Securing and Enhancing the Sydney Rock Oyster Breeding Program

W.A. O'Connor, D.A. Raftos, M.C. Dove, A. Kan & K. Johnston

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Securing and Enhancing the Sydney rock oyster breeding program.

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Authors:	Wayne A. O'Connor, David A. Raftos, Michael C. Dove, Alison Kan & Kyle Johnston.
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NON-TECHNICAL SUMMARY

2006/226 SECURING AND ENHANCING THE SYDNEY ROCK OYSTERS: BREEDING PROGRAM

PRINCIPAL INVESTIGATOR: Wayne O'Connor

ADDRESS: NSW Department of Investment and Infrastructure
Port Stephens Fisheries Institute
Private Bag 1
Nelson Bay, NSW, 2315
Tel ephone: 02 49821232 Fax: 02 4981107

OBJECTIVES:

1. To establish pair mating protocols necessary for the development of selectively bred oyster lines
2. To confirm the association between PO and QX resistance using pair matings and test the performance of PO-selected family lines in QX-prone estuaries
3. To identify and characterise additional genetic markers of disease resistance
4. To assess the value of cryopreservation to secure family lines for later use
5. To assess the use of non-chemical means for the induction of triploidy in SRO
6. To make the family lines produced in this research available to SOCo for incorporation in future breeding plans

NON TECHNICAL SUMMARY:

Outcomes Achieved

The Select Oyster Company breeding plan has been amended to include a comprehensive manual of protocols for pair mating Sydney rock oysters (4.1). The association between phenoloxidase (PO) and QX resistance has been confirmed and a sensitive test for PO genotypes has been developed so that the breeding program has a tool to genotype parents and large numbers of their progeny to assist breeding (4.2-4.8). Protocols for cryopreservation of gametes have been developed and documented as a potential strategy to reduce SOCo's overall maintenance costs for the lines and increase genetic security (4.9). Non-chemical means of triploidy induction in SRO (temperature and pressure shock) were tested (4.10). While these techniques were not as effective as existing chemical induction techniques, they have formed the basis of ongoing research to see if efficacy can be increased. A total of 60 pair mated family lines, including 31 lines with differing PO phenotypes, have been produced and are undergoing performance assessment in the field. These families will be made available to SOCo for incorporation in the industry breeding program.

The NSW, Sydney rock oyster (SRO) industry has undergone major structural change in the past five years that has seen it begin the adoption of selectively bred SRO hatchery seed and establish a company (Select Oyster Company, SOCo) to manage commercialisation of the oyster breeding program. To foster the process, this program has continued investigation and development of oyster husbandry techniques to secure QX resistant breeding lines, and to identify genes associated with QX disease resistance.

Previous success in SRO breeding has been based on mass selection in which a comparatively small number of family lines (4-5) have been produced on a large scale. To permit alternative

breeding approaches, protocols to produce pair mated SRO families from two single adults have been developed in which 30 or more families can be produced concurrently. These protocols using small scale rearing vessels (200L) have been documented in detail for inclusion the SOCo practical operations manual and appended to this report (Section 10.3). Central to these protocols are practices to ensure the gentic intergrity of each line. To demonstrate their efficacy, these techniques have now been used to produce over 60 family lines that will be available for incorporation within future SOCo breeding plans once full performance evaluations have been completed (Objectives 1 & 6). It is envisaged that SOCo will select the best performing of these lines to form the basis of the Sydney rock oyster industry's ongoing breeding program.

Investigations of oysters surviving QX outbreaks indicated that the a particular enzyme, phenoloxidase (PO), is associated with disease resistance and that suppression of this PO activity leads to disease susceptibility. This research has confirmed that association. First by characterising the PO enzyme in SRO, showing that a precursor, proPO, exists in oyster hemocytes (blood cells) and demonstrating how it can be activated to become PO (4.2). Then the link between PO and QX disease resistance was shown, illustrating that PO is a critical component of the cellular defences of oysters against infection by *Marteilia sydneyi* (the organism responsible for QX disease; 4.3). We found SRO hemocytes can recognise and phagocytose (engulf) *M. sydneyi*, and that resistance against QX disease may be associated with enhanced phagocytosis and heightened phagolysosomal activity in QX resistant oysters.

Among the 60 family lines produced were 30 families created from crosses within and between QX resistant and non-selected wild type (WT) oysters. When these families were grown in a QX disease prone area, oysters from QX × QX crosses had significantly lower mortality compared to QX × WT or WT × WT families. Mortality in the different crosses was strictly correlated with infection by *M. sydneyi*. This suggested that the majority of mortality observed in the QX disease-prone growing area was the result of QX disease. And that there was strong association between parentage and mortality resulting from QX disease. Among the families, five different forms of PO were identified. One form of PO (PO^d) was much more common among family lines which survived QX, while another variant (PO^b) was associated with increased mortality. The data strengthen the association between the PO variants and mortality associated with QX disease, but also suggested that other genetic factors contribute to survival (4.4, 4.5).

In addition to PO, this research has used proteomics to identify other proteins associated with QX disease resistance and thus has identified new potential molecular markers of QX disease resistance (4.6, 4.7, 4.8). Six proteins were found that were clearly associated with resistance and were characterized by mass spectrometry. Two of the proteins were extremely similar to superoxide dismutase-like molecules from the Pacific oyster, *Crassostrea gigas*, and the Eastern oyster, *Crassostrea virginica.* The remaining four proteins had no obvious similarities to known molecules.

Current PCR tests for *M. sydneyi* are extremely sensitive and are capable of detecting the organism irrespective of whether it goes on to cause QX disease. As the QX organism is found in a number of estuaries in NSW where QX disease has not been observed, there is a use for a test that can detect the later sporulating stages of *M. sydneyi* that are associated with the disease. While investigating markers for resistance, several of the proteins were isolated that seem to represent potential markers of sporulating *M. sydneyi* infection and so may be effective candidates for protein based diagnostic assays (4.8).

Cryopreservation of gametes (eggs and sperm) is a valuable tool for breeding programs that has a number of potential benefits. Cryopreserved gametes can be used to improve hatchery operations by providing eggs or sperm on demand, eliminating the need to maintain live broodstock. These gametes can be held to protect valuable genetic lines and can be stored well beyond the natural life span of any particular oyster so it can be used in future breeding exercises. Here we assessed the

potential for cryopreservation of SRO gametes. Reliable protocols for the cryopreservation of sperm were developed and a Standard Operating Procedure has been incorporated in the SOCo practical operations manual. Batches of SRO D-larvae, large enough for family line production, have been produced using cryopreserved sperm. Successful cryopreservation of SRO eggs was also achieved; although fertilisation success has been low (5-9%) and further development is required (Objective 4).

To assess the use of non-chemical means for the induction of triploidy in SRO, a number of trials were undertaken in which the individual and synergistic effects of temperature and pressure shock on SRO ploidy were investigated. Temperature shock trials were conducted in which the onset of temperature shock, the duration of the temperature treatment and magnitude of the temperature shock were assessed in terms of ploidy and larval development. The highest levels of triploidy achieved (>90%) arose from treatments in which eggs were shocked at 36^{o}C, 15 mins post fertilisation for a period of 20 mins. However, as the ploidy induction efficacy of treatments increased, the larval development percentages fell to the point at which no development beyond trochophore stage occurred in the most effective treatments. Pressure trials were done in which the impact of pressures between 5000 and 8000 psi on ploidy in SRO larvae were assessed. Overall the percentage triploidy was low, with the best results achieved at 5000-6000 psi. Attempts to use temperature and pressure synergistically are ongoing but to date have failed to provide significant increases in triploidy over and above that achieved with temperature alone. Further, development to D-veliger stage larvae remains poor in treatments in which favourable results are obtained (Objective 5).

KEYWORDS: Sydney rock oyster; *Saccostrea glomerata*; Breeding; Disease Resistance, Phenoloxidase; Cryopreservation; Triploidy

1. BACKGROUND

Oyster farming is one of Australia's top five aquaculture industries. However, its largest contributor, the Sydney rock oyster (SRO) industry, has undergone two decades of decline in which production has fallen by 40%. Two of the greatest challenges to SRO farming have been competition from faster growing oyster species and the catastrophic effects of QX disease. To counteract these impacts, industry, I&I NSW and FRDC have invested 15 years of research and millions of dollars to breed lines of fast growing, disease resistant rock oysters.

FRDC-sponsored research, conducted by I&I NSW, was undertaken to reduce the reliance of SRO farmers on spat caught on sticks and slats from the wild. As a result, a commercial hatchery was established. In 2003 and 2004, approximately 25 million fast growing/disease resistant SRO spat were produced for sale to farmers. This figure has now increased to between 25-30 million spat per annum, which constitutes as much as 25% of total industry spat demand.

In 2003, progress in hatchery technology encouraged farmers to form the Select Oyster Company (SOCo) to supply selectively bred oysters. FRDC is helping to formulate management plans that will guide SOCo's operations over the next ten years. The development of low-cost methods to breed oysters based on selection for reliable genetic markers will be critical to SOCo's plans. Already, strong evidence suggests that the defensive enzyme, phenoloxidase (PO), represents a genetic marker of QX disease resistance, and additional disease resistance genes should be identifiable (Newton et al., 2004; Bezemer et al., in press).

2. NEED

The SRO industry has the potential to exploit new export initiatives and increase production. Any resurgence in the industry will depend on SOCo's ability to manage the development and supply of selectively bred oysters. FRDC has fostered this process by supporting breeding programs and hatchery research, whilst encouraging industry management of technology. The Benzie Report, Oyster Research Advisory Committee (ORAC) R&D Strategic Plan and the Action Plan developed at the FRDC-funded QX workshop in June 2005 have all indicated that effective management strategies for the SRO industry must be underpinned by; 1) the continued development of oyster husbandry techniques to secure QX resistant breeding lines, and, 2) the identification of genes associated with QX disease resistance that can be used for marker-assisted selection. These complementary research goals represent a critical step in "QX proofing" NSW estuaries against ongoing and catastrophic QX disease outbreaks. Continued research may also allow farming to re-establish in areas abandoned because of QX disease. Integration of genetic markers and single pair mating into the hatchery-based breeding program will increase the efficiency of selection, and decrease the number of generations required to establish true resistant lines. Without marker assisted selection, the breeding program may not reach its full potential in time to prevent further losses.

3. OBJECTIVES

1. To establish pair mating protocols necessary for the development of selectively bred oyster lines

2. To confirm the association between PO and QX resistance using pair matings and test the performance of PO-selected family lines in QX-prone estuaries

3. To identify and characterise additional genetic markers of disease resistance

4. To assess the value of cryopreservation to secure family lines for later use

5. To assess the use of non-chemical means for the induction of triploidy in SRO

6. To make the family lines produced in this research available to SOCo for incorporation in future breeding plans

4. RESEARCH & DEVELOPMENT

4.1 "Hatchery production of Sydney rock oyster breeding lines"

The Sydney rock oyster (SRO) industry has undergone major structural change in the past five years that has seen it begin the adoption of selectively bred SRO hatchery seed and establish a company (Select Oyster Company, SOCo) to manage commercialisation of the oyster breeding program. To foster this process, the continued investigation and development of oyster husbandry techniques to secure QX resistant breeding lines has been required.

Previous success in SRO breeding has been based on mass selection in which a comparatively small number of family lines (4-5) have been produced on a large scale. To permit alternative breeding approaches, protocols to produce pair mated SRO families were needed in which 30 or more families could be produced concurrently.

The basic principles and techniques used for the hatchery production of Sydney rock oysters, *Saccostrea glomerata* have been described previously (O'Connor et al., 2008). This manual has been developed to extend those methodologies and techniques to improve the ease and efficiency of the production mass selected SRO breeding lines and to permit the production of pair mated family lines. In particular, it acknowledges the need for larger numbers of smaller cultures to be run concurrently, while ensuring the genetic integrity of each separate breeding line through the use of specific rearing protocols and implementing stringent isolation measures. In addition, the manual has also been designed to assist SOCo with issues specific to the breeding program, such as broodstock management, translocation protocols and stock delivery that would not normally be faced in commercial production.

A system is described that has allowed the concurrent culture of up to 60 batches of SRO larvae. Larvae are produced solely from strip spawned gametes and then isolated to a pair of 200L tanks that will house a single family line for the duration of their culture until settlement. Along with their isolation in dedicated tanks, a dual tagging system is applied. This system allows a tag to remain on the tanks to indicate which family they house and for a tag to be attached to containers holding larvae during water changes to prevent confusion. Protocols for the use of replicate screening systems to prevent cross contamination of batches during water exchanges are described.

Settlement is achieved entirely through the use of epinephrine and families are retained in downwelling systems specifically adapted to allow spat retention in their original larval culture tanks. Spat within families remain isolated in these systems well beyond the time at which settlement has ceased and larvae have all been removed. It is not until larvae have reached a size at which they can be retained on 1 mm mesh that they are eventually moved to communal sytems. Here they remain in separate downwelling screens until large enough to be taken to the field.

The nursery process departs slightly from the normal SRO nursery process. Spat are retained in the hatchery until a mean shell length of approximately 3 mm is reached and they are retained on 2mm mesh. Family lines are placed in mesh bags (1100 µm) secured within a section of a 8 mm mesh plastic tray. All mesh bags are labelled using numbered plastic tags allowing identification of the family and only one mesh bag is opened and worked through at any time to reduce the risk of cross contamination.

The full breeding manual has been appended to this report (Appendix 10.3).

4.2 Characterization of phenoloxidase activity in Sydney rock oysters (*Saccostrea glomerata*)

Saleem Aladaileh, Peters Rodney, Sham V. Nair and David A. Raftos[1]

Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia

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Abbreviations: CPIC, complete protease inhibitor cocktail; DDC, DOPA-decarboxylase; FSW, sterile filtered sea water; HBHZ, *m*-hydroxybenzylhydrazine; HL, hemolymph lysate; 4HA, hydroquinine monomethyl ether; LPS, lipopolysaccharide; L-DOPA, L-3,4-dihydroxyphenylalanine; MAC, marine anti-coagulant; MBTH, 3-methyl-2-benzothiazolinone hydrazone; NADA , N-acetyldopamine; OD, optical density; PAMPs, pathogen associated molecular patterns; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PO, phenoloxidase; ProPO, prophenoloxidase; PRR, pattern recognition receptor; PTU, phenylthiourea; SDS, sodium dodecyl sulfate.

ABSTRACT

Phenoloxidase (PO) activity was studied in Sydney rock oysters (*Saccostrea glomerata*). As in other molluscs, PO was found to exist as a pro-enzyme (proPO) in hemocytes. ProPO could be activated to PO by exogenous proteases (trypsin and chymotrypsin), exposure of hemocytes to pathogen-associated molecular patterns (PAMPs) and by the detergents, Triton X-100 and sodium dodecyl sulphate (SDS). Inhibition studies confirmed the proPO activating system of Sydney rock oysters is a proteinase cascade in which Ca^{2+} dependent serine proteinases proteolytically convert proPO into active PO. Activated PO was found to be a tyrosinase-like enzyme that is responsible for both monophenolase and diphenolase activity. The bifunctional PO had higher affinity for the monophenol, hydroquinine monomethyl ether (4HA) (K_m = 4.45 ± 1.46 mM) than for the diphenol, L-DOPA (K_m = 10.27 ± 1.33 mM). Maximum enzyme activity was evident at 37 º C, pH 8 and at salinities of between 30 and 37 ppt. Melanogensis catalysed by the active enzyme is a composite of eumelanin and the product of a sclerotin pathway combining DOPA-decarboxylase with PO activity.

Keywords: phenoloxidase; tyrosinase; *Saccostrea glomerata*, DOPA-decarboxylase; melanin.

1. Introduction

The prophenoloxidase (proPO) system is an important component of immune responses in many invertebrates. It has been found in a range of molluscs, such as *Crassostrea gigas, Mytilus edulis, Perna viridis, Illex argentinus* and *Perna perna* (Coles and Pipe 1994; Asokan et al. 1997; Hellio et al. 2007). In these species, proPO has been detected in both the plasma component of hemolymph and in hemocytes (Ashida et al. 1983; Saul et al. 1987; Hernandez-Lopez et al. 1996). Many studies have shown that proPO, which is an inactive pro-enzyme, can be activated by low concentrations of elicitors such as lipopolysaccharide (LPS), β-1,3-glucans and peptidoglycans (Söderhäll and Hall 1984; Ratcliffe et al. 1991; Sritunyalucksana et al. 1999). ProPO activation is undertaken by specialized phenoloxidase (PO)-activating enzymes (PPAs), which themselves are activated by a cascade of serine proteases (Marmaras et al. 1996).

In most species, active PO is a bifunctional enzyme that catalyzes the *o*-hydroxylation of monophenols to catechols and the oxidation of *o*-diphenols into *o*-quinones (Yamamoto et al. 2000). The first reaction involves monophenolase activity, which converts tyrosine to 3,4-hydroxyphenylalanine (L-DOPA). L-DOPA is then oxidized to quinone by PO's diphenolase

activity (Bai et al. 1997; Yamamoto et al. 2000). The resulting quinones are converted to melanin by a series of intermediate steps involving enzymatic and non-enzymatic reactions (Sritunyalucksana and Söderhäll 2000; Fang et al. 2002). In one of these enzymatic reactions, dopachrome is decaboxylated by dopachrome isomerase (also called dopachrome tautomerase or Dopachrome Conversion Factor) to form dihydroxyindole, which is then converted to melanin (Sugumaran 1996; Shelby et al. 2000; Olivares et al. 2001).

In arthropods, a second pathway incorporating two additional enzymes contributes to the formation of melanin. The first of two additional enzymes, DOPA-decarboxylase (DDC), acts on the L-DOPA molecules formed by PO's monophenol oxidase activity. DDC decarboxylates DOPA to form dopamine, which is then acetylated to N-acetyldopamine (NADA) by the second enzyme, dopamine N-acetyltransferase. NADA undergoes polymerization, and non-enzymatic reactions lead to the formation of melanin (Saul and Sugumaran 1988; Shelby et al. 2000).

In addition to its role in pigmentation, PO is involved in many cellular defence responses, including melanisation, self / non-self recognition, phagocytosis, nodule and capsule formation, and hemocyte locomotion (Leonard et al. 1985; Johansson and Söderhäll 1989; Söderhäll et al. 1996; Asokan et al. 1998). Our interest in PO comes from its apparent role in defence against infectious QX disease in Sydney rock oysters (*Saccostrea glomerata*). We have shown that PO-like activity is associated with QX disease resistance, and that suppression of this PO-like activity leads to disease susceptibility.

The purpose of the current study was to thoroughly characterize the PO-like activity that we have identified in *S. glomerata*. We compare PO activity in the presence of several inhibitors, including serine protease inhibitors; phenylthiourea (PTU), a dopamine β-hydroxylase or diphenolase inhibitor; tropolone, a phenoloxidase inhibitor; and *m*-hydroxybenzylhydrazine (HBHZ), a DOPA decarboxylase inhibitor. We also measure the ability of sodium dodecyl sulphate (SDS), Triton X-100, pathogen-associated molecular patterns (PAMPs) and exogenous proteases to activate proPO, and study the kinetics of PO's monophenolase and diphenolase activities. Finally, native- and SDS- polyacrylamide electrophoresis are used to identify the different enzymatic components of the prophenoloxidase pathway.

2. Materials and Methods

2.1 Oysters

Sydney Rock oysters (*S. glomerata*), 40 - 60 mm long from the hinge to the frontal edge of shell, were purchased from the Sydney Fish Markets (Sydney, Australia). They were held in aerated aquaria containing seawater (20 L) at room temperature (25º C and pH 7.4). The animals were acclimated for 48 hours before experiments were carried out. Oysters were feed every two days with 10 ml / 20 L mix of four marine microalgae (*Isochrysis*, *Pavlova*, *Thalassiosira weissflogii* and *Tetraselmis*, Shellfish Diet 1800, USA).

2.2 Hemolymph collection

Before hemolymph collection, oysters were removed from the aquaria and allowed to air dry for 30 minutes. After cleaning the shell hinge, a notch was created in the estimated location for the adductor muscle and hemolymph was collected through that notch. Hemolymph was withdrawn from the heart using a 22-gauge needle fitted to a 5 ml sterile syringe. The oysters were then shucked and the rest of the hemolymph was collected from the shell cavity. The hemolymph was immediately mixed with an equal volume of ice-cold marine anticoagulant (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 0.45 M NaCl, pH 7.0) and centrifuged at 400 x *g* for 3 minutes (4 °C). After centrifugation, hemocytes were resuspended in 0.1 M phosphate buffer saline (PBS, pH 7.4) at $1x10^6$ / ml. When hemocyte lysates (HL) were required, hemolymph was

frozen and thawed three times (-80 °C / room temperature) to lyse hemocytes. The lysates were then centrifuged at 5,000 × *g* for 30 minutes (4° C) to remove cellular debris.

2.3 Phenoloxidase assays

PO activity was assayed spectrophotometrically by recording the formation of *o*-quinones. L-3,4-dihydroxyphenylalanine (L-DOPA, ICN, Irvine, CA, USA) and dopamine (Sigma Aldrich) were used as diphenolase substrates, whilst hydroquinine monomethyl ether (4HA, Fluka, Switzerland) was used to measure monophenolase activity. The chromogenic nucleophile, 3-methyl-2-benzothiazolinone hydrazone (MBTH, Sigma Aldrich) was added to both types of substrate. To measure activity, 100 µl of HL was added per well to 96 well flat bottom microtiter plates (Sarstadt, Technology Park, SA) followed by the addition of 100 µl of L-DOPA (4 mg / ml in FSW), dopamine (2 mg / ml in FSW) or 4-HA (5 mM in FSW) and 1 mM MBTH. A microplate spectrophotometer (BioRad, Regents Park, NSW) was used to measure absorbance at 490 nm immediately after the substrates were added. Plates were then incubated for one hour at room temperature in the darkness before a second reading was taken. PO activity was calculated by subtracting the initial readings from measurements made after 60 minutes. Enzyme activities are expressed as the change in optical density (OD) at 490nm (OD_{490}).

2.4 In vitro activation with PAMPs, detergents or serine proteases

Zymosan A, a yeast cell wall antigen (Sigma Aldrich, Castle Hill, NSW) and lipopolysaccharide (LPS) from *Escherishia coli* (055:B5, Sigma Aldrich) were suspended in sterile filtered seawater (FSW; 0.22 □m filtration; Millipore, North Ryde, NSW). The effect of these PAMPs on the activation of proPO system was tested by mixing 100 µl of LPS or 100 µl of zymosan with 300 µl of hemocytes. The mixtures were incubated for 30 minutes before their PO activities were measured as described above. Control samples were incubated with 100 µl sterile FSW without PAMPs. Alternatively, detergents sodium dodecyl sulphate; SDS, Sigma Aldrich or Triton X-100; (Bio-Rad, Castle Hill, NSW) or proteases (trypsin or chymotrypsin, Sigma Aldrich) were used to activate proPO. Thirty µl of SDS (2.5 %, 5 % or 10 %), Triton X-100 (2.5 %, 5 %, or 10 %), trypsin (2mg / ml) or chymotrypsin (0.1mg / ml) were mixed with 300 µl of hemocytes or HL. Again these mixtures were incubated for 30 minutes before being tested for PO activity.

2.5 Kinetic analyses

PO activity was measured over time at different concentrations of L-DOPA (5, 10, 20, 30, 40, 60 mM) or 4HA (2.5, 5, 10, 20, 30, 60 mM) using hemolymph lysates. The Michaelis–Menten constant (K_m) and the maximum reaction velocity (V_{max}) were then determined using the GraphPad Prism 4 software (GraphPad Software, Inc., San Diego).

2.6 Inhibition of phenoloxidase activity

A protease inhibitor cocktail containing aprotinin, leupeptin and 4-[2-aminoethyl]benzenesulfonyl fluoride (Complete Protease Inhibitor Cocktail, CPIC, Roche Applied Science, USA), phenylmethylsulphonylfluoride (PMSF, Sigma Aldrich), tropolone (a PO inhibitor), phenylthiourea (PTU; a diphenolase inhibitor), *m*-hydroxybenzylhydrazine (HBHZ; a DOPA decarboxylase inhibitor) and the chelating agent, EDTA, were tested for their ability to inhibit the conversion of proPO into PO. Thirty µl of CPIC, PMSF, tropolone, PTU, HBHZ, a mixture of PTU and HBHZ, or EDTA were added to 300 µl of hemolymph lysates prepared from hemocytes that had been incubated for 30 minutes with LPS (50 µg / ml). These mixtures were incubated for 30 minutes before being tested for PO activity. In some cases, three hundred µl of hemolymph lysates were preincubated with 100 µl of 16 mM tropolone and 16 mM H_2O_2 for 15 minutes at room temperature in the darkness to test for peroxidase activity.

2.7 Effects of pH, temperature and salinity on PO activity

The influence of pH on PO activity was determined by preparing the substrates (L-DOPA and 4HA) in Tris buffer (0.2 M Tris-HCl and 0.2 M NaCl) adjusted to different pH (5, 6, 7, 8 and 9). The effect of temperature on PO activity was measured at pH 7.4 over a temperature range of 4 - 45 ºC, whilst the role of salinity in PO activity was tested by preparing substrates in seawater adjusted to different salinities using deionized water and sea salt to give final salt concentrations of 10, 20, 25, 31, 37 and 40 parts per thousand (ppt).

2.8 Dopachrome isomerase activity

Dopachrome isomerase activity was assayed according to Aroca et al. (1990) by using dopachrome as a substrate. Dopachrome was generated by the oxidation of L-DOPA with periodate (1:2 molar ratio of L-DOPA : periodic acid). One hundred µl of freshly prepared dopachrome solution was mixed with 100 µl hemolymph lysates in 96 well plates. The depletion of dopachrome was then monitored at 490 nm every 3 minutes.

2.9 Gel electrophoresis

To associate PO enzyme activities with individual proteins, and estimate the molecular weights of the enzymes, SDS-PAGE and native-PAGE were used. Three hundred µl of hemocyte suspensions were mixed with 300 µl of 2 % v/v Nonidet-P40 (NP-40; Fluka, Switzerland) in 10 mM Tris HCl (pH 7.4). Samples were then centrifuged at 5000 *g* for 10 minutes to remove cellular debris. Twenty µl of the lysate was mixed with 5 µl of sample buffer (0.313 M Tris HCl, pH 6.8, 70 % Glycerol, 0.05 % bromophenol blue). Samples were then applied to 6 % native-PAGE gels or 7.5 % SDS-PAGE gels. Electrophoresis was carried out according to Newton et al. (2004). Bio-Rad molecular weight standards and a High Molecular Weight Electrophoresis Calibration Kit (17-0445-01, Amersham Biosciences) were used on the SDS-PAGE and native-PAGE gels respectively. After electrophoresis, gels were stained with a solution containing 5 mM MBTH in 0.1 M PBS (pH 7.4) and either the diphenolase substrates, dopamine (10 mM) or L-DOPA (20 mM), or the monophenolase substrate, 4-HA (20 mM) (Newton et al. 2004). Some gels were incubated with a solution containing the inhibitors, 20 mM tropolone and 10 mM PTU, for 15 minutes followed by extensive washing with distilled water for 20 minutes before being stained for PO activity as described above.

To detect dopachrome isomerase activity after electrophoresis, a solution of 20 mM L-DOPA and 1 % (w/v) sodium *meta*-periodate in 0.1 M PBS (pH 7.4) was used to stain gels. This solution was prepared fresh and added to the gel for 10 minutes with shaking. The gel was then washed with 1% (w/v) sodium *meta*-periodate in 0.1 M PBS for 10 minutes followed by PBS to clear the background (Nicklas and Sugumaran 1995).

2.10 Statistical analysis

All experiments were conducted in triplicate. Five oysters were analyzed in each experiment at each time point. Data were analyzed using the Microsoft Excel package with pop tools (Version 2.7.1). One way analysis of variance (ANOVA) was used to determine the significance of differences between mean values. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Activation with LPS and zymosan

Both LPS and zymosan significantly ($p < 0.05$) increased PO activity relative to negative controls prepared without these PAMPs (Figure 1). The stimulatory activity of both elicitors was dose dependent. Figure 1A shows PO activities induced in hemocyte suspensions by a range of LPS

concentrations (10, 20, 40, 80, 160 µg LPS / ml in FSW). The data show that LPS concentrations of 10 µg / ml to 160 µg / ml significantly ($p < 0.05$) increased both monophenolase (4HA) and diphenolase (L-DOPA and dopamine) activities. When hemocytes were treated with 160 µg / ml LPS, monophenolase activity increased by almost three fold, and diphenolase activity increased by almost two fold relative to FSW treated samples.

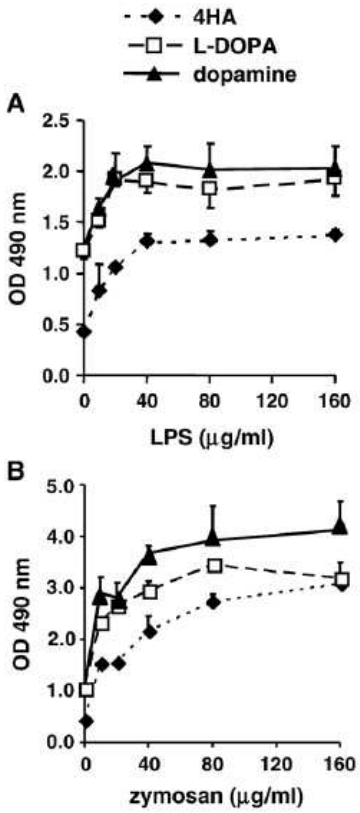

Figure 1. Effect of varied LPS and zymosan concentrations on phenoloxidase activity in oyster hemocyte suspensions. (A) LPS. (B) zymosan. Phenoloxidase activities were measured using 4HA as a monophenolase substrate, and L-DOPA and dopamine as diphenolase substrates. n = 5, bars = SEM.

Similar patterns were evident for zymosan treated samples. Figure 1B shows the PO activities induced by different zymosan concentrations (10, 20, 40, 80 and160 µg / ml). All of these concentrations caused a significant increase ($p < 0.05$) in PO activity compared to FSW controls. Like LPS, the response to zymosan was dose dependent, and it seemed to be more effective activator than LPS. Zymosan elevated monophenolase activity by up to seven fold and diphenolase activity by up to four fold compared to FSW treated samples.

3.2. Activation with detergents

Detergents were also effective elicitors of PO activity. Figure 2A shows the effect different concentrations of Triton X-100 (2.5, 5 and 10 %) on the PO activities of hemocyte suspensions. All three concentrations of Triton X-100 caused a significant increase ($p < 0.05$) in PO activity compared to negative controls. The ability of Triton X-100 to activate proPO was dose dependent. 10% (v/v) Triton X-100 increased monophenolase activity seven fold, from 0.04 ± 0.01 OD_{490} to 0.27 ± 0.04 OD_{490}. Diphenolase activity with L-DOPA as a substrate also increased, from 0.06 ± 0.01 OD_{490} to 0.27 ± 0.02 OD_{490}, in samples treated with 10 % (v/v) Triton X-100. Similar patterns were evident for hemocyte suspension tested with dopamine as a substrate, and Triton X-100 was also able to enhance the PO activities of hemolymph lysates in a dose dependent fashion (data not shown).

Like Triton X-100, SDS significantly induced ($p < 0.05$) the PO activities of hemocyte suspensions when compared to FSW treated samples (Figure 2B). Again, the activity generated by incubation of hemocytes with SDS was dose dependent. 10 % SDS (w/v) increased monophenolase activity five fold, from 0.05 ± 0.03 OD_{490} to 0.23 ± 0.03 OD_{490}, and diphenolase activity by up to six fold.

However, unlike Triton X-100, SDS inhibited PO activity in hemolymph lysates (Figure 2C). Monophenolic activity in lysates was decreased from 0.18 ± 0.03 OD_{490} to 0.02 ± 0.003 OD_{490} after the addition of 2.5 % SDS. Diphenolic activity measured with L-DOPA decreased by ten fold in 2.5 % SDS, while activity against dopamine was almost abrogated.

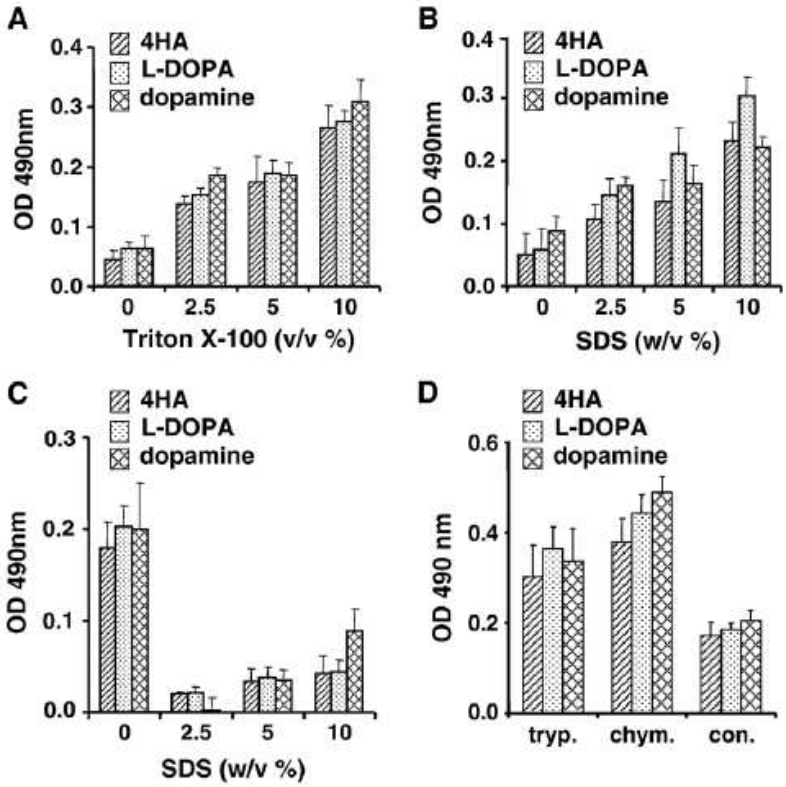

Figure 2. Activation of prophenoloxidase by different serine proteases and detergents (A) Triton X-100 incubated with hemocyte suspensions, (B) SDS incubated with hemocyte suspensions, (C) SDS incubated with hemolymph lysates and (D) serine proteases, trypsin (tryp.;1mg/ml) and chymotrypsin (chym.; 0.1mg/ml), incubated with hemocyte suspensions. Phenoloxidase activities were measured using 4HA as a monophenolase substrate, and L-DOPA and dopamine as diphenolase substrates. n = 5, bars = SEM. con., control.

3.3. Activation with serine proteases

Figure 2D shows that PO activities of hemocyte lysates were increased significantly ($p < 0.05$) by treatment with trypsin and chymotrypsin. Trypsin increased monophenolase activity by two fold compared to untreated controls and enhanced diphenolase activity, using L-DOPA as a substrate, by 1.6 times. Chymotrypsin induced significantly ($p < 0.05$) more PO activity in hemolymph lysates than trypsin, increasing activity by up to three fold relative to controls.

3.4. Phenoloxidase inhibition

Both CPIC and PMSF inhibited PO activity significantly ($p < 0.05$) relative to hemocytes prepared in the absence of inhibitors (Figure 3). The response to both inhibitors was strongly dose dependent. Sixteen mM PMSF decreased diphenolase activity by 70 %, and monophenolase activity by 59 % (Figure 3B). CPIC caused even greater inhibition than PMSF. Figure 3A shows that 4X concentrated CPIC decreased diphenolase activity by 95 % and monophenolase activity by 88 % relative to controls prepared without inhibitor.

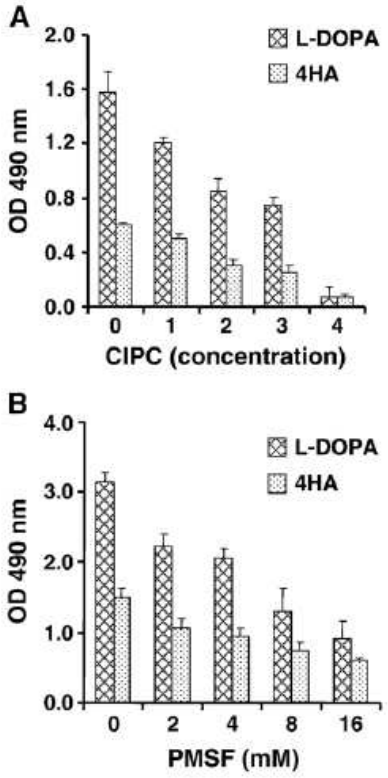

Figure 3. Effects of proteases inhibitors on phenoloxidase activity (A) complete protease inhibitor cocktail (CPIC). The concentrations of CPIC are indicated by the number of tablets / 10 ml (B) PMSF (mM). Phenoloxidase activities were measured using 4HA as a monophenolase substrate and L-DOPA as a diphenolase substrate. n = 5, bars = SEM.

Figure 4 shows the effects of different PO-associated enzyme inhibitors on monophenolase and diphenolase activity. Figure 4A reveals that the PO inhibitor, tropolone, caused dose dependent inhibition of PO activity when incubated with hemolymph lysates. Sixty four mM tropolone decreased monophenolase activity by 98 % and diphenolase activity by 97 % compared to untreated controls.

PTU, which is an *o*-diphenolase inhibitor, cause a significant decrease in the diphenolase activity when L-DOPA was utilized as the substrate (Figure 4B). All concentrations of PTU significantly inhibited activity against L-DOPA. However, PTU was less effective at inhibiting diphenolase activity when dopamine was utilized as the substrate. Only higher concentrations of PTU (25 and 50 mM) significantly ($p < 0.05$) inhibited the diphenolase activity against dopamine (Figure 4B).

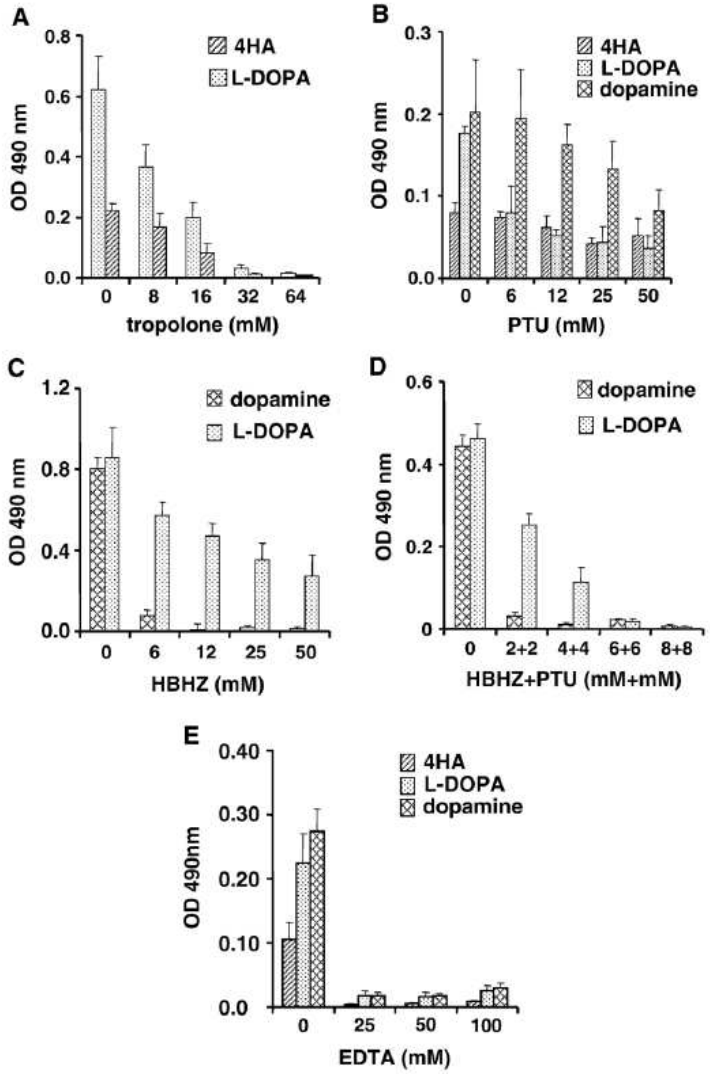

Figure 4. Effects of different inhibitors on phenoloxidase activity in hemolymph lysate samples. Inhibitors were (A) tropolone, (B) PTU, (C) HBHZ, (D) mixtures of PTU and HBHZ and (E) EDTA. Phenoloxidase activities were measured using 4HA as a monophenolase substrate and L-DOPA and dopamine as diphenolase substrates. n = 5, bars = SEM.

The DOPA decarboxylase inhibitor, HBHZ, significantly inhibited PO activity when both L-DOPA and dopamine were used as substrates (Figure 4C). HBHZ at its lowest concentration (12 mM) caused almost complete inhibition of PO activity when dopamine was used as the substrate, but inhibition was less pronounced when L-DOPA was used.

Figure 4D shows that a combination of PTU and HBHZ (8 mM / 8 mM) significantly ($p < 0.05$) inhibited diphenolase activity, and was far more effective at inhibiting activity against dopamine than that against L-DOPA.

PO activity was also strongly inhibited by divalent cation chelating agent, EDTA. Figure 4E shows that PO activity was inhibited significantly ($p < 0.05$) after the addition of EDTA. Even the lowest concentration of EDTA (25 mM) caused a 96 % decrease in monophenolase activity. It also decreased diphenolase activity by 92 % when L-DOPA was used as a substrate, and by 94 % when dopamine was used as a substrate compared to untreated samples.

3.5. Effect of altered pH, temperature and salinity on phenoloxidase activity

Figure 5 shows the effects of various temperatures, pH and salinities on PO activity using 4HA and L-DOPA as substrates. To determine the optimum temperature for PO activity, reactions were performed at a range of temperatures between 5 – 45 °C at pH 7.4. PO activity peaked at 37 º C with both substrates (Figure 5A). The activity of PO was also measured at various pH values ranging between 5 and 9. As shown in Figure 5B, maximum diphenolase activity occurred at pH 8 and decreased substantially at pH 9.

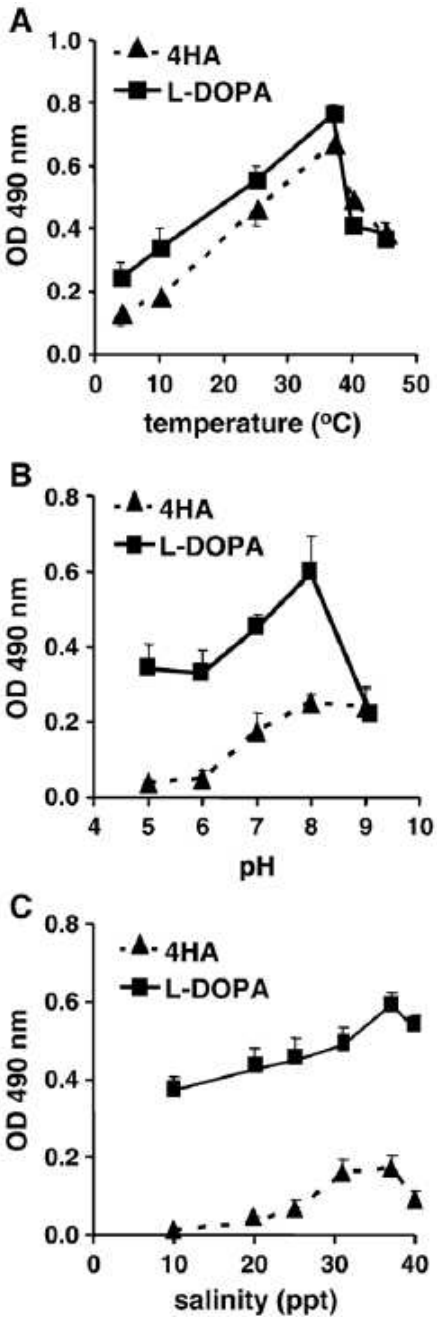

Figure 5. Effects of various (A) temperatures, (B) pH and (C) salinities on phenoloxidase activity. Phenoloxidase activities were measured using 4HA as a monophenolase substrate, and L-DOPA as diphenolase a substrate. n = 5, bars = SEM.

The maximum activity for monophenolase was also obtained at pH 8, but in this case it remained relatively stable between pH 8 – 9. For both substrates, PO activity was lowest at pH of less than 6. The effect of different salinities on PO activity is shown in Figure 5C. Enzyme activity reached a maximum at 37 ppt for L-DOPA, whereas with 4HA the maximum was between 30 and 37 ppt. The lowest PO activities using both substrates were at 10 ppt.

3.6. Enzyme kinetics

Michaelis-Menten curves were constructed so that K_m and V_{max} values could be calculated according to the Lineweaver-Burk equation. The K_m and V_{max} of PO using 4HA were 4.45 ± 1.46 mM and 1.12 ± 0.08 µM / min respectively (Figure 6A). PO had significantly ($p < 0.05$) different K_m and V_{max} values when L-DOPA was used as a substrate (Figure 6B). The K_m and V_{max} values for the diphenolase reaction were 10.27 ± 1.33 mM and 2.4 ± 0.09 µM / min L-DOPA, respectively.

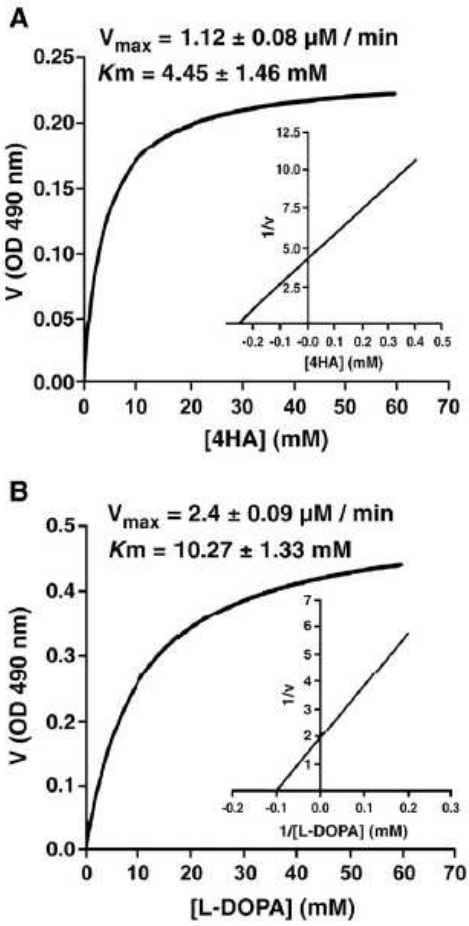

Figure 6. Kinetics of phenoloxidase (A) 4HA was used as a monophenolase substrate and (B) L-DOPA was used as a diphenolase substrate. K_m and V_{max} values were calculated from data for 5 oysters.

3.7. Gel electrophoresis

PO activity was associated with individual protein in hemocyte lysates by zymography using native-PAGE and SDS-PAGE. SDS-PAGE zymographs revealed the existence of two bands using all substrates (Figure 7A). The estimated molecular weight for the upper band was 90.6 kDa and the lower band was 84.3 kDa (Figure 7C). Most of the PO activity appeared in the higher molecular weight band. After treatment with a mixture of tropolone and PTU, the bands completely disappeared, except when 4HA was used as a substrate (Figure 7A). Native- PAGE also revealed the presence of two bands. The estimated molecular weight for the upper band was 219 kDa and the lower band was 192 kDa. Again most activity was evident in higher molecular weight band. Treatment with tropolone and PTU totally inhibited PO activity on Native-PAGE against all three substrates. Figure 7D shows that incubating native-PAGE gels in a solution of L-DOPA and sodium *meta*- periodate, yielded bands with similar electrophoretic mobilities to those identified by L-DOPA alone.

3.8. Dopachrome isomerase activity

Figure 8 shows that absorbance of dopachrome at 490 nm diminished within the first 21 minutes of mixing this substrate with hemocyte lysates. After that, another product began to form, increasing absorbance. The new product was detectable at the same wavelength (490 nm) as dopachrome, which made it difficult to monitor dopachrome depletion for more than 21 minutes.

