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Accelerated Sydney Rock Oyster (SRO) Breeding Research

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Abbreviations

BP: family-based breeding program

CRC-P: Cooperative Research Centres Projects

CSIRO: Commonwealth Science and Industrial Research Organisation

DIIS: Department of Industry, Innovation and Science

DNA: deoxyribonucleic acid

DPI: Department of Primary Industries

EBV: estimated breeding value

EMAI: Elizabeth Macarthur Agricultural Institute

NSW: New South Wales

PCA: principal component analysis

PCR: polymerase chain reaction

PSFI: Port Stephens Fisheries Institute

QX: Queensland unknown disease

RNA: ribonucleic acid

SOCo: The Select Oyster Company

SRO: Sydney Rock Oyster

TC: Technical Committee

WM: winter mortality

Executive Summary

This project focussed on increasing genetic resistance of Select Oyster Company (SOCo) breeding program Sydney rock oyster (*Saccostrea glomerata*, SRO) families to QX disease and winter mortality (WM) disease. NSW DPI has worked collaboratively with SOCo to develop a SRO family-based breeding program (BP) to replace the mass selection program used to develop fast growth and disease resistance since 1991. Family-based breeding has a number of distinct advantages over mass selection including; increased genetic gains, ability to select for disease resistance under biosecure conditions, improved selection methods for multiple traits, better estimates of genetic gains and trends as well as control over inbreeding. Annual family breeding runs commenced in 2014 to establish the SOCo breeding program. An FRDC project (2015-230) provided genetic expertise to establish and refine breeding methodology for a family-based breeding program.

The next step was greater understanding of the genetic parameters for QX and WM disease and how these related to other traits under selection, growth and meat condition. Genetic progress could be achieved by increasing the numbers of families available for selection, improved understanding of the genetic architecture of traits and reducing the length of breeding cycles for disease resistance. NSW DPI, SOCo, genetic specialists at CSIRO and oyster researchers at Macquarie University developed a multidisciplinary research program to deliver genetic progress for the SOCo breeding program.

The aims of this project were to:

1. double the number of families produced for the SOCo breeding program from 80 to 160;
2. halve the generation time for selection to QX and winter mortality resistance;
3. determine the heritability of the trait of winter mortality resistance in SROs; and
4. determine whether marker-assisted selection using reverse transcription can be incorporated into the SOCo breeding program.

Annual family production was approximately 40 when this project started and was increased to nearly 80 as a consequence of this project. Families in the 2014 and 2015 year classes continue to rank highly and are available for breeding runs taking the total number of families available to industry to more than 250.

Commercial hatchery production of SROs is more difficult compared to other species such as Pacific and eastern oysters. Further complexities are encountered when producing SRO families due to fertilisation deficiency in particular crosses, low rates of success when fertilisation occurs and poor levels of larval development in the period immediately after fertilisation. The increase in annual family production in this project was achieved through numerous stepwise improvements to protocols, facilities, husbandry and staff training. Whilst significant advances have been made, this area remains a high priority for further research. Improvements to guarantee better fertilisation success rates and larval yield in the early stages of a breeding run will further boost the number of SRO families possible in a single breeding run to a number well beyond 80. The ability to efficiently produce more families can provide increased genetic gains and program reliability in the future.

A one-year breeding cycle was successfully introduced for QX resistance, effectively doubling the rate of genetic progress for this trait. This required the use of quantitative genetics to understand the genetic architecture of traits, and knowledge of the reproductive and QX disease cycles to determine a design that considered the logistics of all breeding program operations, including those for the other primary traits of growth and meat condition. This understanding allowed the production of SRO families to be advanced so that spat from SOCo families could be challenged to QX during the season following production allowing data to be collected and breeding decisions to be made within 12 months.

During this project, WM disease expression through field exposures was low and inconsistent across multiple year classes and sites. This reduced the level of discrimination between family survival. WM resistance in SROs has low to moderate heritability, and thus would potentially respond to genetic selection. However, the rate of gain is likely to be lower for this trait than for traits such as QX resistance and growth rate. No correlations were found between WM resistance and other primary traits under

selection (QX resistance, meat condition and growth). The best estimates were obtained from the Quibray Bay site using survival data measured in December from 1 yr-old oysters. It is recommended that the SOCo breeding program continue WM field exposure trials of 1 yr-old oysters at Quibray to obtain further data if resources permit. A decision about including WM resistance as part of the SOCo breeding objective is required. This decision will need to be made in consultation with industry and with consideration of the impact of how this will influence gains in other traits. Whilst that decision has not been made, the knowledge generated from this project has enabled this process to be done.

This project developed a method to sample oyster tissue for genetic analysis that does not affect oyster reproduction or survival. Seven gene expression markers were identified that could be incorporated into the breeding program once further validation steps are completed. qPCR is a cost-effective method for screening large numbers of oysters and can provide results in sufficient time to make breeding decisions. Marker-assisted selection has the potential to increase genetic gains for QX resistance by enabling within family selection, and this project has identified candidate markers. Further development is required to determine if these genetic markers can identify susceptible individuals and to incorporate marker-assisted selection into the current logistics and schedule of the breeding program.

Major changes have been made to breeding logistics and are now used in the SOCo breeding program to increase genetic gains for QX disease resistance. The SOCo breeding program is operational using targeted multi-trait selection to increase the rate of genetic gain. Breeding goals have been set by industry based upon the need for SOCo to have a commercially viable product that provides clear benfits to oyster growers. The breeding goals are for SOCo stock to demonstrate 70% survival through a QX disease outbreak, 30% growth advantage compared to wild oysters, and no difference in condition compared to wild oysters. Estimates have been made on the timelines to achieve these goals, using data generated from this project and elsewhere, and these goals will be met in one to five years, depending on the trait in question. The commercial release of oysters with 70% QX disease resistance is expected in March 2020, 30% growth advantage over wild oysters is in March 2021 and oysters with no change in condition is in March 2024. These time frames are conservative estimates and can be reduced by using a more aggressive selection strategy if required.

This project changed the scale, logistics and schedule of the SOCo BP to reduce overall operating costs yet increase the genetic gains for QX disease resistance and now offers industry a viable risk mitigation strategy against disease impacts.

Keywords

Sydney Rock Oyster, *Saccostrea glomerata*, selective breeding, QX disease, *Marteilia sydneyi*, winter mortality disease, marker-assisted selection, resistance

Introduction

Background

Sydney Rock Oysters (SRO) are native to New South Wales (NSW) and their cultivation is the largest and most valuable seafood industry in this state, generating about $50 million a year (NSW Department of Primary Industries, 2018). The SRO mass selection breeding program was established in 1990 in response to a 60% production decline after the mid-1970s caused by disease, water quality declines and competition from the faster growing Pacific Oyster, *Crassostrea gigas.* The program's aim was to produce oysters that grew faster and had superior resistance to QX (Queensland unknown) disease and winter mortality (WM) disease (Nell *et al.*, 2000), thereby increasing productivity and profitability for growers.

QX disease occurs in estuaries in south eastern Queensland, northern NSW as well as the Georges and Hawkesbury Rivers (Green *et al.*, 2011). In estuaries where QX outbreaks have occurred it has invariably led to the total abandonment of cultivation of non-resistant SRO (Dove *et al.* 2013a). The parasite responsible for QX, *Marteilia sydneyi*, has been detected in nearly all estuaries tested to date across over the production range of SROs on the east coast of Australia, consequently, all estuaries are thought to be at risk of this disease (Adlard and Wesche 2005). Oyster deaths from QX disease usually commence in late summer or early autumn months (February to March) and can continue through to the spring months (September to November) (Nell *et al.*, 2000). The onset of QX disease does vary dependent on season and geographic location (Rubio *et al.*, 2013).

Mass selection methods successfully developed oyster lines with superior growth and resistance to QX disease (Nell 2006; Dove *et al.*, 2013a; Dove *et al.*, 2013b). Mass selection was also successful in reducing the impacts caused by WM disease. WM disease was first observed in Georges River in 1924 (Roughley, 1926) and occurs in estuaries south of, and including, Port Stephens (Nell and Perkins 2006). The cause has been attributed to a parasite, *Bonamia roughleyi* (Cochennec-Laureau *et al.*, 2003), however, evidence now exists that this parasite is not solely responsible (Carnegie *et al.*, 2014; Spiers *et al.*, 2014). WM outbreaks generally occur in downstream areas of estuaries and are more severe when average rainfall in autumn is low resulting in elevated estuarine salinities (Wolf, 1967; Nell, 2001). Oyster deaths usually commence in the winter months (June to August) and can continue through the spring months (September to November) (Nell and Smith 1988).

During the mass selection program the trade-off for superior growth and disease resistance was reduced meat condition affecting oyster marketability (Dove and O'Connor, 2012). Mass selection did not enable selection for other commercially important traits in combination with disease resistance and faster growth. There are limitations when using mass selection to target traits where individuals are sacrificed for the measure, such as meat condition and separating environmental and genetic effects on the trait being measured (Camara and Symonds, 2014). Mass selection over six generations has caused a substantial loss of genetic diversity in SRO mass selected lines to the extent that this method of breeding is not a viable long term option (In *et al.*, 2016). Consequently, family-based breeding was recommended for the SRO breeding program by two separate reviews in 2002 and 2012 (Benzie *et al.*, 2002; Rye, 2012). The primary reasons provided for changing from mass selection methods to a family-based model were: improved selection methods, better estimates of genetic gains and future improvement as well as control over inbreeding.

SRO family-based breeding research commenced in 2007 to develop hatchery techniques to create 20 or more single pair-mated families in one breeding run and to understand more about the mechanisms for QX disease resistance (O'Connor *et al.*,2010; Kan *et al.*, 2011). Additional research continued in 2009 to investigate meat condition measures and to determine if this trait could be incorporated into a multi-trait breeding program (Kube *et al.*, 2014). Meat condition responded to selection, however, a family-based breeding program (BP) was necessary to incorporate this trait alongside the other commercially important traits of disease resistance and growth.

In 2014, annual family production commenced at the Port Stephens Fisheries Institute (PSFI) to enable multi-trait selection. Founder families were created from the mass selection lines and wild oysters selected for shell shape. In 2016, an FRDC funded project (2015-230) commenced developing operational and

industry-focused family based breeding, with the goal to allow Industry to access oysters with superior disease resistance, growth and meat condition (SOCo *et al.*, 2019). This project captured historical data related to SRO families, developed a data management system and established a progeny testing regime. A technical committee consisting of representatives from SOCo, NSW DPI and CSIRO was created to allow industry to inform decisions and establish objectives for SRO breeding. A breeding manual was also developed, which covers family production, progeny tests and data management protocols that are presently used. This manual was prepared to assist with the development of an industry management and commercialisation plan for the SRO BP and contains the key information pertinent to operating the SRO family program.

