- ...G....-- -.........
12/11/G1/019   .....----- .C........ .......-.. ..........- ...G....-- -.........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  185        195        205        215        225        235
1999-FRA-001   TCAATGTTAA TTCTATGAGA TTTGTTAAAT CAAGACGACT TCCCTGACAG GCAGACTTTA
2007-CHN-001   .......... .......... .......... .......... .......... .....T....
2010-FRA-014   .......... .......... .......... .......... .......... ..........
11/11/G1/001   .......... .......... .......... .......... .......... ..........
11/11/G3/012   .......... .......... .......... .......... .......... ..........
14/01/G5/066   .......... .......... .......... .......... .......... ..........
14/12/G4/102   .......... .......... .......... .......... .......... ..........
14/01/G5/069   .......... .......... .......... .......... .......... ..........
12/02/G2/006   .......... .......... .......... .......... .......... ..........
11/11/G3/024   .......... .......... .......... .......... .......... ..........
14/01/G5/067   .......... .......... .......... .......... .......... ..........
13/11/G5/100   .......... .......... .......... .......... .......... ..........
15/01/G2/104   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... .......... .......... ..........
13/02/H2/040   .......... .......... .......... .......... .......... ..........
14/01/G5/065   .......... .......... .......... .......... .......... ..........
11/11/G3/011   .......... .......... .......... .......... .......... ..........
13/11/G5/098   .......... .......... .......... .......... .......... ..........
12/11/G2/026   .......... ......A... .......... .......... .......... ..........
13/03/G3/030   .......... ......A... .......... .......... .......... ..........
11/11/G3/013   .......... .......... .......... .......... .......... ..........
12/11/G1/019   .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  245        255        265        275        285        295
1999-FRA-001   ATGTCTATGA GAGTATCGCC AGGGAACATT GTTTAAGACA ACTTCATTGC CAGGCATATT
2007-CHN-001   .......... .......... .......... .......... .......... ..........
2010-FRA-014   .......... .......... .......... .......... .......... ..........
11/11/G1/001   .......... .......... .......... .......... .......... ..........
11/11/G3/012   .......... .......... .......... .......... .......... ..........
14/01/G5/066   .......... .......... .......... .......... .......... ..........
14/12/G4/102   .......... .......... .......... .......... .......... ..........
14/01/G5/069   .......... .......... .......... .......... .......... ..........
12/02/G2/006   .......... ......T... .......... .......... .......... ..........
11/11/G3/024   .......... .......... .......... .......... .......... ..........
14/01/G5/067   .......... .......... .......... .......... .......... ..........
13/11/G5/100   .......... .......... .......... .......... .......... ..........
15/01/G2/104   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... .......... .......... ..........
13/02/H2/040   .......... .......... .......... .......... .......... ..........
14/01/G5/065   .......... .......... .......... .......... .......... ..........
11/11/G3/011   .......... .......... .......... .......... .......... ..........
13/11/G5/098   .......... .......... .......... .......... .......... ..........
12/11/G2/026   .......... .......... .......... .......... .......... ..........
13/03/G3/030   .......... .......... .......... .......... .......... ..........
11/11/G3/013   .......... .......... .......... .......... .......... ..........
12/11/G1/019   .......... .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  305        315        325        335        345        355
1999-FRA-001   TTAAATGTCT ATGGAAAATA GAATATTTAA GAGCGGCAAG GAATTAATTT GTGTTCACTC
2007-CHN-001   .......... .......... .......... .......... .......... ..........
2010-FRA-014   .......... .......... .......... .......... .......... ..........
11/11/G1/001   .......... .......... .......... .......... .......... ..........
11/11/G3/012   .......... .......... .......... .......... .......... ..........
14/01/G5/066   .......... .......... .......... .......... .......... ..........
14/12/G4/102   .......... ...A...... .......... .......... .......... ..........
14/01/G5/069   .......... .......... .......... .......... .......... ..........
12/02/G2/006   .......... .......... .......... .......... .......... ..........
11/11/G3/024   .......... .......... .......... .......... .......... ..........
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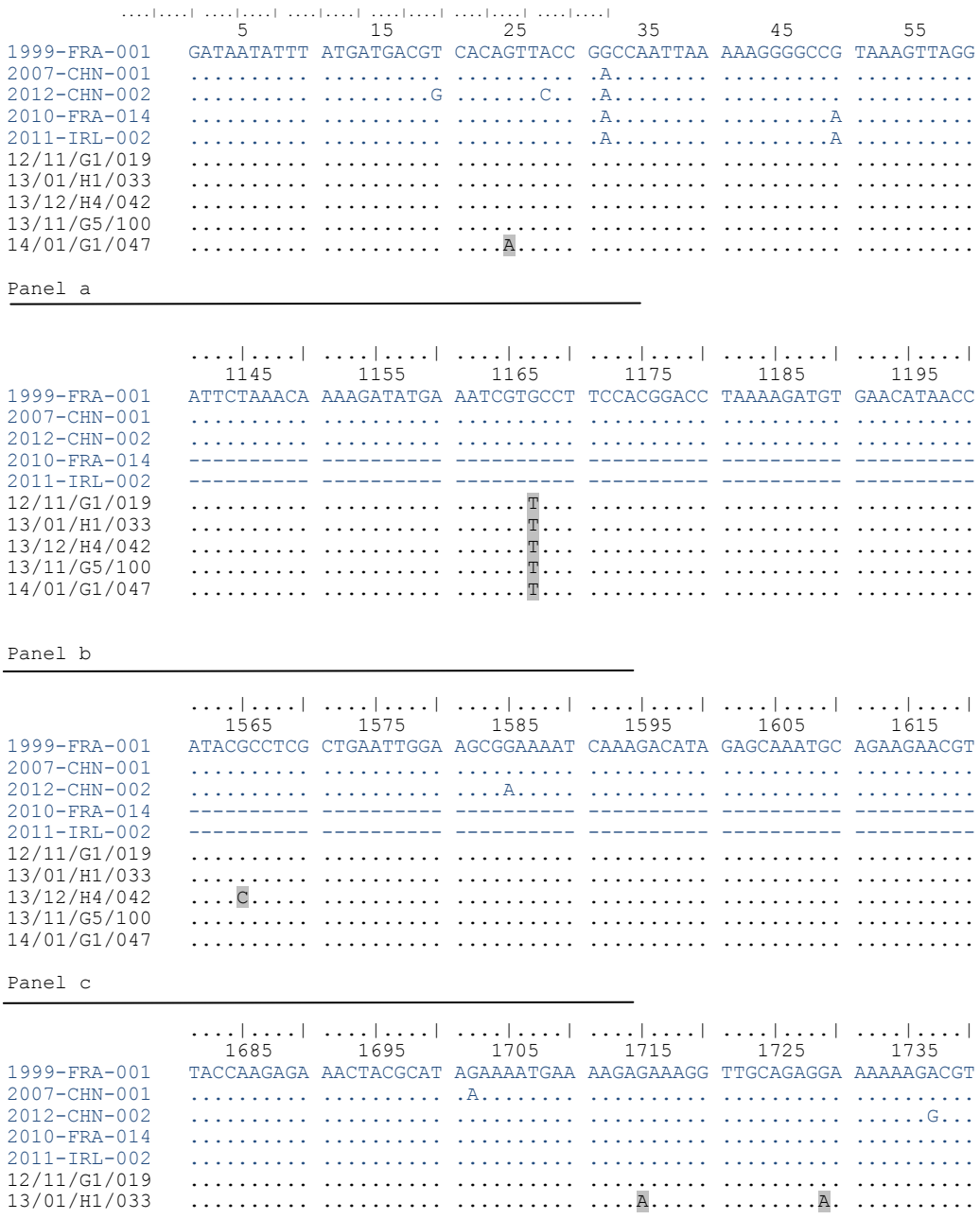
14/01/G5/067   .......... .......... .......... .......... .......... ..........
13/11/G5/100   .......... .......... .......... .......... .......... ..........
15/01/G2/104   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... .......... .......... ..........
13/02/H2/040   .......... .......... .......... .......... .......... ..........
14/01/G5/065   .......... .......... .......... .......... .......... ..........
11/11/G3/011   .......... .......... .......... .......... .......... ..........
13/11/G5/098   .......... .......... .......... .......... .......... ..........
12/11/G2/026   .......... .......... .......... .......... .......... ..........
13/03/G3/030   .......... .......... .......... .......... .......... ..........
11/11/G3/013   .......... .......... .......... .......... .......... ..........
12/11/G1/019   .......... .......... .......... .......... .......... ..........

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  365        375        385        395        405        415
1999-FRA-001   AATATACCCA TATTGTTCCA AGATGTCTGG TATAAAAATT TTCTTTT-CG GTGACGGCAC
2007-CHN-001   .......A.. .......... .......... .......... .C.....-.. ..........
2010-FRA-014   .......... .......... .......... .........A .......-.. ..........
11/11/G1/001   .......... .......... .......... .........A. .......-.. ..........
11/11/G3/012   .......... .......... .......... .........A. .......-.. ..........
14/01/G5/066   .......... .......... .......... .........A. .......-.. ..........
14/12/G4/102   .......... .......... .......... .........A. .......-.. ..........
14/01/G5/069   .......... .......... .......... .........A. .......-.. ..........
12/02/G2/006   .......... .......... .......... .........A. .......-.. ..........
11/11/G3/024   .......... .......... .......... .........A. .......-.. ..........
14/01/G5/067   .......... .......... .......... .........A. .......-.. ..........
13/11/G5/100   .......... .......... .......... .........A. .......-.. ..........
15/01/G2/104   .......... .......T.. .......... .........A. .......-.. ..........
13/01/H1/031   .......... .......... .......... .........A. .......-.. ..........
13/02/H2/040   .......... .......... .......... .........A. .......-.. ..........
14/01/G5/065   .......... .......... .......... .........A. .......-.. ..........
11/11/G3/011   .......... .......... .......... .........A. .......-.. ..........
13/11/G5/098   .......... .......... .......... .........A. .......-.. ..........
12/11/G2/026   .......... .......... .......... .........A. .......-.. ..........
13/03/G3/030   .......... .......... .......... .........A. .......-.. ..........
11/11/G3/013   .......... .......... .......... .........A. .......-.. ..........
12/11/G1/019   .......... .......... .......... .........A. .......-.. ..........
```

Figure 4.1d. Sequence Region 5, ORF -11.

The deletion identified as being indicative of OSHV-1 µvar (Table 1) was not present in any of the NSW isolates, which had sequence consistent with the OsHV-1 reference genome. Panels below show the portions of this region in which 5 unique sequence types from NSW samples are aligned against OSHV-1 reference sequence (1999-FRA-001), AVNV (2007-CHN-001) and OSHV-1 µvar sequences from France (2010-FRA-0014), China (2012-CHN-002) and Ireland (2011-IRL-002). Identical sequences are indicated by a dot. Deletions are indicated by a dash. Sequence base changes are highlighted grey. Each panel represents a variable region in the 2kb of sequence analysed. Panels b and panel c show part of the large deletion found in some variants designated as microvariant OSHV-1.



```
13/12/H4/042   .......... .......... .......... ....A..... .........A. ..........
13/11/G5/100   .......... .......... .......... .......... .......... ..........
14/01/G1/047   .......... .......... .......... .......... .......... ..........

Panel d
```

```
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  1805       1815       1825       1835       1845       1855
1999-FRA-001   CGTGCAGCAG AGGAAAAACG CCGCGCCGAT GCCGCTATTG AGATTGCAAA AATGAAAGCA
2007-CHN-001   .......... .......... ......A... .......... .......... ..........
2012-CHN-002   .......... .......... .......... .......... .......... ..........
2010-FRA-014   .......... .......C.. .......... .......... .......... ..........
2011-IRL-002   .......... .......C.. .......... .......... .......... ..........
12/11/G1/019   .......... .......... .......... .......... .......... ..........
13/01/H1/033   .........A ..A....... .......... .......... .......... ..........
13/12/H4/042   .........A ..A....... .......... .......... .......... ..........
13/11/G5/100   .......... .......C.. .......... .......... .......... ..........
14/01/G1/047   .......... .......... .......... .......... .......... ..........

Panel e
```

Figure 4.1e. Sequence Region 5, ORF -11.

The portions of the sequence containing unique sequence types from NSW isolates are aligned against OSHV-1 reference sequence (1999-FRA-001), AVNV (2007-CHN-001) and OSHV-1 µvar sequences from France (2010-FRA-0014), China (2012-CHN-002) and Ireland (2011-IRL-002) . Identical sequences are indicated by a dot. Deletions are indicated by a dash. Sequence base changes are highlighted grey.

```
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  305        315        325        335        345        355
1999-FRA-001   TCCGCATTTG ATTTATACAG ACTTCGATAT GTGTACGTTG GTCTTAGAGA CGCCATAAAT
2010/FRA/014   .......... .......... .......... .......... .......... ..........
2011/IRL/002   .......... .......... .......... .......... .......... ..........
2012/CHN/002   .......... .......... .......... .......... .......... ..........
2007-CHN-001   .......... .......... .......... .......... .......... ..........
12/11/G3/027   .......... .......... .......... .......... .......... ..........
13/04/G1/028   .......... .......... .......... .......... .......... ..........
13/03/G3/030   .......... .......... .......... .......... .......... ..........