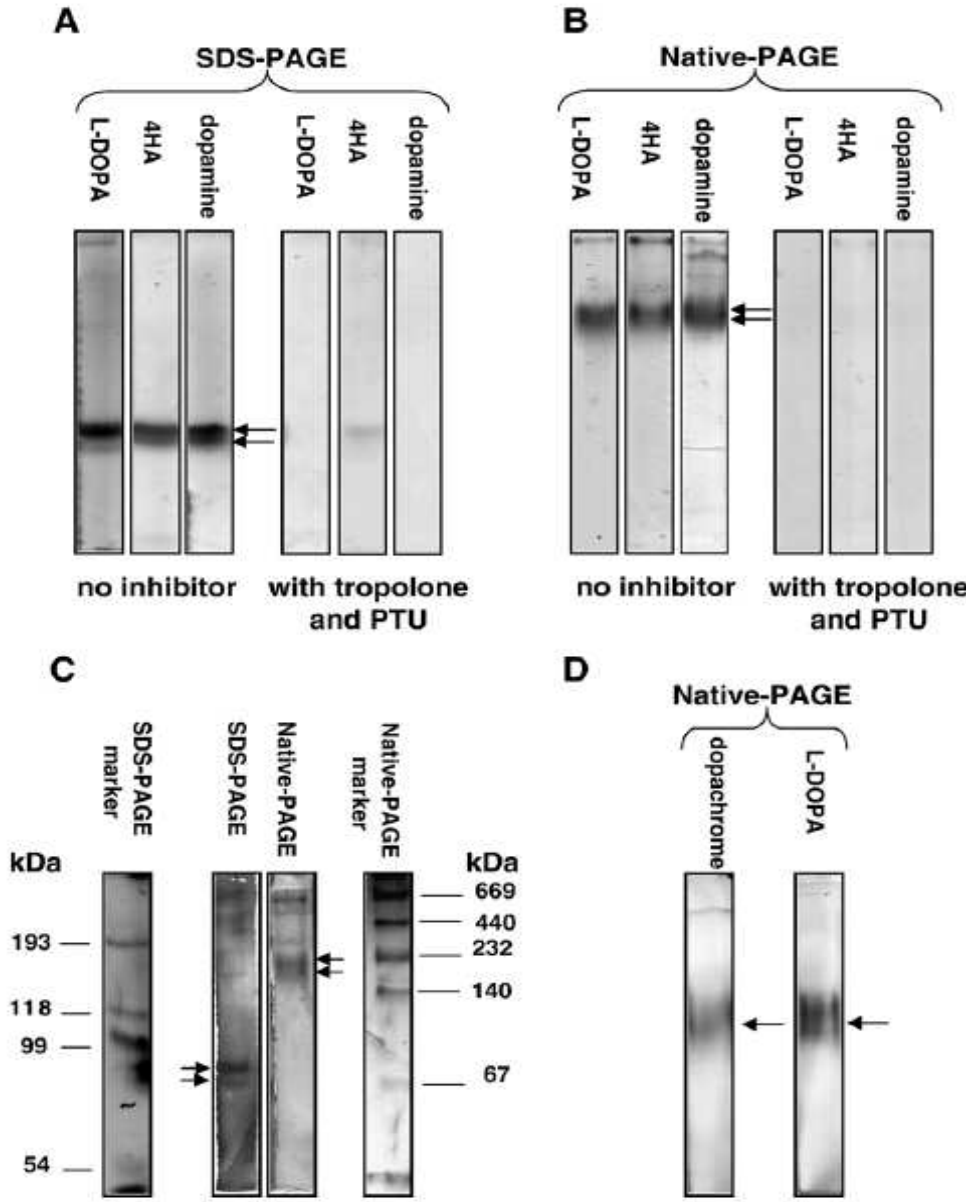

Figure 7. Electrophoresis of the phenoloxidase in Sydney rock oysters. (A) SDS-PAGE, (B) native-PAGE, (C) comparison with SDS-PAGE and native-PAGE molecular weight markers. (D) native-PAGE for dopachrome isomerase activity. A mixture of tropolone and PTU was used to inhibit phenoloxidase.

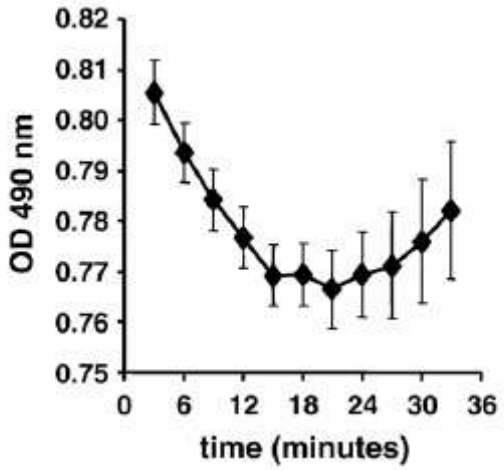

Figure 8. The depletion of dopachrome, measured by absorbance at 490 nm, by hemolymph lysates over time. n = 5, bars = SEM.

4. Discussion

PO is widely distributed in both plants and animals (Yunusov and Yuldashev 1976; Smith and Söderhäll 1991; Alba et al. 2000) and its activity has been extensively studied in many arthropods (Söderhäll et al. 1990; Söderhäll and Aspan 1993). However, the enzyme has not been thoroughly characterized in molluscs, even though PO-like activity has been detected in many bivalve species such as, *M. edulis*, *P. viridis*, *Ruditapes decussates*, *Mytilus galloprovincialis*, *C. gigas* and *Nodipecten subnodosus* (Coles and Pipe 1994; Carballal et al. 1997; Lopez et al. 1997; Asokan et al. 1998; Luna-Gonzalez et al. 2003). The current study rectifies this lack of knowledge. We have shown that the Sydney rock oyster has a PO system comparable to that of arthropods.

As in other species, our study suggests that PO in Sydney rock oysters exists as latent proenzyme, which has to be activated by a molecular system incorporating pattern recognition proteins, proteinases and regulatory factors (Cerenius and Söderhäll 2004). In arthropods, proPO is converted to active PO by specialized serine proteinases, particularly proPO activating enzyme (ppA). This proteinase has been identified in many species, including *Holotrichia diomphalia* and *Pacifastacus leniusculus* (Lee et al. 1998; Wang et al. 2001). In the current study, the addition of exogenous proteases (chymotrypsin and trypsin) increased PO activity when compared to non-activated samples. This indicates that, as in other species, PO activity in *S. glomerata* results from the proteolytic cleavage of a zymogen into active PO.

The role of proteolysis in activating Sydney rock oysters PO was confirmed using protease inhibitors. The level of activity in samples preincubated with two protease inhibitors, CPIC and PMSF, was significantly lower than controls in which hemocytes were stimulated with LPS in the absence of inhibitors. PO activity also strongly inhibited by the chelator, EDTA, suggesting that either PO itself, or the proteases that activate proPO, are divalent cation dependent enzymes.

Our previous work has shown that proPO is stored hemocytes (Aladaileh et al. 2007). Here we found that treatment with Triton X-100 and SDS increased the PO activities of hemocyte suspensions. However, SDS inhibited PO activity in hemolymph lysates. This indicates that PO activation by SDS resulted from hemocyte lysis releasing cellular stores of the enzyme rather than direct conformational changes in the presence of the detergent. In contrast, PO activities were enhanced by Triton X-100 in both hemocyte suspensions and hemolymph lysates. This might be explained by the ability of Triton X-100 to change the enzyme's conformation, which causes direct activation of proPO, as well as its ability to lyse cells and release the pro-enzyme from hemocytes.

The direct activation of PO due to conformational changes in the presence of detergents has been reported frequently in other systems (Wittenberg and Triplett 1985).

As in many other invertebrates, PAMPs (LPS and zymosan) could also induce the release of PO from Sydney rock oyster hemocytes, probably via the stimulation of hemocyte surface pattern recognition receptors. Such receptors have been found in many invertebrate species, such as tobacco hornworm, *Manduca sexta*, Silkworm, *Bombyx mori* and Zhikong Scallop, *Chlamys farreri* (Ma and Kanost 2000; Ochiai and Ashida 2000; Yu and Kanost 2004; Qiu et al. 2007).

Tropolone, a specific inhibitor of PO in other species, when combined with hydrogen peroxide, prevented the conversion of L-DOPA and 4HA to melanin in both hemocyte suspensions and hemolymph lysates from rock oysters. Other experiments could not detect the formation of yellow products that form in the presence of tropolone, hydrogen peroxide and the enzyme, peroxidase (data not shown). This suggests that PO, and not a peroxidase-H_2O_2 system, was responsible for the increase in the absorbency detected by our monophenolase and diphenolase assays. PO is a term that has been applied to both tyrosinases (EC 1.14.18.1) and catechol oxidases (EC 1.10.3.1). Tyrosinases are type-3 copper binding proteins that are part of a protein superfamily that includes hemocyanins and catechol oxidases. Tyrosinases catalyse both monophenolase and diphenolase activities. In contrast, catechol oxidases catalyse only diphenolase activity (Tepper et al. 2005). Hemocyanins found in the hemolymph of many arthropods and molluscs (Nagai et al. 2007; Paoli et al. 2007). Hemocyanin exhibit many properties that similar to those found in phenoloxidase (Paoli et al. 2007).

According to native- and SDS-PAGE, PO activity resulted from two proteins with different molecular masses. Activity staining with monophenolic (4HA) and diphenolic (dopamine and L-DOPA) substrates demonstrated that these proteins undertook both the monophenolase and diphenolase reactions of the PO pathway. This suggests that the Sydney rock oyster POs are of the generic tyrosinase-type. Tyrosinase-like phenoloxidases are copper containing enzymes that have been found not only in animals, but also in plants and fungi (Cameron and Greg 1997; Omiadze et al. 2004; Cong et al. 2005). SDS-PAGE zymography estimated the molecular masses of the two proteins to be 90.6 kDa and 84.3 kDa. Whilst it remains unclear why two PO bands were resolved by electrophoresis, it may be that they represent different proteolytic forms of the same protein. In many invertebrates, proPO is activated to PO by sequentially removing peptides with molecular mass of 5 -15 kDa (Gollas-Galvan et al. 1999). The estimated molecular weights of the reduced *S. glomerata* POs are quite different from those reported from other molluscs, such as, *Crassostrea virginica* (133 kDa), *I. argentinus* (70 kDa) and *Biomphalaria glabrata* (35 kDa) (Bai et al. 1997; Naraoka et al. 2003; Jordan and Deaton 2005), but are close to those found in the clam, *Ruditapes philippinarum* (77 kDa and 84 kDa for PO and proPO respectively) (Cong et al. 2005). Native-PAGE indicated that the native molecular weights of the proteins were approximately 219 kDa and 192 kDa, indicating that the native proteins are oligomers. Oligomerisation is common among POs in other species (Naraoka et al. 2003).

The *S. glomerata* PO system has maximum activity at pH 8 using L-DOPA or 4HA as substrates. This optimal pH is higher than those reported in *C. virginica* (pH 6 - 7.5) and *R. philippinarum* (pH 7.0), but lower than that found in *B. glabrata* (pH 9.5) and *Heliothis virescens* (pH 9) (Lockey and Ourth 1992; Bahgat et al. 2002; Cong et al. 2005; Jordan and Deaton 2005). PO showed maximum activity at 37 º C, which is lower than the temperature optimum for *R. philippinarum* (40 º C), *Charybdis japonica* (40 º C), larvae of *Pieris rapae* (42 º C) and *H. virescens* (45 º C) (Lockey and Ourth 1992; Cong et al. 2005; Liu et al. 2006; Xue et al. 2006), but higher than that for *Drosophila melanogaster* (30 º C) (Asada et al. 1993). PO activity of *S. glomerata* was also optimal at 37 ppt with L-DOPA as a substrate, whereas for 4HA it was the maximum between 30 and 37 ppt.

K_m values for 4HA and L-DOPA were found to be 4.45 ± 1.46 mM and 10.27 ± 1.33 mM respectively. This indicates that *S. glomerata* PO has lower affinity for L-DOPA than for 4HA. In contrast, *R. philippinarum* PO, has a K_m value for L-DOPA of 2.2 mM and 6.0 mM for the monophenolase substrate, tyrosine (Cong et al. 2005). The differences in enzyme kinetics using different substrates could be explained by the ability *S. glomerata* PO, and other downstream enzymes, to catalyze at least two distinct enzymatic pathways leading to melanization. Tropolone inhibited both the monophenolase and dihydroxyphenolase activities of *S. glomerata*. However, the inhibition was not complete, even after the addition of hydrogen peroxide indicating that an additional form of tropolone resistant enzyme activity was present. The activity that was retained in tropolone-treated samples did not appear to be catalyzed by peroxidase. The color change in samples prepared with tropolone was consistent with oxidation of L-DOPA and not oxidation of tropolone by peroxidase (Kahn and Andrawis 1985). So, the retention of activity in the presence of tropolone suggests that an alternative system of melanogensis is present in Sydney rock oysters.

This alternative pathway was further investigated using PTU, a specific *o*-diphenolase activity inhibitor. PTU was able to inhibit the PO activity when dopamine and L-DOPA were used as diphenolase substrates in the presence of hydrogen peroxide. However, the inhibition of PO activity was not complete, indicating that an additional enzyme, other than PO, may have been acting on these diphenolic substrates. The results suggested that L-DOPA could undergo decarboxylation and dopamine could be acetylated. These types of reactions are typical of the sclerotin pathway of melanin formation that is catalyzed by DOPA decarboxylase (DDC) rather than PO (Saul and Sugumaran 1988; Sugumaran 2002). The activation of DDC is a necessary step in the sclerotin pathway (Sugumaran 1991). DDC converts DOPA to dopamine, which then is acetylated by N-acetyltransferase to N-acetyldopamine. DDC has been reported to be involved in many defense reactions, such as defensive melanization and encapsulation, and it has been identified in addition to PO in a number of species, including *Tenebrio molitor* and *Drosophila melanogaster* (Nappi et al. 1992; Kim et al. 2000; Shelby et al. 2000). HBHZ, a decarboxylase inhibitor, was used to confirm the involvement of a DDC pathway in melanin formation by Sydney rock oysters. The addition of HBHZ to samples containing L-DOPA or dopamine decreased the formation of dopachrome and mixtures of HBHZ and PTU caused almost a complete inhibition to the dopachrome formation. This suggests that both the PO and DDC pathways contribute to melanization in *S. glomerata.*

4.3 Phenoloxidase-assocated cellular defence in the Sydney rock oysters (*Saccostrea glomerata*) provides resistance against QX disease infections

Daniel Butt and David Raftos*

Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia

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Abstract

The enzyme phenoloxidase is a critical component of the immunological defence of invertebrates. Previously, we have shown that the activity of phenoloxidase in Sydney rock oysters (*Saccostrea glomerata*) correlates with the severity of QX disease outbreaks. The etiological agent of QX disease is the opportunistic protozoan parasite, *Marteilia sydneyi*. In this study we examined the response of oyster haemocytes to challenge with *M. sydneyi*. Granular haemocytes were able to rapidly phagocytose parasite sporonts. Phagocytosis stimulated intracellular associated phenoloxidase activity that led to the complete melanisation of phagosomes. Significant differences in phagocytic indexes and phenoloxidase activities were observed between oysters selected for resistance to QX disease (QXR) and non-selected wild-type oysters. The data suggest that phagocytosis and cellular melanisation are critical defensive responses of Sydney rock oysters infected with *M. sydneyi*.

(**Keywords;** disease resistance, *Marteilia sydneyi*, oysters, phagocytosis, phenoloxidase)

1. Introduction

Phenoloxidase is a key enzyme involved in the immunlogical defence of invertebrates. The role of phenoloxidase in host defence is best characterised in arthropods, although recent studies have also demonstrated its importance in a number of bivalve mollusc species (1-5). The phenoloxidase enzyme was originally only thought to become activated by serine proteases upon its release from haemocytes. The enzyme then facilitates the formation of the pigment melanin, which is important in the sequestration of foreign material during defensive encapsulation (6). This was recently supported by the work of Hellio *et al.* (3) who found high levels of phenoloxidase-like activity in the cell free haemolymph of the Pacific oyster, *Crassostrea gigas*. Even after the addition of the known phenoloxidase activator, trypsin, phenoloxidase activity was minimal in the cellular component of haemolymph. However, other studies have demonstrated that phenoloxidase is equally active in both the cell free haemolymph and haemocytes of other marine bivalves, including mussels, clams and scallops (1, 5). A study of the mussel, *Perna viridis*, showed that phenoloxidase activation tended to be higher in haemocytes than in plasma (7), whilst phenoloxidase activity has also been associated with the membrane fraction of haemocytes from the Eastern oyster, *Crassostrea virginica* (2).

Previously, we have monitored phenoloxidase activity in the haemolymph of Sydney rock oysters (*Saccostrea glomerata*) during laboratory and field trials to demonstrate that decreased phenoloxidase activity is associated with increased susceptibility of oysters to infection by the protozoan parasite, *Marteilia sydneyi*. *M. sydneyi* is the etiological agent of QX disease, which causes mortality rates of up to 98% in previously unexposed Sydney rock oyster populations (8). Such high mortality has contributed to a 40% decline in Sydney rock oyster production since the 1970's (9). Field trials have shown that oysters from areas with high levels of *M. sydneyi* infection have low pre-infection phenoloxidase activities (10, 11). Conversely, it appears that increased phenoloxidase activity may be associated with greater resistance to *M. sydneyi* infection. Oysters

bred for resistance to QX disease by the New South Wales Department of Primary Industries (NSW DPI) have significantly higher phenoloxidase activities than oysters from wild-type populations that have never been exposed to the disease (12). QX resistance breeding lines have been developed since 1996 by interbreeding survivors of QX disease epizootics in the Georges River, Sydney (13). By the fourth generation of selection, resistance breeding had reduced QX-associated mortality to 63% compared to over 98% among unselected controls (14).

The current study is a continuation of work examining the direct association between phenoloxidase and *M. sydneyi* in the haemolymph of *S. glomerata*. The study was undertaken to further our understanding of cellular activity that underlies resistance to QX disease in Sydney rock oysters.

2. Materials and Methods

2.1 Oysters

Oysters were taken from wild-type, non-selected populations and from strains that had been selectively bred for resistance to QX disease by NSW DPI. Non-selected oysters came from three separate growing areas in the state of New South Wales, Australia. Uninfected wild-type oysters were from Port Stephens (32°42'S) 200 km north of Sydney and the Crookhaven River in southern NSW (34°53'S). Neither site has ever been subjected to a QX disease outbreak. QX-infected wild-type oysters were collected from Neverfail Bay, Georges River, Sydney (33°59'S). Infection in these oysters was diagnosed using tissue smears of digestive glands (15). QX-resistant (QXR) oysters were from the fourth generation of oysters selectively bred in the Georges River, Sydney. They were kindly supplied by Dr John Nell (NSW DPI).

2.2 Isolation of Marteilia sydneyi *sporonts*

M. sydneyi sporonts were isolated from infected oyster digestive glands. Blocks of digestive gland tissue were homogenised in filtered seawater (FSW; 0.45 µm) and passed through a 150 µm sieve (Sigma, USA). Sporonts were then isolated using modified methods of Robledo *et al*. (16). Sieved digestive gland homogenates were diluted in FSW and layered on to a discontinuous Percoll (Pharmacia, Sweden) gradient (20%, 30%, 40% and 50% in FSW). After centrifugation (2,500 x g, 4^{o}C, 30 min) sporonts were collected from the 30%-40% interface and rinsed by gentle centrifugation and resuspension in FSW. After washing, no extraneous proteins could be detected in the supernatant (data not shown).

2.3 Haemolymph collection and sample preparation

Haemolymph was withdrawn from the pericardial cavity of shucked oysters. Approximately 2 mL of whole haemolymph was withdrawn from each oyster. Samples were immediately placed in polypropylene tubes and adjusted to a concentration of 1 x 10^6 cells mL^{-1} using FSW.

One millilitre of whole haemolymph, to be used for phenoloxidase assays, was separated into haemocytes and cell free haemolymph by gentle centrifugation (2500 x g, 5 min). Cell free haemolymph was aspirated from the haemocyte pellet, which was then resuspended in 1 mL FSW. The resulting cell suspensions and cell free haemolymph samples were divided equally in separate tubes and stored on ice for subsequent use in assays of phenoloxidase activity and melanin formation.

2.4 Total haemocyte counts

One hundred microlitres of whole haemolymph were mixed with 100 µL marine anticoagulant (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M sodium chloride, pH 7.5). This haemocyte suspension was then fixed with 100 µL paraformaldehyde (4%

w/v). The number of haemocytes per mL was estimated using an Improved Neubauer haemocytometer.

2.5 Phenoloxidase assays

One set of haemocyte and cell free haemolymph aliquots, from QXR and wild-type oysters, were mixed with *M. sydneyi* by adding 100 µL FSW containing 5 x 10^5 sporonts. One hundred microlitres FSW without sporonts were added to replicate aliquots to act as controls. Samples were left for 30 minutes at room temperature before 500 µL of the phenoloxidase substrate, hydroquinine monomethyl ether (4-HA; Fluka, Switzerland; 20mM in FSW), and the chromogenic nucleophile, 3-methyl-2-benzothiazoline hydrazone (MBTH; Sigma Aldrich, Castle Hill, NSW; 1mM), were added to all samples (17). Samples were then incubated at room temperature for a further 3 hours. After incubation, haemocyte samples were centrifuged (2500 x g, 5 min) to generate haemocyte pellets. The pellets were rinsed with FSW by centrifugation (2500 x g, 5 min) and finally resuspended in 500 µL FSW containing 1% v/v Nonidet-P40 (NP-40; Fluka). One hundred microlitres of resulting haemocyte lysates and cell free haemolymph samples were then added in triplicate to 96-well microtitre plates (Sarstadt, Germany) so that absorbance could be measured at 492 nm. Data were adjusted to account for absorbance in samples containing phenoloxidase substrate in the absence of haemolymph (negative controls).

2.6 Phenoloxidase cytology

Two methods were used to assess the cellular activation of phenoloxidase in the presence of *M. sydneyi*. The first involved incubating 500 µL whole haemolymph (0.5 x 10^6 cells) with 5 x 10^5 *M. sydneyi* sporonts for 30 minutes before adding 4-HA (20mM) with MBTH (1mM), or L-β-3,4-dihydroxyphenylalanine (L-DOPA; 4mg/mL; ICN Biomedicals, USA) also with MBTH (1mM). These samples were incubated for 3 hours before the haemocytes were separated from cell free haemolymph by gentle centrifugation (2500 x g, 5 min). The supernatant was removed and haemocytes were rinsed with FSW by centrifugation (2500 x g, 5 min) before being resuspended in glycerol (60%). Haemocytes were examined using an Olympus BH-2 light microscope. In controls, haemocytes were immediately separated from cell free haemolymph by centrifugation (2500 x g for 5 min) before being resuspended in FSW. Tropolone (10mM; Sigma, USA), a known phenoloxidase inhibitor, was also added to some samples (3).

The second method used to study cellular activation involved adhering *M. sydneyi* sporonts to glass microscope slides rather than incubating them with haemocytes in suspension. Twenty microlitres of FSW containing purified *M. sydneyi* sporonts (5 x 10^6 mL^{-1}) were added to poly-L-lysine (1% w/v in distilled water; Sigma) coated glass slides. Sporonts were left for 20 min to adhere before excess FSW was drawn off. Ten microlitres of whole haemolymph, or rinsed haemocytes resuspended in FSW, were then added to the adhered sporonts, followed by 10 µL of 4-HA (20mM) and MBTH (1mM). Slides were examined at regular intervals for three hours to monitor the development of a red to pink colouration, reflecting phenoloxidase-associated cytological staining.

2.7 Melanin staining

In some of the samples containing haemocytes suspended in FSW, a combination of both 4-HA (20mM) and L-DOPA (4mg/mL) was added in the absence of MBTH to activate the phenoloxidase cascade and stimulate the formation of melanin without providing chromogenic staining. These samples were incubated at room temperature for 18 hrs. Two hundred microlitres paraformaldehyde (4% w/v) were then added to fix the haemocytes. Once fixed, samples were centrifuged (2500 × g, 4°C, 5 min) to remove the cell free hamolymph. Haemocytes were then rinsed with distilled water by centrifugation (2500 x g, 4°C, 5 min) and stained for the presence of melanin using a modified Fontana-Masson stain (18). The haemocytes were incubated in silver nitrate (20% in distilled water) at 56°C for one hour. They were then rinsed three times with

distilled water and incubated with 5% sodium thiosulphate (BDH Chemicals, Australia) for 2 minutes. Finally, the cells were washed with distilled water by centrifugation (2500 x g, 4°C, 5 min), and resuspended in glycerol. The haemocytes were assessed for melanisation using an Olympus BH-2 light microscope.

2.8 Phagocytic activity

M. sydneyi sporonts (5×10^6 mL^{-1}) were adhered to areas of poly-L-lysine coated glass microscope slides. Twenty microlitres of whole haemolymph were then added to the sporonts. Slides were incubated for 1 hr before 5 µL paraformaldehyde (4% w/v) was added to terminate phagocytic activity and fix the cells. Phagocytic activity was quantified by examining 100 cells from each slide and recording the number that had ingested at least one sporont. The number of sporonts ingested by each of 30 randomly selected phagocytic haemocytes was also recorded for each slide.

2.9 Size and staining intensity in phenoloxidase-positive haemocytes

Haemocytes were stained for phenoloxidase activity in suspension, as described above. They were then fixed with paraformaldehyde (4% w/v) and 10 digital images of randomly selected fields of view (x 40 magnification) were captured using a Nikon Digital DXM1200F camera fitted to an Olympus BH-2 light microscope. Images were analysed using Image-J software (v1.36, 2006). Twenty-five randomly selected PO-positive (red to pink stained) cells were analysed for size (diameter) and staining intensity. The intensity of PO staining was taken as the inverse of the mean gray scale value within each cell.

2.10 Data analysis

Data were analysed using SPSS v.15 software (SPSS Inc. 2006). Tests for normalcy and Levene's test for homogeneity of variance were carried out on all data sets. One way analyses of variance were performed to determine whether significant differences existed between experimental treatments. Tukey's post hoc test was used to establish where significant differences occurred within the data set. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Phagocytosis, phenoloxidase activity and melanisation in haemocytes challenged with M. sydneyi

Cytological staining was evident in haemocytes exposed to *M. sydneyi* both on slides and in suspension. Intense phenoloxidase activity was induced by *M. sydneyi* in oyster haemocytes using either L-DOPA or 4-HA. Intracellular phenoloxidase activation occurred both in the presence and absence of cell free haemolymph. After initial contact with the parasite, granular haemocytes were observed ingesting *M. sydneyi* sporonts by phagocytosis. Phenoloxidase activity intensified throughout the 3 hr incubation, often until ingested sporonts were completely obscured by the phenoloxidase staining. Some phenoloxidase activity was also detected in non-phagocytising cells, but to a far lesser extent than after phagocytosis (data not shown). Figure 1A depicts the initial level of intracellular phenoloxidase activity in an oyster haemocyte making first contact with an *M. sydneyi* sporont. Pink colour development, indicative of phenoloxidase activity, is evident in perinuclear granules of the haemocyte. Figure 1B shows an *M. sydneyi* sporont within a granular haemocyte. Phenoloxidase cytological staining is concentrated within granules in the cytoplasm of the oyster cell and deposited directly onto the surface of the phagocytosed sporont. The addition of tropolone inhibited colour generation associated with phenoloxidase activity but did not inhibit phagocytosis (data not shown).

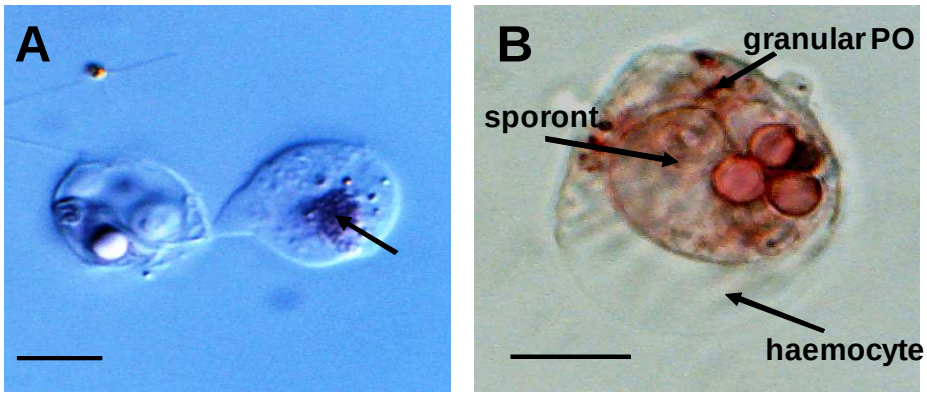


Figure 1. Phenoloxidase cytology of granular haemocytes mixed with *M. sydneyi* sporonts *in vitro* and stained with 4-HA and MBTH. Phenoloxidase activity is shown as red to pink staining. (A) Enzyme activity is generated from the time of initial contact between haemocytes (right) and *M. sydneyi* (left) on glass slides, arrows show phenoloxidase activity. (B) Once *M. sydneyi* are ingested, intense phenoloxidase activity is directly associated with sporonts. Bars = 5 µm.

In addition to cytological phenoloxidase staining, haemocytes that had ingested sporonts underwent partial to complete melanisation within 18 hrs. Initially, melanin production was focused on sporonts within phagosomes (Figure 2A). However, as the process of melanisation continued, entire haemocytes became melanised (Figure 2B).

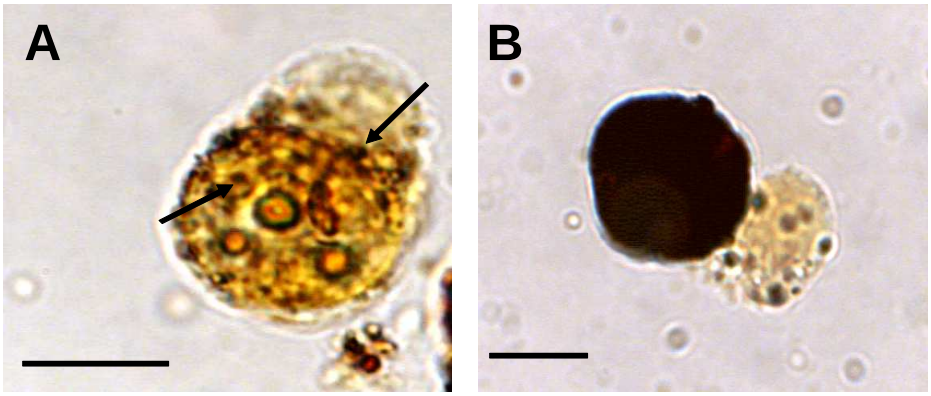


Figure 2. Phenoloxidase activity catalyses the formation of melanin. (A) Initially melanisation is focused around phagocytosed *M. sydneyi* sporonts. Arrows indicate granular melanin deposition around ingested parasite. (B) As melanisation continues, entire haemocytes, including the internalised sporont(s), become melanised. Bars = 5 µm.

3.2 Phenoloxidase activities in haemolymph from QXR and wild-type oysters challenged with M. Sydneyi

Phenoloxidase activities were significantly higher in both cell free haemolymph (F = 9.408, df = 3, 138, $p < 0.05$) and washed haemocytes (F = 21.267, df = 3, 137, $p < 0.05$) from QXR oysters challenged with *M. sydneyi* compared to challenged wild-type oysters (Figure 3). Comparative increases in phenoloxidase activities after *M. sydneyi* challenges were greater in haemocytes than in cell free haemolymph. Haemocytes from challenged QXR oysters had phenoloxidase activities more than two times higher than those of challenged wild-type oysters (Figure 3B). This was a substantially greater difference than that seen in cell free haemolymph (Figure 3A). Differences between challenged and unchallenged QXR oysters were greater in haemocytes (75% increase) compared to cell free haemolymph (55% increase). Phenoloxidase activities in haemocytes were

also significantly higher before challenge in QXR oysters than in wild-type oysters before challenge (F = 16.194, df = 1, 84, $p < 0.05$).

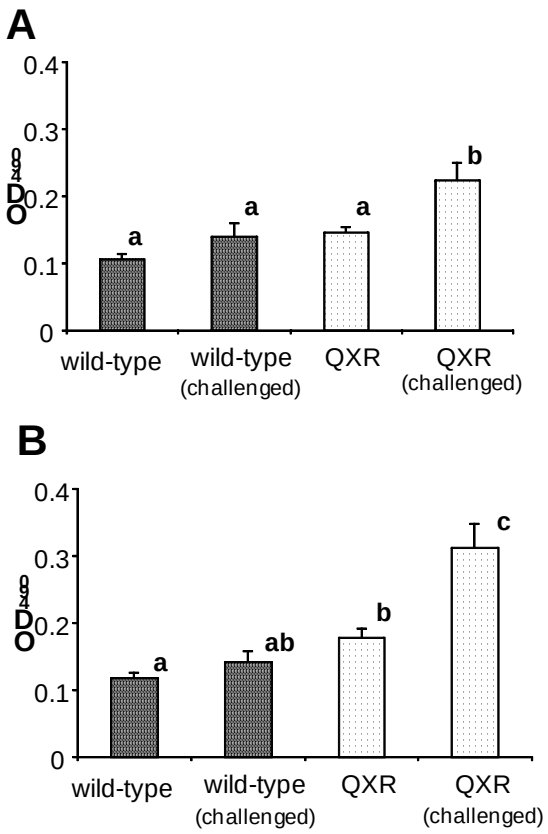
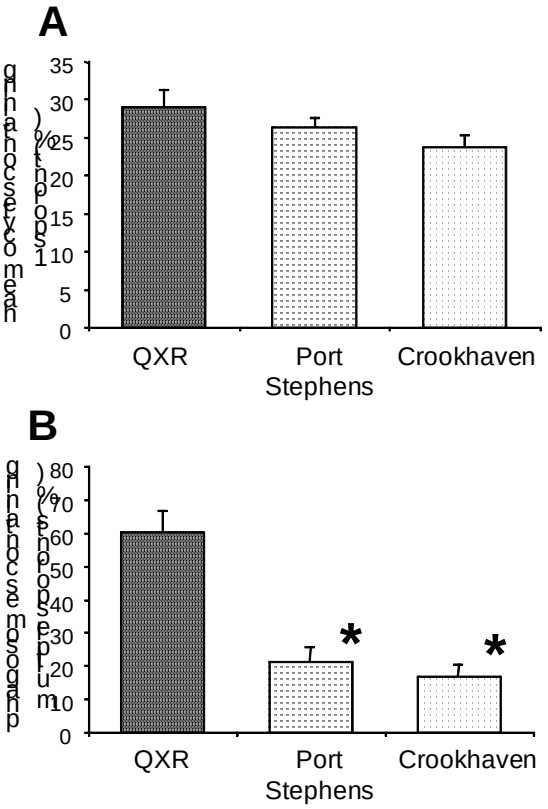


Figure 3. Differences in mean phenoloxidase activities (OD_{492}) in (A) cell free haemolymph and (B) haemocytes from oysters selected for resistance to QX disease (QXR) and wild-type oysters (never previously exposed to QX disease), before and after *in vitro* challenge with *M. sydneyi* sporonts. Like letters indicate comparisons that did not differ significantly (p>0.05). n > 30, bars = 1 S.E.M.

3.4 Phagocytic activities of haemocytes from QXR and wild-type oysters

Almost 30% of haemocytes from QXR oysters phagocytosed *M. sydneyi* sporonts. This was slightly, but not significantly ($p > 0.05$) higher than for haemocytes from wild-type Port Stephens (26.3% ± 1.33) and Crookhaven River (23.7% ± 1.7) oyster populations (Figure 4A). However, significant differences were evident in the average number of sporonts ingested per cell. More phagocytic haemocytes from QXR oysters ingested multiple sporonts compared to both of the wild-type populations (F = 25.513, df = 2, 57, $p < 0.05$) (Figure 4B). Over 60% of phagocytic haemocytes from QXR oysters ingested more than one sporont, with some cells containing up to 5 mature sporonts (Figure 5A). In contrast, the majority of wild-type phagocytes contained one sporont, and only about 20% ingested more than one parasite (Figure 5B).

Figure 4. Differences between (A) the percentage of haemocytes that had phagocytosed at least one *M. sydneyi* sporont and (B) the percentage of phagocytic cells that had ingested more than one sporont. Haemocytes were taken from oysters selected for resistance to QX disease (QXR) and two populations of unselected oysters from Port Stephens and the Crookhaven River, NSW. Like letters indicate comparisons that did not differ significantly (p>0.05). n = 20, bars = 1 S.E.M.



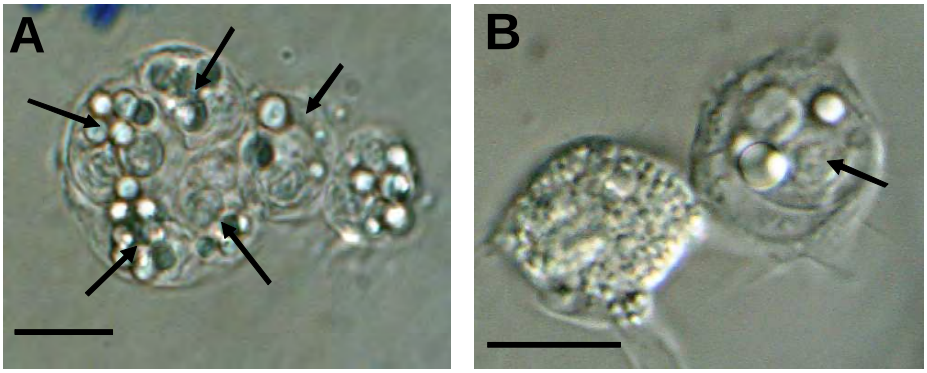


Figure 5. Phagocytosis of *M. sydneyi* sporonts by granular haemocytes from (A) a QXR oyster and (B) a wild-type oyster. Arrows show sporonts ingested by granulocytes. Bars = 5 µm.

3.5 Morphological comparisons of phenoloxidase-positive haemocytes from resistant and wild-type oysters

Phenoloxidase-positive haemocytes from resistant oysters that were exposed to *M. sydneyi* sporonts *in vitro* were larger and had more intense phenoloxidase staining than haemocytes from wild-type oysters. When cluster plots of size vs staining intensity of phenoloxidase-positive haemocytes in wild-type oysters from the Crookhaven River (Figure 6A) and Port Stephens (Figure 6B), were compared with QXR oysters, substantial differences in both staining intensity and cell size were observed. QXR oysters had significantly larger phenoloxidase-positive haemocytes than wild-type oysters from both the Crookhaven River and Port Stephens (F = 115.815, df = 2, 750, p < 0.05), but there were no differences in cell size between the two wild-type populations. Phenoloxidase-positive haemocytes from QXR oysters also had significantly more intense phenoloxidase staining than both wild-type populations (F = 413.609, df = 2, 750, p <

0.05) (Figure 6C). In this case, however, oysters from the Crookhaven River had significantly higher haemocyte phenoloxidase activities than oysters from Port Stephens (F = 130.160, df = 1, 500, $p < 0.05$).

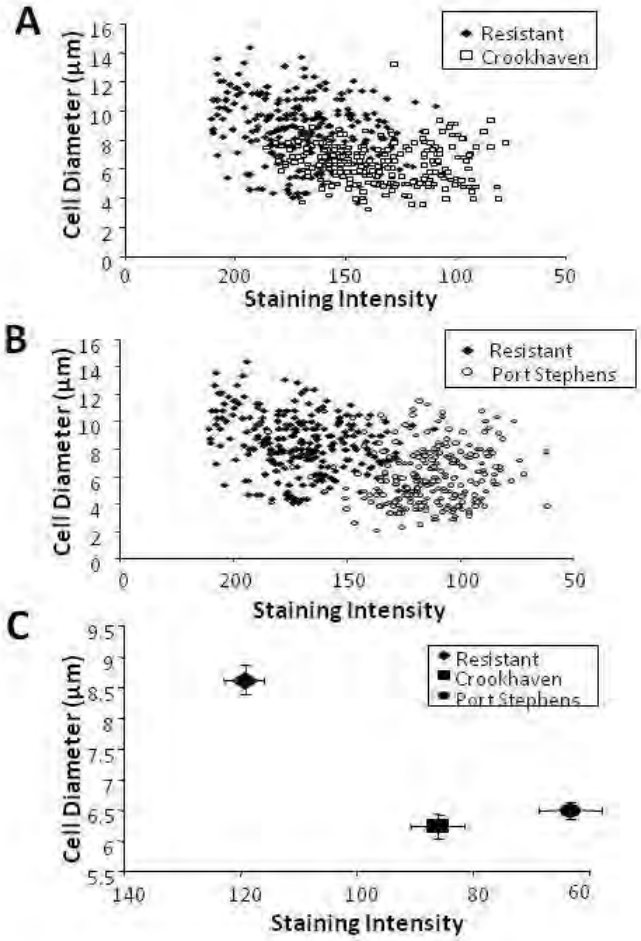


Figure 6. Scatter plots showing cell diameter (µm) plotted against phenoloxidase staining intensity in (A) phenoloxidase-positive haemocytes from QXR oysters and wild-type oysters from the Crookhaven River, (B) phenoloxidase-positive haemocytes from resistant oysters and wild type Port Stephens oysters and (C) mean values for cell diameter versus phenoloxidase staining intensity of haemocytes from each oyster population. Bars = 1 S.E.M. In (A) and (B) each data point represents values for a single haemocyte.

4. Discussion

We have identified phenoloxidase activity in both the cell free haemolymph and haemocytes of Sydney rock oysters. The activity detected in the cellular haemolymph fraction is of particular interest because it appears to be strongly associated with defence against the pathogen, *M. sydneyi*. In arthropods, phenoloxidase has usually been described as inactive whilst contained within haemocytes, only being activated by serine proteases once released into the serum (19). This was confirmed in a recent study of the tobacco hornworm, *Manduca sexta*, which found that phenoloxidase activation and subsequent melanisation only occurred when haemocytes were maintained in the presence of plasma (20).

These findings in arthropods differ substantially from our results. Data from the current study indicate that phenoloxidase in *S. glomerata* can be activated within haemocytes in the absence of serum. Even though a number of other studies have shown that marine bivalves have active phenoloxidase associated with their haemocytes (1, 2, 5, 7), this is the first time that intracellular

phenoloxidase has been associated with anti-parasite defences. We have previously demonstrated the existence of different isoforms of phenoloxidase in the haemolymph of *S. glomerata* (21). It remains to be investigated whether the active enzyme found in the cell free haemolymph and haemocytes are representative of different phenoloxidase isoforms.

Prior research into the relationship between Sydney rock oysters and QX disease has suggested that phenoloxidase is associated with anti-*M. sydneyi* defence. Data from field trials in the Georges River, Sydney, indicated that infection intensities were highest at upriver sites where phenoloxidase activity had been inhibited by low salinity associated with heavy summer rainfall (10). Similar studies in the Hawkesbury River, also near Sydney, again found that phenoloxidase activities decreased significantly in oysters prior to *M. sydneyi* infection (11). These findings imply that phenoloxidase is normally involved in the suppression of *M. sydneyi*, and that it is only when phenoloxidase's control over the parasite is lost that QX disease outbreaks occurred.

The current study is the first time that this implied link between phenoloxidase and defensive responses against *M. sydneyi* in *S. glomerata* has been confirmed. We have demonstrated that phenoloxidase activity is associated with phagocytosed *M. sydneyi* sporonts within granular haemocytes, and that these haemocytes can undergo complete melanisation after ingesting parasites. The data suggest that intracellular phenoloxidase activity and subsequent melanisation are components of *S. glomerata's* immunological defence against *M. sydneyi*.

In response to increasing concern over the impact of QX disease on the oyster industry, an oyster breeding program selecting for resistance to QX disease began in 1997. The identification of intracellular phenoloxidase reactions to *M. sydneyi*, in the current study, suggest that phenoloxidase is a cellular mechanism with the potential to confer QX disease resistance. This view is supported by Newton *et al.* (12) who found that third generation QXR oysters had significantly higher phenoloxidase activities than wild type oysters, and that these differences in phenoloxidase activities were significantly greater in haemocytes than in haemolymph serum (12).

Our results further strengthen this association between phenoloxidase and QX disease resistance. QXR oysters have higher *M. sydneyi*-induced phenoloxidase responses than wild-type oysters. This is true of both cell free haemolymph and haemocytes, but the differences were more pronounced in haemocytes. Phenoloxidase activities in haemocytes from QXR oysters were higher than both challenged and unchallenged wild-type oysters. These differences were due in part to the size of haemocytes. The oyster haemocyte populations of QXR oysters contain significantly more large haemocytes with high phenoloxidase activities relative to wild-type oysters. The same population of large granular haemocytes with active intra-cellular phenoloxidase were responsible for phagocytosing multiple *M. sydneyi* sporonts. This combination of enhanced phagocytosis and higher intracellular phenoloxidase activities is likely to explain much of the QX disease resistance in selected Sydney rock oysters.

Studies of disease resistance in other oyster species have also found that resistance is related to differences in haemocyte sub-populations. Ford *et al.* (22) found that the percentage of large granular haemocytes was significantly lower in Eastern oysters that were susceptible to the protozoan, *Haplosporidium nelsoni* (MSX). Rather than being directly involved in defence, they concluded that these large granulocytes were involved in tissue repair and debris removal, thereby improving oyster survivorship. Similarly, Allam *et al.* (23) found that susceptibility to brown ring disease in clams, caused by *Vibrio tapetis*, is related to the frequency of granular haemocytes. Resistant animals had higher percentages of granular haemocytes in their haemolymph and higher resultant phagocytic activities. European flat oysters, *Ostrea edulis*, that show resistance to infection from the protozoan parasite *Bonamia ostreae* have also been shown to have different haemograms to susceptible populations (24, 25).

These data suggest that resistance to protozoan infections in oysters is linked to increased frequencies of large granulocytes with higher phagocytic clearance rates than other haemocytes.

Our results emphasise the importance of this resistance mechanism, and highlight the role of previously undescribed intracellular phenoloxidase activity. The activity of this enzyme within granulocytes and the cellular melanisation that it catalyses may provide a critical killing and clearance mechanism for *M. sydneyi*.

We still do not know if there is a direct relationship between increased phenoloxidase activities and high phagocytic indexes, or whether the two function independently. However, it is clear that the combination of improved parasite clearance and high intracellular phenoloxidase activities in large granulocytes provides an explanation for resistance in QXR Sydney rock oysters.

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4.4 Phagocytosis of the protozoan parasite, *Marteilia sydneyi*, by Sydney rock oyster (*Saccostrea glomerata*) hemocytes

Rhiannon. P. Kuchel*, Saleem Aladaileh, Debra Birch, Nicole Vella and David A. Raftos

Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia

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Abstract

QX disease is a fatal disease in Sydney rock oysters caused by the protozoan parasite *Marteilia sydneyi*. The current study investigates the phagocytosis of *M. sydneyi* by Sydney rock oyster hemocytes. It also compares the *in vitro* phagocytic activities of hemocytes from oysters bred for QX disease resistance (QXR) with those of wild-type oysters. As reported previously, hemocytes from QXR oysters were more efficient phagocytes. 36% ± 3% of these hemocytes engulfed ≥ 2 *M. sydneyi* sporonts, compared with 16% ± 2% of wild-type hemocytes. Phagocytosis of *M. sydneyi* by hemocytes from both oyster lines was associated with the redistribution of F-actin during the formation of pseudopodia. After ingestion, hemocyte granules fused with phagosome membranes and the pH of phagosomes decreased. 46% ± 3% of phagosomes in QXR hemocytes showed obvious changes in pH within 40 minutes of phagocytosis, compared with 33% ± 3% in wild-type hemocytes. Phenoloxidase deposition was also evident in phagosomes after the *in vitro* phagocytosis of *M. sydneyi*. Most importantly, ingested and melanised *M. sydneyi* were detected *in vivo* among hemocytes from infected oysters. Overall, the data suggest that Sydney rock oysters hemocytes can recognise and phagocytose *M. sydneyi*, and that resistance against QX disease may be associated with enhanced phagocytosis and heightened phagolysosomal activity in QXR oysters.

