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Future Oysters CRC-P:

Enhancing Pacific Oyster Breeding to Optimise National Benefits

Matthew Cunningham, Peter Kube, Andrew Trotter, Xiaoxu Li, Peter Kirkland, Nick Robinson, Greg Smith and Chris Carter

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Researcher Contact Details

Name:	Matthew Cunningham
Address:	Tech 3, Innovation Drive
	Technopark TAS 7010
Phone:	03 6274 7741
Fax:	N/A
Email:	matt@asioysters.com.au

FRDC Contact Details

Address:	25 Geils Court
	Deakin ACT 2600
Phone:	02 6285 0400
Fax:	02 6285 0499
Email:	frdc@frdc.com.au
Web:	www.frdc.com.au

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1. Executive Summary

This report details the research undertaken as part of the Fisheries Research and Development Corporation (FRDC) project 2016-801 Enhancing Pacific Oyster Breeding to Optimise National Benefits. This was undertaken as part of the Future Oysters Cooperative Research Centre Project (CRC-P 2016-553805; Future Oysters - FO CRC-P), conducted as part of the Australian Government's Cooperative Research Centres Program. The project was led by the Australian Seafood Industries (ASI) with collaborating researchers from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Institute of Marine and Antarctic Studies (IMAS) / University of Tasmania (UTAS), South Australian Research & Development Institute (SARDI), Flinders University (FU) and New South Wales Department of Primary Industries (NSW DPI). Project activities occurred across the three major oyster producing states of Australia –New South Wales (NSW), South Australia (SA) and Tasmania (TAS).

The research was conducted as a direct consequence of the 2016 Pacific Oyster Mortality Syndrome (POMS) outbreak TAS which decimated parts of this State's Pacific Oyster (*Crassostrea gigas*) industry and caused numerous flow on effects throughout the entire Australian industry. The project was aimed to allow continuation and improvement of the work that had been undertaken prior to the 2016 outbreak, which was not only a major disruptor to the industry but also the breeding program. New techniques needed to be established to allow continued breeding in TAS in the new POMS paradigm and operations were required to be established in SA due to the biosecurity restrictions brought about by the TAS outbreak. Aspects of the project also looked to increase the rate of genetic gains for POMS resistance by developing additional supporting technologies.

The project was conducted across multiple areas that reflected the objectives of the project. Researchers worked collaboratively to conduct research across breeding strategy development, capacity building in SA, genetic improvement, laboratory and field challenges, accelerated maturation and developing an identification tool.

The results have allowed the ASI selective breeding program to most importantly improve genetic resistance to POMS and allowed these results to be available to all Pacific Oyster growing regions. Specifically, the results of this project have allowed:

1. Continued breeding in TAS by development of a biosecure breeding facility at the IMAS aquaculture facility. Family production has continued at the same level prior to POMS and enabled ASI to breed from Pacific Oysters which have been expose to the virus. The facility now has an approved biosecurity plan which has been externally audited and signed off by the Tasmanian Chief Veterinary Officer (CVO).
2. Production of 160 families in SA to enable POMS resistant broodstock to be deployed to the SA oyster industry. A new project has been developed which allows further breeding in South Australia to deliver breeding outcomes for the medium term.
3. Improved rates of genetic improvement in the TAS population to fast track POMS resistance. The program has now developed and implemented spat POMS challenge trials that have increased gains.
4. Improvement in laboratory challenges which have increased correlations to field survival and a laboratory challenge at IMAS, which has been an important precursor to future work on genomics.
5. Accelerated maturation that has allowed increased usage of one-year old animals in the ASI breeding program, which have been shown to offer increased rates of genetic gains in POMS resistance.
6. Development of a single nucleotide polymorphism (SNP) identification tool which can differentiate between ASI and non-ASI stock and different ASI families.

The results have had a massive impact on the Australian Pacific Oyster farming industry. Since the 2016 POMS outbreak, there has been a significant recovery of the TAS industry, and this can be substantially attributed to the POMS resistant broodstock developed as part of this project. Prior to the outbreak, the TAS oyster industry generated $26 million per annum and employed in the order of 300 people.

Immediately following the outbreak, the TAS industry lost in excess of $12 million and 80 jobs with subsequent losses each year since. There has also been substantial losses due to minimal sat suppliers to the SA industry over the past 3 years. The recovery in TAS is almost complete and has resulted in the industry recovering to full stocking and employment levels within three years of the outbreak. This is internationally unprecedented with other international industries taking much longer to recover. The growers in TAS are returning to profitability and now have a very positive outlook for the future. In SA the production and commercialization of ASI family lines will see that industry partially insulated from the effects of POMS if/when the disease reaches the growing regions. Whilst POMS resistance is currently lower in farm stock in SA than TAS, the stocking of these oysters on farms can avoid the crippling losses experienced in TAS during the 2016 outbreak. The knowledge of this "insurance policy" has allowed the industry in SA to invest in their businesses with greater confidence.

The other aspects of this project have allowed the breeding program to increase the rate of genetic gains and these have had direct beneficial impacts for Australian Pacific Oyster growers.

Keywords

Pacific Oyster, Crassostrea gigas, Pacific Oyster Mortality Syndrome (POMS), Selective Breeding

2. Introduction

This project commenced in response to the 2016 POMS outbreak in Tasmania which caused significant disruption to the Australian Pacific Oyster industry. The issues were experienced in Tasmania as devastating stock losses and in South Australia as an extreme disruption to seed supply. The project aimed to build on efforts to date to ensure that POMS resistance breeding was able to continue, be accelerated and be available to all Pacific Oyster producing states.

Throughout this report the specific areas of research focus are introduced to describe how they contribute to the overall aim of breeding a POMS resistant pacific oyster for the Australian industry.

3. Objectives

1. Design and implement a selective breeding strategy for ASI that meets the immediate and medium term (5 year) needs of the national Pacific Oyster industry

2. Identify biosecurity constraints to the movement of ASI stock and develop a strategy to permit optimal flow of benefits across the national industry.

3. Review, document and communicate protocols and procedures for the use of OsHV-1 exposed broodstock by hatcheries and the transfer of the resulting progeny compliant with state regulations.

4. Redefine the protocols for the laboratory spat challenge model to improve the predictability of field survival, with the goal of 70% correlation between the laboratory and field tests and to extend the application of the challenge model to include challenges to larvae.

5. Develop a system, supported by general purpose algorithms that will allow ASI to routinely benchmark the estimated breeding values of ASI POMS resistant families against commercial performance of hatchery stock of known pedigree after exposure to OsHV-1 at different life stages.

6. Document and implement a strategy to allow use, within the breeding program, of male and female broodstock at one year of age.

7. Develop and verify an SNP based genetic test that can discriminate ASI oysters from non-ASI oysters, to identify oysters to family and implement a plan for this test to be commercially available to stakeholders.

4. Research

4.1: POMS Genetic Variation in Field and Laboratory Trials

Peter Kube [a], Peter Kirkland [b], Lewa Pertl [c] and Matthew Cunningham [c]

[a] CSIRO Agriculture and Food, Castray Esplanade, Hobart, TAS 7000

[b] Elizabeth Macarthur Agricultural Institute (EMAI), New South Wales Department of Primary Industries (NSW DPI), Woodbridge Road, Menangle NSW 2568

[c] Australian Seafood Industries (ASI), Tech 3, Tasmanian Technopark, Innovation Drive, Dowsing Point TAS 7010

4.1.1 Executive Summary

Breeding for Pacific Oyster Mortality Syndrome (POMS) resistance continues to be the primary objective of the Australian Seafood Industry (ASI) breeding program and field trials to evaluate family performance are a mainstay of this breeding effort. During the three years of this project, a total of 22 field trials were deployed containing nearly 360,000 Pacific Oysters (*Crassostrea gigas*) from nearly 400 families. The emphasis of the field trails has now shifted from using one-year old stock to deploying trials with spat, aged between two and three months. This was prompted by the need to directly improve spat resistance, the logistical difficulties with deploying one-year olds in trials in the disease affected Tasmanian (TAS) environment, and the opportunity to accelerate gains with the rapid receipt of data when using spat trials.

Spat trials presented challenges and it is more difficult to obtain high quality data from spat trials compared to adult trials. However, through incremental improvements, good results from spat trials are now being routinely achieved. The heritability of spat resistance to POMS on the underlying scale was moderately high and estimated to be $h^2 = 0.45 \pm 0.04$. This compares to a value of $h^2 = 0.48 \pm 0.03$ for adult heritability resistance to POMS. There was a moderate genetic correlation between spat resistance and adult resistance ($r_g = 0.60 \pm 0.07$) suggesting there are some different mechanisms influencing spat and adult resistance and they should be treated as different genetic traits.

Laboratory spat trials also presented challenges and a process to improve these trials was undertaken. Improvements were made by upscaling the numbers of animals challenged, and through attention to animal husbandry and size. However, correlations between laboratory trials and field performance were variable and sometimes near zero. Other studies have demonstrated that POMS mortality is caused by secondary infections of bacteria after Ostreid Herpes Virus Variant (OsHV-1 µVar) compromises the immune state of oysters and, due to the use of highly treated water in the nursery, it appears that oysters used in laboratory trials in this study were not always exposed to the natural microbiome and, therefore, did not express POMS as it manifests in field trials. Further trials are needed to verify this as the main cause but, going forward, a focus will be placed on allowing all challenged animals to be exposed to raw seawater and exposure to opportunistic bacteria prior to a laboratory challenge. Laboratory trials done under such protocols provided good correlations with field performance for both spat and adult resistance.

The resistance of adult stock in the TAS population appears to be approaching maximal values, with top families for the last three-year classes all having greater than 90% POMS survival. However, spat

resistance remains a work in progress with the best families of the latest year class (2018) having a predicted survival of 70% with a moderate POMS infection and 50% for a severe infection. Stock from this year class will be available for commercial release in 2021. The South Australian (SA) population has much lower levels of POMS resistance, due to the much later start of the breeding program, and is probably at least four years behind the TAS population. The best SA stocks currently have POMS survival predictions of 70% as adults and near zero values as spat, meaning a POMS outbreak in SA would cause moderate losses in adult stock and severe losses of spat. Current procedures for POMS resistance breeding in SA are very unlikely to achieve gains comparable to those achieved in TAS and improving gains in SA is now the major issue for POMS resistance breeding.

4.1.2 Introduction

Breeding for resistance to Pacific Oyster Mortality Syndrome (POMS) has been a focus of the Australian Seafood Industry (ASI) breeding program, and the highest priority for oyster genetics research, since late 2010 (nearly 9 years). Resistance breeding of Pacific Oysters (*Crassostrea gigas*) commenced in 2012, with the deployment of existing families from the 2011-year class, and families specifically selected for POMS resistance were first made in November 2012 with the spawning of the 2012-year class. Resistance breeding has been an uninterrupted activity since that time with annual production of families, annual deployment of field tests, and ongoing data collections and analyses.

The first phase of the POMS resistance breeding work, up to and including the 2015-year class, has been fully reported in Kube et al. (2018). This work built the body of knowledge to implement applied breeding for this trait and resulted in the move to operational breeding. The key findings and outcomes from the first phase, which was prior to this Future Oysters Cooperative Research Centre Program (FO CRC-P) project, were as follows:

1) POMS resistance has a moderate to high heritability in field trials and a moderate heritability in laboratory trials;

2) field challenges are a reliable way to get family performance data for one-year old stock;

3) laboratory challenges can also be used to get family performance data; and

4) genetic gains were obtained in the breeding population, those gains accumulated over successive year classes and resistant stock was delivered to industry for commercial deployment.

There were, however, some gaps in this first phase and breeding for spat resistance was foremost. Spat trials were difficult for a variety of reasons and the breeding program was forced to rely on trials with one-year old (adult) stock. In addition, the need for improvements in the laboratory challenge were noted. This current project was, therefore, a continuation of the previous breeding work and represents phase 2 of the breeding program development. The primary aims were to develop processes to select for spat resistance, and to continue to improve the delivery of commercially resistant stock. In addition, work continued on the development of the laboratory challenge for POMS resistance, and the importance of improving the laboratory challenges was heighted by the need to apply such a system to South Australian (SA) families.

The commencement of the second phase coincided with the arrival of POMS in Tasmania (TAS) and that caused severe disruption to the workflows for all parts of the industry. The disruptions extended to the breeding program and produced an additional set of requirements, which included the development of the SA breeding population (Section 4.2), the need to develop new biosecure facilities and protocols for producing and deploying families in a disease affected environment Section 4.3), and the need to implement and capitalise on the ability to breed from survivors of POMS.

4.1.3 Methods

4.1.3.1 Field trials

This study used families routinely produced from the ASI selective breeding population and the history and management of this population up to the 2015-year class (YC) is described in Kube et al. (2018). This current study was based on four YCs (2015 to 2018), 397 families, 22 field trials, and 367,038 individual oysters (Table 4.1.1). The families are, in effect, from two populations which are the TAS and SA populations. The largest part of the study was done on the Tasmanian population with four YCs, 304 families, 20 trials, and 358,921 oysters. The volume of work on the SA populations was small in comparison, with two YCs, 93 families, two trials, and 8,217 oysters.

Table 4.1.1. Summary of field trials deployed as part of this project.

Year class	Life Stage	Trial no.	Age (mon)	Deployment Date	Measure Date	Site	Pop.	Sur.	No. fam	No. oysters
2015	Adult	15-1	13	24-Oct-2016	22-Dec-2016	Pitt Water	TAS	50%	73	11,672
2015	Adult	15-2	14	20-Sep-2016	27-Dec-2016	Georges R	TAS	22%	78	9,791
2015	Adult	15-3	14	07-Nov-2016	24-Jan-2017	Pipeclay	TAS	30%	78	11,633
2016	Adult	16-1	13	15-Nov-2017	19-Dec-2017	Pitt Water	TAS	27%	73	10,703
2016	Adult	16-2	15	12-Jan-2018	28-Feb-2018	Pitt Water	TAS	95%	76	11,485
2016	Adult	16-3	14	20-Sep-2017	09-Jan-2018	Georges R	TAS	40%	32	4,427
2016	Spat	16-4	3	07-Feb-2017	07-Feb-2017	Pipeclay	TAS	23%	40	14,132
2016	Spat	16-5	3	28-Feb-2017	28-Feb-2017	Pipeclay	TAS	12%	36	12,631
2016	Adult	16-6	15	28-Nov-2017	05-Mar-2018	Pipeclay	TAS	78%	75	11,374
2016	Adult	16-7	18	04-Apr-2018	09-May-2018	Port River	TAS	91%	19	1,711
2017	Spat	17-1	3	13-Dec-2017	24-Jan-2018	Pitt Water	TAS	15%	36	25,877
2017	Spat	17-2	7	12-Apr-2017	23-May-2018	Georges R	TAS	15%	77	12,806
2017	Spat	17-3	4	13-Dec-2017	05-Feb-2018	Pipeclay	TAS	11%	36	21,807
2017	Spat	17-4	4	16-Jan-2018	05-Feb-2018	Pipeclay	TAS	3%	36	18,311
2017	Spat	17-5	5	07-Feb-2018	11-Apr-2018	Pipeclay	TAS	23%	40	20,258
2017	Adult	17-6	17	03-Sep-2018	25-Mar-2019	Pipeclay	TAS	82%	71	10,164
2017	Adult	17-7	19	11-Dec-2018	30-Apr-2019	Port River	TAS	33%	74	6,506
2018	Spat	18-1	4	20-Dec-2018	31-Jan-2019	Pitt Water	TAS	15%	74	46,078
2018	Spat	18-2	5	07-Jan-2019	21-Feb-2019	Pitt Water	TAS	32%	74	36,682
2018	Spat	18-3	7	07-Feb-2019	11-Apr-2019	Pitt Water	TAS	47%	74	18,210
2018	Spat	18-4	7	16-Jan-2019	15-Apr-2019	Pitt Water	TAS	50%	74	15,914
2018	Spat	18-5	6	17-Jan-2019	18-Mar-2019	Pipeclay	TAS	30%	74	34,866

The split into two populations occurred at the 2016 YC and after the spread of POMS to TAS when the SA Government implemented biosecurity regulations that prohibited the movement of oysters between States. These populations have good links for the SA 2016 and 2017 YCs, where parents have sibling parents in the TAS population, and this allows POMS data from TAS population trials to enhance SA data. However, those linkages will diminish with each generation of breeding and, from a quantitative genetics' viewpoint, these will become discrete populations.

All field trials were deployed as part of the operational ASI breeding program which has POMS resistance as the primary breeding goal. The TAS population was deployed in TAS and NSW, however, New South Wales (NSW) deployments stopped after the spread of POMS to TAS due to NSW Government implemented biosecurity regulations. TAS and NSW sites were commercial leases and were locations where the OSHV-1 virus responsible for POMS was known to be present. The SA

site, the Port River in the Adelaide metropolitan area is not an oyster farming region, but was used as it is the only location where the Ostreid Herpes Virus (OSHV-1) is known to occur in SA.

TAS and NSW trials were deployed as both spat, aged between 3 and 7 months, and adults, aged between 13 and 17 months (Table 4.1.1). SA trials were deployed only as adults, aged 18 to 19 months, due to the logistical problems with conducting spat trials at the SA site. The 2017 spat test in NSW was not intentional. Oysters were deployed at that site in mid-April when the POMS window was thought to be well past, based on experience over the previous 5 years, only to find them subject to a severe POMS infection. For the 2016 and 2017 TAS YCs, trials were deployed using stock with spat and adult life stages to compare the performance. This required the adult trials to be deployed 12 months after the spat trials, in the following POMS season. All trials were deployed with three replicates and used an incomplete block design as described in Kube et al. (2018). The numbers of oysters per replicate varied in spat trials. On average, there were 171 oysters per family per replicate, with the 10^{th} and 90^{th} percentile values being 89 and 235 respectively. The numbers per family per replicate in one-year old trials were lower and far more consistent, with an average value of 50 and with the 10^{th} and 90^{th} percentile values being 30 and 53.

4.1.3.2 Laboratory trials

The laboratory trials were done at the NSW Department of Primary Industries Elizabeth Macarthur Agriculture Institute (EMAI). Oysters were shipped to EMAI using airfreight, with no more than 24 hours total transit time. The 2016 YC oysters were challenged in 12 well plates with an average of 19 oysters per well and the 2017 YC oysters were challenged in 24 well plates with, mostly, 5 oysters per well. The challenge period was 7 days, and oysters were not fed during that period. Oysters were challenged at two doses, one low and the other high, together with a control where no virus was added. A significant change to the protocols used for the 2017 YC trials was the upscaling in the numbers of individuals challenged per family. The 2016 YC trials, and the earlier trials described in Kube et al. (2018) generally had about 20 animals per family per dose. However, in the 2017 YC trials, this was increased to either 100 or 120. The 2017 YC trials for both TAS and SA populations involved two separate challenges, approximately 7 weeks apart. This was necessary because the families were produced as separate spawning runs and there were significant size differences at the early life stage. Details of the trials are given in Table 4.1.2 and further details of the methodology, including the virus preparation and the changes made to the protocols previously described in Kube et al. (2018) are given in Section 4.5.

Table 4.1.2. Summary of laboratory trials deployed at EMAI as part of this project.

Year class	Life Stage	Trial no.	Age (mon)	Deployment Date	Measure Date	Virus doses	Pop.	Sur.	No. fam	No. oysters
2016	Spat	16-8	4	22-Mar-2017	29-Mar-2017	0, 10^{-1}, 10^{-2}, 10^{-3}	Tas	68%	60	3,256
2017	Spat	17-8	2	06-Dec-2017	13-Dec-2017	0, 10^{-2}, 10^{-3}	Tas	74%	35	10,633
2017	Spat	17-9	3	10-Jan-2018	17-Jan-2018	0, 10^{-2}, 10^{-3}	SA	77%	29	9,025
2017	Spat	17-10	4	31-Jan-2018	07-Feb-2018	0, 10^{-2}, 10^{-3}	Tas	95%	34	12,139
2017	Spat	17-11	5	21-Feb-2018	28-Feb-2018	0, 10^{-2}, 10^{-3}	SA	75%	31	9,465
2017	Spat	17-12	5	19-Apr-2018	26-Apr-2018	0, 10^{-2}, 10^{-3}	Tas	51%	10	1,475

4.1.3.3 Data collection and analyses

Data from all trials was collected at a single time point. This was determined by regular inspection of the trials to see when an infection had run its course. Previous experience had shown that POMS mortalities would occur over an approximately 3-week period from the start of infection and that was used as the basis for inspection and scheduling assessments (Kube et al. 2018). At assessment, all

oysters were counted, dead oysters removed, and survivors returned to grow-out site. No further measurements were done, or scheduled, but survivors were retained as a source of broodstock.

Data were uploaded to the CSIRO Oyster Selective Breeding Database. This database stores all pedigree and performance data related to the breeding program. For each replicate, the number of oysters and survival is recorded. Routine checks are done at loading, and reports generated ready for analyses. Reports include a pedigree file and data file. The pedigree file has all pedigree records through to founders, which is important for these data given the depth of pedigree and the extent of relationships in the population. The data file converts survival data to binary records, where 0 is dead and 1 is live, and these are the records used for the analyses. Parents of families used in the 2016 to 2018 YCs of the Tasmanian population had been exposed to POMS and were, obviously, survivors. Records of the trial from which these individuals had originated from were retained and these individuals had a phenotypic record that was used in the analyses, albeit with a notional record for replicate. Prior to the 2016 YC, and for SA trials, all parents had no phenotypic record for POMS resistance.

The data were analysed using ASReml (Gilmour et al., 2015) to fit univariate and/or bivariate individual animal models. Trials were first analysed individually and, then in a final analysis, all trials were included in a bivariate analysis which included both spat survival and adult survival as separate traits. The model terms for field trials included the trial as a fixed effect, and random terms for the block, unit and animal, as described in Kube et al. (2018).

The model terms for laboratory trials included the trial as a fixed effect, and random terms for the individual plate well (equivalent to the unit in the field trials) and animal. There were, at times, significant mortalities in the control treatments and observations of the data at all doses indicated that this mortality was common to all animals of that family, and it appeared to be additive to the mortality at each dose. Therefore, the average mortality for each family was fitted as a covariate in the model. Additive genetic effects were inflated by up to 100 percent if the covariate was not included. The effects of spatial variation in laboratory trials was investigated in Trial 17-9 by deploying this trial with families randomly allocated within plates, and plates laid out in a blocking design. However, there were no spatial effects in this trial with variance components for those terms being very small and not significantly different from zero. Such a design is extremely laborious and potentially error prone when done at the scale of these trials. Consequently, all subsequent trials were deployed without a blocking design, meaning each family formed a contiguous block on the laboratory bench. Within each plate, wells were assigned as controls, low dose and high dose and the same wells were used as such on every plate to streamline the logistics of trial deployment.

Hertabilities were calculated using the variance components from the analyses on both the observed and underlying scale, as described in Kube et al. (2018), and the genetic correlation between spat and adult survival was calculated as a routine output from the bivariate analysis. Genetic correlations between field trial sites were estimated using bivariate analyses, where each site is analysed as a separate trait. This was done for the three 2015 YC trials (Table 4.1.1) as three pairwise comparisons. Analyses were also done to compare each of the SA trials with the NSW/TAS trials, where one variable was the SA trials (analysed separately) and the other variable was the combined set of all other trials.

Genetic correlations between laboratory trials and field trials were estimated using a multivariate model with three variables. One variable was the laboratory trial, and separate analyses were done for each individual trial. The other two variables were the combined data sets for all spat and adult trials, with spat and adults being treated as separate variables. These were the data sets used for routine estimated breeding values (EBV) estimation and they were used to get a measure of laboratory performance between field trial sites using bivariate analyses, where each site is analysed as a separate trait. In this report, it is the correlation between the laboratory trial and adult (one year old) resistance that is reported because the adult resistance is expected to be a better and more robust benchmark for the comparisons made in this analysis.

The genetic trend for POMS resistance for spat and adult survival was calculated using the EBV, which are also routine outputs from the analysis. Family EBV were used for this part of the analysis and they were estimated as the average of the sire and dam EBV for each individual family. Family EBV were then averaged for each YC and plotted as the genetic trend. Two plots were created for each trait. One was for the average of all families in a YC, which represents the genetic progress within the breeding population. The second was for the best 10 families in a YC, which represents the genetic stock made available for commercial deployment.

An additional process was undertaken to express the spat EBV on a scale which is meaningful to growers and provide them with better expectations of stock performance with respect to POMS, a process that will be termed benchmarking in this report. It was decided to express the EBV on two scales, one representing what was assumed to be a severe POMS infection and a second scale representing a moderate POMS infection. The Best Linear Unbiased Prediction (BLUP) process used for EBV calculations does not scale values in this way and this was done as a separate step following the ASReml analysis using the following method. First, the EBV were scaled to units of genetic standard deviations by dividing the EBV output from ASReml by the square root of the additive variance. Second, the EBV were rescaled to a distribution assumed appropriate for a severe and moderate POMS infection. This requires an estimate of the genetic standard deviation for that infection, which is then multiplied by the standardised EBV. The genetic standard deviation (GSD) for each POMS infection was calculated as:

GSD = square root (variance x heritability)
= $[p(1-p).h^2]^{0.5}$
(p = survival. h^2 = expected heritability)

The assumed survival for a severe and moderate infection was based on the 2018-year class spat trials where Trial 18-1 represented a severe infections and Trials 18-2 and 18-5 represented moderate infections (Table 4.1.1). The heritability was assumed to be 0.4, which is an estimate on the underlying scale from a combined analysis. The third part of the process was to put the EBV onto an appropriate baseline. The EBV generated by ASReml are “effects” and are not expressed on an absolute scale showing population survival. A scalar is added to the EBV to achieve this, and that scalar was chosen so that the average for the 2018-year class families matched the trial average for the severe and moderate trials. The process of benchmarking EBV is limited by insufficient trial data, with the 2018-year class trials being the only useful data. Therefore, the constants used will be revised as more trial data becomes available.

4.1.4 Results and discussion

4.1.4.1 Adult trials

Trials of adult (one year old) oysters have generally been reliable, as was found with earlier trials (Kube et al. 2018). Heritabilities (on the observed scale) mostly followed the same pattern as earlier trials with high heritabilities typically near 0.5 (Table 4.1.3). There were, however, exceptions which were the 2016 YC trials in TAS, the 2017 YC in TAS, and SA trials.