13/01/H1/031   .......... .......... .......... ..C....... .......... ..........
13/01/H1/032   .......... .......... .......... ..C....... .......... ..........
13/01/H1/033   .......... .......... .......... ..C....... .......... ..........

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  665        675        685        695        705        715
1999-FRA-001   AAAACCACTC CCCAGGAAGA TTGCATTCAG CCCTTTTGCA CCAAAGGAAC AGTGTATGGA
2010/FRA/014   .......... .......... .......... .......... .......... ..........
2011/IRL/002   .......... .......... .......... .......... .......... ..........
2012/CHN/002   .......... .......... .......... .......... .......... ..........
2007-CHN-001   .......... .......... .......... .......... .......... ..........
11/11/G3/016   .......... .......... .......... .......... ..G....... ..........
```

4.5.2 Phenotypic studies of diverse OsHV-1 genotypes

Preliminary amplification of a fresh OsHV-1 stock was possible for 3 selected isolates that were stored as fresh tissue at -80°C (Table 4.4), but not for clinical specimens stored as clarified tissue culture supernatants. Thus, to enable future studies of this nature require samples stored as whole tissue or as tissue homogenates with the addition of cryopreservatives.

The selected isolates had differences in the multi-locus sequence targets: an extra triploid deletion in the microsatellite repeat and a single SNP in 540 bases of sequence in ORF -4; Four SNPs in at 550 base pair non-coding region between ORF 49 and 50. Otherwise there was 100% homology in a 6 region multi-locus study.

Mortality peaked between 48 and 144h after challenge with a lag of 60 hours between injection and cohabitation. Total cumulative mortality for each isolate with OsHV-1$_{_Georges_2011}$ (90.3%), OsHV-1$_{_Georges_2014}$ (74.3%) and OsHV-1$_{_Hawkesbury_2015}$ (74.8%). The mortality of oysters challenged by injection was higher than by cohabitation, irrespective of isolate and dose (Figure 4.2a). Mortality was dose responsive with the lowest dose (10^2 viral copies per injection) close to the minimum infectious dose for OsHV-1 under this experimental setting (Figure 4.2b). Replicate tanks had similar total cumulative mortalities expect in Dose 3 (10^2 viral copies per injection), which had variable mortality among replicates. The survivor probability was lower for Isolate 1 (OsHV-1$_{_Georges_2011}$) compared to Isolate 3 (OsHV-1$_{_Hawkesbury_2015}$).

A Cox proportional hazards model indicated that the hazard of mortality was lower for the lowest dose of OsHV-1 that was injected (Table 4.5). The hazard was also lower for challenge by cohabitation compared to injection. The hazard of death for the Isolate OsHV-1$_{_Georges_2011}$ was significantly higher than for OsHV-1$_{_Hawkesbury_2015}$. The two isolates from the Georges River in 2011 and 2014 did not have a discernibly hazard for mortality of oysters in this standard infection model.

There was a high viral load at the time of mortality (n=100), and in the absence of mortality and negative qPCR tests for OsHV-1 in control oysters, the cause of all deaths of challenged oysters in this trial was attributed to OsHV-1. The prevalence of OsHV-1 in oysters at the cessation of the trial was 12% and the viral load in these positive oysters was less than half compared to oysters which died. There was no difference in the quantity of OsHV-1 DNA at time of death for the three different isolates (Figure 4.3a). For those oysters that died, the initial injection dose did not impact the amount of viral DNA at the time of mortality (Figure 4.3b). However, oysters challenged by cohabitation had a higher viral load (3.36 $x10^3$) compared to those that died after injection (2.30 $x10^2$) (Figure 4.3c; $P < 0.001$).

The present experiment demonstrated that there is phenotypic variation in OsHV-1 isolates present in Australia. The virulence of the OsHV-1 isolate needs to be taken into account as a factor that influences variable mortality in outbreaks of disease. In the longer term, the potential for evolution of OsHV-1 needs to be considered. This pathogen is under extreme selection pressure with high mortality disease and impacts on farm stocking reducing access to susceptible host. Further evaluation of OsHV-1 isolates for virulence, and particularly a study design which targets avirulent isolates might reveal an isolate with potential practical relevance. Further work is required to determine if infection with a strain of OsHV-1 that causes low mortality provides protection from subsequent infection with more virulent strains.

Table 4.4. Isolates of OsHV-1 amplified to compare for differences in virulence and transmission.

Note that preliminary amplification of several more isolates was attempted but the mortality did not occur and OsHV-1 was not amplified from clinical specimens archived at -80°C as clarified tissue homogenates with cryopreservative.

Region	Date collected	Oysters per treatment	Total Cumulative Mortality (%)	Isolate name (sequence ID)	OsHV-1 qPCR positive (%)	Quantity OsHV-1 *
Georges River	24/11/11	9	100	OsHV-1_Georges_2011 (1111G1126)	100	1.15×10^4
Georges River	24/2/14	8	100	OsHV-1_Georges_2014 (1402G3127)	100	4.01×10^4
Hawkesbury River	11/11/15	9	100	OsHV-1_Hawkesbury_2015 (1511H3128)	100	4.76×10^4
Negative control (Shoalhaven) V172	01/2014	9	0	Negative control	0	0

* Mean of genome copies per mg tissue for OsHV-1 positive oysters.

Table 4.5. Hazard ratios predicted from a Cox proportional hazards model.

Data are point estimates with 95% confidence interval. Comparisons with 95% confidence containing the value 1.00 are not significant. *Reference category

Factor	Level	Hazard Ratio		
		Point Estimate	95% Confidence Interval	
			Lower	Upper
Isolate	OsHV-1_Georges_2011	1.37	1.01	1.86
	OsHV-1_Georges_2014	1.06	0.63	1.79
	OsHV-1_Hawkesbury_2015 *	-	-	-
Challenge Method	Injected	1.30	1.02	1.65
	Cohabitated*	-	-	-
Dose 3	10^2	0.44	0.24	0.79
	10^3	0.78	0.57	1.07
	10^4 *	-	-	-

Figure 4.2. Kaplan Meyer survival curves for a standard experimental infection challenge with 3 isolates of OsHV-1.

Figure 4.2a. Challenge by injection compared to cohabitation.

Replicate tanks were pooled for this analysis (n= 20). Each factor was significant, log rank test $p<0.05$. (a) Challenge by injection compared to challenge by cohabitation at a ratio of 50% injected. The survival time for cohabited oysters is adjusted by subtracting 60 hours to account for the different time at risk.

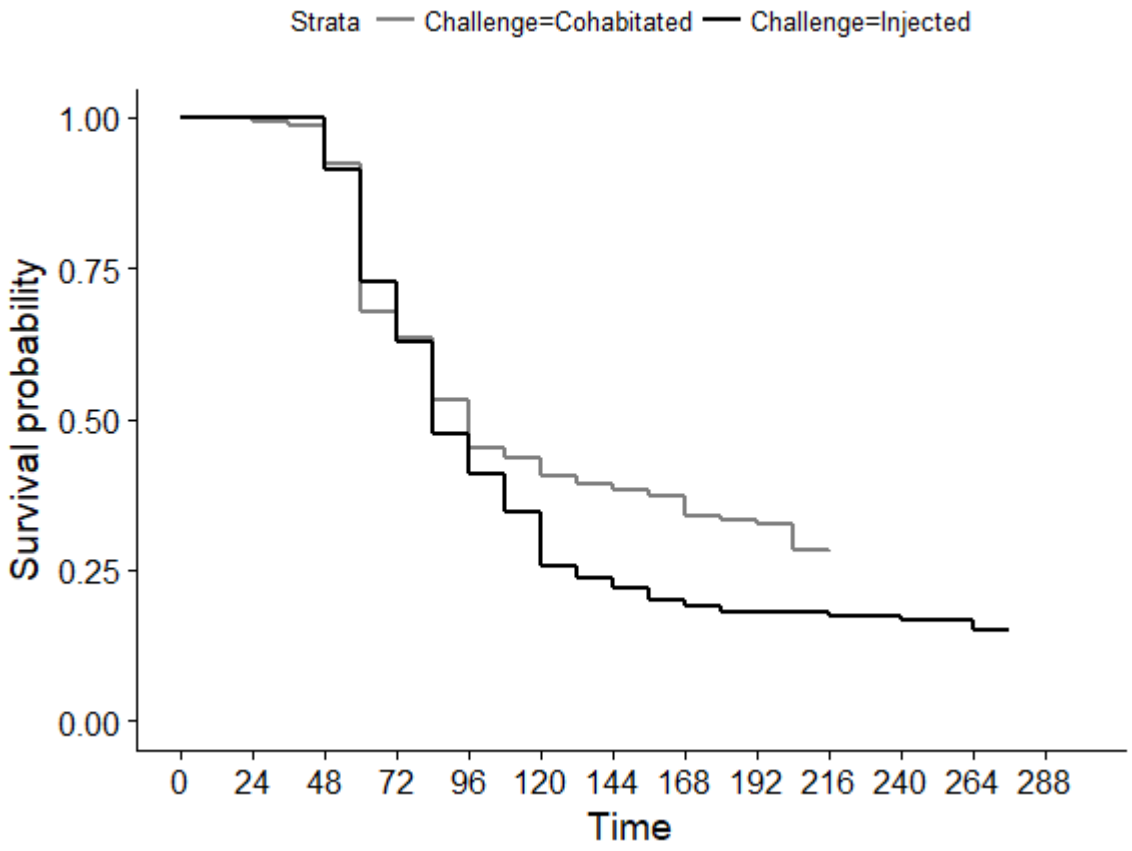


Figure 4.2b. Comparison of isolates

(b) Isolate 1 = OsHV-1$_{_Georges_2011}$ (black line); Isolate 2= OsHV-1$_{_Georges_2014}$ (lightest grey line); Isolate 3 = OsHV-1$_{_Hawkesbury_2015}$ (mid-grey line). Replicate tanks were pooled for this analysis (n= 20). Each factor was significant, log rank test $p<0.05$.

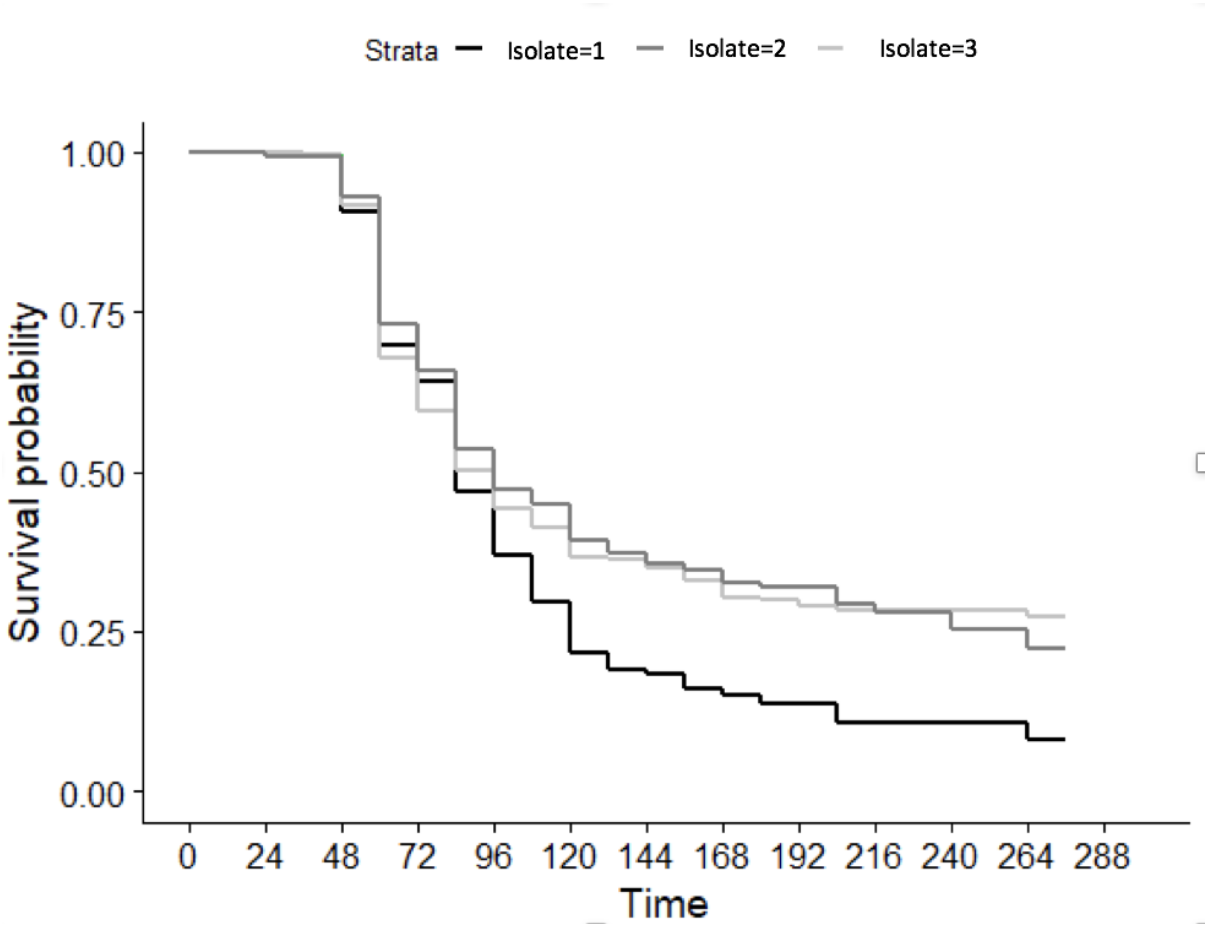


Figure 4.2c. Dose

(c) Dose 1 (10^4 OsHV-1 DNA copies per injection), Dose 2 (10^3), Dose 3 (10^2). Replicate tanks were pooled for this analysis (n= 20). Each factor was significant, log rank test $p<0.05$.