Key words: *Saccostrea glomerata*, *Marteilia sydneyi*, phagocytosis, phenoloxidase, QX disease.

Abbreviations: ANOVA, one-way analyses of variance; DIC, differential interference microscopy; FSW, filtered seawater; L-DOPA, L-ß-3,4-dihydroxyphenylalanine; LPS, lipopolysaccharide; MAC, marine anticoagulant; MBTH, 3-methyl-2-benzothiazoline hydrozone; MSX, multinucleated sphere unknown; NSW DPI, New South Wales Department of Primary Industries; PBS, phosphate buffer saline; PIPES, 1, 4-piperazinediethanesulfonic acid; PO, phenoloxidase; ROS, reactive oxygen species; SEM, standard error of the mean; TEM, transmission electron microscope; QX disease, Queensland unknown disease; QXR, resistant strain oyster; 4-HA, hydroquinine monomethyl ether.

1. Introduction

The Sydney rock oyster industry has suffered recurrent outbreaks of fatal QX disease since the 1970's (Adlard and Ernst, 1995; Bezemer et al., 2006). QX disease is caused by the protozoan, *Marteilia sydneyi* (Perkins and Wolf, 1976; Roubal et al., 1989). Epizootics occur in Summer and Autumn (January to May), resulting in up to 94% mortality (Berthe et al., 1999; Kleeman and Adlard, 2000).

M. sydneyi is a paramyxean protozoan, classified by its distinctive method of sporulation (Berthe et al., 2004). Sporonts are generated by several internal (or cell-within-cell) cleavages that germinate from sporont bodies within the tissues of infected hosts (Berthe et al., 2004; Kleeman et al., 2002). *M. sydneyi* enters *S. glomerata* through the gills and palps as a result of the oysters' filter-feeding regime (Roubal et al., 1989; Bower 1995). Infection is initially established in the gill and palp epithelia and spreads systemically, via the hemolymph and connective tissues, into the

lumen of the hepatopancreas (digestive gland) (Perkins and Wolf, 1976; Desportes and Perkins 1990). *M. sydneyi* finally establishes in the digestive gland as sporangiosorae, which contain between eight to 16 sporonts, or spore progenitors (Berthe et al., 2004). The sporonts undergo further cleavage to form two, and in some cases, three multinucleated spores (Perkins and Wolf, 1976). The mature spores walls are generally between 16 and 27 nm thick and contain a thick myelin whorl (140 to 420 nm) (Perkins and Wolf, 1976; Hine and Thorne, 2000).

Despite our detailed understanding of the lifecycle of *M. sydneyi* within oysters, there is still only limited information regarding intermediate hosts for the parasite, and there are no effective management strategies based on altered farming practices (Nell et al., 2000). To control the disease, a QX disease resistance-breeding program was established in 1996 by the NSW Department of Primary Industries (NSW DPI) (Nell et al., 2000; Newton et al., 2004). This program has successfully reduced mortality among the selectively bred oysters, which are now being farmed in many areas (Nell et al., 2000).

A number of studies have indicated that oyster hemocytes are involved in controlling *M. sydneyi* infection, particularly among QX-disease resistant (QXR) oysters (Newton et al., 2004; Nell and Hand, 2003; Butt and Raftos, 2008; Aladaileh et al., 2007). Sydney rock oysters have three main classes of hemocytes; stem cells, hyalinocytes and granulocytes (Aladaileh et al., 2007; Auffret, 1998). Both hyalinocytes and granulocytes are phagocytic (Auffret, 1998), with granulocytes being more phagocytically active than hyalinocytes (Aladaileh et al., 2007). Cytochemical assays have demonstrated that the granules within granulocytes contain intracellular antimicrobial molecules, such as superoxides and melanin (Aladaileh et al., 2007). They also contain the defensive enzymes, phenoloxidase (PO), acid phosphatase, and peroxidase.

The existence of intracellular PO activity in oysters sets them apart from most invertebrates. PO is usually a key component of humoral (extracellular) defense in invertebrate immune systems rather than intracellular systems (Smith and Söderhall, 1991; Deaton et al., 1999). In both locations, PO is activated by proteolytic cleavage initiated by a cascade of serine proteases (Asokan et al., 1997; Cerenius and Söderhall, 2004). Active PO adheres to the surface of foreign particles, where it catalyses a cascade of monophenolase and *o*-diphenolase reactions to form *o*-quinones (Ashida and Brey, 1998). Polymerization of *o*-quinones results in the formation of the polymer, melanin (Cerenius and Söderhall, 2004). Melanin and many of its precursors have powerful antimicrobial activities, both independently and in conjunction with the formation of reactive oxygen species (ROS) (Nappi and Christensen, 2005).

A study by Newton et al. (2004) demonstrated that novel forms of PO have been selected during the breeding of QXR oysters. These authors also showed that QXR oysters have PO enzyme activities that are up to three times greater than those of wild-type oysters (Bezemer et al., 2006; Newton et al., 2004). Such conclusions complement the findings of Butt and Raftos (2008), who demonstrated that hemocytes from QXR oysters are larger and have increased phagocytic capacities compared with wild-type oysters. Butt and Raftos (2008) concluded that phagocytosis and PO-mediated phagolysosomal killing are key host defence responses against *M. sydneyi*.

The current study supports this conclusion with ultrastructural data on the phagocytic interactions between *S. glomerata* hemocytes and *M. sydneyi*. It focuses on phagolysosomal activity (specifically the involvement of PO) and compares phagocytosis between non-selected wild-type and QXR oysters.

2. Materials and Methods

2.1 Oysters

Oysters were from non-selected wild-type populations and the QX disease resistant (QXR) breeding line. Wild-type oysters that were free of *M. sydneyi* infection were purchased from the Sydney Fish Markets (Sydney, NSW, Australia). Additional wild-type *S. glomerata* that were infected with *M. sydneyi* were sourced by Ms Jane Frances (Biosecurity Manager, NSW DPI) and were kindly supplied by Mr Greg Tunningley, an oyster farmer from South West Rocks (NSW, Australia). QXR oysters were kindly supplied by Dr John Nell (NSW DPI). They were from the fourth generation that had been selectively bred by NSW DPI in the Georges River, Sydney (33º59'S). QXR oysters were grown to "bistro" grade in trays at Lime Kiln Bar (33°59'08" S, 151°03'10" E) and were then transferred to Neverfail Bay (33°59'41" S, 151°04'21" E) during 2003. After collection from the field, oysters were maintained in aerated recirculating aquaria (40 L) at 25°C in Macquarie University's seawater facility (North Ryde, NSW, Australia).

2.2 Hemolymph extraction

Hemolymph was extracted by levering the shell hinge to expose the flesh, whilst not rupturing the pericardial cavity. Hemolymph was withdrawn from the pericardium (near the adductor muscle) using 22 gauge needles fitted to 1 mL syringes. To determine the abundance of hemocytes, 100 µL of hemolymph was mixed with 100 µL marine anticoagulant (MAC; 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 50 mM EDTA, 0.45 M NaCl, pH 7.5). This hemocyte suspension was then fixed with 100 µL paraformaldehyde (4% w/v) and hemocytes were counted with an Improved Neubauer hemocytometer (Sigma-Aldrich, CA, USA).

2.3 Light microscopical analysis of haemolypmh from M. sydneyi in infected oysters

Wild-type oysters with early QX infections were cultured in an aerated recirculating aquarium (40 L) at 25°C for 7 months to allow the infection to develop. Four hundred µL of whole hemolymph was then extracted from the pericardial cavity. The hemolymph was centrifuged (400 × g, for 4 min) and the cells were re-suspended in FSW. Twenty µL aliquots of these cell suspensions were inspected at high magnification (100 × objective, oil immersion) with an Olympus BH-2 microscope equipped with differential interference contrast (DIC) optics.

2.4 Isolation of M. sydneyi sporonts

Oysters infected with *M. sydneyi* were identified using tissue smears of the digestive gland (Berthe et al., 1999). *M. sydneyi* were isolated from infected digestive glands that had been excised using a sterile razor blade. Cubes ($2mm^3$) of digestive gland tissue were homogenized in filtered seawater (FSW; 0.45 µm) and passed twice through a 150 µm sieve (Sigma-Aldrich). The filtered homogenates were further diluted with FSW and layered onto discontinuous Percoll gradients (10%, 20%, 30%, 40%, and 50% Percoll in FSW; Pharmacia, Sweden). Percoll gradients were centrifuged at 2,500 × g, (4ºC) for 20 mins. Purified sporonts were collected from the 30% - 40% Percoll interface. Isolated parasites were then centrifuged (3,000 × g, 4ºC, 10 mins), re-suspended in FSW and stored at - 4ºC.

2.5 Preparation of M. sydneyi and yeast for phagocytosis experiments

Purified *M. sydneyi* or Congo red-stained yeast were used as targets for phagocytosis experiments. Two hundred and fifty mg of baker's yeast (*Saccharomyces cerevisiae*; Sigma-Aldrich) or *M. sydneyi* sporonts (5×10^6 mL^{-1}) were suspended in 5 mL of FSW. Five mL of filtered 0.8% w/v Congo red prepared in FSW was added to the suspension and autoclaved at 90ºC for 20 min. The

suspensions were then centrifuged and washed twice at 3,000 × g, 4ºC, for 5 min. The final pellet was re-suspended in 10 mL FSW and stored at 4ºC.

2.6 Epi-fluorescence and differential interference contrast microscopical analysis of phagocytosis

Two hundred µL Congo red stained yeast or *M. sydneyi* sporonts were dotted onto poly-L-lysine-coated coverslips at room temperature (25ºC) in a moist chamber. Twenty µL of whole hemolymph from QXR or wild-type oysters were then added to the yeast or *M. sydneyi* and allowed to mix for 15 min. Free yeast or sporonts that had not been engulfed by adherent hemocytes after 15 min were removed by dipping the coverslips twice in FSW. The coverslips were then mounted on slides and viewed at high magnification (60 × objective, oil immersion) with an Olympus BH-2 microscope equipped with epi-fluorescence and DIC optics. pH changes in phagosomes were quantified by examining 400 cells from both QXR and wild-type oysters and recording the number of ingested Congo red stained yeast cells that had changed colour from red to blue. Congo red-stained yeast were used for this purpose because the thick wall of *M. sydneyi* sporonts prevented the discoloration of Congo red associated with acidification.

2.7 Phalloidin Staining

Intracellular actin polymerization was analyzed by adhering 1×10^5 *M. sydneyi* sporonts to acid-alcohol cleaned microscope slides for 10 min followed by the addition of 50 µL of whole hemolymph for 20 min. Cells were then fixed in 10% w/v paraformaldehyde for 30 min. Cell membranes were permeabilized with 0.1% Triton–X in phosphate buffered saline (PBS; pH 7.5) for 5 min before being stained with 5 µL of phalloidin Alexa® (Invitrogen, Mount Waverley, VIC) solution in 200 µL PBS for 30 min. Nuclei were then counter stained with To-POR-3 (Invitrogen, Mount Waverley, VIC) for 8 min. Hemocytes were examined using an Olympus Fluoview 300 confocal microscope.

2.8 Cytochemical analysis of phenoloxidase activity after phagocytosis

To stain hemocytes for phenoloxidase activity, 500 µL whole hemolymph was mixed in suspension with 5×10^5 *M. sydneyi* sporonts and incubated for 30 min. 3-methyl-2-benzothiazoline hydrozone (MBTH; Sigma Aldrich, Castle Hill, NSW) and L-ß-3,4-dihydroxyphenylaline (L-DOPA; 8 mg mL^{-1} in FSW; ICN Biomedicals, CA, USA) were then added to the suspension, which was further incubated for 1.5 hours. Tropolone (10 mM; Sigma) was then added to prevent further phenoloxidase activity. Cells were pelleted by centrifugation (2500 × g, 5 min) and the supernatant was aspirated. The hemocytes were rinsed in FSW by centrifugation (2500 × g, 5 min) before being re-suspended in fresh FSW. The hemocytes were examined using an Olympus BH-2 microscope.

2.9 Transmission electron microscopy (TEM) of phagocytosis

Five hundred µL of whole hemolymph from QXR or wild type oysters were mixed with 200 µL FSW containing 5×10^5 *M. sydneyi* sporonts. Aliquots (100 µL) were removed from these mixtures 0, 2, 5, 30 60, 120 and 180 min later. These aliquots were fixed for 10 min at 4ºC in 4% paraformaldehyde, 2.5% glutaraldehyde and 0.3 M sucrose in 0.1 M 1, 4-piperazinediethanesulfonic acid (PIPES) buffer (Sigma-Aldrich, pH 7.2). After fixation, the cells were centrifuged at 400 × g for 10 min. The supernatant was then aspirated and the pellet was re-suspended in fresh fixative for a further 4 hours at room temperature. The cells were again centrifuged (2000 × g, 2 min) and the pellets were embedded in melted 2% agar before being centrifuged again (2000 × g, 2 min). Pellets were washed in PIPES for 2 × 1 hour and post-fixed in osmium tetroxide (OsO_4 in 0.1 M PIPES buffer) for 1 hour, followed by two 10 min washes in 0.1 M PIPES. The pellets were then immersed in 2% aqueous uranyl acetate for 20 min (*en bloc*

staining) and rinsed in PIPES for 10 min. Pellets were dehydrated through a graded ethanol series (50%, 70%, 80%, 90%, 100%) before being embedded in L.R. White resin (80% polyhydroxy substituted bisphenol, A dimethacrylate resin 19.6%, C12 methacrylate ester, 0.9% benzoyl peroxide) for 48 hours. This was followed by 2 × 1 hour washes in pure L.R White resin at 24 hour intervals. Each pellet was then placed on the bottom of an oven-dried gelatin capsule, and left in an oven (60ºC) for a further 24 hours to set. Semi-thin sections were cut, stained with methylene blue, and examined with an Olympus BH-2 microscope. To quantify phagocytic activity, hemocytes from 5 wild type and 5 QXR oysters were mixed with *M. sydneyi*. Hemocytes in methylene blue stained sections of these mixtures were then inspected (200 cells per oyster) so that the frequency of hemocytes that had ingested 2 or more sporonts could be determined.

Ultrathin sections were cut using a Reichert Ultracut-S ultramicrotome and mounted onto pioloform coated, 300 copper mesh grids. Ultrathin sections were stained with filtered saturated uranyl acetate for 40 min and filtered Reynolds lead citrate for 5 min. Ultrathin sections were examined with a Philips CM 10 transmission electron microscope.

TEM was also used to examine *M. sydneyi* in the guts of infected oysters. Digestive gland tissue from seven *M. sydneyi* infected oysters prepared for TEM using the methods described above.

2.10 Data Analysis

Numerical data from *in vitro* phagocytosis experiments (percentage of hemocytes engulfing >2 *M. sydneyi* sprotonts and the percentage of Congo red-stained yeast exhibiting colour changes after phagocytosis) were analyzed using SPSS v.15 software (SPSS Inc. II, USA, 2006). One-way analyses of variance (ANOVA) were performed to determine whether significant differences existed between data for QXR and wild-type oysters. Differences were considered significant when $p < 0.05$.

3. Results

3.1 M. sydneyi purification

Light microscope analysis of methylene-blue stained sections and TEM identified various developmental stages of *M. sydneyi* within the digestive gland tubules and endothelial cells of infected oysters (Figure 1A). Parasites located within the endothelial cells were present as solitary sporonts, while sporonts within the digestive gland tubules appeared either alone or as clusters within sporangiosori (Figure 1A). *M. sydneyi* purified from infected gut tissue by density gradient centrifugation appeared as individual sporonts, suggesting that homogenising intact the gut tissue ruptured sporangiosori, releasing individual sporonts (Figure 1B).

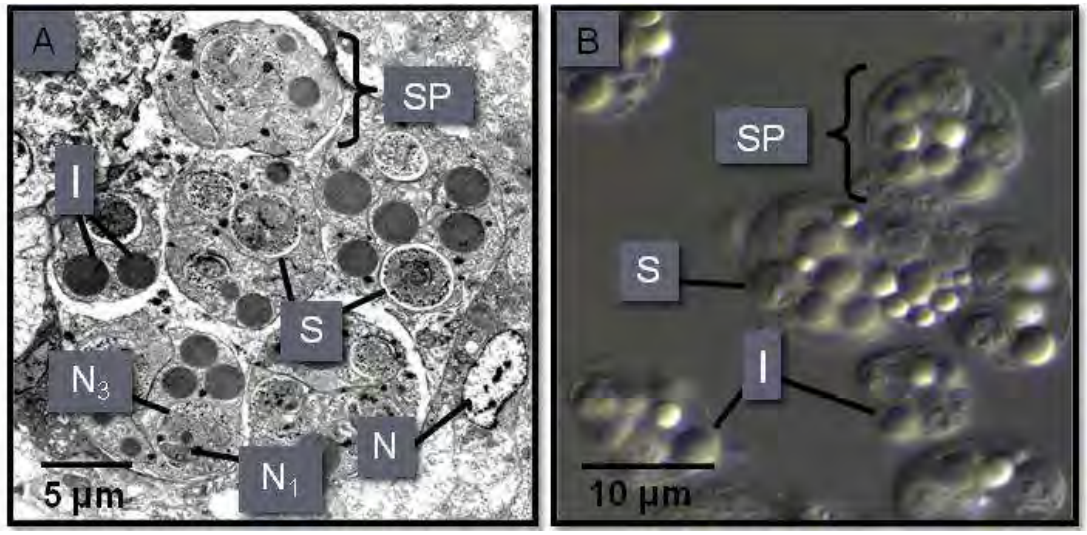

Figure 1. **A**; An *M. sydneyi* sporangiosorus in the digestive gland of an infected oyster (TEM, 5,200 ×). This sporangiosorus contains six individual reproductive bodies (sporonts or secondary cells, SP). Cleavage of the sporangium (cytoplasm of the sporont) leads to the development of two to three multinucleated (N_1, N_3) spores (S), surrounded by inclusion bodies (I). The nucleus (N) of an oyster hemocyte adjacent to the sproangiosorus is also shown. **B**. A DIC micrograph of *M. sydneyi* sporonts purified by density gradient centrifugation (100 × objective, oil immersion). Note the presence of spores within the sporonts.

3.2 Morphology of S. glomerata hemocytes

Three distinct hemocyte types were identified by TEM and DIC microscopy: stem cells (hemoblast-like), hyalinocytes, and granulocytes (Aladaileh et al., 2007). These cell types were classified based upon the presence or absence of cytoplasmic granules, their nucleus/cytoplasm ratio, and by the presence of vacuoles.

Stem cells were the smallest of the three hemocyte classes averaging 4.0 µm in diameter (Figure 2A and D). They were either ovoid or spherical in shape and had a high nucleus:cytoplasm ratio (Figure 2A and D). Stem cells extended only very small filopodia and rarely adhered to glass slides (Figure 2D).

Hyalinocytes were characterized as having no, or very few, granules (Figure 2B, and E). Their diameter ranged from 5-9 µm and they had either oval or spheroidal nuclei. When attached to glass slides, hyalinocytes often projected long filopodia (Figure 2B) and were highly amoeboid.

Granulocytes were identified by the presence of numerous granules, the size and number of which varied between cells (Figure 2C and F). Granulocytes ranged in diameter from 5-9 µm. Nuclei in granulocytes were either ovoid, spheroidal, or bificated. When exposed to ultra-violet light (448nm), the granules autofluoresced, and the intensity of autofluorescence varied between granules and cells (data not shown). Granulocytes also moved with an ameboid motion and extended filopodia on glass slides. Intracellular degranulation formed vacuoles, which contained particles that exhibited Brownian motion.

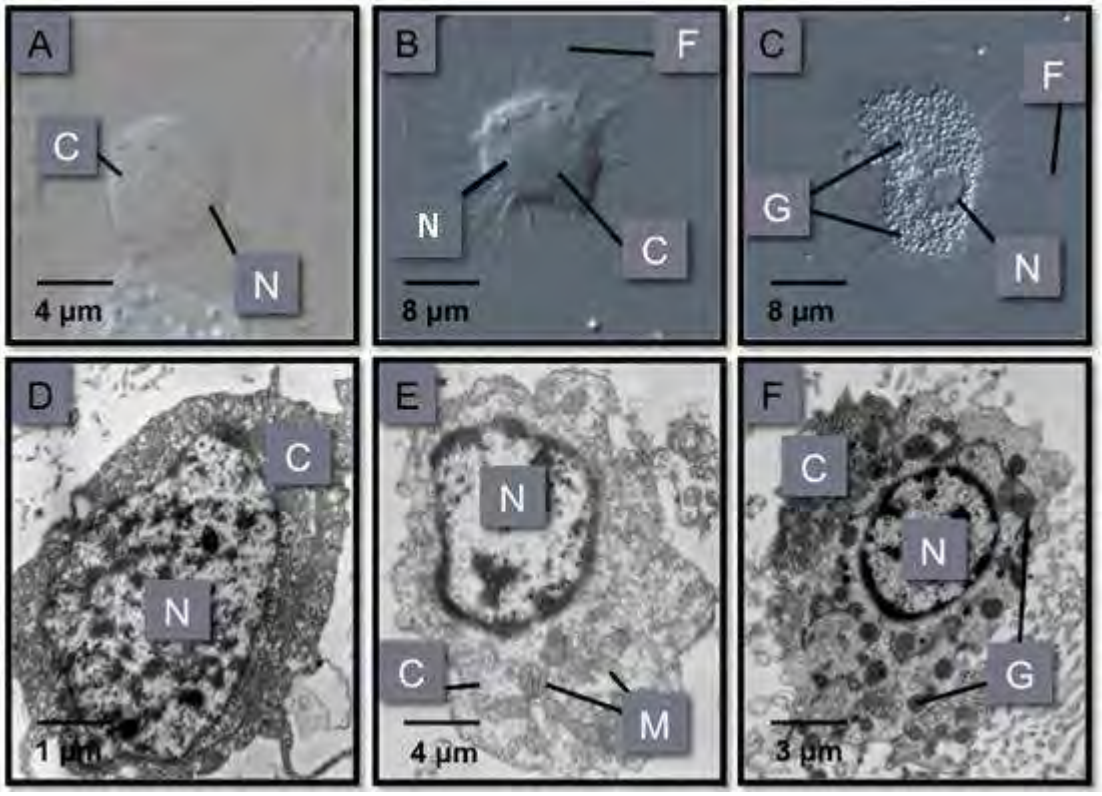

Figure 2. A-C. DIC micrographs of hemocytes from the hemolymph of *S. glomerata* (100 ×, oil immersion) **A**, Stem cell. Stem cells typically have large nuclei (N) and limited cytoplasm (C) compared with granulocytes and hyalinocytes. **B**, Hyalinocyte. These cells often project long filopodia (F). **C**, Granulocyte. Granulocytes were easily identified by the presence of cytoplasmic granules. Their nuclei are spheroidal in shape and they also extend filopodia. **D-F**. TEM micrographs of the same cell types. **D**, Stem cell (8,900 ×). **E**, Hyalinocyte (6,600 ×). Nuclei of hyalinocytes appear either round or oval in shape and mitochondria (M) can be seen throughout the cytoplasm. **F**, Granulocyte (6,600 ×).

The distribution of nuclear material within hemocytes varied between cell types. Stem cells appeared to have a more even distribution of chromatin throughout the nucleus (Figure 2D), while the nuclear material of granulocytes and hyalinocytes was often localized around the periphery of the nucleus (Figure 2E and F).

3.3 Phagocytosis of M. sydneyi by oyster hemocytes

In initial experiments, DIC microscopy was used to observe phagocytic interactions between Congo red-stained *M. sydneyi* and hemocytes from both wild-type and QXR oysters.

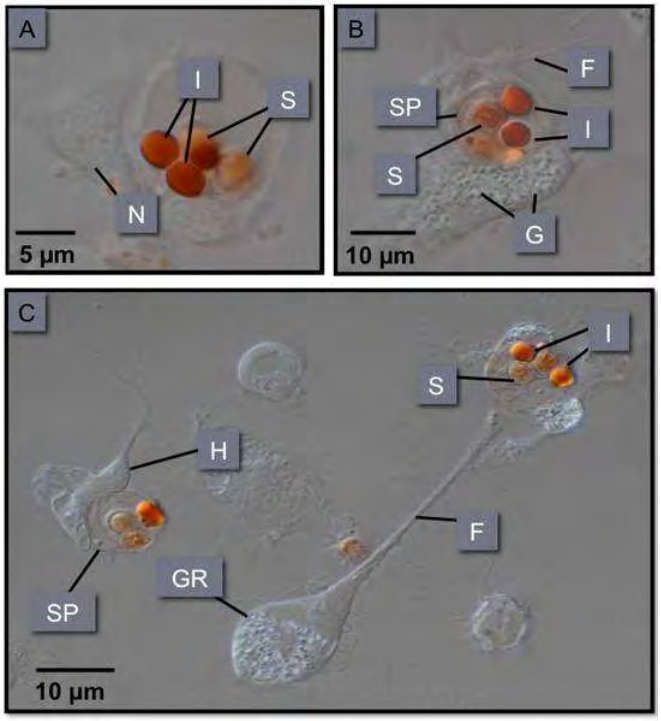

Figure 3. Phagocytosis of *M. sydneyi* sporonts by *S. glomerata* hemocytes. **A**, *M. sydneyi* sporont phagocytosed by a hyalinocyte from a QXR oyster (DIC, 100 × objective, oil immersion). **B**, A granulocyte engulfing an *M. sydneyi* sporont (SP). **C,** DIC micrograph of *M. sydneyi* sporonts engulfed by hemocytes from a wild-type oyster (100 × objective, oil immersion). A hyalinocyte (H) is shown engulfing a sporont (SP) after approaching the parasite by ameboid motion. A granulocyte (GR) can be seen extending a filopodium (F) towards and around an *M. sydneyi* sporont. The inclusion bodies (I) and spores (S) of sporonts were stained with Congo-red. N, nucleus; G, intracellular granules.

Both granulocytes and hyalinocytes engulfed *M. sydneyi* sporonts (Figure 3A and B). Two distinct types of phagocytosis were apparent. In the first, hemocytes approached the parasite by ameboid movement surrounding and engulfing the target cell (Figure 3C). Alternatively, long filopodia were extended from the main body of the hemocyte towards and around *M. sydneyi* (Figure 3C). Once the parasite was engulfed, the filopodium retracted into the body of the hemocyte.

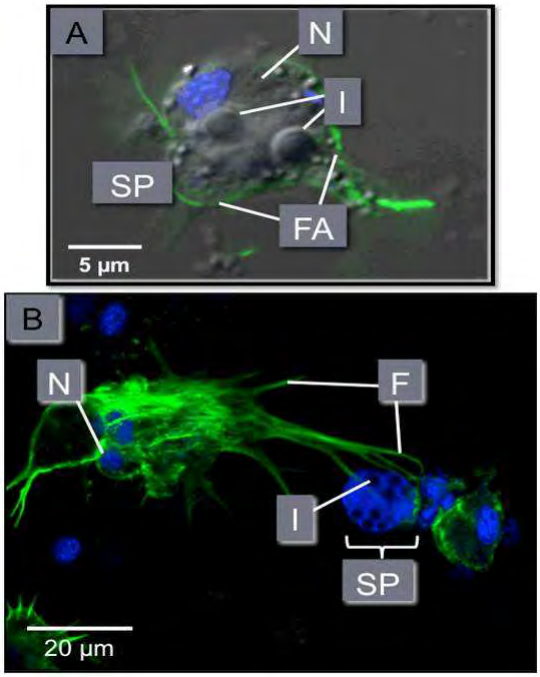

Figure 4. Engulfment of sporonts by hemocytes. F-actin (FA) green, nuclei (N), blue. **A**, *M. sydneyi* engulfed by a *S. glomerata* hemocyte. Note the localised development of FA around the periphery of the sporont (SP). **B**, A fluorescence confocal micrograph showing filopodia (F) development that moved towards and around a sporont (100 × objective, oil immersion). Inclusion (I) bodies did not fluoresce.

Fluorescence confocal microscopy showed that F-actin polymerisation was closely associated with filpodia formation. Filopodia were observed as bright green F-actin-positive extensions of the cell body (Figure 4A and B). Filopodia stained with phalloidin-Alexa were seen to extend towards and around *M. sydneyi* sporonts (Figure 4B). The intensity of F-actin staining was often greatest around the periphery of the engulfed parasites (Figure 4A).

Granulocytes were often seen to engulf more than one *M. sydneyi* (Figure 5A). This was common in both wild-type and QXR hemocytes. However, hemocytes from QXR oysters engulfed significantly more sporonts than hemocytes from wild-type oysters. 36% ± 3% of QXR hemocytes engulfed two or more sporonts, compared with 15% ± 2% of wild-type hemocytes (Figure 5 B; $F = 6.68$, $df = 1, 9$, $p = 0.032$).

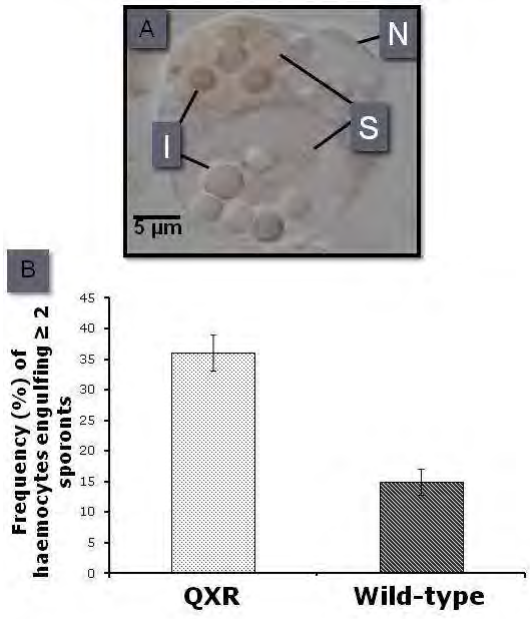

Figure 5. **A**, DIC micrograph (100 × objective, oil immersion) of an oyster hemocyte that has engulfed two *M. sydneyi* sporonts (S). I, sporont inclusion bodies, N, hemocyte nucleus. **B**, Mean percentages of hemocytes from QXR and wild-type oysters that had engulfed ≥ 2 sporonts. N = 6; bars = SEM.

3.4 Ultrastructure of M. sydneyi phagocytosis

As with DIC, TEM showed that sporonts were frequently phagocytosed by both granulocytes and hyalinocytes. Initial contact with sporonts and pseudopodial development by hemocytes began between 0 - 5 min after sporonts and hemocytes were mixed. During this time, pseudopodia were seen to extend toward and around parasites, with the leading edge of pseudopodia appearing tapered (Figure 6A and B). A distinct gap was evident between the membranes of the phagosome and parasite in early stages of phagocytosis (Figure 6C & D). As parasites were engulfed, phagosomes formed around the sporonts (Figure 6C). After 30 min, most hemocytes had engulfed at least one sporont. Phagosomes were evident as continuous membranes surrounding ingested sporonts that were external to the outer membrane of the parasite. Phagosome membranes appeared thinner than those of the parasites (Figure 6D).

Fusion of round to ovoid hemocyte granules to the phagosome membrane was detected within 5 min of sporont engulfment. Granules were observed adhering to the phagosome membrane and then fusing with the membrane (Figure 7A, B and C).

No ultrastructural differences were detected in phagocytosis between wild-type and QXR oysters.

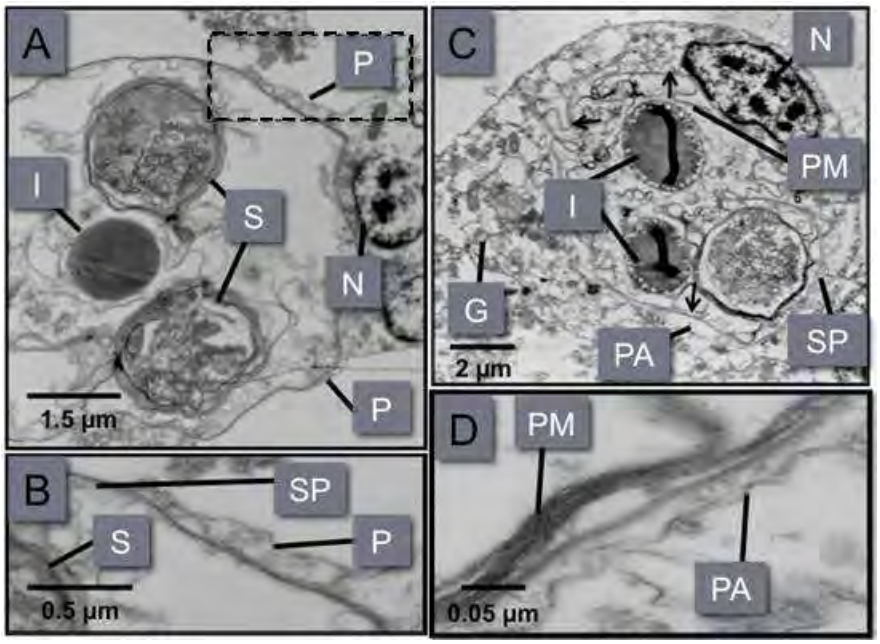

Figure 6. Early stages of *M. sydneyi* phagocytosis by a QXR hemocyte. **A**, Transmission electron micrograph of a QXR granulocyte engulfing an *M. sydneyi* sporont (5,200 ×). Pseudopodia (P) develop around the parasite. The parasite depicted here contains two spores (S) and a single inclusion body (I). **B**, Higher resolution image of the boxed area shown in **A**. Pseudopodia appear tapered during the process of phagocytosis. **C**, transmission electron micrograph of wild-type hemocyte that has engulfed an *M. sydneyi* sporont (6,6100 ×). A phagosome membrane (PA) extends around the periphery of the parasite membrane. Arrows show the parasite/hemocyte interface. **D**, High resolution image of the phagosome/parasite interface. G, intracellular granules; S, spore. SP, sporont.

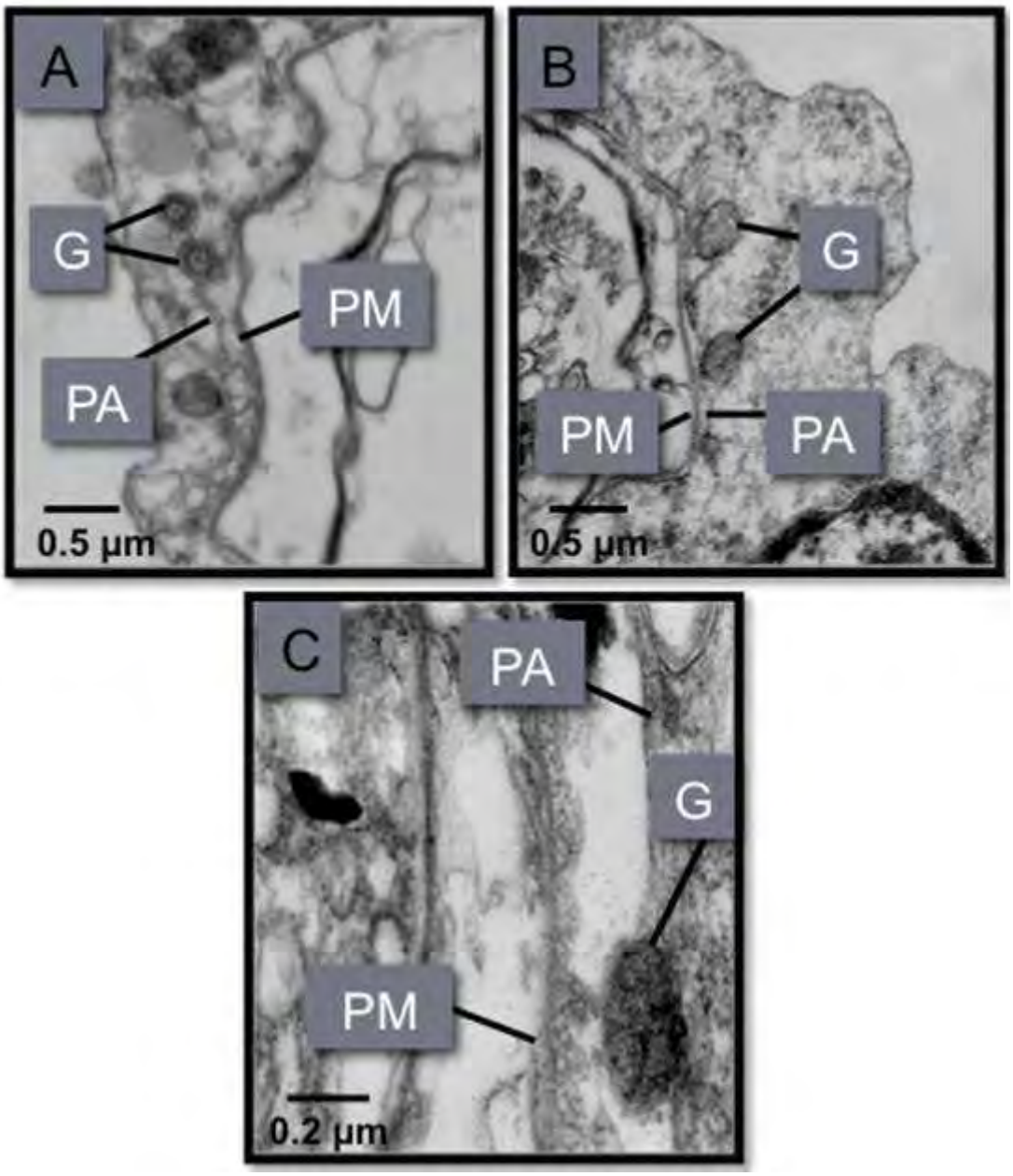

Figure 7. Granular fusion to phagosomes. **A**, hemocyte granules (G) approaching and fusing to the phagosomal membrane (PA) that surrounds an *M. sydneyi* sporont in a QXR hemocyte (TEM, 21,000 ×). PM, parasite membrane; PA, phagosome membrane. **B**, Granules fusing to the phagosome containing an *M. sydneyi* sporont in a wild-type oyster hemocyte (TEM, 21,000 ×). **C**, high resolution image of a granule fusing with the phagosome membrane around an *M. sydneyi* sporont (TEM, 28,500 ×).

3.5 *Acidification of phagosomes*

Alteration in colour among Congo red-stained yeast cells was used to monitor pH changes that were associated with phagosome formation (Figure 8A, B, C and D). Yeast were used for this purpose because the cell walls of *M. sydneyi* sporonts prevented discolouration of Congo red. Figures 8A and C show the formation of phagosomes around engulfed yeast in hemocytes from QXR and wild type oysters (Figure 8A). The percentage of engulfed yeast that changed from red to blue (Figure 8B, D, and E), reflecting acidification of phagosomes, differed significantly between hemocytes from wild-type and QXR oysters (one-way ANOVA, F = 10.72, *df* = 1, 5, *p* = 0.031). In

QXR oysters, 44% ± 3% of yeast underwent detectable colour changes, compared to 32% ± 3% in wild-type hemocytes (Figure 8E).

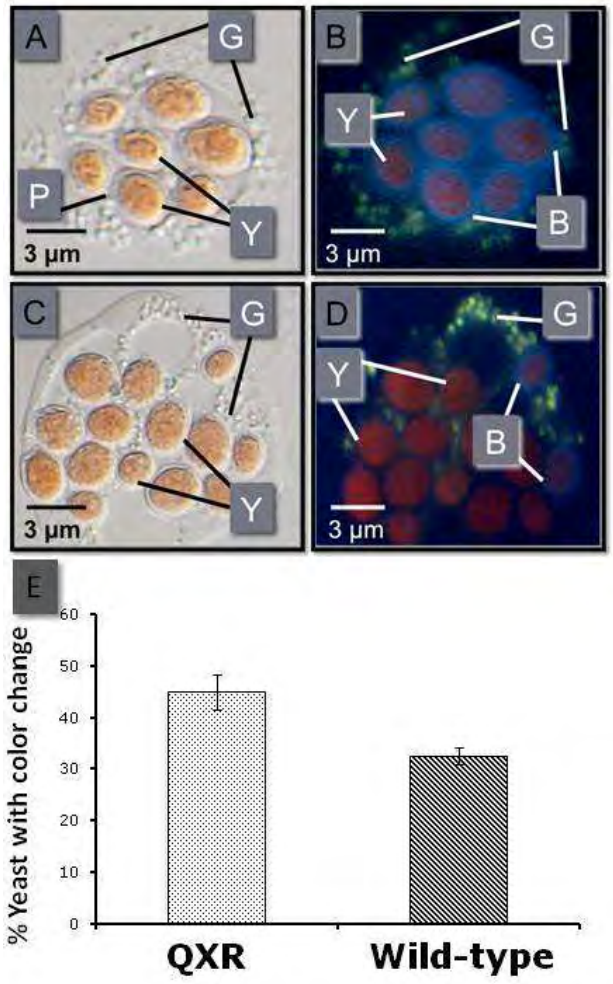

Figure 8. Phagocytosis of Congo red-stained yeast cells by *S. glomerata* hemocytes. **A**, DIC micrograph of a QXR hemocyte (100 × objective, oil immersion) with individual phagosomes (P) around each of seven ingested yeast cells (Y). **B**, Fluorescence micrograph of the same QXR granulocyte showing a colour change from red to blue (B) in all seven yeast (100 × objective, oil immersion). Granules (G) within the granulocyte autofluorescence green. **C**, DIC image of 14 yeast cells engulfed by an wild-type granulocyte (100 × objective, oil immersion). **D**, Fluoroesence micrograph of the same wild-type hemocyte (100 × objective, oil immersion) showing colour change in only two of the fourteen engulfed yeast cells. **E**, The mean percentage of yeast exhibiting colour changes after being phagocytosed by QXR or wild-type hemocytes. N= 5, bars = SEM.

3.6 PO activity associated with M. sydneyi phagocytosis

The deposition of pink-coloured products of MBTH/L-DOPA oxidation was evident in hemocytes from both QXR and wild-type oysters immediately after the *in vitro* ingestion of *M. sydneyi*. Colour development was evident within granules that surrounded phagosomes containing *M. sydneyi* sporonts (Figure 9A). The distribution of PO-positive granules was patchy over the surface of most phagosomes (Figure 9A and B). PO activity intensified over a 3-hour incubation period (Figure 9B) extending from granules in the cytoplasm of granulocytes into phagosomes, resulting in a brown-pink discolouration of the ingested *M. sydneyi* (Figure 9B).

Discolouration consistent with PO activity was also evident in hemocytes taken from *M. sydneyi* infected oysters. Hemocytes from infected oysters were found to have engulfed *M. sydneyi*, and demonstrated various stages of darkening, consistent with melanization (Figure 9C and D). Melanization appeared to be localised around and on the surface of the parasites (Figure 9C and D). In many cases, parasites had degenerated within hemocytes, leaving only inclusion bodies (Figure 9D).

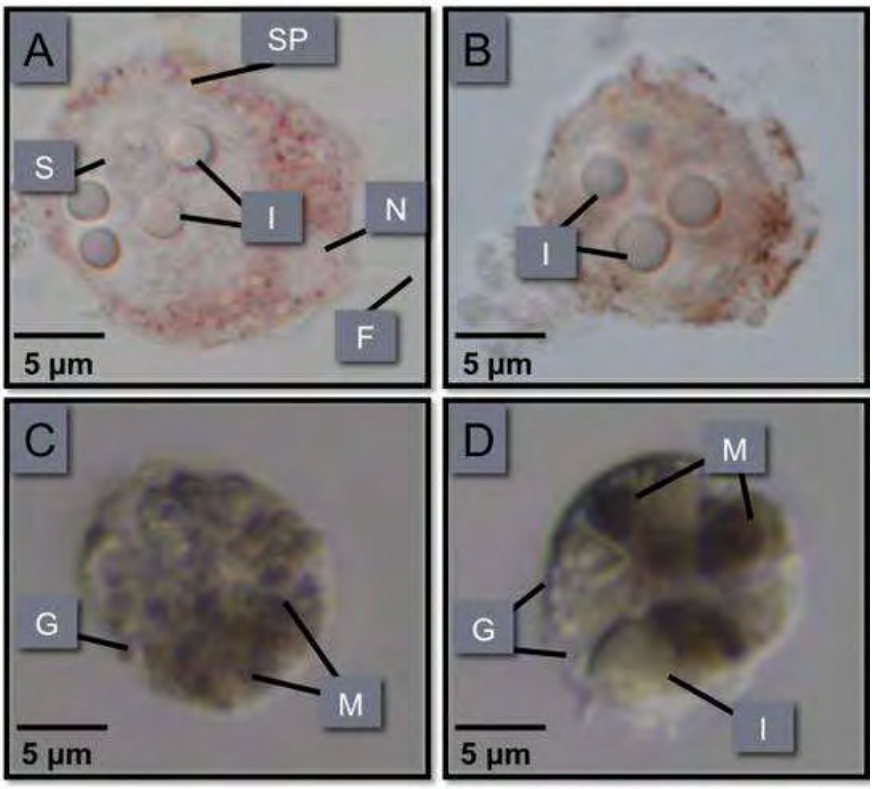

Figure 9. A and **B**, intracellular PO activity after mixing *S. glomerata* hemocytes and *M. sydneyi* sporonts (SP) in the presence of L-DOPA and MBTH (DIC, 100 × objective, oil immersion). **A**, Hemocyte from a QXR oyster containing a single *M. sydneyi* sporont 2 hour after mixing. PO activity in granules appears pink. **B**, An *M. sydneyi* sporont engulfed by a wild-type hemocyte 3 hours after mixing. Note the development of pink/brown colouration within the phagosome containing the *M. sydneyi* sporont and the patchiness of stained granules associating with the phagosome. I, inclusion body; S, spore; N, hemocyte nucleus. **C**-**D**, DIC micrographs of putative melanin deposition in hemocytes from *M. sydneyi* infected wild-type oyster hemocytes (100 × objective, oil immersion). Note the absence of a phagosome membrane and the melanization (M, brown/black colouration) of inclusion bodies within the oyster hemocytes. G, denotes granules.

4. Discussion

Since 1972, the Sydney rock oyster industry has experienced a significant decline in production as a result of infectious diseases, most notably winter mortality and QX disease (Nell et al., 2000). In response, NSW DPI initiated a selective breeding program for disease resistance in 1996 (Nell et al., 2000). The current study investigated the phagocytosis of *M. sydneyi* and compared the phagocytic activities of selectively bred, QXR, oysters with those of unselected wild-type oysters. This work expands on previous data (Butt and Raftos, 2007) by; providing an ultrastructural description of phagocytosis, particularly the fusion of cytoplasmic granules to phagosomes; identifying higher levels of acidification in the phagosomes of QXR hemocytes compared to those of wild-type oysters; and confirming that phagocytosis and apparent melanisation of *M. sydneyi* occurs *in vivo* among infected oysters.

Previous studies have highlighted the importance of hemocytes in host defence and the role they play in resistance or susceptibility to disease (Ratcliffe and Gagen, 1976; Ratcliffe et al., 1985; Söderhall and Cerenius, 1992). An investigation by Ford et al., (1993) showed that resistance to the protozoan, *Haplosporidium nelsoni*, (causative agent of MSX disease) in the American eastern oyster was associated with a circulating sub-population of granulocytes. Resistant oysters were found to have a significantly higher percentage of large granulocytes, which are known to be involved in tissue repair and debris removal (Ford et al., 1993). Similarly, data from the current study indicated that *S. glomerata* hemocytes engage in the active phagocytosis of *M. sydneyi*. Both granulocytes and hyalinocytes were observed engulfing the parasite using either ameboid movement, or by the extension of filopodia toward and around sporonts. Initial phagocytic activity was recorded as early as 2 min after *M. sydneyi* were mixed with hemocytes. The fusion of pseudopodia around *M. sydneyi* sporonts resulted in the formation of phagosomes within hemocytes, characterised by a distinct hemocyte-derived membrane surrounding the engulfed *M. sydneyi*.