This project was developed in consultation with SOCo and utilised genetic specialists at CSIRO and oyster experts at Macquarie University to conduct a multidisciplinary approach to accelerate breeding program progress. The aims of this project were to: double the number of families produced for the SOCo breeding program from 80 to 160; halve the generation time for selection to QX and WM resistance; determine the heritability of WM resistance; and, determine whether marker-assisted selection using reverse transcription can be incorporated into the SOCo breeding program. To achieve the project goals: the genetic parameters for resistance were determined; family production capacity was increased; the operation of the breeding program was redesigned; and, markers were developed to identify highly QX disease resistant individuals. The outcomes of this research were directly applied to SRO breeding and provide the SRO industry with a risk mitigation strategy for disease impacts.

Need

In 2009, after seven generations of mass selection it was apparent that there were a number of shortcomings using mass selection for future breeding. Meat condition of oysters selected for superior growth was lower than wild oysters cultivated in the same conditions (Dove and O'Connor, 2009). A study of the reproductive cycle of faster growing mass selected oysters at three sites in NSW found that faster growing oysters and wild oysters developed and spawned synchronously, but in most instances, the faster growing oysters had a lower condition index and reduced gonad area (Dove and O'Connor, 2012).

While mass selection can improve given traits quickly, the selection of individuals on the basis of the best performance for certain traits without regard to pedigree information can result in inbreeding and loss of potentially valuable alleles and net additive genetic variation. Inbreeding can adversely affect genetic breeding programs in a number of ways. The fast growth and disease resistance mass selection lines were a 'closed' population and wild oysters were not added to the breeding nucleus. By closing the nucleus, having high selection intensities for fast growth or disease resistance, conducting selection over six generations and not controlling the crosses at fertilisation, resulted in an unsustainable level of inbreeding in the mass selection lines (In *et al.*, 2016).

Reviews by Benzie *et al.* (2002) and Rye (2012) as well as the findings by In *et al.* (2016) contributed to a fundamental shift in SRO breeding where traditional mass selection breeding methods were replaced with methods to facilitate production of single-pair mated families and to use among-family selection. These methods record the parentage of individuals used as broodstock so that genetic correlations and genotype-by-environment interactions can be evaluated. Consequently, the BP can now address impacts to meat condition caused by selection for superior growth over many generations. The focus of the SRO BP is to develop a breeding strategy that incorporates disease resistance, growth and meat condition for Industry.

Development and delivery of a SRO BP has economic benefits for Industry and social outcomes for the community of NSW. The SRO BP provides a risk mitigation tool for industry to safeguard against disease. It also incorporates commercial traits that increase productivity gains and on-farm returns needed to improve the profitability of regional oyster enterprises. Additionally, the BP provides an excellent platform to respond to unidentified future problems and needs, and therefore ensure the long term sustainability of this industry.

Objectives

1. By 2019 to have doubled the number of family lines currently planned for the SOCo breeding program
2. To reduce the generation time for QX and winter mortality resistance to 1 year
3. To have confirmed the value of “stress markers” in selective breeding of Sydney Rock Oysters

1. Double the number of families in the SOCo Breeding Program

Michael Dove, Peter Kube, Curtis Lind and Wayne O'Connor

1.1 Introduction

The first objective of this project was to have doubled the number of families currently planned for the SOCo breeding program by 2019. A family is a single male crossed with a single female. Producing more families in three annual breeding runs scheduled during this project was one of the strategies used to accelerate genetic gains for the SOCo Breeding Program. The other strategy was to reduce the breeding cycles for disease resistance breeding.

The selection decisions for all families produced for this project were guided by industry advice provided through SOCo. QX disease resistance is the most important trait identified by industry and the economic weight for this trait is twice that for growth and meat condition. QX disease resistance is a risk mitigation strategy for Industry. Fast growth is important both to reduce impacts caused by QX disease, by enabling a commercial sized oyster to be produced with only a single QX exposure, as well as increase productivity and on-farm returns. Meat condition is also an important commercial trait because it can significantly decline when selecting for fast growth if not properly managed.

Prior to this Future Oysters CRC-P project, 80 SRO families were planned for production; 40 families for the 2016 year class and 40 families for the 2017 year class. The most families produced in a single SRO family breeding run prior to this project was 47 for the 2015 year class. The first year class produced in this project (2016 year class) used only broodstock from families that comprised the SOCo breeding program. All other year classes created prior to this were a mix of wild stocks and the mass selected lines.

This objective was undertaken together with the second project objective to reduce the generation time for QX disease resistance to one year. To achieve this, the SRO breeding run was brought forward in time to October from January. However, October is prior to the peak reproductive window for SROs, meaning spawning is effectively being done out of season. Therefore to achieve this, hatchery conditioning of broodstock was tested to prepare broodstock for spawning.

1.2 Method

The strategy employed to increase the number of families produced each breeding run was to: commission an additional bivalve hatchery to increase larval and spat rearing capacity, increase algal production capacity; acquire more hatchery equipment; and train additional staff to assist with breeding runs. To achieve the second objective of this project (to reduce the generation time for QX and winter mortality resistance to one year) hatchery conditioning was done and the timing of the breeding operation was brought forward to October to enable exposure of spat (4 mo) to QX disease.

The procedures used to condition broodstock and produce families of SROs are included in The Sydney Rock Oyster Family Based Breeding Program Manual (Dove *et al.*, 2019). The manual includes strip spawning, larval rearing and settlement methods as well as nursery and grow-out management for families.

A SRO BP technical committee was formed in December 2015 as part of FRDC project 2015-230. This committee had representatives from NSW DPI, CSIRO and SOCo. The goal of the technical committee was to facilitate effective communication and decision making concerning the technical aspects of the breeding program. The planning for all of the breeding runs related to this project was developed by this committee. The breeding goal was set in consultation with the SOCo Board. A desired gains approach was used where the TC applied economic weights in accordance with the relative trait gains decided by the Board.

1.3 Results

The estimated breeding values (EBV) and resulting broodstock candidate list was compiled in early August, after which broodstock condition commenced. Broodstock conditioning continued for approximately 10 weeks and concluded when broodstock had viable gametes. Breeding decisions for the 2016, 2017 and 2018 year classes were made by the SOCo technical committee with a goal to increase QX resistance and growth rate without causing a difference in condition compared to wild oysters. The spawn date and number of families produced for the 2016, 2017 and 2018 year classes are listed in Table 1.1. The broodstock used in each breeding run is also listed in this table.

Table 1.1: Total number of families per year class produced for this project (2016, 2017 and 2018 year classes) and year classes that existed in the SOCo breeding program prior to this project (2014 and 2015).

Year class	Spawn Date	Number of families	Broodstock source
2014*	8/01/2015	47	2006YC, Port Stephens and Georges River mass selection lines
2015*	22/10/2015	44	2009YC, 2010YC (including QX survivors), Georges River mass selection lines, wild oysters from Nambucca River, Hastings River, Camden Haven River, Wallis Lake, Port Stephens and Tuross Lake
2016	1/11/2016	68	2009YC, 2010YC, 2014YC and 2015YC
2017	9/10/2017	78	2014YC, 2015YC and 2016YC
2018	30/10/2018	33	2014YC, 2015YC, 2016YC and 2017YC

* breeding strategy developed prior to this project in consultation with CSIRO, NSW DPI and SOCo

The 2014 and 2015 year classes were produced prior to the start of this project and consisted of 91 families in total (Table 1.1). The main sources of broodstock for these families were the mass selection lines and wild stocks. The 2014 year class was produced mostly from broodstock chosen from the Georges River disease resistant mass selection lines and the Port Stephens fast growth mass selection lines. Some broodstock were sourced from the 2006 year class families because these families had QX survival data collected from Lime Kiln Bar, Georges River in 2008. The broodstock for the 2015 year class were mostly wild oysters selected on the basis of shell shape from six estuaries across the SRO growing range. In addition, some broodstock were sourced from Clarence River (QX disease survivors from the 2010 year class). More details regarding the genetic groups that comprise the SRO breeding program are given in Dove *et al.* (in prep).

Broodstock conditioning and out-of-season spawning were effective. Furthermore, family broodstock management accelerated early growth and permitted the use of one-year-old broodstock which had viable gametes following the conditioning period. This facilitated the use of the most recent year class in all breeding runs conducted for this project (Table 1.1) and was fundamental in reducing the breeding cycle for QX disease to one year.

The 2016, 2017 and 2018 year classes were produced exclusively from families within the SRO BP. The rate of success of a single pair cross producing a family with sufficient spat was 29% in 2016, 25% in 2017 and 27% in 2018 (average ± SD = 27 ± 2%).

The SOCo breeding program produced more than double the 80 planned families to 179 families available in 2019. Sixty families were settled as spat in the hatchery for the 2018 year class. Unfortunately, 27 families

were lost on the nursery lease in early January due to a combination of persistent hot weather and high winds that were atypical and never previously experienced. This reduced the total number of families in the 2018 year class to 33. Growing spat to a larger size within the hatchery would reduce the risk of family losses at the nursery lease site. However, deploying spat earlier to the nursery lease increases their size and total weight when they are sent to the Georges River for QX disease field trials. Using larger spat in this trial delivers better QX survival data.

1.4 Discussion

The strategy to increase family numbers in an annual family run was effective. The number of families planned for the SOCo breeding program was more than doubled. Eighty families were planned prior to the start of this project and 179 families were available for selection in 2019. Families in the 2014 and 2015 year classes continue to rank highly in the breeding program. These families are still available for breeding runs and take the total number of families available to industry to more than 250.

A hatchery manual for the SRO BP has been produced which captures improved spawning and larval rearing techniques for family production developed through this project. This manual represents the initial step to transfer hatchery skills from NSW DPI to industry. All breeding runs conducted for this project were done in collaboration with SOCo staff thereby further facilitating transfer of the hatchery breeding skills to industry.

Shifting breeding runs before SROs have attained their reproductive peak was successful when done in conjunction with hatchery conditioning. This made it possible to achieve the second objective of this project and reduce the breeding cycle for QX disease to one year. Further research effort to improve fertilisation success during spawning would increase the total number of families that can be produced in a single breeding run and would also reduce the number of broodstock that are sacrificed during each breeding run.