The 2016 TAS trials were affected by the POMS outbreak that spread to TAS in January 2016 and that disrupted normal nursery grow-out for these families during their first 6 months. They were held in an indoor system for an extended period, until the POMS window of infection passed, but that system was not designed for extended use and the growth of the animals was suboptimal and variable. In addition, one trial (Trial 16-2) was deployed late, mostly missing the POMS infection and experiencing little mortality. The 2017 trial was deployed in September 2018 (Trial 17-6 in Table 4.1.1) after another extended period of indoor nursery rearing. When deployed, it experienced low mortality, which is not ideal for family screening, and had lower than expected correlations with spat

trials (r_g between 0.21± 0.18 and 0.33± 0.20) and a low correlation with all other adult trials ($r_g = 0.46 \pm 0.18$).

Table 4.1.3. Heritabilities of POMS resistance field trials.

Year class	Life Stage	Trial no.	Age (month)	Measurement Date	Site	Pop.	Sur.	h^2	(se)
2015	Adult	15-01	13	22-Dec-2016	Pitt Water	Tas	50%	0.56	(0.07)
2015	Adult	15-02	14	27-Dec-2016	Georges R	Tas	22%	0.53	(0.07)
2015	Adult	15-03	14	24-Jan-2017	Pipeclay	Tas	30%	0.47	(0.07)
2016	Adult	16-01	13	19-Dec-2017	Pitt Water	Tas	27%	0.12	(0.04)
2016	Adult	16-02	15	28-Feb-2018	Pitt Water	Tas	95%	0.21	(0.05)
2016	Adult	16-03	14	09-Jan-2018	Georges R	Tas	40%	0.33	(0.08)
2016	Spat	16-04	3	07-Feb-2017	Pipeclay	Tas	23%	0.10	(0.05)
2016	Spat	16-05	3	28-Feb-2017	Pipeclay	Tas	12%	0.09	(0.03)
2016	Adult	16-06	15	05-Mar-2018	Pipeclay	Tas	78%	0.31	(0.05)
2016	Adult	16-07	18	09-May-2018	Port River	SA	91%	0.23	(0.10)
2017	Spat	17-01	3	24-Jan-2018	Pitt Water	Tas	15%	0.36	(0.08)
2017	Spat	17-02	7	23-May-2018	Georges R	Tas	15%	0.24	(0.05)
2017	Spat	17-03	4	05-Feb-2018	Pipeclay	Tas	11%	0.14	(0.05)
2017	Spat	17-04	4	05-Feb-2018	Pipeclay	Tas	3%	0.21	(0.05)
2017	Spat	17-05	5	11-Apr-2018	Pipeclay	Tas	23%	0.21	(0.05)
2017	Adult	17-06	17	25-Mar-2019	Pipeclay	Tas	82%	0.51	(0.08)
2017	Adult	17-07	19	30-Apr-2019	Port River	SA	33%	0.11	(0.03)
2018	Spat	18-01	4	31-Jan-2019	Pitt Water	Tas	15%	0.33	(0.05)
2018	Spat	18-02	5	21-Feb-2019	Pitt Water	Tas	32%	0.39	(0.06)
2018	Spat	18-03	7	11-Apr-2019	Pitt Water	Tas	47%	0.45	(0.08)
2018	Spat	18-04	7	15-Apr-2019	Pitt Water	Tas	50%	0.40	(0.09)
2018	Spat	18-05	6	18-Mar-2019	Pipeclay	Tas	30%	0.35	(0.06)

A decision has been made to cease adult POMS trials for the TAS population. This is due to the sub-standard data now being generated from these trials (described above) and the logistical difficulties and costly infrastructure needed to fix these problems. Another reason is the refocus of the breeding program on spat resistance, which is discussed in Section 4.1.3.4. There is a moderately strong correlation between spat and adult resistance and, therefore, adult resistance is expected to be maintained at current levels through spat testing alone and without the need for adult trials.

The POMS trials deployed in SA were also suboptimal and the Port River site continues to be a difficult site to manage for POMS field testing. Both SA trials had relatively low heritabilities for POMS, which suggests non-genetic factors influenced mortality. The trial site available was not a commercial oyster farming site and this resulted in logistic and management difficulties with the trials. Furthermore, the SA family production was done in a new hatchery (Section 4.2) and on a different time schedule to the TAS family production. Consequently, the 2016 trial was deployed outside what would likely have been the POMS window of infection (although there is very little experience with that site) and had low levels of POMS. The 2017 YC trial had high mortality but the low heritability ($h^2 = 0.11$) indicates that much of the mortality could not be explained.

Genetic correlations (r_g) between sites for the 2015-year class trials were all high, ranging between 0.78 ± 0.06 and 0.91 ± 0.03. This indicates that POMS resistance expresses in a very similar manner in both NSW and TAS, and it indicates that the expression is similar across a range of severities (with mortalities of 22%, 30% and 50%, Table 4.1.1).

Genetic correlations between the SA and TAS trials were estimated and these estimates benefited from the close genetic links that currently exist between the two populations. In an analysis that combined the 2016 and 2017 SA trials, the genetic correlation was $r_g = 0.91 \pm 0.16$. This indicates a high similarity in POMS expression between SA and TAS/NSW. It also indicates that the data in SA is, currently, strengthened by the very large POMS data set accumulated in TAS. However, the value of the TAS data to SA families will diminish quickly as populations diverge genetically, as will happen if there are total restrictions to the flow of genetic material into SA.

4.1.4.2 Spat trials

A focus of this project for the TAS population has been on developing spat trials, with 77% of total TAS population deployments being as spat. These have been more difficult to perfect than adult trials, but with stepwise improvements good results now appear routinely possible. A comparison of the heritabilities for each trial illustrates that progression (Table 4.1.3). Trials of the 2016 YC had low heritability with values near 0.1. The 2017 YC trials had variable heritability with values ranging from 0.14 to 0.36, and with survival that was sometimes exceptionally (and unusually) low. In contrast, the 2018 YC trials consistently had a moderate heritability, ranging from 0.33 to 0.45. Whilst survival was variable, it was within expected ranges and was thought to reflect different severities of POMS, a result that has also been experienced by commercial oyster growers.

The improvements were due to incremental changes in hatchery/nursery procedures and the husbandry associated with trials. This included earlier family spawning to ensure stock is available to better fit the POMS window, condensing spawning runs so there was less variation in the size of stock, improved nursery management, more attention (consistency) to the size of animals deployed, aligning deployments with tidal movements to decrease deployment stress, and better hardening of stock prior to POMS exposure. Another factor in the success of the spat trials has been the level of POMS resistance that has accumulated in this population. In early trials, detailed in Kube et al. (2018), death was mostly rapid and complete meaning no family discrimination was possible, thus rendering the trials useless for genetic selection. In current trials, mortality is relatively high but discrimination between families is possible, as the heritability estimates indicate.

Heritabilities of spat trials have not been as high as those of adult trials and ongoing improvements will be sought. It is, however, unlikely that the consistently high heritabilities found in adult trials will be achieved for spat trials because spat are inherently more vulnerable and will always be susceptible to mortality for other reasons. The heritability in a combined analysis of all trials was $h^2 = 0.23 \pm 0.02$ on the observed scale and $h^2 = 0.45 \pm 0.04$ on the underlying scale, with the latter underlying scale value being likely to be the better estimate and more likely to reflect the response to selection. This compares to values of $h^2 = 0.31 \pm 0.02$ and $h^2 = 0.48 \pm 0.03$ for adult resistance from a combined analysis, on the observed and underlying scales respectively. Heritability estimates are converted to the underlying scale because estimates made on the measured data (i.e. the observed scale) are based on binomial data and are likely to be underestimates. The conversion to the underlying scale used the method of Dempster and Lerner (1950) and this type of conversion, or an equivalent, is typically done when using binomial data.

The genetic correlation between spat resistance and adult resistance was estimated using a combined data set of all trials. The estimated value, $r_g = 0.60 \pm 0.07$, indicates a moderate correlation. This is based on the complete data set which has 284,502 spat records, 186,430 adult records, 584 families, and 8 YC. It is therefore likely to be a robust estimate. It is a lower value than that reported in Kube et al. (2018) but that was based on a very limited data set. The moderate genetic correlation suggests there are some different mechanisms influencing spat and adult resistance and they should be treated as different genetic traits. Selecting for one will confer resistance for the other, but the correlated response will only provide partial resistance (60% of maximal resistance in this case).

4.1.4.3 Laboratory trials

A series of laboratory POMS challenge trials were done to further refine the challenge model (fully described in Chapter 6), and to begin a process of routine POMS screening for the SA families. The routine screening was particularly important for SA families due to the absence of a proven field challenge. TAS families were also challenged in the laboratory with the aim of measuring the correlation between field and laboratory challenges as a validation of the laboratory model.

All laboratory family challenge trials used spat between 2 and 5 months of age and between 2 and 4 mm in length. The actual mortality at each dose was variable from trial to trial (Table 4.1.4). For example, the survival at the high dose (10^{-2} dilution) varied from 3% to 87%, and the low dose (10^{-3}) was between 44% and 100%. Reasons for this variation are unclear. It may be related to oyster size, although that did not seem to explain all variation. Differences may also be related to handling or related to the exposure to other microorganisms (discussed further below).

Heritabilities in each trial ranged from 0.11 to 0.28. They are comparable to the field spat challenges for the 2016 and 2017 YC trials (Table 4.1.3) but lower than the 2018 spat field trials. The genetic correlations between the laboratory and field trials were an important part of this study and, for these, results were variable. Correlations were estimated between individual laboratory trials and the combined data set from all adult field trials. The field trials used the combined data set because this is the best and most accurate estimate of field performance. Correlations ranged from very good and near unity to very poor and not significantly different from zero, and these are shown in Table 4.1.4. Of note are the extremely poor correlations between the trials of the TAS families for the 2017 YC (Trials 17-8 and 17-10) where there was no relationship between laboratory performance and adult field resistance (correlations were not significantly different from zero). When excluding these trials there appears to be a good correlation between laboratory and field trials, with genetic correlations between laboratory - spat resistance and laboratory - adult resistance being $r_g=0.83 \pm 0.14$ and $r_g=0.56 \pm 0.12$ respectively.

There is no certainty about reasons for the variability of relationships between laboratory trials and field performance. However, it is possibly associated with the husbandry practices used for nursery rearing and associated with the use of highly filtered water in the nursery. This was necessary due to the arrival of POMS in TAS and a consequence has been that oysters used in the laboratory challenge are taken directly from this very clean environment. The spectrum of microorganisms that the spat were exposed to is likely to have been altered and these oysters may have a very different microbiome to those in the field. Lorgeril et al. (2018) have demonstrated that POMS mortality is caused by secondary infections of bacteria after OsHV-1 µVar compromises the immune state of oysters. This suggests that the mortality observed in the laboratory in these trials may have different causes to that in the field due to the likely absence of those opportunistic bacteria. It accords with observations on the TAS 2017 YC which had been grown in a nursery with significantly improved water treatment compared to the 2016 YC and to the 2017 SA families.

To test the hypothesis that the microbiome was a critical factor in the relationship between laboratory and field trials, an additional trial was undertaken (Trial 17-12 in Tables 4.1.2 and 4.1.4) in which the oysters were placed on a field site before shipment to EMAI for the laboratory challenge. This trial was limited by its small scale but, nonetheless, the results suggest the microbiome may indeed be the critical factor in the expression of POMS in the laboratory challenge. In this trial, the genetic expression was slightly stronger as indicated by the heritability, the average mortality was higher at both the low and high doses, and correlation between the laboratory and combined field tests was very high ($r_g = 0.95$) (Table 4.1.4).

Overall, the laboratory challenge trials done in this project produced variable results. Significant improvements were made through changes to the protocols which included; 1) a five-fold increase in numbers of animals challenged compared to previous trials, 2) more extensive use of controls across all families, which received same procedures but no virus; and 3) attention to oyster size with a focus

on consistent sizes across all families. However, a setback was the unforeseen importance of the microbiome and the likely influences of using highly treated seawater in the nursery. These factors are the likely cause of the variable results. Further trials are needed to verify the importance of the microbiome as the main cause and such trials were not possible within the scope of this project. Nonetheless, a focus should be placed on allowing all challenged animals to be exposed to untreated (raw) seawater and exposure to opportunistic bacteria in future trials and, with this, there appears to be good correlations between laboratory and field resistance. Ideally, research should be done to better identify protocols that guarantees bacteremia (i.e. the presence of bacteria in the bloodstream) if POMS laboratory challenges are to be done with confidence and in the absence of field trials as a verification.

Table 4.1.4. Heritabilities of POMS resistance laboratory trials, and genetic correlations with the combined data set of all field spat trials. Survival is shown for both the low dose (10^{-3} dilution) and high dose (10^{-2} dilution) infection treatments.

Year class	Life Stage	Trial no.	Age (month)	Measurement Date	Pop.	Sur. (low)	Sur. (high)	h^2	(se)	r_g (se) with adult field	
2016	Spat	16-8	4	29-Mar-2017	Tas	100%	78% *	0.28	(012)	0.99	(0.28)
2017	Spat	17-8	2	13-Dec-2017	Tas	74%	60%	0.21	(0.05)	-0.21	(0.23)
2017	Spat	17-9	3	17-Jan-2018	SA	89%	45%	0.11	(0.03)	0.50	(0.18)
2017	Spat	17-10	4	07-Feb-2018	Tas	98%	87%	0.12	(0.03)	-0.17	(0.26)
2017	Spat	17-11	5	28-Feb-2018	SA	90%	37%	0.22	(0.06)	0.99	(0.02)
2017	Spat	17-12	5	26-Apr-2018	Tas	44%	3%	0.20	(0.13)	0.95	(0.08)

* Trial 16-8 also received a very high dose with a dilution of 10^{-1} which had 25% survival.

4.1.4.4 Genetic progress for adult resistance

Resistance of adult (one year old) stock was the focus of the breeding program between 2011 and 2017 and the genetic progress, as demonstrated by the annual accumulation of gains, is shown in Figure 4.1.1. Whilst gains are shown for the 2018 YC, that was largely achieved via a correlated response from selection for spat resistance. Overall, the key message from the genetic trend is that adult resistance has been achieved. As at the 2018 YC, the breeding population has an average POMS adult survival of greater than 90% (the blue line in Figure 4.1.1), and commercial deployments have had that level of resistance since the 2016 YC (the red line in Figure 4.1.1). The flattening of the trend for commercial selections after the 2015-year class appears to be due to smaller differences between top families and the YC average, indicating that the entire population is approaching good levels of adult POMS resistance.

The genetic trend tells the story of obtaining POMS resistance. Selections during the period up to and including the 2015 YC were based on sib selection (or family selection) in NSW trials, and this period delivered annual gains in the order of 10%. The 2016 YC was spawned after the arrival of POMS in Tasmania and was able to use survivors from POMS trials as broodstock, thus enabling some within family selection. Details of the benefit of breeding from survivors is provided in Kube et al. (2018) and the extra gains were predicted to average 9% for the families selected as broodstock. The genetic trend suggests that improvement was realised. The flattening of the genetic trend following the 2016 YC is likely to be due to the poor quality of the adult trials for the 2017 YC, and the absence of adult trials for the 2018 YC, as explained in Section 4.1.3.1.

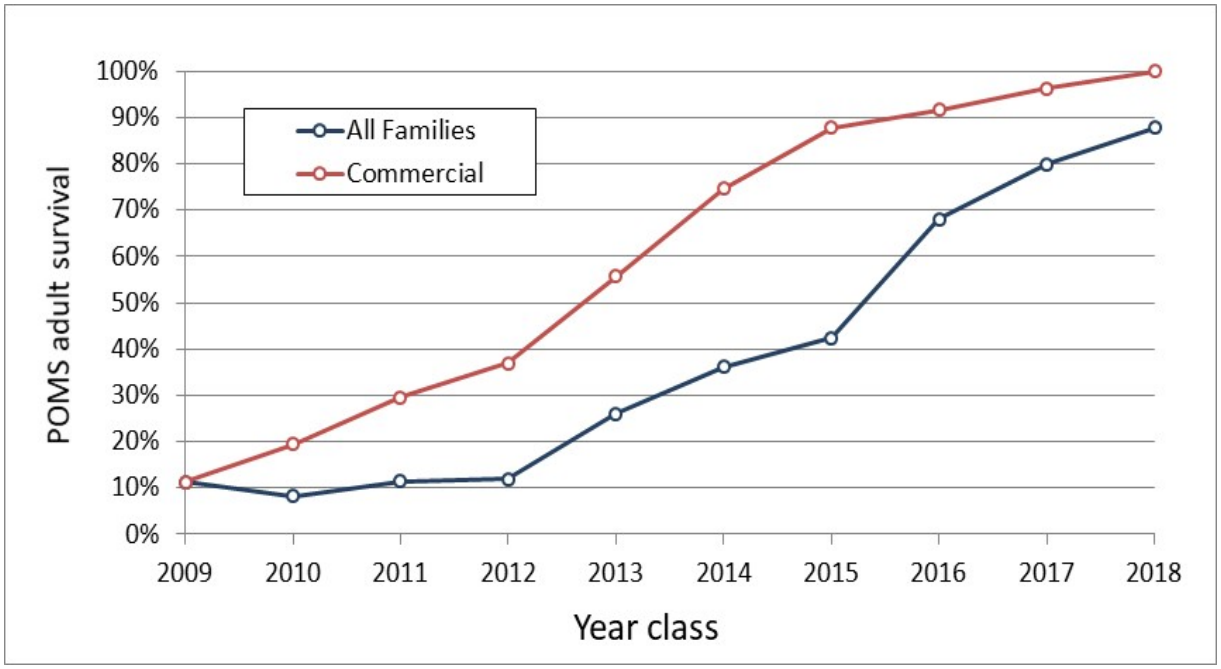

Figure 4.1.1. Genetic trend for POMS resistance for adult (one year old) stock in the TAS population. Cumulative gains are shown for each year class of the ASI population. The blue line (all families) represents the genetic gain in the breeding population. The red line (commercial) represents the gains of the best 10 families, which is typically the number of families made available to hatcheries for use as broodstock for commercial production.

There is a risk that the high levels of adult resistance may not be maintained in future year classes given that one-year old trials will no longer be deployed. However, that risk is probably low given that there will be high selection for spat resistance, which is moderately correlated with adult resistance. Nevertheless, there may be a need to consider occasional deployments of families as one-year old stock to ensure that there is no drift in this trait.

The genetic progress for POMS resistance in the SA population is shown in Table 4.1.5. The SA population was founded using the 2013 and 2014 YCs and benefited from the extensive POMS resistance testing done on those families, with a baseline POMS adult survival of 31%. Annual genetic gains are approximately 10%, which is the rate of gain achieved with family selection in the TAS population between 2011 and 2015. The SA POMS trials have been difficult to manage (Section 4.1.3.1), however, gains in this population are assisted by close genetic relationships between the SA and TAS populations for the parents of the SA 2016 and 2017 YCs. These relationships will diminish rapidly in future YCs because state regulations prohibit oyster movements from POMS affected States into SA. Consequently, the rate of gains will be lower unless SA trials are improved. Genetic gains are directly proportional to heritability and given no improvement in the Port River trials then the rate of gain in SA is likely to be in the order of 3% per year. Discussions are currently underway to address this issue.

Table 4.1.5. Genetic progress for POMS resistance in the SA population. Values are shown for the cumulative gains for adult and spat resistance. No spat trials have been deployed in SA and the spat gains are estimated using the genetic correlation between these trails.

Year class	Adult survival (all families)	Adult survival (best 10 fam)	Spat survival Moderate POMS (all families)	Spat survival Moderate POMS (best 10 fam)	Spat survival Severe POMS (all families)
SA baseline	31%	-	-	-	-
2016	59%	64%	9%	11%	0%

2017	52%	72%	1%	13%	0%

4.1.4.4 Genetic progress for spat resistance

Resistance for spat has now become the breeding focus of the TAS population, and that process has been fully implemented with the 2018 YC. POMS resistance EBV are now routinely calculated for spat resistance, and EBV are separately provided for both a moderate and severe POMS infection. Spat EBV will now be used to select parents for families and spat EBV are also supplied to hatcheries to be used for decisions about commercial deployments.

The genetic trend for spat resistance in the TAS population, for both moderate and severe infections, is shown in Figure 4.1.2. Breeding for spat POMS resistance remains a work in progress and spat resistance is much less than that for adults. Spat are inherently more susceptible to POMS than adults and the differences in susceptibility can be seen by comparing Figures 4.1.1 and 4.1.2. The annual rate of gain in spat resistance for a moderate and severe infection is approximately 13% and 8% respectively.

Commercial releases of the best current stock, which are selections from the 2018 YC, are likely to have adequate resistance to a moderate infection, with 70% survival predicted, but they will suffer high losses from a severe infection, with only 50% survival. Whilst these are families that are currently available, they are immature and will not be commercially available to growers until about mid-2021. More selection is required to produce stocks with high levels of resistance to a severe POMS infection. With the current rate of genetic progress three more years are needed, with top families from the 2021-year class projected to have 75% survival. These would be commercially available to growers in about mid-2024.

The spat resistance of the current SA population is very low in comparison to the TAS population. The best of current stocks are expected to suffer very heavy losses with a moderate infection, with about 10% survival, and total loss with a severe infection. It is difficult to accurately project a time for even moderate spat resistance in the SA population. Without deploying spat trials, gains in spat resistance will occur via a correlated response with adult trials but these will be scaled by the genetic correlation between those traits, giving an expected response of no more than 2% per year for spat. Alternately, spat trials could be deployed, however, the experience in TAS has been that these are more difficult than adult trials and, therefore, are likely to present significant challenges at the relatively difficult Port River site.

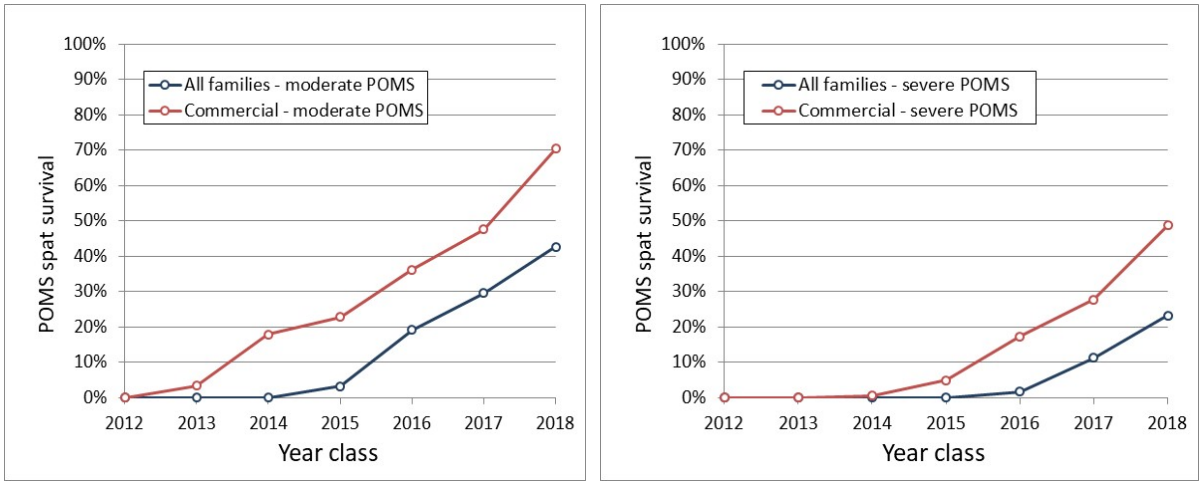

Figure 4.1.2. Genetic trend for POMS resistance for spat (two to three months old) in the TAS population. The left-hand chart shows spat gains with a moderate POMS infection and the right-hand chart shows a severe POMS infection. Cumulative gains are shown for each year class of the ASI population. The blue line (all families) represents the genetic gain in the breeding population. The red line (commercial) represents the gains of the best 10 families, which is typically the number of families made available to hatcheries for use as broodstock for commercial production.

4.1.5 Future direction

The major gap in the ASI POMS resistance breeding program is the ability to achieve gains in SA at the same rate as has been achieved in TAS. This gap represents a significant risk to SA oyster growers if POMS spreads from the Adelaide region to commercial oyster growing sites in SA. Whilst field testing has been done at the Port River site, it does not provide data of sufficient quality to support an ongoing POMS breeding program. At best the annual rate of gain will be about 5% per year in adult resistance and 2% per year for spat resistance, given the *status quo* with regard to family performance tests.

Work is needed to improve the POMS family performance testing process in SA as a priority. The existing procedures for adult trials need improvement and consideration then needs to be given to implementing spat trials if rapid gains are required for spat resistance. An alternate approach is to rely on the laboratory challenge for the POMS resistance data. However, whilst improvements have been made in laboratory challenges during this project, there remain risks that need to be addressed. Foremost of these are the very low correlations that sometimes occur between laboratory challenges and field challenges. Checks of those correlations were possible during this project due to the close genetic links between SA and TAS populations, but that will not be possible going forward. Therefore, some means of verification is necessary. It has been hypothesised in this report that the oyster microbiome is the cause of this variation and further investigation of this offers a potential pathway to remove the risk of laboratory challenges.

A second alternative to improving POMS resistance in SA is to develop processes to transfer POMS resistant germplasm from TAS to SA. There are a range of possible options being evaluated as part of a separate process and, given the biosecurity implications are beyond the scope of this report, they will not be detailed here. However, from an applied breeding perspective, they offer a valid alternative to developing SA based POMS performance testing and have the advantage of capitalising on the substantial gains achieved in TAS.