This process of phagocytosis seemed to be actin dependent. The redistribution of F-actin within hemocytes appeared to drive the formation of pseudopodia towards and around *M. sydneyi* sporonts, and in some cases F-actin was closely associated with the phagosome formation. In other species, this type of F-actin redistribution results from the phosphorylation of membrane-associated proteins, most significantly profilin and gelsolin (Underhill and Ozinsky, 2002; Stuart and Ezekowitz, 2005). These molecules regulate the interactions between actin filaments and the plasma membrane to facilitate engulfment (Lee and Söderhall, 2002), resulting in an intimate association between F-actin microfilaments and developing phagosomes.

After phagosome formation around *M. sydneyi* sporonts, hemocyte granules were often found to fuse with the phagosome membrane. Studies by Aladaileh et al. (2007) have shown that hemocyte granules contain high levels of PO and acid phosphatase, and have the capacity to form superoxides and peroxides. The presence of acid phosphatase indicates that some granules function as lysosomes, and other data suggest that PO co-localises in these lysosomal granules (Aladaileh et al., 2007). Granule fusion, particularly involving lysosomes, has been thoroughly documented in the process of phagocytosis in other species and is a key mechanism involved in phagolysosomal killing (Cheng 1983; Cronin et al., 2001). Lysosomal contents degrade the cellular membranes of internalised pathogens (Cheng, 1983) and promote the formation of an acidic environment with the phagosome (Cheng 1983; McDade and Tripp, 1967). A similar process is evident in the current study. Colour changes in the pH-sensitive Azo dye, Congo red, were associated with the fusion of granules onto phagosomes containing Congo red-stained yeast. In many cases, distinct colour changes from red to blue were detected, suggesting that acidified lysosomal contents are deposited into the phagosomes of *S. glomerata*. There was also evidence that the phagocytosis of *M. sydneyi* led to the activation of intracellular PO and deposition of both PO and its products onto parasites within phagolysosomes. This suggests that PO may be directly involved in the intracellular killing of *M. sydneyi*.

No obvious differences in the ultrastructural processes of phagocytosis between QXR and wild-type oysters could be discerned by TEM in the current study. However, there were substantial differences between the two oyster strains in other parameters. 36% ± 3% of hemocytes from QXR oysters could engulf two or more *M. sydneyi* sporonts compared with just 15% ± 2% of unselected wild-type hemocytes. This result agrees with that of Butt et al. (2008) who also identified differences between phagocytic activity of hemocytes from QXR and wild-type oysters.
It was also clear that higher rates of acidification, perhaps reflecting an increase in frequency of granules fusing onto phagosomes, occurred in hemocytes from QXR oysters, when compared to wild-type hemocytes. 46 % ± 3% of Congo red-stained yeast cells engulfed by QXR hemocytes demonstrated a marked change in colour, compared to 33% ± 3% in wild-type hemocytes.

These data suggest that QX resistance in Sydney rock oysters may be associated with enhanced parasite clearance through increased phagocytic activity and heightened intracellular degradative

mechanisms. The biological relevance of these data from *in vitro* phagocytosis experiments was confirmed by the identification of hemocytes from *M. sydneyi*-infected oysters that had phagocytosed *M. sydneyi* sporonts *in vivo*. Apparent melanisation and degeneration of the parasite was also evident *in vivo*. This suggests that hemocytes have the capacity to recognise, ingest and destroy *M. sydneyi* during the course of an *M. sydneyi* infection.

4.5 Mortality in single pair mated families of QX-disease resistant and wild type Sydney rock oysters (*Saccostrea glomerata*)

Alison Kan[a], Michael C. Dove[b], Wayne A. O'Connor[b] and David A. Raftos[a]*

[a] *Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia*
[b] *Industry & Investment NSW, Port Stephens Fisheries Institute, Taylors Beach, NSW, 2316, Australia.*

Abstract

QX disease causes mass mortalities among Sydney rock oysters (*Saccostrea glomerata*). To overcome commercial production losses, the New South Wales Department of Primary Industries has been developing mass selected QX-disease resistant breeding lines since 1997. This breeding program has significantly reduced QX-associated mortality in the Lime Kiln Bar (LKB) breeding line relative to non-selected, wild type (WT) oysters. The current study assesses mortality in families produced by single-pair mating between LKB and WT oysters. When these families were grown in a QX disease prone area, the progeny of LKB × LKB crosses had significantly lower mortality compared to LKB × WT or WT × WT families. Mortality in the different crosses was strictly correlated with infection by the parasite responsible for QX disease (*Marteilia sydneyi*). This suggested that the majority of mortality observed in the QX disease-prone growing area was the result of QX disease. Overall, the study identified a strong association between parentage and mortality resulting from QX disease.

Keywords: Sydney rock oysters, pair mating, QX disease, mortality, *Saccostrea glomerata*

1. Introduction

The Sydney rock oyster (*Saccostrea glomerata*) is known for its gourmet taste and long shelf life. This species has been farmed in the eastern Australian states of Queensland and New South Wales (NSW) since the 1870s (Nell 2005). Sydney rock oyster farming is the oldest and most valuable component of the aquaculture industry in NSW, generating an income of more than AUD $36 million per year (NSW DPI 2008).

Since the 1970s, an paramyxean parasite, *Marteilia sydneyi* (Perkins & Wolf 1976), has seriously decreased productivity in a number of Sydney rock oyster farming areas. *M. sydneyi* infection can result in fatal QX disease, which affects oysters during the warmer months of the year from late summer (February) to early autumn (April/May). In the Georges River, NSW, yearly outbreaks of QX disease between 1994 and 2000 resulted in up to 85% mortality (Adlard & Ernst 1995), leading to the abandonment of Sydney rock oyster farming in that area in 2001 (Nell *et al.* 2000). More recently, an outbreak of QX disease in the Hawkesbury River, NSW, in 2004 resulted in almost 100% mortality in some areas of that river (Nell 2007). Despite the severity of these epizootics, the frequency and intensity of QX disease outbreaks is highly variable, and many areas have never experienced an epizootic.

In a bid to combat QX disease, the NSW Department of Primary Industries (NSW DPI) has been breeding oysters for QX-disease resistance since 1997 (Nell *et al.* 2000). This breeding program has been based on mass selection with large numbers of parents contributing to each generation. Mass selection was initiated from a base parental population that incorporated oysters from four major growing estuaries (Wallis Lake, Port Stephens, Hawkesbury River, and Georges River) (Nell *et al.* 1996). In 1997, 216 oysters from breeding lines that had originally been selected for fast-growth were chosen as parental broodstock for QX disease resistance breeding (Nell *et al.*

2000). The Lime Kiln Bar (LKB) line is the main QX resistance breeding line produced since 1997. Successive generations of the LKB line have been exposed to QX disease at Lime Kiln Bar in the west of the Georges River where QX-disease outbreaks are most severe. Survivors of those outbreaks have been used as parents for subsequent generations of the LKB line

This mass selection strategy has significantly decreased mortality in QX-prone growing areas. Mortality among the 2nd generation of LKB oysters in the Georges River was reduced by 22% relative to non-selected wild type (WT) oysters (Nell & Hand 2003). By the 3rd generation, LKB oysters suffered only 22% mortality during their first year of growth in the Georges River, compared to 80% among WT controls (Nell & Perkins 2006). 5th generation hatchery bred LKB oysters are now available to the Sydney rock oyster industry, and are being grown in a number of QX-prone areas.

Similar mass selection programs to develop disease resistant oysters have also been successful in France and the USA. European flat oysters (*Ostrea edulis*) have been selected for resistance to Bonamiasis (caused by *Bonamia ostreae*) in France (Naciri-Graven *et al.* 1998) and American eastern oysters (*Crassostrea virginica*) have been bred for resistance to MSX disease caused by *Haplosporidium nelsoni* (Haskin & Ford 1979; Allen 1998).

To enhance their breeding program, NSW DPI is now assessing the performance of oyster families produced from single pairs of parents (single-pair families), as opposed to mass selected lines. In mass selection programs, the genetic contribution of individual parents is unknown, and inaccurate heritability estimates can result (Newkirk & Haley 1983; Toro & Newkirk 1990 Newkirk 1996). Single-pair families give far more reliable genetic information and provide the capacity to more rapidly select for desirable traits. The incorporation of single pair mating into breeding programs has been used successfully in other oyster species, including *O. edulis* (Baud *et al.* 1997), *C. virginica* (Ford and Haskin 1987) and *C. gigas* (Guo *et al.* 1996). Selective breeding based on pedigreed families is a cornerstone of the breeding program for *C. gigas* in Australia (Kube & Parkinson 2007).

The current study tests mortality in single-pair families derived from different combinations of LKB and WT parents. Our aim was to compare differences in mortality between families in a QX disease prone area.

2. Materials and Methods

2.1. Localities

Single-pair families were grown in two locations – Lime Kiln Bar in the Georges River, Sydney (33°59’41” S, 151°04’21” E), and Cromarty Bay, Port Stephens (32°72’38” S, 152°06’27” E). Lime Kiln Bar suffers recurrent severe QX disease outbreaks annually (Adlard & Ernst 1995). In contrast, the disease has never been reported at Cromarty Bay, although *M. sydneyi* has been detected at low levels in Port Stephens.

2.2. Single-pair matings

Two-year old 5th generation LKB oysters from the Georges River and 3-year old wild-type (WT) oysters from Port Stephens were used as parents for single-pair mating. WT oysters were collected from naturally-caught spat in Port Stephens. Three different types of crosses were performed using single-pairs of parents: LKB × LKB, LKB × WT, and WT × WT. Each cross was created by striping gametes from individual parents and performing the desired fertilisations in isolation from other oysters. Each cross was then cultured in individual 200L rearing containers to ensure cross contamination did not occur between larval batches.

Families from 31 single-pair matings were successfully generated in the oyster hatchery at the NSW DPI Port Stephens Fisheries Institute. They included 19 LKB × LKB families (family numbers 1 – 19), 8 LKB × WT families (family numbers 20 – 27), and 4 WT × WT families (family numbers 28 – 31). Approximately 800 spat were obtained for each family, except for families 11 and 20 which had approximately 200 spat per family.

2.3. Exposure of oysters to M. sydneyi

Two replicates of approximately 100 oysters each from 29 families, and two replicates of 50 and 20 oysters from families 11 and 20 respectively, were transplanted from the hatchery to Lime Kiln Bar in September 2007. The remaining oysters from each family were retained at Cromarty Bay. Oysters were housed in 1 mm mesh bags (30 x 65 cm) held in compartments of plastic trays measuring 0.9 x 1.8 m. Each tray had 8 compartments (2 bags per compartment) and the trays were covered with 8 mm plastic mesh. In March 2008, the oysters at Cromarty Bay were removed from the mesh bags due to mortality resulting from overcrowding, and the contents of each bag were placed into a single tray compartment. In the Georges River all families remained in the mesh bags for the duration of the study.

2.4 Mortality assessment

The oysters were retrieved in March, May/June, August and October/November, 2008. Mortality was calculated at each of these times based on the number of dead (empty and open shells) vs. live oysters (closed shells) in both replicate bags for each family in the Georges River, as well as in the single replicate of each family at Port Stephens.

2.5 Histological detection of M. sydneyi

In April 2008, when QX disease is usually reflected by the presence of sporulating *M. sydneyi* in the digestive diverticulae of oysters (Lester, 1986), 102 oysters from Port Stephens and 102 oysters from the Georges River were randomly selected from the 3 types of crosses. These included 2 oysters from each of the 19 LKB × LKB families (38 oysters), 4 oysters from each of the 8 LKB × WT families (32 oysters), and 8 oysters from each of the 4 WT × WT families (32 oysters). The presence of *M. sydneyi* in the digestive tract of these oysters was tested by the method of Kleeman *et al*. (2003). Briefly, imprints of the digestive glands were made on microscope slides and allowed to air dry before being fixed in methanol. The slides were then stained in modified Wright's stain (6% w/v Wright's stain, 0.6% w/v Giemsa stain in methanol; Kleeman *et al.* 2003) and allowed to air dry before being mounted with Ultramount No. 7 (Fronine, Riverstone, Australia). The presence of *M. sydneyi* was determined by inspecting tissue imprints with an Olympus BH-2 microscope.

2.6 Data analysis

Two-way ANOVA and Fisher's exact probability tests (2 x 3 contingency tables) were performed using the online statistical computation website, VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). Two-way ANOVA (factor 1, cross; factor 2 site) were used to determine the statistical significance of differences in cumulative mortalities between the three types of single-pair crosses (LKB × LKB, LKB × WT, and WT × WT). Fisher's exact probability test was used to determine the significance of differences between the numbers of oysters from each cross that exhibited *M. sydneyi* infections and the final cumulative mortality levels for each cross in the Georges River. For all of these tests, differences were considered to be significant when $P \leq 0.05$. All of the P values shown are two-tailed.

Hierarchical clustering analysis (with Euclidean distance measurement using an average clustering linkage algorithm; Expression Profiler tool, European Bioinformatics Institute,

http://www.ebi.ac.uk/) was performed to identify distinct clusters of mortality (low, intermediate and high mortality) among the families.

3. Results

3.1 Cumulative mortalities in the 3 types of crosses

The cumulative mortalities among progeny from the three different types of crosses (LKB × LKB, LKB × WT, and WT × WT) in the two locations are shown in Figure 1. In Port Stephens (Figure 1A), no significant difference in cumulative mortality was found between the crosses ($P > 0.05$), and cumulative mortality remained constant at approximately 17% over the sampling period. The relatively high mortality recorded at the first sampling point (March) was ascribed to overcrowding in oyster bags. The final (November) cumulative mortality at Port Stephens among the progeny of the LKB × LKB families was 16.7% ± 2.3%, compared to 13.8% ± 3% for the LKB × WT cross and 11.2% ± 2.5% for WT × WT families.

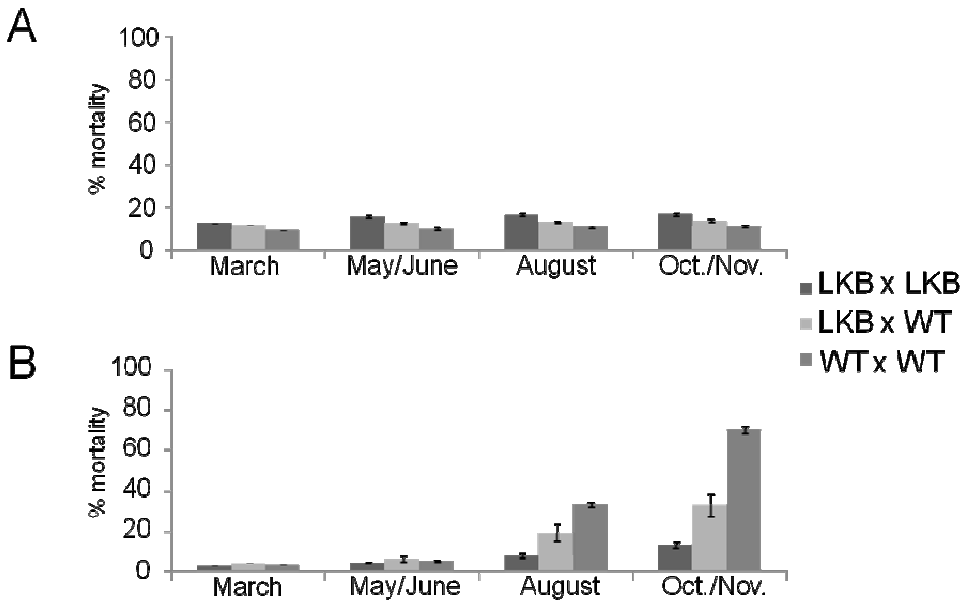

Figure 1. Mean cumulative mortalities (% ± SEM) among single pair families from 3 crosses (LKB x LKB, n = 19; LKB x WT, n = 8; and WT x WT, n = 4) in **(A)** Port Stephens and **(B)** Georges River between March and October/November, 2008.

Unlike Port Stephens, significant differences in cumulative mortality between the crosses were observed in the Georges River (Figure 1B). For all three crosses, mortalities remained relatively low (2.7 ± 0.8% to 4.0 ± 1.3%) until May. However, mean cumulative mortality increased significantly between August and November with significant differences found between LKB × LKB and WT × WT ($P < 0.01$) for both months. Mean mortality in the LKB × LKB families was significantly lower compared to the other 2 crosses in November ($P < 0.01$). The final (November) cumulative mortality for the WT × WT families was the highest at 70.1% ± 11.1 %, compared to 32.8% ± 5.1% for LKB × WT ($P \leq 0.01$) and 13.2% ± 2.0% for the LKB × LKB families ($P \leq 0.01$).

Significant differences in mean cumulative mortalities were observed between Port Stephens and the Georges River across the 4 sampling points. In the first two months (March and May/June), mortality was significantly ($P \leq 0.05$) higher in the LKB × LKB families held at Port Stephens compared those in the Georges River. Again, this was ascribed to overcrowding at Port Stephens.

Later, in August and October/November, mean cumulative mortalities in the LKB × WT and WT × WT crosses were significantly higher ($P \leq 0.05$) in the Georges River compared to Port Stephens.

3.2 Cumulative mortalities among individual single pair families

The final (November) cumulative mortalities for all 31 single pair families in the Georges River are shown in Figure 2. Mortalities among the 31 families ranged from 2.9% to 89.2%. The lowest mortality was in an LKB × LKB family (family 19) and the highest was in a WT × WT family (family 31). Individual mortalities were lowest in the LKB × LKB families, ranging from 2.9% to 34.5%, whilst mortality in the WT × WT families ranged from 39.6% to 89.2%.

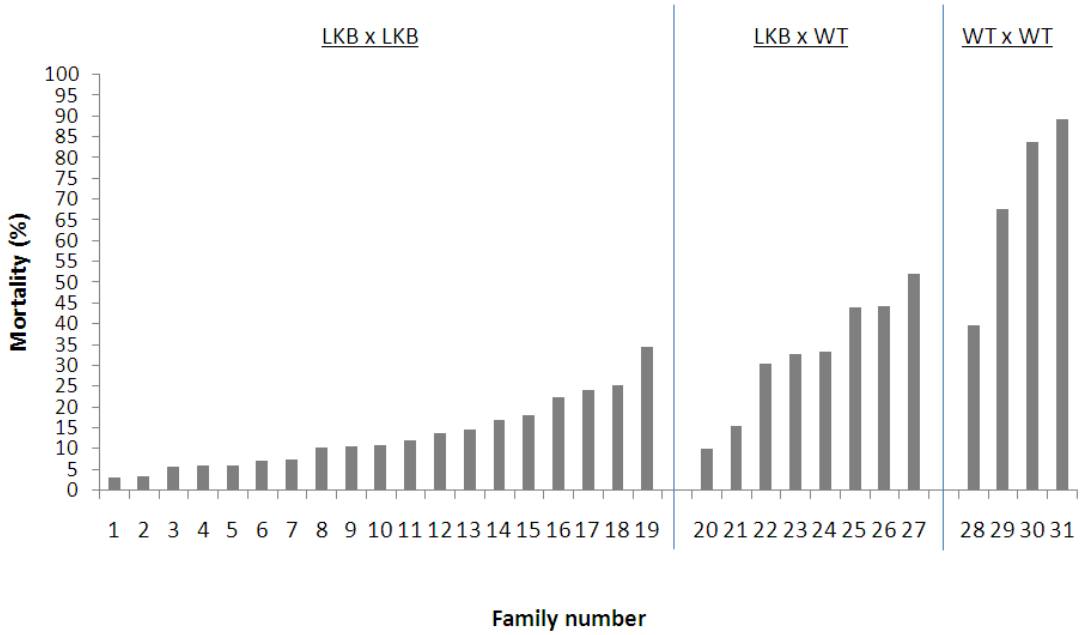

Figure 2. Final cumulative mortality (%) in November 2008 for each of the 31 families from the 3 types of crosses (LKB × LKB, LKB × WT, and WT × WT) in the Georges River. Data for each type of cross is ranked in order of ascending percentage cumulative mortality.

Cluster analysis showed that the 31 families were grouped into three discrete primary categories representing low, intermediate and high mortalities (Clusters 1, 2, and 3, respectively) (Figure 3a). The mean cumulative mortalities for the families in the three primary clusters were 12.1% ± 1.5% (n = 20), 38.9% ± 2.7% (n = 8) and 80.2% ± 6.5% (n = 3) for the low, intermediate and high mortality clusters, respectively. Generally, families from the same cross fell within the same category. Eighteen of the 19 LKB × LKB families (94.7%) were in the low mortality cluster, whilst 6 of the 8 LKB × WT families (75%) clustered in the intermediate mortality group. Three of the 4 WT × WT families (75%) were among the high mortality cluster. However, mortality in one of the LKB × LKB families (family 10) was higher than the other families with the same parentage, and fell into the intermediate mortality cluster, whilst families 24 and 25 had mortalities that were lower than other LKB × WT families, and were categorised in the low mortality cluster. Family 29 from the WT × WT cross had intermediate mortality, whilst the other three WT × WT families were in the high mortality group.

It was also clear that the low and intermediate mortality clusters were each comprised of two further strictly hierarchical secondary categories, designated clusters 1a and 1b; and 2a and 2b (Figure 3a). Secondary clusters 1a and 1b comprised 12 and 8 different families respectively. These secondary clusters included those families with both LKB × LKB and LKB × WT parentage. Cluster 2a comprised four LKB × LKB families, whilst cluster 2b contained four LKB

× WT families. The mean cumulative mortalities for each of the clusters (1a, 1b, 2a, 2b & 3) differed significantly from each other (Figure 3b; $P \leq 0.01$).

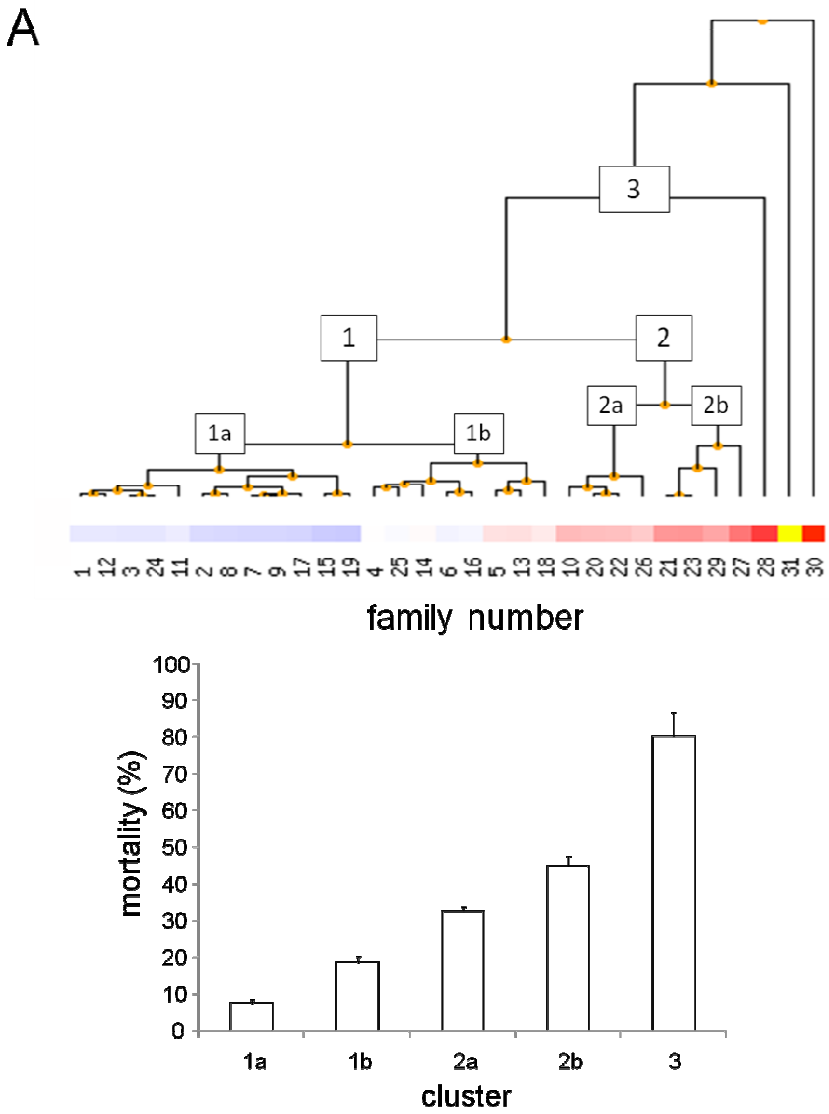

Figure 3. (A) Dendograms showing results of Euclidian hierarchical clustering for final cumulative mortality (%) in each of the 31 single pair families in the Georges River (families 1-19 = LKB × LKB; 20-27 = LKB × WT; 28-31 = WT × WT). The 31 families were grouped into five clusters – 1a, 1b, 2a, 2b, and 3 as denoted by boxes. **(B)** Mean cumulative mortalities for the five clusters shown in A.

3.3 M. sydneyi infection and mortality

Examples of the *M. sydneyi* sporonts identified in the digestive glands of infected oysters are shown in Figure 4. These correspond with the diagnostic images of Kleeman *et al.* (2003). A total of 102 oysters from the 3 crosses in Port Stephens were analysed for the presence of *M. sydneyi* sporonts in April 2008. No sporonts were detected in any of these oysters. However, in the Georges River, sporonts were detected in a substantial percentage of oysters from the LKB × WT and WT × WT crosses. Significantly more oysters from the W × W crosses contained sporonts (42.1%) than in the LKB × WT crosses (13.3%) ($P \leq 0.05$; Table 1). No sporonts could be detected in any of the 38 LKB × LKB oysters analysed. Only 26 of 32 LKB × WT oysters and 11 of 32 WT

× WT oysters collected from Georges River could be analysed because the remainder died before tissue samples could be collected.

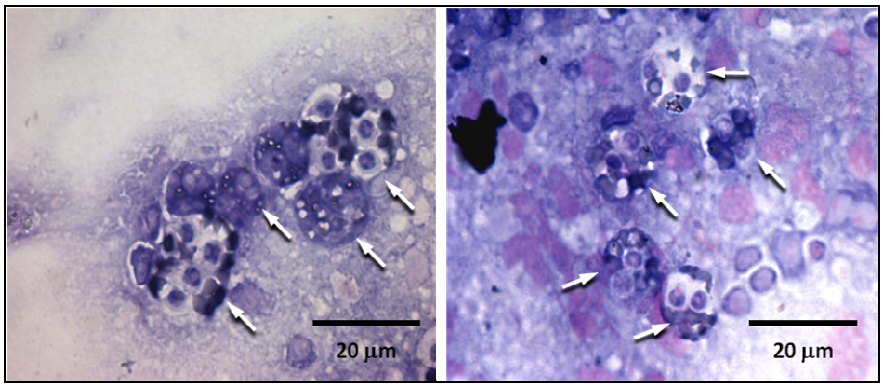

Figure 4. Stained digestive gland imprints of *M. sydneyi* infected oysters. Immature and mature *M. sydneyi* sporonts are denoted arrows.

Table 1. Percentage of oysters in the 3 different crosses from the Georges River in which *M. sydneyi* were detected in the digestive diverticulum in April, 2008.

cross	% infected	% mortality
LKB × LKB	0 (n = 36)	13.2
LKB × WT	13.3 (n = 26)	32.8
WT × WT	42.1 (n = 11)	70.1

Figure 5 shows the relationship between the final (October/November, 2008) mean cumulative mortalities for the 3 types of crosses and the frequency of oysters from these crosses in which *M. sydneyi* sporonts had been detected. Based on this data, a significant relationship was evident between the number of infected oysters in each cross and the number of oysters were infected with *M. sydneyi* infection (Table 1; Fisher exact test, $P \leq 0.05$).

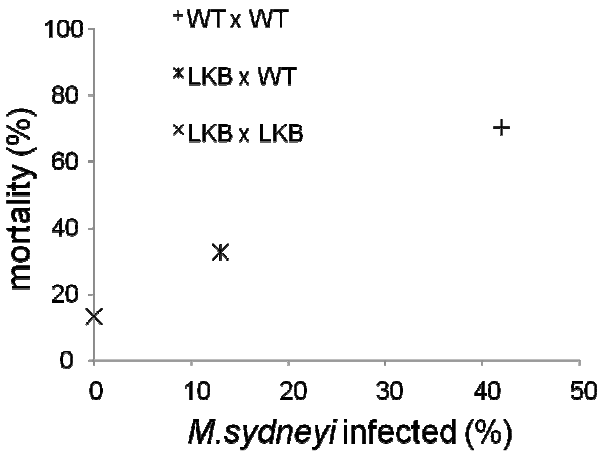

Figure 5. Percentage mean cumulative mortality in the three different crosses (LKB × LKB, LKB × WT, and WT × WT) versus percentage of *M. sydneyi*-infected oysters in each cross from the Georges River.

4. Discussion

This study demonstrates that QX disease-associated mortality in single-pair families of Sydney rock oysters is determined by parentage. There was a clear relationship between parentage and mortality among progeny grown in a QX-disease prone estuary. Significantly higher mortality was observed in both LKB × WT and WT × WT crosses compared to the LKB × LKB cross in the QX disease-prone Georges River. Mortality in the Georges River was far higher than at Port Stephens in the later stages of the study (August – November) when QX disease is usually at its height. The substantial differences in cumulative mortalities observed between oysters grown at Port Stephens and those in the Georges River between August and November suggest that the mortalities in Georges River were primarily due to QX disease. This is supported by the strong correlation evident between the presence of sporulating *M. sydneyi* infection in oysters from the Georges River (tested in April 2008) and subsequent mortality rates in that location between August and November 2008.

This is a typical pattern for QX disease. *M. sydneyi* infection is usually detected in middle to late summer and mortalities occur some months later (Nell & Smith 1988; Adlard & Ernst 1995). *M. sydneyi* is a widespread parasite of Sydney rock oysters. It has been detected throughout the cultivation range of Sydney rock oysters on the Australia east coast, and infection often occurs at low levels that do not result in QX disease outbreaks (Adlard & Wesche 2004). It is possible that QX disease occurs only when oysters cannot control *M. sydneyi* infections. Susceptibility to the disease may result from normal physiological changes exacerbated by environmental stress. In summer and early autumn, when *M. sydneyi* infection is at its highest, oysters are reproductively active. Spawning usually takes place in mid- to late- summer. During this period, at least 25% of metabolic energy is used for reproductive processes (Honkoop 2003), and the number of circulating haemocytes are at their lowest (Butt & Raftos 2007). These decreases in haemocyte frequencies may make oysters more susceptible to disease because haemocytes have a critical role in combating infection (Roch P. 1999; Arumugam *et al.* 2000; Pancer & Cooper 2006; Aladaileh *et al.* 2007; Butt & Raftos 2007). This underlying susceptibility is critically enhanced by external stressors, such as low salinity (Butt *et al.* 2006; Burge *et al.* 2007; Hégaret *et al.* 2007). We have found that low salinity levels, which can often occur in the Georges River during summer after heavy rainfall, inhibit the immunological activity of oysters and are strongly correlated with QX disease outbreaks (Butt *et al.* 2006). In years when salinity does not decrease substantially, QX outbreaks are not as severe (Butt *et al.* 2006). This fits with a growing realisation that environmental stress enhances disease susceptibility. In marine environments, a large number of external stress factors affect host-parasite relationships (Lafferty & Kuris 1999) and it is clear that opportunistic infections occur when hosts are immunosuppressed by such environmental factors (Lafferty 1997; Wang & Chen, 2005, 2006; Liu *et al.* 2006).

Euclidian distance analysis identified three distinct primary clusters of mortality (low, intermediate and high) among the single pair families. These clusters primarily contained families from a particular type of cross. Not surprisingly, families derived from WT × WT parents were in the high mortality cluster, most LKB × WT families fell within the intermediate mortality cluster and the majority of LKB × LKB families clustered in the low mortality group. This pattern is consistent with hybridisation between mass selected or outbred lines and does not differentiate between phenotypes derived from polyfactorial or monofactorial inheritance. However, the low and intermediate mortality clusters could be further sub-divided each into two statistically robust sub-groups, with each subgroup of a particular cluster containing approximately the same number of families. Given that these sub-groups were comprised primarily of families from different crosses (LKB×LKB and LKB x WT), the data suggest that there are some discrete genetic factors that contribute substantially to disease resistance.

It is already known that two genetic systems contribute substantially to QX disease resistance. We have shown that the defensive enzyme, phenoloxidase (PO), is strongly associated with QX

disease resistance (Peters & Raftos 2003; Bezemer *et al.* 2006; Butt *et al.* 2006; Butt & Raftos 2007; Butt & Raftos 2008). Five different PO isotypes have been identified in Sydney rock oysters. Among these, the PO^b form of the enzyme has decreased in frequency during mass selection for QX disease resistance in the LKB breeding line (Butt *et al.* 2006). The frequency of this form of PO also decreases during the course of QX disease outbreaks, suggesting that oyster bearing PO^b are more susceptible to QX disease than those carrying other forms of the enzyme. Butt & Raftos (2007) have also shown that PO activities in haemocytes are higher in LKB oysters than in wild-type oysters after challenge with *M. sydneyi*, and that haemocytes from LKB oysters have higher phagocytic activities than WT oysters.

More recent studies have identified other genes that are associated with QX disease resistance (Green *et al.* 2009; Nair *et al.* 2009). Green *et al.* (2009) used subtractive suppression hybridisation to identify two anti-oxidant genes, extracellular superoxide dismutase (ecSOD) and non-selenium glutathione peroxidise (Prx6) that are differentially expressed in LKB oysters compared to WT oysters. In a complementary proteomic analysis, Nair *et al.* (2009) identified six different proteins, including two homologues of the ecSOD found by Green *et al.* (2009) that are expressed at far higher levels in the LKB line than in WT oysters.

All of these data suggest that breeding for QX disease resistance in the LKB line has selected components of the phagolysosomal and oxidative stress response systems of oyster haemocytes, such as PO and ecSOD. Selection for these systems may provide LKB oysters with a greater capacity to control *M. sydneyi* infections via phagocytosis and oxidative intracellular killing.

Acknowledgements

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4.6 Expression of phenoloxidase variants is associated with mortality in families of Sydney rock oysters (*Saccostrea glomerata*) produced by single pair mating

Alison Kan[a], Daniel Butt[a], Michael C. Dove[b], John A. Nell[b], Wayne A. O'Connor[b] and David A. Raftos[a]*

[a] *Department of Biological Sciences, Macquarie University, North Ryde, NSW, 2109, Australia*
[b] *New South Wales Department of Primary Industries, Port Stephens Fisheries Centre, Taylors Beach, NSW, 2316, Australia*

Abstract

Variants of the defensive enzyme, phenoloxidase (PO), have previously been linked to disease susceptibility in Sydney rock oysters (*Saccostrea glomerata*). In this study, we compared the frequencies of the different forms of PO with mortality among oyster families produced by single-pair mating. Five different forms of PO were identified by native polyacrylamide gel electrophoresis in crosses between two generations of oysters bred for resistance to QX disease (LKB lines) and non-selected wild type (WT) oysters. The frequencies of PO variants differed significantly between LKB × LKB, LKB × WT and WT × WT crosses. One form of PO (PO^b) was positively correlated with the mortality among family lines, whilst another variant (PO^d) was negatively correlated with mortality. The data strengthen the association between the PO variants and mortality associated with QX disease, but suggest that other genetic factors also contribute to survival.

Keywords: pair mating, superoxide dismutase, phenoloxidase, *Saccostrea glomerata*

1. Introduction

QX disease afflicts Sydney rock oysters (*Saccostrea glomerata*) in a number of estuaries along the Eastern seaboard of Australia. The etiological agent of QX disease, the protozoan parasite *Marteilia sydneyi* (Perkins and Wolf, 1976), is thought to be endemic in many rock oyster growing areas (Adlard and Ogburn, 2003; R. Adlard, Queensland Museum, personal communication). Once established, epizootics can result in stock losses of over 95%. QX-resistant oysters have been bred by the New South Wales Department of Primary Industries (NSW DPI) since 1997 by interbreeding the survivors of QX disease outbreaks. Breeding has been based on mass selection, where gametes from large numbers of oysters that have survived QX-disease outbreaks are pooled to generate the subsequent generation. So far, the selective breeding program has decreased QX-associated mortality to less than 63% in fourth generation selected oysters from the Lime Kiln Bar (LKB) breeding line (Nell and Perkins, 2006).

Our previous studies indicate that QX disease outbreaks are restricted to areas where oysters have become immunosuppressed allowing opportunistic *M. sydneyi* infections to develop (Butt and Raftos, 2006; Butt et al., 2007). Other studies have shown that resistance to QX disease in Sydney rock oysters is correlated with the increased expression of extracellular superoxide dismutase (ecSOD) (Green et al., 2009) and the possession of particular variants (isotypes) of PO (Bezemer et al., 2006; Newton et al., 2004). These two enzymes play important roles in the immunological and oxidative stress responses of many invertebrates, including molluscs (Soderhall and Cerenius, 1998; Gonzalez et al., 2005; Aladaileh et al., 2007a,c).

SOD is a metalloenzyme involved in oxidative stress responses. It may also be involved in immunological reactions involving phagocytosis and oxidative, intracellular killing. SOD catalyzes the dismutation of superoxide to hydrogen peroxide (H_2O_2), which is involved in the defense against invading pathogens via respiratory bursts in phagocytes. H_2O_2 may also act as second

messenger for NF-κB (Schreck et al., 1991), a transcription factor involved in regulation of immune responses to infection. SODs are found across many vertebrate and invertebrate species. In bivalves, ecSOD has been isolated in the hemolyph and tissues of the mussel *Mytilus edulis* (Manduzio et al., 2003), the oyster *Crassostrea gigas* (Gonzalez et al., 2005), the Zhikong scallop, *Chlamys farreri* (Ni et al., 2007), and the Sydney rock oyster (Green et al, 2009). Green et al (2009) used subtractive expressed sequence tag (EST) and real-time PCR analyses to identify significantly higher levels of ecSOD expression in QX-disease resistance breeding lines of Sydney rock oysters, when compared to non-selected (WT) oysters.

PO is an oxidative enzyme involved in the formation of the pigment melanin (Soderhall and Cerenius, 1998). Both melanin and a number of its metabolic precurors have powerful anti-microbial activities. PO converts phenolic compounds to quinone, which spontaneously converts to toxic quinone intermediates and melanin. We have shown that low PO enzyme activity is strongly correlated with the severity of QX disease outbreaks (Peters and Raftos, 2003; Butt and Raftos, 2006), and that oysters selectively bred for QX-disease resistance have significantly higher PO activities than WT animals when challenged *in vitro* with *M. sydneyi*. Other studies have demonstrated that PO activity is associated with phagolysosomal activity and intracellular killing by *S. glomerata* hemocytes (Butt and Raftos, 2008; Kuchel al, in press). Five discrete phenotypic forms of PO have been identified in *S. glomerata* hemocytes (Bezemer et al., 2006) by native polyacrylamide gel electrophoresis (native-PAGE). One phenotypic variant of the enzyme, designated PO^b, has been negatively selected during mass selection for QX-disease resistance. PO^b is also lost from both mass selected and WT oyster populations during the course of QX disease epizootics. These data suggest that PO^b is directly associated with disease susceptibility (Bezemer et al., 2006).

In this study, we use single-pair mated crosses to further test the relationships between mortality, SOD and PO. A total of 35 oyster families were produced from single pairs of parents taken from both QX-disease resistant and WT populations. These families were assessed for mortality, SOD enzymatic activity and the expression of different PO variants.

2. Materials and Methods

2.1 Chemicals, Reagents, and Enzymes

All chemicals were obtained from Sigma-Aldrich (NSW, Australia) except for 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium salt (WST-1), which was from Dojindo Laboratories (Kumamoto, Japan), and nitroblue tetrazolium chloride (NBT), which was from AMRESCO Inc (Ohio, USA). The enzymes used were as follows: catalase from bovine liver (4.54 kU/mg; C1345), superoxide dismutase from bovine erythrocytes (30 kU; S2515), and xanthine oxidase from bovine milk, grade III (1 U/mg; X4500).

2.2 Single-pair crosses

Brood stock used for single pair crosses were chosen at random from mass selected QX-disease resistant lines and WT (non-selected) populations. QX-disease resistant oysters were from the LKB breeding line, which has been exposed to successive QX disease outbreaks at Lime Kiln Bar, in the upper reaches of the Georges River, Sydney (33°59'S, 151°10'E), since 1997. WT oysters were from the Merimbula River, NSW, Australia. This site has never experienced an outbreak of QX-disease.

Two rounds of single pair mating were undertaken. In 2004, LKB × LKB crosses were performed using parents from the fourth generation of the LKB line (LKB_4). In 2006, LKB × LKB, LKB × WT and WT × WT crosses were established using sixth generation LKB parents (LKB_5) and WT oysters. Strip-spawning, fertilization, spat selection and rearing were performed as described by

Kan et al (2009 submitted). 17 fertilizations between separate pairs of parents were carried in the 2004 mating trial, and 60 were performed in 2006. Hemolymph from the parents used in the 2006 mating trail was collected for PO phenotyping (see below). The resulting families were kept separate from each other for the remainder of the experiment.

In the 2004 mating trial, spat had reached an average size of 25-30 mm (antero-posterior measurement) 11 months after fertilization (November 2005). However, problems associated with viability of larvae due to inefficient larval diets (Nell et al., 1996) and the difficulty of maintaining relatively small numbers of spat in the hatchery meant that spat from only 5 of the 17 original families survived (approximately 200 oysters per family). For the 2006 mating trial, oysters had grown to 30 – 50 mm by December 2007. The 2006 trial produced 19 $LKB_5 \times LKB_5$, 7 $LKB_5 \times$ WT and 4 WT × WT families, each comprising approximately 600 oysters. The families were grown out in Port Stephens, NSW, which has never suffered a QX-disease outbreak.

2.3 Assessment of mortality

To assess mortality in a QX disease prone estuary, subsets of each family were transferred from Port Stephens to Neverfail Bay (2004 mating trial) or Lime Kiln Bar (2006 mating trial) in the Georges River, Sydney, in November 2005 and November 2007. Both Neverfail Bay and Lime Kiln Bar have been the sites of severe QX disease outbreaks since the discovery of the disease on the Georges River in 1994 (Nell and Perkins, 2006). Two replicates of 100-200 oysters each were held separately in mesh bags placed on 800 x 1500 mm oyster trays. Racks were set at a depth of approximately 500 mm above the low tide mark. Oysters were checked every two to three months for one year so that the number of live and dead oysters could be recorded for each of the families. Cumulative mortalities were calculated for each family over the course of the year. Histopathological examination confirmed that oysters in the Georges River were subject to severe QX disease infections during early to mid 2006 and 2008. *M. sydneyi* were detected in digestive gland tissue imprints from 20 LKB_4 oysters tested in July, 2006, and in both the $LKB_5 \times$ WT and WT × WT families from the 2006 trial (data not shown).

2.4 Hemolymph collection

Twenty to 50 oysters from each family were collected from Port Stephens and held in either a recirculating seawater aquarium at Macquarie University or in a flow-through seawater system at the Sydney Institute of Marine Science, Chowder Bay, Sydney. The oysters were allowed to acclimatize to the aquaria for 7 to 14 days before hemolymph was extracted for PO phenotyping and SOD enzymatic assays. Oysters were shucked and between 1.5 – 2.0 mL whole hemolymph was collected per oyster. For PO phenotyping, hemocytes from an aliquot of each sample were pelleted by centrifugation at 4°C for 10 s at 13 000 x g and the supernatant discarded. The hemocyte pellets were the resuspended in 40 µL of lysis buffer (4% CHAPS in 50 mM Tris-HCl, pH 7.4). Hemolymph aliquots for SOD assays and total protein quantitation were mixed with 4 µL of 25% NP-40. Samples were stored at -80°C.

2.5 Protein quantitation

Five µL of whole hemolymph in 1% NP-40 (v/v) was diluted 10-fold in 50 mM potassium phosphate buffer (pH 8.0). Total protein quantitation was performed using a 250 µL microplate protocol according to manufacturer's instructions (Quick Start™ Bradford Protein Assay, Biorad, CA, USA). The total protein content of each sample was interpolated from standard curves generated with bovine serum albumin. Triplicate readings at 595 nm were performed for each standard, control (blank), and sample.

2.6 SOD enzymatic assay

SOD enzymatic activity was measured using a Superoxide Dismutase Assay Kit, (Cell Technology Inc, California, USA). One hundred µL of whole hemolymph in 1% NP-40 (v/v) was thawed on ice and immediately centrifuged at 4°C for 5 min at 16 000 x g. The supernatant was assayed for SOD activity according to the method of Peskin and Winterbourn (2000), with the exception that EDTA was used instead of diethylenetriamine-pentaacetic acid. Twenty µL triplicates of hemolymph samples (diluted 20-fold in 50 mM potassium phosphate, pH 8.0), standards (SOD from bovine erythrocytes), and controls (xanthine oxidase buffer, 50 mM potassium phosphate, pH 8.0; or Milli-Q water) were added to the wells of 96-well microtitre plates. Twenty µL of xanthine oxidase solution (4.5 mU/mL) and 200 µL of assay buffer (0.1 mM EDTA, 0.1 mM hypoxanthine, 0.386 mg/mL catalase, 10 mM WST-1 in 50 mM sodium phosphate buffer, pH 8.0) were then added and the plates were incubated at 37°C for 20 min before SOD activity was measured at 450 nm on a microplate spectrophotometer. SOD standards were prepared in accordance to the manufacturer's instructions. The concentrations of SOD standards ranged from 0.001 to 200 U/mL.

2.7 PO phenotyping

For PO phenotyping, hemocyte pellets in lysis buffer vortexed (20–30s; room temp.) to disperse the pellets. The samples were then centrifuged at 4°C for 5 min at 16 000 x g. Forty µL of the resulting supernatants were mixed with 10 µL sample buffer (0.5 M Tris-HCl; 10% v/v glycerol; 0.1% w/v bromophenol blue, pH 6.8) and then loaded onto 8% NUView® LongLife native PAGE gels (NuSep, NSW, Australia). Two µL of native-PAGE markers (High Molecular Weight Electrophoresis Calibration Kit, Amersham Biosciences, NJ, USA) were run on each gel. The gels were electrophoresed at 120 V for 3 h at 4°C in a Mini-PROTEAN TetraCell system (Bio-rad Laboratories, NSW, Australia) using Tris-glycine running buffer (pH 8.3). Tyrosinase (Sigma-Aldrich, Castle Hill, NSW) was used as a positive control. Negative controls included wells that did not contain hemolymph lysates and wells that were not incubated with PO substrates. After electrophoresis, the marker lane was cut from each gel and stained separately with blue silver according to the method of Candiano *et al.*, 2004. The remaining portions of the gels were stained for PO with 20 mM hydroquinone monomethyl ether in PBS containing 5 mM 3-methyl-2-benzothiazoline hydrazone (in 70% ethanol) for up to 1 h. Gels were then imaged (Epson Stylus Photo RX630) at 300 dpi. The PO phenotypes of oysters were determined by the criteria of Bezemer et al. (2006) with the phenotypic variant PO^a having the slowest electrophoretic mobility and PO^e being the fastest. Phenotyping was based on the retention factor (Rf) values (Gallagher, 1995) for PO bands relative to the eletrophoretic migration of the native-PAGE markers.

2.8 Data analysis

Data were analyzed using either SPSS v.15 (SPSS Inc., 2006, Chicago), Primer v. 5 (Primer-E, 2001, Plymouth), or the online statistical computational website, VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). ANOVA with Tukey's HSD post hoc test was used to determine the significance of differences between SOD enzymatic activities in the 3 types of crosses (LKB × LKB, LKB × WT and WT × WT). Fisher's exact test was used to test differences in the proportions of different PO variants between crosses. Linear regression analyses were performed to assess the correlation between mortality and phenoloxidase phenotypes. Differences were considered significant when $P < 0.05$. All P values were two-tailed.