Increased family production places additional logistical pressures on progeny testing in order to gather data from each family. Additional staff were used at critical time points to collect all of the necessary data in a timely manner so that stress to animals in these experiments was minimised.

The SOCo Breeding Goal was set in June 2018 and is 70% survival through a QX disease outbreak, 30% growth advantage compared to wild oysters, and no difference in condition compared to wild oysters. These are targets endorsed by industry and are considered to be requirements for SOCo to have a commercially viable and distinguishable product. Those targets will be met in one to five years, depending on the trait in question. The commercial release of oysters with 70% QX disease resistance is expected in March 2020, 30% growth advantage over wild oysters is in March 2021 and oysters with no change in condition is in March 2024. These time frames are conservative estimates and can be reduced by using a more aggressive selection strategy if required.

A limitation on the efficiency of SRO family production is the low success rate of families, averaging only 27% during this project. This is substantially lower than that for other oyster breeding programs, such as for Pacific and eastern oysters. The low rate of success of single pair crosses occurs due to no fertilisation occurring in particular crosses, low rates of success when fertilisation does occur and poor levels of larval development in the period after fertilisation. The consequences of the low family production success rate are that broodstock requirements and hatchery time are greatly increased to produce a targeted number of families. The increase in annual family production in this project was achieved through numerous stepwise improvements to protocols, facilities, husbandry, and staff training. Whilst significant advances have been made, this area remains a high priority for further research. Improvements to guarantee better fertilisation success rates and larval yield in the early stages of a breeding run than current levels will further boost the number of SRO families possible in a single breeding run to well beyond 80.

A SRO family breeding run has been conducted each year over the last 5 years and started with production of the 2014 year class. Continuing annual breeding runs are crucial to delivery of the SOCo Breeding Goal. Annual breeding has been assisted through Department of Industry, Innovation and Science (DIIS) funding support via the Future Oysters Cooperative Research Centres Projects (CRC-P) and an associated FRDC

funded project to secure genetic services (FRDC 2015-230). The future of SRO breeding was explored in a NSW DPI commissioned report to determine feasible options for resourcing breeding activities and commercialisation of outputs. Interruptions to the current breeding schedule will have implications on genetic trends and delivering a commercial product corresponding with the time frames stated in the SOCo Breeding Goal.

1.5 Conclusion

The number of families produced for the SOCo breeding program was more than doubled. Annual family production was approximately 40 when this project started and was increased to nearly 80 as a consequence of this project. Families in the 2014 and 2015 year classes continue to rank highly in the breeding program. These families are still available for breeding runs and take the total number of families available to industry to more than 250. Hatchery conditioning facilitated successful out-of-season breeding runs to occur which was essential to reduce the breeding cycle for QX disease resistance to one year effectively doubling the rate of progress achievable within the SRO BP. The major limitation on family production is the poor family production success rate. During this project, the probability that a single pair mated cross resulted in a viable SRO family was 27%. This is substantially lower than that for other oyster breeding programs, such as for Pacific and eastern oysters. Increasing single pair mated fertilisation success remains a high priority for future research.

2. One year breeding cycle for QX disease resistance

Peter Kube, Michael Dove, Curtis Lind and Wayne O'Connor

2.1 Introduction

The second objective of this project was 'to reduce the generation time for QX and winter mortality resistance to one year'. SRO breeding has never exposed spat to QX disease for breeding selections in its 30 year history. Small oysters are highly susceptible to QX disease and field trials to expose oysters can be problematic due to rapid and high mortality rates after infection with the QX disease agent, *Marteilia sydneyi*. The mass selection breeding cycle for QX disease was three years, and included a dual exposure of 1- and 2-year-old animals. Mass selection lines were produced in January, sent to the Georges River in the following July (after the QX disease infection period) and QX survivors were used as broodstock 30 months after the deployment. Dual exposure was used because the second exposure compounds QX mortalities.

The mass selection program was very successful in delivering faster growing and QX disease resistant oysters to industry. The survival and growth of fourth generation SROs selected for resistance to QX and winter mortality was assessed (Dove *et al.*, 2013b). QX disease mortality in control oysters at Lime Kiln Bar was 97% compared to 28% measured in the fourth generation QX resistant line. Oysters selected for disease resistance were also selected for superior growth. Fast growth is important because it means that commercial stocks only receive one exposure to QX disease prior to harvest. The relationship between QX disease resistance and oyster growth is not presently understood (Nell and Perkins, 2006; Dove *et al.*, 2013b).

A fundamental shift in SRO breeding occurred in 2014 when mass selection breeding was terminated and replaced with family-based breeding. It was important to capture the genetic gains made through mass selection, establish sound methods to facilitate family-based breeding and to use among-family selection for QX disease and other commercial traits. This was needed to determine an accurate heritability of QX disease, understand oyster resistance at different life stages and investigate options to reduce the breeding cycle for QX disease to one year. This chapter presents the results of exposing spat and adults to QX disease and the changes to the design of the breeding program so that the breeding cycle for QX disease can be reduced to one year.

2.2 Method

A total of 7 field trials were deployed to address the aims of the QX breeding component of this project. Trials were from two year classes, they were deployed at two sites, and they were exposed to the QX pathogen at two different ages. QX disease was confirmed through polymerase chain reaction (PCR) testing of samples from the field trial for the presence of *M. sydneyi* DNA by veterinarians at the Elizabeth Macarthur Agricultural Institute (EMAI).

The ages at exposure were 4 months, hereafter referred to as spat trials, and 16 months, referred to as adult trials. Table 2.1 provides a summary of these trials. All trials contained families from the SRO BP, where a family is a single male crossed with a single female, and the source material for these families was a mix of wild stocks and the mass selected lines (Table 1.1). Further details of the population structure and the breeding program are given in Dove *et al.*, (in prep). A total of 106 different families were used, of which 44 were from the 2015 year class and 62 from the 2016 year class, and the total number of oysters deployed was 43,338. Family rearing and the QX field trial used the standard protocols for the SRO BP and these are described in Dove *et al*. (2019).

Table 2.1: A summary of the QX trials deployed as part of this project.

Trial	Year class	Site	Number families	Number oysters deployed	Age at QX exposure (months)	Age at assessment (months)	Survival
1	2015	Georges R	44	7,713	4 (spat)	10	26%
2	2015	Georges R	42	5,902	16 (adult)	21	31%
3	2015	Yamba	38	6,833	4 (spat)	10	81%
4	2015	Yamba	39	3,958	16 (adult)	21	45%
5	2016	Georges R	52	7,298	4 (spat)	8	69%
6	2016	Georges R	62	8,579	16 (adult)	20	81%
7	2016	Yamba	43	3,055	4 (spat)	8	84%

The trials were measured at up to three time points to determine the optimal time for assessment. However, in this report data is only shown for a single time point, which is the July assessment. This was the time point that combined optimal discrimination between families and fitted the logistic timeframe for allowing a one-year breeding cycle, which was the principal aim of this part of the study. Details on the effects of different measurement times are given in Dove *et al.* (in prep). The measurements were survival counts and these were converted to binary data (zeros and ones), representing dead and alive individual animals, for the statistical analyses. All pedigree and performance data were uploaded to the CSIRO Selective Breeding Program Database and data reports were generated from this database for the analyses.

The data were analysed to address the following questions: (1) Is there genetic variation for QX resistance and how does this variation compare at different ages of exposure, at different sites, and in different seasons (year classes)? (2) How does genetic expression of QX resistance compare (i.e. correlate) on different sites? (3) How does genetic expression compare (i.e. correlate) at different ages of exposure?

The analyses were done by fitting a series of individual animal mixed model equations using ASReml. The models used genetic groups to account or the prior selection in different founder stocks (i.e. wild oysters compared to mass selected lines) and the use of genetic groups was found to be important for accurate estimates of genetic variation. More details of the genetic group effects are given in Dove *et al.* (in prep).

Two sets of analyses were done. Firstly, between site effects were analysed using models that combined the data from the Georges River and Yamba sites where each site was treated as a separate variable in a bivariate analysis. These analyses were done independently for each exposure age. Secondly, age effects were analysed using models that combined spat and adults trials where each exposure age was treated as a separate variable in bivariate analyses. These were done within year classes and within sites. A combined analysis was done using both year classes from the Georges River site but Yamba was not included due to the low correlations and questionable nature of some of the Yamba data (explained in results). The main metrics used to address the above questions were the heritabilities and genetic correlations, both of which are calculated from the standard outputs from ASReml. The heritabilities reported here are on the underlying scale and were calculated using the method of Dempster and Learner (1950).

2.3 Results

Genetic variation for QX resistance was present in all 7 trials, although there was large variation from trial to trial. Heritabilities, the metric used to measure genetic expression, ranged from 0.12 ± 0.05 to 0.67 ± 0.06 (values are $h^2 \pm$ se on the underlying scale) and appeared to vary between sites, and the age of exposure. The experimental design included a structure to allow estimates of spatial effects but these were negligible for the trials presented here, suggesting the disease was uniformly expressed within a trial.

Georges River trials had higher heritabilities and higher disease severity (as indicated by average survival) than those at Yamba. The range of heritabilities at Georges River and Yamba was, respectively, 0.16 to 0.67 and 0.12 to 0.38 and in an analysis combining all trials for each site the heritabilities were 0.26 ± 0.04 and 0.18 ± 0.04. Importantly, the genetic correlations between the two sites for the spat exposures were low indicating that the genetic expression of survival differed between sites. Values for the 2015 and 2016 year classes were $r_g = 0.20 \pm 0.18$ and 0.08 ± 0.24 and in an analysis that combined year classes the value was $r_g = 0.15 \pm 0.14$. It was suspected that the mortality at Yamba may have been caused by agents in addition to QX disease and that is likely to be the reason for the very low genetic correlations. The genetic correlation for adult exposure was moderately high ($r_g = 0.76 \pm 0.18$), which is a better result, but no further combined site analyses were done due to the uncertainty of the Yamba result.

Heritabilities were generally higher for animals exposed as spat than for adults, with heritability values ranging from 0.12 to 0.67 in spat trials and 0.16 to 0.35 in adult trials. In a combined analysis at the Georges River site, the heritabilities (± se) for spat and adult QX exposure were $h^2 = 0.42 \pm 0.07$ and $h^2 = 0.24 \pm 0.04$, respectively. The genetic correlation between spat and adult exposure was $r_g = 0.70 \pm 0.07$ indicating a moderate correlation between these traits. This suggests 70% of the genetic effects of QX resistance are commonly expressed at spat and adult exposure. Results from the Yamba site are unlikely to be meaningful due to the uncertainty of QX expression on that site but, unsurprisingly, the genetic correlation between spat and adult exposure was low and not significantly different from zero.