4.1.6 Acknowledgements

Families were produced over three-year classes in support of this project, and those families were the basis of this project. This was done at both the IMAS hatchery in Taroona, TAS, and at the SARDI facility in SA. This project could not have been possible without the generous help from growers who gave their time and knowledge in helping ASI manage its stock. In particular, we acknowledge and thank Josh Poke, Hayden Dyke, Tony Byrnes, Justin Goc, Michael Riley, Craig Lockwood, Carl Jaeschke, Bruce Zippel, Gary Zippel and Brendan Guidera for their assistance and support. There were numerous ASI field staff that assisted with this project; Lewa Pertl, Nick Griggs, John Wright, Charlotte Levi, Zoe Byrne, Paige Potter, Jarintzin Mones, Oliver Sargent, Joe Mangan, Geoff Endo, Ethan Bowditch-Wharton, Sophie Broomhall, Katrina Alter, Mary Woodward and Tommy Males are all acknowledged for their efforts. We also thank Mike Dove (NSW DPI) for the work on the NSW trials and Curtis Lind (CSIRO) for advice. Xiaoxu Li, Penny Ezzy and Marty Deveney are also acknowledged for their work on producing and managing ASI family lines in SA.

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4.2: National Breeding Strategy, South Australian Breeding

Xiaoxu Li[a] and Penny Ezzy[a]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, 2 Hamra Avenue, West Beach, SA 5024

4.2.1 Executive Summary

Since the outbreak of Pacific Oyster Mortality Syndrome (POMS) in Tasmania (TAS) in 2016, the SA Government through Primary Industries and Regions South Australia (PIRSA) has banned the import of all live oysters from TAS, including POMS resistant Pacific Oysters (*Crassostrea gigas*) produced by Australian Seafood Industries (ASI). To address the inaccessibility of POMS resistant oysters to the South Australian (SA) Pacific Oyster industry a breeding strategy was developed and implemented at the South Australian Aquatic Sciences Centre (SAASC) by the South Australian Research and Development Institute (SARDI). In total, 97 full-sib Pacific Oyster families were established over the 2016/17 and 2017/18 spawning season and 63 families over the 2018/19 season, exceeding the project's targeted production level. These families captured all the genetic variation currently available in the ASI Pacific Oysters in SA and provided the basis for the development of a new project, which has now been funded, to target increasing POMS resistance to over 90% for ≥one year old oysters over the next three years.

4.2.2 Introduction

Australian Seafood Industries (ASI) is owned by the South Australian Oyster Growers Association and Oysters Tasmania. Its current goal is to produce Pacific Oyster Mortality Syndrome (POMS) resistant Pacific Oysters (*Crassostrea gigas*) to help to sustainably rebuild the regions that have been affected by POMS and protect the areas that are currently free from it. In the past, all the families used in the ASI selective breeding program were established in TAS and then distributed to growers in TAS, SA and New South Wales (NSW) for performance evaluations. However, the ban of importation of live oyster material from TAS by the SA Government through Primary Industries and Regions South Australia (PIRSA) in response to the identification of POMS in February 2016, has since prohibited the SA oyster industry access to POMS resistant Pacific Oysters developed by ASI.

In 2012-13, the SA oyster industry had an on-farm value of $35 million and employed about 250 people directly; with indirect and downstream aspects increasing these figures to $250 million and 1,240 people (Econsearch 2014). Farming in SA is regionally based (i.e. Denial Bay, Smoky Bay, Streaky Bay, Coffin Bay, Louth Bay, Franklin Harbour and Nepean Bay) and therefore very important to the economy and social structures within these coastal communities. In February 2018, POMS was detected, in wild Pacific Oysters in the Port River, metropolitan Adelaide, SA, thus increasing the threat to SA oyster farming regions. Having resistant oysters stocked onto SA Pacific Oyster farms prior to any potential outbreak will hopefully avoid the crippling losses suffered following outbreaks of POMS in NSW and Tasmania.

The primary objective of this study was to design and implement a selective breeding strategy for ASI that meets the immediate and medium term (5 years) needs of the national Pacific Oyster industry. This study aimed to develop the capacities and infrastructure to support a SA breeding node and established a genetic base for a long-term breeding strategy in SA through the production of a targeted 90 Pacific Oyster families over the 2016/17 and 2017/18 seasons, and 60 families over the 2018/19 season.

4.2.3 Methods

A breeding strategy was developed through collaboration with ASI, ASI's quantitative geneticist, the Pacific Oyster aquaculture industry (including the South Australian Oyster Research Council (SAORC)/South Australian Oyster Growers Association (SAOGA)) and project participants. This strategy took into account the estimated breeding values (EBVs) for POMS resistance and other traits of current ASI genetic material available in SA. The aim was to maximise genetic diversity to increase the longevity of the breeding program, whilst increasing POMS resistance.

Family establishment followed standard protocols currently used by ASI (Kube et al., 2018) adapted for the South Australian Research and Development Institute (SARDI) facilities.

4.2.3.1 Microalgae production

Four species of microalgae (*Tisochrysis lutea*, *Pavlova lutheri*, *Chaetoceros calcitrans* and *Chaetoceros muelleri*) were cultured semi-continuously in 50 L bags using Walne medium (Lavens and Sorgeloos, 1996). A diet of ~ 50% diatoms and ~ 50% flagellates were fed to larvae and spat with ~ 50,000 cells mL^{-1} effluent rate.

4.2.3.2 Broodstock

Upon arrival, the ASI broodstock were either strip spawned within 24 hours or maintained in the SARDI Pacific Oyster broodstock conditioning system, supplied with 1 µm filtered 18 - 20 ºC seawater, and fed with an algal mixture of up to four species (Section 4.2.1.1). Prior to spawning, the broodstock were cleaned, sexed and sorted into mating pairs.

4.2.3.3 Spawning

Broodstock were strip-spawned with the gametes collected and maintained in individual containers to avoid cross-contaminations. Fertilisation was conducted according to the mating structure provided by ASI.

4.2.3.4 Larval rearing, metamorphosis induction and settlement

Pacific Oyster larvae were reared in a flow-through (20 exchanges day^{-1}) system using 22 L conical tanks, one family per tank, supplied with 1 µm filtered seawater. Larvae were graded and the tanks cleaned daily. Over this period, the larvae were fed daily with up to four species of microalgae (Section 4.2.2.1). Approximately 16 - 20 days post spawning, when at least a third of the larvae were competent, metamorphosis was induced once daily, up to three times per family, using 0.022 g L^{-1} epinephrine hydrochloride.

4.2.3.5 Spat rearing

After settlement, > 300 µm screen size spat were initially transferred to a bottle system, one family per bottle, approximately 100,000 - 200,000 spat per bottle, until they reached > 500 µm screen size. They were then transferred to an upweller system and graded (750 µm, 1000 µm, and 2000 µm) as required. The bottle and upweller systems were supplied with 1 µm seawater, fed as described in Section 4.2.2.1 and cleaned daily.

4.2.3.6 POMS challenge testing

After approximately 1.5 months each family was graded with 2 and 3 mm screens and 300 spat (> 2 mm and < 3 mm in size) per family were selected and sent to Elizabeth Macarthur Agriculture Institute (EMAI), NSW for laboratory POMS challenge test over the 2016/2017 and 2017/2018 seasons. Given the outbreak of POMS in the Port River in 2018, it was decided that a POMS field

challenging system would be established at the SARDI facility in the north arm of the Port River, Port Adelaide for testing of the 2018/2019 families once they are one year old.

4.2.3.7 Delivery of spat

Prior to delivery of spat to the SA oyster industry, 1 - 10 spat were sampled from each family, combined and sent for POMS assessment (Gribbles Veterinary Pathology, Adelaide, SA, Australia).

When enough (~ 10, 000 spat per family) 2 mm screen size spat (~ 2 months post metamorphosis) were available across all families in each run, they were counted, packed and sent to the SA oyster grower(s) determined by ASI.

4.2.4 Results discussion and conclusion

In total, 97 full-sib Pacific Oyster families were established over the 2016/17 and 2017/18 spawning season and 63 families over the 2018/19 season, thus exceeding the project's targeted production level. The breeding strategy was designed so that the 160 families produced captured as much of the genetic variation available in the SA ASI Pacific Oysters as possible in order to increase the longevity of the breeding program, whilst still maintaining high POMS resistance.

The outbreak of POMS in the Port River, metropolitan Adelaide, SA in 2018 provided an opportunity to complete field challenge tests of the families as opposed to sending them to EMAI in NSW for laboratory testing. Previous research has shown discrepancies between laboratory and field challenge tests for POMS, with the data suggesting that field tests provide a better prediction of EBV (Kube et al. 2018). The 2018/19 families produced will be challenge tested in the Port River when they reach one year of age.

The primary objective of this study has been achieved, as the 160 families produced will provide POMS resistant broodstock for the Pacific Oyster aquaculture industry to use in upcoming years, thus meeting their immediate needs. The breeding strategy, capacities and infrastructure developed will in turn meet their medium-term needs.

4 2.5 Future direction

Following completion of this project a new three year project "2019-039 - South Australian Pacific Oyster selective breeding program: Building POMS resistance to reduce risk for the South Australian oyster industry" has been approved by the Fisheries Research and Development Corporation (FRDC), which is jointly funded by FRDC, ASI, PIRSA/SARDI, Flinders Ports and SAOGA. This new project will use the breeding strategy, capacity and infrastructure developed in the current CRC-P study to breed 180 new Pacific Oyster families over three years with the aim to increase POMS resistance to over 90% for ≥one-year old oysters.

4.2.6 Acknowledgements

The authors would like to acknowledge the support of Mark Gluis and the many other SARDI staff that provided technical support to this sub-project and to Dr Marty Deveney, SARDI and Dr Shane Roberts, PIRSA for biosecurity advice. Also, to ASI and the SAORC/SAOGA for coordinating the delivery and collection of Pacific Oyster broodstock and spat.

4.2.7 References

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4.3: Production of OsHV-1 µVar Free Pacific Oyster Spat

Andrew Trotter [a], Greg Smith [a], Lewa Pertl [b] and Matthew Cunningham [b]

[a] Fisheries and Aquaculture, Institute for Marine & Antarctic Studies (IMAS), University of Tasmania, Private Bag 49, Hobart, TAS 7001

[b] Australian Seafood Industries (ASI), Tech 3, Tasmanian Technopark, Innovation Drive, Dowsing Point TAS 7010

4.3.1 Executive Summary

Since the commencement of the project in 2016, all spat and larvae screened for the ostreid herpes virus variant (OsHV-1 µVar) have tested negative for the virus. The numbers of families produced have been near to the target of 80 per year. These families have been produced in the absence of health issues and deployed to leases for selective breeding trials. Given the lack of evidence of any obvious health issues in the spat produced since the outbreak of OsHV-1 µVar in Tasmania (TAS), it is likely that spat produced in the Institute for Marine and Antarctic Studies (IMAS) facility since 2016 have been negative for the virus. The IMAS Biosecure Oyster Hatchery has been in operation throughout the 2016, 2017 and 2018 breeding seasons under strict biosecurity protocols, primarily as outline in the IMAS-ASI Biosecurity Plan. Approval for translocation of spat from the IMAS Biosecure Oyster Hatchery to non-infected areas of TAS was given by the Chief Veterinary Officer of Tasmania on 3 January 2019. The aims of this work were to produce OsHV-1 µVar free Pacific Oyster spat for the Australian Seafood Industry (ASI) selective breeding program; and to obtain formal accreditation from Biosecurity Tasmania for a designated biosecurity plan for the Biosecure Oyster Hatchery at the Institute for IMAS; allowing translocation of ASI spat to non-OsHV-1 µVar infected parts of TAS.

4.3.2 Introduction

The Pacific Oyster (*Crassostrea gigas*) is listed by the Food And Agricultural Organisation (FAO) as a principal marine aquaculture species, with global production of 583,000 tonnes in 2015 (FAO 2015). The species is a native of the north-east Pacific Ocean where it is widely cultured; it has also been successfully introduced to Europe, North America, Australia and New Zealand for the purpose of aquaculture (Park et al. 1989; Helm 2005; Pernet et al. 2016;). In recent decades mortality caused by Ostreid Herpes Virus (OsHV-1) and a variant (OsHV-1 µVar) has severely impacted aquaculture production in Europe, Australia and New Zealand. This disease is multi-factorial, driven by host physiology, husbandry practices, environmental stressors and secondary pathogens (Barbosa-Solomieu et al. 2005; Garnier et al. 2007; Samain et al. 2007; Petton 2015; Azéma et al. 2017; Burioli et al. 2017). Disease outbreaks are most common in grow-out culture (typically bays and estuaries), where there is no control over the dispersal of the virus, greater difficulty in managing biosecurity and little opportunity to regulate stress induced by adverse environmental conditions. Hatcheries have also experienced mortality events, particularly during the initial emergence of the disease and in times of translocation; but as environmental conditions and biosecurity in hatcheries can be controlled, the presence of disease in a hatchery setting is rare, even where the disease is established in the surrounding environment (Whittington et al. 2012; Hick et al. 2016; Pernet et al. 2016).

A large body of research has been undertaken since the initial outbreak of OsHV-1 in France during in the early 1990's (Nicolas et al. 1992; Renault et al. 1994; Pernet et al. 2016; Rodgers et al. 2018). Integrated approaches have been developed to mitigate the impacts of the disease through greater understanding of the epidemiology, improved husbandry, selective breeding for disease resistance, surveillance and biosecurity policy (Pernet et al. 2016; Lafferty and Hofmann 2016; Hick et al. 2018; Rodgers et al. 2018). Biosecurity policies are developed and revised based on the current state of knowledge and when gaps occur, precautionary polices are usually adopted. Biosecurity measures designed to control the spread of the disease are intrinsic to mitigation of further disease impacts. Restriction of stock movement is central to maintaining biosecurity protocols to restrict the spread of the disease, but this can have considerable negative impact on previous farm/industry management practices. Grow-out operations commonly move stocks between growing areas for husbandry and business management reasons based on life-stage, environmental conditions and economics. These activities represent an extremely high risk for the translocation of disease through the movement of stocks between infected and non-infected areas (Pernet et al. 2016). In oyster hatcheries, broodstock are commonly collected from grow-out sites and conditioned prior to spawning. The segregation of a biosecure oyster facility into three exclusion zones (i.e. broodstock, larval rearing and nursery) is common with physical and biosecurity boundaries that operate during the culture period, with new spat delivered to grow-out sites at the completion of the breeding cycle. However, there is some risk that the disease will be translocated by these types of movements (Degremont and Benabdelmouna 2014; Pernet et al. 2016).

In Tasmania (TAS) in order to move spat from hatcheries located in the infected part of the state (the southeast from Little Swanport to Cygnet) to non-infected areas; the facilities must have an approved biosecurity plan for the premises, and the spat must be screened for OsHV-1 µVar by a NATA[1] accredited laboratory prior to stocking. Restrictions in place in South Australia (SA), where OsHV-1 µVar is limited to one site (Port River, metropolitan Adelaide) that is remote from *C. gigas* farming locations, forbid importation of stock from either New South Wales (NSW) or TAS, where hatcheries are located within infected areas of these states. These restrictions have had significant economic impact to the SA industry due to a shortage of spat, as about 80% of the spat used by SA Pacific oyster farmers was imported from TAS prior to the OsHV-1 µVar outbreak in January 2016 (Ugalde et al. 2018). Additional hatcheries have now been built in SA to compensate for this shortfall, but production by them is only now (2019) beginning to meet demand. Should the presence of OsHV-1 µVar be detected at more sites in SA, the inability of SA growers to use the most disease resistant family lines produced by the Australian Seafood Industries (ASI) TAS selective breeding program will disadvantage this group of growers. This breeding program has been selecting for OsHV-1 µVar resistance since the disease first occurs in NSW in 2010 (Paul-Pont et al. 2013). As a response to this, ASI with the support of the Future Oysters CRC-P project established a selective breeding node in SA, which over the last two years has begun to increase the genetic resistance of Pacific Oyster in this state. The aims of this work were to produce virus free *C. gigas* spat for the ASI TAS selective breeding program, to ensure the survival and health of spat to deployment on oyster leases; and to obtain accreditation from Biosecurity Tasmania to allow translocation of ASI spat to non-infected parts of TAS. Two objectives were set to meet these aims: 1) develop a biosecurity plan for the IMAS hatchery facilities and obtain accreditation; and 2) breed *C. gigas* families in the new IMAS facility and assess the status of spat produced via testing in a NATA accredited laboratory.

[1] National Association of Testing Authorities, Australia

4.3.3 Methods

4.3.3.1 Biosecure Oyster Hatchery and biosecurity management plan development and certification

Funding for some of the equipment required to establish a biosecure oyster facility at the Institute for Marine and Antarctic Studies (IMAS) has been met by this CRC-P project 2016-801 - Enhancing Pacific Oyster Breeding to Optimise National Benefits. Following the outbreak of POMS in southeast TAS in January 2016, Biosecurity Tasmania implemented several biosecurity measures to mitigate the risk of the disease spreading to other parts of the State. This included strict guidelines on the movement of oysters within TAS. Importantly hatcheries are now required to develop certified biosecure facilities to permit them to supply oyster spat to non-infected areas of the state. Biosecurity Tasmania administers this certification according to the Animal Health Act (1995), Section 40A. Furthermore, as the operation of a biosecure oyster hatchery is also intrinsic to the practicalities of the production of OsHV-1 µVar free spat, the establishment of a biosecure facility at IMAS is fundamental to this project. The IMAS facility was upgraded to meet the new biosecurity requirements at the commencement of this Future Oysters CRC-P project in September 2016. Since the initial upgrade to meet the challenges of operating in an area known to contain OsHV-1 µVar, a number of modifications have been implemented to improve biosecurity infrastructure and protocols as part of the on-going certification process.

The newly developed facility comprises three rooms physically isolated from one-another: 1) the Broodstock Room; 2) the Larval Rearing Room; and 3) the Nursery. The rooms are zoned as Red, Transitional (Red, Amber or Green) and Green respectively for the Broodstock Room, the Hatchery and the Nursery, according to the level of pathogen risk assigned to each room (Figure. 4.3.1). Water flow and staff movement within the Biosecure Oyster Facility has been regulated to prevent the spread of the pathogen between differing biosecurity zones.

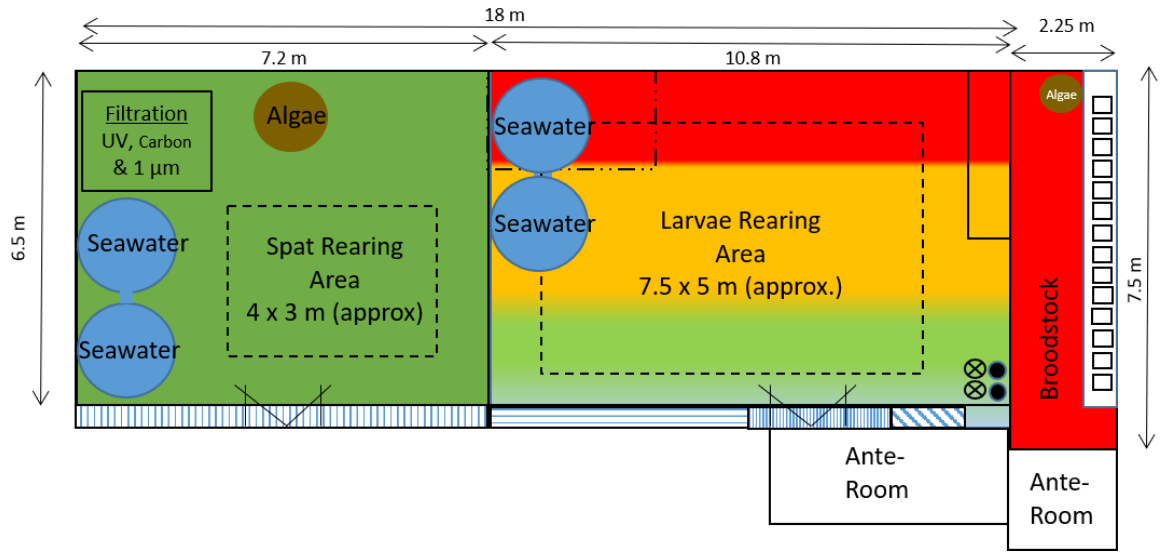

Figure 4.3.1. Floorplan of the IMAS Biosecure Oyster Hatchery. Broodstock Room (right) Hatchery (middle) Nursery (left). The rooms are zoned as Red, Transitional (Red, Amber or Green) and Green respectively for the Broodstock Room, the Hatchery and the Nursery, according to level of pathogen risk assigned to each room.

The IMAS Biosecure Oyster Hatchery is supplied highly treated seawater (TSW) that has undergone, sand and carbon filtration (40 µm nominal), foam fractionation, ozonation (> 750 mV for > 20 min) from the IMAS Seawater Treatment Plant, plus heating (15 – 21 °C), 1 µm nominal bag filtration and UV treatment upon entry into the IMAS oyster hatchery facility. Wastewater exiting the facility is transferred to the IMAS Wastewater Treatment Plant where it is processed through 1 µm nominal bag filtration, ozonation (> 750 mV for > 20 min) and UV sterilisation before being discharged to waste.

Both IMAS treatment plants are fully automated with logging of oxidation-reduction potential and operate under a 24 h alarm system. Ozone disinfection was chosen for treatment of both incoming seawater and wastewater because of its effectiveness against viruses and bacteria, and the reliability and practicality of controlling the disinfection process (Read and Arnold 1994; Liltveld et al. 1995; Anon 2002). This ensures that pathogens do not enter or egress the Biosecure Oyster Hatchery in systems water. Live microalgae were initially supplied to the Biosecure Oyster Hatchery from an existing static batch culture algal system located in the IMAS Algal Culture Laboratory. Upon the commencement of the project a continuous algal system was established using project support for equipment, to produce a high-density algal product that meets the needs of the animals located in the broodstock, hatchery and spat nursery facilities.

Broodstock room

The Broodstock Room is always considered to be infectious, as broodstock are sourced from infected waters. The floor area of the Broodstock Room is approximately 17 m^2, plus an anteroom of 6.75 m^2. Seawater supply is flow-though, which is treated before entry to the room and exits to the wastewater treatment plant only. The Broodstock Room has capacity for 17, 35 L tanks, where up to 500 broodstock can be held and conditioned prior to spawning (Figure 4.3.2A, B).

Hatchery

The Hatchery has a floor space of 70 m^2, plus an anteroom of 7.5 m^2, with a capacity to operate 42, 140 L larval rearing tanks (Figure 4.3.2C). Seawater is used on an intermittent basis for batch water exchanges for larval cultures; and therefore, in the normal operating state, two 5,000 L sumps are used to hold TSW whilst it is brought to temperature using a heat-chill unit, prior to daily water exchanges. Seawater exits directly to the wastewater treatment plant. The Hatchery operates at three biosecurity zone levels according to the stage of oyster production. On the day of spawning when hand-stripped gametes are taken from the Broodstock Room and passed through the one-way cabinet between the Broodstock and Hatchery rooms, the Hatchery is classified as a Red zone. Water is held in batch cultures that is changed regularly, making the water from this area essentially flow-through. From the day after the spawning procedure, the Hatchery is then classified as Amber (potential infectious) until the results of OsHV-1 µVar screening are supplied by a NATA accredited laboratory. If the results are negative, the Hatchery is then zoned Green and animals can be moved to the Nursery.

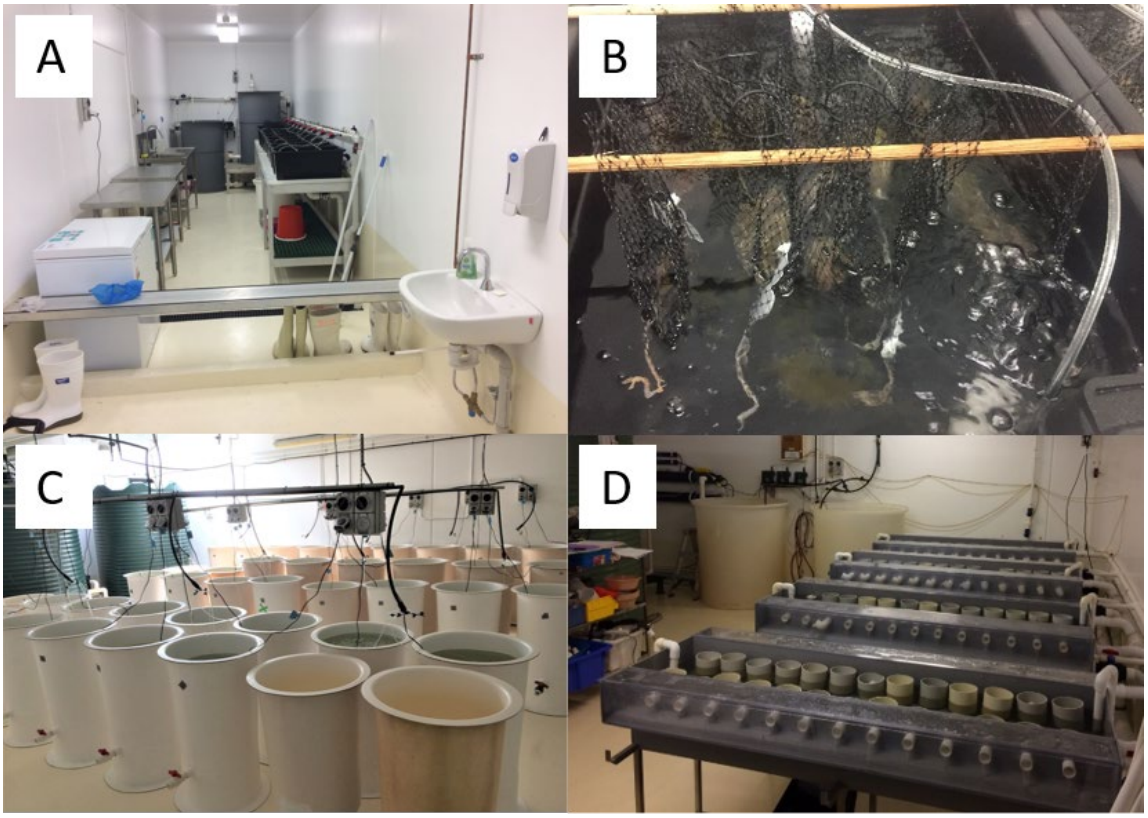

Figure 4.3.2. (A) Broodstock Room, from the ante-room; (B) broodstock being held in mesh bags in a 35 L tank; (C) Hatchery in operation using 150 L larval rearing tanks; (D) Nursery and upwelling spat system.