3. Results

3.1 Oyster mortalities

Cumulative mortalities for the 2006 trial have been described in detail elsewhere (Kan et al. 2009 submitted). Briefly, they ranged from 2.9% in an $LKB_5 \times LKB_5$ family to 83.9% in one of the WT × WT families. Mean mortalities were highest in families from the WT × WT crosses (35.8% ± 4.7 %), compared to 16.4% ± 2.5% for $LKB_5 \times$ WT ($P \leq 0.05$) and 6.71% ± 1.2% for the $LKB_5 \times LKB_5$ families ($P \leq 0.05$). For the 2004 trial, mortalities among the $LKB_4 \times LKB_4$ families ranged from 5.0% to 57.6%, with a mean of 24.8% ± 10.0% (data not shown).

3.2 Frequencies of PO phenotypes

Five different electrophoretic forms of PO were identified by native-PAGE (Figure 1A). These were designated $PO^{a\text{-}e}$. In most families, individual oysters expressed just one of these 5 types, but two or more of the variants were identified in most of the families. For instance, all five types of PO were evident in family 8 (Table 1).

The frequencies of the PO variants differed significantly between the different types of cross (Figure 1B). PO^a, PO^b and PO^c were detected in all 4 types of cross ($LKB_4 \times LKB_4$, $LKB_5 \times LKB_5$, $LKB_5 \times$ WT and WT × WT), but PO^b was significantly ($P \leq 0.05$) more common in the $LKB_5 \times$ WT and WT × WT crosses than in $LKB_4 \times LKB_4$, $LKB_5 \times LKB_5$ families. Conversely, PO^d was more frequent in $LKB_5 \times LKB_5$ oysters than in either the $LKB_5 \times$ WT or WT × WT families ($P \leq 0.05$). PO^c was the most common variant in $LKB_5 \times LKB_5$ families (47.5%), followed by PO^d (39.0%) and PO^b (38.0%). The frequencies of the different PO forms in the WT × WT cross were similar to those in the $LKB_5 \times$ WT cross with the exception of PO^a, which was more frequent in $LKB_5 \times$ WT oysters and in the WT × WT cross. The least common form in all families was PO^e.

Among the individual families, the frequency of PO^b ranged from zero (in 3 of the LKB × LKB families) to 78.6% in an $LKB_5 \times$ WT family (Figure 1C). The frequency of PO^d ranged from zero (in 11 different families from all 4 types of cross) to 68.8% in one of the $LKB_5 \times LKB_5$ families.

Table 1. PO phenotypes of parents and progeny from the 30 different single pair families in the 2006 trial (families 1-19, $LKB_5 \times LKB_5$; 20-26, $LKB_5 \times$ WT; 27-30 WT $\times$ WT. The numbers shown under "progeny" are the number of oysters tested in each line that expressed that PO variant.

	Parental phenotype		Phenotypes of progeny				
family #	male	female	a	b	c	d	e
1			0	1	2	5	0
2			1	0	3	11	1
3			0	3	6	0	7
4	a	a	10	6	11	12	0
5	b + d		2	5	9	1	0
6			3	11	2	8	0
7	a		0	5	9	3	0
8	a	a	10	6	11	17	2
9			1	6	10	17	6
10			1	2	5	2	0
11	b	a	0	2	7	2	0
12			0	4	0	6	0
13	b	b	6	20	12	4	3
14	b	b	0	6	5	2	0
15	a		0	3	6	5	0
16		b + c	2	8	14	5	0
17		b	5	5	10	15	1
18	b	b	3	21	18	0	0
19			0	2	5	4	1
20			1	11	2	0	0
21			0	8	2	2	0
22		a	10	6	1	0	0
23		d	14	1	0	0	0
24			3	16	4	3	1
25		b	0	11	7	3	0
26		a	3	6	2	4	0
27			2	4	5	4	0
28	a	a	11	11	21	2	0
29	a		4	6	5	3	1
30	a		1	5	1	0	3

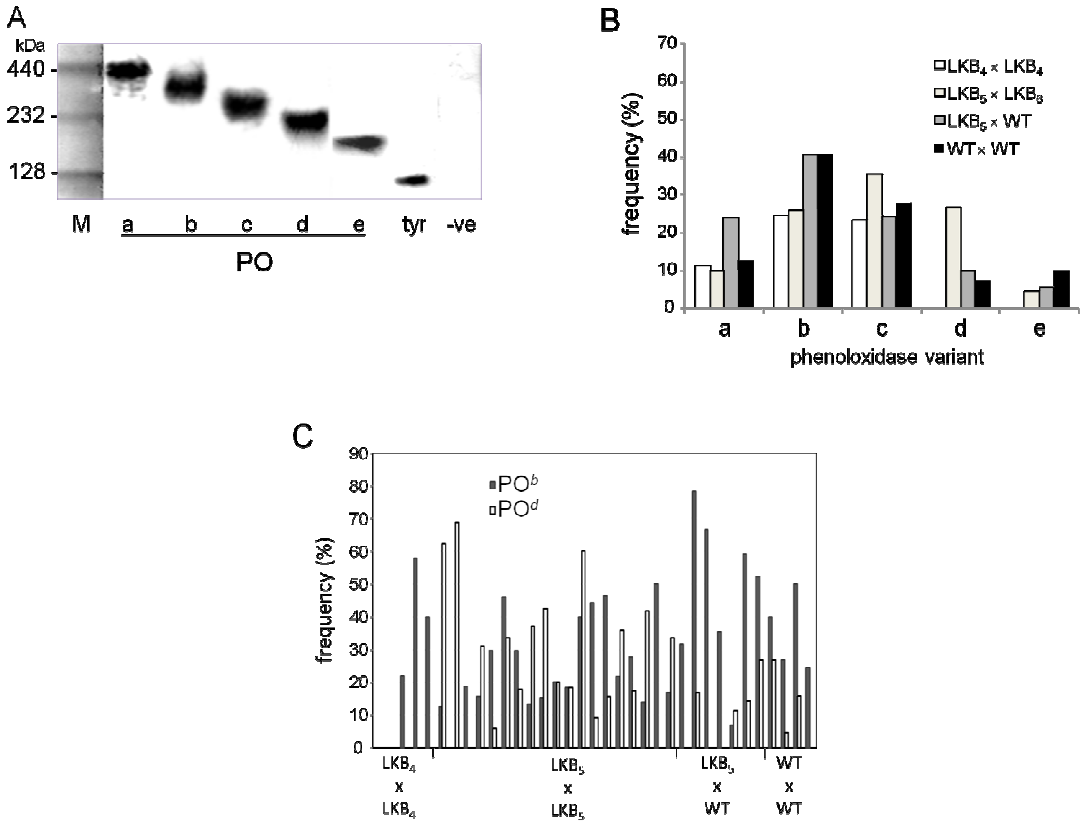


Figure 1. PO variants in single pair family lines. **A.** Native-PAGE electrophoretic mobilities of the 5 different phenotypes (PO^{a-e}) identified in single pair families. The position of native-PAGE markers is shown on the left, and controls (insect tyrosinase, tyr; no PO sustrate, -ve) on the right. **B.** Frequencies (%) of the 5 different PO variants in single pair mating families from the four different crosses ($LKB_4 \times LKB_4$; $LKB_5 \times LKB_5$; $LKB_5 \times WT$; and $WT \times WT$). **C.** Frequencies of PO^b and PO^d in individual single pair mating families for the four crosses.

3.3 PO phenotypes of parents compared to their progeny

PO phenotypes were determined for 24 of the parents used to generate 17 of the families produced in the 2006 trial. Both parents for families 4, 8, 9, 11, 13, 14, 18 and 28 were successfully phenotyped (Table 1). The relationships between these parental PO phenotypes and those of their offspring did not fit the expectations of a simple model for Mendelian inheritance in which each of the PO variants (PO^{a-e}) represents a different allele at a single PO gene locus. The minimum expectations of this model are that "alleles" appearing in the parents should be detected in their progeny, and that only "alleles" appearing in the parents should be found in their progeny groups. There were numerous exceptions to these minimum expectations. For instance, the male parent in family 7 had the PO^a phenotype but this variant was not detected in any of its 17 progeny tested. In family 8, both parents expressed the PO^a phenotype, but their progeny group contained all 5 PO variants (PO^{a-e}). The same pattern was evident in family 13, in which both parents had the PO^b phenotype.

Even though the PO phenotypes for parents and offspring did not fit this simple Mendelian model, there were recurring patterns in the data. In families where both parents had the PO^b phenotype (families 13, 14 and 18), PO^b was the most common (47% ± 2%) phenotype among their progeny, and the relative frequencies of each form of PO among the progeny did not differ significantly between the 3 families ($\chi_8^2 = 12.8$, df = 8, p = 0.12). By contrast, in the three families where both parents were PO^a, the majority (81% ± 3%) of their progeny had the PO^a, PO^c or PO^d phenotypes,

and the relative frequencies of each form of PO among the progeny did not differ significantly between the 3 families ($\chi_8^2 = 20.8$, df = 8, p = 0.28).

3.4 Relationships between PO phenotypes and mortality

Linear regression analysis was used to test the significance of correlations between mortality and the frequencies of the 5 different PO variants in all families. Frequencies of two of the variants, PO^b and PO^d, were significantly ($p<0.05$) correlated with mortality (Figure 2). PO^b was positively correlated with mortality, whilst there was a negative correlation between mortality and the frequency of PO^d. When all 35 families were included in the analysis, the relationship between the frequency of PO^b and mortality had a correlation coefficient (r^2) of 0.18 and a slope of 0.43(p=0.009). This correlation was stronger ($r^2 = 0.38$, slope = 0.82, p = 0.001) when only the 24 LKB × LKB (19 $LKB_5 \times LKB_5$ and 5 $LKB_4 \times LKB_4$) families were regressed against cumulative mortality. The relationship between mortality and the frequency of PO^d had a correlation coefficient (r^2) of 0.27 and slope of -0.51(p = 0.003) when all families were included in the analysis, and an r^2 of 0.26 and slope of -0.21(p = 0.026) if only LKB × LKB families were included.

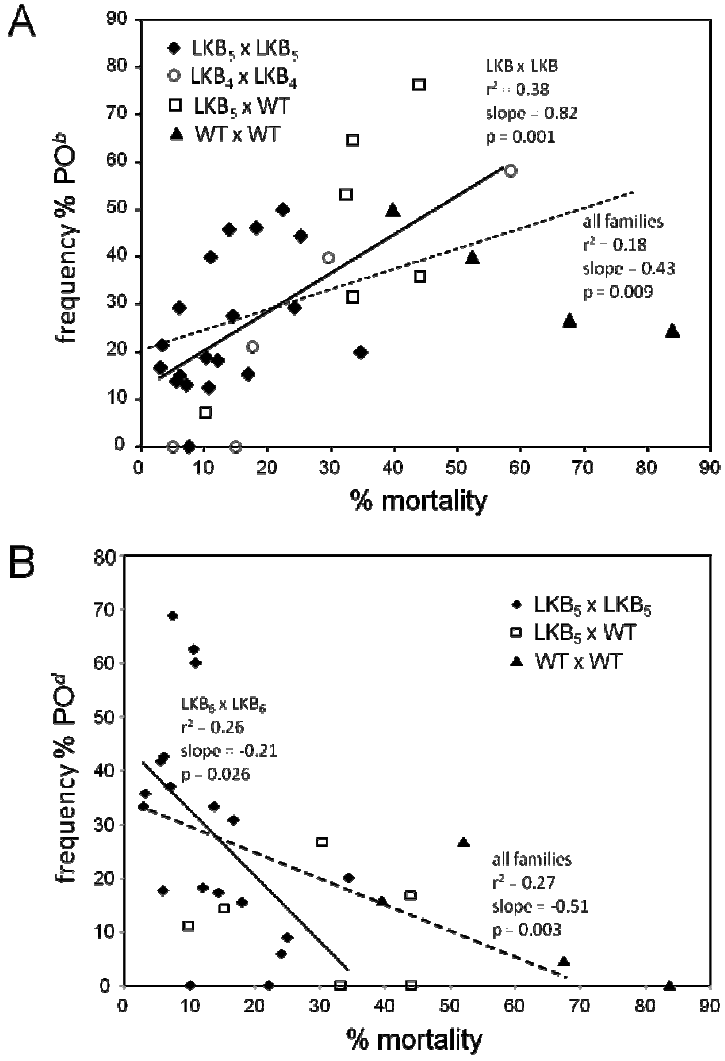


Figure 2. Associations between mortality and the frequencies of two different PO variants (A. PO^b; B. PO^d) in single pair families. Each point is data for one of the 35 single pair family lines (5 $LKB_4 \times LKB_4$; 19 $LKB_5 \times LKB_5$; 7 $LKB_5 \times WT$; and 4 WT ×WT). Two linear regressions (all families and LKB × LKB) are shown in each panel.

3.5 SOD activity

Mean SOD activity was significantly ($p<0.05$) higher in the $LKB_5 \times WT$ cross (8.0 ± 0.1) than in both the $LKB_5 \times LKB_5$ (6.1 ± 0.1) and the WT × WT (6.4 ± 0.3) crosses (Figure 4A; SOD activity was not measured in $LKB_4 \times LKB_4$ families). There was no significant difference in the mean SOD activities of the $LKB_5 \times LKB_5$ and WT × WT crosses ($p>0.05$). SOD activities among the 30 families tested (19 $LKB_5 \times LKB_5$, 7 $LKB_5 \times WT$ and 4 WT × WT) ranged from 2.8 ± 0.5 in one of the $LKB_5 \times LKB_5$ families to 10.7 ± 1.3 in an $LKB_5 \times WT$ family (Figure 4B).

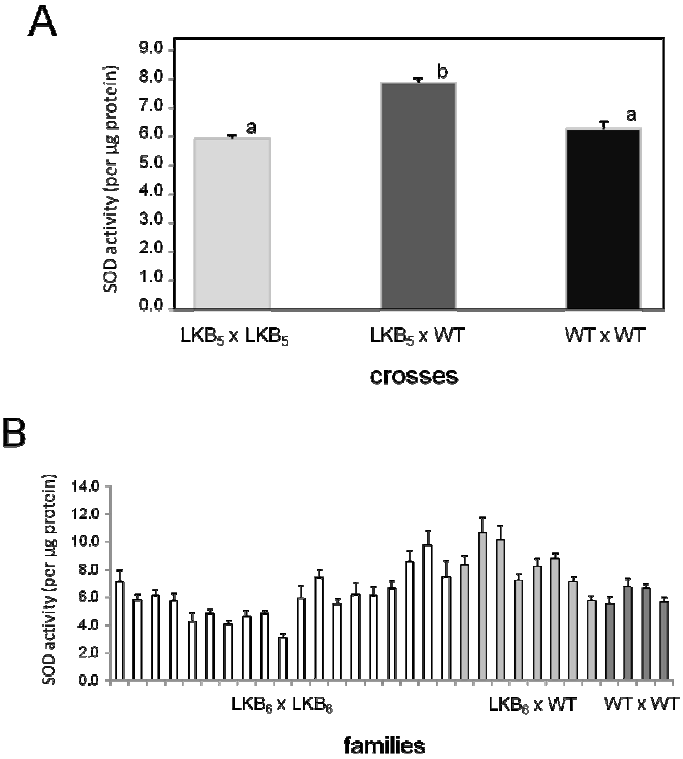


Figure 3. SOD enzymatic activities in single pair families. A. Mean SOD activities for each type of cross ($LKB_5 \times LKB_5$; $LKB_5 \times WT$; and WT × WT) in the 2006 single pair mating trial. Like letters indicate no significant ($p<0.05$) difference. B. SOD activities for each of the single pair families in each cross.

There was no significant correlation when the SOD activities of all 30 families were regressed against cumulative mortality ($r^2 = 0.0027$; $p = 0.78$). However, a weak (not significant, $p = 0.094$) relationship, with a correlation coefficient (r^2) of 0.46, was apparent when the SOD activities of just the 7 $LKB_5 \times WT$ families were regressed against their mortality data. There were no significant relationships between the SOD activities of the families and their frequencies of either PO^b or PO^d (data not shown).

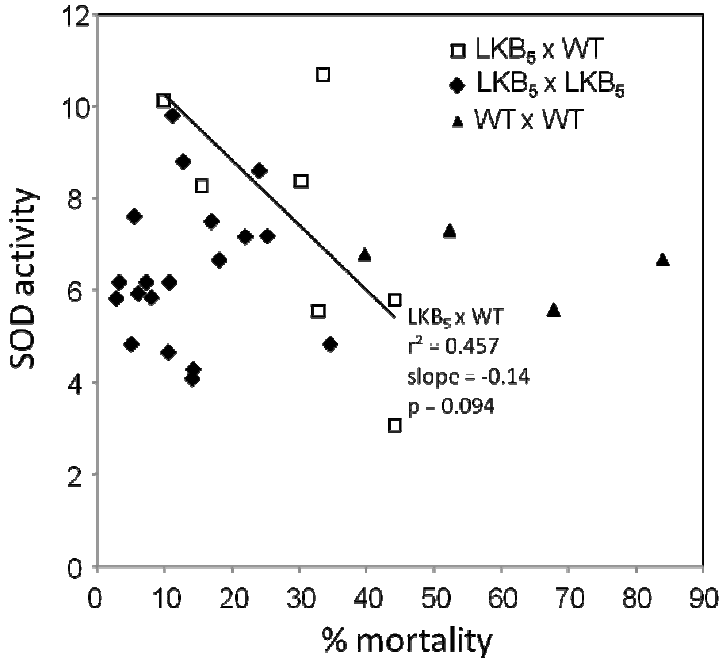


Figure 4. Association between mortality and the SOD activities of single pair families. Each point is data for one of the 30 single pair family lines tested (19 $LKB_5 \times LKB_5$; 7 $LKB_5 \times WT$; and 4 $WT \times WT$). The linear regression shown is for the 7 $LKB_5 \times WT$ families.

4. Discussion

This study provides further support for the association between PO and mortality in Sydney rock oysters. Differences in the frequencies of PO phenotypes among single pair families were correlated with differences in mortality. In the 2004 single pair mating trial, the two $LKB_4 \times LKB_4$ families that suffered the highest mortalities in the Georges River, Sydney, had significantly greater frequencies of the PO^b variant than the other three families. Families from the 3 different types of crosses performed in 2006 ($LKB_5 \times LKB_5$, $LKB_5 \times WT$ and $WT \times WT$) also had significantly different frequencies of the different forms of PO, and the frequency of PO^b in these families was positively correlated with mortality.

These data agree with our previous analyses of PO in mass selected breeding lines. Bezemer et al (2006) reported that PO^b is significantly less frequent in mass selected LKB_4 oysters compared to wild-types. The frequency of PO^b also decreased in mass selected LKB_4 and WT oyster populations over the course of a QX disease outbreak, and PO^b was less frequent in wild caught oysters collected from a QX-disease prone estuary. Bezemer et al (2006) concluded from these results that exposure to QX disease negatively selects PO^b, and by corollary that possession of PO^b is associated with susceptibility to QX disease. They also showed that there were compensatory increases in the frequency of two other PO variants (PO^c and PO^d) in populations that had been mass selected for QX disease resistance.

A similar pattern was evident in the current study. PO^d was significantly more frequent in $LKB_5 \times LKB_5$ oysters when compared to the $LKB_5 \times WT$ and $WT \times WT$ families. More importantly, there was a significant negative correlation between the frequency of PO^d in individual families and their cumulative mortalities. This suggests that possession of PO^d is associated with enhanced survival.

The implication in the current study of single pair families is that the mortalities evident when families were grown in the Georges River were due primarily to QX disease, so that the positive and negative correlations between mortality and the frequencies of PO^b and PO^d represent QX-

disease susceptibility and resistance, respectively. This implication is supported by our previous analysis of mortality among the same single pair families (Kan et al, 2009, submitted), which identified the etiological agent of QX disease (*M. sydneyi*) in the $LKB_5 \times WT$ and $WT \times WT$ families but not in $LKB_5 \times LKB_5$ oysters. Significant levels of *M. sydneyi* infection were also evident in oysters co-cultured with the $LKB_4 \times LKB_4$ families during the 2004 single pair mating trial. Moreover, the sites where the families were held in the Georges River (Neverfail Bay and Lime Kiln Bar) are known to suffer severe annual QX outbreaks (Nell and Perkins, 2006), and so most of mortalities observed among the families were probably due to QX disease resulting from *M. sydneyi* infection (R. Adlard, Queensland Museum, personal communication). However, establishing a definitive post-mortem association between mortality and *M. sydneyi* infection in the families was not possible due to the rapid degeneration of oyster tissues after death.

Even though the mortalities identified in this study cannot be definitively ascribed to QX disease, there is a strong physiological foundation for a link between the PO system of oysters and their resistance or susceptibility to QX disease. In Sydney rock oysters, PO is localized in the membrane bound vesicles of granular hemocytes (Aladaileh et al, 2007b). These PO-positive granular hemocytes are responsible for the rapid phagocytosis of *M. sydneyi* sporonts (Butt and Raftos, 2008). The membrane bound vesicles containing PO then fuse with phagosomes containing *M. sydneyi* (Kuchel et al., in press), and the phagsomes containing parasites undergo complete PO-mediated melanization (Butt and Raftos, 2008). This suggests that PO is an important component of defense against *M. sydneyi* infection and that changes in the PO system will affect QX-disease resistance.

The control of PO expression and subsequent melanization is also an important disease resistance factor in other species. Resistance to microfilariae in mosquitoes (Li et al., 1994; Shiao et al., 2001) and defense against parasitoid wasps in *Drosophila* (Nappi et al., 2000) both rely upon the killing ability of cytotoxic molecules produced by the PO cascade. Genetic analysis of resistance in both of these host-parasite relationships has found that discrete genes influence pathogen susceptibility and that resistance can be pathogen specific (Carton et al., 2005). These and other studies of anti-parasite defense in invertebrates have shown that resistance is generally determined by one major gene locus with high penetrance and at least partial dominance (Carton et al., 2005).

Our data also suggest that PO is a major genetic factor associated with QX disease resistance. However, we still do not understand the genetics of this system. Bezemer et al. (2006) concluded that the different PO variants detected as discrete bands by native-PAGE represented electrophoretically distinct alleles of a single PO gene locus. However, the data presented in this study do not support this conclusion. Bezemer et al (2006) suggested that oysters expressing two different PO bands on native-PAGE gels were heterozygous at a single PO locus. But very few of these individuals simultaneously expressing two different forms of PO were detected in the progeny of the 2006 single pair matings so that their frequencies within individual families were not consistent with the ratios typical of simple Mendelian inheritance of a single locus.

It was also clear that the relationships between the PO phenotypes of the parents used for single pair mating and their offspring did not fit the simple Mendelian model proposed by Bezemer et al (2006). The current data suggest that the genetic control of PO phenotypes is more complex than previously thought. It seems that the PO variants detected by native-PAGE are not simple alleles, but might represent different proteolytic forms produced during the activation of PO from its inactive state (proPO), or they may be different oligomerisation states of the enzyme.

Even though these explanations do not fit a simple Mendelian model, there were distinct patterns that emerged when comparing parental PO phenotypes with those of their progeny groups. Most importantly, it appeared that mating two PO^b parents produced a preponderance of PO^b phenotypes among their offspring, whilst two PO^a parents generated mostly PO^a, PO^c or PO^d progeny. This has important ramifications for selective breeding. Given the positive correlation between PO^b and

mortality (discussed above), breeding programs could target parents with the PO^a phenotype, and exclude those with PO^b, in an effort to minimize the penetration of PO^b into subsequent generations.

However, it is also clear that PO alone cannot explain all of the mortalities reported in this study. Correlation co-efficients for the relationships of mortality with the frequencies of PO^b and PO^d were 0.18 and 0.27 respectively, even though the r^2 value for the relationship between mortality and PO^b rose to 0.38 when only LKB × LKB families were considered. This suggests that other factors contribute substantially to mortality/survival. We expected this result because there is already evidence for the contribution of other factors. Butt and Raftos (2008) reported that, in addition to their altered PO systems, LKB oysters also have larger hemocytes that are more actively phagocytic than those of WT oysters. At a molecular level, both Nair et al. (2009, in press) and Green et al (2009) have identified a number of genes and proteins that differ significantly between the mass selected LKB and WT populations. In particular, these molecular analyses found that SOD transcripts and proteins were more abundant in the mass selected LKB line than in WT oysters.

This led us to measure SOD enzymatic activity in the single pair families from the 2006 trial. However, no clear correlation was apparent between the SOD activities of these families and mortality, apart from a weak (not significant) association in LKB_5 × WT crosses. We suspect that this lack of correlation with SOD activity may be due to the diversity SOD-like proteins expressed in oysters. Oysters have multiple versions of this enzyme that are distinct in terms of both sequence and two-dimensional electrophoretic mobility (Nair et al., 2009). The enzymatic assay that we used may not have differentiated between these different forms of SOD and so may not have been sufficiently sensitive to distinguish between the SODs that are differentially expressed in the LKB and WT lines.

Overall, this study confirms the relationship between PO and mortality in Sydney rock oysters, with the implication that PO may protect oysters from the effects of QX-disease. Our next step will be to characterize the PO variants in an effort to understand their genetic origins.

4.7 Proteomic analysis of QX-disease resistance markers in Sydney rock oysters (*Saccostrea glomerata*)

Sham V. Nair [a]*, Margaret Simonian [a], John A. Nell [b], David A. Raftos [a]

[a] *Department of Biological Sciences, Macquarie University, North Ryde NSW, 2109, Australia*
[b] *New South Wales Department of Primary Industries, Port Stephens Fisheries Centre, Taylors Beach, NSW, 2316, Australia.*

J. Proteomics, In press.

Abbreviations:
2DE, two dimensional electrophoresis; DPI, Department of Primary Industries; NSW, New South Wales; QXR_5, fifth generation oysters bred for QX disease resistance; QXR_6, sixth generation oysters bred for QX disease resistance; SOD, superoxide dismutase; WB, fifth generation multi-disease resistant oysters from Woolooware Bay.

Abstract

The Sydney rock oyster, *Saccostrea glomerata*, is susceptible to infection by the protozoan parasite, *Marteilia sydneyi*, causative agent of QX disease. *M. sydneyi* infection peaks during summer when QX disease can cause up to 95% mortality. The current study takes a proteomic approach using 2-dimensional electrophoresis and mass spectrometry to identify markers of QX disease resistance among Sydney rock oysters. Proteome maps were developed for QX disease -resistant and -susceptible oysters. Six proteins in those maps were clearly associated with resistance and so were characterized by mass spectrometry. Two of the proteins (p9 & p11) were homologous to superoxide dismutase-like molecules from the Pacific oyster, *Crassostrea gigas*, and the Eastern oyster, *Crassostrea virginica*. The remaining S. *glomerata* proteins had no obvious similarities to known molecules in sequence databases. p9 & p11 are currently being investigated as potential markers for the selective breeding of QX-disease resistant oysters.

***Keywords**:* QX disease; *Marteilia sydneyi*; proteomics; *Saccostrea glomerata*; Sydney rock oysters; superoxide dismutase; two dimensional electrophoresis.

1. Introduction

QX disease is caused by the protozoan parasite, *Marteilia sydneyi*. The parasite causes epizootics among Sydney rock oysters (*Saccostrea glomerata*) that occur during summer and can result in up to 95% mortality. The disease is more common in the northern, warmer estuaries on Australian's eastern seaboard, in the states of New South Wales (NSW) and Queensland. QX was responsible for a decline of the oyster industry in southern Queensland and in the Tweed, Richmond and Clarence Rivers of northern NSW during the 1970's [1]. The disease was later identified around Sydney, in the Georges River in 1994 and in the Hawkesbury River in 2004. These most recent outbreaks have had devastating effects on rock oyster production, which declined by 80% between 1994 and 2000 in Georges River, and was largely abandoned on many areas of Hawkesbury River within 18 months of first outbreak in 2004 [2].

M. sydneyi was initially characterized by Wolf in 1972 [3], and Perkins and Wolf in 1976 [4]. Oysters that are heavily infected with *M. sydneyi* are usually in poor condition with their gonads completely resorbed [2]. Infection results in death from starvation less than 60 days after the initial infection. The parasite invades digestive gland epithelial cells, leading to complete disorganization of the infected gut tissues [5,6,7]. *M sydneyi* sporonts pass into the environment via the alimentary

canal before the death of the oyster [8]. A variety of developmental stages of the parasite in *S. glomerata* have been characterized by Kleeman et al [9,10], and a putative intermediate host for the parasite has recently been identified (R. Adlard, Queensland Museum, personal communication).

In the 1990's, the New South Wales Department of Primary Industries (NSW DPI) began a rock oyster breeding program for fast growth and resistance to Winter Mortality Syndrome. Winter mortality is caused by the protozoa parasite, *Bonamia roughleyi*, which can kill up to 80% of oysters [11]. In 1994, an outbreak of QX disease in the Georges River, Sydney, caused 85% mortality in 2 out of the 3 breeding lines established by NSW DPI. As a result, the program was altered in 1997 to include breeding for QX disease resistance. After 2 generations, the mortality rate from QX disease was reduced to 62% in the best QX resistance (QXR) breeding lines. It was further decreased to 50% in fourth generation (QXR_4) and to 30% in fifth generation resistant (QXR_5) oysters [11,12,13; and unpublished data].

Our laboratory has been investigating the role of the defensive enzyme, phenoloxidase, as a potential marker of QX disease resistance. In 2003, we showed that *M. sydneyi* infects oysters when their phenoloxidase system is suppressed [14]. Phenoloxidase is not affected in rivers that do not suffer QX disease outbreaks. However, in oysters transferred from non-infested to QX-infested rivers, phenoloxidase activity decreases rapidly immediately before oyster are infected by *M. sydneyi* [14]. We concluded that the suppression of phenoloxidase-mediated defense decreases the ability of oysters to control *M. sydneyi*, leading to the development of QX as an opportunistic disease. The implication of phenoloxidase in QX disease has been further supported by studies of the QX-resistant oysters being bred by NSW DPI. The breeding program has selected oysters with enhanced phenoloxidase activities and novel forms of the enzyme [15].

Although QX resistance breeding has reduced mortality, one remaining limitation in the breeding program is that genetic factors, in addition to phenoloxidase, that might enhance disease resistance during selection have not been identified. This continues to limit the scope for marker-assisted selection. Despite the potential of phenoloxidase as a marker of QX disease resistance, NSW DPI and the Sydney rock oyster industry are eager to identify additional genetic factors that contribute to disease resistance. Here, we use proteomics to identify new markers of QX disease resistance. This is the first time that proteomics has been employed to study the expression of proteins in *S. glomerata*. Differences in the proteomes of wild-type, QXR and *M.* sydneyi-infected oysters are examined using 2-dimensional electrophoresis (2DE) to identify proteins that differ in expression between oyster populations. These proteins are then characterized by mass spectrometry.

2. Experimental Procedures

2.1 Oysters

Four types of *S. glomerata* were used in this study; uninfected wild-type oysters, *M. sydneyi*-infected wild-type oysters and two strains of selectively bred oysters. The two breeding lines tested were; the QXR line from Lime Kiln Bar on Georges River, which was bred specifically for QX resistance [13 and unpublished data], and the Woolooware Bay (WB) line, which has been selected for resistance to both QX and Winter Mortality Syndrome [13 and unpublished data]. Within the QXR line, QXR_5 oysters were from the fifth generation of selection and QXR_6 were from the sixth generation. QXR_6 oysters were the survivors of a QX disease outbreak in the Georges River, while QXR_5 oysters had never been exposed to QX disease. Non-selected wild-type oysters were purchased from the Sydney Fish Markets and transferred to Neverfail Bay on the Georges River (34º 01´S, 151º 10´E) between March and September 2006. When required, oysters were collected from the Georges River and held in 30L aquaria filled with UV-sterilized seawater for at least 7 days before being subjected to proteomic analysis.

2.2 Collection of hemolymph

Oysters were removed from aquaria 30 min prior to hemolymph extraction so that excess seawater could drain from their mantle cavities. Oysters were then shucked and the exuding hemolymph was removed from the mantle cavity. Approximately 3ml of hemolymph was collected from each oyster. Hemolymph was immediately transferred to 10 ml polypropylene tubes containing an equal volume of marine anticoagulant (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10mM EDTA, 0.45 M NaCl, pH7.0). Protease inhibitor 10µl (Mini Cocktail Tablets EDTA-free, Boehringer Mannheim, Germany) was added before the hemolymph was stored at -80ºC

The total hemolymph protein concentrations of samples were determined using the Bradford assay with a Bio-Rad Protein Assay Kit (Bio-Rad, Castle Hill, NSW). Bovine serum albumin was used to generate standard curves. The density of the hemocytes in hemolymph and the frequency of *M. sydneyi* in infected oysters were determined using an improved Neubauer hemocytometer.

2.3 Cytological assessments of M. sydneyi infection.

Tissue imprints were prepared by excising approximately $2mm^3$ of digestive gland from each oyster. Excess water was removed by blotting the sample on absorbent paper. A series of imprints from each samples were made onto microscope slides, sufficient to fill an area of 20 x 40mm. The slides were air-dried, then fixed in 100% methanol for 2-3 min. The imprints were stained according to Kleeman & Adlard [10] using modified Wright's stain (Wright's stain 6g/l , Giemsa stain 0.6 g/l in methanol) for 5 min. After staining, the slides were washed in phosphate buffered saline (13.7 mM sodium chloride, 0.2 mM potassium chloride, 1 mM phosphate buffer pH 7.2), rinsed in tap water and left to air-dry. Slides were mounted with a cover-slip using Ultramount No7 (Fronine, NSW). *M. sydneyi* spores were identified in imprints using an Olympus BH-2 microscope (Figure 1).

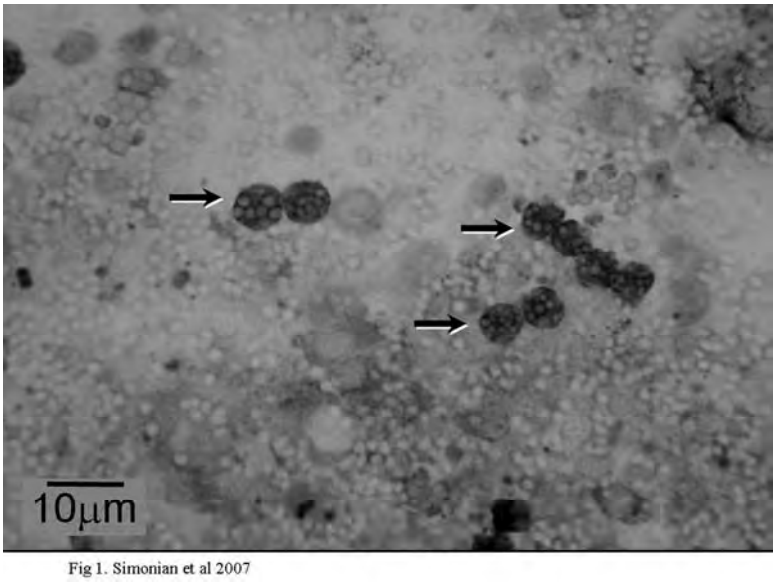


Figure 1. Wright's stain of infected oyster gut. Arrows show *M. sydneyi* sporonts.

2.4 Phenoloxidase assay

Three hundred microliters of whole hemolymph was freeze-thawed (-80ºC to room temperature) to rupture hemocytes and then centrifuged for 1 min (5000xg, 4 ºC) to remove cell debris. Phenoloxidase activity was measured as described by Butt et al [16]. The assay was carried out in

96-well microtitre plates (Sarstadt, Germany) using the substrate, hydroquinone mono-methyl ether (4-HA; Fluka, Switzerland ; 5mM in filtered seawater), and the chromogenic nucleophile, 3-methyl-2-benzothiazoline hydrazone (MBTH; Sigma Aldrich, Castle Hill, NSW; 1mM) [17]. Absorbances of triplicate wells were measured at 492 nm immediately after the addition of the substrates and again after 1 hr incubation at 22ºC. The change in absorbance per min was calculated and adjusted for the total protein content of hemolymph. Phenoloxidase activities are presented as the change in absorbance per min per microgram of protein (ΔOD_{490} min^{-1} μg^{-1}).

2.5 Two dimensional electrophoresis (2DE)

Protein precipitation and quantification were performed on whole hemolymph using 2-D Clean-Up kits and 2-D Quant kits according to manufacture's instructions (Amersham Biosciences, NJ, USA). Two hundred µg of precipitated proteins were re-suspended in 125µg of rehydration buffer containing 8 M urea, 2% 3-cholamydopropyl dimethyl-ammonio, 1-propane sulphate (CHAPS), 2.8 mg dithiothreitol (DTT), 0.002% w/v bromophenol blue and 0.5% v/v 3-10 pharmalyte or IPG buffer (Bio-Rad, Castle Hill, NSW).

Samples were subject to 2DE as described by O'Farrell et al. [18]. Proteins were separated by isoelectric focusing (IEF) using 7 cm immobilized pH gradient strips (IPG strips; pH 3-10; Bio-Rad). The IPG strips were rehydrated overnight with the protein samples that had been prepared in rehydration buffer. Isoelectric focusing was performed using an IPGPhor Isoelectric Focusing Cell (Amersham Biosciences). After isoelectric focusing, the IPG strips were equilibrated for 15 min in 10 ml reducing buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% w/v SDS, 20% v/v glycerol, 2% w/v DTT, 1% bromophenol blue) and for a further 15 min in alkylation buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% w/v SDS, 20% v/v glycerol, 2.5% w/v idodoacetamide, 1% w/v bromophenol blue). Second dimension SDS-PAGE was performed on 15% polyacrylamide gels for approximately 2 hrs using Mini Protean II electrophoresis cells.

After 2DE, protein spots were visualized by silver staining according to the method of Blum et al [19]. Image analysis software (Progenesis; PG 240, version 2006, UK) [20] was used to plot the position of proteins on proteome maps and to calculate their relative intensities. The amount of each protein visualized was calculated as a percentage of total pixel density (normalized density), where higher normalized values indicated higher protein expression.

2.6 Mass spectrometry

Proteins of interest were excised from gels using sterile scalpel blades. Excised proteins were de-stained by soaking in 100 mM sodium thiosulfate and 30 mM potassium ferricyanide for 20 min before being washed with water (4x 250µl). After de-staining, the proteins were digested with 20 µl of trypsin (20 µg/ml in 100 mM ammonium bicarbonate, Promega, WI.) at 37ºC overnight. The resulting tryptic peptides were extracted from the gel slices by washing with water (50µl), followed by 2 washes with 75µl 5% formic acid in 50% acetonitrile and one wash in 100% acetonitrile with the aid of sonication. The extracts were combined and concentrated to 10 µl by vacuum centrifugation.

Electrospray ionization tandem mass spectrometry (ESI MS/MS) was performed at the Bio Molecular Sequencing Facility (BMSF; University of NSW) using LTQ FT Ultra Hybrid Mass Spectrometer (Thermo Finnigan). Peak lists were generated using Mascot Daemon/extract_msn (Matrix Science, London, England, Thermo) using the default parameters, and submitted to the database search program Mascot (version 2.1, Matrix Science). Search parameters were: Precursor tolerance ± 1.4 Da and product ion tolerances ± 0.6 Da; Met(O) and Cys-carboxyamidomethylation specified as variable modification, enzyme specificity was trypsin, 1 missed cleavage was possible and the NCBInr database (15_7_07; 5303346 sequences; 1837221997 residues; September 2007) was searched. Individual ions scores > 56 indicate

identity or extensive homology ($p<0.05$). No species restrictions were applied for homology searches.

2.7 Non-Metric Multidimensional Scaling

Non-metric multidimensional scaling (MDS) was employed to construct a numerical "map" of combined expression data for individual oysters using PRIMER v5 analysis software (PRIMER-E Ltd, Plymouth, 2001) [21]. Bray-Curtis similarity matrixes comparing oysters were generated using square root transformed abundance data of the six proteins associated with QX resistance, and incorporated phenoloxidase activity as an additional factor. “Bubble plots” were generated from this analysis to give a visual representation of the pooled protein expression data for individual oysters. In these plots, larger bubble sizes indicate greater expression levels of the superimposed variables. The distance between bubbles indicates how closely individual oysters were related to each other in multidimensional plains, where each of the dimensions represents the expression of one of the six resistance-associated proteins, or phenoloxidase activity [21].

Cluster analyses were also performed on the same data set. Group average linkage in a hierarchical, agglomerative clustering algorithm was performed by PRIMER to plot dendrograms. The dendrograms indicated the distance between clustered groups and sub-groups in the different populations (uninfected wild-type, infected wild-type, QXR_5, QXR_6 and WB lines).

2.8 Other statistical analysis

Differences in mean values for individual factors were tested for statistical significance by one-way ANOVA using Microsoft Excel with Pop Tools.

3. Results

3.1 Hemolymph protein concentrations

The hemolymph protein concentrations of wild-type and QXR_5 oysters were compared using a one–way analysis of variance (ANOVA). Total protein concentrations in the wild-type population were found to be significantly higher than those of the QXR_5 population *($df=1$, $n=80$, $p < 0.05$).* Wild-type oysters had approximately 25% more protein in their hemolymph than QXR_5 oysters (Figure 2).

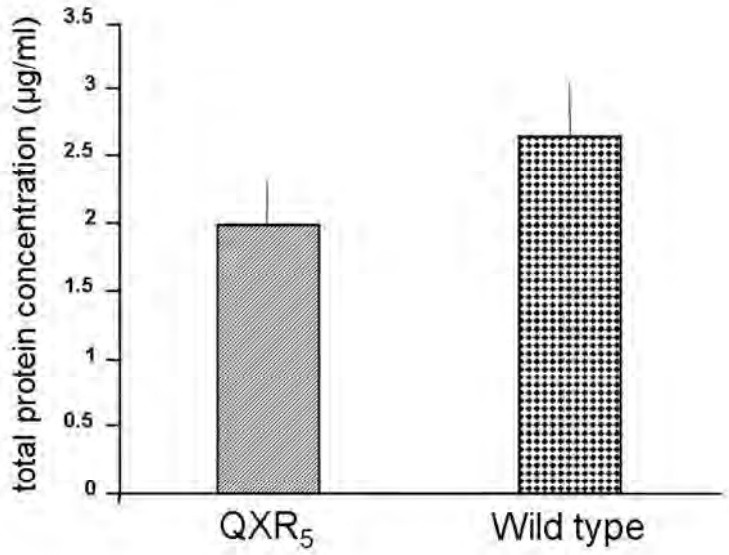


Fig 2. Simonian et al 2007

Figure 2. Total protein concentration of hemolymph (µg/ml) from uninfected wild-type and QXR_5 oysters. n = 40 from each population, bars = SEM.

3.2 Phenoloxidase activities

In contrast to hemolymph protein content, mean PO activities of QXR_5 were significantly higher than those of uninfected-wild-type oysters (one-way ANOVA, *n=5, df=1, p<0.05).* Four times more PO activity was detected in the hemolymph of QXR_5 oysters compared to wild-type (Figure 3).

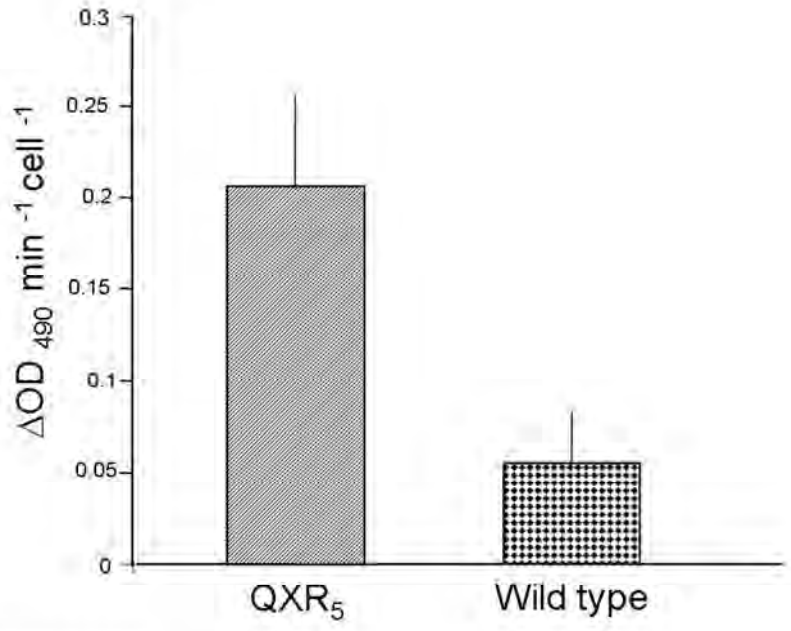


Fig 3. Simonian et al 2007

Figure 3. Phenoloxidase activities (ΔOD_{490} min^{-1} $cell^{-1}$) of QXR_5 and wild uninfected oysters. n=5 from each population, bars=SEM.

3.3 Proteome maps of wild-type, QXR and WB oysters.

Figure 4 shows a representative proteome map of individuals from the QXR_5 population, as well as uninfected wild-type and *M. sydneyi*-infected wild-type oysters. In total, five proteome maps of total hemolymph proteins from different QXR_5 were compared with five maps from uninfected wild-type and five maps from infected wild-type oysters. Progenesis software detected 60 protein spots in proteome maps of QXR_5 oyster hemolymph, 80 spots in uninfected wild-type oysters, and 140 spots in QX-infected wild-type oysters. Proteins that differed in frequency and intensity between populations and within the populations were further evaluated. An additional nine proteome maps of hemolymph proteins from QXR_6 oysters and five from WB individuals were also studied.

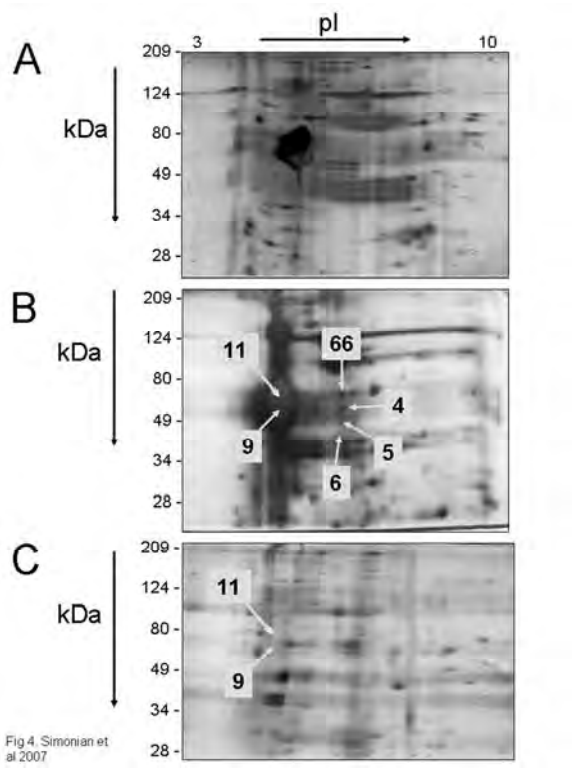


Figure 4. Proteome maps of (A) uninfected wild-type, (B) QXR_5 and (C) *M. sydneyi* infected wild-type oyster hemolymph proteins. Arrows in (B) indicate proteins present primarily in QXR_5 oysters. Arrows in (C) show the expected position of proteins p9 & p11.

Six proteins with molecular weights ranging from 48-68 kDa and pI's from 5.6-6.7 were expressed more frequently and at higher concentrations in QXR_5 oysters compared to wild-type oysters (Figure 4B). Two of these proteins, p9 (49 kDa, pI 5.6) and p11 (60 kDa, pI 5.7), were expressed in all oyster strains, but had higher expression levels in QXR and WB oysters than in wild-types (Figure 5A and B).