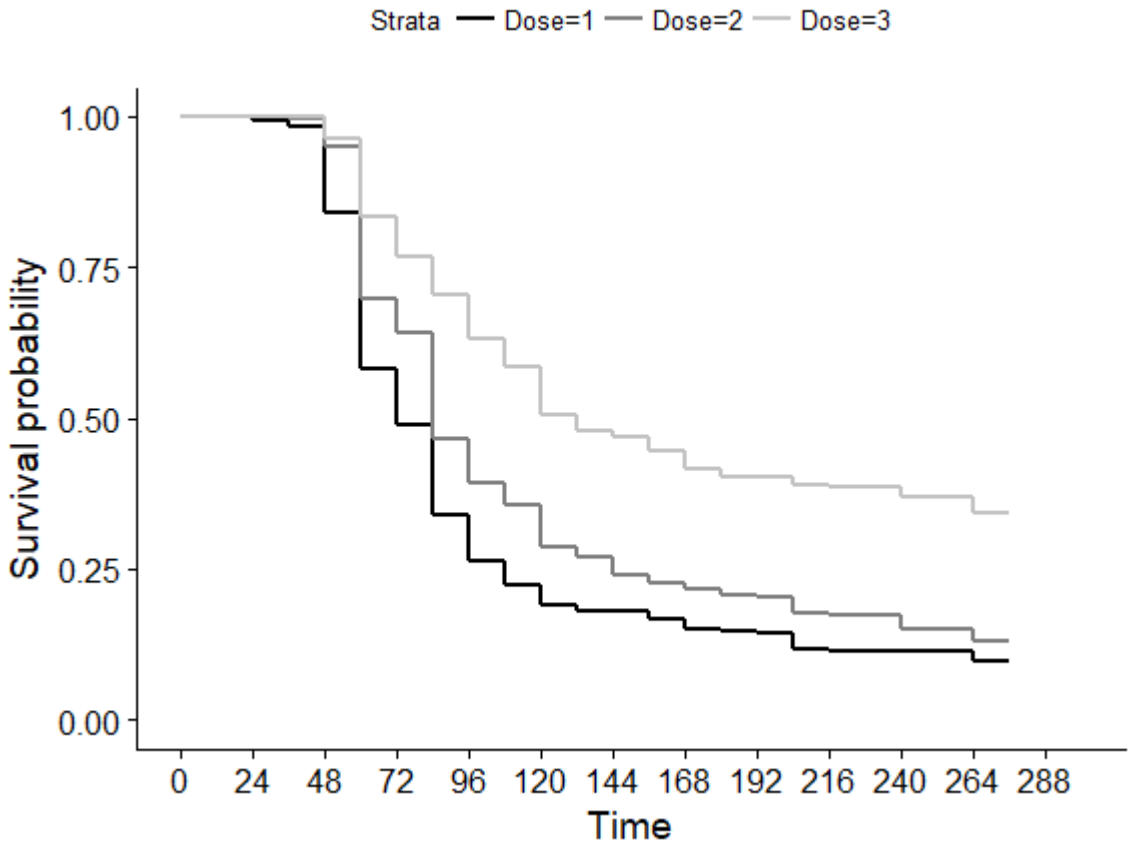


Figure 4.3a. The quantity of OsHV-1 DNA in oysters after a standard experimental challenge by injection and cohabitation with 3 different isolates of OsHV-1.

Figure 4.3a. Isolate.

Oysters challenged with 3 different isolates: OsHV-1$_{_Georges_2011}$, OsHV-1$_{_Georges_2014}$, OsHV-1$_{_Hawkesbury_2015}$. The data are estimated mean and standard error of the mean from the general mixed model.

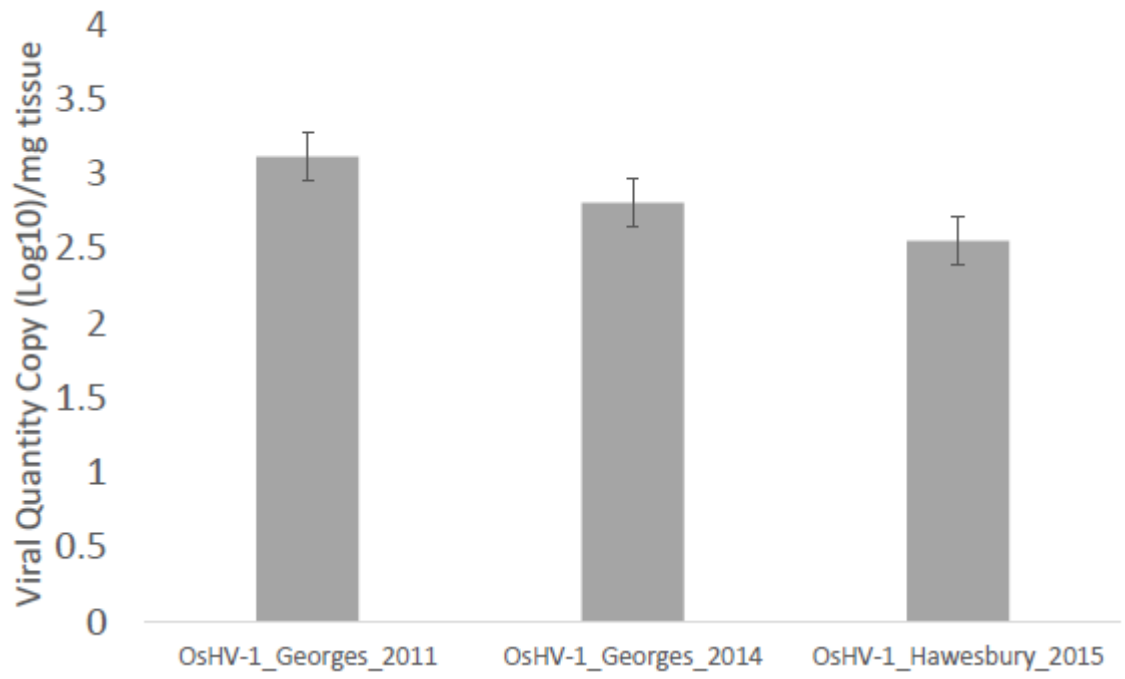


Figure 4.3b. Dose.

b) dose injected Dose 1 (10^4 OsHV-1 DNA copies per injection), Dose 2 (10^3), Dose 3 (10^2). The data are estimated mean and standard error of the mean from the general mixed model.

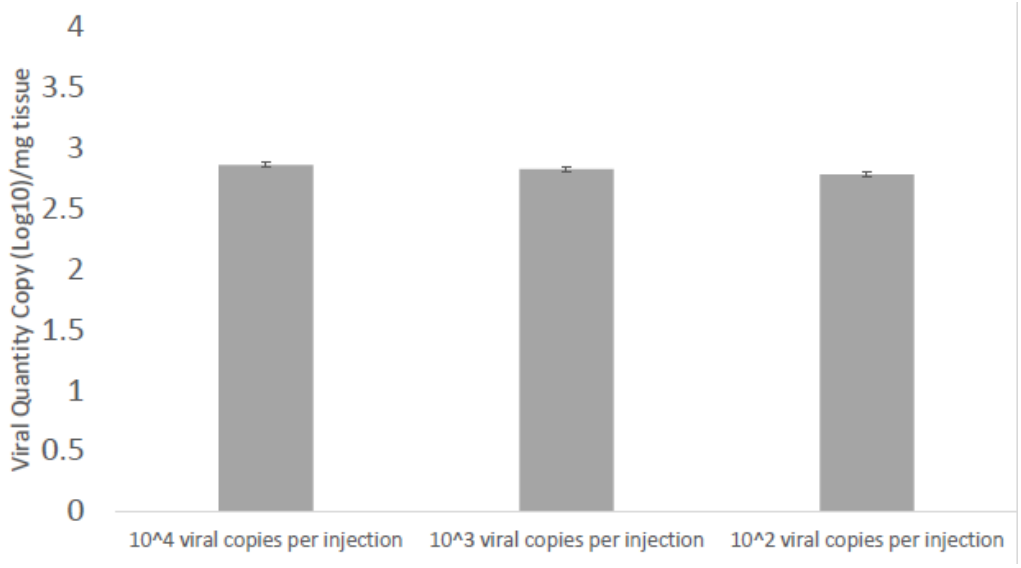
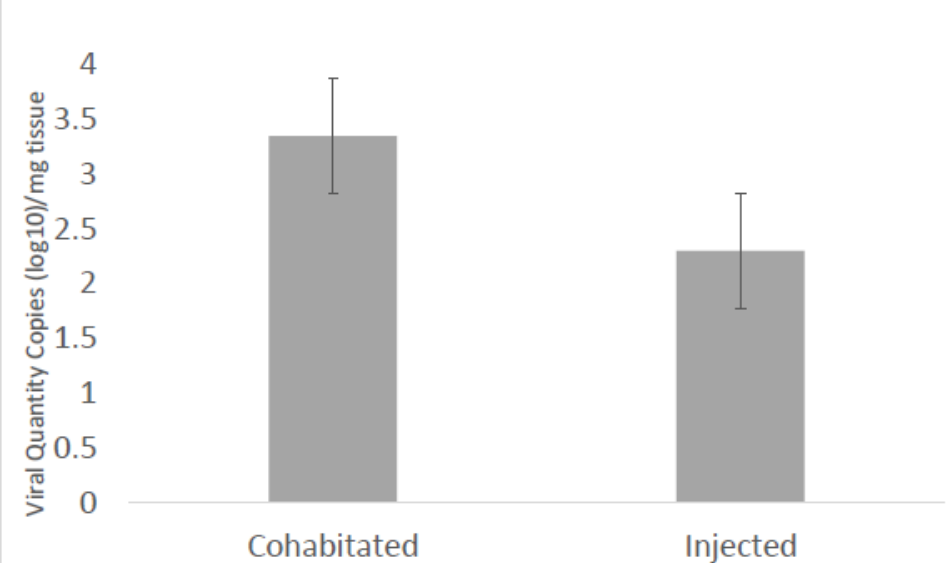


Figure 4.3c. challenge method.

(c) challengeby injection comapred to cohabitation. The data are estimated mean and standard error of the mean from the general mixed model.



4.6 Evaluation of an integrated OsHV-1 disease control strategy

Trial 1. Commercial Grow-out Monitoring Trial 2013-2014

Spat were supplied by Shellfish Culture to oyster growers in 2013. The locations of these spat are shown in Table 5.1. Normal cultivation practices were followed. Oyster farmers monitored the health of the spat approximately weekly, coordinated by Bruce Alford, who notified researchers if there was any mortality so that appropriate samples could be collected to determine the cause.

POMS was observed at all locations except Patonga Creek. The mortality rate was very high, with few survivors reported, and there was complete loss from a commercial perspective. Between 7 to 10 pools of 2 to 12 oysters were tested from each location, and in each case OsHV-1 viral load exceeded 10^5 copies per mg of tissue, confirming the role of the virus in the mortality event.

Table 5.1. Oyster stock in Trial 1, Commercial Growout Monitoring Trial 2013-2014.

Grower	Location	Date commenced	Batch	No.	Date of onset of POMS
SJ	Kimmerikong	22 Aug 13	SPL13A	100,000	24 Oct 13
RM	Coba Bay	22 Aug 13	SPL13A	35,000	5 Nov 13
RM	Marra Marra	22 Aug 13	SPL13A	35,000	30 Oct 13
RM	Kimmerikong	22 Aug 13	SPL13A	35,000	24 Oct 13
RM	Mooney Mooney	15 May 13	SPL13A	2,000	Nil*
BA	Patonga		SPL12FT	60,000	Nil

*lost to follow-up in January 2014

Trial 2. Commercial grow-out Monitoring Trial 2014-2015

Spat were purchased by oyster growers from Shellfish Culture, Tasmania and stocked into an estuary on the far south coast of NSW during the high risk period for POMS in spring 2013. They were agisted/grown during the summer and autumn then they were transferred to the Hawkesbury River in batches commencing in May 2014. An observational study was then conducted: each batch was monitored to determine whether oysters reached the market and to confirm the cause of mortalities.

From May to July 2014 stock were brought up from the south coast estuary by truck in several loads and placed on trays at Coba Bay and Marra Marra Creek at standard growing height. In order to avoid potential losses due to POMS the stock were moved downriver to Porto Bay in October 2014. This was based on a risk assessment: POMS occurred at these locations in October 2013 whereas POMS did not appear in oysters in research trials in Porto Bay until February in both 2013 and 2014. After the movement to Porto Bay sufficient stock were sold into the retail market prior to cover the costs of agistment owed to the south coast grower. The remainder of the stock remained in Porto Bay. The water temperature increased steadily during November 2014 (Figure 5.1) On 26th November 2014 mortality was observed among stock in about 100 trays of oysters (Figure 5.2). The oysters were 80-110 mm in length with occasional very large or small oysters present. qPCR confirmed high OsHV-1 viral load in a sample of dead oysters. The mortality rate was approximately 70%. No stock were able to be sold after this outbreak.