2.4 Discussion

The results from these trials confirm previous results on the genetic control of QX resistance, indicating that this is a trait under moderate genetic control and a trait suitable for selective breeding. Mass selection has made significant changes to this trait (Dove *et al.*, 2013a) and results from the broader range of family trials on QX resistance have found similar heritabilities, albeit for adult exposure (Dove *et al.*, in prep). These trials have addressed QX resistance for stock exposed as spat, which is something not previously done, and have indicated common aspects to resistance at different ages, although these traits are not perfectly correlated.

The results to these trials address the primary purpose of this part of the study, which is to determine ways to reduce the QX breeding cycle to a single year. There are two main parts to addressing this question. One is to understand the genetic control of QX resistance at different ages using quantitative genetic metrics. The second aspect is to structure the logistics of breeding in a way that makes a one-year breeding cycle possible, and effectively uses the quantitative genetics information.

The quantitative genetics measures indicate that a one-year breeding cycle is a practical alternative. Not only is spat resistance heritable, it appears to be a more highly heritable trait than resistance as a one-year-old oyster. Genetic gains are directly proportional to heritability meaning gains are potentially greater. The moderate genetic correlation between spat and one-year-old resistance is also encouraging. Selection as spat will confer resistance as one-year-olds, although the imperfect correlation means maximal gains will not be achieved at both age classes with a single progeny test. A question for the breeding program will be whether to run with a single test or to test both spat and one-year-old oysters. There is no standard answer to this question, and the chosen approach will depend on the needs of industry, the true value of additional gains, and the costs of implementation.

One question not addressed as part of this study is how QX resistance manifests with an exposure over two seasons, or a double exposure. Whilst managing the grow-out to limit oysters to a single exposure is logistically possible, especially with selection for faster growth, it is inevitable that some oysters will periodically get a double exposure. Studies are underway to address this question and will be presented in Dove *at al*. (in prep). Another question that has not been completely addressed in this study is the correlations between sites. The correlations between the two sites in this study were low for a spat exposure. This was thought to be due to other factors causing mortality but there is no definite answer from this study. More trials across multiple sites would be an advantage to ensure selections are for a generalised QX resistance and not a site-specific resistance.

The second major factor in implementing a one-year breeding cycle is about designing the logistics of the breeding operation. This considers the quantitative genetic aspects but it also needs to consider the biological cycles for reproduction and disease, and to be able to balance the workflow to fit within these cycles. All this has been successfully done as part of this project. The chosen breeding cycle is depicted in Figure 2.1 and is as follows:

(1) Spawning is done annually in mid to late October using broodstock that were selected in August and which have been in conditioning since selection. Ideally, families are set before the Christmas/New Year break to fit with the availability of staff and to avoid excessive work during a period when workplaces seek to shut-down.
(2) Nursery rearing is done between mid-December and mid-February. The goal is to have stock at a suitable size for field deployment by mid-February to ensure the oysters were exposed to the QX pathogen. This is an essential requirement for the disease to express, and spawning and conditioning are essentially scheduled by working back from this window.
(3) Disease expression (mortality) occurs between late May and late September, and measurements need to be done in this period. A later measure appears to give better genetic expression (see Dove *et al*. in prep), but scheduling must be such that selections can be completed by August in order to meet the conditioning window. Scheduling assessments in Mid-July has been found to be optimal with regard to disease expression and conditioning time.

Reproductive conditioning is done between mid-August and mid-October. A suitable period of conditioning is essential for successful family production and, without appropriate conditioning, the ability to target selected families is compromised. Conditioning is done in an indoor facility with heated water and it has been necessary to find the right balance between effective use of the facilities available (they are currently a given), the number of families to spawn (which dictates numbers in the conditioning system), and the time available for conditioning.

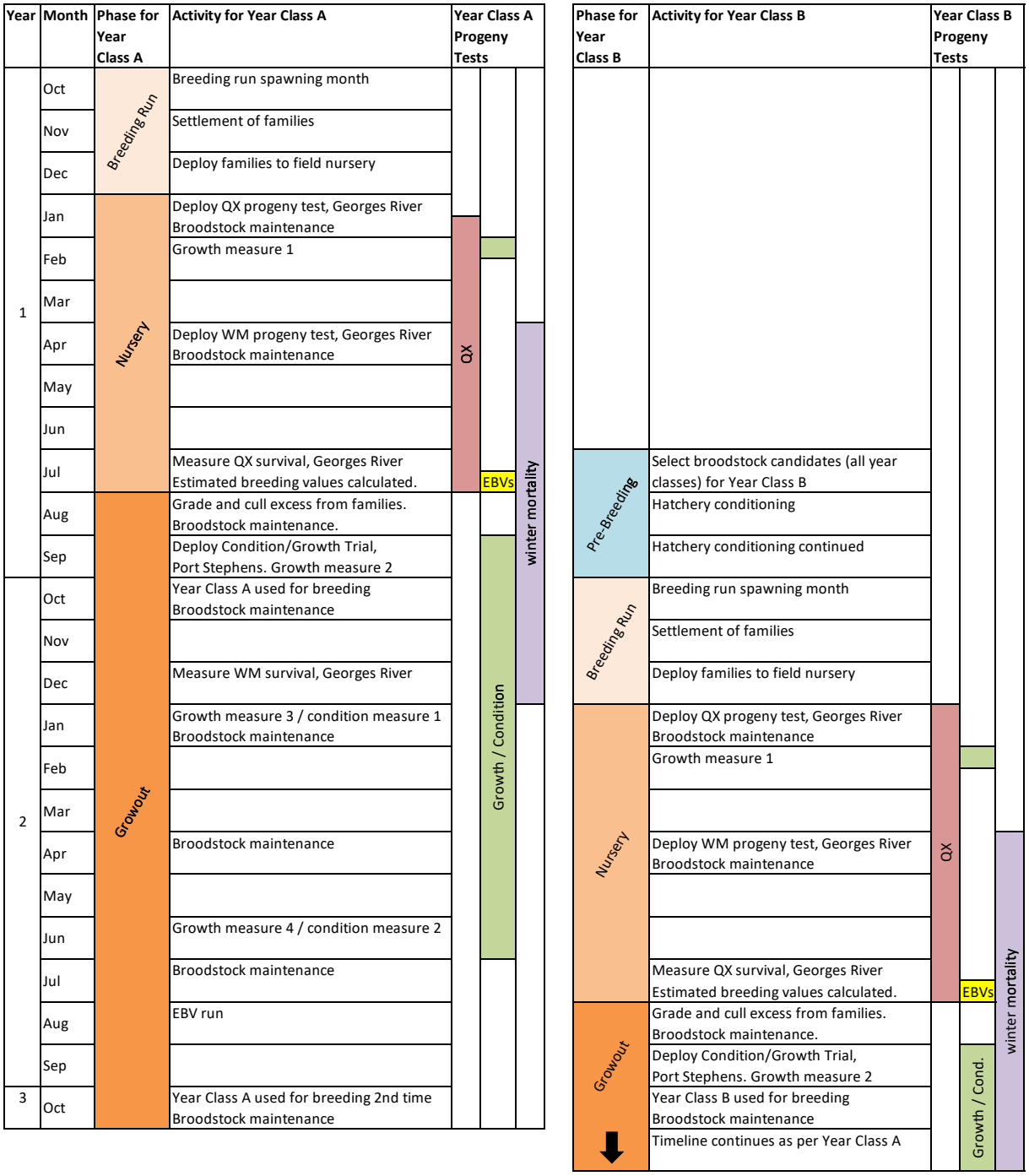

Figure 2.1: Sydney Rock Oyster family breeding program schedule of activities as at July 2019.

Another factor considered was the ability to breed from survivors of QX. This would provide an increased rate of gain by allowing for a within family selection component. However, it has not been possible for two main reasons. One reason is the regulatory restrictions on the movement of oysters. Georges River is a site that has been used for QX screening for many years and has recurrent QX disease outbreaks. Ostreid herpesvirus outbreaks that affect *Crassostrea gigas* also occur in the Georges River which has led to a quarantine closure (Pacific oyster Mortality Syndrome Movement Protocol, 2016) that prevents translocation of SRO broodstock to other non-affected regions. The Yamba site was added as a potential work-around but that site has lacked the consistency of QX expression, meaning the information from Yamba is likely to be sub-optimal. The second and more important reason limiting our ability to breed from survivors is the poor reproductive condition of QX exposed animals. This alone has effectively excluded this as an option, particularly given the desire to introduce a one-year QX selection cycle. At best, there would be a low spawning success rate of QX exposed animals and a need to condition large numbers of

candidates (to cover spawning failures). Additionally, conditioning would need to occur in a biosecure facility with the capacity to treat the water used for conditioning. Large numbers of broodstock and post-treatment of effluent make conditioning of QX survivors impractical.

2.5 Conclusion

A one-year breeding cycle has been successfully introduced for QX resistance in the SRO BP. QX resistance is one of the primary traits for the breeding program and a one-year breeding cycle provides a significant step up in the rate of genetic gains, effectively doubling the rate of genetic progress for this trait. Conceptually this is a simple step, but it has required the use of quantitative genetics to understand the genetic architecture of traits, and knowledge of the reproductive and QX disease cycles to determine the logistics of all breeding program operations.

3. Heritability of winter mortality resistance in Sydney Rock Oysters

Curtis Lind, Michael Dove, Peter Kube and Wayne O'Connor

3.1 Introduction

Winter Mortality (WM) is an economically important disease for the Sydney Rock Oyster (SRO) industry, however, the genetic characteristics of WM expression are not well understood. Improving the resistance to WM through selective breeding has been identified as desirable by the industry and is therefore, a trait of interest to the SOCo breeding program (BP). Earlier efforts have been made to improve WM resistance through mass-selection breeding strategies. Four generations of mass selection breeding reduced losses in the winter mortality resistant line to less than half that measured in the non-selected controls (23% mortality compared to 52%).