Figure 5A shows that four QXR_5 oysters expressed p11 at higher intensities than the five wild-type individuals. The maximum expression intensity of p11 among these four QXR_5 oysters was 15.7 and the minimum was 10.9, while the maximum intensity of p11 expression in wild-types was 8.7. One of the QXR_5 oysters analyzed had a level of p11 expression comparable to that of wild-type oysters (relative intensity 3.1). The expression of p11 in four out of five WB oysters was higher than in QXR_5 oysters, and QXR_6 had higher levels of p11 than all other oyster strains.

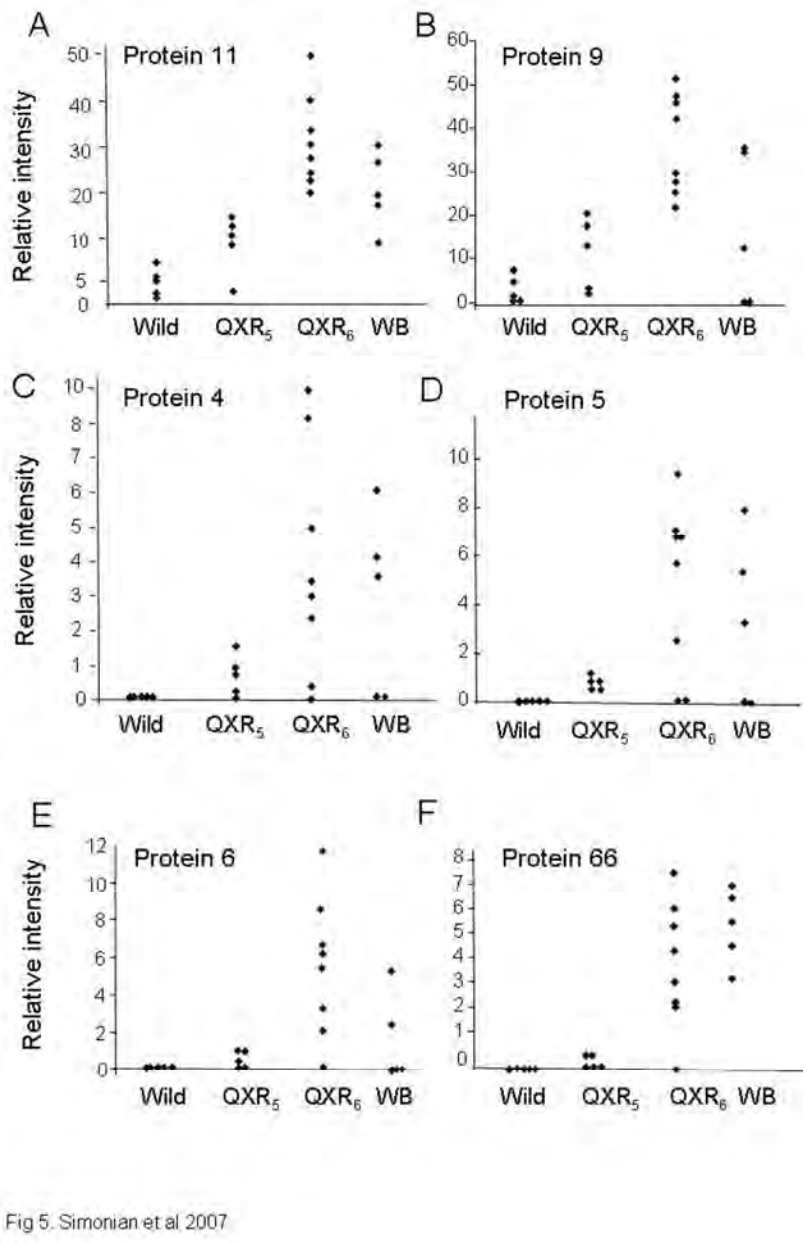


Figure 5. Relative expression levels for the six proteins (p4, p5, p6, p9, p11 and p66) that were associated with QX disease resistance in the uninfected wild-type, QXR_5, QXR6, and WB populations. Each (♦) represents expression data for a single oyster.

The expression of p9 in three out of five QXR_5 oysters was greater than that of wild-type oysters. These three QXR_5 individuals expressed p9 with an average intensity 16.8 ± 2.7 compared to 2.4 ± 1.5 in the five wild-type oysters ($p<0.05$). p9 was entirely absent in two wild-type oysters. The remaining two QXR_5 oysters had expression levels for p9 (relative intensities 2.3 and 3.4) that fell within the range of the wild-type oysters (0 - 4.2). QXR_6 oysters expressed p9 at much higher intensities compared to the other oyster strains with a maximum intensity in QXR_6 oysters of 51.2 and a minimum of 24.3. Only three out of five WB oysters expressed detectable levels of p9 (34.4 and 35.46) (Figure 5B).

Both p11 and p9 were entirely absent in all five of the infected wild-type oysters tested (Figure 4C).

The other four proteins that differed in expression between QXR and wild-type oysters were p4 (65 KDa, pI 6.68), p5 (58 KDa, pI 6.69), p6 (48 KDa, pI 6.68) and p66 (68 KDa, pI 6.69). These proteins were only present in QXR oysters and were not expressed by any of the uninfected or infected wild-type oysters tested. The frequency with which they appeared and their intensity varied substantially within the QXR_5 population (Figure 5). Figure 5 show that p4 was expressed

by 4 out of 5 QXR_5 oysters, while p5 was present in all five QXR_5 individuals. P6 was expressed by three out of five QXR_5 individuals and p66 was present in only two of the QXR_5 individuals.

The QXR_6 population expressed p4, p5, p6 and p66 at much higher intensities than QXR_5 and WB oysters. Six oysters from QXR_6 expressed p4 and p5 at higher intensities compared to all five QXR_5 oysters, with relative expression ranging from 2.3-10.1 for p4 and 2.5-9.6 for p5. Only three out of five WB oysters had expression levels of p4 and p5 that fell within the range of the QXR_6 individuals. The remaining two WB oysters did not express p4 and p5 (Figures 5C & 5D and supplementary Table 2). Seven QXR_6 oysters expressed p6 and p66 at higher intensities compared to all five QXR_5 oysters, with relative expression ranging from 2.1-12.0 for p6 and 2.1-7.4 for p66. Only two of WB oysters expressed detectable quantities of p6 (Figure 5E & F and supplementary Table 2).

3.6 Mass spectrometry

All six of the differentially expressed proteins (p11, p9, p4, p5, p6 and p66) associated with QX disease resistance were subjected to mass spectrometry. P11 was found to incorporate a tryptic peptide that was homologous to a protein from the Pacific oyster, *C. gigas*, designated cavortin (Table 1). Three ion fragments (p11.1, p11.2 and p11.3) matched a predicted tryptic peptide of 2492 Da from cavortin that was bounded by two R/S tryptic proteolysis sites. An additional ion fragment from p11 (p11.4) matched a slightly larger (2924 Da) predicted peptide from *C. gigas* cavortin that incorporated an additional three amino acids (HAH) on the carboxyl end of the 2492 Da peptide (Table 1).

Two tryptic fragments of p9 (p9.1 and p9.2) matched a predicted peptide from an uncharacterized EST from the Eastern oyster, *C. virginica*, designated, dominin (Genbank non-redundant database accession number BAF30874; Itoh et al., unpublished data). The matching predicted peptide from dominin had a molecular weight of 2492. It was identical in amino acid sequence to the cavortin peptide that matched p11, except for the presence of an isoleucine instead of a valine at reside 15. The remaining *S. glomerata* proteins (p66, p4, p5 and p6) had no obvious similarity to any known proteins in sequence databases.

Table 1. Mascot search results for MS/MS analysis of S. *glomerata* proteins p9 and p11, showing the m/z (mass-to-charge) ratios and the observed (calculated) molecular weights (Mr observed) for four ion fragments from p11 and two fragments from p9. Also shown are the expected molecular weights (Mr expected) and amino acid sequences of the closest matching peptides to the *S. glomerata* fragments in the NCBInr database.

Ion fragments from p11	**m/z**	**Mr (observed)**	**Mr (expected)**	**Score**	**E value**	**Matching peptide sequences** **Best match: *Crassostrea gigas* cavortin (gi|30039400; Mass: 19276); Mascot Score: 111; Significant score cutoff: >56; Coverage: 16.7%**
p11.1	832.0222	2493.044	2492.2627	37	3.5	R.SLAILQGDHTSHTAVIACCVIGR.S+Acrylamide(C);C
p11.2	1247.731	2493.447	2492.2627	57	0.036	R.SLAILQGDHTSHTAVIACCVIGR.S+Acrylamide(C);C
p11.3	832.2092	2493.605	2492.2627	28	23	R.SLAILQGDHTSHTAVIACCVIGR.S+Acrylamide(C);C
p11.4	976.2299	2925.667	2924.4497	54	0.053	R.SLAILQGDHTSHTAVIACCVIGRSHAH.+Acrylamide(C)
Ion fragments from p9	**m/z**	**Mr (observed)**	**Mr (expected)**	**Score**	**E value**	**Matching peptide sequences** **Best match: *Crassostrea virginica* dominin (gi|113928362 Mass: 21042); Mascot Score: 94; Significant score cutoff: >56; Coverage: 13%**
p9.1	1247.6580	2493.301	2492.2627	94	7.2e-6	R.SLAILQGDHTSHTAIIACCVIGR.S+2(C)
p9.2	832.1196	2493.337	2492.2627	69	0.002	R.SLAILQGDHTSHTAIIACCVIGR.S+2(C)

3.7. Non-Metric Multidimensional Scaling (MDS)

Figure 6A shows a 2-dimensional MDS plot, in which pooled expression data for all six resistance-associated proteins (p11, p9, p66, p4, p5 and p6) and phenoloxidase activity, are shown for individual oysters *(stress=0.05).* Distinct clusters representing the different populations are evident on the plot. These clusters agree with those generated by the matching cluster analysis (Figure 6B). Wild-type oysters formed a relatively dispersed cluster to the right of the MDS plot. Two oysters ($QXR_5$1 and $QXR_5$5) fell within the region of the wild-type cluster. The other three QXR_5 oysters formed a discrete group between the wild-type and QXR_6 populations. Cluster analysis also grouped $QXR_5$1 and $QXR_5$5 with the wild-type oysters, whilst $QXR_5$2, $QXR_5$3 and $QXR_5$4 were grouped with the QXR_6 oysters. The two QX resistant populations still formed discrete clusters, with QXR_5 clearly separated from the QXR_6 population. The larger "bubble" sizes of QXR_6 oysters relative to QXR_5 and wild-type oysters also reflects their increased expression of the resistance-associated proteins. Two of the oysters from the WB line (WB2 and WB 3) clustered closely with the QXR_6 population, whilst the remaining two WB individuals were distinct from all of the other populations tested.

Figure 6C shows the same type of MDS analysis for six randomly chosen proteins other than the resistance-associated proteins, p4, p5, p6, p9, p11 and p66,. The proteins selected for this "control" plot were not obviously associated with QX disease resistance. No clear clustering was evident in this plot. QXR_5 and wild-type oysters were equally dispersed *(stress=0.07)* and cluster analysis failed to identify any discrete populations (data not shown).

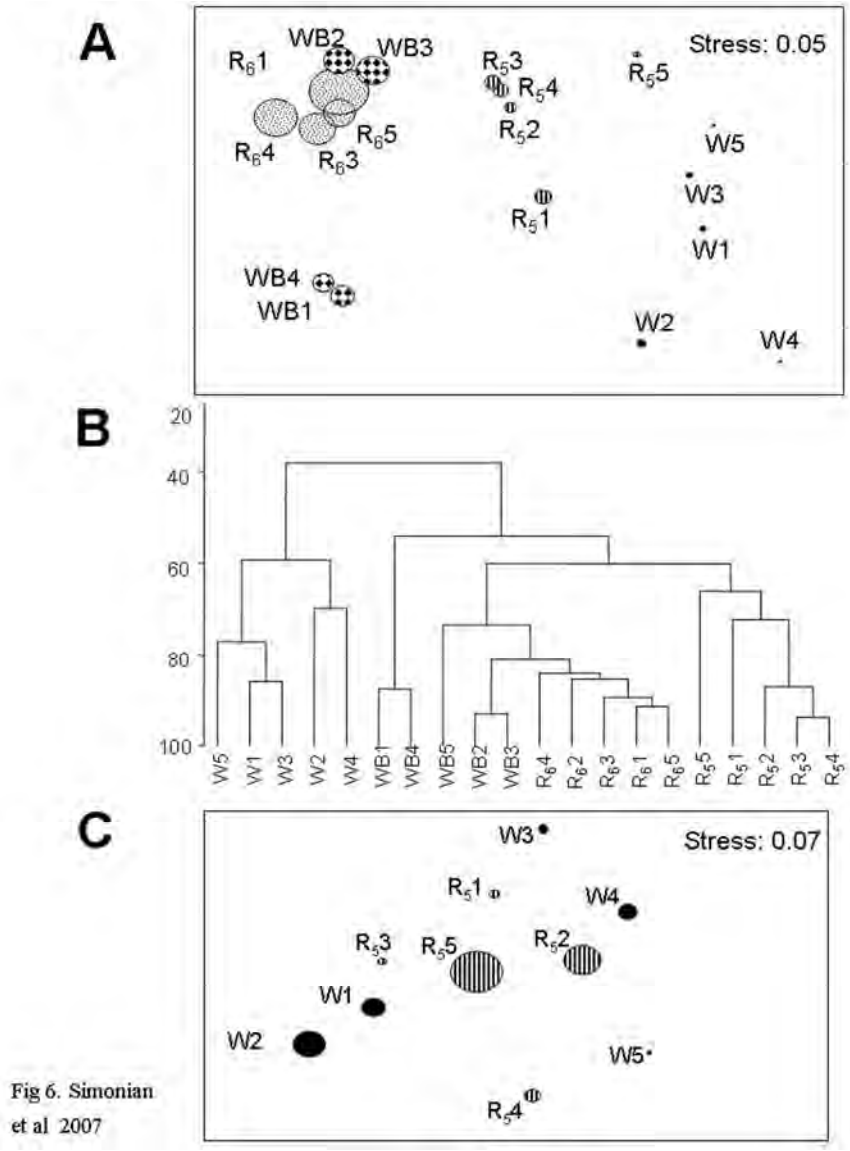


Figure 6. (A) Non-metric MDS bubble plot of combined expression data for six proteins associated with QX disease resistance in the uninfected wild-type, QXR_5, QXR_6, and WB populations *(stress=0.05)*. Each bubble represent data for a single oyster. (B) Dendrogram of combined expression data for six proteins associated with QX disease resistance in uninfected wild-type, QXR_5, QXR6, and WB populations. (C) Non-metric MDS bubble plot of combined expression data for six randomly chosen proteins (not obviously associated with resistance) in the QXR_5 and wild-type populations *(stress=0.07)*. W=uninfected wild-type, R_5=QXR_5, R_6= QXR_6, WB= Woolooware Bay line.

4. Discussion

The productivity of Sydney rock oyster farming in Australia has fallen by over 40% in the past 30 years [22]. This decline has resulted in part by two infectious diseases, Winter Mortality Syndrome and QX disease [23]. Selective breeding undertaken by NSW DPI has reduced the mortality rates of both diseases. However, the introduction of marker-assisted selection is still required to increase the efficiency of the breeding program and to maintain resistance in the absence of high level selection in the field.

This study employed proteomics to identify new markers of QX disease resistance. Our aim was to identify proteins that are differentially expressed between QXR and wild-type oysters. It was immediately clear that overall protein expression differed between the two populations. Total hemolymph protein concentrations were found to be significantly higher in wild-type oysters when compare to the resistant strain. This agrees with our proteomic analysis of the two populations, which identified more proteins on proteome maps of wild-type oysters compared to QXR animals.

In addition to the overall change in protein expression, there were also clear differences in the expression of individual proteins by the two populations. 2DE showed that six proteins with molecular weights ranging from 49-65 kDa and pIs from 5.6-6.69 were specifically associated with resistance breeding. Two of those proteins, p11 (60 kDa, pI 5.7) and p9 (49 kDa, pI 5.6) occurred more frequently and were expressed at greater intensity in QXR oysters compared to wild-types. Interestingly, not all QXR_5 oysters expressed p9 and p11 at enhanced levels compared to wild-types. This suggests that there is still allelic heterogeneity among the genes that encode these proteins in QXR_5 population, which fits with expectations for QX resistance-associated molecules. Current mortality data indicate that only 50% - 60% of the QXR_5 populations have genetic resistance to the disease and so resistance-associated molecules should not be found in all QXR_5 individuals (J Nell unpublished data). Similar heterogeneity was not evident among the QXR_6 oysters tested in this study, all of which expressed p11 and p9 at high levels. Again this fits expectations, because the QXR_6 oysters used here were the survivors of previous QX disease exposure, which would presumably have eliminated genetically susceptible individuals.

The expression of p9 and p11 also varied between the two selective breeding regimes tested here. QXR oysters, which were bred specifically for QX-disease resistance, expressed these proteins more frequently and at higher titers than oysters from the WB line, bred for combined Winter Mortality and QX-disease resistance. This matches expectations based on mortality rates for the QXR_5 and WB populations. According to the latest NSW DPI data, QX-disease associated mortality rate for QXR_5 in Georges River is lower than that of the WB line (J Nell unpublished data). Most significantly, p9 and p11 were entirely absent from infected wild-type oysters (Figure 4c), which indicates a direct association between these proteins and infection by *M. sydneyi*, the etiological agent of QX disease.

ESI MS/MS showed that p9 & p11 incorporate peptides that closely resemble regions of the proteins cavortin and dominin from the Pacific oyster, *C. gigas*, and the Eastern oyster, *C. virginica* (Table 1). Both cavortin and dominin are closely related to superoxide dismutases (SODs) [27], suggesting that p9 and p11 from *S. glomerata* incorporate SOD-like domains. The SOD-like domains of the *Crassostrea* proteins have a copper binding site with sequence similarities to SODs from other species that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. This is an important antioxidant defense mechanism in almost all cells, including those of mollusks [24]. SODs in most eukaryotic cells exist as dimeric copper or zinc containing proteins [25]. However the SOD-like domains of cavortin and dominin are highly modified with numerous insertions and deletions, which suggests that they may have modified enzymatic activities relative to true SODs [24].

Summer mortality has been reported in the Pacific oyster, *C. gigas*, for many years and is mainly associated with high temperature and the oyster reproductive period [26]. A previous study of *C.*

gigas by Huvet et al. showed that cavortin is associated with resistance to summer mortality. The protein is more highly expressed in oysters that are resistant to summer mortality compared to susceptible animals [27].

One intriguing possibility from our results is that, in *S. glomerata,* SOD-like proteins have adopted phenoloxidase-like enzymatic activities or otherwise contribute to oxidative processes associated with parasite killing. Our previous work has found a strong association between QX disease resistance and the phenoloxidase system, and that phenoloxidase activities are higher in QXR oysters compared to the susceptible population [14, 15, 28]. The potential role of the proteins p9 & p11 in oxidative reactions fits well with this known resistance mechanism. We are currently investigating the potential p9 and p11 as genetic markers of QX-disease resistance, and further studying their relationship to the phenoloxidase activities that are known to be associated with disease resistance.

The remaining four QX resistance-associated proteins detected in *S. glomerata,* p4 (65 KDa, pI 6.687), p5 (58 KDa, pI 6.69), p6 (48 KDa, pI 6.68) and 66 (68 KDa, pI 6.69), were present only in QXR and WB oysters, with different intensities of expression within each population. Given that mortality data predict the appearance of QX disease susceptible oysters in both the QXR_5 and WB lines, their expression in all resistant oysters suggests that these proteins may not be reliable markers of disease resistance when compared to p11 and p9. Having said this, the relatively low sample sizes used in this study warrant more extensive analyses of these proteins before definitive conclusions can be made about their usefulness as genetic markers for selective breeding.

In this study, the most effective predictor of QX resistance came from combining expression data for all six resistance-associated proteins. MDS and associated cluster analysis revealed clear distinctions between different oyster populations. In MDS bubble plots, significant distances were evident between the wild-type oysters and all of the resistant lines. However, two QXR_5 oysters had MDS profiles that were more consistent with the wild-type population than with other QXR oysters. Again this suggests that genetically susceptible oysters remain in the QXR_5 population. Despite this, most QXR oysters clustered tightly together and there was a clear progression from wild-type, through QXR_5, to QXR_6 phenotypes. This is consistent with continuing selection of QX disease resistance. It was also evident that at least some oysters bred for combined resistance to Winter Mortality Syndrome and QX disease (WB oysters) had phenotypes that closely resembled to QXR_6 population, suggesting that different selection regimes may lead to similar genetic outcomes.

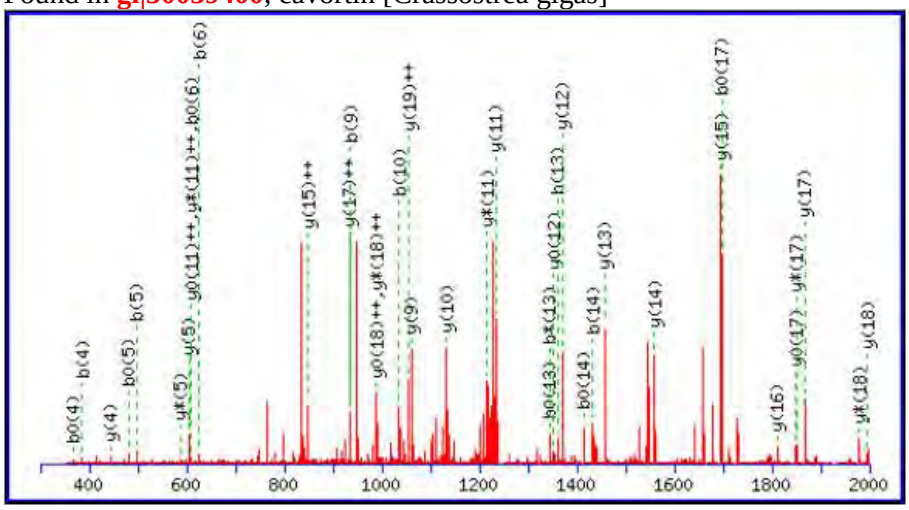
Overall the data presented here suggest that numerous genetic factors contribute to QX-disease resistance in Sydney rock oysters and that proteomic analysis is a useful way of identifying disease resistant oysters for selective breeding.

Appendix

A. Protein p11

MS/MS Fragmentation of **SLAILQGDHTSHTAVIACCVIGR**

Found in **gi|30039400**, cavortin [Crassostrea gigas]



Monoisotopic mass of neutral peptide Mr(calc): 2924.4498

Variable modifications:

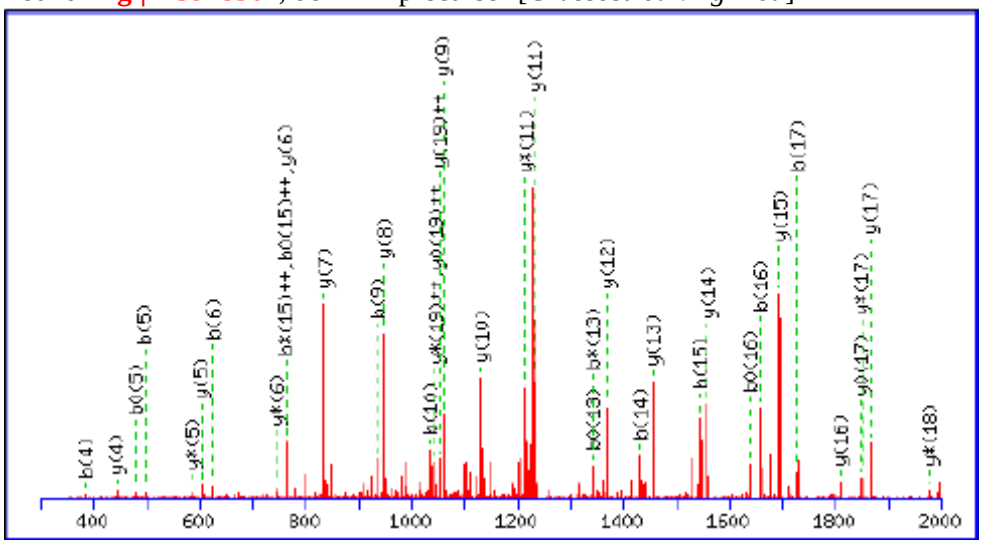
C18 : Acrylamide (C), **C19 :** Carbamidomethyl (C), **Ions Score:** 43 **Expect:** 0.92

Matches (Bold Red): 75/288 fragment ions using 165 most intense peaks

#	b	b++	b*	b*++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	88.0393	44.5233			70.0287	35.5180	S							27
2	201.1234	101.0653			183.1128	92.0600	L	2838.4250	1419.7161	2821.3985	1411.2029	2820.4144	1410.7109	26
3	272.1605	136.5839			254.1499	127.5786	A	2725.3410	**1363.1741**	2708.3144	1354.6608	2707.3304	1354.1688	25
4	**385.2445**	193.1259			**367.2340**	184.1206	I	2654.3038	1327.6556	2637.2773	1319.1423	2636.2933	1318.6503	24
5	**498.3286**	249.6679			**480.3180**	240.6627	L	2541.2198	**1271.1135**	2524.1932	1262.6002	2523.2092	1262.1082	23
6	626.3872	313.6972	609.3606	305.1840	**608.3766**	304.6919	Q	2428.1357	1214.5715	2411.1092	**1206.0582**	2410.1251	1205.5662	22
7	683.4087	342.2080	666.3821	333.6947	**665.3981**	333.2027	G	2300.0771	**1150.5422**	2283.0506	**1142.0289**	2282.0666	**1141.5369**	21
8	**798.4356**	399.7214	781.4090	391.2082	780.4250	390.7162	D	2243.0557	**1122.0315**	2226.0291	1113.5182	2225.0451	1113.0262	20
9	935.4945	468.2509	918.4680	459.7376	917.4839	459.2456	H	2128.0287	**1064.5180**	2111.0022	**1056.0047**	2110.0182	**1055.5127**	19
10	**1036.5422**	518.7747	1019.5156	510.2615	1018.5316	**509.7694**	T	1990.9698	**995.9885**	1973.9433	**987.4753**	1972.9592	**986.9833**	18
11	**1123.5742**	562.2907	1106.5477	553.7775	1105.5636	553.2855	S	1889.9221	**945.4647**	**1872.8956**	936.9514	1871.9116	936.4594	17
12	1260.6331	630.8202	1243.6066	**622.3069**	1242.6226	621.8149	H	**1802.8901**	901.9487	1785.8636	893.4354	1784.8795	892.9434	16
13	1361.6808	**681.3440**	1344.6543	**672.8308**	1343.6702	672.3388	T	**1665.8312**	**833.4192**	**1648.8046**	824.9060	1647.8206	824.4140	15
14	1432.7179	**716.8626**	1415.6914	**708.3493**	**1414.7074**	**707.8573**	A	**1564.7835**	782.8954	**1547.7570**	**774.3821**	**1546.7729**	**773.8901**	14
15	1531.7863	766.3968	1514.7598	757.8835	1513.7758	757.3915	V	**1493.7464**	747.3768	1476.7199	738.8636	1475.7358	738.3716	13
16	1644.8704	822.9388	1627.8438	814.4256	1626.8598	813.9336	I	**1394.6780**	697.8426	**1377.6514**	689.3294	**1376.6674**	688.8373	12
17	1715.9075	858.4574	1698.8810	849.9441	1697.8969	849.4521	A	**1281.5939**	641.3006	1264.5674	632.7873	1263.5834	632.2953	11
18	1889.9538	**945.4805**	**1872.9273**	936.9673	1871.9432	936.4753	C	1210.5568	605.7820	1193.5303	597.2688	1192.5462	596.7768	10
19	2049.9845	1025.4959	2032.9579	1016.9826	2031.9739	1016.4906	C	**1036.5105**	518.7589	1019.4840	510.2456	1018.4999	**509.7536**	9
20	2149.0529	**1075.0301**	2132.0263	**1066.5168**	2131.0423	**1066.0248**	V	876.4799	438.7436	859.4533	430.2303	858.4693	429.7383	8
21	2262.1369	**1131.5721**	2245.1104	**1123.0588**	2244.1264	**1122.5668**	I	**777.4114**	389.2094	760.3849	380.6961	759.4009	380.2041	7
22	2319.1584	**1160.0828**	2302.1319	**1151.5696**	2301.1478	**1151.0776**	G	**664.3274**	332.6673	647.3008	324.1541	646.3168	323.6620	6
23	2475.2595	**1238.1334**	2458.2330	1229.6201	2457.2489	1229.1281	R	**607.3059**	304.1566	590.2794	295.6433	589.2954	295.1513	5
24	2562.2915	**1281.6494**	2545.2650	**1273.1361**	2544.2810	**1272.6441**	S	**451.2048**	226.1060			**433.1942**	217.1008	4
25	2699.3505	**1350.1789**	2682.3239	**1341.6656**	2681.3399	**1341.1736**	H	**364.1728**	182.5900					3
26	2770.3876	**1385.6974**	2753.3610	**1377.1841**	2752.3770	**1376.6921**	A	227.1139	114.0606					2
27							H	156.0768	78.5420					1

B. Protein p9

MS/MS Fragmentation of **SLAILQGDHTSHTAIIACCVIGR**
Found in **gi|113928362**, dominin precursor [*Crassostrea virginica*]



Monoisotopic mass of neutral peptide Mr(calc): 2492.2628

Variable modifications:

C18: Carbamidomethyl (C)

C19: Carbamidomethyl (C)

Ions Score: 97 **Expect:** 3.5e-006

Matches (Bold Red): 38/234 fragment ions using 59 most intense peaks

#	b	b++	b*	b*++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	88.0393	44.5233			70.0287	35.5180	S							23
2	201.1234	101.0653			183.1128	92.0600	L	2406.2381	1203.6227	2389.2115	1195.1094	2388.2275	1194.6174	22
3	272.1605	136.5839			254.1499	127.5786	A	2293.1540	1147.0806	2276.1274	1138.5674	2275.1434	1138.0754	21
4	**385.2445**	193.1259			367.2340	184.1206	I	2222.1169	1111.5621	2205.0903	1103.0488	2204.1063	1102.5568	20
5	**498.3286**	249.6679			**480.3180**	240.6627	L	2109.0328	**1055.0200**	2092.0063	**1046.5068**	2091.0223	**1046.0148**	19
6	**626.3872**	313.6972	609.3606	305.1840	608.3766	304.6919	Q	1995.9488	998.4780	**1978.9222**	989.9647	1977.9382	989.4727	18
7	683.4087	342.2080	666.3821	333.6947	665.3981	333.2027	G	**1867.8902**	934.4487	**1850.8636**	925.9355	**1849.8796**	925.4434	17
8	798.4356	399.7214	781.4090	391.2082	780.4250	390.7162	D	**1810.8687**	905.9380	1793.8422	897.4247	1792.8581	896.9327	16
9	**935.4945**	468.2509	918.4680	459.7376	917.4839	459.2456	H	**1695.8418**	848.4245	1678.8152	839.9112	1677.8312	839.4192	15
10	**1036.5422**	518.7747	1019.5156	510.2615	1018.5316	509.7694	T	**1558.7829**	779.8951	1541.7563	771.3818	1540.7723	770.8898	14
11	1123.5742	562.2907	1106.5477	553.7775	1105.5636	553.2855	S	**1457.7352**	729.3712	1440.7086	720.8580	1439.7246	720.3659	13
12	1260.6331	630.8202	1243.6066	622.3069	1242.6226	621.8149	H	**1370.7031**	685.8552	1353.6766	677.3419	1352.6926	676.8499	12
13	1361.6808	681.3440	**1344.6543**	672.8308	**1343.6702**	672.3388	T	**1233.6442**	617.3258	**1216.6177**	608.8125	1215.6337	608.3205	11
14	**1432.7179**	716.8626	1415.6914	708.3493	1414.7074	707.8573	A	**1132.5966**	566.8019	1115.5700	558.2886			10
15	**1545.8020**	773.4046	1528.7754	**764.8914**	1527.7914	**764.3993**	I	**1061.5594**	531.2834	1044.5329	522.7701			9
16	**1658.8860**	829.9467	1641.8595	821.4334	**1640.8755**	820.9414	I	**948.4754**	474.7413	931.4488	466.2281			8
17	**1729.9232**	865.4652	1712.8966	856.9519	1711.9126	856.4599	A	**835.3913**	418.1993	818.3648	409.6860			7
18	1889.9538	945.4805	1872.9273	936.9673	1871.9432	936.4753	C	**764.3542**	382.6807	**747.3277**	374.1675			6
19	2049.9845	1025.4959	2032.9579	1016.9826	2031.9739	1016.4906	C	**604.3235**	302.6654	**587.2970**	294.1521			5
20	2149.0529	1075.0301	2132.0263	1066.5168	2131.0423	1066.0248	V	**444.2929**	222.6501	427.2663	214.1368			4
21	2262.1369	1131.5721	2245.1104	1123.0588	2244.1264	1122.5668	I	345.2245	173.1159	328.1979	164.6026			3
22	2319.1584	1160.0828	2302.1319	1151.5696	2301.1478	1151.0776	G	232.1404	116.5738	215.1139	108.0606			2
23							R	175.1190	88.0631	158.0924	79.5498			1

4.8 Protein markers of *Martielia sydneyi* infection in Sydney rock oysters (*Saccostrea glomerata*)

Margaret Simonian [1], Sham V. Nair [1], Wayne A. O'Connor [2], David A. Raftos [1]*

[1] *Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia*
[2] *New South Wales Department of Primary Industries, Port Stephens Fisheries Centre, Taylors Beach, NSW, 2316, Australia*

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Abstract

Marteilia sydneyi is the causative agent of QX disease in Sydney rock oysters (*Saccostrea glomerata*). It is responsible for disease outbreaks among oysters that occur during summer and can result in up to 95% mortality. QX disease has significantly decreased *S. glomerata* production in some areas of Australia's eastern seaboard over the past 30 years. *M. sydneyi* sporulates in the digestive gland of oysters leading to complete disorganization of the infected tissues. The current study used proteomics to identify potential molecular markers of sporulating *M. sydneyi* infection during a field trial undertaken in the Georges River, Sydney, between December 2006 and May 2007. Early stages of *M. sydneyi* infection were detected by PCR, whilst cytological examination was used to identify sporulating *M. sydneyi* in the gut. Protein expression in oyster hemolymph was assessed during the *M. sydneyi* infection period by two dimensional electrophoresis. Proteome maps identified significant differences in the expression of four proteins in oysters with sporulating *M. sydneyi* infections.

Keywords*: Marteilia sydneyi*, QX disease, Sydney rock oysters, *Saccostrea glomerata*, hemolymph protein, proteomics.

1. Introduction

The Sydney rock oyster (*Saccostrea glomerata*) industry is worth approximately US $33.5 million to Australian growers annually (NSW DPI, 2008). It is the fourth largest edible oyster industry in the world and the largest aquaculture industry in the state of New South Wales (NSW). Production levels have been falling since the mid 1970's (O'Connor and Dove, in press) with much of the reduction due to recurrent loses from QX disease. For instance, production in the Georges River, NSW, declined by 94% between 1994 and 2001 due to annual QX disease outbreaks, and production was reduced by almost 100% in Hawkesbury River, NSW, by a severe QX epizootic in 2004 (O'Connor and Dove, in press).

QX disease is caused by the protozoan parasite, *Marteilia sydneyi* (Perkins & Wolf 1976; Roubal et al. 1989). Parasites enter oysters through the gills and progress to the digestive gland where they release spores (Kleeman et al. 2002a). Death results from the complete disorganization of the infected gut tissues as a result of parasite sporulation (Kleeman et al. 2002a). Spores are short-lived outside oysters, usually surviving for between 7 and 35 days (Wesche et al. 1999). *M. sydneyi* has also been identified in benthic polychaetes, which may act as the parasite's intermediate host (Adlard and Nolan 2007).

The development of QX disease resistant strains of *S. glomerata* became a priority for the NSW Department of Primary Industries (DPI) in 1996 to alleviate the effects of QX disease on oyster production. NSW DPI's selective breeding program has been based on interbreeding the survivors of annual disease outbreaks (Nell et al. 2000; Nell 2006).

Even though the selective breeding program has substantially reduced mortality, there remains a need to develop new diagnostic tests for *M. sydneyi*. The Sydney rock oyster industry relies on the translocation of oysters between growing areas to take advantage of favorable local conditions. To allow translocation without the threat of spreading QX epizootics, oyster farmers require rapid, inexpensive tests to detect sporulating *M. sydneyi* infections. Current diagnostic protocols for *M. sydneyi* are time consuming and expensive. They rely either on detailed histological examination of sectioned tissue, cytological identification of sporulating parasites in gut tissue imprints, or on polymerase chain reaction (PCR) analysis targeting 16S ribosomal DNA from the parasite (Kleeman and Adlard 2000).

The aim of the current study is to identify protein markers of *M. sydneyi* infection that can be used to develop high throughput assays for the presence of sporulating parasites. To identify potential markers, changes in protein expression were studied in QX disease resistant and wild type oysters by two dimensional electrophoresis (2DE) during the course of a *M. sydneyi* infection cycle. Concurrent PCR and cytological tests were used to identify oysters with different stages of *M. sydneyi* infection.

2. Materials and methods

2.1. Oysters

Three types of Sydney rock oysters were used in this study; non-selected wild type (WT) oysters, which were purchased from the Sydney Fish Markets, NSW; selectively bred oysters from the Georges River, NSW (GR_6); and non-selected wild type oysters from the Macleay River (MR) NSW. The MR oysters were collected from a population undergoing a severe QX disease outbreak, which resulted in substantial (greater than 50%) mortality (C. Tunningly, personal communication). GR_6 oysters were originally bred by NSW DPI for fast growth, and subsequently have been selected for QX disease resistance.

2.2. Field trial

A field trial was conducted in the Georges River, Sydney, NSW, from December 2006-May 2007. Four hundred non-selected WT oysters and 300 GR_6 oysters were transplanted to Neverfail Bay (33º 59' 41"S, 151º 03' 10") in December 2006. Ten oysters from each population were collected fortnightly from 21 January, 2007 (time 1, T1) to 24 May, 2007 (T10). The oysters were held at Macquarie University in 30 L aquaria filled with UV-sterilized seawater for 4 days before further analysis.

2.3. Cytological assessment of M. sydneyi infection

M. sydneyi were identified in imprints of oyster tissue by the method of Kleeman & Adlard (2000). Imprints were prepared by dissecting approximately $2mm^3$ of digestive gland from each oyster. Tissues were blotted on absorbent paper before a series of imprints from each explant was made onto glass microscope slides, sufficient to fill an area of 20mm x 40mm. Imprints were air-dried and then fixed in 100% methanol for 2-3 min. The slides were stained with modified Wright's stain (Wright's stain 6 g/L, Giemsa stain 0.6 g/L in methanol) for 5 min. Stained slides were washed in phosphate buffered saline (13.7 mM sodium chloride, 0.2 mM potassium chloride, 1 mM phosphate buffer pH 7.2), rinsed in tap water and air-dried. Slides were cover-slipped using Ultramount No7 (Fronine, NSW), before *M. sydneyi* spores were identified using an Olympus BH-2 microscope.

2.4. PCR assessment of M. sydneyi infection

M. sydneyi 16S ribosomal DNA was detected in oyster tissues by a modified method of Kleeman and Adlard (2000). Genomic DNA was extracted from gut and gill tissues using a DNeasy kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instruction. PCR reactions were performed under the following conditions: Go-*Taq* buffer (pH 8.5 and 1.5 mM $MgCl_2$ with proprietary formulation); dNTP's 200 µM; primer1: ITSF, 400 nM; primer 2: ITSR 400 nM; *Taq polymerase* (Promega) 1U; DNA template 2 µL ; ultra-pure water to 25 µL total. Amplification was carried out using a PTC-100 thermal-cycler (Gene Works) with a step-up PCR program (initial denaturation at 94˚C for 3 min, then cycles of denaturation at 94˚C for 20 sec; primer annealing at 55˚C for 20 sec; chain extension at 65˚C for 20 sec; repeated for 30 cycles with a final extension at 65˚C for 5 min). Primers 1 and 2 were the same as those used by Kleeman and Adlard (2000). Purified *M. sydneyi* sporonts were used as positive controls (see below).

2.5. M. sydneyi sporont purification

M. sydneyi sporonts were isolated from infected oyster digestive glands by density gradient centrifugation using modified methods of Robledo et al. (1995) and Butt et al. (2007). Incised sections of the digestive gland were homogenized in filtered sea water (FSW; 0.45 µm) and passed through a 150 µm sieve (Sigma Aldrich). Sieved digestive gland homogenates were diluted in FSW and layered onto a discontinuous Percoll (Pharmacia, Sweden) gradient (20%, 30%, 40% and 50% Percoll in FSW) and centrifuged at (2,500xg, 10 min 4ºC). Sporonts were collected from the 20%-40% Percoll interfaces with a lumbar puncture syringe. After centrifugation (2,500xg, 10 min 4ºC), the pellets were re-suspended in FSW. *M. sydneyi* sporonts were then counted with an improved Neubauer hemocytometer and stored at -20ºC.

2.6. Collection of hemolymph

Oysters were removed from aquaria 30 min prior to hemolymph extraction to drain excess seawater from their mantle cavities. They were then shucked so that the exuding hemolymph (2-3 mL per oyster) could be removed from the mantle cavity. Hemolymph was immediately mixed with an equal volume of marine anticoagulant on ice (MAC, 0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10mM EDTA, 0.45 M NaCl, pH7.0). Hemocyte frequencies were calculated using an improved Neubauer hemocytometer. Hemolymph from the ten WT and ten GR_6 oysters collected each fortnight were pooled using equal numbers of hemocytes from each oyster. Aliquots of hemolymph from each individual were also collected. All samples were stored at -20 ^{o}C prior to 2DE analysis.

2.7. Two dimensional electrophoresis

Two dimensional electrophoresis was performed on whole hemolymph. Hemocyte lysis, protein precipitation and quantification were performed using 2-D Clean-Up kits and 2-D Quant kits according to manufacturers instructions (Amersham Biosciences, NJ, USA). Two hundred µg of precipitated hemolymph protein were re-suspended in 125 µg of a rehydration buffer containing 8 M urea, 2% 3-cholamydopropyl dimethyl-ammonio, 1-propane sulphate (CHAPS), 2.8 mg dithiothreitol (DTT), 0.002% w/v bromophenol blue and 0.5% v/v 3-10 pharmalyte or IPG buffer (Bio-Rad, Castle Hill, NSW). Samples were subject to 2DE by the method of O'Farrel et al. (1975). Proteins were separated in the first dimension by isoelectric focusing (IEF) using 7 cm immobilized pH gradient strips (IPG strips; pH 3-10; Bio-Rad). The IPG strips were rehydrated overnight with the same rehydration buffer described above. Isoelectric focusing was performed using an Amersham Biosciences IEF cell. After isoelectric focusing, the IPG strips were equilibrated for 15 min in 10 mL of reducing buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% w/v SDS, 20% v/v glycerol, 2% w/v DTT, 1% bromophenol blue) and for a further 15 min in alkylation buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% w/v SDS, 20% v/v glycerol, 2.5% w/v

idodoacetamide, 1% w/v bromophenol blue). Second dimension SDS-PAGE was performed on 15% polyacrylamide gels for approximately 2 h using Mini Protean II electrophoresis cells (BioRad).

After 2DE, protein spots were visualized by silver staining according to the method of Blum et al. (1987). Image analysis software (Progenesis; PG 240, version 2006, UK) was used to plot the position of proteins on 2DE gels and to calculate relative intensity of individual proteins. The amount of each protein was calculated as a percentage of total pixel density (normalized density), where higher normalized values indicated higher protein expression (Progenesis; PG 240, version 2006, manual, UK).

2.8. Mass spectrometry

Proteins of interest were excised from gels using sterile scalpel blades. Excised proteins were de-stained by soaking in 100 mM sodium thiosulphate and 30 mM potassium ferricyanide for 20 min before being washed with water (4x 250 µL). The proteins were then digested with 20 µL of trypsin (20 µg/mL in 100 mM ammonium bicarbonate, Promega, Annandale, NSW) overnight at 37ºC. The resulting tryptic peptides were extracted by immersing gel slices in water (50 µL), followed by 2 washes with 5% formic acid in 50% acetonitrile (75 µL) and one wash in 100% acetonitrile (75 µL) with the aid of sonication. The extracts were pooled and concentrated to 10 µL by vacuum centrifugation.

Electrospray ionization, tandem mass spectrometry (ESI MS/MS) was undertaken at the Bioanalytical Mass Spectrometry Facility (BMSF; University of NSW) and at the Australian proteome Analysis Facility (APAF), using Q-STAR and Q-TOF mass spectrometers. Peptide mass/charge ratios were compared to known proteins in the Genbank non-redundant and SWISSPROT databases using the MASCOT search engine set for carbomethylation (C) and oxidation (M), with fragment mass tolerances of ± 0.25 Da and a maximum missed cleavage of 1.

2.9. Statistical analysis

SPSS v.13 software (SPSS Inc. 2004) was used for statistical analyses. Differences in mean values for protein expression and hemocyte frequencies were tested for statistical significance by one-way ANOVA, whilst differences in the presence or absence of proteins in infected and uninfected oysters were assessed using Fisher's exact two-tailed test. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Identification of M. sydneyi infection by PCR and cytology

No 16S ribosomal *M. sydneyi* DNA was detected in WT or GR_6 oysters by PCR before oysters were transplanted to the Georges River on 24 December, 2006 (T0; Figure 1B). *M. sydneyi* DNA was first detected in both the gut and gills of WT oysters at T2 (4 February, 2007; Figure 1B). GR_6 oysters first tested positive for *M. sydneyi* DNA in their gills at T4 (2 March, 2007) and in the guts at T5 (19 March, 2007) (data not shown). Seventy percent of WT oysters were positive for *M. sydneyi* DNA in gill tissue at T3 (14 February, 2007; Figure 1A). All gill samples from WT and GR_6 oysters were PCR-positive for *M. sydneyi* between T4-T10 (2 March, 2007 - 24 May 2007), except for T7 (16 April, 2007) when 3 GR_6 oysters tested negative (data not shown).
Cytological examination of gut tissue imprints from WT and GR_6 oysters first detected *M. sydneyi* sporonts at T8 (1 May 2007; Figure 1C). One out of the ten GR_6 oysters was positive for *M. sydneyi* sporonts at this time, compared to 2 of the 10 oysters from the WT population. In total, *M. sydneyi* sporonts were detected in the guts of five WT and one GR_6 between T8 and T10. Twenty out of 42 oysters sampled from the Macleay River in August, 2007, had *M. sydneyi* sporonts in their gut tissues.