Figure 5.1. Daily temperature readings in intertidal trays.

The probes are named based on the bay in which the trays were initially located, but the most recent period of measurement is for Porto Bay.

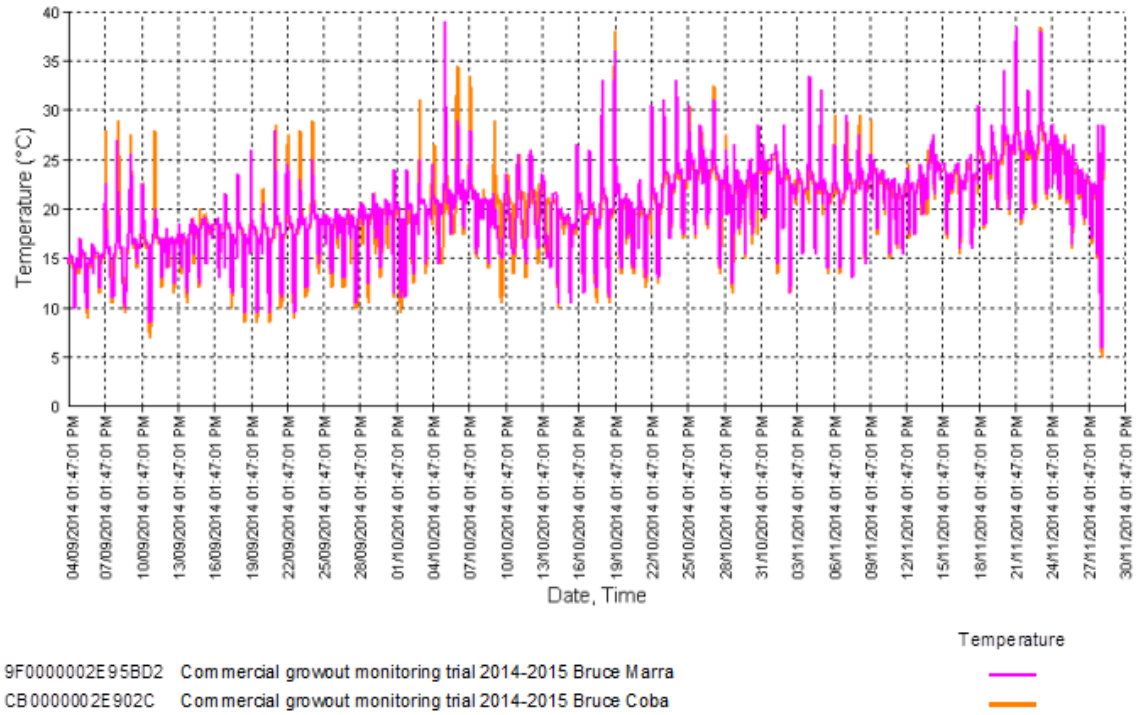


Figure 5.2. Mortality due to POMS in November 2014.

Mortality occurred in 80-100 mmm stock in November 2014 progressed from a patchy 20% mortality to 70% total cumulative mortality between inspections on 28 November and 12 December.

Trial 3. POMS Commercial grow-out trial 2016-2017

Triploid Pacific oyster spat (100,000) were supplied by Shellfish Culture, delivered to Sydney 20th December 2016 as 2240 spat. The batch SPL16A was spawned on 10 October 2016 from female diploids (YC13.01, EBV 51% resistance to POMS) and tetraploid males (TN13A and TN14A, both lines exposed to POMS in February 2016).

Spat were deployed in floating baskets on 21/12/16 on long lines at the entrance to Patonga Creek, a location where there has never been any sign of POMS. A basic plan was agreed where the spat would remain at the safe location in Patonga Creek until the end of the window of infection, and then be moved to a growing site during the non-POMS season, with marketing as soon as oysters were large enough prior to the next POMS season. A range of husbandry options were encouraged to be explored to maximize commercial opportunity and minimise costs. The collaborating farmer was asked to record major husbandry events and outcomes in a diary.

Grading was conducted as infrequently as possible to reduce management costs. Most of the stock were handled only once or twice over the entire production run between December 2016 and October 2017, compared with every 3 weeks with conventional husbandry. Low stocking rates were used to reduce the need for grading to thin out stock. Rates used were 10 dozen in 6 mm baskets, graded into 12 mm baskets at 5 dozen per basket; the final transfer was into 12 mm trays at 2 dozen per segment or 12 dozen per tray. On July 1 2017, after the close of the window of infection (deemed to be late May 2017), the largest grown spat (front runners) were moved up-river to Coba Bay on intertidal trays at standard growing height. About 10% of the cohort was moved.

Sales of stock from Coba commenced in mid-September and were complete by the end of October 2017, before the anticipated window of infection for 2017-2018 (deemed to be from early November 2017). The size range sold was bistro to large (80-100mm shell length), with bistro to buffet size (50-70 mm) predominating. About 90% of the stock moved to Coba were sold; the remainder were too small to sell and were kept at Coba on about 40 trays, with the objective to see how they survive the summer.There was no significant mortality observed during the trial.

The remainder of the cohort of spat were kept at upper Patonga Creek on intertidal trays and long-line baskets subtidal and were still there at the end of the trial in December 2017; they will be sold from that location when large enough.

During this trial commercial numbers of *C. gigas* were present in the Hawkesbury River estuary, but these were limited to attempts to recommence farming by 4 or 5 growers who had 100,000 spat each of the same batch SPL16A acquired in April 2017 and located at Mullet Creek, Porto Bay and the mouth of Mooney Mooney Creek. Bruce Alford also had some of these held in Patonga Creek. Supervision of this trial concluded in December 2017 at the end of the University of Sydney FRDC POMS project 2014-040.

Discussion

The strategy of using a safe location and knowledge of the window of infection allowed commercial quantities of Pacific oysters to be reared from 2240 spat to a marketable table size oyster in the Hawkesbury River estuary, which is endemically infected with OsHV-1. A novel approach was used to minimize production costs, namely use of very low stocking densities to reduce the need for grading oysters, an important labour saving innovation at a time when there were no farm staff or economic capacity to engage them. The experiment was pilot scale, and depended on existing infrastructure. Unfortunately there is insufficient infrastructure in place in Patonga Creek to enable immediate scale up. However, it has been demonstrated clearly how this strategy could work.

The commercial strategy described here depends on a number of factors:

- Cooperation between oyster farmers in several estuaries who would not normally work together. This can be difficult to set up due to geographic distance and might be assisted through communication via oyster farmer associations, to identify opportunities for growers in different regions to cooperate

- Mutual commercial advantage to growers in different estuaries. At present this is based on unequal growth potential for C. gigas stocked into different estuaries, presumably due to environmental (nutritional) differences which are poorly understood

- Restricted geographic distribution of POMS

- Biosecurity policies in NSW which permit movement of oysters between estuaries under certain conditions (including risk-based consideration of the geographic distribution of endemic diseases)

- Biosecurity policies which preclude movement of large oysters from Tasmania to NSW. In 2013 growers in NSW commissioned a risk assessment and approached NSW DPI Biosecurity to enable movement of juvenile oysters that had been partially grown in Tasmania directly to the Hawkesbury River for grow-out during the safe window.

The biosecurity implications of window farming are changing with the increased geographical range of OsHV-1 in Australia and the impact of other diseases requiring restriction on translocation. Over time it might be possible to integrate other research findings, such as the benefits of growing oysters at high height during the risk period to reduce losses due to POMS and to evaluate the impact of prior exposure to OsHV-1 either incidentally or as part of a controlled program for disease control.

4.7 Outbreak investigation: Index cases of POMS in Tasmania

A total of 6 farms were surveyed, and there were between 42 and 299 baskets sampled at each farm (Tables 6.1 and 6.2). Of the baskets surveyed, 67.5% contained oysters with a mean length between 41mm and 60mm (Table 6.3, Figure 6.5). Time on farm was between 1 and 39 months with an average of 12.2 months (Figure 6.10). Growing infrastructure varied between farms with sub-tidal, long-line, floating baskets and fixed rack systems all in use. However, the majority of baskets surveyed (92%) were on a height adjustable long-line system. On-growers were present only on Farms 2 and 4 and had been on farm for between 1 and 2 months at the time of the outbreak. Density was greatest and had the largest range in the smallest size category but was relatively consistent across the larger size categories (Figure 6.8).

Total mortality was 78.3% with a range of 0% to 100% between baskets (Figure 6.7). The range of mortality was similar across all 3 bays however overall mortality was higher in Blackman Bay (87.1%) than in Upper or Lower Pitt Water (53.5% and 54.5% respectively) (Table 6.3). Farm level mortality ranged from 37.3% to 92.3%.

Mortality was 62.9% lower in baskets containing the largest compared to the smallest size categories (Table 6.3). Mortality in spat < 20mm top shell length was 95.9%, and decreased with increasing size of the oysters to 33.9% in the largest group (> 71mm top shell length) (Figures 6.4 and 6.5, Table 6.3). Univariable analysis indicated an association between mortality and both time on farm and size category (Figures 6.5 and 6.6). The lowest density groups had the lowest mortality (62.1%) compared to the standard (74.6%) and high (84.6%) density groups (Table 6.3). Mortality in diploid oysters was 82.0% and in triploids was 54.1%. Mortality for oysters handled in the 7 days prior to the outbreak was 35.2% higher than in those which were not handled (Table 6.3). Multivariable analysis was then undertaken to separate factors that were not significant in the presence of variation in mortality that was due to other factors.

Seven out of the nine proposed husbandry or oyster factors had a significant association with mortality at $p < 0.25$ (Table 6.3). The final multivariable model contained four significant ($p < 0.05$) explanatory variables and a single non-significant variable as part of an interaction. The random effects structure detailed above accounted for clustering in the data. This model included time on farm, size category, stocking density, and the recently handled status (Table 6.4). Handling the oysters in the seven days prior to the outbreak was associated with almost double the odds of mortality compared to oysters which were not handled, after accounting for all other effects included in the final model. The odds of mortality in oysters in size categories of <40mm mean length was more than 3 times that of oysters >61mm in length. There was an interaction between time on farm and density indicating the effect on mortality of time on farm was different at each level of density (Figure 6.6). The probability of death was highest for oysters stocked at low density or high density when the time on farm was short (3-6 months, i.e. when oysters were relatively small and young) compared to a long period (21-24 months, i.e. when oysters were relatively large and old), but for oysters kept at standard density the probability of death was the same regardless of how long they had been present on farm (1 to 24 months) (Figure 6.6). The standard density produced the lowest mortality until 18 months on farm, after which both high and low density stocking had slightly reduced mortality.

There were 129 baskets from Farms 1 and 2 included in an analysis of the effect of length variation within a basket on mortality. These baskets had a mean mortality of 46.5% (range 4.1 - 94.9%) the range of lengths in each basket was 27.1mm ±9.9mm. There was no association between mortality and either the range of lengths in a basket ($p = 0.055$) or the standard deviation of lengths in a basket ($p = 0.351$) in univariable analyses. Greater individual oyster length was associated with a greater number of dead

oysters in the two highest and two lowest mortality baskets from each sub-batch counted on Farm 2 ($p < 0.001$). The difference in length between the live (72.7 ±7.7mm) and dead oysters (69.3 ±7.7mm) was 3.4mm.

Table 6.1: Description of risk factors and outcome variables

Variable	Description
Bay	One of three bays containing the farms which were surveyed
Farm	Unique and anonymous number for each farm
Block	Uniquely identifies each block, nested within farm
Batch	Uniquely identifies each group of oysters as they came onto the farm
Sub-batch	Uniquely identifies sub-groups of a batch split across different locations
Dead	Count of dead oysters in a basket, outcome variable
Total	Total count of oysters and shells in a basket, outcome variable
Size	Total top shell length standardized across farms, categorized based on management size categories
Time on farm	Number of months spent on a farm since spat left the hatchery of origin, a proxy for age notwithstanding variable time at the hatchery for some batches
Density	Designated as high for the top quartile, standard for middle two quartiles or low if in the lowest quartile for stocking density for the size category in each basket
On-grower	Whether or not the batch had been bought from another farm for grow-out and sale
Handled	Whether or not the basket had been subjected routine management procedures in the 7 days prior to the outbreak
Ploidy	Genome status, diploid or triploid
Hatchery	The hatchery of origin, one of two local hatcheries
Clip height	Ordinal descriptor of relative basket height, including sub-tidal growing, which determines immersion time

Table 6.2. Number of levels and baskets counted for the factors included as random effects in the GLMM.

Random effect	Description	Number of levels	Variance (SE)	Intra-class correlation coefficient	Number of baskets surveyed at each level			
					Mean	Maximum	Median	Minimum
Farm	Unique and anonymous number for each farm	6	0	0.000	122.0	229	89	42
Block	Uniquely identifies each block, nested in farm	45	0.15 (0.22)	0.036	16.3	154	7	1
Sub-Batch	Uniquely identifies each batch split across each location	131	0.80 (0.16)	0.225	6.1	38	1	1

Table 6.3. Total cumulative mortality (%) from counts of dead and live oysters.