Nursery

The Nursery has a floor space of 47 m^2, with the capacity to operate 80 upwellers for spat culture (Figure 4.3.2D). Seawater supply is flow-though and exits to the wastewater treatment plant only. Seawater is used on an intermittent basis for batch water exchanges for larval cultures; and therefore, in the normal operating state, two 5,000 L sumps are used to hold TSW whilst it is brought to temperature using a heat-chill unit, prior to daily water exchanges. The Nursery operates under a Green zone classification only.

The IMAS-ASI Biosecurity Plan (Pertl et al. 2018) was developed in consultation with Biosecurity Tasmania and is based on the National Biosecurity Plan Guidelines (Spark et al. 2018). This includes 22 standard operating procedures (SOP) and an Emergency Response Plan. The SOPs include detailed methodologies used to produce the ASI families, all biosecurity protocols and some commercial-in-confidence information. A draft plan was completed in August 2018 and was reviewed by Biosecurity Tasmania. A revised biosecurity plan was reviewed by Biosecurity Tasmania on 29 October 2018 and approved by the Chief Veterinary Officer (CVO). Following the second review and site inspection on 19 October 2018, Biosecurity Tasmania recommended the independent audit process commence. The independent audit was undertaken by Joan van Drunen (Biosecurity Tasmania approved auditor for oyster hatcheries) on 5 November 2018. Remediation works were completed following this audit and a final compliance inspection was undertaken on 5 December 2018. An approvals notice was received from the CVO on 3 January 2019 (Appendix 10).

4.3.3.2 Hatchery trials

Broodstock conditioning and spawning

Six to eight weeks prior to spawning, broodstock were selected from oyster framing leases at Pipeclay Lagoon, Pitt Water or Little Swanport based on gamete condition and traits of appearance. Broodstock were transported to the IMAS Biosecure Oyster Hatchery, where they were passed

through a one-way cabinet into the Broodstock Room and held for final conditioning. Broodstock were conditioned in mesh bags (segregating families) suspended in 35 L tanks with approximately 15 to 30 animals per tank depending on animal size. System water was approximately 20 ºC, 34 ppt, pH 7.9 and DO of 85%. Broodstock were fed daily on a combination of *Chaetoceros muelleri*, *Chaetoceros calcitrans* and *Tisochrysis lutea* (T-iso). All of the growing areas where broodstock are held were infected with OsHV-1 µVar. There was no attempt to segregate broodstock other than by pedigree, with equipment shared without restriction within the Broodstock Room.

Spawning was conducted using a strip spawning technique. Briefly, eggs were stripped from mature females by removing the right valve, cutting adductor muscle to remove the animal and then lacerating and scraping the gonad using a scalpel blade. Stripped eggs were collected in a 1 L beaker then rinsed over a 75 µm sieve to remove tissue debris and fluids, screened on a 20 µm sieve and rinsed into a clean 1 L beaker in 300 mL of TSW. Males were treated in the same way with the exception that sperm was screened directly over a 20 µm sieve and collected directly into 200 mL of TSW in a 500 mL beaker. Between handing each animal all equipment was sterilised by chlorination (200 ppm) or disposed of, to prevent genetic contamination between families.

Eggs were held in 300 mL TSW for a minimum of 1 h before fertilisation and gently mixed every few min to prevent them settling out. Sperm was held for up to 30 min prior to fertilisation. For each cross, eggs from a single female were fertilised with sperm from a single male. The fertilisation of eggs was undertaken in 10 L of TSW at 24.5 ºC by gently mixing in 5 mL of sperm. Typically, between 1 x 10^7 - 2.5 x 10^7 eggs are fertilised per cross. Fertilisation was confirmed by the presence of polar bodies and the embryos were then stocked into 150 L conical larval rearing tanks.

Larval rearing

At one day post-fertilisation (dpf), larval stocking density was reduced to 5 x 10^6 150 L tank^{-1} (30 mL^{-1}). Larval counts were conducting by enumerating immobilised larvae (10% ethanol) using a Sedgewick Rafter slide. Well-formed D-larvae only are enumerated. Larvae were reared using a batch water exchange methodology. Briefly, larvae were collected on an appropriate size nylon-mesh screen for grading, with the culture media going to waste and the tank refilled with temperature adjusted TSW. All culture hardware (tank, airline and heater) was re-used after cleaning and chlorination. Between use in tanks housing single families all equipment was sterilised by chlorination (200 ppm) to prevent genetic contamination between families.

Fresh algae (carboy culture) were fed to the larvae daily (*C. calcitrans, C. muelleri*, *T. lutea* and *Diacronema lutheri*) in various combinations and cells counts. Water temperature was kept between 23.5 and 24.5 ºC.

At metamorphosis, eyed-larvae were screened (at 225 µm) for transfer into down-welling pots (180 µm screens). Temperature was maintained at 22.5 ºC during settlement with daily water exchanges. To produce cultchless spat, larvae were bathed in epinephrine (9 g L^{-1}) for 60 to 90 min. Once this procedure was completed, metamorphosed spat were transferred to the Nursery.

Spat rearing

Spat were reared in upwelling pots with temperature maintained at approximately 21 ºC. Nursery system culture water was exchanged daily (TSW) and fresh algae was fed to the spat daily (various combinations of *C. calcitrans, C. muelleri*, *T. lutea, D. lutheri* and *Skeletonema pseudcostatum*). The entire upwelling system was cleaned weekly by scrubbing and chlorination (200 ppm). Spat were washed daily to remove pseudo-faeces and detritus and graded as necessary using various sized nylon mesh screens.

OsHV-1 µVar screening

Data presented here are from OsHV-1 µVar screening of spat tested under operational need by ASI, with targeting samples of the 2018 broodstock and larvae for the purpose of providing more depth to this reporting (Table 4.3.1). Screening was done according to Department of Primary Industries Water and Environment, Animal Health Laboratory (AHL) protocols, based on the World Health Organisation for Animal Health Manual (OIE 2018).

Sampling procedures - larvae

Year Class 2018 larvae were collected for sampling by pooling animals removed at daily grading from 1 – 11 dpf. Sub-sized larvae were added to two 150 L tanks providing representation of all families in the hatchery during both of the 2018 ASI spawning runs (Table 4.3.1). These larvae designated for sampling were reared following standard larval rearing practices described above. At 11 dpf, the larvae were screened on a 63 µm mesh. The larvae were then washed into a 1 L beaker and divided into 1.7-mL Eppendorf tubes (30 samples) with a minimum of 200 mg of larvae in each. Samples were then transported live to AHL (by courier, same day delivery).

Sampling procedures - spat

Year Class 2016 spat: 30 samples were collected (Table 4.3.1); each sample contained five spat randomly selected from the 76 families produced for the year. Samples were collected on 10 April 2017 and received at AHL on 11 April 2017. Spat were sent dry by overnight courier.

Table 4.3.1. Summary of OsHV-1 µVar screening for 2016, 2017 and 2018 ASI year classes.

Case ID	Year Class	Sample Type	Stage	Test	Date Received
17-1329	2016	Pooled samples: 30 bags of <200 mg of spat	Spat	qPCR	11/04/2017
17-12737	2017	Pooled samples: 30 bags of <200 mg of spat	Spat	qPCR	4/4/2018
18-4088	2018	Pooled samples: 30 tubes of 100-200 µm spat	Larvae	qPCR	29/10/2018
18-3768	2018	Pooled samples: 30 tubes of 100-200 µm spat	Larvae	qPCR	5/10/2018
18-4332	2018	Individual broodstock: 90 gill sections from individual animals from the second spawning run of the 2018 season	Broodstock	qPCR	15/11/18
19-0867	2018	Progeny of two broodstock that tested positive in Case 18-4332. Pooled samples: 30 tubes of 100-200 µm spat	Spat	qPCR	19/02/2019

Year Class 2017 spat: 30 samples were collected (Table 4.3.1); each sample contained five spat randomly selected from the 75 families produced for the year. Samples were collected on 3 April 2018 and received at AHL on 4 April 2018. Spat were sent dry by overnight courier.

Year Class 2018 spat: 30 samples were collected (Table 4.3.1); each sample contained five spat randomly selected from the 79 families produced for the year. Samples were collected on 18 February 2019 and received at AHL on 19 February 2019. In addition, progeny of the two broodstock that tested positive in Case 18-4332 were also sampled. Spat were sent dry by overnight courier.

Sampling procedures - broodstock

Year Class 2018 broodstock were sampled at the time of spawning after the gametes had been removed (an aseptic technique was used between each animal for the gamete removal). Using an aseptic technique between animals, a small section of gill was dissected (approximately 5 x 5 mm)

and placed into a cryovial in 95% ethanol (Table 4.3.1). The samples were stored at -80 ºC until being transferred to AHL for screening.

Laboratory analysis

Screening for OsHV-1 µVar was completed via Taqman qPCR assay conducted by the NATA accredited AHL. Primers for the qPCR target the B-region of the OsHV-1 µVar genome (Martenot et al. 2010). Samples are considered positive for OsHV-1 µVar for Ct values of ≤ 37 with a true sigmoidal curve. These assays were conducted on a fee-for-service basis.

4.3.4 Results and discussion

Production for the ASI year classes from 2016 to 2018 has been consistent between years in terms of the number of families produced with 76, 75 and 79 bred respectively for 2016, 2017 and 2018[2] (Table 4.3.2); and essentially the same as prior to the outbreak of OsHV-1 µVar in Tasmania. Families lost from the 2017 and 2018 year classes were higher than the previous two year classes, 14 and 18 lost compared to 8 and 6 lost respectively; noting that spat produced in 2015 were pre-outbreak of OsHV-1 µVar in Tasmanian (February 2016: Table 4.3.2). This increase in the number of families lost is unlikely to be related to OsHV-1 µVar, as mortality associated with this disease in hatcheries is widespread throughout the hatchery population and catastrophic in terms of mortality (Hine et al. 1992; Nicolas et al. 1992; Renault et al. 1994; Le Duff et al. 1996; Pernet et al. 2016; Rodgers et al. 2018), this was not evident in the IMAS hatchery. Loses of the magnitude observed from 2015 to 2018 are not unusually high in *C. gigas* breeding and can be attributed to the larvae either failing to thrive, or more commonly due to abnormal embryonic development, relating to paternal inputs such as poor egg quality resulting from poor maternal health, egg immaturity, low sperm quality (Thiriot-Quiévreux et al. 1988; Helm et al. 2004). In most cases families lost resulted from management decisions to terminate the families due to high incidences of abnormal D-larvae 1-2 dpf, rather than as a result of a mortality event. Occasionally a family was lost later in the larval rearing phase, whereby poorer preforming families kept for operational requirements finally failed and were then terminated. In some cases, chronic bacterial infections were suspected to be further compromising weaker larval cultures. Chronic bacterial infections are common in bivalve culture (Sugumar et al. 1998; Estes et al. 2004; Zannella et al. 2017). The case for the absence of OsHV-1 µVar involvement in any mortality is reinforced by the negative qPCR results in the larvae in 2018 and spat reared 2016, 2017 and 2018. Although unlikely, false-negative results for OsHV-1 µVar cannot be completely ruled out.

The positive result OsHV-1 µVar for some broodstock (6 of 90) of the 2018 Year Class is not unexpected, as asymptomatic carriers of herpes virus *C. gigas* have been reported previously (Arzul et al. 2002; Barbosa-Solomieu et al. 2005; Paul-Pont et al. 2014; Petton et al. 2015). As the broodstock are completely isolated from either the larvae or the spat, this would not be a biosecurity risk unless there was a biosecurity breach or there was vertical transmission from the infected broodstock. A biosecurity breach is considered unlikely as incoming seawater is treated well beyond what has been demonstrated to prevent disease outbreak resulting from a contaminated seawater supply (Whittington et al. 2015), and strict biosecurity protocols have been in place and upheld since the start of the 2016 breeding season. To date the screening for OsHV-1 µVar suggests that there has been no vertical transmission from the ASI broodstock, which were sourced from OsHV-1 µVar infected areas. Although the screening methods are based on OIE guidelines, the lack of understanding of vertical transmission mean the results could be considered ambiguous by some biosecurity stakeholders. Currently two studies indicate there is a likelihood vertical transmission of OsHV-1 µVar can occur in *C gigas*. Barbosa-Solomieu et al. (2005) provided equivocal evidence of vertical transmission across three generations in *C. gigas*, while Sanmartín et al. (2016) demonstrated vertical transmission in the very closely related Portuguese Oyster *C. angulata*.

[2] Eighty families are targeted each year, but loses cannot typically be made up due to logistical constraints.

Table 4.3.2. ASI production statistic for 2016, 2017 and 2018, with corresponding OsHV-1 screening results.

Year Class	Broodstock Used	Crosses Made	Families Produced	Families Lost	Year Class	OsHV-1 µVar Status
2015	174	87	79	8	2015	All negative
2016	164	82	76	6	Spat	All negative
2017	178	89	75	14	Spat	All negative
2018	194	97	79	18	Broodstock	6 of 90 positive
-	-	-	-	-	Larvae	All negative
-	-	-	-	-	Spat	All negative

[#]Spat reared prior to OsHV-1 outbreak in Tasmania (February 2016). Spat were tested for OsHV-1 µVar as they had been held at Shellfish Culture Ltd hatchery when the first outbreak occurred.

Surprisingly, this topic has received little attention, given its importance to oyster biosecurity (Rogers et al. 2018; Pernet et al. 2018). The lack of attention given to vertical transmission of OsHV-1 µvar may suggest the there is little perceived need in these industries to drive such research. We have observed OsHV-1 µvar does not readily vertically transmit (based on routine screening of spat) and does not cause disease in hatcheries when the appropriate measures are in place to prevent horizontal disease transmission. This has been reflected many times elsewhere, based on anecdotes shared to the authors by a number of researchers and culturists from other hatcheries in TAS, France and New Zealand. Six of 90 broodstock screened were positive for OsHV-1 µVar, however screening of the larvae and spat retuned negative results (Table 4.3.2). Development of the IMAS Biosecure Oyster Hatchery and the ASI-IMAS Biosecurity Plan lead to the approval for translocation of spat from the IMAS facility to non-infected areas of TAS. This approval was given the CVO of Tasmania on 3 January 2019 (Appendix 10).

The IMAS Biosecure Oyster Hatchery has been used throughout the 2016, 2017 and 2018 under essentially the same strict biosecurity protocols as outline in the ASI-IMAS Biosecurity Plan. Since the commencement of the project all spat and larvae screened for OsHV-1 µVar have been negative in a NATA accredited laboratory. The numbers of families produced have been very close to the target of 80 per year, and there have been no health issues whereby the spat have not been able to be deployed to leases for selective breeding analysis. Given the excellent health observed for all of the animals produced, it is likely that all of the spat produced since 2016 have been negative for OsHV-1 µVar.

4.3.5 Future direction

In order to provide greater surety of the disease free status of spat produced from broodstock originating from OsHV-1 µVar infected waters, two aspects of uncertainly need to be assessed or investigated further: 1) the probability of obtaining false-negative results based on the current screening protocols; and 2) vertical transmission - does it occur in hatchery settings and if so, how variable is it in terms of likelihood and infectivity, and can measures be developed to prevent it.

In terms of virus detectability, the assay used here is OIE recommended (hence widely used), and is both highly specific and sensitive, detecting as few as three copies of viral DNA mg^{-L} (Martenot 2010; Paul-Pont et al. 2015). Inappropriate choice of tissue may lead to false negatives in broodstock (Bootland et al. 1991; Barbosa-Solomieu et al. 2005), but this does not present a problem for larvae and spat as they are screened whole. Development of a stress test to induce activation of the virus prior to screening could enhance surety of screening for virus free status.

Presently vertical transmission of OsHV-1 µVar has not been clearly demonstrated in *C. gigas* and there has been little recent attention to confirm or deny if this occurs or not. Recent screening data

and anecdotes from other hatcheries in TAS, France and New Zealand suggests that hatcheries strip spawning broodstock sourced prior to the summer morality period, does not lead to vertical transmission. Fully understanding vertical transmission, or lack of it, including mechanisms represents a considerable amount of work, but the benefits would be significant in terms of understanding biosecurity risks.

4.3.6 Acknowledgements

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4.4: Ozonation Trials: Potential use of ozone as a disinfectant for Pacific oyster embryos

Andrew Trotter [a], Geoff Endo [a], Matthew Cunningham [b], and Greg Smith [a]

[a] Fisheries and Aquaculture, Institute for Marine & Antarctic Studies (IMAS), University of Tasmania, Private Bag 49, Hobart, TAS 7001

[b] Australian Seafood Industries (ASI), Tech 3, Tasmanian Technopark, Innovation Drive, Dowsing Point TAS 7010

4.4.1 Executive Summary

Ozone is commonly used to disinfect cultured fish and shrimp embryos for disease control. The tolerance of Pacific Oyster (*Crassostrea gigas*) embryos to ozonated seawater was investigated as a possible disinfection and disease control mechanism. A protocol was developed to successfully collect oyster embryos from the culture water, apply ozone treatment and rinse the embryos without causing handling damage. Pilot studies indicated there was good potential to disinfect *C. gigas* embryos using ozone, suggesting embryos may tolerate ozone exposure up to a CT value of 2 (mg L^{-1} total residual oxidants {TRO} x min exposure time). However, subsequent experimentation demonstrated low exchange of ozonated seawater immediately adjacent to the embryos was responsible for the observed embryo tolerance to ozone during the pilot studies. Both exposure time and TRO significantly affected the proportion of normal larvae at relatively low doses. A CT value of just 0.25 produced 30.6% normal larvae, compared to the control with 63% normal larvae. An ozone CT value of 0.25 is unlikely to have a useful biocidal effect in disinfecting embryos of bacteria and viruses. When this is coupled with the observed high incidence of abnormal larvae, it seems ozone is inapt for the disinfection of *C. gigas* embryos.

4.4.2 Introduction

Mass mortality events in Pacific Oyster (*Crassostrea gigas*) culture in both hatchery and grow-out stocks have been reported from numerous countries and this has caused considerable economic impacts in recent decades (Renault 1995; Estes 2004; Elston et al. 2008; Travers et al. 2015; Azéma et al. 2017; Burioli et al. 2017; Rodgers et al. 2018). The grow-out of spat, juveniles and adults have been impacted by summer mortalities, large-scale mortalities were first recorded in Japan and the USA in the 1960's (Mori 1979; Perdue et al. 1981). Hatcheries also experience mortality events, but as environmental conditions and biosecurity are subject to more stringent control, disease occurrences in hatcheries are far less frequent than those observed during grow-out of stocks. Bacterial infections, with *Vibrio* species (e.g. *V. splendidus*, *V. tubiashii* and *V. coralliilitychus*) (Jeffries 1982; Elston 1993; Sugumar et al. 1998; Elston 2008; Travers et al. 2014; Travers 2015) and Ostreid Herpes Virus (OsHV-1) (Nicolas et al. 1992; Hine 1992; Renault 1995) are known pathogens of *C. gigas* in hatcheries. In recent years, much of the focus on summer mortalities has centred on OsHV-1, which was first identified as being associated with major summer mortality events of spat and juveniles in France in 1993 (Renault et al. 1994). Additionally, *V. aestuarianus* and *V. splendidus* have been implicated in summer mortality of *C. gigas* reared in tidal areas of the French coast (Le Roux et al. 2002; Gay et al. 2004; Garnier et. al 2007; Saulnier et. al 2010). *Nocardia crassostreae* has been associated with summer mortality in *C. gigas* in coastal regions of Canada, USA and the Netherlands (Friedman et al. 1998; Engelsma et al. 2008). Similar disease outbreaks attributed to both viral and bacterial pathogens have been observed in numerous other bivalve species in both hatchery and growout settings (reviewed in Travers et al. 2015; Arzul et al. 2017; Dubert et al. 2017; Zannella et al. 2017).

Aquaculture production of *C. gigas* is commonly impacted by endemic diseases and is likely to be further affected by emergent diseases in the future. Integrated strategies are needed to mitigate the disease impacts by developing greater understanding of epidemiology, improved husbandry, breeding programs,

surveillance, biosecurity and through the development of biocides, vaccines and medicines (Krkošek 2016; Pernet 2016; Lafferty and Hofmann 2016; Stentiford et al. 2017; Kalatzis et al. 2018; Rodgers et al. 2018). Managing the potential for disease spread through translocation of stocks is an intrinsic component of disease control. In oyster hatcheries, broodstock are typically collected from growout sites and conditioned prior to spawning. Hatcheries are segregated into broodstock, larval and spat culture areas to assist in the management of site hygiene, however at the end of this process spat are then delivered back to growout sites. There is considerable risk that disease will be spread by such movements, both within hatcheries and across geographically diverse grow-out sites (Degremont and Benabdelmouna 2014; Pernet et al. 2016). In fish and shrimp aquaculture the risk of disease transmission is often mitigated by egg disinfection, a technique not used in bivalve hatcheries (Coman et al. 2005; Battaglene and Morehead 2006; Coman and Sellars 2007; Ghomi et al. 2007; Ballagh et al. 2011; Can et al. 2012; Powell and Scolding 2016; Swaef et al. 2016). Target pathogens for egg disinfection include viruses, bacteria and eukaryotes. A number of chemicals have been used including; iodophors, hydrogen peroxide, formaldehyde, glutaraldehyde, bronopol among others (Battaglene and Morehead 2006; Swaef et al. 2016). Recently, ozone has emerged as a powerful tool in aquaculture water treatment processes and is being integrated into biosecurity protocols (Powell and Scolding 2016).

Ozone is a powerful oxidising agent and one of the most potent biocides, more effective than chlorine dioxide, chlorine, peracetic acid, hydrogen peroxide (Weavers and Wickramanayake 2001). Ozone reacts quickly with halides in seawater, chiefly bromide and to a lesser extent chlorine, to produce highly oxidative compounds that provide the biocidal action (Sugita et al. 1992; Grguric et al. 1994; Gonçalves and Gagnon 2018). The tolerance of fish and shrimp embryos has been found to be species specific and the maximum dose depends primarily on oxidant concentration, duration of exposure, and stage of embryonic development (Coman et al. 2005; Battaglene and Morehead 2006; Coman and Sellars 2007; Can et al. 2012; Swaef et al. 2016).

The aim of the study was to investigate the potential to use ozone to disinfect *C. gigas* embryos. The objectives were to conduct experimentation to define: 1) a handling technique for ozone exposure (treatment); 2) the tolerances of embryos to ozone; and 3) the optimal developmental stage for exposure to ozone.

4.4.3 Methods

4.4.3.1 Experimental overview

Treatment protocols and equipment were developed during February-March 2017, with pilot studies conducted in September 2017 and January 2018. Pilot studies were conducted subject to logistical constraints that precluded application of treatments at the same time (treatment duration was approximately 1 h. Data from two of the pilot studies that best represent the outcomes of this work are presented here for context and to provide the rationale for the experimentation. This work guided the experiments that were undertaken in August and September 2018. The experiments were designed to confirm the findings from pilot studies, using greater replication and application of the treatments simultaneously.

Disinfection potential of ozone is reported as a combination of concentration (C) of ozone measured as total residual oxidants TOR (mg L^{-1}) and time (T) of exposure to the TOR in min = ozonation CT (Coman and Sellars 2007).

4.4.3.2 Pilot studies

Study 1: effects of ozonation at two embryonic stages

The study was designed to examine the effects of ozone concentrations of 1 mg L^{-1} total residual oxidants (TRO) and ozone exposure times of 1, 2 and 4 min at two developmental time points, blastula, ~ 4 hpf and gastrula, ~ 7 hpf, with CTs of 1, 2, and 4. A no ozone control (OC) was used for both the blastula and gastrula treatments. Treatments occurred between 4 - 5 hpf for the gastrula treatment and between 7 - 8 hpf for the blastula treatment. All treatments and the control had three replicates.

Study 2: effects of ozone CT at gastrulation

As the previous study indicated that the embryos were more tolerant of ozonation at blastula ~ 4 hpf this developmental stage was selected for this study. The study was designed to examine if concentration or time was more important in determining the tolerance to a particular CT. Hence, two ozone concentrations (0.5 min and 2 mg L^{-1} TRO) and two ozone exposure times (1 and 4 min) were used to create CTs of 2, i.e. 4 min x 0.5 L^{-1} TRO (= CT 2, hereafter called 2A) and 1 min x 2 mg L^{-1} TRO (= CT 2, hereafter called 2B). In addition, low and high CT values were also examined for comparison, 1 min x 0.5 mg L^{-1} TRO (= CT 0.5) and 4 min x 2 mg L^{-1} TRO (= CT 8). An OC was also tested. Treatments occurred from 4 to 5 hpf. All treatments and the control had three replicates.

4.4.2.3 Experiments

Experiment 1: effects of ozone CT at gastrulation

As the embryos were found to be more tolerant of ozonation at blastula, this stage was used for the experiments.

This experiment used a factorial design of CxT. The concentrations were 0.5 and 1 mg L^{-1} TRO and ozone exposure times of 1, 3 and 5 min, creating six treatment CTs (Table 4.4.1). An OC was also tested. All treatments and the control had five replicates and treatments were carried out simultaneously.

Table 4.4.1. Experimental design for Experiment 1.

Total residual oxidants (mg L^{-1})	Time (min)	CT	Replicates
0.5	1	0.5	5
0.5	3	1.5	5
0.5	5	2.5	5
1	1	1	5
1	3	3	5
1	5	5	5
0	1	0	5

Experiment 2: ozonation with flowing water

As the results from the first experiment and the pilot studies were not congruent, it was suspected that the exchange of ozonated seawater around the embryos was affecting the earlier results. Hence, the experimental protocol was modified to continuously pump ozonated seawater into the treatment vessels throughout the treatment. This experiment used a factorial design of CxT. The concentrations were 0.25 and 0.5 mg L^{-1} TRO and ozone exposure times of 1, 2 and 3 min, creating six treatment CTs (Table 4.4.2). An OC was also tested. All treatments and the control had five replicates and treatments were carried out simultaneously.

Table 4.4.2. Experimental design for Experiment 2.

Total residual oxidants (mg L^{-1})	Time (min)	CT	Replicates
0.25	1	0.25	5
0.25	2	0.5	5
0.25	3	0.75	5
0.5	1	0.5	5
0.5	2	1	5
0.5	3	1.5	5
0	1	0	5

Animal source and spawning

Pacific Oyster broodstock three to four years of age were collected from intertidal leases at Pipeclay Lagoon, Little Swanport and Pitt Water, Tasmania and brought to IMAS Taroona for conditioning. Broodstock were conditioned in treated seawater (TSW) at approximately 20 ºC, for a minimum of seven weeks. Broodstock were fed daily on a combination of predominately *Chaetoceros calcitrans, Chaetoceros muelleri* and *Tisochrysis lutea*.