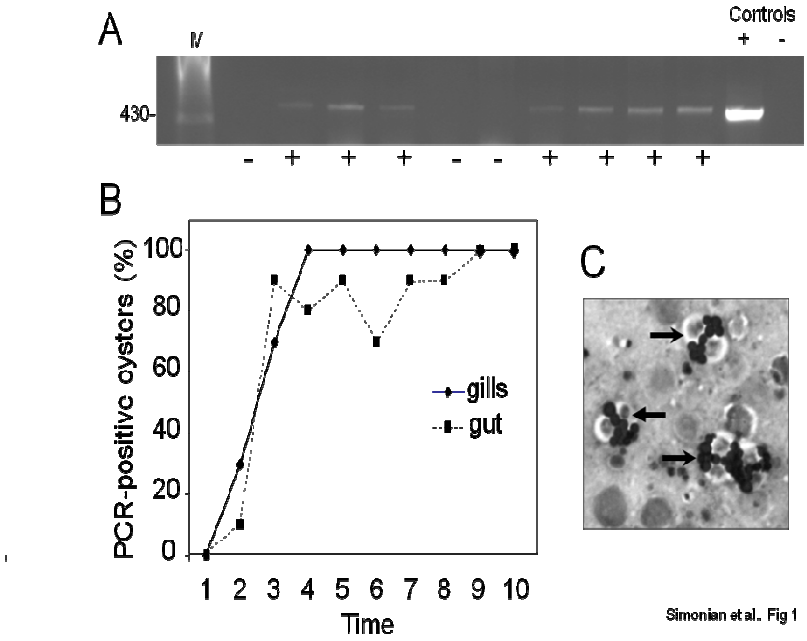


Figure 1. Diagnosis of *M. sydneyi* infection. **A**. 16S *M. sydneyi* rDNA products from WT oysters collected from the Georges River at T3. "+" and "-" identify oysters that were designated PCR - positive or - negative. **B**. Frequency of WT oysters that were identified as PCR-positive or - negative for 16S *M. sydneyi* rDNA in the gills or guts of oysters collected from the Georges River at ten time points over the period Dec, 2006 (T0) - May, 2007 (T10). n = 10 for each time point. **C**. Imprints of oyster gut tissue stained with modified Wright's stain showing *M. sydneyi* sporonts (arrows).

3.2 Hemocyte frequencies

Hemocyte frequencies among both GR_6 and WT oysters were relatively stable at approximately 5 $\times 10^5$ hemocytes/mL between T0 and T5 (Figure 2). From T5 (19 March, 2007) onward, hemocyte frequencies increased, reaching a maximum of between 1.5×10^6 and 2.0×10^6 hemocytes/mL by T10 ($p < 0.05$ vs. T0 for both WT and GR_6 oysters).

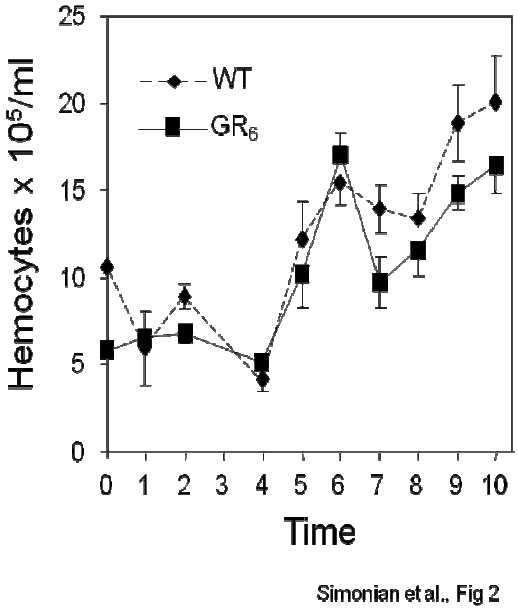
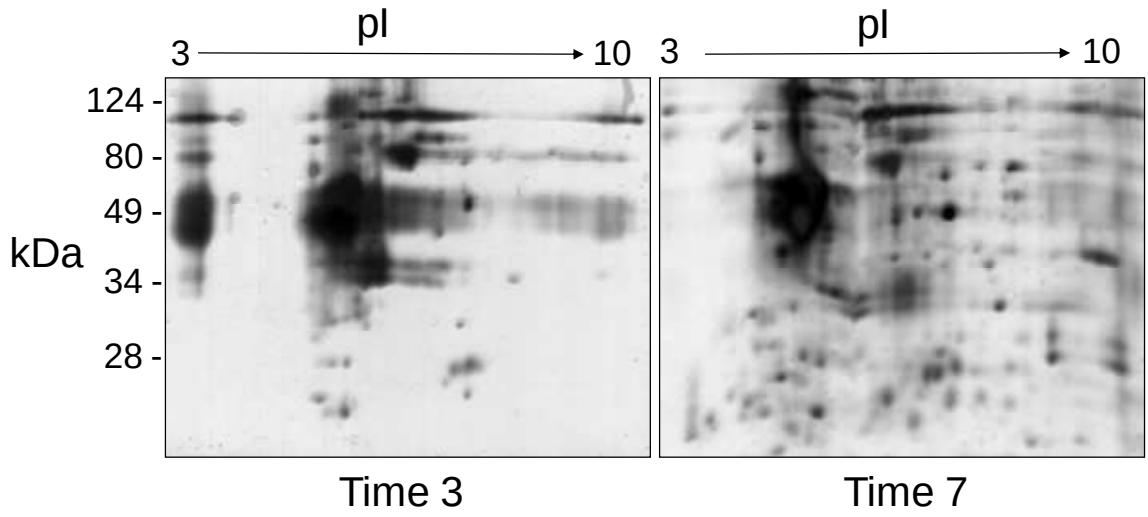


Figure 2. Frequencies of hemocytes in hemolymph of the wild type (WT) and GR_6 oysters collected at various times during the field trial in the Georges River. n = 10 at each time point.

3.3 Comparative proteome maps of WT and GR_6 *oysters*

Proteome maps of pooled hemolymph from WT and GR_6 were similar between T0 and T4 (10 Jan - 2 March 2007). During this period, image analysis identified 61 protein spots on 2DE gels of GR_6 oysters and 72 protein spots in the hemolymph of WT oysters. Additional proteins became visible on 2DE gels of both WT and GR_6 oysters from T5 (19 March 2007) onward. For instance, at T7 (16 April 2007), 136 protein spots were visible on 2DE gels of GR_6 oysters and 122 protein spots were detected in WT oyster hemolymph (Figure 3).

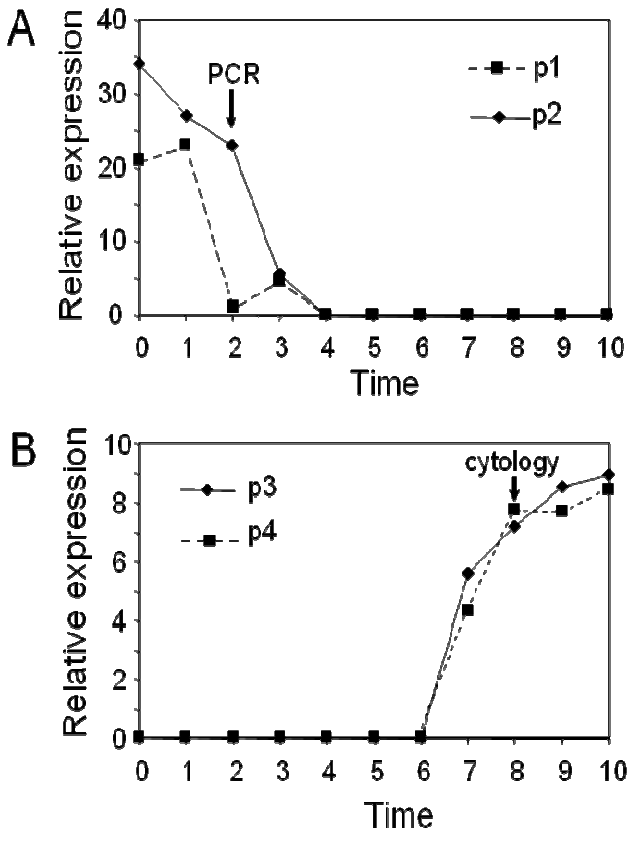
Four proteins (designated p1, p2, p3 and p4) were of particular interest because their expression changed from present to absent, or visa versa, shortly after *M. sydneyi* infection was detected either by PCR or cytological analysis (Figure 4). Protein 1 and p2 had molecular weights of 55 kDa and 49 kDa respectively, with pIs of approximately 3 (Table 1). Protein 3 and p4 had molecular weights of approximately 47 kDa and pI's of 5.8 and 5.9, respectively.



Simonian et al., Fig 3

Figure 3. Proteome maps comparing protein expression in the hemolymph of 10 WT oysters collected from the Georges River at times 3 (T3; 14 Feb, 2007) and 7 (T7; 16 April 2007).

Figure 4. Relative expression of four proteins (p1, p2, p3 and p4) that appeared to be associated with *M. sydneyi* infection. **A**. (p1 and p2) ; **B**. (p3 and p4). Expression intensity values are for hemolymph from 10 oysters pooled at each time points over the period Dec, 2006 (T0) - May, 2007 (T10). The time points at which *M. sydneyi* infection was first detected by PCR or cytology are shown by arrows.



Simonian et al., Fig 4

The relative expression of p1 and p2 decreased from approximately 20 at T0 and T2, to zero by T4 (Figure 4A). This was during the period when oysters started testing positive for *M. sydneyi* DNA by PCR. In contrast, the relative expression of p3 and p4 increased from zero to 5.2 and 4.3 respectively between T6 and T7. This was immediately before *M. sydneyi* sporonts were first detected in the gut of oysters by cytology (Figure 4B). Proteins p3 and p4 continued increasing up to 8.9 and 8.4 respectively by T10.

The relationships between these four proteins (p1, p2, p3 and p4) and sporulating *M. sydneyi* infections were further tested by analyzing proteome maps of whole hemolymph from individual oysters, as opposed to pooled samples. Nine oysters (four WT, one GR_6 and four MR) with sporulating *M. sydneyi* infections were compared to an equal number of oysters sampled at the same time points that had tested negative for *M. sydneyi* spores by cytology. Comparison of proteome maps for these individual oysters revealed no clear relationship between p1 or p2 and sporulating *M. sydneyi* infection. Among the nine oysters with sporulating *M. sydneyi*, four expressed both p1 and p2, one expressed only p2 and one expressed neither p1 nor p2. In comparison, three of the oysters that did not have sporulating *M. sydneyi* infections expressed both p1 and p2, whilst the remainder did not express either protein. Chi-squared analysis reveal no significant association between sporulating infection and the expression of either p1 or p2, *($p>0.05$*; Table 1).

In contrast to p1 and p2, there was an absolute relationship between sporulating *M. sydneyi* infection and the expression of p3 and p4 (Figure 5; Table 1). All nine oysters in which sporulating *M. sydneyi* infections were detected by cytology expressed both p3 and p4, whilst none of nine spore-negative oysters sampled at the same time points expressed these proteins *($p<0.05$).*

Comparisons of the proteome maps for individual oysters also identified two additional proteins (designated p5 and p6) with expression patterns that were significantly

Table 1. Relationships between the six proteins identified in proteome maps of oyster hemolymph that were strongly associated with *M. sydneyi* infection. The data shown here describe the basic physicochemical characteristics of the proteins (molecular weight and pI), and their levels of expression (presence/absence, +/-) in oysters with or without sproulating *M. sydneyi* infections (n = 9 for each category).

Protein	Molecular weight (kDa)	pI	expression	Sporonts in gut (number of oysters)	No sporonts in gut (number of oysters)	Fishers exact test 2-tail *P*
p1	55	3.0	+	2	3	
			-	5	4	0.5
p2	49	3.0	+	4	3	
			-	3	4	0.5
p3	47	5.8	+	9	0	
			-	0	9	2.1E-5
p4	47	5.9	+	9	0	
			-	0	9	2.1E-5
p5	42	5.8	+	7	0	
			-	2	9	0.001
p6	42	5.9	+	7	0	
			-	2	9	0.001

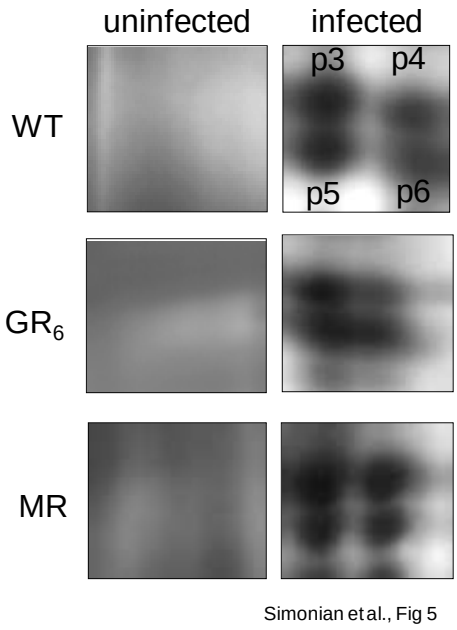


Figure 5. Cropped images of 2DE proteome maps comparing the expression of p3, p4, p5 and p6 in oysters with and without sporulating *M. sydneyi* infections. WT, wild type; GR_6, selectively bred QX disease resistant ; MR, Macleay River. Each panel shows expression in a single oyster.

correlated to sporulating *M. sydneyi* infection. p5 and p6 had comparable pIs to p3 and p4 (5.8 and 5.9 respectively), but lower molecular weights (42 kDa; Figure 6). Both p5 and p6 were present in five out of the nine oysters with sporulating *M. sydneyi* infections, but they were not detected in any oysters that tested negative for sporulating infection by cytology ($p<0.05$; Figure 5; Table 1).

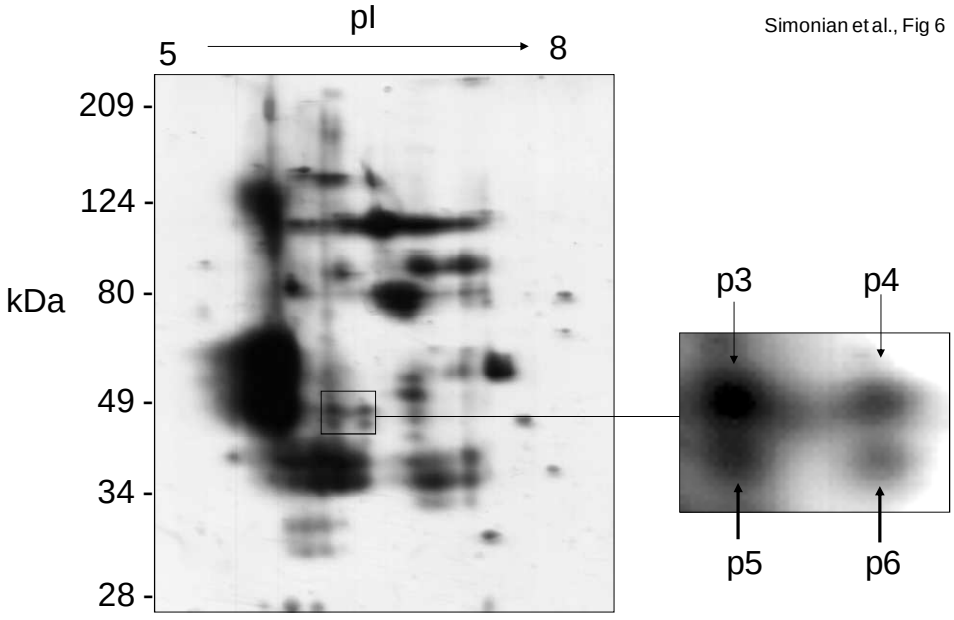


Figure 6. 2DE proteome map of hemolymph from an oyster with sporulating *M. sydneyi* infection. The inset shows the four proteins (p3, p4, p5 and p6) that were strongly associated with sporulating infection.

3.5. Mass spectometry

Mass spectrometric analysis of the four proteins that were associated with sporulating *M. sydneyi* infection (p3, p4, p5 & p6) failed to identify peptides with significant homology to other known proteins in the Genbank non-redundant and SWISSPROT databases.

4. Discussion

The goal of this study was to identify proteins that act as markers of sporulating *M. sydneyi* infection in *S. glomerata*. We sought to provide the Sydney rock oyster industry with a reliable and inexpensive assay of sporulating infection upon which to base decisions regarding the translocation of oysters from QX-prone to QX-free growing areas. *M. sydneyi* appears to be an endemic parasite of Sydney rock oysters. Its presence in oysters does not always result in the development of QX disease. *M. sydneyi* can be detected by PCR in oysters throughout their growing range, not just in areas where QX disease outbreaks occur. Sporulation is more reliable marker of QX than PCR because it corresponds with the disease state, rather than simply reflecting the presence of *M. sydneyi*.

The first significant observation from the current study is the lack of a direct relationship between *M. sydneyi* infection detected by PCR and the subsequent development of sporulating parasites that is associated with QX disease. All oysters from the field trial in the Georges River were PCR-positive for *M. sydneyi* in their gills by T4 (2 March, 2007), and in their guts by T9. However, only six oysters with sporulating *M. sydneyi* were identified by cytological analysis during the course of the field trial in the Georges River. This agrees with previous analyses, which have shown that *M. sydneyi* infection does not necessarily result in sporulating infections resulting in QX disease. The parasite has been detected by PCR analysis in estuaries from the NSW/Victorian border to Moreton Bay in Queensland. However, QX disease occurs in only a few of these estuaries. Within NSW, *M. sydneyi* was identified by PCR analysis in as many as seven in which QX disease outbreaks had not occurred (Adlard and Wesche 2004). This supports the contention that QX is an opportunistic disease that occurs only when the immune system of oysters, specifically their phenoloxidase cascade, is suppressed by environmental factors, such as low salinity associated with heavy rainfall (Peters and Raftos, 2003; Butt et al., 2006; 2007).

Since there seems to be little relationship between the detection of *M. sydneyi* infection by PCR and the cytological identification of sporulating QX disease, it is not surprising that the expression of two proteins (p1 and p2), that at first appeared to be associated with the PCR-based detection of *M. sydneyi* infection, was not correlated with sporulating infection. In pooled hemolmyph samples, the expression of p1 and p2 was significantly down-regulated immediately prior to the detection of *M. sydneyi* by PCR. However, analysis of individual oysters showed that there was no significant relationship between the expression of these proteins and the presence of sporulating *M. sydneyi*.

Changes in the expression of p1 and p2 are probably indicative of normal physiological variability in oysters that occurred during the course of the field trial conducted in the Georges River. Substantial variation, not associated with QX disease, was evident during the field trial. More proteins were detected by 2DE in both the wild type and GR_6 populations over time and there was a substantial increase in the frequency of hemocytes in the hemolymph. This suggests that seasonal factors have a significant influence on both hemocyte frequencies and protein expression. Such results are in accordance with data obtained previously, which shows that total hemolymph protein concentrations and hemocyte frequencies have varied over time (Peters and Raftos 2003; Butt and Raftos 2007; Duchemin et al. 2007; Samain et al. 2007).

Even though none of the proteins identified on proteome maps were associated with PCR-based detection of *M. sydneyi*, strict correlations were observed between protein expression and the occurrence of sporulating infections. The expression of four proteins (p3, p4, p5 and p6), with molecular weight ranging from 42-47 kDa and pI's of 5.8-5.9, was strongly associated with sporulation in the gut (Figure 5 & 6). The sample sizes for comparison between oysters with sporulating infections and those without were low (n = 9 for both categories), primarily because infection in the Georges River during this trial was less intense than in previous years. However, the relationships between the expression of p3, p4, p5 and p6 were still highly significant, particularly for p3 and p4.

The expression of p3 and p4 in pooled samples of 10 oysters increased significantly immediately before sporulating *M. sydneyi* were detected in the gut (Figure 4B). Subsequent analysis of individual oysters confirmed that p3 and p4 were expressed only in oysters from the Georges River trial with sporulating infections. None of the individuals without sporulating *M. sydneyi* tested at the same time points expressed p3 or p4.

Significant changes in the expression of p5 and p6 were not detected in pooled samples at the time of sporulation, probably because the association between the expression of these two proteins and sporulating infection was less strong than for p3 and p4. Protein 5 and p6 were expressed in most (5/7) but not all oysters with sporulating *M. sydneyi*, but were entirely absent from oysters (9/9) without sporulating infections.

A similar pattern was evident in proteome maps of oysters from the Macleay River. All oysters with sporulating infections from the Macleay River expressed p3, p4, p5 and p6 at high levels, whilst these proteins were absent from all of the non-sporulating oysters tested.

In our previous studies of *M. sydneyi* infection, we have shown that the activity of the defensive enzyme, phenoloxidase, is suppressed in oysters immediately before the detection of sporulating parasites (Peters and Raftos 2003; Butt et al. 2006; Butt and Raftos, 2007). In the Georges River, this suppression was strongly associated with decreases in salinity associated with heavy summer rainfall (Peters and Raftos 2003; Butt and Raftos, 2007). However, phenoloxidase was not identified as a marker of infection in the current study. There are two explanations for this. Data from other experiments suggests that phenoloxidase is only expressed at low levels in *S. glomerata* hemocytes (unpublished data), and so may have been below the limits of sensitivity of 2DE. Alternatively, while the enzymatic activities of phenoloxidase are substantially decreased at the time of sporulating infection, expression levels for this protein may remain unchanged.

The nature of the proteins associated with sporulating infection remains unclear because mass spectrometric analysis failed to identify any homologues in the available databases. This is not surprising since there are very little sequence data available for *S. glomerata* genes and proteins. There are large expressed sequence tag databases available for oysters of the genus *Crassostrea*, but our experience suggests that mass spectrometric analysis of *S. glomerata* only provides matches to the *Crassostrea* databases in about 50% of cases (Simonian et al. 2007, in press). All four proteins had similar molecular weights and isolectric points (42 or 47 kDa; pI 5.8 or 5.9), suggesting that they may be related to each other.

Given the lack of positive identification, it is impossible to tell whether the proteins that were strongly associated with sporulating infection were derived from the parasite itself, or were oyster proteins expressed in response to the infection. Attempts to extract proteins from purified parasites in sufficient quantities for 2DE were not successful (data not shown). So it was not possible to test whether p3, p4, p5 and p6 were derived from *M. sydneyi* as opposed to oysters.

Regardless of their origin, these proteins (particularly p3 and p4) seem to represent potential markers of sporulating *M. sydneyi* infection and so may be effective candidates for protein based diagnostic assays. Our next step will be to develop rapid assays for the expression of p3, p4, p5 and p6 so that their expression can be tested in far larger numbers of oysters with and without sporulating infections in an effort to validate their use as infection markers. These rapid tests will be based on enzyme-linked immunosorbent assay (ELISAs) using antibodies against p3, p4, p5 and p6. Such ELISA assays might also provide a basis for the development of rapid diagnostic tests for sporuating *M. sydneyi* infection that can be used by the oyster industry. Another key factor that we will test is whether the expression of these proteins is specific to sporulating *M. sydneyi* infections, or if they represent more generic markers of disease that might respond to a range of different pathogenic infections.

Acknowledgments

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4.9 Asssessing the potential for cryopreservation of Sydney rock oyster gametes

As part of Seafood CRC/FRDC program 2006/226, cryopreservation techniques suitable for Sydney rock oysters (SRO) have been investigated by NSW DPI in collaboration with Dr Serean Adams (Cawthron Institute, New Zealand). This work demonstrated that cryopreservation of SRO sperm and eggs are possible and show significant potential for integration into future SRO breeding programs. Reliable techniques for sperm have been developed and cryopreserved eggs have been successfully fertilised.

A major outcome from this work is a 'standard operating procedure' (SOP) for cryopreservation of SRO sperm based on the most successful method developed to date. The most effective cryoprotectant (CPA) was 1.0 M dimethyl sulphoxide combined with 0.08-0.51 M trehalose and sperm is frozen by holding 3 cm above liquid nitrogen for 10 minutes followed by plunging directly into liquid nitrogen. At least 1,000 cryopreserved sperm should be added for each egg to ensure maximum rates of fertilisation.

Cryopreserved sperm from a single male has been used to produce D-veliger stage larvae. Fertilisation success of cryopreserved eggs has ranged between five and nine percent. However, future research direction has been identified to improve cryopreservation techniques for SRO eggs which is expected to increase fertilisation rates. This research will investigate the freezing and cooling method along with different CPAs, CPA concentrations and diluents. The progress in cryopreservation techniques for SRO so far is significant, particularly for use in future SRO breeding programs. Irrespective of further developments in cryopreservation of SRO eggs, the methods developed to date will deliver a number of safeguards for future breeding programs and allow breeding and hatchery procedures to be operated more efficiently.

We are currently awaiting the outcomes of a CRC communal project (2008/733) looking at potential cryobanking service options.

The full Cryopreservation report has been appended to this report (Appendix 10.4)

4.10 Asssessing the Potential for Heat/Pressure Induction of Triploidy in Sydney rock oysters

1. Introduction

Production of the Sydney rock oyster (*Saccostrea glomerata*) has been in decline since the mid 1970s and has resulted, among other things, from disease and competition by faster growing species (White, 2001). A breeding program for *S. glomerata* was established by Industry & Investment NSW in 1990 to improve growth rates, increase the length of the marketing season and enhance disease resistance. The success of the program has seen the production of oysters that are ready for market 12 mo earlier and have a high level of resistance to QX disease and winter mortality. The program is now focused on further enhancing the existing breeding lines and improving marketability traits.

The induction of triploidy in edible shellfish has become a popular tool to increase growth rates and lengthen the marketing season of stock (Nell, 2002). Additional benefits in disease resistance have been observed, including reduced losses in *S. glomerata* caused by the disease winter mortality (Hand et al., 1998). Triploid oysters can be produced by inhibiting meiosis I or meiosis II using a chemical or physical stress (Nell, 2002). The chemicals cytochalasin B (CB) and 6-dimethylaminopurine (6-DMAP) have been the primary agents used to inhibit meiosis in oyster eggs and while they continue to be successful, the toxicity of CB in particular has raised health concerns (Guo et al., 1994). It is considered that a physical stress such as heat shock or hydrostatic pressure offers a more user friendly method of triploidy induction and would be more suitable for commercial hatchery use.

Heat shock and hydrostatic pressure have both been employed to inhibit polar body extrusion in a range of shellfish species with varying success. Beaumont and Fairbrother (1991) summarised ploidy manipulation in shellfish using physical shock and reported that temperatures ranging from 25-38°C were successful for triploidy induction, while hydrostatic pressure levels ranging from 6000-8000 psi produced the best results.

The success of triploid inductions is most easily determined using flow-cytometry (FCM). Access to a flow-cytometer can sometimes be limited which creates a need for suitable preservation techniques of larval samples. Yang et al., (2000) outlined a method for preserving Pacific oyster gill tissue, which included a pre-treatment in a hypotonic solution (0.075M KCL solution) followed by fixation in 70% ethanol, however attempts to preserve SRO larvae for FCM using this technique have so far been unsuccessful.

This study aims to: 1) investigate the success of a range of temperature shocks and hydrostatic pressures on triploid induction in the Sydney rock oyster (*Saccostrea glomerata*) and 2) develop techniques for preserving SRO larvae for analysis using FCM.

2. METHODS

2.1 General methods

Trials were conducted at the Port Stephens Fisheries Institute (PSFI) using wild *S. glomerata* broodstock sourced from various NSW estuaries. Broodstock were strip spawned at the PSFI mollusc hatchery and gametes were rinsed and screened through a 100µm sieve to remove shell fragments. The sperm was then placed immediately on ice and the eggs were left to water harden for a minimum of 1 h. Egg suspensions were fertilised with 10^6 sperm ml^{-1}.

After each trial, a subsample (50%) of larvae (24 h post fertilisation [HPF]) from each replicate was transferred to a piece of 20 µm nylon mesh and packaged in a 10 ml vial which contained only tissue paper dampened with filtered seawater. The vials were then packed on ice and sent to a laboratory for ploidy testing the following day (48 HPF) using flow cytometry (FCM). The remaining larvae (50%) were incubated until 48 HPF and were then preserved with 10% formalin and seawater and assessed for the proportion of abnormalities at the D-veliger stage.

2.2 Temperature shock trials

Temperature shock trials were conducted in an aluminium block heated at one end by recirculating heated water and cooled at the opposite end by recirculating chilled water, the apparatus is described by Dove and O'Connor (2007). Sample jars (120 ml) containing 30 ml of filtered seawater were inserted into the block to equilibrate seawater to each of the treatment temperatures before the trial began. Embryos (10,000) were stocked into the treatment vials 15 minutes post fertilisation (MPF), and after 20 min of treatment time the vials were removed from the aluminium block and filled with 25°C filtered seawater to immediately reduce the temperature of the treatment water. Experimental jars were then transferred to an incubator and maintained at 25°C.

2.3 Hydrostatic Pressure trials

Pressure trials were conducted using a 600 ml hydrostatic pressure chamber (Aquatic Ecosystems, Inc. Florida, USA). Embryos (15 MPF) were placed in a 70 ml sample jar filled with filtered seawater (25°C), which was fitted with a 20µm mesh lid to prevent embryos from escaping while allowing water to move freely in and out of the sample jar. The sample jar was then submerged into the chamber containing filtered seawater. The desired pressure was applied to the chamber using a 12,000 kg hydraulic shop press for 10-20 minutes. Sample jars were then removed from the chamber, embryos were transferred to a 120ml sample jar, topped up with filtered seawater and incubated at 25°C.

2.4 Sydney rock Oysters

2.4.1 Sydney rock oyster temperature Trial 1

Temperature Trial 1 was designed as a 1-factor experiment to determine the effects of temperature shock on triploid induction in the SRO. Embryos (15 MPF) from three mating pairs (fertilised at 25°C) were exposed to heated seawater (25, 31, 32, 33, 34, 35 or 36°C) for 20 minutes. Each treatment was replicated 3 times.

2.4.2 Sydney rock oyster temperature Trial 2 – Magnitude of temperature change

Results from Temperature Trial 1 indicate that a temperature shock of 36°C was effective in inducing triploidy in SROs. Temperature Trial 2 was a 2-factor experiment to determine whether the temperature shock (i.e. the magnitude of temperature change from 25 to 36°C [11°C change]) or the treatment temperature was responsible for inducing triploidy . Three of the nine mating pairs were fertilised at each of 18, 21 or 25°C, and fertilised eggs (15 MPF) from each pair were then exposed to heated seawater (32, 34 or 36 °C) for 20 minutes.

2.4.3 Sydney rock oyster pressure Trial 1 (3 attempts)
Pressure Trial 1 was designed as a 1-factor experiment to determine the effects of 3 hydrostatic pressures (5000, 6000, 7000 psi) on triploid induction in the SRO. Embryos (15 MPF) from three broodstock pairs were exposed to each pressure level for 20 min (attempt 1 and 2) or 10 min (attempt 3), with the result that each treatment was replicated 3 times and water hardening times varied from 1-4 h.

2.4.5 Sydney rock oyster - preservation for flow-cytometry
The preservation trial was designed as a 2-factor experiment to determine an appropriate technique for preserving SRO oyster larvae (24 HPF) for flow cytometry (FCM). Firstly, 12 groups of 10,000 oyster larvae (diploid and obtained by strip spawning wild SRO broodstock) were exposed to a pre-treatment of 0.075M KCl solution (hypotonic solution) or seawater (control). Each batch of larvae was then preserved using one of six preservation techniques: 1) Carnoy's fixative 2) 10% formalin and seawater, 3) Davidson's fluid, 4) 70% Ethanol, 5) an ascending ethanol series (20, 50 and 70% ethanol; EtOH series a), 6) an ascending ethanol series, followed by a descending ethanol series (70, 50, 20% ethanol; EtOH series b). Before analysis with FCM, larvae were rinsed with sterilised seawater. The experiment was replicated 3 times with 3 different broodstock mating pairs.

3. Results

3.1 Sydney Rock Oysters

3.1.1 Sydney rock oyster temperature Trial 1
Analysis by FCM produced diploid peaks for all treatment replicates and a triploid peak for one replicate (no % recorded), which was exposed to heated seawater of 36°C. No significant differences between the treatments existed for the mean percent development, which ranged from 4.7 ± 1.5 % to 11.1 ± 4.7 % (mean ± SEM) (Figure 1.). However, the replicate that produced a triploid peak (exposed to 36°C seawater) had no survival after incubation to 48 HPF.

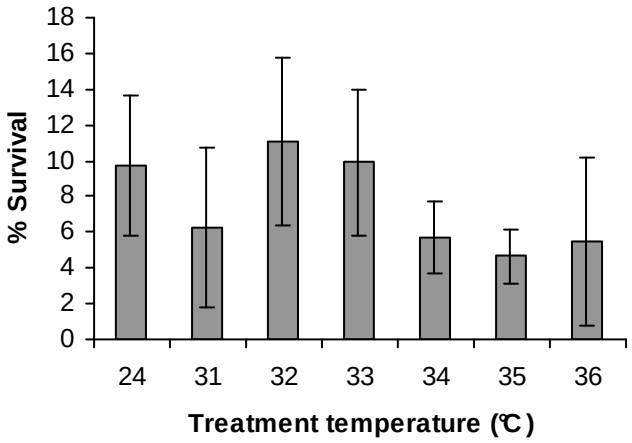

Figure 1. Percent survival (mean ± SEM; n=3) of SRO D-veliger larvae (48 h post fertilisation) exposed to heated seawater 15 minutes post fertilisation.

3.1.2 Sydney rock oyster temperature Trial 2 – Magnitude of temperature change
Analysis by FCM detected diploid animals for all treatment replicates (Figure 2). Triploid larvae were detected in samples fertilised at 25°C and exposed to a heat shock of 32, 34°C and 36°C; and in larvae fertilised at 21°C and exposed to a heat shock of 34°C and 36°C (Figure 2). Pentaploid animals were detected in larvae fertilised in 18°C and exposed to a heat shock of 36 °C; and in

larvae fertilised in 25°C and exposed to a heat shock of 36°C (Figure2). There was no significant difference between development for any of the treatments (Figure3.).

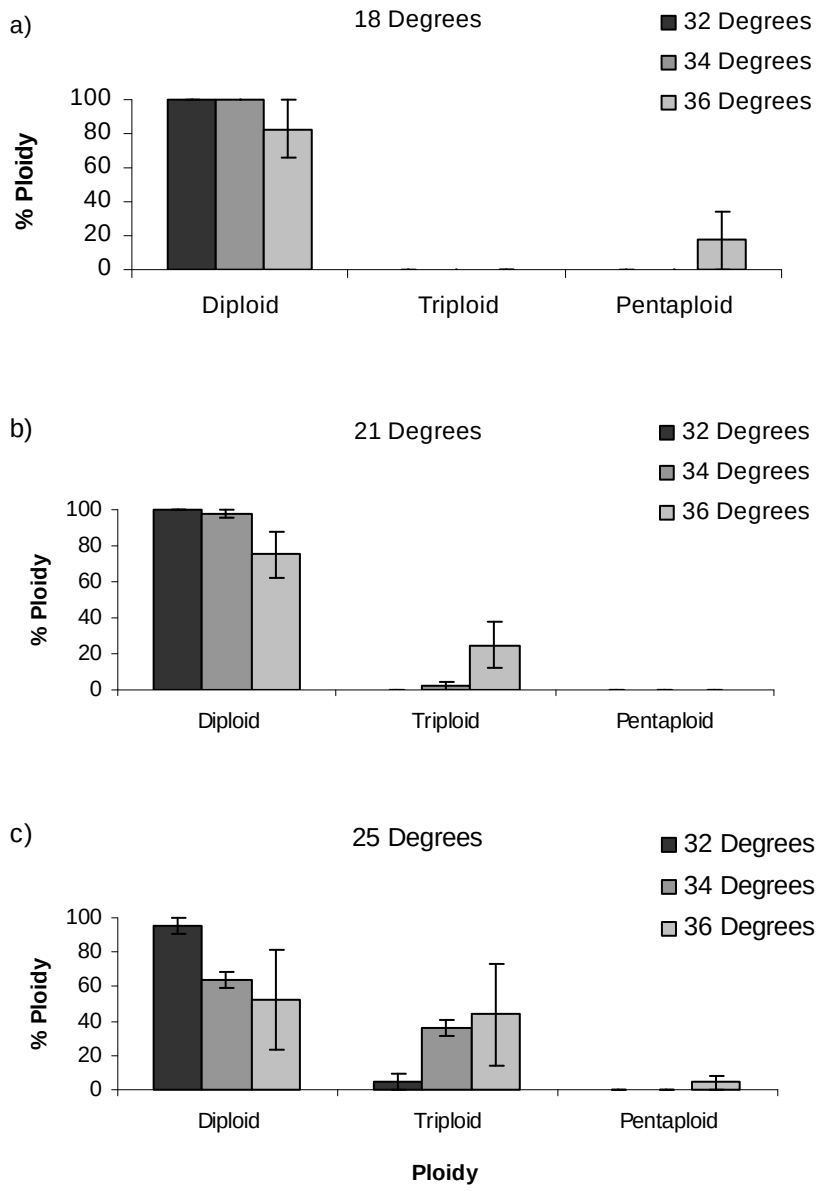

Figure 2. Percent ploidy (mean ± SEM; n=3) of SRO larvae fertilised in seawater of 18, 21 or 25°C (Figures a, b, c, respectively) and exposed to a heat shock of 32, 34 or 36°C, 15 minutes post fertilisation.

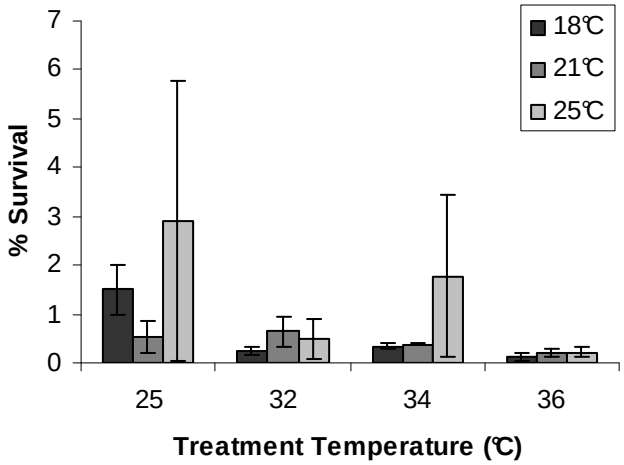

Figure 3. Percent survival (mean ± SEM; n=3) of SRO larvae fertilised in seawater of 18, 21 or 25°C and exposed to a heat shock of 32, 34 or 36°C, 15 minutes post fertilisation.

3.1.3 Sydney rock oyster pressure trial

In the first attempt of the pressure trial, one replicate of the 5000 psi hydrostatic pressure treatment produced a small amount of triploid SRO larvae (no % recorded). In the second attempt, one replicate of the 6000 psi hydrostatic pressure treatment produced triploid SRO larvae (14.5 % triploid). In the third attempt, each hydrostatic pressure level produced triploid larvae (Figure 4). 1-factor ANOVA determined no significant differences between the percentages of triploid larvae detected in the third attempt, however the survival data has not yet been analysed.

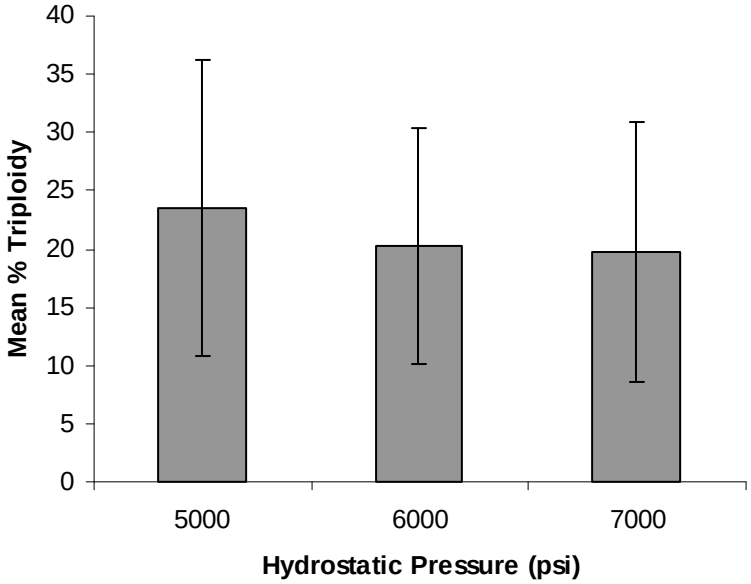

Figure 4. Percent triploidy (mean ± SEM; n=3) of SRO larvae exposed to hydrostatic pressure (15 MPF) of 5000, 6000 or 7000 psi for 10 min.

3.1.4 Sydney rock oyster - Preservation for flow-cytometry

Only one peak was detected in larvae that were exposed to a saltwater pre-fixation treatment, those larvae were fixed with an ascending ethanol series (EtOH series a). The presence of a diploid peak was more frequent for samples pre-treated with the KCl hypotonic solution. All of the fixative treatments exposed to the KCl pre-treatment contained replicates that produced a diploid FCM peak, except for those treated with Davidson's fluid (Figure 5). However, no statistically significant differences were detected between the treatments.

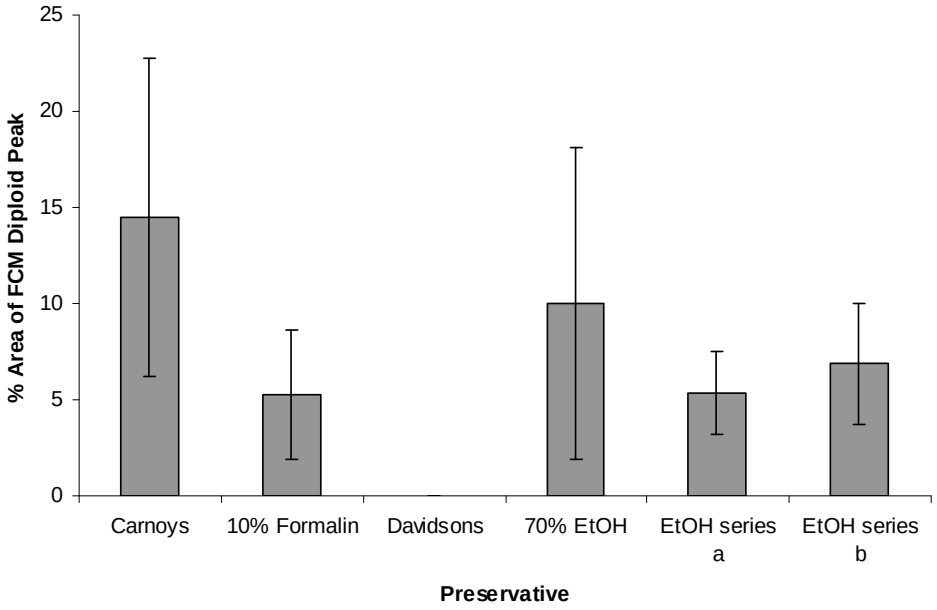

Figure 5. Percent of area (mean ± SEM; n=4) of the FCM diploid peak for larvae preserved with Carnoys fixative, 10% formalin, Davidsons fluid, 70% ethanol, an ascending ethanol series (EtOH series a) or an ascending ethanol series followed by a descending ethanol series (EtOH series b).

4. Discussion

So far this study has determined that triploid induction in the SRO can be achieved using temperature shock and hydrostatic pressure. However, results have been variable and further investigations are required. Preliminary results suggest that temperatures ranging from 34 to 36°C and hydrostatic pressures of 5000-7000psi can induce triploidy in the SRO; however development is usually poor. Some problems identified likely to cause the variability observed in results include a lack of synchronous development and poor development rates, which is common for strip spawned SRO gametes.

A method for preserving SRO larvae for analysis using FCM was investigated. Results suggest that samples must be pre-treated in a hypotonic solution (0.075 M KCl) before preservation, which was also reported by Yang et al., (2000). The only preserved samples not to produce a FCM diploid peak were those treated with Davidsons's fluid. No significant differences have been detected between the remaining preservative types; however it is likely that 70% ethanol will be the safest and most suitable preservative for hatchery operators to use. This preservation technique has been trialled with limited success and could be the result of a reduced sensitivity of the test using preserved samples and, therefore, a larger volume of sample material is required to detect a result. This procedure requires further investigation.

Future experiments will investigate ways of increasing survival in triploidy trials, the number of preserved larvae required for detection using FCM and the optimum temperature and pressure that repeatedly produce the largest percentage of triploid oysters.

5. BENEFITS

The SRO industry is now seeing the benefits of research conducted over the past 15 years. However, The Select Oyster Company (SOCo), which will manage those benefits, is still in its development phase. The Seafood CRC, FRDC and I&I NSW acknowledge the importance of SOCo in securing the future viability of the industry. As a result, they are supporting the preparation of a plan to guide SOCo over the next decade. The research described here will provide critical information for SOCo's management. It will allow protocols in the management plan to be adapted over time as new findings become available. The outcomes of this work will underpin SOCo's ongoing capacity to manage SRO breeding on behalf of industry as well as providing the family lines that will form the basis of the future breeding program, significantly reducing the start-up cost to industry.

There are a number of specific benefits arising.

1. The inclusion of pair mated family lines within the program will overcome potential inbreeding risks identified in previous reviews of SRO breeding (Benzie Report).
2. Tools are in place to use marker assisted selection to accelerate selection for disease resistance in SRO, which could be of significant assistance as the number of traits under selection increases.
3. SOCo's ability to further the breeding program through alternate breeding strategies has been enhanced and greater levels of genetic security through cryopreservation of material is now possible.
4. During the course of this research, "marketability" of oysters has been highlighted as a key factor for ongoing breeding program development within Australa. The family lines developed in this program will form the basis of proposed assessments of the potential to select for marketable traits to be undertaken under the auspices of the Seafood CRC.

Overall the major beneficiary of this program will be NSW, as it is the focus for the Sydney rock oyster industry, but SRO are farmed in QLD and their farmers have access to SOCo hatchery produced lines and the benefits that ensue.

6. FURTHER DEVELOPMENT

From inception this program was designed to enhance Sydney rock oyster breeding through the development of a series of complementary technologies that targeted shortfalls in our understanding of production techniques or specific opportunities to improve breeding. Accordingly the work has highlighted a number of potential areas for further development.

Studies of mechanisms underlying resistance to QX disease have had significant implications for our understanding of disease resistance in molluscs. While this understanding is already being harnessed to address disease issues in other species, further opportunities exist to build on this work with SRO. Beyond QX, the NSW oyster industry also suffers significant losses from other diseases, most notably "winter mortality"(WM). The proteomic techniques developed and applied here could be adapted to focus on WM. Winter mortality resistant oyster lines exist and thanks to this program so to do pair mated winter mortality resistant lines. Collectively these resources could become the basis for further research.

Work on cryopreservation of SRO gametes demonstrated feasibility of the process and showed significant potential for integration into future SRO breeding programs. Reliable techniques for sperm were developed and cryopreserved eggs have been successfully fertilised. While the latter is encouraging, improvement of cryopreservation techniques for SRO eggs to increase fertilisation rates has been identified as an area for future research. This research would investigate the freezing and cooling method along with different CPAs, CPA concentrations and diluents. Additionally, the SRO industry and many others could benefit from the development of avenues for centralised storage of cryopreserved material. Whether it be an aquaculture dedicated facility, or perhaps more likely, access to an existing facility serving other agricultural sectors, remains to be evaluated, but this question is being considered in a current CRC project investigating potential for a communal cryobanking project (2008/733).

Triploid induction trials in the SRO were undertaken using temperature shock and hydrostatic pressure; however, results were variable and further investigations are required. Preliminary results suggest that temperatures ranging from 34 to 36°C and hydrostatic pressures of 5000-7000psi can induce triploidy in the SRO, but development is usually poor. Some problems identified likely to cause the variability observed in results include a lack of synchronous development and poor development rates, which are common for strip spawned SRO gametes. Future experiments will investigate ways of increasing survival in triploidy trials and the optimum temperature and pressure that repeatedly produces the largest percentage of triploid oysters.

As indicated earlier, during the course of this research, "marketability" of oysters was highlighted by the Australian Oyster Consortium as a key factor for ongoing breeding program development. The family lines developed in this program will form the basis of proposed assessments of the potential to select for marketable traits to be undertaken under the auspices of the Seafood CRC. This research is intended to:

1) Establish heritability estimates for market condition, reproductive condition and non-specific mortality.

2) Inclusion of market condition and survival (in terms of non specific mortality) in breeding programs

3) Definition of the relationship between growth and the attainment of market condition, and refinement of the Pacific oyster Economic Weights Model (CRC 2006/227).

4) Establishment of rapid and low cost methods for chemical analysis of Pacific and Sydney rock oysters using Near Infra-red Spectroscopy (NIRS) for use in this and other projects.

5) Comparison of sensory attributes of selected and standard commercial Pacific and Sydney rock oysters.

7. PLANNED OUTCOMES

Each of the planned outcomes of this program has been completed:

Initially, the SOCo Breeding plan has been amended, through the addition of a comprehensive manual, to show the protocols for pair mating and the incorporation of QX resistance through phenoloxidase genotype selection.