SRO breeding activities now focus on multi-trait, family-based approaches. There is a need to better understand the genetic inheritance of WM resistance in greater detail. This section outlines work done to address the following aims:

1) Estimate the heritability of WM resistance in SRO after field exposure to a disease event

2) Estimate the genetic correlation between WM resistance measured at different times during the disease exposure period

3) Estimate the genetic correlation between WM resistance of different age classes of SRO

Results will be used to inform the possibility of including WM resistance as a future trait for genetic improvement in the SOCo selective breeding program.

3.2 Method

A total of 11 field trials were conducted between 2015 and 2018. The goal of each trial was to expose SRO families from SOCo's BP to field conditions and sites most likely to result in winter mortality disease. Two sites were used for the trials, Quibray Bay in the Georges River, and Crookhaven River which is in the Shoalhaven Region on the NSW South Coast. These sites were selected as being prone to regular WM events (Spiers *et al.*, 2014). Trials were deployed between March and April (except one, in August) as either 5-7 month- or 14-17 month-old animals. In this report these age class animals are referred to as 1-year and 2-year olds, respectively, reflecting the approximate age at which they were exposed to WM.

Full-sibling families with known pedigree were produced as described in Dove et al (in prep), and were deployed to the trial sites in three replicates of 50 individuals per replicate. Each replicate was a single basket, herein referred to as "units". Replicate units were distributed among three separate racks using an incomplete block design to allow analyses to account for potential spatial patterns of winter mortality disease within the trial site.

A 'summer measure' of mortality was taken by counts of dead and live oysters within each unit between November and December, after exposure to the winter months. In some trials a 'winter measure' was taken between August and October (Table 1).

All data were uploaded and stored in the CSIRO Selective Breeding Program database system. The statistical software R was used for data manipulation, cleaning and summarising. ASReml v4.0 was used for statistical analyses. Survival was analysed at the individual level as a binary trait, where animals alive at assessment were denoted as 1, and those being dead were denoted as 0.

The following analyses were done to compare genetic parameters and variance component estimates of WM:

1. Univariate analyses of each trial and each measurement period separately

2. Bivariate analysis assuming WM measured in summer and WM measured in winter are different traits.

3. Bivariate analysis assuming WM measured as 1-year old animals and measured as 2-year old animals are different traits.

Survival was analysed as a binary trait using a linear mixed animal model. The model used was:

$$y = Trial + Unit + Trial(Block) + Individual$$

where y is WM survival, trial (PRT_ID) is fitted as a fixed effect, and unit (UNT_ID), block (BLOCK) within trial, and individual (IND_ID) are fitted as random effects.

Heritability (observed scale) was calculated using the variance components estimated in the above models, using the following formula:

$$h^2 = Vadditive / Vunit + Vblock + Vresidual$$

Heritability estimates were transformed to the underlying scale using the method of Dempster & Lerner (1950). All heritabilities are reported on the underlying scale.

3.3 Results

Survival was generally high across all trials, with an overall average survival of 88% and a range from 76% to 99% for individual trials. Survival was higher at Quibray Bay (avg. 92%) compared to Crookhaven River (avg. 81%). Differences in survival between age classes were small, with 1-year-old trials having average survival of 86% and 2-year old trials slightly higher with 90%.

When analysed as individual trials, heritability for WM at the summer measure (i.e. final counts between November and January) ranged from 0.02 ± 0.02 to 0.24 ± 0.08 (h^2 ± s.e., underlying scale). This indicates there was detectable genetic variation for WM, however, trial success was variable. No evidence of spatial influences on WM was observed, as inferred from block variances within a trial site.

A combined bivariate analysis using data from all trials within each site showed that heritabilities of WM for 1-year old and 2-year old oysters at Quibray Bay were 0.35 ± 0.06 and 0.31 ± 0.06, respectively. The genetic correlation (r_g) between 1-year and 2-year olds at Quibray Bay was high and close to unity (r_g = 0.98 ± 0.06), indicating the genetic expression of WM at this site is the same for these two age classes. For trials at Crookhaven River, however, h^2 was 0.02 ± 0.03 and 0.02 ± 0.04 for 1-year old and 2-year old oysters, respectively, indicating no genetic variation for WM at this site.

The consistently poor results from Crookhaven trials, whether analysed as individual trials or combining trials together, indicate these were failed trials. For this reason, any site-site analysis attempting to compare WM expression across Quibray Bay and Crookhaven River would be meaningless and was therefore not carried out.

The genetic correlations between winter and summer measures of WM were consistently very high (near unity), and will be fully reported in Dove *at al*. (in prep, b). However, survival at the winter measurement was always very high, indicating a very low expression of WM resistance. As a consequence, these measurements are unlikely to discriminate families across the full range of WM resistance and unlikely to give precise estimates of genetic merit. Therefore, it appears that there is no benefit to conducting an earlier measurement and that maximising the opportunity for WM to express is preferred.

There was no evidence that genetic expression of WM is correlated to other commercially significant traits in SROs (total weight, condition, QX resistance) based relationships between family estimated breeding

values (EBVs). Further analyses of the genetic correlations between traits will be presented in Dove *et al.* (in prep).

Some broodstock for the families in this study were sourced from the WM mass-selected lines and, therefore had a prior history of selection. The origins of this material and the effects of the prior selection will be reported in Dove *et al.* (in prep). However, a preliminary analysis using a genetic groups model suggests the effects of prior selection were not strong, which provides further evidence of the low heritability and variable expression of this trait.

Table 3.1: Summary of winter mortality trials for the SOCo selective breeding program

Trial	Year class	Site	Number of families	Number of oysters	Age deployed (months)	Event	Event date	Time (days)	Mean survival
1	2014	Quibray	47	12191	7	Deployed	1/08/2015	0	100%
						Summer Measure	9/12/2015	130	99.7%
2	2014	Quibray	45	5258	14	Deployed	15/03/2016	0	100%
						Winter Measure	17/08/2016	155	98.6%
						Summer Measure	22/11/2016	252	94.1%
3	2015	Quibray	31	5640	5	Deployed	15/03/2016	0	100%
						Winter Measure	17/08/2016	155	99.0%
						Summer Measure	22/01/2016	252	95.0%
4	2015	Quibray	37	5231	17	Deployed	17/03/2017	0	100%
						Winter Measure	19/09/2017	186	96.4%
						Summer Measure	7/12/2017	265	91.9%
5	2015	Crookhaven	35	5231	17	Deployed	17/03/2017	0	100%
						Summer Measure	30/11/2017	258	94.2%
6	2016	Quibray	49	7301	5	Deployed	17/03/2017	0	100%
						Winter Measure	19/09/2017	186	95.6%
						Summer Measure	7/12/2017	265	93.4%
7	2016	Crookhaven	47	7006	5	Deployed	17/03/2017	0	100%
						Summer Measure	30/11/2017	258	76.5%
8	2016	Quibray	53	7453	17	Deployed	10/04/2018	0	100%
						Summer Measure	11/12/2018	245	89.6%
9	2016	Crookhaven	47	6535	17	Deployed	10/04/2018	0	100%
						Winter Measure	10/10/2018	183	92.9%
						Summer Measure	15/01/2019	280	77.7%
10	2017	Quibray	65	9616	6	Deployed	10/04/2018	0	100%
						Summer Measure	11/12/2018	245	77.2%
11	2017	Crookhaven	63	9521	6	Deployed	10/04/2018	0	100%
						Winter Measure	10/10/2018	183	91.3%
						Summer Measure	15/01/2019	280	76.9%

3.4 Discussion

WM disease occurs at an unknown time point between July and October. The field exposure trial is deployed in April and is finalised in the following December. The timing of WM disease and the current SRO BP schedule does not enable the breeding cycle for this disease to be reduced to one year. However,

annual breeding and the current SRO BP schedule does make it possible to respond to WM resistance data within 12 months (Figure 2.1).

Significant effort was expended to determine an accurate estimate for the heritability of WM disease. Eleven trials were conducted and the overall success of these was mixed. The results of these trials suggest data collected from the Quibray Bay site are most reliable, and that exposure to WM at roughly 1-year of age gives a greater expression of the disease (i.e. greater mortality). Based on the most reliable data collected it appears that susceptibility to WM disease in SROs has a low to moderate heritability, and therefore potentially improved via selective breeding.

A high genetic correlation between expression of WM at different ages of exposure means that selection based on resistance as a 1-year old should confer resistance as a 2-year old animal. The practical implication for this in a breeding program is that families can be progeny tested at earlier ages, reducing the need for extended field trials or animal holding. In addition, the higher heritabilities combined with greater mortality observed in 1-year old animals indicates that greater genetic gains are likely when basing selection decisions on data collected at this earlier age.

However, consistently high survival of trial oysters deployed in areas known for episodes of WM furthers the perception that this disease is somewhat enigmatic. Effective selective breeding depends on there being measurable variation in the trait of interest. If survival is too high, there is insufficient discrimination between families and selection decisions become ineffective. It is clear from the data that trials at the Crookhaven site were not effective, despite having relatively higher mortalities. The near zero heritability estimates from these trials indicate that mortality is not explained by genetic factors and is most likely due to random "noise" caused by environmental and farming influences. Trials at Quibray Bay were more reliable, but the mostly high survival (low expression of WM) is not ideal. Continued investigation is likely to be required to further refine how best to maximise the genetic expression of WM on a routine basis.

Resistance to WM is not currently part of the long term breeding objective of the SOCo BP. The observation that there are no strong genetic correlations between WM and other commercial traits has direct implications. Firstly, it means that genetic improvement in any of the current traits will not result in a correlated response in WM (in neither positive or negative direction). Consequently, if resistance to WM were to be included in SOCo's BP, it would need to be directly included in its breeding objective, and therefore given an economic weighting together with the other traits of interest. This will have direct implications on the rate of genetic gain achievable.

In a simplified scenario where WM is the only trait under selection and the top 30% of families are selected each generation, estimates for population genetic gain are expected to be within the range of 3.7 – 6 % per generation, translating to an annual genetic gain of 1.5 – 3% per year based on the results of these trials. This is assuming data is collected on 1-year old animals and the severity of WM is similar to that observed here. Under a more realistic scenario where multiple traits are being selected, this rate will be lower. Such a scenario would require a revision of the SOCo Breeding Goals, and the relative selection emphasis placed on each objective trait.

Given WM is likely to remain a significant issue for sections of the SRO producers, the continuation of a routine WM field challenge progeny test for SOCo could be considered as an opportunistic activity if it does not incur a significant additional cost. If future episodes of WM become more frequent and more severe, SOCo would then be well placed to respond in a timely fashion.