Eggs were stripped from mature females by removing the right valve, cutting adductor muscle to remove the animal and then lacerating the gonad using a scalpel blade. Stripped eggs were collected in a 1 L beaker then rinsed over a 75 µm sieve to remove tissue debris, collected on a 20 µm sieve and rinsed into a clean 1 L beaker in 300 mL of TSW. Males were treated in the same way with the exception that a sperm was screened directly over a 20 µm sieve and collected directly into 200 mL of TSW in a 500 mL beaker. Eggs were held in 300 mL TSW for a minimum of 1 h before fertilisation and gently mixed every few minutes to prevent them settling out. Sperm was held for up to 30 min prior to fertilisation. For each female or male the gametes were checked under a microscope for even, tear-shaped eggs and for motile sperm, respectively. For each cross, eggs from a single female were fertilised with sperm from two to four males. The fertilisation of between 5 to 10 million eggs per female was undertaken in 10 L of TSW by gently mixing in 3 - 4 mL of sperm. Fertilisation was confirmed by the emergence of polar bodies in the embryos at 20 min post-fertilisation. At approximately 1 h fertilisation success was scored, crosses with greater than 85% fertilisation were used in the pilot studies and experiments.

Embryos from two to four females were stocked at 50 mL^{-1} into a 450 L rectangular fibreglass tank (the 'holding tank'), where they were held until treatment. Aeration (coarse bubbles) was provided at five points in the tank to keep the embryos in suspension, temperature was set at approximately 24 ^{o}C, with ambient salinity of the Derwent estuary, ~ 34 ppt.

Ozone generation and measurement

Ozone was produced using a corona discharge ozone generator (OZ2BTU; Ozotech, Yreka, CA, USA), supplied with oxygen by an AirSep Intensity 10 oxygen concentrator. Ozone generation was achieved using an oxygen flow of 6 standard cubic feet h^{-1} and a voltage of 240 volts. Ozone was diffused directly into 20 L bucket or 30 L tub of TSW using a submersible pump and a venturi. Total residual oxidants were measured using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (Franson 1976) with a Palintest 1000 ozone meter (Palintest Australia, Peakhurst, NSW, Australia) for each CT value used.

The ozonated seawater was then transferred to 35 L rectangular tubs (treatment vessels) where it was used to treat the embryos.

Embryo treatment

Pilot Studies

To treat the embryos at the prescribed CT value a treatment-rinsing technique was developed that minimised handling damage. It was observed that it is critical to avoid external pressure to the embryos, i.e., embryos are very susceptible to damage from coming into contact with the screen in the first few hours after fertilisation. The protocol involved removing 400 mL of water and embryos from the holding tank using a plastic jug and wet sieving the contents into a mesh sieve (75 mm high, 100 mm diameter, 20 µm mesh covered PVC pipe submerged in the holding tank). Immediately prior to obtaining the 400 mL of water from the holding tank, the vessel was thoroughly mixed by oscillating a secchi disc (19 cm diameter) up and down to homogenise the embryos distribution within the holding tank. To move the embryos to the treatment vessel (containing the designated TRO concentration) the mesh sieve was loosely sealed with a 100 mm PVC cap to minimise water loss and avoid forcing the embryos against the screen. Approximately 1 - 3 cm of water was left in the sieve as it was moved and lowered into the treatment vessel at which time the cap was removed. The sieve was slowly lowered and allowed to fill to a depth of 70 mm and then lifted slowly to 1 cm depth, this procedure takes 15 s, and was repeated twice. Testing the TRO concentration with the sieve demonstrated that the holding water was replaced with the ozonated treatment water. The sieve and embryos were maintained in the ozone treatment vessel for the prescribed exposure time with slight oscillation (the mesh sieve was manually held during this time). At the end of the treatment period the sieve was removed from the ozonated water using the PVC cap to control the water and the embryos rinsed sequentially through two 10 L buckets of TSW. Each rinse involved slowly filling and draining the sieve twice, with 1 cm of water always left covering the screen. Earlier work had demonstrated all TRO are removed after two rinses. Total TRO of the treatment vessels was measured at the start and end of each treatment. After the final rinse was completed, the sieve was suspended in a 15 L bucket containing TSW to a depth of 50 mm to give volume approximately 400 mL and an embryo density of 50 mL^{-1}. Each treatment was conducted individually, by one operator, with subsequent treatment immediately following the previous one. Gentle aeration was provided to keep the embryos in suspension in the sieves after treatment. Once the treatment

was completed, the embryos were transferred to the incubation vessels to provide technical replication to allow for any environmental effects on development throughout the incubation period (Coman and Sellars 2007). For Study 1, six well tissue culture plates (10 mL) were used for the incubation and for Study 2, 50 mL centrifuge tubes were used. Larvae were stocked into the replicates at 50 mL^{-1}. Incubation vessels were placed in a water bath and supported by a rack (centrifuge tubes laying down), temperature was regulated at 24 ºC by a Ratek TH5 heater (Ratek Instruments, Boronia, VIC, Australia), salinity was approximately 34 ppt and a 12L:12D photoperiod used.

Experiments

The same technique was used for the experiments with the following exceptions. As the experimental treatments were applied at the same time, this required three sieves to be moved at once to a treatment vessel at the designated TRO concentration. Custom 'capping' plates were made to fit and hold the sieves, in order to move three sieves at once to the treatment vessels. The three sieves then provided the three treatment times used. A single sieve was used for the no ozone control which was left in non-treated water for 1 min before undergoing the rinsing procedure. During the exposure times, all sieves were left resting on a rack within the treatment vessel, which supported the sieves in place. The sieves were submerged to allow filling to a volume of 400 mL. There was no movement of the ozonated water in Experiment 1, but for Experiment 2 submersible pumps (Aquapro AP500) were used to pump water back into the sieves (~ 200 mL min^{-1}), providing a continuous flow of ozonated water around the embryos.

Sampling

Larvae (D-stage) were scored for normal development and the effects of the applied treatments were scored 24 - 28 h post-fertilisation; applying the criteria of Mottier el al. 2013 to define normal larvae. Observations of larvae were undertaken using either a Leitz Biomed (Pilot studies) or a Leica DM500 (Experiments) compound microscope at 40 or 100x magnification. Larvae were held in Sedgewick Rafter slides immobilised in a 10% ethanol TSW solution for scoring. For the pilot studies, 20 larvae were scored per replicate, in a few cases 20 could not be retrieved (presumably dead and decomposed). For the experiments 30 larvae were scored per replicate.

Statistical Analysis

The pilot studies were not analysed statistically due to the limitations in their implementation, as described earlier. For Experiment 1 and Experiment 2, two-way ANOVA was used to analyse treatment effects. If both factors were significant and there was a non-significant interaction, the replicates were pooled and re-analysed separately by one-way ANOVA. Means were compared using Tukey's HSD test. Homoscedasticity was evaluated by Levene's test and normality was tested by the Shapiro – Wilk W-test. Percentage data were arcsine square-root transformed only if the assumptions of ANOVA were violated. Significance was accepted at $P < 0.05$. Analyses were performed using SPSS statistics software v. 24.

4.4.4 Results

4.4.4.1 Pilot studies

Study 1: effects of ozonation at two embryonic stages

Treatments at blastula (4 - 5 hpf) using 1 mg L^{-1} TRO under 1 and 2 min exposure (CTs of 1 and 2, respectively) had very similar numbers of normal larvae compared to the OC (ranging from 93.3% to 96.7%), while treatments using 1 mg L^{-1} TRO under 4 min exposure (CT of 4) were negatively impacted with only 10.6% ± 3.4 S.E of normal larvae (Figure 4.4.1). Treatments at gastrula (7 - 8 hpf) using 1 mg L^{-1} TRO under 1 and 2 min exposure (CTs of 1 and 2) returned 73.3% ± 4.4 S.E. and 58.3% ± 3.3 S.E normal larvae compared to those treated under similar conditions at blastula, resulting in 93.3% ± 4.4 S.E. and 97.6% ± 1.7 S.E. normal development. While larvae treated with 1 mg L^{-1} TRO with 4 min exposure (CT of 4) at blastula were all abnormal.

Study 2: effects of ozone CT at gastrulation

Treatment with a CT value of 0.5 (1 min exposure x 0.5 mg L^{-1} TRO) returned lower numbers of normal larvae than the OC (80% ± 17.6 S.E compared to the OC with 93.3% ± 1.7); this was largely the result of one replicate with 45% normal larvae (Figure 4.4.2). Treatment with CT value of 2A (4 min exposure x 0.5 L^{-1} TRO) were negatively impacted, with 41% ± 4.5 S.E of larvae normal, while embryos treated with a CT value of 2B (1 min exposure x 2 mg L^{-1} TRO) returned a much higher proportion of normal larvae (85% ± 2.9 S.E), despite the same CT (CT = 2). Treatment with a CT value of 8 (4 min exposure x 2 mg L^{-1} TRO) produced 100% abnormal larvae.

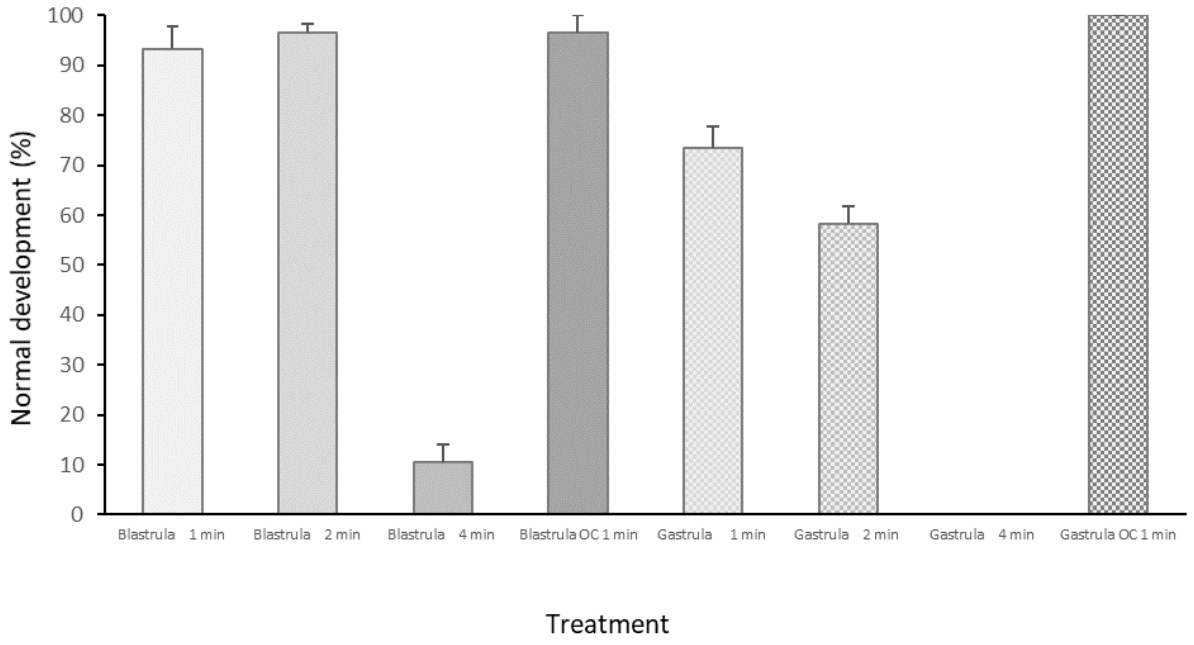

Figure 4.4.1. The effect of ozonation (1 mg L^{-1} TRO) at either blastula (~ 4 h post-fertilisation), or gastrula (~6 h post-fertilisation) exposed for 1, 2 or 4 min (CTs of 1, 2 and 4); and the ozone controls (OC) (0 ozone, 1 min) at both stages. Values are mean ± SE.

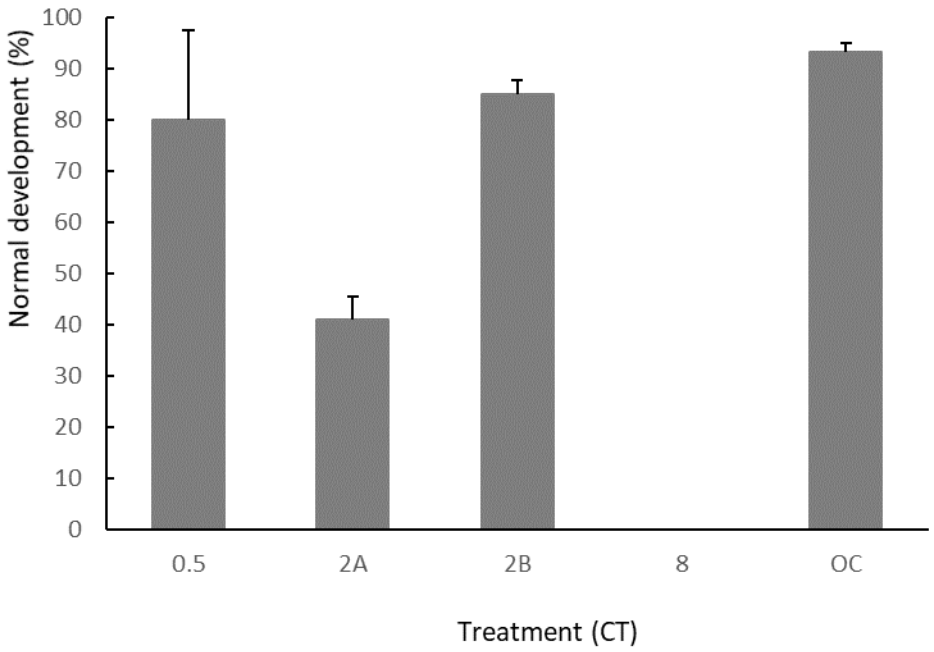

Figure 4.4.2. The effect of different ozone treatment (CT): 0.5 (1 min exposure x 0.5 mg L^{-1} TRO); 2A (4 min exposure x 0.5 L^{-1} TRO); 2B (1 min exposure x 2 mg L^{-1} TRO); 8 (4 min exposure x 2 mg L^{-1} TRO); and OC (the ozone control, 0 ozone, 1 min). Values are mean ± SE.

4.4.4.2 Experiments

Experiment 1: effects of ozone CT at gastrulation

In contrast to the results observed in the pilot studies, there was no effect of CT value (ranging from 0.5 to 5) on the proportion of normal D-stage larvae. No significant difference was observed: interaction F (2,28) = 0.188, P = 0.830; time F (2,28) = 1.471, P = 0.247; concentration F (2,28) = 0.147, P = 0.864, using transformed data. Means of the treatments ranged from 74% ± 6.9 S.E and 74% ± 3.9% for the 2.5 and 3 CT value treatments, respectively to a high of 83.3% ± 3.9 S.E for the CT value of 0.5, compared to the OC with 83.3% ± 3.3 S.E (Figure 4.4.3).

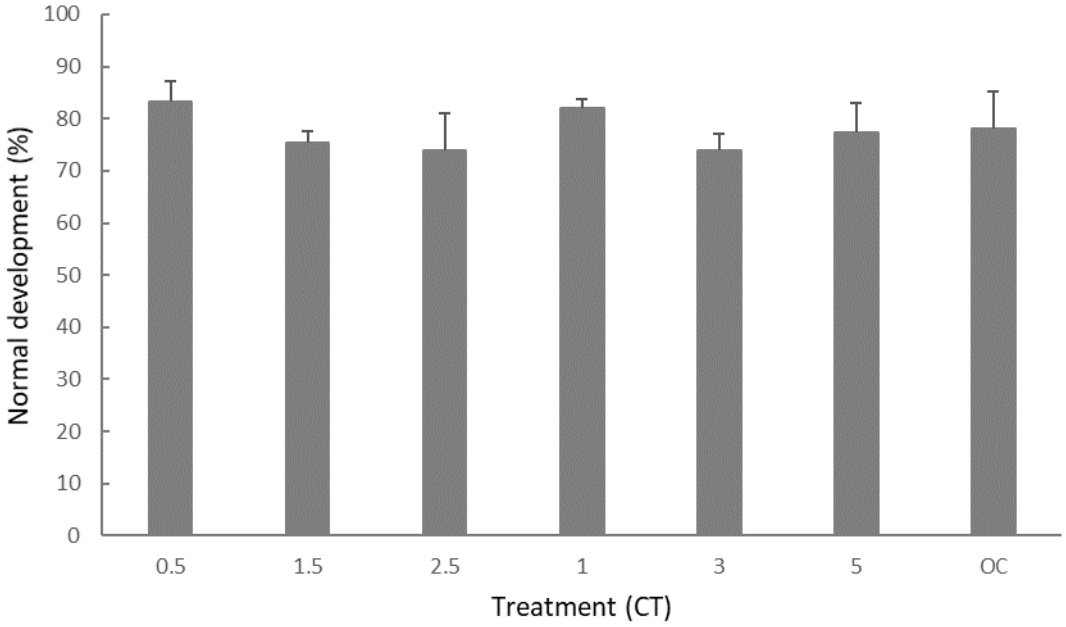

Figure 4.4.3. The effect of different ozone treatment (CT): 0.5 (1 min exposure x 0.5 mg L^{-1} TRO); 1.5 (3 min exposure x 0.5 L^{-1} TRO); 2.5 (5 min exposure x 0.5 mg L^{-1} TRO); 1 (5 min exposure x 1 mg L^{-1} TRO); 3 (3 min exposure x 1 mg L^{-1} TRO); 5 (5 min exposure x 1 mg L^{-1} TRO); and OC (the ozone control, 0 ozone, 1 min). Data were arcsine square-root transformed prior to analysis. Values are mean ± SE. Means are not significantly different ($P > 0.05$).

Experiment 2: ozonation with flowing water

Flowing water appeared to greatly increase the effect of ozone treatment. Even though the CT values were lower than used in Experiment 1, a significant effect of ozone was observed for both concentration: F (2,28) = 8.23, P = 0.002; and time F (2,28) = 22.748, P = 0.000. The interaction effect however, was non-significant F (2,28) = 0.674, P = 0.518. Embryos treated with 0.25 and 0.5 mg L^{-1} TRO had significantly lower proportion of normal larvae compared to the OC (F 2,32 = 12.987, P = 0.000) (Figure 4.4.4A). Progressively longer exposure time produced significantly lower proportions of normal larvae with each minute of exposure (F 2,27 = 37.947, P = 0.000) (Figure 4.4.4B).

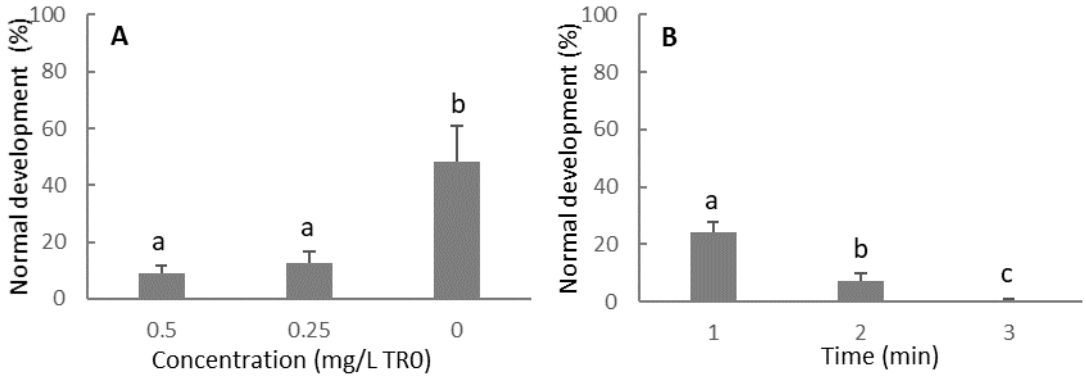

Figure 4.4.4. The effect of different ozone concentration and exposure time. Note: time analysis does not include no ozone control data. Values are mean ± SE. Data were arcsine square-root transformed prior to analysis. Different superscript indicates significantly different means ($P < 0.05$).

4.4.5 Discussion

The pilot studies indicated there could be potential to disinfect *C. gigas* embryos using ozone, with the embryos apparently tolerant of ozone exposure with a CT value up to 2. Using similar CT values in treating eggs of three fish species, Can et al. (2012) demonstrated that bacterial sterility could achieved using ozone with CT values of ≥ 2. While Ben-Atia et al. (2001), reported improved larval culture performance and zero bacterial growth of bacteria on marine agar after treating fish eggs (*Sparus aurata*) with a CT value of 0.6 (0.3 mg L^{-1} with 2 min exposure). Chang et al. (1998) demonstrated ozone concentrations as low as 0.2 mg L^{-1} for 2 min exposure (CT value of 0.4) reduced the infectivity of white spot syndrome virus. In the pilot studies we observed relatively high numbers of normal larvae (very similar to no ozone controls) exposed to CT values of 2, when embryos were treated at blastula (~ 4 - 5 hpf). The time of exposure appeared important, as a CT value of 2 created by 4 min exposure x 0.5 mg L^{-1} TRO caused more abnormal larvae than higher concentrations and shorter exposures, i.e. 1 mg L^{-1} TRO x 2 min and 2 mg L^{-1} TRO x 1 min.

However, the same result was not observed in Experiment 1, with CT values from 0.5 to 5 not significantly different to the no ozone control treatment, with the proportion of normal larvae ranging from (74 to 83.3%). This result was perplexing as CTs of 5 have adversely impacted on the hatchability of fish eggs that are encased and presumably protected by an acellular chorion. Hence, such a high level of tolerance to ozone was considered unlikely to be true for *C. gigas* embryos. It was suspected that changing the method of ozone exposure from the pilot studies to the experiments was responsible for the contrasting results, specifically it appeared likely the stillness of the embryos during treatment could be responsible. During treatment application of Experiment 1, the sieves containing the embryos rested on a supporting rack within the treatment vessel, whereas previously during the pilot studies one sieve was held by hand in the treatment vessel, with gentle manual oscillation of about 1 cm applied. This slight movement of the sieve would have caused an exchange in the ozonated water around the embryos. Hence, in Experiment 2 submersible pumps were installed into the treatment vessels so ozonated water could be pumped into the top of the submerged sieves to ensure an exchange of ozonated seawater around the embryos. The results of Experiment 2 appear to confirm the supposition, as the effect of exposure to ozonated seawater was much greater than observed in Experiment 1 and also greater than the pilot studies, despite lower CT values. Both exposure time and ozone concentration significantly impacted the proportion of normal larvae. A CT value of 0.25 retuned only 30.6% of normal larvae compared to the no ozone control with 63% normal larvae. The stark difference in the results observed between the pilot studies, Experiment 1 and Experiment 2 is attributed to the differences in the exchange of ozonated water around the embryos. It is likely the oxidative compounds are depleted by the organic load immediately against the embryos. This highlights the need for appropriate mixing of embryos/eggs and the treatment water when using ozone as a disinfectant, even with very small embryos and a low biological load.

In conclusion, an ozone CT of 0.25 is unlikely to have a useful biocidal effect in disinfecting *C. gigas* embryos of bacteria and viruses (Chan et al. 1998; Ben-Atia et al. 2001; Can et al. 2012). With this pairing of

high incidence of abnormal larvae and limited biocidal potential with a CT value of 0.25, it seems ozone is inapt for the disinfection of *C. gigas* embryos.

4.4.6 Future direction

Our studies have indicated that ozone is unlikely to be useful in disinfecting *C. gigas* embryos in hatchery settings and no follow up work appears warranted.

4.4.7 Acknowledgements

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4.5: Refinement of Laboratory Challenge Model

Peter Kirkland [a] and Xingnian Gu [a]

[a] Elizabeth Macarthur Agricultural Institute, Woodbridge Road, Menangle, NSW 2568

4.5.1 Executive Summary

The refinements to the challenge model that have been adopted during this project should provide a more robust assessment of genetic resistance to ostreid herpes virus variant (OsHV-1 µVar) and with less within family variability. There is now a capacity to work with very large numbers of animals, and with many separate replicates within a family. This system provides a capacity to manage an extremely large number of individuals under highly controlled conditions.

4.5.2 Introduction

ASI selective breeding program relies on field challenge experiments where healthy Pacific Oysters are exposed to the caused by OsHV-1 µVar is commonly known as Pacific Oyster Mortality Syndrome (POMS). Whilst field challenges have been instrumental in progressing genetic gains in POMS resistance, lab trials offer a standardized challenge system by allowing exposure of oysters to a known concentration of virus and, most likely, in the absence of other microorganisms that may contribute to mortalities. Additionally, challenge experiments can be undertaken at any time of the year whereas field challenge assessments can only be undertaken when water temperatures exceed a threshold of approximately 18 °C. This usually limits the range of oyster ages that can be challenged under field condition and there can be uncertainty of the presence of virus in the environment to allow the outcome of exposure to be assessed.

In a previous research project by Kirkland et al. (2015), an experimental challenge model was developed for OsHV-1 µVar. This model had 2 main attributes: long term cryopreservation of OsHV-1 µVar that would provide a stable virus preparation; and a model in which oysters of various ages and developmental stages could be exposed to OsHV-1 and the impact could be measured.

The objectives of this project were to:

- refine the challenge model with the goal of a 70% correlation between the laboratory and field tests;
- define relationships between spat size and viral dose; and
- continue the development of a larval challenge model and identify opportunities where larval challenges can improve genetic progress for both the breeding population and for commercial deployment.

4.5.3 Methods

A series of studies were undertaken to generate data to meet the project objectives. Due to time restrictions over which oysters were available at particular developmental stages and ages, these studies were not undertaken in the order of the objectives. Rather, experiments were undertaken in-line with the availability of suitable oysters (from early larval stages through to spat of varying ages).