Data include 732 baskets across 3 bays and 6 farms surveyed. Odds ratio estimates from univariable logistic regression are presented for each level of categorical variables measured following a POMS outbreak in Tasmania, January 2016. Reference categories are indicated with an odds ratio of 1. Variables significant at $p < 0.25$ were considered for multivariable analysis.

Variable	Levels	Mortality % (95% CI)	Odds Ratio (95% CI)	P value	Number of baskets
Bay	Upper Pitt Water	53.51 (53.62 - 53.40)	1.00	< 0.001	86
	Lower Pitt Water	54.53 (54.58 - 54.48)	0.78 (0.44 - 1.39)		391
	Blackman Bay	87.12 (87.16 - 87.08)	2.55 (1.42 - 4.57)		255
Farm	1	53.51 (53.62 - 53.40)	-	-	86
	2	50.70 (50.76 - 50.64)	-		299
	3	92.28 (92.32 - 92.24)	-		165
	4	76.79 (76.91 - 76.67)	-		48
	5	37.31 (37.46 - 37.16)	-		42
	6	59.53 (59.63 - 59.43)	-		92
Size	0-20mm	95.88 (95.93 - 95.83)	3.93 (1.89 - 8.15)	< 0.001	70
	21-30mm	78.67 (78.77 - 78.57)	3.80 (1.83 - 7.89)		71
	31-40mm	69.68 (69.82 - 69.54)	3.55 (1.75 - 7.19)		40
	41-50mm	49.49 (49.55 - 49.43)	2.35 (1.11 - 4.94)		233
	51-60mm	44.35 (44.41 - 44.29)	1.17 (0.59 - 2.32)		261
	61-115mm	32.94 (33.06 - 32.82)	1.00		57
Clip height	Sub-tidal	23.37 (23.71 - 23.03)	0.23 (0.05 - 0.97)	0.043	6
	Low clip	56.83 (56.92 - 56.74)	2.24 (0.61 - 8.25)		128
	Middle clip	82.28 (82.32 - 82.24)	1.52 (0.89 - 2.60)		455
	High clip	48.21 (48.29 - 48.13)	1.00		143
Handling	Not handled	50.20 (50.25 - 50.15)	0.49 (0.26 - 0.90)	0.021	414
	Handled	85.38 (85.42 - 85.34)	1.00		318
On-grower	Non on-grower	80.53 (80.56 - 80.50)	0.45 (0.15 - 1.40)	0.169	503
	On-grower	54.47 (54.53 - 54.41)	1.00		229
Hatchery	Unknown	34.12 (34.37 - 33.87)	-	< 0.001	14
	A	88.82 (88.86 - 88.78)	3.38 (2.02 - 5.67)		224
	B	52.37 (52.41 - 52.33)	1.00		494
Genotype	Unknown	34.12 (34.37 - 33.87)	-	0.007	2
	Diploid	81.98 (82.01 - 81.95)	1.99 (1.21 - 3.28)		514
	Triploid	54.03 (54.10 - 53.96)	1.00		216
Density	Low	62.10 (62.17 - 62.03)	0.94 (1.11 - 0.80)	< 0.001	176
	Standard	74.59 (74.63 - 74.55)	0.81 (0.90 - 0.72)		376
	High	84.56 (84.61 - 84.51)	1.00		180

Table 6.4. Odds ratio from the multivariable model for the number of dead oysters in a basket.

Variable	Level	Estimate	Std. Error	Odds Ratio (95% CI)	P value
Intercept	-	0.488	0.441	-	-
Time on farm	-	-0.028	0.013	-	0.135
Density	Low	-0.169	0.132	-	< 0.001
	Standard	-0.549	0.084	-	
	High	-	-	-	
Density*Time on farm	Low	0.002	0.013	*	0.001
	Standard	0.028	0.008		
	High	-	-		
Handling	Not handled	-0.619	0.265	0.54 (0.32 - 0.91)	0.020
	Handled	-	-	1	
Size Category	(0-20mm)	1.347	0.371	3.85 (1.85 - 7.97)	< 0.001
	(21-30mm)	1.165	0.396	3.21 (1.47 - 6.98)	
	(31-40mm)	1.254	0.357	3.50 (1.73 - 7.06)	
	(41-50mm)	0.690	0.362	1.99 (0.98 - 4.06)	
	(51-60mm)	-0.045	0.341	0.96 (0.49 - 1.87)	
	(61-115mm)	-	-	1	

*The interaction between density and time on farm is presented graphically in Figure 6.

Figure 6.1. Sampling locations in oyster growing areas in Upper and Lower Pitt Water (A), Blackman Bay (B).

Also shown are growing areas in which mortality occurred in January-February 2016 (Biosecurity Tasmania, 2016) in the first POMS outbreak in Tasmania, and the sampling locations for this study which were representative of these.

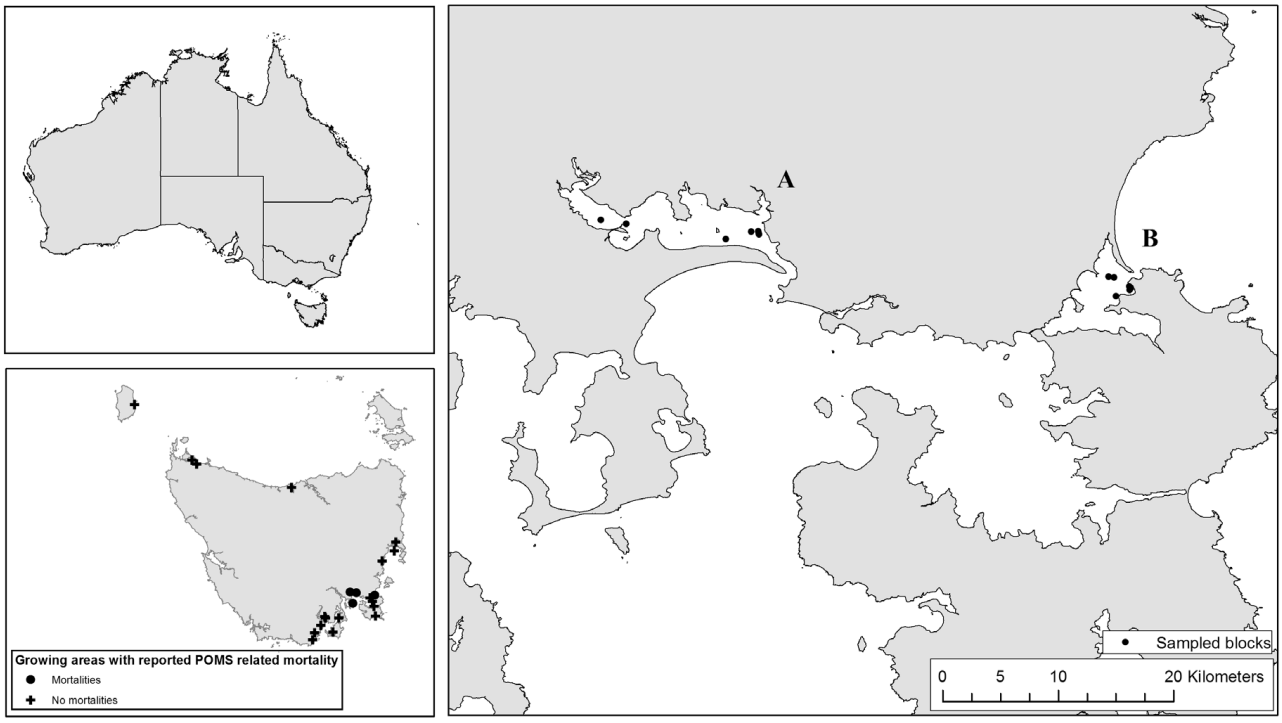


Figure 6.2. Illustration of the plastic basket on long-lines surveyed in Tasmania at the time of the POMS outbreak.

Figure 6.3. Sampling locations within each farm; grey scale indicates mortality % in each basket sampled.

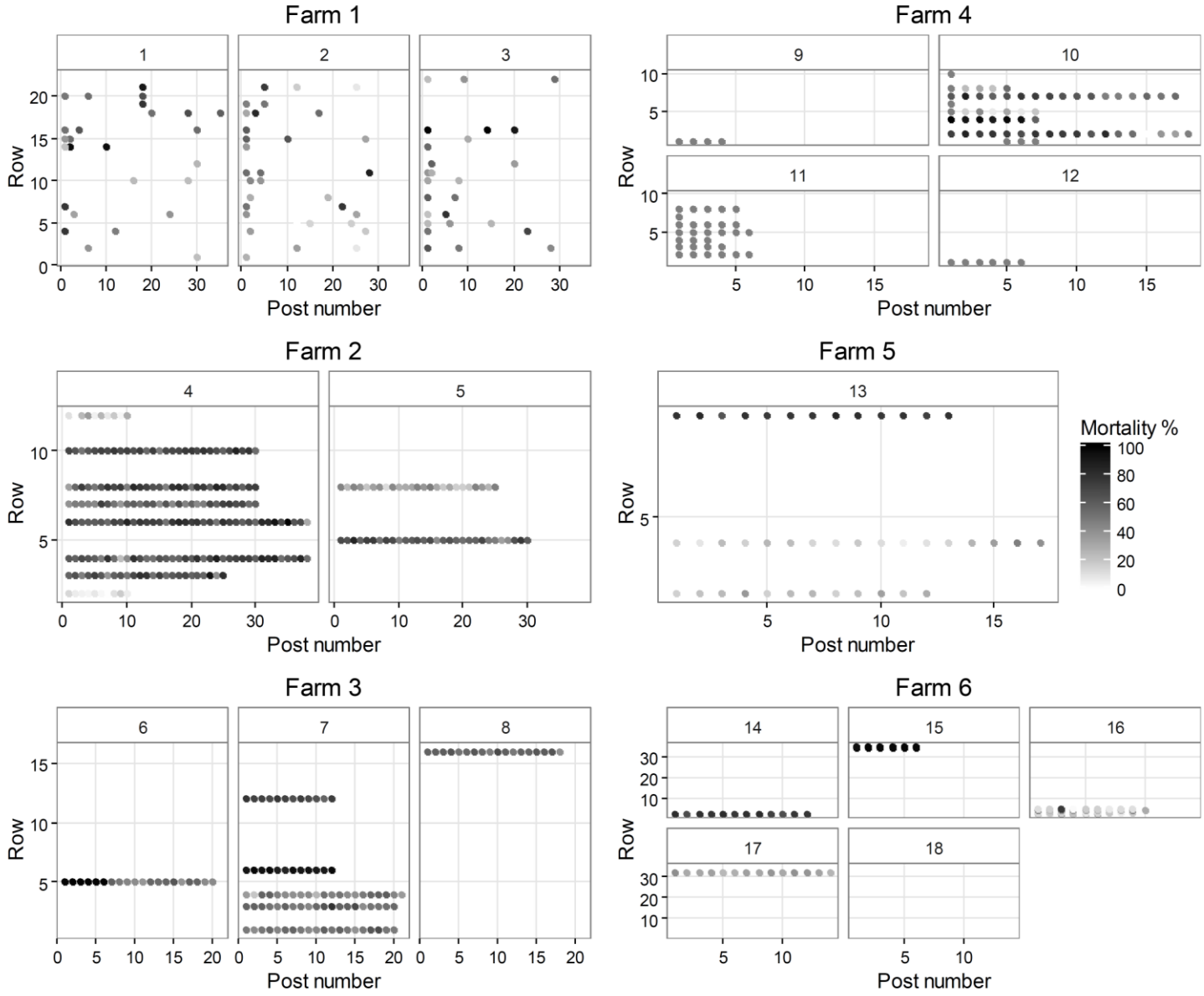


Figure 6.4. Mortality (%) for baskets of oysters that were present on the farm for different times.

Baskets were sampled across 6 farms following an outbreak of POMS, the size category of each basket also shown. Points have been jittered around each time. Time on farm reflects the age of the oysters.