3.5 Conclusion

WM of SRO has low to moderate heritability, and thus would potentially respond to genetic selection. The genetic correlations between ages was high at Quibray Bay ($r_g = 0.98 \pm 0.06$) but low at Crookhaven ($r_g = 0.35 \pm 0.87$). There were no obvious correlations between WM resistance and other significant traits, such as QX resistance, condition and growth (primary traits in the SRO BP). Expression of WM through field exposure was low and inconsistent across multiple year classes and sites which reduced discrimination between family survival. Low levels of WM at field trial sites significantly dampen response to selection. The most reliable estimates were obtained from Summer measurement of 1-year-old oysters after exposure

at Quibray Bay site. It is recommended that the SRO BP continue WM field exposure trials of 1-year-old oysters at Quibray if resources permit. This will mean that useful data can be collected if a significant winter mortality outbreak occurs.

4. Incorporation of marker assisted selection utilising stress markers into the SOCo breeding program

Vivian Cumbo, David Raftos, Michael Dove and Wayne O'Connor

4.1 Introduction

The overarching aim for this objective was to determine if genetic marker-assisted selection could successfully be incorporated into the Sydney Rock Oyster (SRO) breeding program (BP) to increase QX disease resistance of families. Additionally, greater knowledge about the mechanisms for QX disease resistance in individual oysters safeguards the SRO BP if families are lost from the breeding nucleus.

To be successfully incorporated into the SRO BP the chosen molecular method had to be fast and cost-effective so that large numbers of oysters could be screened prior to a breeding run. Additionally, the technique to obtain enough genetic material from a single oyster had to be non-lethal and not impact gonadal development so that oysters sampled could be used as broodstock in the subsequent breeding run.

For marker-assisted selection, we targeted the expression of key genes. With this novel approach, we hoped to capture the molecular level phenotypic differences between QX disease resistant and susceptible oyster. To successfully achieve this, a literature search extracting gene expression markers with different expression profiles in QX disease resistant and disease susceptible SROs was performed. Only expression levels that differed in the oyster populations under normal ambient conditions were included in the final list of markers because these captured differences in resting state gene expression levels. Additionally, sampling oysters under normal ambient conditions was important to allow this method to be incorporated into the BP because it enables the technique to be standardised each time that it is performed. Another benefit is it does not require broodstock to be exposed to QX disease, which has implications for gametogenesis and hatchery production of families needing to be done under quarantine protocols.

This work was completed in three stages. The first stage was to devise a procedure to sample broodstock tissue without impacting oyster health and gametogenesis. The second stage was to determine the markers that best separate disease resistant and susceptible oysters. The final stage was to verify if the selected markers successfully determined if an oyster was disease resistant or susceptible to QX.

4.2 Method

4.2.1 Non-lethal Tissue Sampling

To obtain tissue samples, oysters were narcotised with magnesium chloride using the techniques described in Butt *et al.* (2008). While there were some reports of narcotising SROs (Butt *et al.*, 2008), none had successfully narcotised and sampled large numbers of oysters for genetic analysis. A method to non-lethally sample oysters was created and tested on oysters from the five most QX disease resistant and disease susceptible families from the 2015 year class (Table 4.1). Oyster family lines were selected based on their survival results from QX affected estuaries, however, the oysters used were collected from NSW DPI leases in Port Stephens so were QX naïve animals. Oysters were separated into three different treatments to determine the effect narcotising and tissue sampling had on oyster survival (narcotised only, narcotised + tissue sampled, seawater control). Forty oysters from each of the families were acclimated in 750L tanks containing filtered seawater for 2 weeks prior to sampling. Oysters were removed from the tanks and placed in experiment trays and exposed to air overnight, after which a relaxant bath was added to the narcotised only and narcotised + tissue sampled groups of oysters. Between 1-2hr after exposure to the relaxant bath, mantle tissue from the narcotised + tissue sampled group of oysters was biopsied using a biopsy forcep (Tischler Baby 4.2 x 2.3 mm Jaw 21cm Shaft ARMO) and placed in RNA*later* for genetic analysis. After sampling, the oysters were placed back in the 750L tanks and their survival was monitored. One-week post

sampling the oysters were transferred onto the NSW DPI leases at Port Stephens and survival was recorded 49 days later.

Table 4.1: A list of the selected disease resistant and susceptible family lines from the 2015 year class

Resistant	**Susceptible**
2015006	2015020
2015007	2015032
2015008	2015038
2015022	2015040
2015025	2014042

4.2.2 Selected markers that best separate disease resistant and susceptible oysters

Total RNA was extracted from the biopsied oyster mantle using an optimized RNAzol method. Total RNA concentration and purity were checked with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000). Reverse transcription was performed for each oyster on 2µg of total RNA using ImProm-II Reverse Transcription System (Promega) in 40µl reactions with 0.5µg Oligo(dT), following the manufacturer's protocol.

Gene expression of the target genes and reference genes were investigated using qPCR to find the markers that best separated the different populations of oysters. qPCR analyses were run on a LightCycler® 480 II (Roche) following the protocol in Goncalves *et al.* (2016). Each sample was run in triplicate and the LightCycler® 480 II software analyzed the amplification data to produce Cq values, with values averaged among the triplicate curves to provide a single value for each oyster per gene. Reference gene stability was assessed using the web-based RefFinder platform (Xie *et al.*, 2012), and the most stable gene combination included all 5 reference genes. Cq values for each gene were normalized to references genes to give ΔCT.

Four statistical methods were used to determine which genes best separated the disease resistant and susceptible oysters. These included: (1) Monte Carlo simulations to determine the probability that the expected ΔCT values are predicted, (2) Univariate analysis of each gene, (3) Calculation of the log fold change in expression for each gene between the disease resistant and susceptible oyster populations (ΔΔCT), and (4) The linear correlation between gene expression and oyster survival for each family line. A principal component analysis (PCA) plot containing the ΔCT values for the selected genes of each individual oyster was produced.

4.2.3 Verifying selected markers on broodstock oysters

To verify if the selected markers successfully determined if an oyster was resistant or susceptible to QX disease, mantle tissue from 103 broodstock oysters used in the 2018 breeding season were sampled and placed in RNA*later*. The broodstock oysters were from the 2014, 2015, 2016 and 2017 year classes. QX survival rates of the sampled oysters were unknown during the initial sampling to ensure randomized sampling and to not bias downstream results. RNA was extracted from the biopsied mantle tissue, cDNA was synthesized and qPCR was performed on the target and reference genes. Results were overlaid on the PCA plot from above. Some of the oysters used to produce offspring during the 2018 breeding run were biopsied in the non-lethally sampling of oysters experiment.

4.3 Results

A list of 66 published markers, including 5 reference genes was compiled from the literature search (Green *et al.* 2009; Goncalves *et al*., 2016, 2017; McAfee *et al*., 2018).

A method to non-lethally sample SRO was successfully developed. No oyster mortality was detected from any treatment including those biopsied, and the oysters produced sufficient gametes so that they could be used as broodstock in the subsequent breeding run. Furthermore, sufficient amounts of RNA were extracted from the biopsied mantle tissue for qPCR analysis of the 66 selected gene markers.

Statistical analysis of the 61 target genes resulted in a list of 7 potential target markers. The PCA plot of these 7 genes for each individual oyster indicated good separation between the QX diseases resistant and susceptible individuals (Fig. 4.1). QX survival ranged from 51.3 – 76.0% in the five most disease resistant families from the 2015 year class (Fig. 4.1, green), while the susceptible family lines had between 4.0-16.0% survival (Fig. 4.1, blue).

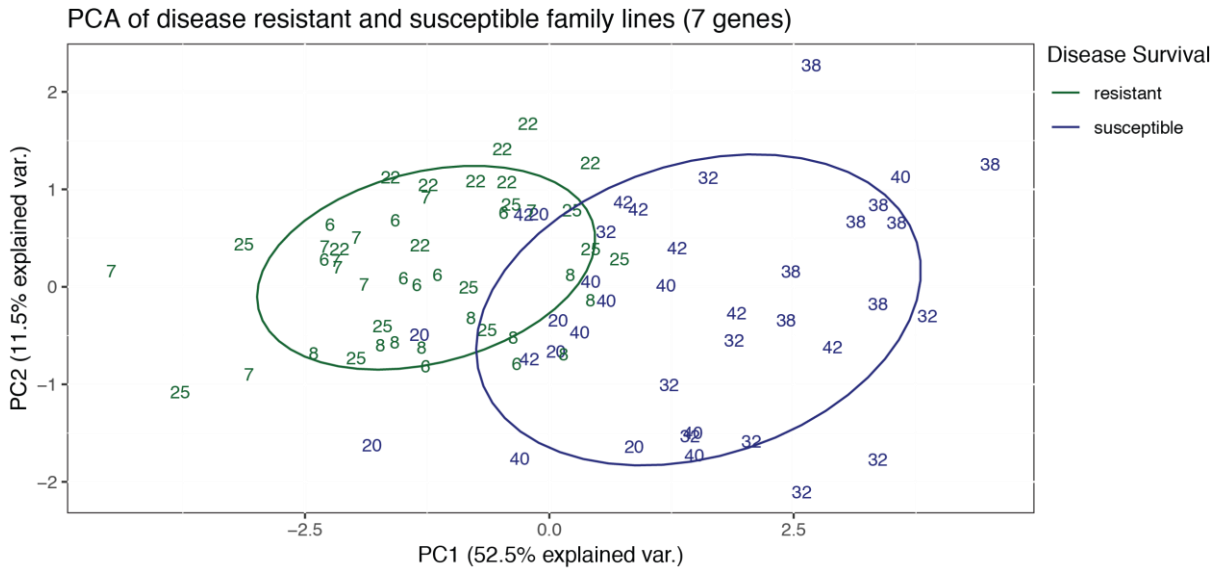

Figure 4.1: A principal component analysis plot of ΔCT values of the 7 target genes for the QX disease resistant and susceptible oysters from the 2015 year class.

Overlaying ΔCT of the 7 genes from each of the 103 broodstock oysters sampled during the 2018 breeding season indicated that the majority of oysters were resistant. These results were confirmed by the NSW DPI QX survival data which revealed that the majority of oysters sampled were from families with QX survival of 50% or above (Fig. 4.2). Only 6 of the oysters sampled in the 2018 breeding season were from families with low survival of less than 15%. Genetic analysis of these 6 individuals revealed that 4 (67%) were susceptible to QX (Fig. 4.3).