4.5.3.1 Source of oysters

Pacific Oysters were obtained from the IMAS-ASI Biosecurity Facility in Tasmania (TAS) to determine the outcome of spat to exposure to OsHV-1 virus at different ages. Stock from highly susceptible families was sourced from one to two distinct genetic lines ranging in size from 500 µm to 5 mm. Stock was tested on the

2016 and 2017 year classes and were airfreighted to Elizabeth McArthur Agricultural Institute (EMAI) by a 'same day' courier service or overnight services. They generally arrived within 6 to 12 h of collection. Initially, challenges were carried out on stock within the larval or pediveliger stage using animals obtained from the New South Wales (NSW) Department of Primary Industries hatchery at Port Stephens, NSW. This area contained ASI stock that were highly susceptible to OsHV-1 µVar and animals used were transported directly to EMAI by road transport (approximately 4 h travelling time).

4.5.3.2 Virus preparation

Cryopreserved OsHV-1 µVar was used for all experiments in this project. The virus was derived directly from the prototype Australian virus obtained from the original outbreak in the Georges River, NSW. Two batches of virus were used throughout these studies and were prepared from a master stock to ensure consistency of the virus stock with the original strain. As there are no *in vitro* cell culture systems available for oysters, OsHV-1 µVar virus was amplified by a single round of *in vivo* replication. These batches were obtained from one round of amplification from the original field strain. Oysters were "relaxed" by submersion in magnesium chloride solution then inoculated by intramuscular injection. Inoculated oysters that died between 36 - 72 h after inoculation were collected and a small portion of mantle or gill was taken to quantify the virus concentration by real time PCR (qPCR). Subsequently, mantle and gill tissues were collected and pooled from all oysters with high virus concentrations (Ct value < 15 by qPCR). The tissues were homogenized, cell debris removed by centrifugation and the clarified supernatant frozen at -80 ^{o}C after the addition of cryoprotectants. After virus had been stored frozen at -80 ^{o}C for several weeks, the concentration of infectious virus was determined by titration by immersion of susceptible oysters in artificial seawater (ASW) containing OsHV-1 µVar. The concentration of infectious virus was determined by the highest virus dilution of virus that caused mortalities in 50% of the oysters that were exposed to the virus. When there was a need to produce a new batch of virus, the titration of the new batch was tested in parallel with the existing batch to ensure reproducibility and standardization.

4.5.3.3 Virus detection assays

When oysters are being used in a challenge study (whether in the field or the laboratory), as there are no grossly visible or distinctive signs of infection with OsHV-1 µVar, it is necessary to use a laboratory assay to confirm that the deaths were due to this virus. This is achieved by collecting and testing samples of mantle and gill or, where the spat are too small to easily dissect specific tissues, a portion of the animal that would include mantle and/or gill. The tissue samples are disrupted by mechanical bead beating or by enzymatic digestion, or, when larvae or spat < 5 mm are tested, the whole animal is processed, usually by enzymatic digestion. The nucleic acid is subsequently purified from the disrupted tissue and then tested by "real time" polymerase chain reaction (qPCR) assay that is specific for OsHV-1 µVar. This qPCR both detects and quantifies the amount of OsHV-1 µVar DNA that is present in the sample.

4.5.3.4 Virus exposure studies

During previous research projects, this laboratory challenge model had been used to infect oysters ranging in age from spat (approximately three months old) through to juvenile and adult oysters (up to 15 months old). One of the principle objectives was to establish whether oysters could be reliably infected at much earlier development stages and younger age to allow genetic selections to be made at an earlier time. To achieve this objective, a range of investigations were undertaken and included infections of: (1) larval stages from soon after fertilization and at weekly intervals thereafter for five weeks; and (2) spat ranging in size including less than 0.5 mm, approximately 1 mm, 2 - 3 and 5 mm.

To support the handling and management of extremely small stock (many of which required microscopic examination), animals were held in cell culture dishes containing 96, 24, 12 or 6 wells. Corresponding well diameters ranged from 6.96 mm to 34.8 mm and each well held volumes of water from 0.34 mL to 16.8 mL. Depending on the size of the oysters, different sized wells and/or differing numbers of oysters were placed in each well. For example, 15 - 20 motile larvae could be held in 96 well plates whereas five or 10 larger (5 mm) spat might be placed in a 12 or 6 well plate. To achieve this, larvae were placed in a sufficiently large volume of water to reduce the density and were then collected by aspiration using a modified pipette while spat were individually collected by holding with wooden skewers (as miniature "chopsticks"). Each well contained artificial seawater (ASW - Ocean Nature sea salt, (Aquasonics), in de-chlorinated tap water). In early experiments, water was changed at two to three intervals and oysters were fed with algae appropriate

for their age. Algae were supplied by staff at the NSW DPI hatchery at Port Stephens Fisheries Centre (PSFC), Port Stephens.

For infection with OsHV-1 µVar, ten-fold dilutions of the standard virus preparation were made in ASW and separate lots of oysters were exposed to one of two or three different virus concentrations. This was achieved by first placing the required number of oysters in appropriately sized wells. They contained the virus suspension at one of the selected dilutions; approximately 50% of the standard volume of water relative to the well size. Oysters were immersed in the virus suspension for 24 h before additional ASW was added to maximise each well volume. The model was later refined to minimize handling of large numbers of potentially infected spat. As soon as all spat had been placed into the plates the virus was added to all wells (except controls).

Although attempts were made to standardize the challenge virus dose expressed as "oyster infective doses" by including stock from a highly susceptible genotype as controls, this only provided a guide to the dose of infectious virus used due to the variation in susceptibility of oysters at varying ages. However, a more exact measure of the virus concentration used had been established in Kirkland et al. (2015). by determining the number of virus particles present, as calculated using qPCR and expressed as genome equivalents of OsHV-1 µVar. A Ct value of 12 was shown to be equivalent to approximately 10^7 copies of OsHV-1 µVar DNA μL^{-1}. Since there are two copies of the qPCR target in each OsHV-1 µVar genome, a Ct of 12 equates to approximately 5 x 10^6 OsHV-1 µVar genomes μL^{-1}. It had been established that some oysters could become infected using a virus concentration as low as 10^4 OsHV-1 µVar genome copies per mL (10^{-5} dilution) while minimum infective concentrations for other oysters was 10^6 – 10^7 OsHV-1 µVar genome copies per mL (10^{-3} and 10^{-2} dilutions respectively). In order to achieve an outcome on each occasion that the model was used, ideally with deaths in only a proportion of the oysters at one of the concentrations, two or three different virus doses were used for each challenge. The range of concentrations was adjusted depending on the age of oysters under study, with young spat (less than 4 months) usually exposed to 10^6 – 10^7 OsHV-1 µVar genome copies per mL and older spat and juvenile oysters 10^4 – 10^5 OsHV-1 µVar genome copies per mL. Larvae were sometimes exposed to even higher virus concentrations. Confirmation of the virus dose used in each challenge experiment was confirmed by testing a series of $\log_{10}$ dilutions of virus by qPCR.

The impact of OsHV-1 µVar infection was routinely assessed by determination of the mortality rate in different groups of oysters relative to unexposed stock of the same age and genotype. For larval stages and very small spat, this required microscopic examination or examination under an illuminated magnifier. If there was no clear evidence of disease (e.g. lack of motility for larvae, open or gaping shells for spat), a response to stimuli (touching with a disposable wooden skewer) was also used. However, because there are no grossly observable and characteristic changes associated with OsHV-1 µVar infection, during the refinement of the challenge model for larvae and very young spat, tissues from dead oysters were also tested by qPCR to confirm that deaths were due to this virus. This was also used to confirm that biosecurity had been maintained and any higher than expected deaths in unexposed (negative control) groups were not due to OsHV-1 µVar. Nevertheless, testing of large numbers of individual oysters by qPCR is both labour intensive and expensive. Consequently, with increasing experience with the model for young stock, it was possible to monitor the presence or absence and level of virus replication in animal groups by testing of the ASW in which individual groups were being held.

Over time, the laboratory challenge model was refined by evaluating potential sources of variability and repeatability and, if considered to be reducing the accuracy of the data, alternative steps were evaluated. Ultimately, the optimal challenge model for young spat involved the assessment of a moderately large number of animals contained (depending on size) in 24 or 12 well plates. These animals were held in small groups, with a large number of replicates. The different treatments, consisting of different virus doses and unexposed controls were also distributed across multiple plates. Mortalities were assessed at days 5 and 7 after challenge. The raw mortality data was provided to the project geneticist for evaluation and comparison with field data.

4.5.3 Results, discussion and conclusion

The various experiments that were undertaken showed that there was an increase in susceptibility of spat as the age and size increased. While larvae can be infected with OsHV-1 µVar, in general they require exposure to much higher concentrations of virus than settled spat and juvenile animals. Further, there was a stage

around settling where they appeared to have a very high but short-lived level of resistance. Collectively, these observations created some uncertainty about the practical application of challenge during early (larval) stages of development. Consequently, comparisons were undertaken in a longitudinal study where animals from the same families were assessed at different stages of growth. It was shown that there was an increase in susceptibility in animals that were an average of 1 mm compared to 500 µm (0.5 mm). In turn, 1 mm stock tended to be less susceptible than 2- or 3-mm animals. There was evidence of variability in responses with spat that were less than 2 mm in size but spat that were 2 mm or larger gave consistent, reproducible responses within families.

In addition to variation in susceptibility, there were practical aspects that must be considered in the operation of such a model. The handling steps to place very small spat into wells required considerable manual dexterity and care to avoid damage to the animals, particularly when a challenge across all family lines might involve handling of > 10,000 individual animals. Additional considerations were that there were difficulties with assessing the viability of oysters at very early/young developmental stages. To resolve the status of animals, sometimes it was necessary to screen by qPCR individuals or ASW in which they had been held. This substantially increased workload as well as costs for testing.

Taking all these factors into account, a refined challenge model was established, based on the following considerations:

- ideally spat are between 2 - 4 mm;
- spat are managed in 24 or 12 well plates;
- spat are placed in groups of five with at least 10 (often 20) replicates for each virus dose or as controls;
- animals in each of the treatment and control groups are divided equally across multiple plates;
- animals are exposed to virus as soon as practical after receipt;
- at least two concentrations of virus are used;
- virus dilutions are routinely tested by qPCR to confirm the accuracy of titrations;
- once challenged, there are no water changes or feeding; and
- generally, the outcome is determined on the basis of mortality, with qPCR testing restricted to the resolution of uncertain results and usually by testing of ASW from the group under investigation.

During these evaluations, 61 families from the 2016-year class from TAS were challenged on two different occasions and on each occasion at several different sizes. In the summer of 2017/18 a total of 69 of the 2017 families bred in TAS and 60 of the 2017/18 2017 families bred in South Australia (SA) were challenged in the laboratory model. Each challenge treatment group involved approximately 100 spat per treatment. To put the capacity of the system into perspective, these challenges included a total of more than 38,000 individual spat. An example of the differences in mortality rates observed between families and at the two challenge virus doses is shown in Figure 4.5.1.

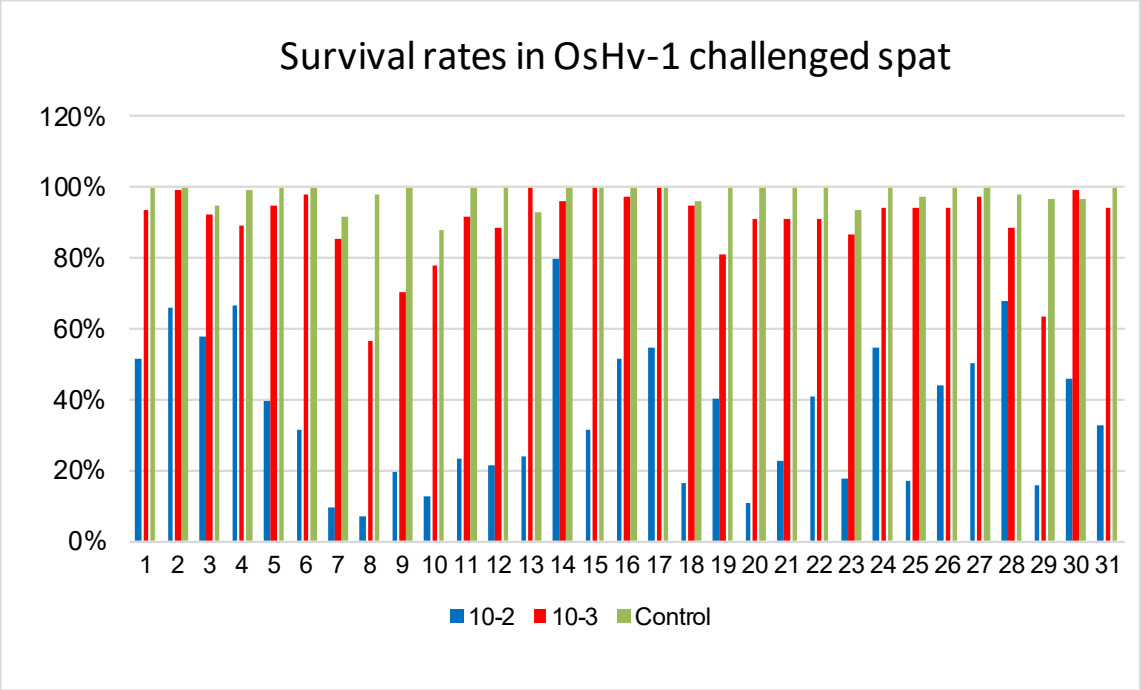

Figure 4.5.1. Differences in mortality rates observed between families and at the two challenge virus doses.

Additional challenges were undertaken to assess variability in the challenge model and to optimize group sizes. Despite most families having few losses in the unchallenged stock, some experienced significant mortalities in the negative controls; rendering the virus challenge data unsuitable for analysis. This occurred more frequently in SA stock compared with TAS.

Comparisons of the data obtained from the laboratory challenge model with field challenges are described in section 4.1.

These studies have demonstrated the advantages of the laboratory challenge model. These include:

- standardising controlled dose of the same virus over a long period of time;
- virus challenges can be undertaken at any time without the adverse influences of environmental factors such as low temperature and other weather elements;
- challenges are not dependent on ongoing virus transmission in the field;
- the risk of an excessive challenge resulting from local amplification of the virus can be averted;
- with the availability of appropriate facilities, biosecurity risks associated with handling of OsHV-1 µVar can be alleviated; and
- the observed mortalities are likely to be the impact of OsHV-1 µVar virus without additional impacts of other microorganisms.

The refinements to the challenge model that have been adopted during this project should provide a more robust assessment of genetic resistance to OsHV-1 µVar virus and with less within family variability. There is now a capacity to work with very large numbers of animals, and with many separate replicates within a family. For example, challenges have been undertaken with groups of up to 120 spat being exposed in groups of five animals to three different treatments that have been replicated across up to five separate plates. This separation of oysters into small groups also reduces the impact that a small number of highly susceptible individuals may have on their peers as a result of a high level of virus amplification and lateral transmission. This system provides a capacity to manage an extremely large number of individuals under highly controlled conditions. Factors such as fine temperature control are readily achieved by using large laboratory incubators where there is more precise control of the temperature. When challenges involve large oysters, fine temperature control of water is more difficult because this can only be achieved on a large scale by holding containers in a controlled temperature room.

The current laboratory challenge model provides a mechanism for pathogen interactions to be investigated and potentially incorporated into a future challenge system. The challenge of very young spat with OsHV-1

µVar virus can be successfully undertaken and allows the identification of OsHV-1 µVar resistant stock at an early age.

4.5.4 Future direction

The role of other microorganisms as secondary factors that inevitably contribute to the severity of disease remains unclear and controversial. As with almost all viral diseases, secondary microbial infection will usually exacerbate the clinical outcome, but the microorganisms involved can vary widely, depending on the local flora to which animals are exposed. Further work is required to understand these complex interactions.

4.5.5 Acknowledgements

NIL

4.5.6 References

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4.6: IMAS Laboratory Challenge Development

Andrew Trotter [a], Geoff Endo [a], Matthew Cunningham [b], Chris Carter [a] and Greg Smith [a]

[a] Fisheries and Aquaculture, Institute for Marine & Antarctic Studies (IMAS), University of Tasmania, Private Bag 49, Hobart, TAS 7001
[b] Australian Seafood Industries (ASI), Tech 3, Tasmanian Technopark, Innovation Drive, Dowsing Point TAS 7010

4.6.1 Executive Summary

Australian Seafood Industries (ASI) operates a Pacific Oyster selective breeding program. Presently the focus is on breeding spat with resistance to Pacific Oyster Mortality Syndrome (POMS); the viral disease caused by Ostreid Herpes Virus Variant (OsHV-1 µVar). As a component of this work, Institute of Marine and Antarctic Studies (IMAS) has been tasked with developing a POMS laboratory challenge in Tasmania (TAS); including the development of an aquatic PC2 laboratory. This undertaking is designed to ease logistical constraints associated with translocation of spat of selected families from TAS to New South Wales, where challenges have been undertaken previously. The aims of this work were to: 1) develop a laboratory challenge at IMAS; 2) further investigate the findings of Chapter 1 that estuary conditioning is required for normal progression of the disease; and 3) examine the suitability of translocating infected animals from the field to the laboratory for tissue collection. We successfully adapted methodologies of POMS laboratory challenges used elsewhere to develop a laboratory challenge at IMAS. Our laboratory challenge demonstrated some congruence to estimated breeding values (EBV) of families obtained from field trial data, but overall the correlations to the field challenges were moderate to weak. The results demonstrated that spat exposed in the laboratory with OsHV-1 µVar after being 'conditioned' on a commercial oyster lease had lower survival than those held in a hatchery. We also confirmed that spat infected on a lease go on to develop the POMS without further viral exposure and this suggests that field infected spat could be used as a source for laboratory-based collections of tissues. However, the poor correlation with field EBV observed in the lease controls, indicates that there is variability induced through the translocation process and/or holding spat in the laboratory during the disease process and this would require further investigation to determine the worth of this methodology.

4.6.2 Introduction

Since the 1990's mass mortalities caused by Ostreid Herpes Virus (OsHV-1) and a variant (OsHV-1 µVar) has severely impacted aquaculture production of the Pacific Oyster in Europe, New Zealand and Australia. Extensive research has been undertaken since the original outbreaks of OsHV-1 in France (Nicolas et al. 1992; Renault et al. 1994; Pernet et al. 2016; Rodgers et al. 2018). This disease is multi-factorial, driven by environmental stressors, host physiology, husbandry practices and secondary pathogens (Arzul et al. 2001; Barbosa-Solomieu et al. 2005; Garnier et al. 2007; Samain et al. 2007; Petton 2015; Azéma et al. 2017; Burioli et al. 2017; Hick et al. 2018; de Lorgeril et al. 2018). The disease caused by OsHV-1 µvar is commonly known as Pacific Oyster Mortality Syndrome (POMS) (Paul-Pont et al. 2013).

Selective breeding has made significant gains towards producing POMS resistant family lines, particularly in France but also in New Zealand and Australia (Samain et al. 2007; Kube et al. 2018; Camara and Symonds, 2014; Dégremont et al. 2015a; Camara et al. 2017). Both field and laboratory challenges for resistance to POMS have been used in this research (Kube et al. 2018; Dégremont et al. 2015b; Camara et al. 2017). Selective breeding in Australia has been implemented and run by Australian Seafoods Industries (ASI) in collaboration with CSIRO, Hobart, Tasmania (TAS) and the Elizabeth Macarthur Agricultural Institute (EMAI), Menangle, New South Wales (NSW). This breeding program has enabled the Australian Pacific Oyster industry to move into a POMS recovery phase, by breeding stocks that have substantial resistance to

the disease as juveniles. Developing resistance in spat, which are more susceptible to the disease, is now the primary focus of the program. As a component of the *Future Oysters CRC Project 2016-801 Enhancing Pacific Oyster Breeding to Optimise National Benefits,* IMAS has been tasked with developing a POMS laboratory challenge in TAS. This undertaking was done to ease logistical constraints associated with translocation of spat from TAS to NSW where the challenges have previously been undertaken due to a lack of facilities in Hobart where infectious studies could be undertaken.

ASI plans to transition to a genomic platform for selective breeding in the near future. To use genomic selection for POMS resistance, DNA needs to be collected from both dead and living oysters after exposure. It is difficult to collect DNA from dead spat during field challenges for POMS, due to the rapid decay of tissue and the unpredictability of when and where the disease will occur. Hence, genomic selective breeding will rely heavily on a laboratory challenge; or a method to translocate infected spat from the field and collect tissue from dead and surviving spat in the laboratory. Intrinsically, a laboratory challenge needs to closely mimic a field challenge in order to capture the complexities of the disease and this is likely to be inherently difficult (Petton et al. 2015; de Lorgeril et al. 2018; King et al. 2019). Since the outbreak of POMS in TAS in 2015 the ASI breeding program has operated in the Biosecure Oyster Hatchery at IMAS (Pertl et al. 2018; Chapter 3). Hence, larvae and spat are kept in an environment where hygiene is very high and the incoming culture water is treated by a combination of filtration and sterilisation by ozonation and UV irradiation (Pertl et al. 2018; Chapter 3). In some laboratory challenges at conducted at EMAI, spat that were not exposed to estuarine environmental conditions prior to laboratory experimentation, resulted in weak correlation to field challenges (Chapter 1). It is likely that spat held in such an environment have depauperate microbiomes compared to a natural estuarine environment (Lokmer et al. 2016) and may well lack some of the pathogenic bacteria that complete the infection process of this disease, leading to death (de Lorgeril et al. 2018). Hence, in developing a laboratory challenge at IMAS, we wanted to further examine the finding of Chapter 1 that 'conditioning' of spat in an estuary is essential to allow the natural progression of the disease in laboratory challenged spat.

The aims of this work were to: 1) develop a laboratory challenge at IMAS; 2) further investigate the findings of Chapter 1 that estuary conditioning is required for normal progression of the disease; and 3) examine the suitability of translocating infected animals from the field to the laboratory for tissue collection.

4.6.3 Methods

4.6.3.1 Development of a PC2 laboratory

On 25 April 2017 works commenced to establish a PC2 laboratory at Institute of Marine and Antarctic Studies (IMAS), with the animal holding systems, microbiology and microscopy facilities completed on 10 November 2017 and the wastewater treatment plant completed on 21 June 2018. Following completion of the wastewater treatment plant an physical containment (PC) audit was completed on 4 August 2018 by Neil Wall Consulting. A number of items were identified as non-compliant to either AS/NZS 2243.3:2010 and AS/NZS 2982:2010. Remediation was completed by 2 August 2018 and the facility was audited again on 7 August and reported as PC2 compliant. Approval to operate disease challenges was given by the Chief Veterinary Officer of Tasmania on 2 November 2018.

4.6.3.2 Experimental animals

Spat used in the challenge experiments were reared by ASI in September and October 2018 for the Year Class 2018 families of the ASI breeding program. All breeding was undertaken in the IMAS Biosecure Oyster Hatchery (approved by Biosecurity Tasmania) (Pertl et al. 2018; Chapter 3). This facility is supplied with treated seawater (TSW) from the IMAS Seawater Treatment Plant (sterilised by ozone contact time of 6 h at an oxidative reduction potential (ORP) of 750 mV, particle filtration to 5 µM and UV irradiation). This treatment procedures ensured no live OsHV-1 µVar were introduced to the challenge systems from the water source (Derwent estuary) (Whittington et al. 2015; Powell and Scolding, 2016). Spat were reared in upwelling pots with temperature maintained at approximately 21 ^{o}C. Nursery system culture water was exchanged daily (with TSW) and fresh algae was fed to the spat daily (various combinations of *Chaetoceros calcitrans, Chaetoceros muelleri*, *Tisochrysis lutea, Diacronema lutheri* and *Skeletonema pseudcostatum*) with occasional supplementary feeding with Shellfish Diet 1800® (Reed Mariculture), at a rate of 2% of the

animals dry body weight per day. The entire upwelling system was cleaned weekly by scrubbing and chlorination (200 ppm). Spat were washed daily to remove pseudo-faeces and detritus. Prior to use in the IMAS laboratory challenges spat were tested for the presence of OsHV-1 µVar under the routine operational procedures of ASI. Testing was conducted by the NATA accredited Department of Primary Industries Water and Environment, Animal Health Laboratory. The testing undertaken did not detect OsHV-1 µVar in any of the cultures.

4.6.3.3 Detection of OsHV-1 µVar

Tissue homogenisation and DNA extraction

Approximately 20 mg of oyster tissue (mantle and gill) was added to 180 µL Qiagen ATL buffer and 20 µL Proteinase K (Sigma-Aldrich) in a 1.8 mL Eppendorf tube. The suspension was incubated at 56 ºC for 2 - 6 h (depending on the sample being fresh or fixed) to digest the oyster tissue (Jenkins et al. 2013). OsHV-1 µVar DNA extraction was done using a MagMax™-96 Viral RNA Isolation Kit (ThermoFisher Scientific) manually according to the manufacturer's protocol, with a 96 well magnetic-ring stand (ThermoFisher Scientific) and orbital shaker, with elution into 50 µL of the kit buffer.

RT-qPCR analysis

Quantitation and detection of OsHV-1 µVar was done via a TaqMan® quantitative PCR (qPCR) assay using oligonucleotide primers and probe originally developed by (Martenot et al. 2010). Briefly, amplification was performed on a Rotor-Gene Q thermal-cycler in 20 µL reactions containing 2 µL of template DNA, 0.8 µL of forward primer and reverse primer (10 µM) (Integrated DNA Technologies, IDT), 0.4 µL of probe (10 µM) (IDT), 10 µL of PrimeTime® Gene Expression Master Mix (IDT) and 6 µL of molecular biology grade water. Thermocycling parameters were: 95 °C for 5 min; 50 cycles of 95 °C for 15 s and 60 °C for 1 min, with fluorescence data analysed with Roto-Gene Q Software 2.3.1.49 (Qiagen).

4.6.3.4 OsHV-1 µVar preparation

Virus stocks were prepared based on methodologies of Schikorski et al. (2011) and Kirkland et al. (2015). Briefly, mantle and gill (~ 25 mg) was dissected from individual moribund or dead oysters and placed in 95% ethanol prior to extraction (either stored at -20 ºC or processed on the same day), and screening for OsHV-1 µVar using qPCR. Mantle and gill were dissected from oysters confirmed to have high viral loads and pooled prior to being homogenised using an Ultra-Turrax mixer (4 x 15 s at 30,000 rpm), followed by centrifugation (3000 g, 10 min, 4 ºC), with the supernatant filtered using syringe filters at 5, 1.2, 0.45 and 0.22 µm. The concentration of OsHV-1 µVar in the viral suspensions were estimated via qPCR. The same procedure was used to prepare a negative control stock from virus-free oysters confirmed by qPCR.