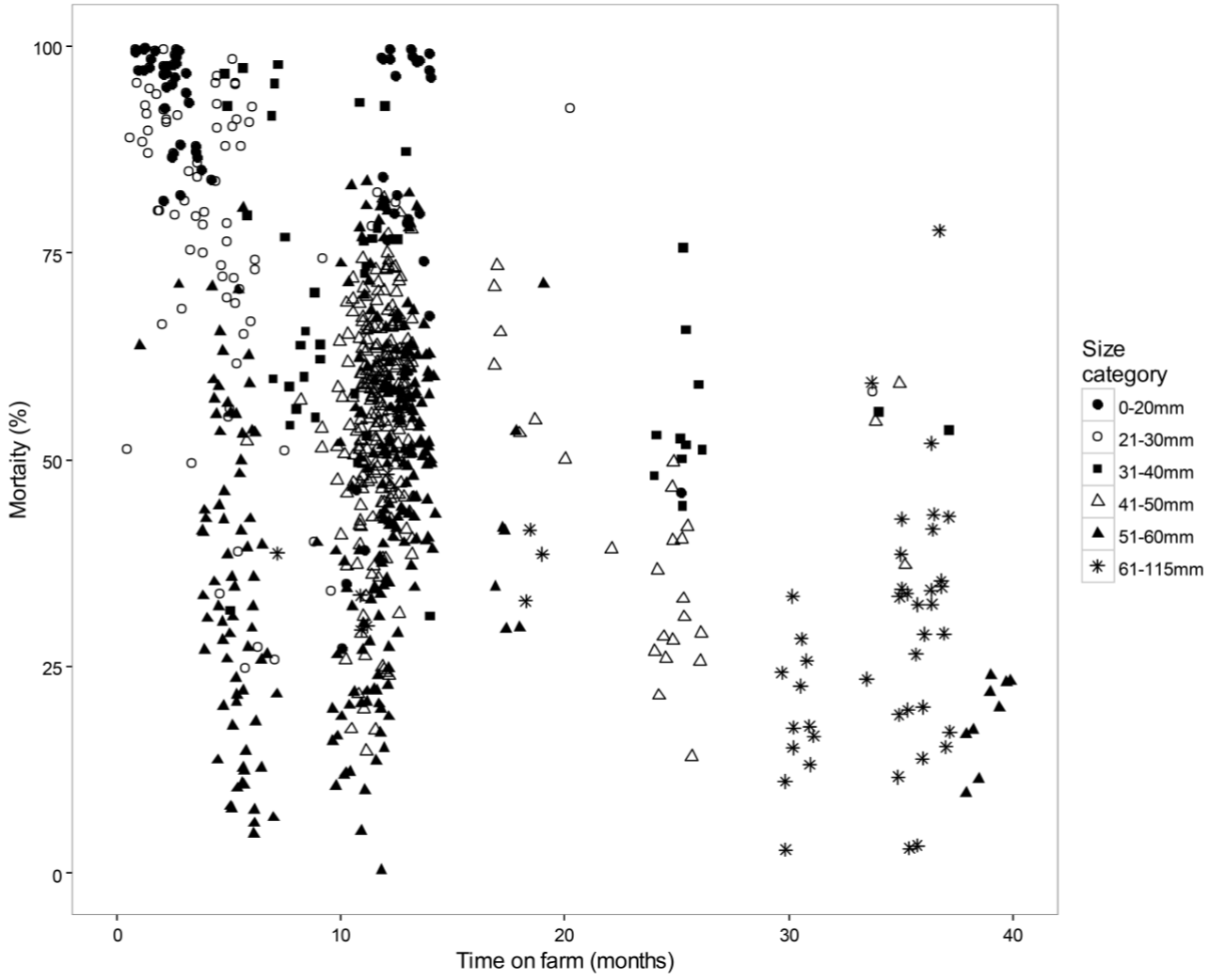


Figure 6.5. Distribution of mortality attributed to POMS across size categories.

Size categories are based on the average total shell length of oysters within each basket. Whether or not oysters handled in the 7 days prior to the outbreak is also shown. Points have been jittered around each size category.

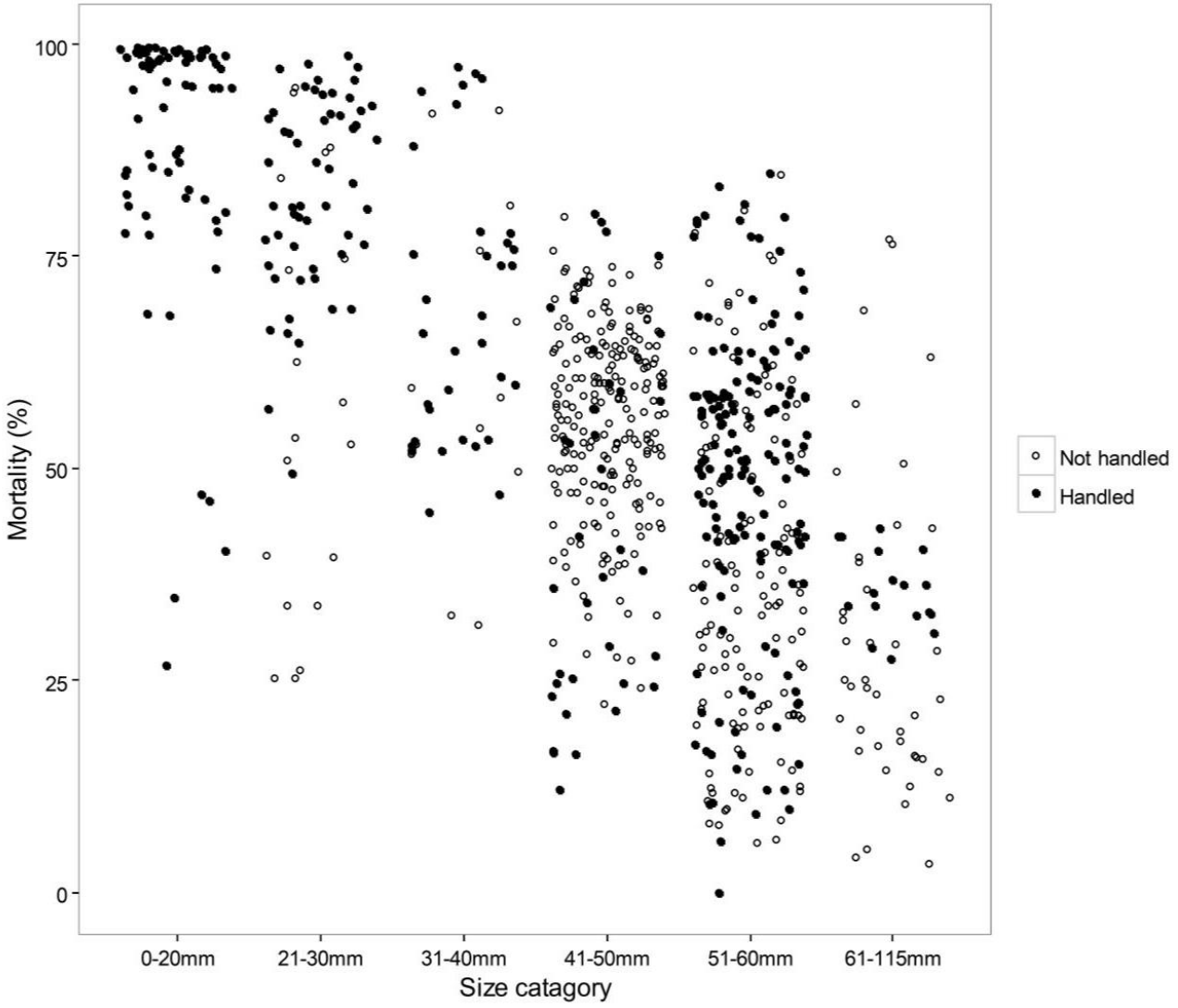


Figure 6.6. Predicted mean mortality at different stocking density.

Date are derived from the multivariable logistic regression model for oysters which had been on a grow-out farm for 3 – 24 months at each of 3 growing densities following the outbreak of POMS in Tasmania.

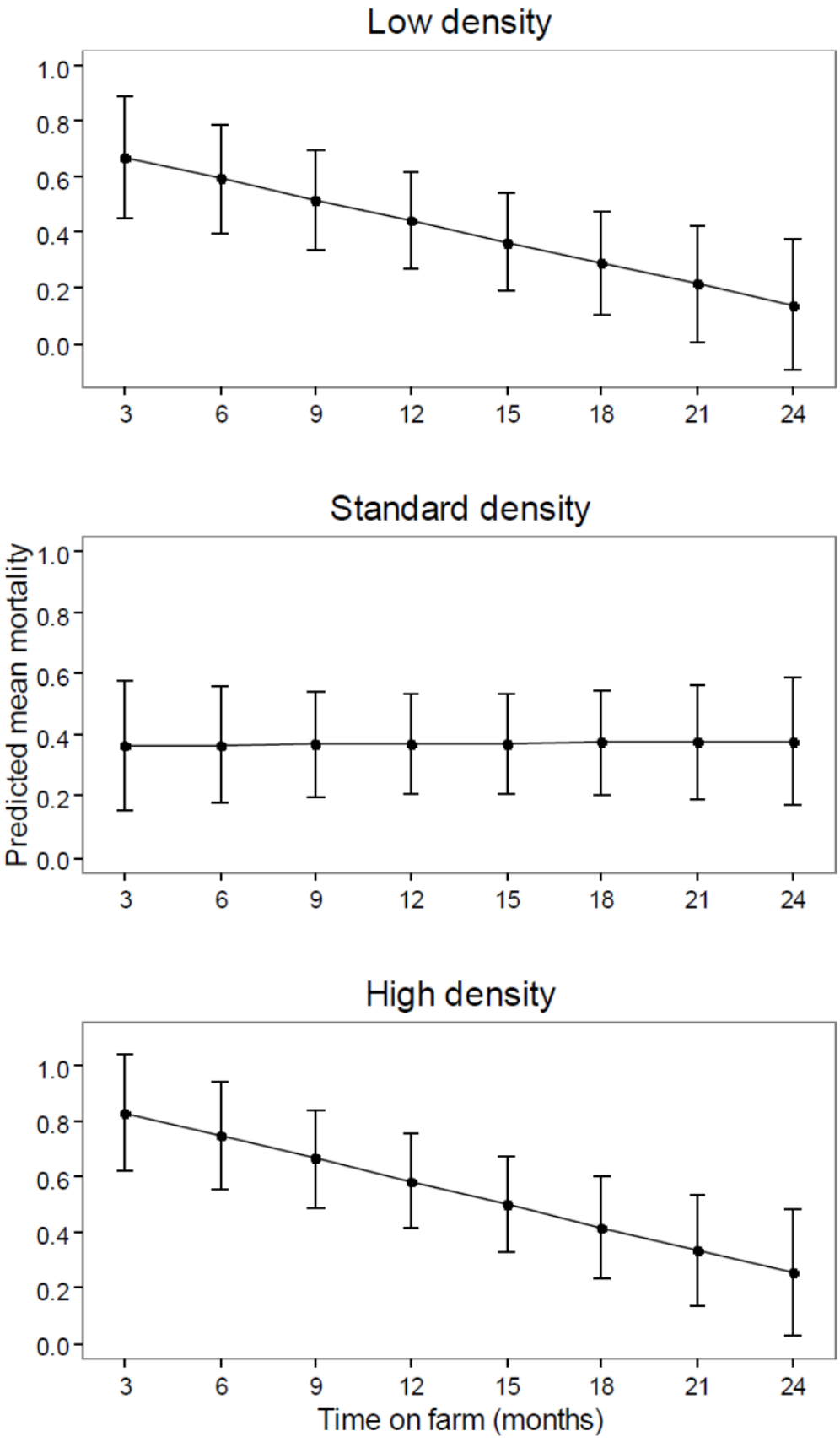


Figure 6.7. Number of baskets sampled for mortality counts.

Mortality counts according to (A) bay, (B) farm, (C) time on farm, (D) average length of oysters.

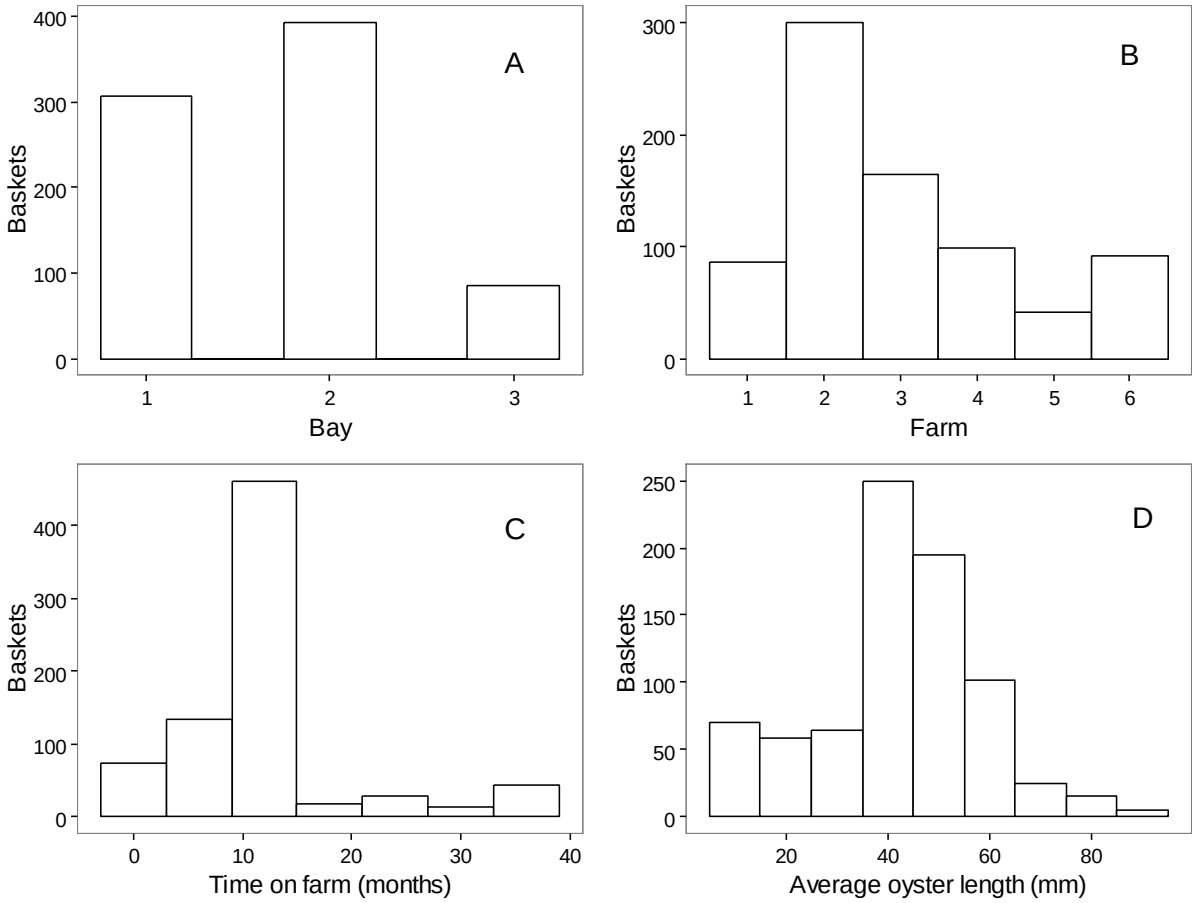


Figure 6.8. Number of oysters per basket sampled in each size category.