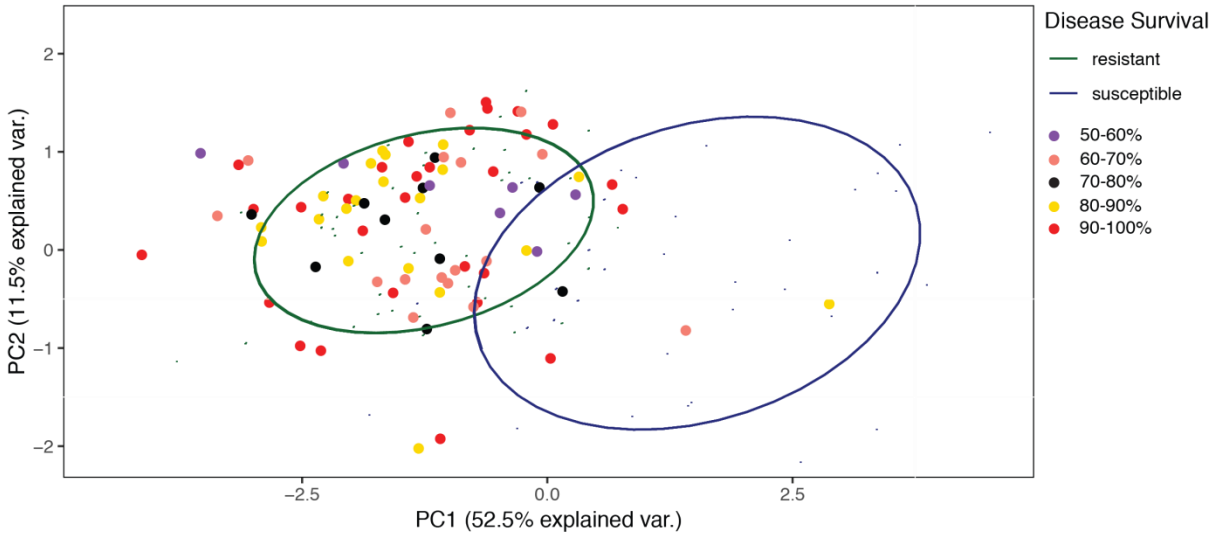

Figure 4.2: Individuals from resistant family lines (50-100% survival) overlaid on the reference principle component analysis plot

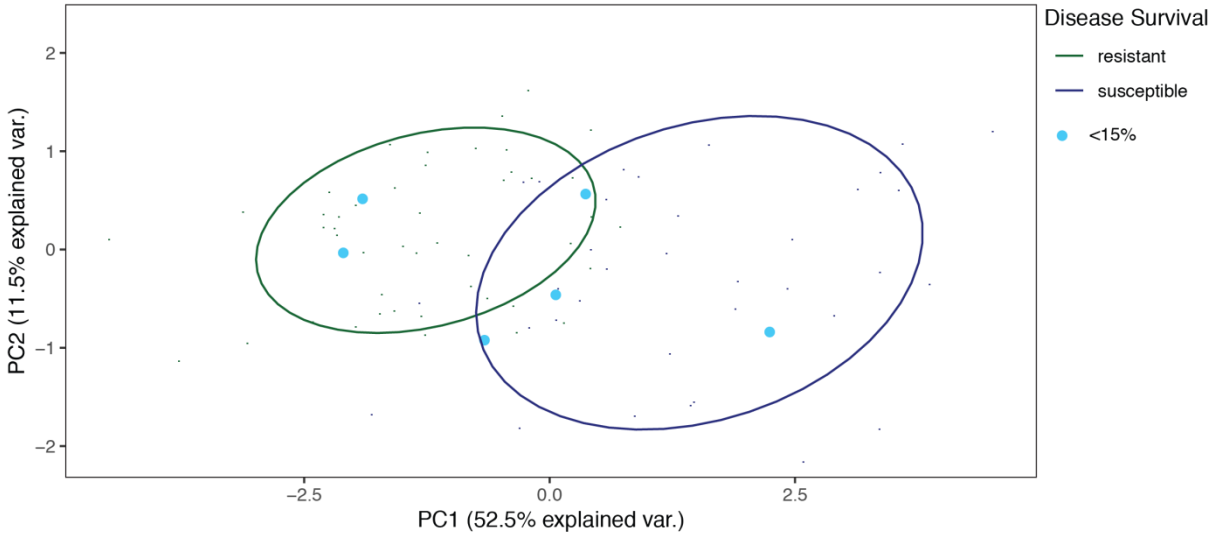

Figure 4.3: Individuals from susceptible family lines (<15% survival) overlaid on the reference principle component analysis plot

4.4 Discussion

The results from these experiments have successfully addressed the primary aims of this study, including:

1) Successful development of a method to sample oyster tissue for genetic analysis that does not affect their reproduction cycle or survival.
2) Generating a list of 7 gene expression markers that could be incorporated into the breeding program to accelerate QX disease resistant selection.
3) Using qPCR as a fast and cost-effective method for screening large numbers of oysters.

Unfortunately, because so few susceptible oysters were sampled in the second experiment it is currently unclear if these genetic markers are successfully capturing susceptible individuals. To rectify this, more sampling of susceptible oysters from a range of year classes is required.

Ideally, to further increase the robustness of these results, the resistance or susceptibility of individual oysters should be determined using the gene expression markers. After sampling, the individuals should be placed in a QX affected estuary over a QX outbreak and their survival should be recorded. Comparing individual oyster survival to their predicted disease resistance obtain from the genetic markers will unequivocally validate or invalidate this method.

This project demonstrated that marker-assisted selection has the potential to identify highly QX resistant oysters within families. Tissue samples can be collected from individuals in families that have a high EBV ranking for QX disease survival. This is an additional step after candidate families have been identified for the subsequent breeding run. The broodstock candidate list is produced in early August and identifies the highest-ranking families that are used as parents for the next generation. There is sufficient time to screen, analyse, tag and condition animals before the subsequent breeding run so that individuals meeting criteria for high QX disease resistance are targeted as broodstock.

An objective of this project was to reduce the generation time for QX disease resistance to one year. Further testing is required to determine if tissue samples can be collected for screening from the youngest year class due to their smaller size. If the youngest year class can be effectively screened this method will complement the one-year breeding cycle for QX disease and increase genetic gains for QX disease resistance.

Marker-assisted selection has the potential to increase genetic gains for QX resistance by enabling within family selection, and this project has identified candidate markers. However, there are still additional validation steps that are needed before this technology is integrated into the logistics of the breeding program. Things that still need to be considered are the:

- integration of these data into the EBV model
- additional costs associated with screening large numbers of oysters
- optimal time to screen animals (to get maximum discrimination between susceptible and resistant oysters and have data available for breeding decisions)
- other activities in the BP schedule; management of tagged broodstock prior to spawning, and
- screening of sufficient animals to account for the poor single pair mating success rate.

The increase in QX resistance provided by marker assisted selection needs to be at a certain threshold to substantiate the additional costs of adding this process to the BP. Further research is needed to quantify the increase in QX resistance resulting from marker assisted selection and to consider the technical and cost benefits of incorporating this step into the existing BP logistics.

4.5 Conclusion

This project developed a method to sample oyster tissue for genetic analysis that does not affect survival or the reproduction cycle. Seven gene expression markers were identified that could be incorporated into the BP to accelerate disease resistant selection. qPCR was a cost-effective method for screening large numbers of oysters and could provide results in sufficient time to make breeding decisions. Marker-assisted selection has the potential to increase genetic gains for QX resistance by enabling within family selection, and this project has identified candidate markers. Further development is required to determine if these genetic markers can identify susceptible individuals. Additional validation steps are needed before maker assisted selection can be fully incorporated into the logistics of the BP.

Conclusion

The aims of this study were:

- to double the number of families for the SOCo BP
- understand resistance and determine heritability for QX and WM disease at different life stages,
- reduce the breeding cycle for QX and WM disease to one year, and
- incorporate marker assisted selection using stress markers into the SOCo BP

The number of families planned for the SOCo BP was more than doubled. Eighty families were planned prior to the start of this project and 179 new families were available for selection in 2019. Families in the 2014 and 2015 year classes continue to rank highly and are still available for breeding runs taking the total number of families available to industry more than 250.

A one-year breeding cycle has been successfully introduced for QX resistance effectively doubling the rate of genetic progress for this trait. To do this required the use of quantitative genetics to understand the genetic architecture of traits, and knowledge of the reproductive and QX disease cycles to determine the logistics of all BP operations.

Expression of WM through field exposure was low and inconsistent across multiple year classes and sites which reduced discrimination between family survival. SRO disease resistance to WM has low to moderate heritability, and thus would potentially respond to genetic selection. No obvious correlations between WM resistance and QX resistance, condition and growth were found. The best estimates were obtained from the Quibray Bay site using survival data measured in December from 1-year-old oysters.

This project developed a non-lethal method to sample oyster tissue for genetic analysis that does not affect the reproduction cycle. Seven gene expression markers were identified that could be incorporated into the SOCo BP to increase QX disease resistance. qPCR was a cost-effective method for screening large numbers of oysters to enable within family selection of QX resistant individuals. However, further development is required to determine if these genetic markers can identify susceptible individuals and additional validation steps are needed before maker assisted selection is incorporated into the logistics of the BP.

Implications

Significant gains in QX disease resistance have been made over the course of this project. Additionally, more is known about the mechanisms of QX disease resistance in individual oysters. The new BP design has been implemented and will continue to step up QX resistance of families providing annual breeding continues.

The Sydney Rock Oyster industry remains vulnerable to QX disease. The SRO BP is operational and is delivering QX disease resistant oysters to industry. These oysters provide both a risk mitigation strategy for oyster enterprises and the NSW Government. Socio-economic implications of a QX disease outbreak are lessened through a more resilient oyster industry and reduced potential of abandoned oyster lease remediation which is costly to tax payers.

The robust methodology and design developed for the SOCo BP provides an excellent platform to respond to unidentified future problems and needs, for example, emergent diseases and climate change, to ensure the long term sustainability of this industry.

Recommendations

The time frames to deliver the SOCo Breeding Goal are dependent on continued genetic services, annual breeding runs and QX disease outbreaks during progeny testing. Future resourcing of the SOCo BP is presently uncertain. This includes breeding runs, progeny tests, genetic services and access to the data management system. Future funding is required to properly resource all components of the SOCo BP including commercialisation activities so that industry can continue to access the benefits of improved stock.