4.6.3.5 Laboratory challenges

Laboratory challenge systems

Two challenge holding systems were used during for the laboratory challenges: 1) mesh baskets in 35 L troughs (Basket System); and 2) 10 mL tissue culture plates (TCP System). All laboratory challenges were conducted with TSW from the IMAS Seawater Treatment Plant. Treatment procedures would ensure no live OsHV-1 µVar are present in the water source (Whittington et al. 2015; Powell and Scolding, 2016).

The Basket System comprised nine rectangular plastic troughs (holding tanks), approximately 75 cm long, 46 cm wide, 17.5 high (Orion Australia), each filled with 35 L of TSW. Each trough housed four plastic baskets (1.8 mm mesh), approximately 31 cm long, 11 cm wide, 7 cm high, with each basket partitioned with a plastic divider to make five compartments per basket. This configuration provided 180 individual compartments. Baskets were suspended in the troughs on a plastic frame, whereby each basket protruded above the water surface by approximately 10 mm, giving each compartment a volume of about 450 mL. Water circulation within the troughs was maintained via submersible pumps (AP210, Aquapro). Gentle aeration was provided immediately in front of the outlet of the submersible pump. This system was operated with batch water exchange of 50% per day, water exchange was facilitated by removing an internal standpipe and draining to a set level, replacing the standpipe and refilling with TSW via a flexible hose. Spat were fed a combination of live algae (*T. lutea* and *C. muelleri*) and Shellfish Diet 1800® daily, at a rate of 2% of

animals dry body weight per day. Water quality parameters were recorded daily, prior to the water exchange.

The TCP System is based on laboratory challenges developed at EMAI (Chapter 1; Kirkland et al. 2015). Six-well tissue culture plates (ThermoScientific) were filled with 10 mL TSW and incubated on a benchtop within a temperature-controlled laboratory. Spat were not fed in this system. Water temperature was approximately 25 ^{o}C.

Twenty of the 74 ASI Year Class 2018 families were used in each challenge. Families were selected for use in the challenges based on whether their families had previously had demonstrated either high, medium or low resistance to POMS (based on the first field trial on the 2018 Year Class families: 1801), this allowed testing of a representative spread of the entire year class (Table 4.6.1). To compare the IMAS laboratory challenges to the ASI field challenges for 2018-2019, the May 2019 estimated breeding values (EBV) (provided by P. Kube, CSIRO) and survival (%) (at termination) for each challenge were analysed using Pearson's *r*.

Challenge 1

Challenge 1 (C1) was undertaken in the Basket System. Three triplicate treatments were examined: 1) lease conditioned and exposed to 3.5 x 10^4 copies mL^{-1} of OsHV-1 µVar (HD); 2) lease conditioned, exposed to 3.5 x 10^3 copies mL^{-1} of OsHV-1 µVar (LD); and 3) lease conditioned, exposed to a control (homogenate made from oysters negative for OsHV-1 µVar) (C), (Table 4.6.2). Twenty families were used in the challenge, selected to have either high (25.9 to 37.5%), medium (10.7 to 15.1%) or low survival (0.7 to 7.2%) based on the first field trial conducted by ASI for the 2018 Year Class (ASI trial 1801) (Table 4.6.1). This was considered to be a hard impact of the disease (Table 4.6.3). The age of spat ranged between 127 to 151 days post-fertilisation on the day of treatment (Table 4.6.1). Families selected for the challenge were graded to a uniform size (~7 mm) and kept in separate upwellers for approximately one week prior to deployment to the oyster grow-out lease. The day before deployment spat of each family was placed into mesh bags. Spat for the lease conditioned treatments were taken from the IMAS hatchery on 15 February 2019 to Pitt Water (lease 46, -42.7998404, 147.4756622) and returned to IMAS six days later on 21 February. During the lease conditioning period, POMS was being recorded in Pitt Water (ASI trial 1805 received at light hit after being deployed 7 February). Twenty spat were sampled from each family to test for OsHV-1 µVar when the animals were returned from exposure on the lease.

Table 4.6.1. Family identification number and survival data (%) for ASI field trial 1801 and age of each family used for IMAS Laboratory Challenges 1 to 3 on the day of treatment.

Family ID	Survival in ASI Field Trial 1801 (%)	Age (day post-fertilisation)		
		Challenge 1	Challenge 2	Challenge 3
2018004	50.8	-	167	193
2018007	42.2	-	167	193
2018009	12.8	151	-	-
2018024	37.5	149	165	191
2018029	14.3	149	165	191
2018032	2	149	165	191
2018033	12.1	149	165	191
2018034	37.5	149	165	191
2018039	10.7	149	165	191
2018041	0.7	149	165	191
2018048	7.2	147	163	189
2018058	30.2	129	145	171
2018061	2	129	145	171
2018062	15.1	129	145	171
2018064	6.3	129	145	171
2018065	1.7	129	145	171
2018067	2.3	129	145	171
2018077	28.8	127	143	169
2018080	25.9	127	-	-
2018081	14.8	127	143	169
2018087	1.7	127	143	169
2018088	14.3	127	-	-
2018095	66.7	-	141	167

Table 4.6.2. Challenges system, viral concentration, number of families and replicates, treatments and number of spat for each replicate for IMAS Laboratory Challenges 1 to 3 on the day of treatment.

Challenge	System	Copies of OsHV-1 µvar mL^{-1}	Number of ASI families	Replicates	Treatments	Spat per replicate
Challenge 1	Basket System	3.5×10^4/3.5×10^3	20	3	HD, LD, C	~50
Challenge 2	TCP System	7.5×10^5	20	3	L+, L-, H+, H-	10
Challenge 3	TCP System	6×10^5	20	3	L+, L-, H+, H-	10

HD (lease conditioned exposed to a moderate dose of OsHV-1 µVar); LD (lease conditioned, exposed to a low dose of OsHV-1 µVar); C (lease conditioned, exposed to a control {a suspension made from oysters negative for OsHV-1 µVar}); L+ (lease conditioned, exposed to OsHV-1 µVar); L- (lease conditioned, exposed to a control); H+ (hatchery conditioned, exposed to OsHV-1 µVar) H- (hatchery exposed to a control).

Table 4.6.3. Mean survival for all families for ASI field trials in Pitt Water for the 2018 Year Class.

ASI Trial ID	Site	Deployment Date	Survival	POMS Impact
1801	Pitt Water (lease 46)	20-Dec-2018	14%	Hard
1802	Pitt Water (lease 81)	07-Jan-2019	31%	Moderate
1803	Pitt Water (lease 46)	17-Jan-2019	29%	Moderate
1805	Pitt Water (lease 46)	07-Feb-2019	41%	Light

Samples were processed as described above for DNA extraction with all 20 spat from each family pooled into one sample for qPCR testing. Eighteen of the 20 families tested positive for OsHV-1 µVar (cycle-threshold {Ct} values ≤37, considered positive, Kirkland et al. 2015). The Ct values of positive samples ranged from 20.9 to 34.1. The remaining spat were held in a recirculation system in the PC2 laboratory overnight before allocation to the Basket System. Treatment replicates comprised one 35 L holding tank, each with 20 basket compartments for the 20 families challenged (Table 4.6.2). Families were randomly allocated within each treatment replicate. The following day (day 0), approximately 50 spat (by weight) were allocated into each treatment replicate and held in the Basket System prior to treatment. No mortalities were observed in the spat at this time. At 1400 h the baskets were lifted from the holding tanks and the TSW was replaced with 10 L of MgCl solution (50 g L^{-1} in 35% TSW, 65% fresh water) to anesthetise the spat (Suquet et al. 2009). The baskets were returned to the holding tank without the plastic frame in place to allow suspension of the spat in the 10 L anaesthetic solution for two hours. At 1600 h each basket was placed in a 1 L viral suspension in rectangular plastic containers, 38.5 cm long, 15 cm wide, 15 cm high at the designated concentrations for 16 h (Camara et al. 2017), after which the baskets were returned to the holding tanks. Survival was recorded on day 3, day 4 and day 6 of the challenge with dead animals removed when recorded. Dead spat (five specimens from a high, medium and low resistance family) were collected from each treatment and fixed in 95% to test for the presence of OsHv-1 µVar. Mean temperature was 25.0 ± 0.1 (S.D), 25.0 ± 0.4, and 24.8 ± 0.6 ºC for C, LD and HD, respectively, during the challenge.

Challenge 2

Challenge 2 (C2) was undertaken in the TCP system. Four triplicate treatments were examined: 1) lease conditioned, exposed to OsHV-1 µVar (L+); 2) lease conditioned, exposed to a control (homogenate made from oysters negative for OsHV-1 µVar) (L-); hatchery conditioned, exposed to OsHV-1 µVar (H+); and hatchery conditioned, exposed to the control (H-). The OsHV-1 µVar concentration was 7.5×10^5 mL^{-1} (Table 4.6.2). Twenty families were used in the challenge, selected to have either high (28.8 to 66.7%), medium (10.7 to 15.1%) or low survival (0.7 to 7.2%) when challenged by OsHV-1 µVar, based on the first field trial conducted by ASI for the 2018 Year Class (ASI trial 1801) (Table 4.6.1). The age of spat ranged between 141 to 167 days post-fertilisation on the day of treatment (Table 4.6.1). Families selected for the challenge were graded to a uniform size (~10 mm) and kept in separate upwellers for approximately one week prior to deployment to an oyster growout lease. The day before deployment spat of each family were placed into mesh bags. Spat for the lease conditioned treatments were taken from the IMAS hatchery on 4 March 2019 to Pitt Water (lease 81, -42.8076011, 147.4864306) and returned to IMAS three days later on 7 March. During the lease conditioning period farmers were recording incidences of POMS in Pitt Water (N. Griggs ASI, pers. comm.). Twenty spat were sampled (stored in 95% ethanol) from each family to test for OsHV-1 µVar when the animals arrived back from the lease. Samples were process as described above for DNA extraction with all 20 spat from each family pooled into one sample for qPCR testing. All of the 20 families tested positive for OsHV-1 µVar. The Ct values ranged from 27.5 to 34.9. The remaining spat were held in a recirculation system in the PC2 Aquarium Facility before allocation to the TCP System two days later. The following day (day 0), ten spat were placed into each treatment replicate with 10 mL of TSW. The TSW was removed at 1400 h and the spat were anesthetised by immersion in 50 g L^{-1} MgCl in TSW for

two hours. No mortalities were observed in the spat at this time. The anaesthetic solution was then removed and replaced with TSW and the OsHV-1 µVar suspension (Table 4.6.2). The spat were held in the viral suspension for 16 h, after which the suspension was replaced with TSW. Survival was recorded daily during the challenge, with dead animals removed as recorded. Dead spat (five specimens from a high, medium and low resistance family) were collected from each treatment and fixed in 95% to test for the presence of OsHV-1 µVar.

Challenge 3

Challenge 3 (C3) was undertaken in the TCP system. Four triplicate treatments were examined, the same as used in C2 (Table 4.6.2). The OsHV-1 µvar concentration was 6 x 10^5 mL^{-1} (Table 4.6.2). The twenty families used were the same as in C2 (Table 4.6.1). The age of spat ranged between 167 to 193 days post-fertilisation on the day of treatment (Table 4.6.1). Families selected for the challenge were graded to a uniform size (~13 mm) and kept in separate upwellers for approximately one week prior to deployment to an oyster growout lease. The day before deployment spat of each family were placed into mesh bags. Spat for the lease conditioned treatments were taken from the IMAS hatchery on 29 March 2019 to Pitt Water (lease 46, -42.7998404, 147.4756622) and returned to IMAS five days later on 3 April. During the lease conditioning period farmers were not recording incidences of POMS Pitt Water (N. Griggs ASI, pers. comm.). Twenty spat were sampled (stored in 95% ethanol) from each family to test for OsHV-1 µVar when the animals arrived back from the lease. Samples were processed as described above for DNA extraction with all 20 spat from each family pooled into one sample for qPCR testing. Nine of the 20 families tested positive for OsHV-1 µvar. The Ct values of the positive samples ranged from 33.9 to 37. The remaining spat were held in a recirculation system in the PC2 Aquarium Facility overnight before allocation to the TCP System. The following day (day 0), ten spat were placed into each treatment replicate with 10 mL of TSW. No mortalities were observed in the spat at this time. The TSW was removed at 1400 h and the spat were anesthetised by immersion in 50 g L^{-1} MgCl in TSW for two h. The anaesthetic solution was then removed and replaced with TSW and the OsHV-1 µVar suspension as given in (Table 4.6.2). The spat were held in the viral suspension for 16 h (Camara et al. 2017), after which the viral suspension was replaced with TSW. Survival was recorded daily during the challenge, with dead animals removed. A water exchange was done on day 5 post-treatment (PT) as the water in some replicates was becoming cloudy, this was not done in C2 and probably relates to the greater size of the older spat used in this challenge. Dead spat (five specimens from a high, medium and low resistance family) were collected from each treatment and fixed in 95% to test for the presence of OsHV-1 µVar.

4.6.4 Results

4.6.4.1 Challenge 1

All treatments had considerable mortality by the first sample point on day 3 PT, with mean survival for all families combined ranging between 33.0 and 52.4%, with the HD treatment returning the best survival (Figure 4.6.1). By day 4 PT survival had dropped to a range of 23.3 to 24.2%, with little difference between the treatments. At termination on day 6 PT, mean survival for all families had dropped to a range of 16.7 to 17.2%. At termination, survival of families of all treatments had weak correlations to the May 2019 EBV, with correlations as follows: HD $r = 0.274$, LD $r = 0.321$, control $r = 0.250$ and all treatments combined $r = 0.291$.

4.6.4.2 Challenge 2

The L+ and L- treatments returned mortality as early as day 1 PT. From day 2 PT onward the mortality observed in L+ was by far the greatest of all treatments, with survival dropping to a mean of 12.5% by d7 (Figure 4.6.2). In contrast L- had mean survival of 62.5% at d7. Mortality was not observed in the H+ until d4, but essentially this was incidental; there was no treatment effect, with final survival of 98.2%. There was no mortality observed in H-. At termination (day 7 PT), there was a moderate correlation between the L+ treatment and the May 2019 EBV, $r = 0.43$, while the correlation with the L- treatment approached zero, $r = -0.05$.

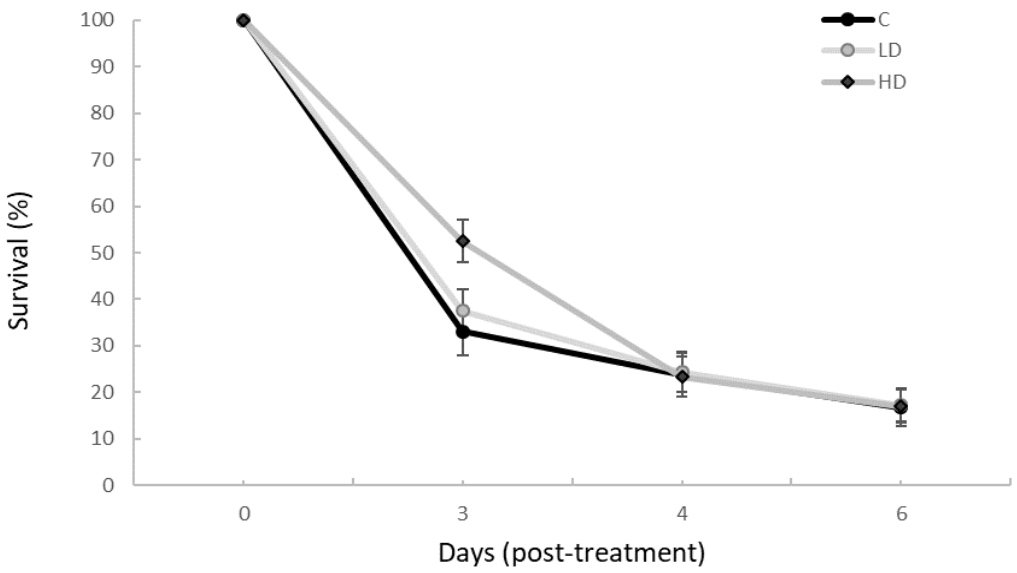

Figure 4.6.1. Mortality curve for all families of Challenge 1. HD (lease conditioned, exposed to 3.5 x 10^4 mL^{-1} of OsHV-1 µVar); LD (lease conditioned, exposed to 3.5 x 10^3 mL^{-1} of OsHV-1 µVar); C (lease conditioned, exposed to a control). Values are mean ± SE for 20 families.

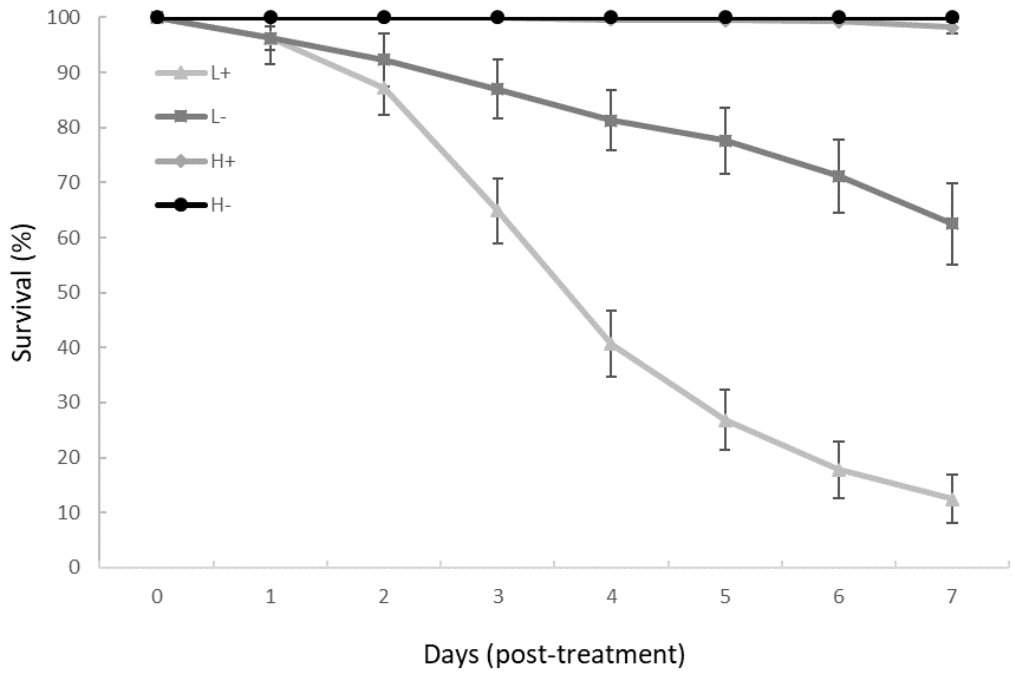

Figure 4.6.2. Mortality curve for all families of Challenge 2. L+ (lease conditioned, exposed to OsHV-1 µVar); L- (lease conditioned, exposed to a control); H+ (hatchery conditioned, exposed to a OsHV-1 µVar); and H- (hatchery conditioned, exposed to a control). Values are mean ± SE for 20 families.

4.6.4.3 Challenge 3

Treatment effects on survival were greatest in L+, followed by L- and H+ in that order. One spat only died in the H- treatment (day 6 PT). By day 7 PT mean survival in the was 26, 72.8, 74.1 and 99.8% for L+, L-, H+ and H- respectively (Figure 4.6.3). The survival observed for L+ by day 6-7 PT was similar to the survival observed for the ASI field trials in Pitt Water (light-moderate), while the L- and H+ recorded less than half the mortality observed for a moderate impact result (Table 4.6.3). At termination (day 7 PT), the correlations between the May 2019 EBV and treatments were H+ r = 0.605, L- r = 0.491, L+ r = 0.605. Lease treatments combined correlation was r = 0.485.

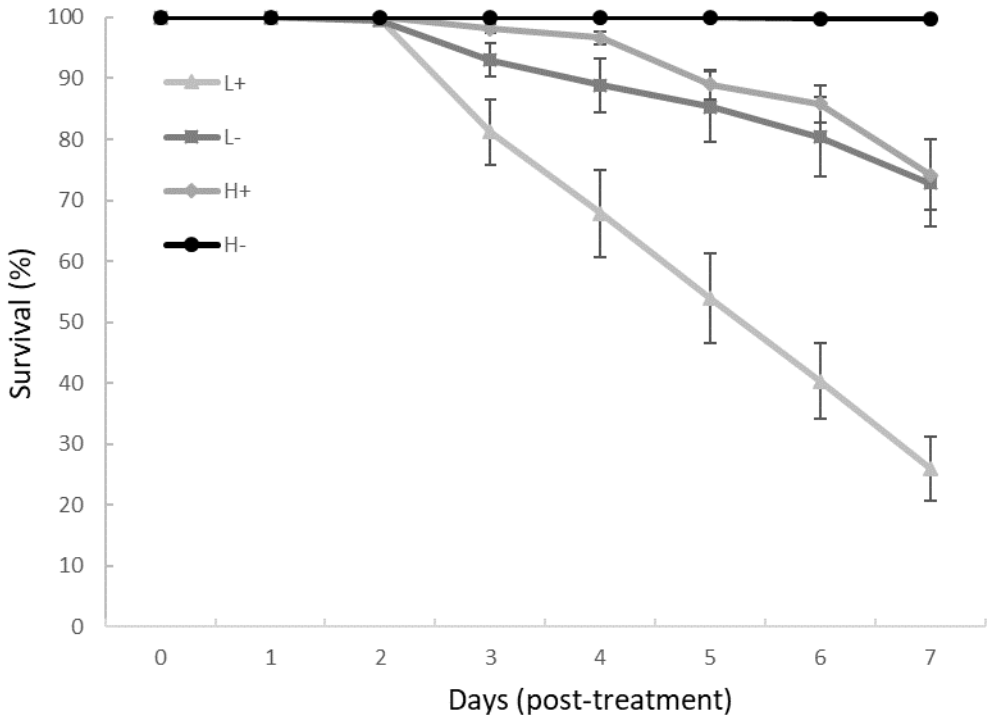

Figure 4.6.3. Mortality curve for all families of Challenge 3. L+ (lease conditioned, exposed to OsHV-1 µVar); L- (lease conditioned, exposed to a control); H+ (hatchery conditioned, exposed to OsHV-1 µVar); and H- (hatchery conditioned, exposed to a control). Values are mean ± SE for 20 families.

4.6.5 Discussion

The development of a POMS laboratory challenge at IMAS was achieved in two phases: 1) the construction and development of a PC2 laboratory; and 2) challenge set up and commissioning. The challenge protocol and commissioning occurred in the final summer (2018-2019) of the CRC-P project and was not fully refined at the end of the project.

Our challenges demonstrated that lease conditioning had a clear effect on the survival of spat. Oyster spat exposed in the laboratory to OsHV-1 µVar after being held on a commercial oyster lease had lower survival than those held in a hatchery prior to exposure. This is likely to be at least in part attributed to the microbiome acquired on the lease, which is known to form an intrinsic part of the disease process (de Lorgeril et al. 2018; King et al. 2019). In addition to effects likely to be under the control of the microbiome, the lower survival observed in the L+ is also likely to be affected by prior infection with OsHV-1 µVar whilst being held in Pitt Water. Testing by qPCR for OsHV-1 µVar on spat returned from the lease showed infection occurred on the lease for all our challenges. The early mortality in C2 and C3 in both lease conditioned treatments (L+, L-) provides further evidence for this. Mortality was recorded on day 1 PT in C2 (1.2% for both L+, L-), while in C3 mortality was recorded on day 2 PT (0.5 and 0.3%, for the L+ and L- respectively). For the L+ spat, mortality on day 1 or day 2 TP would not be caused by the laboratory exposure to OsHV-1 µVar, as the disease takes approximately 66 h to induce mortality (de Lorgeril et al. 2018). The disease process begins with OsHV-1 µVar infecting haemocytes, with viral replication leading to the host becoming immuno-compromised, before bacteraemia causes to death (de Lorgeril et al. 2018). Hence, infection with OSHV-1 µVar whilst being held in Pitt Water is likely responsible for the early onset of death in lease conditioned spat.

Our laboratory challenge demonstrated some congruence to field trial data (EBV), but overall the correlations to the field challenges were moderate to weak. All three challenges had considerable levels of variability compared to the field EBV with some families well correlated and others not. Understanding this variability is inherently difficult due to the complexity of the disease (Barbosa-Solomieu et al. 2005; Garnier et al. 2007; Petton 2015; Hick et al. 2018; de Lorgeril et al. 2018; Rodgers et al. 2018). Challenge 1 gave the poorest results and this most likely lies with the doses used. There was no treatment effect between the lease conditioned control and the two doses used (3.5×10^4/3.5×10^3). These doses are at and below the minimum

effective dose observed by Kirkland et al. (2015). The dosage used in C1 was driven the availability of viral stocks at the time and the volume of the viral suspension vessels (36 x 1 L) in the Basket System. The Basket System has been developed to afford better control of water quality compared to the TCP System and allows for a greater number of spat to be held in each replicate, which has been found to be an important factor in POMS laboratory challenges (Chapter 1). Typically, low EBV families had low survival as expected in our lab challenges, but there were some notable exceptions where these families performed better than expected when compared to the field challenges (data not shown). This most commonly occurred with lease conditioning treatments, without or with low dose laboratory exposure (all C1 treatments and L- treatments); again pointing to insufficient dose. Likewise, for high EBV families there was also inconsistency, i.e. non-congruent low survival (data not shown), particularly in L+ treatments were the dose may have been too high on top of field infection in the TCD System.