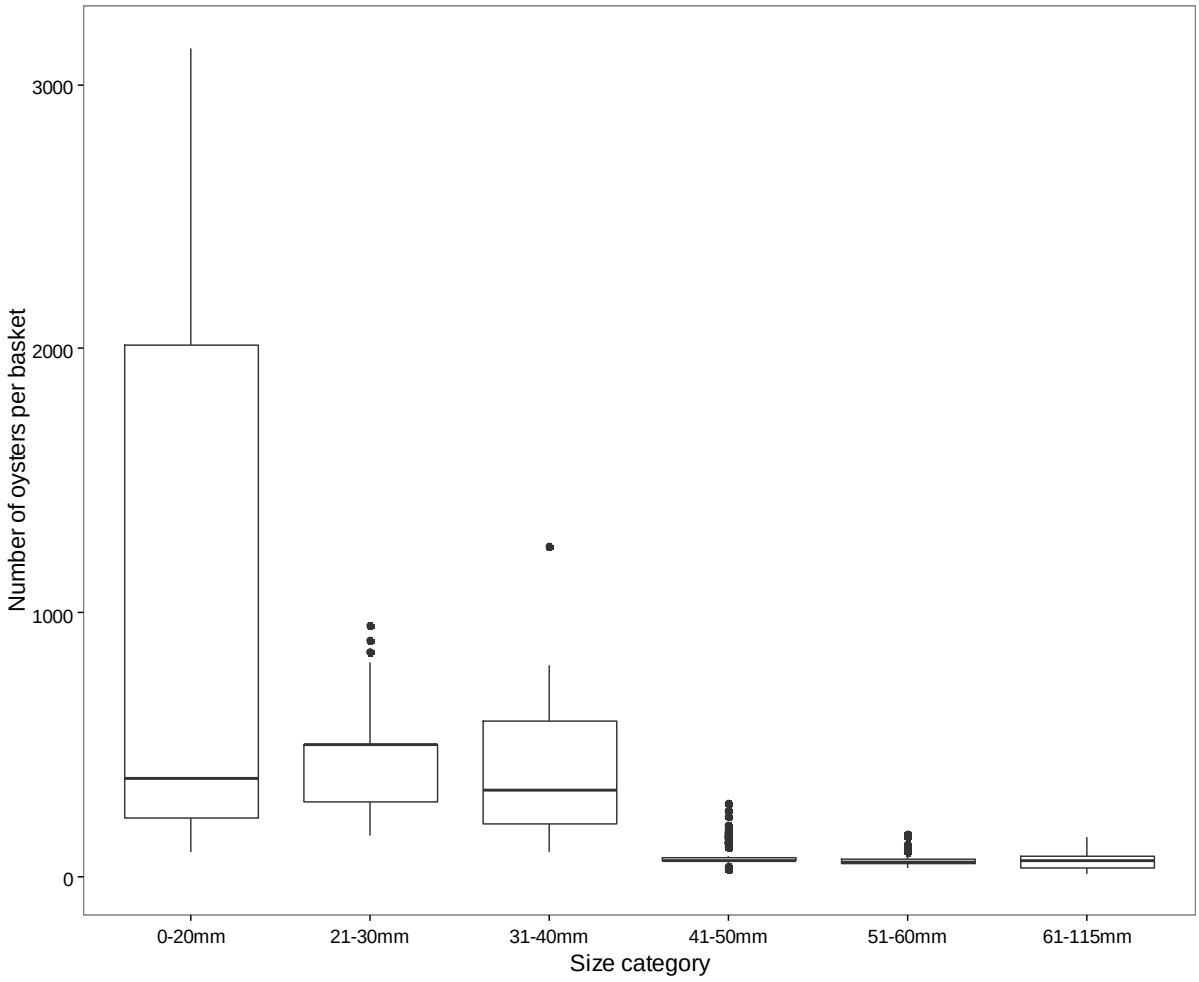


Figure 6.9. Mortality counts for all baskets sampled.

(A) Mortality at basket level (number of dead oysters / total number of oysters) and (B) basket level mortality on each of the 6 farms surveyed following a POMS outbreak in Tasmania, January – February 2016.

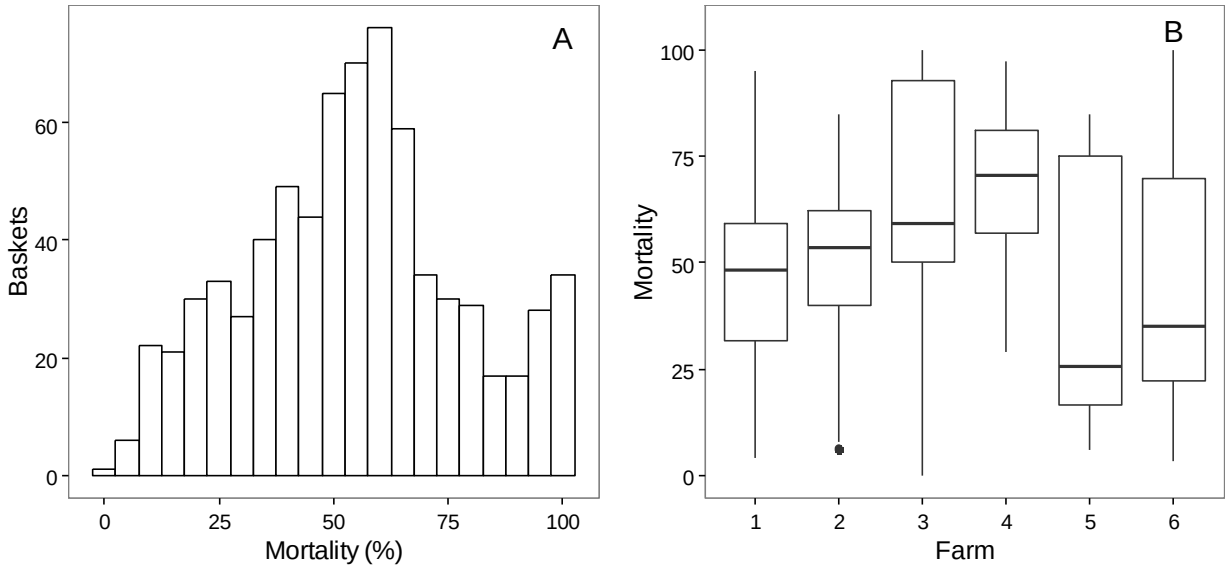
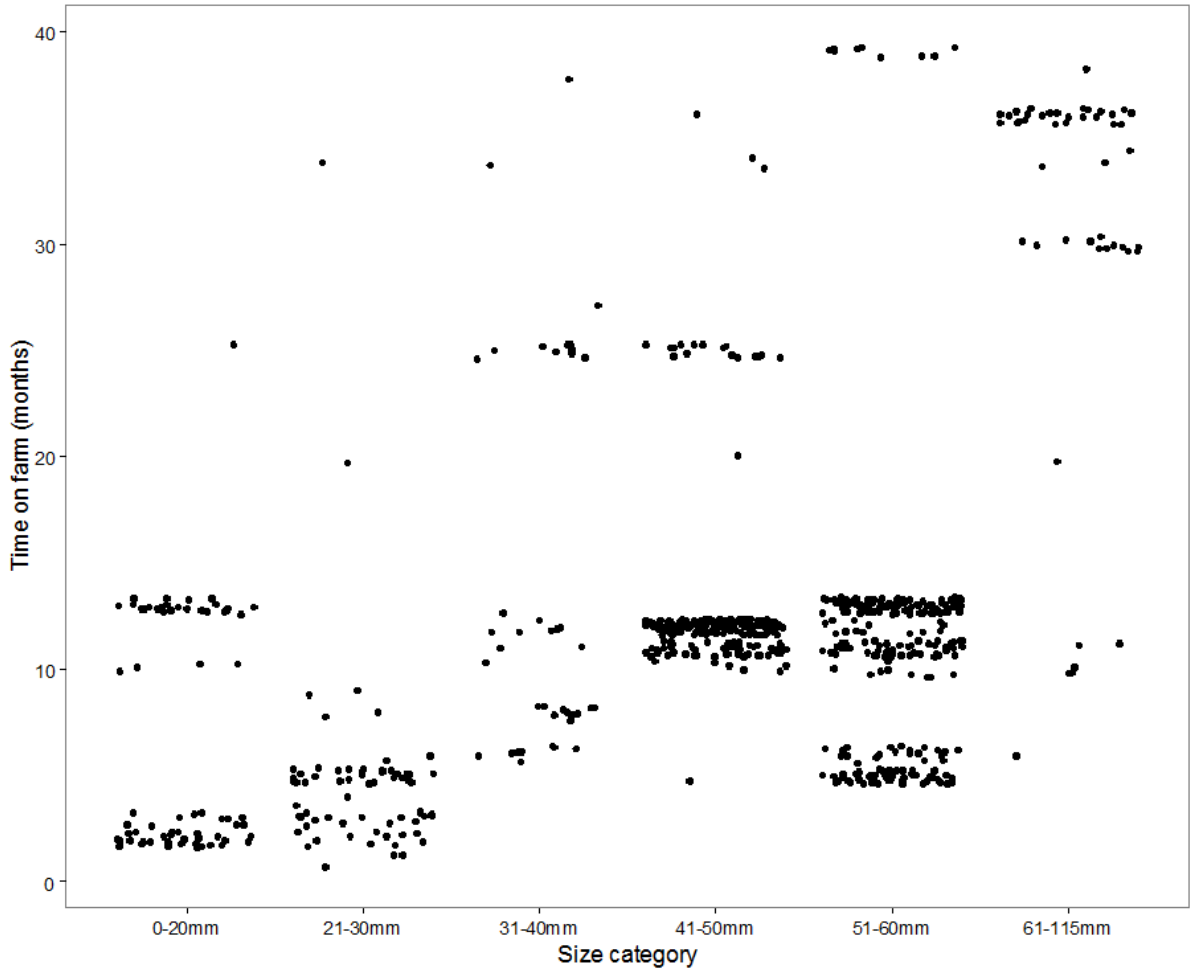


Figure 6.10. Distribution of age within each size category.

Points have been jittered around each size category.



5. Discussion

The following discussion describes the achievements against the objectives that were not directly addressed with by the experiments discussed above.

Objective: To build capacity in aquatic animal health for Australian industry through training a post graduate student.

Mr Max de Kantzow received an Australian Post Graduate Award subsequent to his honours research associated with the previous OsHV-1 project, FRDC 2012-032. His PhD candidature has been supported by association with the present project. Mrs Erandi Pathirana received an Endeavour Scholarship and University of Sydney Post Graduate support to undertake PhD studies and is a valuable member of the OsHV-1 research team. Three honours research students have been trained with the benefit of research opportunities arising from the OsHV-1 research program. Jeremey Zhang has graduated with a BVSc (Hons) for his thesis entitled "Effect of emersion time on the mortality of *Crassostrea gigas* infected with *Ostreid herpesvirus 1* (OsHV-1)". The students undertaking final year honours in the Animal and Veterinary Biosciences program are students are Georgia Cain (Biological diversity of *Ostreid herpesvirus 1* isolates from disease events in Australia)and Rebecca Oliver (Impacts of the factors that alter physiology of Pacific oysters, *Crassostrea gigas* on mortality caused by *Ostreid herpesvirus 1*).

Further capacity development has been facilitated by the employment of former PhD student Dr Olivia Evans to support the present project in the capacity of Research Associate. Dr Evans has subsequently taken a position with the Australian Government in Aquatic Biosecurity.

Objective: Collaboration with University of Tasmania CRC-P Project. Individual items subject to opportunity and need.

Dr Paul Hick participated as a member of the steering committee for CRC-P project at UTAS and travelled to Tasmania for this and other industry meetings in June 2017.

Consultation on experiment design for a sentinel surveillance (wiundow of infection) and disease investigation to identify risk factors for POMS were facilitated by multiple face-to-face meetings and phone conversations between the USyd and UTas research groups. Drs Crawford and Ugalde toured the USyd laboratory and accompanied a field work excursion in October 2016.

A selection of sentinel spat from the University of Tasmania window of infection trial were submitted to the USyd laboratory for testing on the 7th December 2016. Results were issued on the 9th December. This timely information was sought due to an incidence of increased oyster mortality and speculation about recurrence of POMS. Interpretation of the results for tests on pooled sentinel spat including the early detection of subclinical OsHV-1 infection in Blackman Bay were reported to industry via the UTAS-IMAS newsletter, POMS Update 2016 # 2, December. A further submission was tested with short turn in May 2017 to enable presentation of results at the industry meeting at Middle Point Tavern. There was consultation between Drs Hick and Ugalde regarding interpretation of the results and implications for review of activities in future sentinel surveillance projects that were discussed at the project steering committee meeting.