Currently, resistance to WM is not selected for at present in the SOCo BP. The observation that there are no clear genetic correlations between WM and other commercial traits of interest has direct implications. Firstly, it means that genetic improvement in any of the currently selected traits will not see a correlated response in WM (in neither positive or negative direction). Consequently, if resistance to WM were to be included in SOCo BP, it would need to be directly included in its breeding objective, and therefore given its own weighting amongst the other traits of interest. This will have direct implications on the rate of genetic gain achievable. Given WM is likely to remain a significant issue for much of the SRO industry, the continuation of a routine WM field challenge progeny test for SOCo should continue. If future episodes of WM become more frequent and more severe, SOCo would then be well placed to respond in a timely fashion. Therefore it is recommended that the SOCo BP continue WM field exposure trials of one-yr-old oysters at Quibray Bay if resources permit.

Further development

The highest priority for further development of the SRO BP is increasing the success rate of single pair mated crosses. Family production success during this project was 27% which is substantially lower than that for other oyster breeding programs. Improved understanding of SRO gamete quality, optimal fertilisation conditions and embryonic development from stripped spawned individuals will increase family production success. Protocols that induce individuals to naturally spawn in the absence of other oysters could increase family production success and revolutionise SRO hatchery techniques. Increased fertilisation success rates and larval yield will boost the number of SRO families possible in a single breeding run to well beyond 80.

It is not possible to reduce the breeding cycle for both QX and WM disease resistance to one year. This is due to the infection period of these diseases occurring at different times of the year. This is complicated by the fact that winter mortality infection can occur at any time between the months of June and October and the disease agent/s responsible have not been identified. WM field exposure trials of one-yr-old oysters at Quibray Bay should continue so that it is possible to respond to a significant WM disease outbreak within one year.

Due to low numbers of susceptible oysters being sampled, it is unclear if genetic markers developed in this project are able to capture susceptible individuals. To rectify this, more sampling of susceptible oysters from a range of year classes is recommended. Ideally, to make these results more robust, the resistance or susceptibility of individual oysters should be determined using the gene expression markers and then exposed to QX disease for survival measurements. Comparing individual oyster survival to their predicted disease resistance obtain from the genetic markers will unequivocally validate or invalidate this method.

Validation steps are needed before marker-assisted selection is incorporated into the logistics of the breeding program. Things that still need to be considered are the:

- integration of these data into the EBV model
- additional costs associated with screening large numbers of oysters
- optimal time to screen animals (to get maximum discrimination between susceptible and resistant oysters and have data available for breeding decisions)

- other activities in the BP schedule; management of tagged broodstock prior to spawning, and
- screening of sufficient animals to account for the poor single pair mating success rate.

The increase in QX resistance provided by marker-assisted selection needs to be at a certain threshold to substantiate the additional costs of adding this process to the BP. Further research is needed to quantify the increase in QX resistance resulting from marker-assisted selection and to consider the technical and cost benefits of incorporating this step into the existing BP logistics.

Extension and Adoption

The outcomes from this research extend directly to the SRO industry and have already been successfully incorporated in the SOCo BP. Additionally, outcomes have been documented in the Sydney Rock Oyster Breeding Program Manual (Dove *et al.*, 2019). Families created during this project have joined the SOCO BP and will be used to produce the 2019 year class. The one-year breeding cycle for QX disease continues to be used to determine genetic merit of families for QX disease survival and a successful QX disease field trial of 4 mo oysters was completed in July 2019. QX selection markers developed as part of this study will be used to identify resistant and susceptible individuals within SRO families and these animals will be used as broodstock for the 2019 year class. A WM field trial using one-year-old animals at Quibray Bay is in progress to gather the data to improve heritability estimates as well as advance our understanding of WM resistance among families and how this trait relates to QX disease resistance, growth and meat condition.

Project outcomes have directly contributed to:

- A robust SOCo BP focused on delivering industry's breeding objective
- Delivery of improved oysters to industry that have superior QX disease resistance, growth as well as improved meat condition compared to mass selected lines
- A SRO Breeding Program manual updated with improvements from this project to facilitate transfer of SRO breeding knowledge and skills to industry
- 250 pedigreed SRO families available for commercialisation

Publication of this research in international scientific journals is anticipated. Two publications are in preparation. The titles of these manuscripts are: "Genetic selection of Sydney Rock Oysters for resistance to QX disease" and "Haemolymph microbiome of the Sydney Rock Oyster breeding lines for resistance to QX disease from Port Stephens and Wallis Lake". The authors have extended research outcomes from this project at conferences, oyster industry events and sharing knowledge with growers during field work. Extension of outcomes from this project was provided through the following channels:

Conference presentations

Dove, M., Kube, P., Lind, C., Cumbo, V., O'Connor, W., Saowaros, M., Elizur, A., Seeto, R., Gibb, Z., Abramov, T., Raftos, D., Wilkie, E., The Select Oyster Company and Southern Cross Shellfish. New technologies to improve Sydney rock oyster breeding and production. The South Australian Oyster Industry 2019 Seminar, 21-23 August 2019, Streaky Bay, South Australia.

Dove, M., Kube, P., Lind, C., Cumbo, V., O'Connor, W., Saowaros, M., Elizur, A., Seeto, R., Gibb, Z., Abramov, T., Raftos, D., Wilkie, E., The Select Oyster Company and Southern Cross Shellfish. The Sydney Rock Oyster Breeding Program – Update and Lessons for the Australian Oyster Industry. Shellfish Futures 2019, 16-17 August 2019, Orford, Tasmania.

Dove, M., Kube, P., Lind, C., O'Connor, W. and The Select Oyster Company. 2019. Sydney Rock Oyster Breeding Program – Traits, Trials and Achievements. 2019 NSW Oyster Conference, 6-8 August 2019, Wallis Lake, New South Wales.

Dove, M., Kube, P., Lind, C., Cumbo, V., O'Connor, W., Saowaros, M., Elizur, A., Seeto, R., Gibb, Z., Abramov, T., Raftos, D., Wilkie, E., The Select Oyster Company and Southern Cross Shellfish. 2019. Advancement in the Sydney Rock Oyster Breeding Program – Families and New Technologies. 2019 NSW Oyster Conference, 6-8 August 2019, Wallis Lake. New South Wales.

Dove, M. and O'Connor, W. 2019. Refining Sydney rock oyster breeding. 3rd Australia New Zealand Marine Biotechnology Conference, 20-22 May 2019, The University of New South Wales, Sydney Australia

Dove, M. and O'Connor, W. 2018. History, status and future of oyster culture in Australia. Rushan International Oyster Forum, 21-23 April, China.

McOrrie, S., Dove, M. and O'Connor, W.A. 2017. Breeding a "better" disease resistant oyster – the trade-offs. 7th International Oyster Symposium, 11-14 September 2017, Bangor, UK.

Wilkie, E., 2017., 2017. Boosting Sydney Rock Oyster production through an industry owned breeding program. Seafood Directions, 27 September 2017, Sydney, Australia.

Dove, M., Kube, P., O'Connor, W., Johnston, K., Archer, B. and Wilkie, E. 2017. Breeding better oysters: traits and trade-offs. NSW Oyster Conference, 22-24 August, Merimbula, NSW.

Dove, M., Kube, P. and O'Connor, W. 2017. Breeding disease resistant Sydney rock oysters for the future. NSW Oyster Conference, 22-24 August, Merimbula, NSW.

Non-scientific communications

Dove, M. 2018. CRC-P News: Smart Strategies for Sydney Rocks. Oysters Australia website https://www.oystersaustralia.org/blog/smart-strategies-for-sydney-rocks

Dove, M. 2018. Sydney rock oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 23, December 2018.

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Dove, M. 2018. "2016-802 Accelerated Sydney rock oyster breeding research". Presentation at the Oyster Australia R&D Meeting, Sydney Fish Market, Sydney, NSW, 9 April 2018.

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Dove, M. 2017. Sydney rock oyster breeding update. Aquaculture News, NSW Department of Primary Industries, Issue 21, June 2017.

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Dove, M.C. & O'Connor, W.A. 2017. NSW DPI breeding and research update. Oceanwatch and Local Land Services Travelling Workshops for the South Coast Oyster Industry, 30 May – 2 June: Tanja, Pambula, Batemans Bay and Greenwell Point, NSW.

Dove, M.C. & O'Connor, W.A. 2017. Sydney rock oyster breeding: current progress and future directions. FRDC Hatchery Hub Workshop, 8 February. Port Stephens Fisheries Institute, Taylors Beach, NSW.

Project coverage

Information related to this project and the Future Oysters CRC-P was provided to the following ABC programs:

7 August 2019	ABC Mid and North Coast Rural Report (Reporter: Michael Cavanagh). Interview at Barclays Oyster Shed at the NSW Oyster Conference 6:15 am. https://www.abc.net.au/radio/midnorthcoast/programs/mid-and-north-coast-rural-report/mid-and-north-coast-rural-report/11372472
3 October 2018	ABC Landline interview with Sean Murphy, Port Stephens Fisheries Institute for 'Rock Steady: Sydney rock oysters enjoy a resurgence in price and popularity' https://www.abc.net.au/news/2018-11-03/rock-steady:-sydney-rock-oysters-enjoy-a/10463346.
23 February 2017	ABC Rural Report: (Reporter: Bronwyn Herbert) Oyster growers hopeful new genetics boost quality. http://www.abc.net.au/news/rural/2017-02-23/growers-access-to-oyster-genetics/8296764

Project materials developed

Sydney Rock Oyster families

Sydney Rock Oyster Breeding Program Manual updates

Appendices

List of researchers and project staff

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Mr Walter Scifleet, NSW DPI

Mr Chris Stanley, NSW DPI

Mr Nigel Valentine, CSIRO

Dr Mathew Wassnig, SOCo

Dr Emma Wilkie, SOCo

Intellectual Property

Prior intellectual property that project partners brought to this project:

NSW DPI's multi-generational data set on traits of Sydney rock oyster breeding and selection.

NSW DPI's selected family lines of Sydney rock oysters including those supplied by NSW DPI or held/supplied by third parties.

SOCo and NSW DPI has entered into an intellectual property (IP) working agreement which sets out the IP rights of SOCo and NSW DPI in regard to the broodstock and data (available on request).

Oyster Selective Breeding database, owned by CSIRO

Intellectual property arising from this project:

Breeding population animals, the pedigree records and the performance data relating to those animals from the 2016, 2017 and 2018 year classes generated during the life of this project is owned by NSW DPI

IP of the markers is owned by Macquarie University

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