One of the aims of this work was to examine the suitability of translocating infected animals from the field to the laboratory for tissue collection during future genomic selective breeding. All lease controls developed POMS without further viral exposure in the laboratory and this suggests that field infected spat could be used as a source for laboratory based collections of tissues. However, the poor correlation with field EBV observed in the lease controls, indicates that there is variability induced through the translocation process and/or holding spat in the laboratory during the disease process. The general trend for lease control effects was to decrease with each challenge (16.6, 71.2 and 80.3% survival for all families combined, respectively for C1, C2 and C3 on day 6 PT). This could reflect the decreasing load of the virus in the estuary as the POMS 'season' declines with decreasing water temperature (Petton et al. 2013; de Kantzow et al. 2016). The results of qPCR testing from the three challenges also support this notion. The spat returned from the lease had lower ranges of Ct values with each challenge: 20.9 to 34.1, 27.5 to 34.9, and 33.9 to 37 for C1, C2 and C3 respectively. Spat of C1 and C2 were deployed when POMS events were occurring on Pitt Water oyster leases, while for C3 POMS was not being reported on the oyster leases. Understanding these aspects of the disease dynamics, at least pragmatically, would be important in developing this as a methodology tissue collection from following field infection. However, based on Chapter 1, lease conditioning followed by laboratory exposure appears to be the best laboratory method to select for POMS resistance in the laboratory; however, we could not achieve the same level of congruence as Trial 17-12 (Chapter 1). It is likely that the refined protocols of the EMAI laboratory challenge were fundamental to that result.

In conclusion, while we successfully adapted methodologies of POMS laboratory challenges used elsewhere to develop a laboratory challenge at IMAS, there remains considerable work to refine the challenge to a point where it can be used in conjunction with field challenges to select for POMS resistance.

4.6.6 Future direction

Improvement to the IMAS laboratory challenge will focus on development of viral preparations to provide more uniform exposures and further testing of the Basket System in order to challenge greater numbers of spat while maintaining high quality water quality with minimal labour input. We also plan to investigate potential differences between the microbiome of spat held in our hatchery compared to those held on an oyster lease.

4.6.7 Acknowledgements

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4.7: Strategy for Use of One-Year-Old Broodstock Within the Breeding Program

Matthew Cunningham [a], Lewa Pertl [a] and Nick Griggs [a]

[a] Australian Seafood Industries (ASI), Tech 3, Tasmanian Technopark, Innovation Drive, Dowsing Point TAS 7010

4.7.1 Executive Summary

The strategy to be able to use one-year old Pacific Oyster broodstock has been developed and successful implemented into the Australian Seafood Industries (ASI) breeding program. This strategy was adopted to in accelerate the rate of genetic improvement through breeding from one-year old oysters rather than two year old oysters previously used. The process to relies upon careful management of the broodstock oysters to maximise growth and gonadal condition. The use of one-year old broodstock within the ASI program has increased over the course of the project and increased Pacific Oyster Mortality Syndrome (POMS) resistance has been observed in these families. The result will mean that POMS resistance levels can be accelerated, and the benefits will ultimately flow to the Pacific Oyster industry faster.

4.7.2 Introduction

Genetic improvement in selective breeding programs is linked to the generation time of the species being improved. In general terms the quicker that animals with known estimated breeding values (EBV's) can be reproduced, the faster the genetic improvement can be achieved within a breeding program. In Pacific Oysters the breeding age of Oyster broodstock is typically 2 years. With the introduction of spat challenges for POMS resistance we are now able to generate EBV's for this trait very early in the life cycle of the oyster, typically around six months of age. Developing the ability to breed from these animals when they are approximately 12 months of age was identified as an area where genetic gains for POMS resistance could be accelerated. This component of the project aimed to develop a method to rapidly grow and mature Pacific Oysters so that both male and female broodstock could be used at 12 months of age.

4.7.3 Methods

4.7.3.1 Year Classes

Three separate year classes of Pacific Oyster families were produced over the course of this project. The YC16 families were produced in November and December 2016. This year class could not be considered for this study as there were significant biosecurity constraints in place as a result of the 2015 Tasmanian POMS outbreak. This removed the ability to be able to move stock between different growing areas which was one of the primary strategies to allow breeding of one-year old oysters. As a result, the YC15 year class has been included as a point of comparison.

4.7.3.2 Selection of Broodstock

Broodstock were selected from the first POMS trials deployed each season. The first disease outbreak has always been the most severe which means that the broodstock will have had the most selection pressure applied in terms of POMS resistance. For similar reasons the broodstock were selected from the trials that were deployed in Pitt Water, which has been the most affected growing region. Once data was collected estimated breeding values were determined for the families and these were used to select the 30 most resistant families to be targeted for use as one-year old broodstock. The generation of data and selection of animals occurred at the end of January when the animals are approximately 8 - 10 mm.

4.7.3.3 On-Growing

For the purposes of producing one-year old broodstock, Pitt Water has the dual advantages of having the most extreme POMS outbreaks and being an extremely productive waterway over the summer months. Pitt Water is a shallow waterway that has constrained tidal movement and because of this, water temperatures that are higher than other growing regions in POMS affected areas of Tasmania. Oyster experience rapid growth from December to March.

For the reasons highlighted above the spat from the 30 selected families continued to be on-grown in Pitt Water until the end of March. The Oysters were housed in individual 6 mm growing units at a very low density of 100 oysters per unit so that competition for resources was reduced. The spat were placed into an area of the lease where growth performance would be maximised. They were subsequently checked for growth and fouling fortnightly. At the end of March, the oysters had reached a size of 20 - 30 mm.

At this point, the beginning of April, the productivity of the Pitt Water growing region tapers off. The largest 50 oysters from each family were selected and moved to the Little Swanport growing region on the east coast of Tasmania. Little Swanport is one of the most Productive waterways in Tasmania and unlike Pitt Water good growth is achieved even over the winter months. The oysters were housed in 12 mm mesh Seapa tubes on adjustable long lines so that growing height could be used as a tool to manage growth and meat condition. As with Pitt Water the Oysters were grown in the most productive areas of the lease. The broodstock was initially grown lower in the water column to maximise feeding times and hence growth. The Oysters were checked for growth and fouling monthly and growing units were changed if required. The broodstock was lifted higher in the water column approximately four weeks prior to moving into broodstock conditioning. This increased air exposure time reduces shell growth, promotes meat condition and allows the shell to harden. At the end of July between 5 and 15 animals from each family are selected depending on their POMS EBV and moved to broodstock conditioning systems at IMAS at Taroona. At this point the oysters have reached 50 – 60 mm.

4.7.3.4 Broodstock Conditioning and Spawning

Broodstock were transferred to the IMAS facility and cleaned prior to stocking into tubs. The animals were housed in the biosecurity red zone as they had been exposed to POMS. Quarantine management and water sterilisation was in line with the ASI biosecurity plan for the IMAS facility. The one-year old broodstock were stocked at a density of 45 animals per 25 litre tub. The temperature was gradually increased from 16 ˚C to 21 ˚C over a period of 1 week. Feed rates were also gradually increased over the period of one week until a feed rate of at a rate of 2% of the animals' dry body weight per day was achieved. The broodstock were fed a mixed diet of microalgae consisting of various combinations of *Chaetoceros calcitrans, Chaetoceros muelleri*, *Tisochrysis lutea, Diacronema lutheri* and *Skeletonema pseudcostatum*. Broodstock were conditioned for a total of eight weeks before being spawned in late September using genetic data such as estimated breeding values to determine the pairings for POMS resistance.

4.7.4 Results, discussion and conclusion

Contribution of one-year old broodstock as a percentage of total parents used within the ASI breeding program has increased from 10.1% and 7.1% in the YC15 and YC17 year class to 43.2% in the YC18 year class (Table 4.7.1). The percentage of one-year old females used increased from 0% in the earlier year classes to 8.1% in the YC18 year class. The increases are non-linear due to issues with one-year old broodstock held at Little Swanport where mortalities occurred due to high levels of toxic algae in the YC17 year class. Based on these results it can be concluded that the strategy for using one-year old broodstock within the breeding program has been successful.

Table 4.7.1. Contribution of one-year old broodstock as a percentage of total parents used.

Year Class	1 Year old broodstock usage	1 Year old females' usage
YC15	10.1%	0%
YC17	7.1%	0%
YC18	43.2%	8.1%

There is a general trend of increased estimated breeding values for POMS resistance where one-year old broodstock are used to create a new family (Table 4.7.2). The increases are even greater when both parents are one-year old broodstock. The YC18 year class showed little difference between the one-year old crossed with other (1YO x other) as compared with the other crossed with other (other x other) is insignificant. The reasons for this are unclear but may be related to the design of the mating plan where lower EBV 2-year-old stock are predominately crossed with high EBV one-year old animals. It can be concluded that the use of one-year old broodstock within the ASI breeding program has led to increased gains in POMS resistance.

Table 4.7.2. Estimated Breeding Values (EBV) of crosses using different combinations of broodstock. Crosses are denoted y the symbol "x".

Year Class	EBV 1YO x 1YO	EBV 1YO x other	EBV other x other
YC15	NA	-2.4%	-19.1%
YC17	NA	20.6%	9.6%
YC18	30.5%	21.8%	21.6%

In conclusion, this component of the project has been successful and has led to increased usage of one-year old animals within the ASI breeding program. There is little room for error in the management strategies employed to allow one-year old broodstock to be used within the program as witnessed by the issues experienced in both YC15 and YC16 year classes. The result of using one-year old animals has led to increased genetic gains in POMS resistance. The gains have already been commercialised with families derived from one-year old parents distributed to commercial hatchery producers.

4.7.5 Future direction

The strategy for using one-year old broodstock within the breeding program has proved successful for managing POMS. It has led to increased genetic gains in POMS resistance and has informed further research in young animal resistance (2-3-month old). We plan to continue progress in this area until genetic targets are reached in this age group.

4.7.6 Acknowledgements

This project could not have been possible without the generous help from growers who gave their time and knowledge in helping ASI manage its stock. In particular, we acknowledge and thank Josh Poke, Hayden Dyke, Tony Byrnes, Justin Goc, Michael Riley, Craig Lockwood, Carl Jaeschke, Bruce Zippel, Gary Zippel and Brendan Guidera for their assistance and support.

Peter Kube (CSIRO) and Curtis Lind (CSIRO) provided expertise on genetic analysis and advice in conjunction with Nigel Valentine (CSIRO) and Scott Cooper (CSIRO) database assistance. Andrew Trotter (IMAS) and Greg Smith (IMAS) provided support with the IMAS-ASI Biosecurity Facility. We also thank Mike Dove (NSW DPI) for the work on the NSW trials and Xiaoxu Li, Penny Ezzy and Marty Deveney for their work on producing and managing ASI family lines in SA.

There were numerous ASI field staff that assisted with this project; John Wright, Charlotte Levi, Zoe Byrne, Paige Potter, Jarintzin Mones, Oliver Sargent, Joe Mangan, Geoff Endo, Ethan Bowditch-Wharton, Sophie Broomhall, Katrina Alter, Mary Woodward and Tommy Males are all acknowledged for their efforts.

4.7.7 References

NIL

4.8: Development of a SNP-based Genetic Test for Pacific Oysters

Nick Robinson[ab]

[a] The Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima), P.O.Box 5010, N-1432 Ås

[b] Flinders University, Sturt Road, Bedford Park, SA 5042

4.8.1 Executive Summary

A single nucleotide polymorphism (SNP) deoxyribonucleic acid (DNA) testing regime was developed and used to correctly infer familial relationships for the ASI Pacific Oyster breeding program. The panel of some 13 thousand SNP tests detected was shown to give high accuracy for determining genomic relationships and high potential as a tool for guiding selection to effectively limit inbreeding and to predict genomic breeding values for boosting the progress of genetic improvement for traits such as disease resistance. GenomeNZ supplied the SNP test results in the form of a genomic relationship matrix, and this matrix format can be readily utilised by quantitative geneticists for genomic selection and other purposes. The SNP testing is commercially available through GenomeNZ, but other service providers could also be considered.

4.8.2 Introduction

The pedigree of oysters is critical information affecting the performance of animals on farm and the ability of the breeding program to make genetic improvement. A way of double checking the pedigree of stock is needed to ensure that errors are not made during progeny testing of selected families and to enable growers to confirm the stock they are receiving. DNA testing can be used to confirm parentage (even grand parentage), but a set of specific tests needs to be developed for this purpose.

The objectives of this sub-project were to:

1. To identify SNPs existing in the selectively bred Pacific Oyster population in Australia.
2. To develop tests based on these polymorphisms that can be used to confirm parentage using samples from production stock and ASI stock (grandparents).
3. To liaise with companies offering commercial DNA testing services to ensure that the panel of tests can be commercially available.

4.8.3 Methods

To develop a SNP based genetic testing panel, 100 samples from five full-sibling families were sent to be sequenced by GenomeNZ. The samples consisted of 10 parents, 70 offspring provided with identified family names and 20 “blind” samples (family records were not provided so that the accuracy of the SNP panel could be tested).

A genotyping-by-sequencing procedure known as RAD-seq (with PstI and MspI restriction enzyme digestion) was used to identify valid SNPs and genotype the samples. An analysis package developed by GenomeNZ (freely available in R from GITHUB) was used to process and analyse the sequence output.

4.8.4 Results, discussion and conclusion

The sample and DNA quality of the parent samples were very poor due to the preservation techniques of old tissue samples.

In all, 12,871 sequence variants were identified as valid SNP loci (screening out putative SNPs with likely sequence errors and likely variants caused by locus duplications etc). The genotypes from the 12,871 SNPs deemed as valid were used for subsequent analysis.

The results are summarised in the below heatmap of genomic relationships (Figure 4.8.1). Here, the genomic relationship (SNP alleles shared across the entire genome) of every sample is compared to every other sample. The trees at the top and down the left side show the extent of the relatedness of every sample to every other sample (corresponding oyster identification numbers can be provided on request).

The heatmap trees (figure 4.8.1) show five clear major groupings at the highest level, distinguishing the five full-sibling families. These five families are from left to right along the top: 2016121, 2016126, 2016103, 2016122 and 2016123. All the offspring, sires and dams that were said to be of these families, grouped together into these corresponding major branches of the heat map. The “blind” samples that were sent could be clearly allocated to the families as shown in Table 4.8.1.

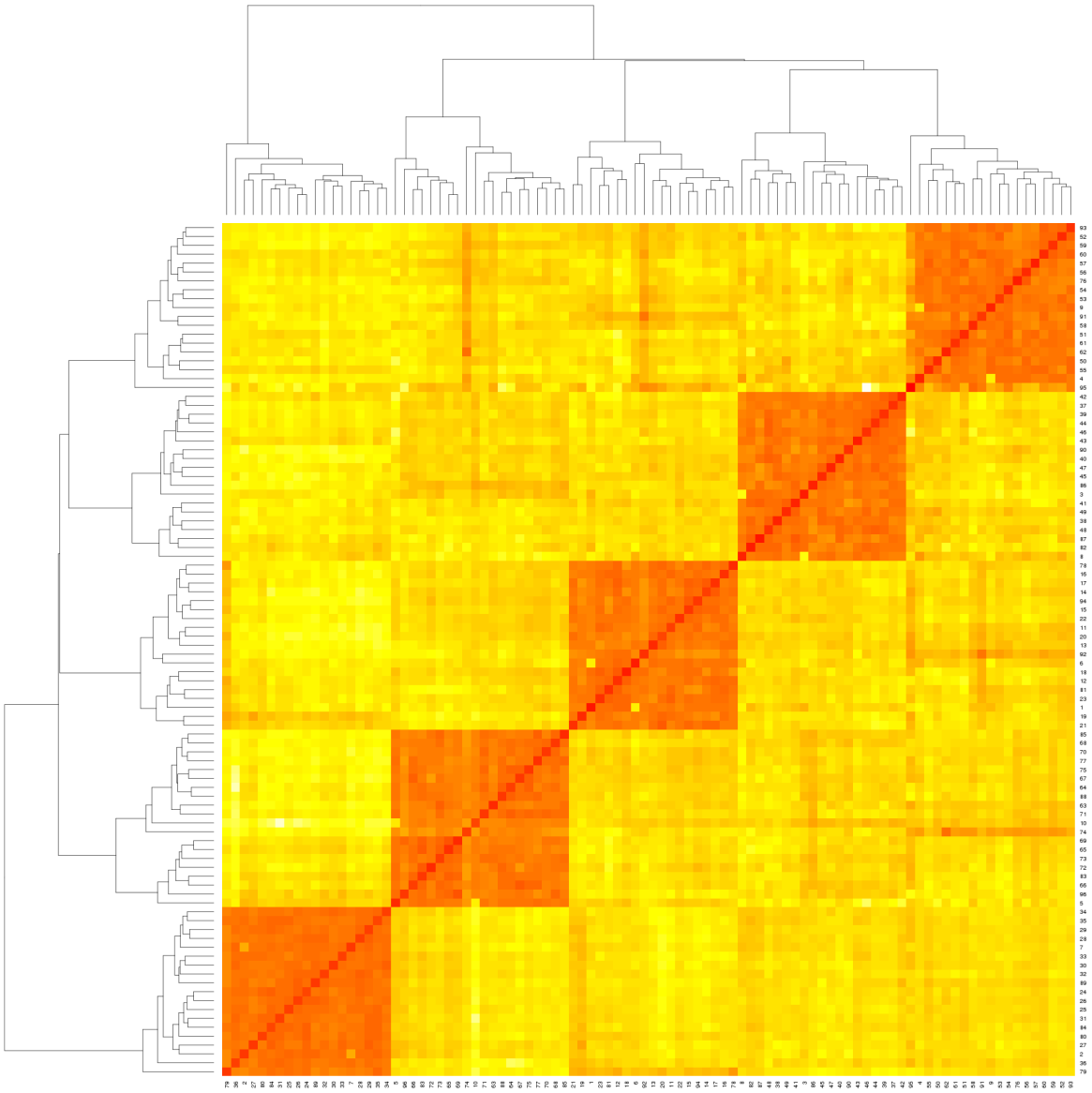

Figure 4.8.1. Heat map of genomic relationships calculated between every sample showing clear family groupings (represented as trees of relationships to the top and left side of the heat map). The greater the relationship the “hotter” (i.e. redder) the colour in the heat map. All samples that were sent with family identifications grouped according to family (including parents). The family grouping of samples where the family identification was withheld (for the blind test) are shown in Table 4.8.1.

Table 4.8.1. Genomic relationship grouping of "blind" samples, sires and dams into families. Numbers shown before each oyster ID correspond with the numbers on Figure 4.8.1.

Grouping/Family				
Family 2016121	Family 2016126	Family 2016103	Family 2016122	Family 2016123
76 A30301	83 A30308	81 A30306	82 A30307	
80 A30305	88 A30313	92 A30317	87 A30312	91 A30316
84 A30309	77 A30302	94 A30319	86 A30311	76 A30301
89 A30314	85 A30310	78 A30303	90 A30315	93 A30318
2 sire A30222	5 sire A30225	1 sire A30221	3 sire A30223	4 sire A30224
7 dam A30227	10 dam A30230	6 dam A30226	8 dam A30228	9 dam A30229

Another interesting feature is that family 2016121 is more distantly related to the other four families (which group together, indicating that these four families may share a distant common ancestor or have been sourced from the same wild stock). Also, some substructure was detected within families (e.g. family 2016126). This is also interesting and needs to be explored further with reference to the known history of these families.

Detailed tables and figures from the analysis can be provided on request.

In conclusion, this component of the project has successfully demonstrated that the SNP genotyping tests developed can be used to improve the performance and consistency of deployment and evaluation of selectively bred oyster stock derived from the ASI Pacific Oyster Breeding Program.

The SNP-based genetic test panel is extremely accurate and reliable for discriminating ASI from non-ASI oysters and for identifying oysters to family. The genomic relationship data from the SNP genotypes could be utilised to limit inbreeding, help predict genomic breeding values for difficult to measure traits such as disease resistance or simply used as a tool to discriminate ASI from non-ASI oysters.

From the known pedigree data (70 animals), the groupings at a family level were 100% accurate. Therefore, it can be assumed that this SNP-test panel will provide an extremely accurate and reliable resource for discriminating ASI from non-ASI oysters, and further to that, tracing oysters to particular families.

To make the testing commercially available to stakeholders, samples could be sent in batches to GenomeNZ (or any other appropriate sequencing service provider) and simply analysed and stored in a database by Nick Robinson, or another scientist with adequate knowledge of SNP test analysis, on behalf of ASI. It is recommended that ASI should coordinate the testing service and that ASI would own all results and inform oyster growers as appropriate.

4.8.5 Future direction

NIL

4.8.6 Acknowledgements

NIL

4.8.7 References

NIL

5. Conclusions

The overall goal of this project was to allow acceleration and improvement of ASI's selective breeding program undertaken prior to the 2016 POMS outbreak. To achieve this the ASI breeding program required: new techniques to continue breeding in TAS; increase its rate of genetic gains for POMS resistance; and, the establishment of a new breeding hub in SA due to the biosecurity restrictions brought about by the TAS outbreak. These goals have been achieved in TAS and progress continues in SA, which has caused numerous positive flow on effects throughout the entire Australian Pacific Oyster industry.

The improvement on the IMAS-ASI Biosecurity Facility has allowed continuation of family production at the same level prior to POMS and enabled ASI to breed from Pacific Oysters which have been expose to the virus. There have been no health issues observed - with PCR testing consistently showing negative POMS results over the past three years. The increase in field and lab trials improved rates of genetic improvement in TAS POMS resistance. The program has now developed and implemented POMS challenge trials for both spat (2-3-month-old) and one-year old animals that have increased gains.

During the three years of this project, a total of 22 field trials were deployed containing nearly 360,000 Pacific Oysters (*Crassostrea gigas*) from nearly 400 families and numerous laboratory challenge trials occurred at EMAI in NSW and IMAS. Trials conducted using ASI's novel methods have not only generated high quality data but have maximised genetic gains for POMS resistance. There also has been improvement in laboratory challenges which have increased correlations to field survival and a laboratory challenge at IMAS, which has been an important precursor to future work on genomics.

The development of an SNP identification tool was successful in differentiating between ASI and non ASI stock and different ASI families. The SNP-based genetic test panel proved to be accurate and reliable for discriminating ASI from non-ASI oysters and for identifying oysters to family. This research could be further used to limit future inbreeding, help predict genomic breeding values for difficult to measure traits such as disease resistance or simply used as a tool to discriminate ASI from non-ASI oysters.

A new SA specific breeding hub has been established to permit optimal flow of benefits across the national industry to overcome biosecurity constraints. Production of 160 families in SA through SARDI allowed ASI to enable POMS resistant broodstock to be deployed to the SA oyster industry. A new project has been developed which allows further breeding in South Australia to deliver breeding outcomes for the medium term.

This project has given the resources to improve genetic resistance to POMS, fast-track positive commercial outcomes and has contributed significantly towards the rapid recovery of the Pacific Oyster farming industry across Southern Australia.

6. Implications

The implications of this project have been substantial. The outcomes of this project have allowed the Pacific Oyster industry in Tasmania (TAS) to fully recover from the 2016 POMS outbreak. The timeframe for recovery has been very quick compared with other international industries which have been affected by POMS. In South Australia (SA) an insurance population of partially resistant oysters has been bred and commercialised. This will result in similar economic benefits to the TAS industry to be realised if/when POMS reaches the SA Pacific Oyster growing regions.

7. Recommendations

It is strongly recommended that growers of Australian Pacific Oysters use the outcomes of this project to insulate themselves from future POMS outbreaks by stocking POMS resistant Pacific Oysters.

8. Further Development

- Specifics discussed under each research section.

- The southern Australian oyster industry is strongly supportive of continuing the breeding programs managed by ASI, both to continue to continue to enhance disease resistance to address priority diseases, but also to once again include the historically important traits of growth, condition and shell shape. With industry in many oyster farming regions experiencing more extreme environmental conditions there is also discussion of selecting for increased temperature, salinity and pH tolerance.

9. Extension and Adoption

Extension has occurred according to the Future Oysters CRC-P extension and communication project; below are a list of the key media communications.

Date	Media	URL
20 August 2019	ABC	https://www.abc.net.au/radio/programs/tas-country-hour/tasmanian-country-hour/11411646
8 July 2019	Mirage News	Celebrating excellence in our Tasmanian seafood industry
27 June 2019	West Coast Sentinel	New disease-resistant oyster to protect SA industry
8 March 2019	ABC	POMS-resistant oyster research brings confidence back to Tasmanian industry
23 May 2019	ABC	Super oyster: the oyster that could save Tasmania's industry
21 May 2017	ABC	Tasmanian 'super oyster' program set to fight crippling mortality syndrome
4 April 2018	ABC	Disease-resistant oysters released in Adelaide's Port River in new trial
28 February 2016	The Mercury	Tasmanian 'super oyster' program set to fight crippling mortality syndrome

10. Appendix

Biosecure Oyster Hatchery Certification Letter

Department of Primary Industries, Parks, Water & Environment
BIOSECURITY TASMANIA

Hobart GPO Box 44, Hobart, Tasmania, 7001
Launceston PO Box 46, Kings Meadows, Tasmania, 7249
Devonport PO Box 303, Devonport, Tasmania, 7310
Ph 1300 368 550
Web www.dpipwe.tas.gov.au

Tasmanian Government

Enquiries: Mary Lou Conway
Ph: (03) 6165 3258
Fax: (03) 6278 1875
Email: Marylou.Conway@dpipwe.tas.gov.au
Our ref: H
Your ref:

3 January 2019

Dr Andrew Trotter and Mr Matt Cunningham
IMAS-ASI Oyster Hatchery
C/-Tech 3, Tasmanian Technopark
Innovation Drive Dowsings Point 7010

Dear Dr Trotter and Mr Cunningham

Thank you for providing me with a copy of the IMAS-ASI Oyster Hatchery Biosecurity Plan. I have also received a copy of the Audit report for this hatchery from your auditor, Ms Joan Van Drunen.

Based on the information provided, I am satisfied that IMAS-ASI has implemented and can maintain a standard of biosecurity suitable to mitigate the risk of Pacific oyster mortality syndrome (POMS) in respect to the premises located at 15-21 Nubeena Crescent, Taroona.

I am pleased to advise that consistent with advice that I have provided to IMAS-ASI previously, the successful auditing of the IMAS-ASI Biosecurity Plan for the Taroona Hatchery now allows me as Acting/CVO to approve the company producing oyster spat for sale directly from your Taroona Hatchery to any oyster growing area of the state.

As with audit programs we will need to develop a schedule of re-auditing. Please discuss this with John Preston.

Congratulations to all involved in what has been a long and arduous journey that has ultimately resulted in such a fine achievement for your organisations.

Kind regards

Mary Lou Conway
Acting/Chief Veterinary Officer