6. Conclusion

Despite the apparent attenuation of POMS in endemic estuaries in NSW, this disease remains an industry limiting threat for Pacific oyster farming. Long term monitoring has demonstrated that OsHV-1 will remain endemic in waterways and cause recurrent seasonal disease. In the presence of commercial stocking density at bay level, the mortality from disease outbreaks in the Hawkesbury River was high. Despite best practice disease mitigation, the outcome of production trials was borderline for economic viability.

Increased understanding of the transmission of OsHV-1 and the risk factors for mortality have helped explain the characteristically unpredictable disease outbreaks which have a clustered spatial and temporal pattern of occurrence. Good information on seasonal disease risk periods is now available. This can be combined with a growing awareness of the local hydrodynamic, farm management practices that influence disease and the risks that can be attributed to oysters of a given size, age and previous OsHV-1 exposure to enable farm management to minimise disease impact.

Disease control recommendations based on minimisation of risk factors will mature

Continued research is required to identify disease control strategies based on a thorough understanding of the immune responses of oysters

Environmental sources of a point source epidemic that see OsHV-1 distributed according to planktonic associations help explain the disease pattern. Water temperature predicts the seasonal disease risk with less predictability of the geographic and hydrodynamic factors that influence bay and lease level occurrence.

Host factors relating to the age, size and exposure history of the oysters provide a predictable mortality overlaid by the influence of physiological and metabolic state at the time of exposure which appears to substantially impact the disease outcome. Understanding all of these risk factors for infection and disease is essential so that farm management can be directed towards disease control, including maximising the genotypic potential of Pacific oysters selectively breed for genetic resistance to POMS.

7. Implications

Improved understanding of the long range spread of OsHV-1 indicates that all growing areas in Australia should be prepared for the occurrence of POMS in the future. Farmers can integrate knowledge about the epidemiology of POMS to minimise losses. This will take the form of stock management calendars that minimise handling, alter stocking density and minimise the proportion of susceptible young and small stock during POMS risk periods. Monitoring of water temperature on-lease will help predict disease risk.

These POMS minimisation recommendations based on understandings of disease epidemiology are important to maximise the performance of the best available genetic stock. Efforts to suppress POMS outbreaks at farm and bay level through stock management will reduce infection pressure. Oysters selected for resistance to POMS are most likely perform with low mortality under mild challenge conditions, whereas disease resilience might break down when the OsHV-1 challenge is high.

The present research has generated new understandings about the unique and specific transmission of OsHV-1. This apparently unpredictable disease can be explained by the spatial and temporal clustering of virus transmission events and the various combinations of risk factors that result in infection progressing to disease. This information needs to be carefully considered in the design and interpretation of further OsHV-1research and surveillance efforts.

Knowledge about the risk factors for POMS remains incomplete and effective disease management requires additional research.

8. Recommendations

The studies undertaken in this project have highlighted the complexity of the transmission OsHV-1 and the epidemiology of the disease it causes. These findings should be considered in management of disease preparation and response measures at individual farms, by industry bodies and in formulating relevant policy. Specifically, models of POMS impact using thermal predictors of disease risk periods in regions of Australia where the pathogen is not yet present should guide decision making.

Continued production of Pacific oysters despite the recurrent occurrence of POMS is possible by incorporating the present research into farm management plans that consider production calendars for stock management based on host risk factors for disease. Gains made in selection for resistance to POMS and other advances in oyster health management should be synthesised with these measures to enhance gains in production.

Key knowledge gaps have been highlighted that require further research. Additional research activities will provide the most positive impact on Pacific oyster farming are:

- Evaluation of the impact of prior exposure to OsHV-1 on survival of subsequent OsHV-1 infection challenge. An age dependant resistance to further infection that may be conferred by previous exposure to OsHV-1 is a key observation that needs to be confirmed and understood in greater depth. The potential to exploit controlled exposure to OsHV-1 is a potential high impact disease control tool.

- Pathogenesis and immunology studies of Pacific oysters that survive OsHV-1 challenge or develop persistent subclinical infection in a controlled laboratory environment are essential to underpin research considering spat conditioning by controlled pre-exposure.

- Integrated disease research that concurrently considers all potential causes of high mortality in Pacific oysters. The present study definitively demonstrated that diseases other than POMS occur concurrent with this disease and would impact farming substantially even if OsHV-1 was controlled. Detection of multiple diseases using sentinel spat for surveillance according to the methods described in this study provides an approach to investigate the aetiology and risk factors for these diseases.

- Further investigation of in genotypic and strain variation of OsHV-1. Isolates of OsHV-1 with different virulence were identified in this study. Further studies should target OsHV-1 isolates associated with low mortality disease. The potential for deliberate exposure to low virulence strains of OsHV-1 should be evaluated as a disease management strategy.

- Additional risk factors that drive the variable in mortality due to OsHV-1 exposure remain to be elucidated. The impact of the metabolic and physiologic status of the oyster on susceptibility to OsHV-1 is important. Additionally, the local hydrodynamic effects on transmission of OsHV-1 should be investigated in new environments where OsHV-1 becomes endemic. Currently understood and newly investigated disease risk factors can be minimised by modification or development of suitable growing infrastructure.

- The mechanisms for reduced in mortality due to selective breeding should be elucidated as understanding the biological basis of the resilience can be leveraged through changes in farm management.

9. Further development

POMS will remain one of the major constraints on farming Pacific oysters. The knowledge generated about the transmission of OsHV-1 and the risk factors for POMS can improve productivity of affected farms.

These findings can be integrated into farm management at individual, industry and policy level. Decision making at each of these levels needs consider the potential for long distance spread of the OsHV-1 and the high likelihood that it will become endemic in regions currently considered free from disease. Further research is required, as outlined in the recommendations, to provide comprehensive disease management tools for farms in affected waterways.

10. Extension and Adoption

Conference presentations and posters:

Olivia Evans, Paul Hick, and Richard J. Whittington, 2017. Detection of Ostreid herpesvirus-1 microvariant in healthy *Crassostrea gigas* and their possible role as reservoirs of infection. 18th International Conference on Diseases of Fish and Shellfish, European Association of Fish Pathologists, 4-8 September, Belfast, Northern Ireland.

Olivia Evans, Ika Paul-Pont, and Richard J. Whittington, 2017. Detection of *Ostreid herpesvirus-1* microvariant DNA in aquatic invertebrate species collected from the Georges River estuary, New South Wales, Australia. 18th International Conference on Diseases of Fish and Shellfish, European Association of Fish Pathologists, 4-8 September, Belfast, Northern Ireland.

Olivia Evans, Ika Paul-Pont, Paul Hick, and Richard J. Whittington, 2017. A simple centrifugation method for improving the detection of *Ostreid herpesvirus-1* (OsHV-1) in natural seawater samples. Poster, 18th International Conference on Diseases of Fish and Shellfish, European Association of Fish Pathologists, 4-8 September, Belfast, Northern Ireland.

de Kantzow MC, Hick PM, Dhand NK & Whittington RJ. Risk factors for mortality during the first occurrence of Pacific Oyster Mortality Syndrome due to *Ostreid herpesvirus – 1* in Tasmania, 2016. 4th FRDC Australasian Aquatic Animal Health & Biosecurity Scientific Conference, 10-14 July 2017, Cairns.

Hick PM, Evans O, de Kantzow MC, Pathirana E, Rubio A, Dhand NK & Whittington RJ. Pacific Oyster Mortality Syndrome: Closing knowledge gaps to continue farming *Crassostrea gigas* in Australia. 4th FRDC Australasian Aquatic Animal Health & Biosecurity Scientific Conference, 10-14 July 2017, Cairns.

Outreach:

Drs Evans and Rubio present at the 2017 NSW Oyster Conference in Merimbula, 22-24th August.

The project website www.oysterhealthsydney.org continues to be well visited.

Professor Whittington is a member of SCAAH and sits on the POMS working group.

Richard Whittington and Paul Hick visited Tasmanian oyster growers, the two major hatcheries in Tasmania, Oysters Tasmania Executive Officer and the Chief Veterinary Officer for Tasmania 15th to 17th February 2016 in response to the POMS outbreak in Tasmania. Richard Whittington attended the South Australian Oyster Growers Association meeting in Port Lincoln on 26-2-16 and participated in an expert panel. Richard Whittington provided information to FRDC for a report in FISH magazine in March and April 2016. Richard Whittington provided a summary of research outcomes to date for the project to Oysters Australia RD&E Manager for presentation to the Oysters Australia meeting in Tasmania on 7-April 2016. Richard Whittington attended the Oysters Tasmania meeting in Campbell Town Tasmania on 8-April 2016 and provided an update on research.

Richard Whittington and Ana Rubio attended the FRDC Board Briefing at Sydney Fish Markets and gave an address on POMS research on 10-May 2016.

Project coverage

n/a

11. Project materials developed

The following scientific publications arising from this project and related research were published at the time of reporting:

de Kantzow, M.C., Hick, P.M., Dhand, N.K., Whittington, R.J., 2017. Risk factors for mortality during the first occurrence of Pacific Oyster Mortality Syndrome due to *Ostreid herpesvirus 1* in Tasmania, 2016. **Aquaculture** 468, Part 1, 328-336.

de Kantzow, M.C., Whittington, R.J., Hick, P., 2019. Prior exposure to Ostreid herpesvirus 1 (OsHV-1) at 18 °C is associated with improved survival of juvenile Pacific oysters (Crassostrea gigas) following challenge at 22 °C. **Aquaculture**.

Evans, O., Hick, P., Alford, B., Whittington, R.J., 2017a. Transmission of Ostreid herpesvirus-1 microvariant in seawater: Detection of viral DNA in seawater, filter retentates, filter membranes and sentinel *Crassostrea gigas* spat in upwellers. **Aquaculture** 473, 456-467.

Evans, O., Kan, J.Z.F., Pathirana, B.E., Whittington, R.J., Dhand, N., Hick, P., 2019. Effect of emersion on the mortality of Pacific oysters (Crassostrea gigas) infected with Ostreid herpesvirus-1 (OsHV-1). **Aquaculture**.

Evans, O., Hick, P., Whittington, R.J., 2016a. Comparison of Two External Tagging Methods Used for the Identification of Individual Adult Pacific Oysters, *Crassostrea gigas*. **Journal of Shellfish Research** 35, 837-840.

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Evans, O., Hick, P., Whittington, R.J., 2017b. Detection of *Ostreid herpesvirus 1* microvariants in healthy *Crassostrea gigas* following disease events and their possible role as reservoirs of infection. **Journal of Invertebrate Pathology** 148, 20-33.

Evans, O., Paul-Pont, I., Whittington, R.J., 2017c. Detection of *Ostreid herpesvirus 1* microvariant DNA in aquatic invertebrate species, sediment and other samples collected from the Georges River estuary, New South Wales, Australia. **Diseases of Aquatic Organisms** 122, 247-255.

Hick, P.M., Evans, O., Rubio, A., Dhand, N.K., Whittington, R.J., 2018. Both age and size influence susceptibility of Pacific oysters (*Crassostrea gigas*) to disease caused by *Ostreid herpesvirus 1* (OsHV-1) in replicated field and laboratory experiments. **Aquaculture**. https://doi.org/10.1016/j.aquaculture.2018.02.013

Hick, P., Evans, O., Looi, R., English, C., Whittington, R.J., 2016. Stability of *Ostreid herpesvirus 1* (OsHV-1) and assessment of disinfection of seawater and oyster tissues using a bioassay. **Aquaculture** 450, 412-421.

Pernet, F., Lupo, C., Bacher, C., Whittington, R.J., 2016. Infectious diseases in oyster aquaculture require a new integrated approach. **Philosophical Transactions of the Royal Society B: Biological Sciences** 371.

Whittington, R.J., Paul-Pont, I., Evans, O., Hick, P., Dhand, N.K., 2018. Counting the dead to determine the source and transmission of the marine herpesvirus OsHV-1 in *Crassostrea gigas*. **Veterinary Research** 49, 34.

A series of factsheets were provided to industry via the website Oyster health Sydney (https://oysterhealthsydney.wordpress.com/). The fact sheet titles were:

1. Water treatments for hatcheries. Provides information to protect a hatchery by removing infectious OsHV-1 (POMS) from incoming seawater, please download this fact sheet.

2. Husbandry to reduce losses. Provides information to minimise mortality due to OsHV-1 (POMS) during growout of oysters, please download this fact sheet:

3. Disinfection. Provides information to disinfect water or equipment to remove infectious OsHV-1 (POMS), please download this fact sheet:

4. Water temperature. Provides information about water temperature and seasonality of POMS risk, please download this fact sheet:

5. Restocking after POMS. Provides information about risks off restocking after a POMS outbreak, please download this fact sheet:

6. Factors to improve survival. Provides information about things to do or things to avoid to improve survival in POMS outbreaks in Tasmania, please download this fact sheet:

12. Appendices

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