A total of 60 pair mated family lines have been produced and are undergoing performance assessment in the field. At the completion of this assessment, these families will be made available to SOCo for incorporation in the industry breeding program.

A total of 31 family lines with different phenoloxidase phenotypes were tested in QX resistance field trials. These family lines are a subset of the 60 lines that will be made available to SOCo for incorporation into the breeding program.

A sensitive test for phenoloxidase genotypes has been developed so that the breeding program has a tool to genotype parents and large numbers of their progeny to assist breeding.

Protocols for cryopreservation of gametes have been developed and documented as a potential strategy to reduce SOCo's overall maintenance costs for the lines and increase genetic security.

Non-chemical means of triploidy induction in SRO (temperature and pressure shock) were tested. While these techniques were not as effective as existing chemical induction techniques, they have formed the basis of ongoing research to see if efficacy can be increased.

8. CONCLUSIONS

This program commenced with six objectives, each of which has been met.

1. To establish pair mating protocols necessary for the development of selectively bred oyster lines
2. To confirm the association between PO and QX resistance using pair matings and test the performance of PO-selected family lines in QX-prone estuaries
3. To identify and characterise additional genetic markers of disease resistance
4. To assess the value of cryopreservation to secure family lines for later use
5. To assess the use of non-chemical means for the induction of triploidy in SRO
6. To make the family lines produced in this research available to SOCo for incorporation in future breeding plans

Detailed protocols have been documented for the production of pair mated family lines and their utility has been demonstrated through the production of over 60 families. Undoubtedly these protocols will be refined as time progresses, but they are currently sufficient to allow the incorporation of pair mated breeding into future breeding plans. The families that have been produced have contributed to an understanding of the role of phenoloxidase in disease resistance and have assisted in the identification of other enzymes involved in the process (Superoxide dismutase). This work has provided a strong basis for investigating other diseases, such as winter mortality, and the existing family lines will also act as a resource in this regard.

The identification of phenoloxidase and other proteins associated with QX disease resistance has provided potential molecular markers of QX disease resistance. These markers serve both to protect the breeding program in the event of catastrophic stock loss and to assist in future breeding programs as additional traits for selection arise. The first of these additional traits, “marketability”, has now been identified and the prospect that this characteristic may be correlated with disease resistance enhances the value of our knowledge of these proteomic markers.

Cryopreservation of SRO sperm to secure family lines for later use is now feasible. Reliable protocols for sperm have been developed and incorporated in the SOCo practical operations manual. On the contrary, successful cryopreservation of SRO eggs, although achieved, remains a challenge that warrants further research. While fertilisation success has been low (5-9%), and this may be acceptable from a breeding perspective given the low numbers of stock required, it has been the variability between batches of cryopreserved eggs that has been the cause for concern. It’s hoped that with further investigation the causes for this variability may be understood and suggestion for the direction of this research have been provided.

Despite more than a decade since triploid SRO were made available to the NSW industry on an experimental basis, there remains a call for more of this stock. Unfortunately, this is becoming increasingly difficult. Techniques used to produce tetraploid Pacific oysters (as the basis for triploid production) have failed with SRO and access to the chemicals used previously to produce triploid SRO is now seriously restricted. Non-chemical stressors do offer a path to triploid production, but this has yet to be achieved however, results to date have been variable and further investigations are required. Some problems identified likely to cause the variability observed in results include a lack of synchronous development and poor development rates, which are common for strip spawned SRO gametes. Future experiments will investigate ways of increasing survival in triploidy trials and the optimum temperature and pressure that repeatedly produce the largest percentage of triploid oysters.

The Sydney rock oyster breeding program and its managers, the Select Oyster Company, are now in a demonstrably stronger position to further the oyster breeding program and face the challenges that may arise in meeting industry breeding needs.

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10. APPENDICES

10.1 Intellectual Property

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10.2 Staff

Dr Wayne O'Connor – Senior Research Scientist, Principal Investigator,
NSW DPI, Port Stephens Fisheries Centre

Dr Michael Dove – Scientific Officer
NSW DPI, Port Stephens Fisheries Centre

Mr Stephan O'Connor
NSW DPI, Port Stephens Fisheries Centre

Ms Lynne Foulkes
NSW DPI, Port Stephens Fisheries Centre

10. 3 Hatchery production of Sydney rock oyster breeding lines

NSW Department of Primary Industries - Fisheries Research Report Series:

Hatchery Production of Sydney Rock Oysters (*Saccostrea glomerata*) breeding lines

Wayne O'Connor, Michael Dove, Stephan O'Connor, Kyle Johnston and Ben Finn

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EXECUTIVE SUMMARY

The basic principles and techniques used for the hatchery production of the Sydney Rock oyster, *Saccostrea glomerata* has been described previously (O'Connor et al., 2008).

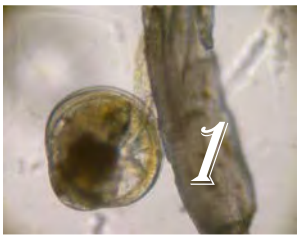
This manual describes the methodology and techniques used by NSW Department of Primary Industries (NSW DPI) for Sydney rock oyster selected family line production and mass selection breeding. Similar larval rearing techniques to commercial-scale production are used for the propagation of breeding lines, however, the need to maintain the genetic integrity of each separate breeding line necessitates the use of specific rearing protocols and implementing stringent hygiene measures. SRO breeding that utilises family lines may require as many as ninety separate breeding lines to be reared simultaneously. This manual has been generated to assist in the larval rearing and, in particular, maintenance of the genetic integrity of bivalves used for breeding purposes.

NSW DPI commenced the SRO breeding program in 1990 with the aim of developing faster growing SROs. Oysters from the program were held in two estuaries, Port Stephens and Georges River. Selection of broodstock oysters in Port Stephens was solely based on weight gain and shell shape whereas the Georges River was used for selection for weight gain and resistance to winter mortality caused by *Bonamia roughleyi*. In 1994, a QX disease epizootic in the Georges River led to the breeding program including exposure to and selection for oysters resistant to this disease which is caused by the parasite *Marteilia sydneyi*. The program successfully developed dual disease resistance against these two major diseases in Georges River. This was achieved whilst increasing the growth rate of the disease resistant oysters. When not exposed to QX disease, the weight of the disease resistant oysters increased by 36% over controls, but when exposed to QX disease this difference was much greater. The Port Stephens breeding lines, which did not suffer major disease problems, had a similar increase in weight and achieved a reduction of ten out of a usual 38 months in time to reach market size (minimum 50 g whole weight). Simultaneous selection for dual disease resistance and fast growth in Georges River was achieved by using large numbers of oysters for each breeding line, allowing for selection of fast growth amongst the survivors.

More recently, there has been rationalisation of the breeding program and a change in emphasis from fast growth and disease resistance alone to other desirable economic traits. This shift has seen the development of selected family breeding lines in 2007. The selected family breeding lines are pair matings of oysters selected for desirable phenotypic traits and these families are run alongside the most recent generations of the mass selected breeding lines.

This manual was prepared to assist with the development of an industry management and commercialisation plan for the Sydney rock oyster breeding program and contains information pertinent to producing and maintaining *S. glomerata* breeding lines at the juvenile stage.

INTRODUCTION



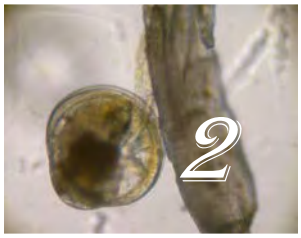
The Sydney rock oyster (SRO), *Saccostrea glomerata* (Gould 1850) is found along much of the eastern Australian coastline as well as across the Tasman Sea in New Zealand (Anderson and Adlard, 1994). Culture of *S. glomerata* is largely confined to estuaries in southern Queensland and New South Wales, where it forms the basis of one of the oldest and largest aquaculture industries in the eastern states. In New South Wales (NSW), the industry currently generates approximately $36 million in sales annually. Overall production of *S. glomerata* has been decreasing since the mid 1970s. The causes of the decline are varied and include competition from other faster growing oyster species, disease and declining water quality (White, 2002).

In the early 1990's, NSW DPI Fisheries commenced a selective breeding program for faster growth and disease resistance in *S. glomerata* (Nell et al., 2000). Mass selected breeding lines were selected for fast growth only and fast growth combined with resistance to winter mortality (*Bonamia roughleyi*) (Cochennec-Laureau et al., 2003) and in 1997, QX disease (*Martellia sydneyi*) (Perkins and Wolf, 1976). Following five generations of selective breeding for fast growth, cultivation time to market had been reduced from 38 to 28 months. In combination with out-of-season hatchery production of spat in early spring (September), cultivation time can be lowered by an additional three months (Nell, 2006). Between January 2004 and December 2009 over 100 million selectively bred oysters were distributed to industry.

To maintain genetic integrity of each breeding line during the hatchery and nursery rearing phases, broodstock, larvae and spat as well as the equipment used to rear them are all labelled with their origin (identifying lineage) and a unique identifying number. All possible precautions are taken to ensure that there is no cross-contamination of the individual breeding lines. This manual documents the equipment and procedures that are in use at the PSFI for the production of Sydney rock oyster breeding lines.

HATCHERY OPERATION

Seawater filtration

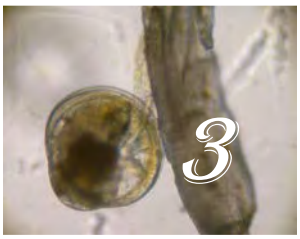


Seawater is transported to PSFI, filtered through 5µm nominal filter bags, stored onsite in 4 x 40,000L storage tanks and allowed to settle for 48h before use. All storage tanks are cleaned with fresh water + Virkon S (concentration = 5 g L^{-1}, Antec International) and allowed to dry before re-use.

Before commencing a production run, seawater delivery lines are dismantled, physically cleaned, by either brushes or by passing a cleaning 'pig' through the line, and then dried for at least five days. All valves in the hatchery are dismantled, cleaned and dried before being reassembled for use. After the delivery lines are reconstructed they are filled with chlorinated water (concentration = 20 ppm) for 24–48 h. The delivery lines are then flushed with freshwater and allowed to dry for a further 24 h.

Seawater is pumped from the storage tanks to the hatchery where it is passed through 1µm nominal filtration before it is used. All seawater delivery lines and valves involved in the pipe-work to the hatchery can be dismantled and cleaned. On a daily basis, all seawater delivery lines and valves are flushed with fresh water, drained and allowed to dry overnight. Additionally the filter housing and filter bags are dismantled washed and placed into chlorinated water (50 mL of chlorine: 13% hypochlorite w/v in 200 L of freshwater: changed weekly). All filter components remain in the chlorine solution until required.

Broodstock maintenance and selection



Production of both mass selection and pair mated breeding lines are scheduled to coincide with optimum seasonal broodstock condition. To facilitate and extend seasonal access to gravid SROs, selected broodstock are maintained within several estuaries along the NSW coast. Broodstock from the different mass selected and pair mated lines are kept separately in labelled oyster trays or baskets. The reproductive condition of oysters is determined by sacrificing the oyster and visual inspection of the gonad. Gonad size, tugor, colour and degree to which gametes fill the gonad are used to assess the oyster's suitability for spawning (Figure 1).

Figure 1. Ripe, ready to spawn Sydney rock oyster

Translocation protocols are adopted when broodstock are transported from a holding location to the hatchery and on return. Filtration and chlorination, autoclaving, ozone treatment or a combination of these processes are used to treat all seawater from spawning/strip spawning.

The following protocol has been developed to minimise the risk of translocation of diseases from estuaries that have experienced QX disease epizootics caused by *Marteilia sydneyi*.

Translocation and hatchery protocols for PSFI Bivalve Hatchery when using broodstock from QX Disease restricted estuaries.

A. Pre-transport treatment of broodstock oysters.

1. Shell to be scrubbed clean of loose material.
2. Oysters to be dried at above 20°C for 24 h.
3. Oysters to be bathed in freshwater containing 200 ppm iodine solution for 2 min.
4. Oysters must not be exposed to raw water during or post treatment. If soaking in saline water is required, it must have been disinfected using a minimum of 30 ppm active chlorine for 12 h. If at any stage during the treatment process the oysters contact raw saline water, the treatment process must be re-started.

B. PSFI broodstock maturation and larval rearing.

1. Water used in the maturation of broodstock and production of larvae must be disinfected with at least 40 ppm active chlorine for a 24 h settlement period prior to 1 µm filtration and discharge. Any remaining sediment is treated with 200 ppm for a further 24 h before disposal as land fill.
2. Equipment used in the production of larvae must be disinfected prior to subsequent use by immersion in a solution containing a minimum of 200 ppm active chlorine for a minimum of 15 minutes, or other equivalent means of disinfection.
3. Broodstock must be disposed of to landfill or returned to their origin estuary once they are no longer required.
4. Larvae must be subject to thorough and regular visual inspection for any signs of disease throughout the rearing period. The NSW DPI Manager, Aquatic Biosecurity & Risk Management must be notified of any suspect mortality or morbidity that occurs during this time, and any such mortalities must be fully investigated. Samples from any such mortality event must be collected and subjected to testing as deemed necessary by the NSW DPI Manager Aquatic Biosecurity & Risk Management.

C. QX testing.

1. Approximately 2 g of fertilised eggs are centrifuged and sent of for PCR screening for *M. sydneyi* (Elizabeth Macarthur Agicultural Institute, Camden Sydney). If the PCR test results indicate that *M. sydneyi* is present, then larvae and culture water must be treated as above. Once all larval cultures have been confirmed PCR clear of QX disease, waste water can be disposed of as normal.

Broodstock oysters that are not collected from QX disease restricted estuaries are pressure cleaned thoroughly and hand scrubbed and washed with iodine or a dilute chlorine solution on arrival. If multiple lines are brought in to the hatchery all containers hold oysters are clearly marked with the line details. Like broodstock are retained in the hatchery in separate, labelled holding tanks (Figure 2a & 2b) if conditioning is required.

Figure 2a Broodstock conditioning system used at the PSFI Bivalve Hatchery.

The broodstock conditioning systems use a submersible pump to recirculate water via the spray-bars (Figure 2b) through black broodstock containers. The standpipe ensures that broodstock are continually immersed. The broodstock container is cleaned daily and every second day the whole conditioning system is cleaned and refilled with an appropriate algal ration and temperature equilibrated water. The bottom reservoir containing the pump is aerated to maintain sufficient levels of dissolved oxygen to and keep algae suspended in the water column.

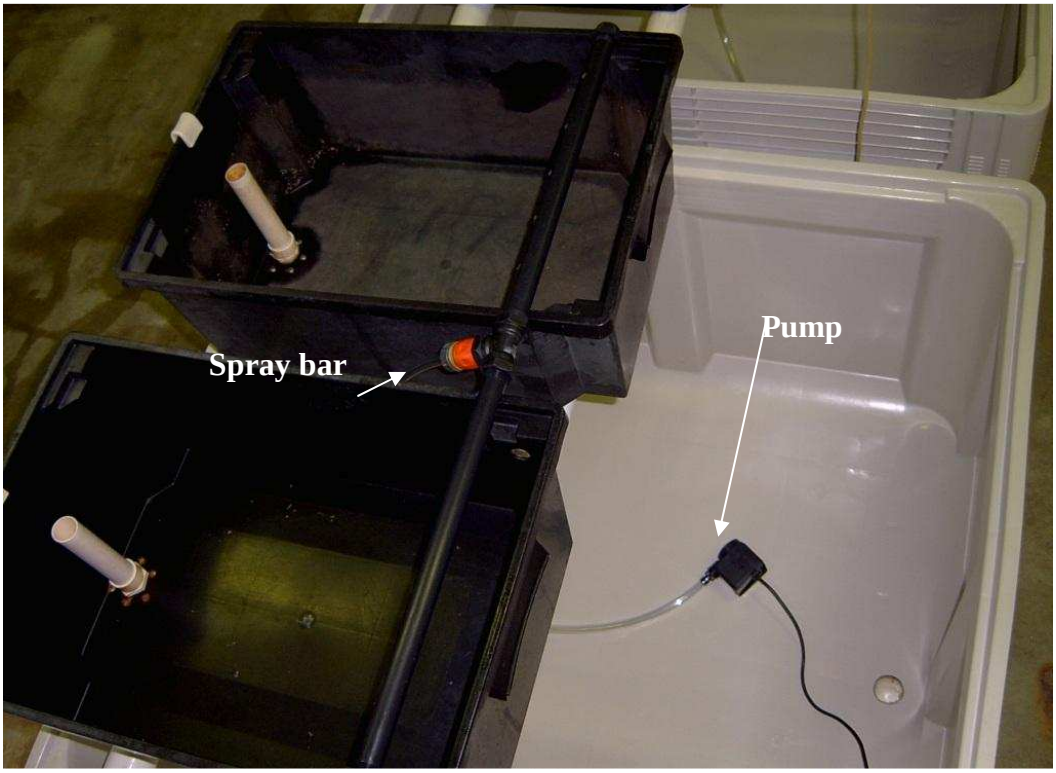


Figure 2b Brood stock conditioning system

As indicated above, as soon as broodstock arrive at the PSFI Bivalve Hatchery a strict tagging protocol is applied so that important information is recorded and broodstock lines are not mixed. The information that is transferred to a tag that then remains with broodstock whilst in the PSFI hatchery is the:

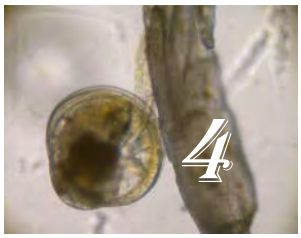
- oyster line
- generation details
- estuary oysters are from
- grower who supplied the stock, and
- date that the oysters arrived at the hatchery.

Broodstock from the same breeding line are maintained in the same conditioning system. If spawning does occur in the conditioning system the gametes may still me useful to the hatchery operator.

NSW DPI have the responsibility of propagating new breeding lines and new generations of existing breeding lines as well as maintaining and grow-out broodstock lines in Port Stephens and Georges River. As such, NSW DPI have developed a tray and basket tagging system that clearly indicates the line and generation of all stock.

Broodstock carers, designated by SOCo, also maintain and grow-out the main commercial lines used for the SRO industry in five estuaries spread from the Hastings River in the north to Pambula Lake in the south to extend the reproductive season of these lines for independent hatchery operators. A tagging system is being implemented that enables broodstook carers to rapidly identify the line and generation of stock in their charge so that information can be conveyed accurately to hatchery operators.

Spawning Induction



The following protocols are used at the PSFI Bivalve Hatchery for thermal induction of spawning.

Preparation

- All equipment used in any spawning attempt is washed and cleaned with Virkon S (5 g L^{-1}), rinsed thoroughly and allowed to dry for 48 h before use.
- Prior to commencing any spawning attempt (mass selection or pair mating) broodstock are cleaned thoroughly (following the protocols listed in Chapter 3) and placed in a separate labelled broodstock container (Figure 2a & 2b).
- All buckets, beakers and containers are clearly labelled for easy identification of broodstock lines to minimise the possibility of cross-contamination between genetically distinct breeding lines.
- During the spawning attempt, when different broodstock are to be used, all equipment and surfaces are washed and cleaned before work commences to spawn a different broodstock line.
- All larval rearing tanks are cleaned thoroughly and taps disassembled and cleaned before reassembly and use.

Mass Selection

Spawning for mass selection is usually conducted using thermal shock and fluctuating salinity levels. For each generation, 216 of the largest survivors of each line are transferred to the hatchery to be used for breeding. A minimum of four independent spawning trays are used for spawning each line stock. This is done to minimise the chance of an early spawning male oyster to fertilise all of the eggs used for a batch. Egg numbers are determined and a common number of eggs pooled, sperm from each tray is pooled following the similar procedures. Fertilisation is conducted to maximise the number of possible combinations.

Cleaned oysters are air exposed at 25ºC for 12 h and then placed in a spawning tray filled with 1µm filtered seawater (FFSW) at 24°C with 35 ppt salinity (Figure 5). Oysters are allowed to acclimate for approximately 15 min or until they have opened and are actively filtering. The temperature is raised gradually to, between 28 and a maximum of 30ºC over a 30 min period. Oysters are held at the elevated temperature for 15 min before fresh water is added to lower the salinity level to approximately 22 ppt and cause a concomitant reduction in temperature (to approx. 25 to 26ºC). Oysters are held at the lower salinity for 15 min, at the end of this period if gamete release has not started, the cycle (beginning from acclimation at approximately 24ºC and 35 ppt salinity) is repeated.

When individual oysters commence spawning, they are immediately removed from the spawning table, rinsed thoroughly in clean fresh water and placed in individual 750-1000 ml plastic food containers filled FFSW (25^{o}C at 33-35 ppt) and allowed to continue to spawn for about 30 seconds before transferred to another 750-1000 ml spawning container. This process of transferring the spawning oyster into a new container of FFSW is repeated four times. Fresh water rinsing and transfer removes residual sperm on the shell or in the oyster cavity and allows for a controlled fertilisation. .

Before pooling gametes, both sperm and eggs are microscopically assessed - poor quality or fertilised eggs and non-motile sperm are discarded. If only a small number of naturally spawned gametes are obtained, the number of eggs from each female is determined and equal numbers of eggs from each parent are pooled in a labelled 20 lt bucket. However, what generally occurs is a large number of naturally spawned gametes are obtained and enumeration of eggs and sperm is not be possible due to time constraints. All gametes released are still inspected, with the first batch of eggs and sperm counted and made up in a known volume. The remaining eggs and sperm are diluted to match the same colour. Then equal volumes of eggs and sperm are pooled separately from each female or male.

Sperm is counted using a haemocytometer and pooled to give equal quantity of sperm from each male. Pooled eggs are gently washed through a 63µm screen and retained on a 15µm screen with FFSW at cultivation temperature to remove debris from the spawning process. The pooled eggs are allowed to "water harden" (taking on a rounded shape, see Figure 6 and 9a) for about 30 min and are checked microscopically to ensure eggs have not already been fertilised and have "rounded up" (round appearance) before fertilisation is attempted.

A general rule, for successful fertilisation enough sperm is added so that approximately one to three sperm are visible at the periphery of each egg when viewed microscopically. After a 5 minutes interval, a second sample is taken to examine the egg/sperm ratio. This sample is retained on the microscope to assess percentage fertilisation. Once the majority of eggs are fertilised (identified by cell cleavage shown in Figure 7) they are stocked into two separate, labelled 1000 L incubation tanks containing FFSW at 25^{o}C. Stocking densities range from 10 to 30 embryos ml^{-1} for the first 24 h and stocking densities are then reduced to 5 D-veliger larvae ml^{-1}. Throughout the larval rearing process two separate 1000 L rearing tanks are maintained for each breeding line.

Figure 5. Mass selection spawning

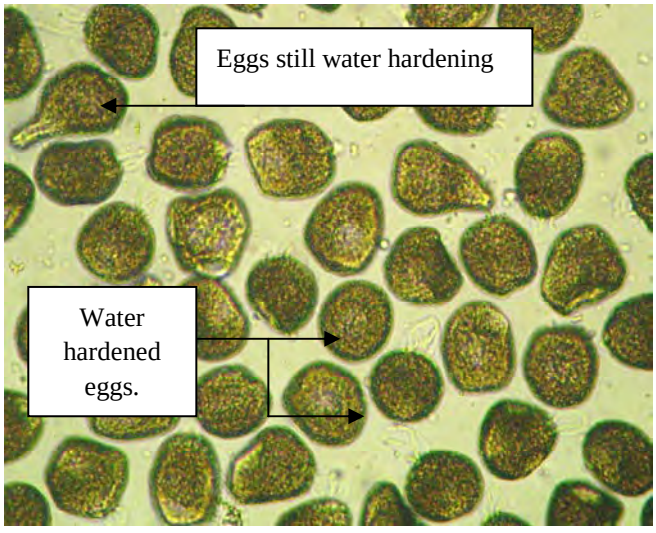


Figure 6. Recently released oocytes undergoing the “water hardening” process

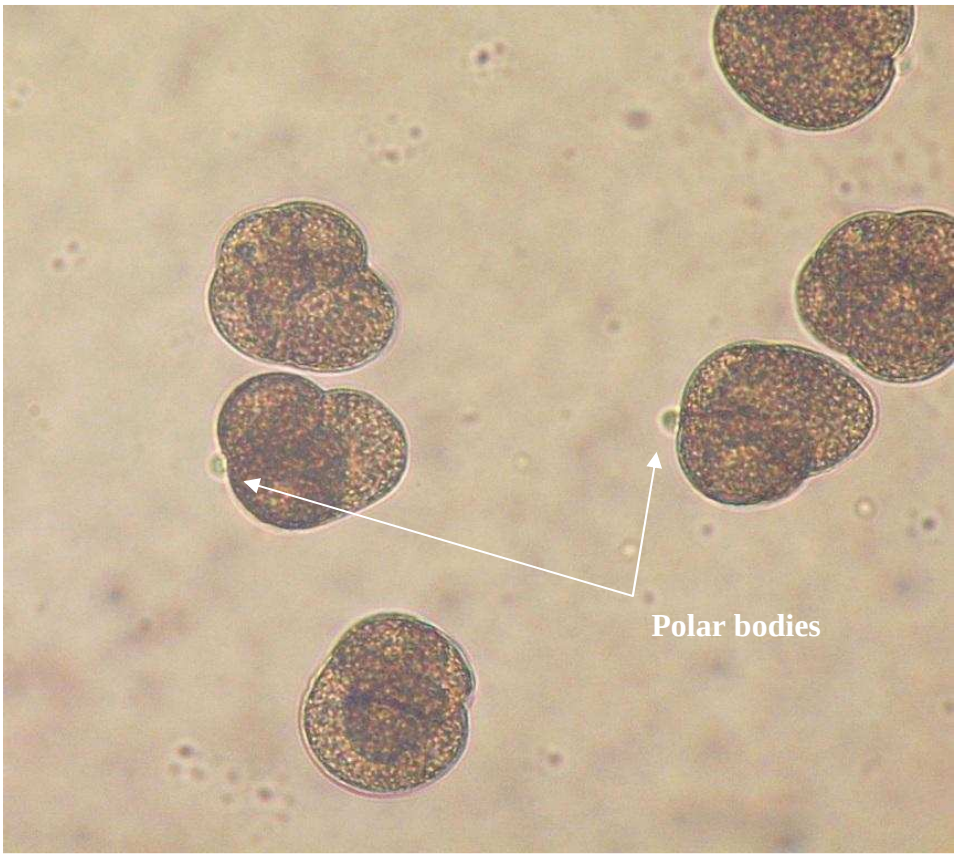
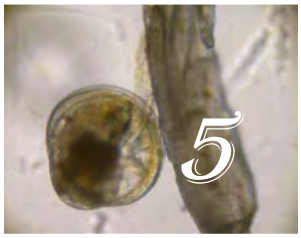


Figure 7. Early stage of cleavage of developing SRO embryo's

Paired Mated Family Lines (strip spawning)



The efficacy of strip spawning varies with species and success with SROs is highly variable. When gametes are physically stripped the percentage of development for this species is generally very low (<10%). However, this technique offers absolute control of parentage where specific crosses are required. Pair mating of oysters is used to create family lines that possess heritable phenotypic traits from their parents (eg. fast growth, disease resistance, shell shape, oyster condition or shell colour) or to cross between family lines. The gametes are physically removed from the gonad of the oyster by scarifying the surface of the gonad and washing the gametes free in FFSW or inserting a Pasteur pipette beneath the gonad epithelium to gently draw the gametes out (Figure 8).

Figure 8. Stripping or physical extraction of gametes from Sydney rock oysters using a Pasteur pipette

To insure that no possibility of cross-contamination of genetic material can occur, only oysters intended for that particular breeding line cross are handled at one time. Following any particular cross all oysters are discarded and all equipment and working surfaces are chemically sterilised and cleaned before any further work is done. As with mass selection, stripped gametes are microscopically assessed and inferior eggs or males that have immotile sperm are immediately discarded. Eggs are gently washed through a 63 µm screen and retained on a 15 µm screen with FFSW at cultivation temperature to remove any debris from the spawning process. All sperm is

placed onto crushed ice once stripped to maintain freshness. Eggs are gently rinsed into individual, labelled 500 ml containers and fertilised. Due to the low viability of stripped gametes from SROs, a minimum of 10 separate pairs are crossed for each new family breeding line. Following fertilisation the developing embryos are stocked in individual, labelled 200 L polypropylene rearing vessels (Figure 4) at up to 50 embryos mL^{-1}.

Through out the larval rearing process two rearing vessels are dedicated to one family line to insure that cross contamination is not possible. If dedicated rearing vessels are not available then the rearing vessel and tap must be completely disassembled, cleaned and scrubbed before a new family is stocked into that tank.

Methodology used for strip-spawning Sydney rock oyster gametes and fertilisation process at the Port Stephens Fisheries institute:

A. Preparation and equipment

- FFSW (filtered 1 µm) at 25°C
- 150 mm glass pipettes
- Lab Serv 70 mL containers
- Haemocytometer
- Kimble glass test tubes
- Sedgewick-Rafter slide and slide coverslips
- Polystyrene esky
- Crushed ice
- Tissues
- Gloves
- If possible and appropriate, work in a temperature controlled room (held at approx. 24°C)

B. Obtaining gametes

1. Open oyster
2. Sample gametes to determine sex
3. For female gametes a wash bottle containing FFSW can be used rinse the gametes from the gonad into a suitable container, however if the male gametes are not for immediate use do not used dilute gametes in FFSW (see point 5 below). Remove gametes either by:

    i. using a scalpel to create a series of incisions across the gonad surface taking care not to intersect the gut,
    ii. and/or using a pipette to extract the gametes from underneath the mantle, and/or
    iii. using a pipette end to exert pressure on the gonad such that gametes are extruded out of the gonad from a scalpel incision.

Note: With any of the above techniques care should be taken to avoid penetrating the gut and mixing the gut contents with gametes.

4. Collect gametes in a plastic container (~70mL) labelled with the unique identifier for the particular breeding line cross, oyster number, sex and the time it was stripped.

Note: If sperm is to be cryopreserved or fertilization of eggs with cryopreserved sperm refer to section 7 “Methods for cryopreserving SRO sperm”.

5. Immediately after stripping filter the sperm through a 15 µm screen into a suitable container and place sperm onto ice (*Do not dilute sperm in FFSW until fertilisation of eggs is to be attempted*).

6. Pass eggs through a 100 µm mesh to remove debris then wash eggs on a 15 µm screen.

C. Gamete evaluation

1. Evaluate the quality of the eggs and ensure sperm is motile on contact with seawater. Exclude females that have poor quality eggs and males with immotile sperm.

D. Determine sperm density

1. Set up and label (1, 2 and 3) Kimble glass test-tubes for 10 fold serial dilutions to enable sperm to be counted.
2. Place 900 µL of sea water into the first 2 test tubes and into the third tube mix 800 µL of seawater with 100 µL of 10% formalin and seawater, then discard the pipette tip to prevent any formalin contamination.
3. Ensuring that the sperm is evenly mixed in suspension, take a 100 µL sample of the sperm suspension and place into the first tube. Mix well using the pipette and flicking the base of the tube.
4. Change the pipette tip and transfer 100 µL from the first tube to the second tube. Mix well.
5. Again change the pipette tip and transfer 100 µL from tube two to tube three. Mix well.
6. In the third tube is a 1000 fold dilution of the original stock.
7. Take a haemocytometer slide and place the cover slip over the grid. Place the pipette tip near the edge of the coverslip and expel sample, allowing it to be drawn under the coverslip by capillary action. Place the haemocytometer slide under the microscope. If there are insufficient sperm to count, the dilutions should be repeated but with only a 200 fold dilution of the original stock (i.e. take 900 µL of sea water + 100 µL of sperm into first test tube, mix well, then into second test tube take 850 µL seawater and add 50 µL of sperm solution from first test tube plus 100 µL of 10% formalin and seawater).
8. Count the number of sperm in a 1mm x 1mm square grid of the haemocytometer. Do not count cells on all of the boundaries. Only count those sperm touching the top and left that are touching the middle lines. Do not count cells touching the bottom and right middle lines. To speed up the process, count only 5 of the smaller squares and multiply the average by 25 to determine the number in the 1 mm x 1mm square.
9. Determine the sperm concentration using the formula: Sperm concentration (per ml) = no. in a 1mm x 1mm square x dilution factor (either 1000 or 200) x 10,000.

E. Determine egg density

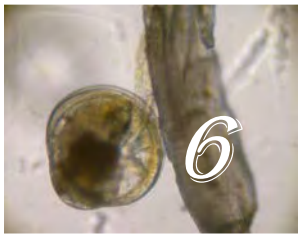
1. Suspend eggs in a known volume of seawater and remove 100 µL into 900 µL of seawater in a Kimble glass test-tube. Take 100 µL and place on a Sedgewick-Rafter slide and count the number of eggs in this volume. Multiply the number of eggs by 100 for the number of eggs mL^{-1} in the initial egg suspension. For total number of eggs multiply the number of eggs mL^{-1} by the total volume of the initial egg suspension.

F. Fertilisation

1. Pool or pair males and females as required.
2. Add sperm to the eggs such that the final concentration of sperm in the egg suspension equates to 10^5 sperm mL^{-1}.
3. Check that sperm is motile and is attaching to the eggs (as a rough guide you want to see at least 3 sperm attached to each egg).

4. After a contact time of 15 - 20 min with continuous mixing, check for first polar body release and transfer the eggs to larval rearing tanks for incubation.

Cryopreservation



As almost without exception, sperm for cryopreservation is obtained by strip spawning male oysters. Successful cryopreservation of SRO sperm and long-term storage offers several advantages to a breeding program.

- Evaluation of progeny and retention of viable gametes for continued future use.
- Store gametes well beyond the normal life span of an oyster.
- Archive of genetic material in the case of breeding lines losses in the field.
- Alleviate some of the biosecurity risks involved in broodstock transport.

Though limited success has been achieved with cryopreservation of SRO eggs further investigation into freezing and thawing regimes and optimum cryoprotectants is required to improve success rates.

Methodology used for cryopreservation of Sydney Rock oyster sperm and for fertilizing eggs with cryopreserved sperm.

A. Preparation and equipment

- D(+) Trehalose (Sigma Product:T5251)
- Dimethyl sulphoxide (DMSO) (Sigma Product: D8779)
- Milli-Q or deionized water
- Lugol's iodine
- 50 ml falcon tubes
- Lab Serv 70 ml pottles
- Haemocytometer
- Steel straw rack attached to 3 cm thick polystyrene
- Polystyrene chilli bin / esky
- Liquid Nitrogen
- Long forceps
- Safety gloves and glasses
- Storage goblets
- 0.5 ml straws
- Sealing powder
- Plastic Comb and Boat
- Storage Dewar
- Container for thawing
- Scissors
- Ethanol (70%)
- Tissues

B. Solution preparation

1. Prepare a trehalose solution (Sigma T5251) by adding 15.2 g trehalose to 20 ml distilled water in a 50 ml falcon.
2. Then combine 15 ml of the trehalose solution with 4.3 ml of DMSO and 10.7 ml of Milli-Q water.
3. Place solution on ice to cool before use (Note: the addition of DMSO will give cause an exothermic reaction when added to the trehalose).
4. Prepare the polystyrene chilli-bin/esky for freezing by adding liquid nitrogen so that the chilli-bin is approximately 2/3 full. Be aware that the liquid nitrogen will bubble and splash initially as it cools the container. Gently float the straw rack attached to polystyrene on the liquid nitrogen. To obtain a more stable environment for freezing, loosely place the lid on the chilli-bin.

C. Obtain sperm an determine sperm density

1. Strip sperm from males into a plastic container using a minimal amount of seawater and hold "dry" on crushed ice.
2. Set up and label (1, 2 and 3) Kimble glass test-tubes for serial dilutions and counting of sperm.
3. Place 900 µl of sea water into the first 2 test tubes and 800 µl of sea water into a third test tube.
4. Ensuring that the sperm is evenly mixed in suspension, take a 100 µl sample of the sperm suspension and place in the first tube. Mix well using the pipette.
5. Change the pipette tip and transfer 100 µl from the first tube to the second tube. Mix well.
6. Again change the pipette tip and transfer 100 µl from tube two to tube three. Mix well.
7. Again Change the pipette tip and add 100 µL of 10% formalin and seawater. Mix well then take a 100 µL sample of the suspension. This is a 1000 fold dilution of the original stock.
8. Take a haemocytometer slide and place the cover slip over the grid. Place the pipette tip near the edge of the coverslip and expel sample, allowing it to be drawn under the coverslip by capillary action. Place the haemocytometer slide under the microscope. If there are insufficient sperm to count, the dilutions should be repeated but with only a 200 fold dilution of the original stock (i.e. take 900 µL of sea water + 100 µL of sperm into first test tube, mix well, then into second test tube take 850 µL seawater and add 50 µL of sperm solution from first test tube plus 100 µL of 10% formalin and seawater).
9. Count the number of sperm in a 1mm x 1mm square grid of the haemocytometer. Do not count cells on all of the boundaries. Only count those sperm touching the top and left that are touching the middle lines. Do not count cells touching the bottom and right middle lines. To speed up the process, count only 5 of the smaller squares and multiply the average by 25 to determine the number in the 1 mm x 1mm square.
10. Determine the sperm concentration using the formula: Sperm concentration (ml^{-1}) = no. in a 1mm x 1mm square x dilution factor (either 1000 or 200) x 104

D. Diluting sperm with cryoprotectant

1. Set up the required number of Kimble disposable glass culture tubes. Mix sperm well then add 1 mL of sperm to each test tube. Keep on ice.
2. Add the cryoprotectant solution to the sperm in 100 µL aliquots in 10 one minute intervals to give a final volume = 2 mL/test tube.

Note: Gently mix each test tube after each addition. After addition 8 and 10 the tubes should be capped and inverted several times to ensure good mixing of the sperm and the cryoprotectant solution.

E. Loading the straws

1. Load 0.5 ml straws with sperm-cryoprotectant mixture, by sucking the mixture up the straws. The flow will stop once the top plug becomes wet. This will seal this end of the straw.
2. One test tube of the mixture will be sufficient to fill 3 straws.
3. Place the straws onto the comb to form an air space in the straw and to allow the coloured sealing powder plug to be formed. It is important to do this as the air space allows room for the mixture to expand without blowing the plug from the straw.
4. Tap in coloured sealing powder to plug the end. Wipe excess powder from the straws and place coloured plug end down into a 70 mL plastic container (Lab serve catalogue number LBS 30002) containing 20 mL of water to seal the plug. Leave straws in the water for approximately 10 seconds then remove, wipe dry with a tissue and lie flat until 10 minutes has elapsed from the completion of the addition of the cryoprotectant solution.

F. Freezing

1. Gently place straws across the rack floating on liquid nitrogen in the polystyrene chilli-bin prepared earlier. Place the lid loosely back on the chilli-bin and wait 10 min.
2. After this time has elapsed, plunge the straws into the liquid nitrogen bath. Place a plastic goblet in the liquid nitrogen bath and pack straws into the goblet for storage. Transfer the goblet to a liquid nitrogen dewar with canisters until ready for thawing.

G. Obtaining eggs

1. Obtain eggs by inducing spawning in Sydney rock oysters using standard procedures of temperature and salinity shock. It is important to ensure that eggs collected remain unfertilized. Separate spawning females into individual beakers and transfer several times into fresh seawater to remove sperm.
2. Keep each lot of eggs collected from each female separate. Take a sample (approximately 200 µL) of the egg suspension at least 30 min after gamete release and insure that no fertilisation has occurred (i.e. that there are no sperm present and/or polar bodies)
3. For each female, pool up eggs that are unfertilised into one container to determine egg density.

H. Determine egg suspension density

1. Use a plastic transfer pipette to pick up settled eggs from the bottom of the beaker and transfer to a 15 mL disposable conical tube.
2. Place the tube in a rack at 4°C for up to 60 minutes to allow the eggs to settle and concentrate.
3. Remove the excess sea water (leave a small amount, approximately 500 µL, of sea water above the eggs) from the surface with a plastic transfer pipette. Take care not to remove the eggs.
4. Set up Kimble glass test-tubes for serial dilutiion and counting of the eggs by placing 900 µL of sea water into the first 3 tubes.
5. Re-suspend the eggs in the conical tube by inverting several times. Make sure that the eggs are well mixed and the concentrated eggs from the tip of the tube have been re-suspended.

6. Take a 100 μL sample of the egg suspension and place in the first tube. Mix well using the pipette.
7. Change the pipette tip and transfer 100 μL from the first tube to the second tube. Mix well.
8. Again change the pipette tip and transfer 100 μL from tube two to tube three. Mix well.
9. Again change the pipette tip and take 20 μL aliquots and make 4 drops in a well of a multi-well plate lid.
10. Count the number of eggs in each drop. Take the mean of the four counts and multiply by 50,000. This is the number of eggs ml^{-1}.

J. Thawing gametes

1. Set up water bath for thawing. Use a thermos or other suitable container filled with tap water adjusted to 25°C. Use a thermocouple or thermometer to monitor the temperature. Increase the temperature with warm water when it drops to 22°C.
2. Fill a plastic thermos with liquid nitrogen for holding straws. Wash a pair of scissors with 70% Ethanol.
3. Select required straw from liquid nitrogen and Check the details are correct. Plunge into water bath and hold until the ice melts. Remove and dry with tissue.
4. Cut off cotton plug with sterile scissors. Place the straw into a test tube and cut off the PVP powder plug. Allow the contents of the straw to slowly run out. Blow the last drop out. The sperm are now ready to be used for fertilization.

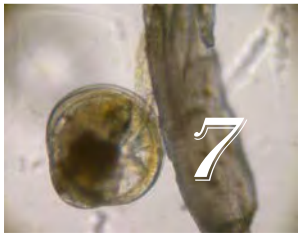
K. Bulk scale fertilization

1. Suspend eggs at a density of approximately 1000 to 5000 mL-1.
2. Add sperm at a ratio of approximately 1000 to 5000 sperm egg^{-1}

Note: the sperm concentration will be half of what was determined earlier allowing for a 1:1 dilution with the cryoprotectant solution).

3. After a contact time of 15 - 20 min with continuous mixing, transfer the eggs to larval rearing tanks for incubation.

Larval rearing and equipment



For larval rearing of the mass selected lines, 1000 L polypropylene rearing vessels (Figure 3a) are used to produce the large number of spat required during the selection process. Alternatively, 200 L polypropylene rearing vessels (Figure 4) are used for rearing the pair mated family lines. Embryo and larval rearing vessels are heated using titanium immersion heaters which are always removed before embryos or larvae are added. To maintain rearing water temperature in the tanks the room is heated to 25.5°C which holds the water temperature between 24 to 25°C.

The larvae are "screened" and moved in a cleaned rearing vessel filled with FFSW every second day, termed water change. The larval rearing vessel is drained through a series of immersed screens (Figure 3b). The mesh size of the screens is selected to retain the larvae but allow excess algae and faecal material to pass through. Larvae are rinsed thoroughly in clean filtered seawater before being moved to a cleaned rearing vessel. Not all larvae will develop and successfully metamorphose, accordingly the different screen sizes are used to "cull" the slower growing or malformed larvae. Particular care must be taken when culling slow growing larvae from the mass selected lines, as this may preferentially select for a particular set of individuals and may compromise genetic diversity (see Taris et al. 2006 for more information on selection pressure on larvae in a hatchery environment).

To insure that cross contamination does not occur, all rearing vessels and taps are dismantled, rinsed in freshwater, cleaned and scrubbed using Virkon S solution (5 g L^{-1}), rinsed again with freshwater and allowed to dry before re-use. Wherever possible two tanks are dedicated to the production of each breeding line. Each tank is labelled (using a waterproof system) and an additional tag is also used that is moved with the larvae through the water change process (ie. this tag is attached to the screen when screening larvae, attached to the bucket when the larvae are rinsed from the screen and then attached to the new tank filled with FFSW). The larvae are identifiable at every stage during the rearing process and any equipment dedicated to that particular breeding line can also be identified.

One particular problem endemic to all hatcheries when producing a large number of breeding lines is the repeated use of the same screens during water changes. Due to the high cost of construction, maintenance and storage problems associated with having a large number screens it is not possible to dedicate a complete set to an individual breeding line. To negate the risk of cross contamination all screens are hosed down in fresh water, placed in a dilute chlorine solution (20 ppm) for a minimum of 10 min, rinsed and hosed down again before re-use. The mesh size of the larval screens ranges from 15 µm (used for washing eggs) to 265µm (used for grading freshly metamorphosed spat from larvae). Several sets of screens allow tanks to be drained concurrently with sufficient time for cleaning to prevent cross contamination of larval batches. Common mesh screen sizes and their diagonal measurement are listed in Table 1. The four main developmental stages of bivalve larvae, and the approximate size at which each of these stages occur, is displayed in Figures 9a to 9e and 10.

Before every water change, screens are checked for any visible holes in the fabric or any degradation of the silicon seals around the screen. Small holes (1-2mm) can be repaired using an

eye-dropper and PVC glue. Larger holes may require complete replacement of the screen material, hence the requirement for spare mesh of each screen size kept on hand so that repairs can be made.

Figure 3a. Larval retention screens and apparatus used during water changes for 1000L tanks

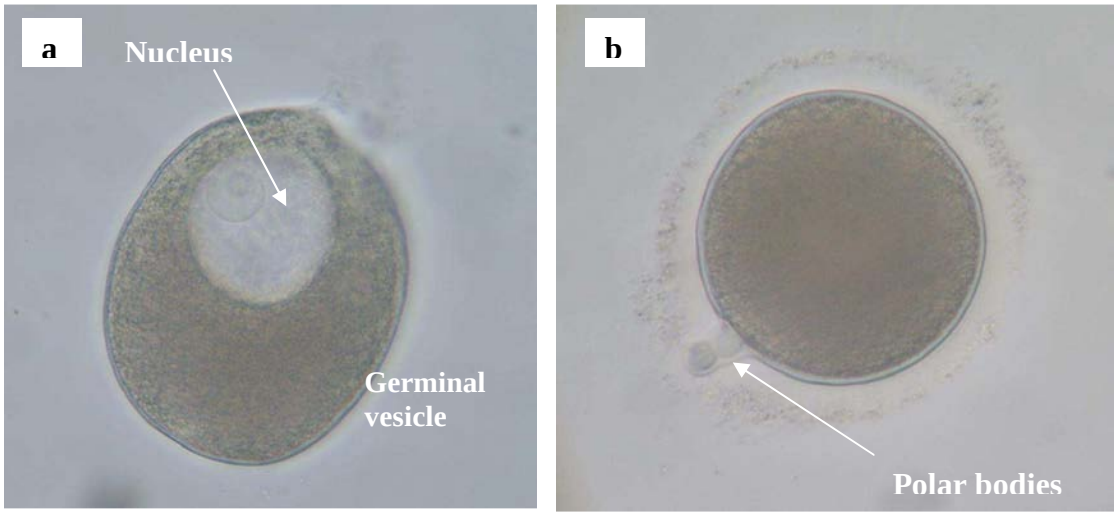
Figure 3b. Sydney rock oyster larvae retained on screens

Figure 4. 200 L polypropylene rearing vessels used for production of the pair mated family lines.

Larval Rearing

Both induced and strip spawned eggs have a characteristic "pear shape" following liberation. After a short time in seawater (15 to 30 min) eggs begin to hydrate and become more rounded in appearance. This process is referred to as "water hardening" (Figure 6 & 9a). When the eggs are viewed microscopically a centrally located, large transparent area (nucleus) bounded by the germinal vesicle and surrounded by densely packed yolk granules is normally visible (Figure 9a). Polyspermy (too many sperm penetrating the egg caused by excess sperm) can be problematic for successful fertilisation in some bivalve species. With SRO's, polyspermy is not generally a problem in water hardened eggs.

Following fertilisation the egg contracts, the germinal vesicle breaks down and the cytoplasm of the egg takes on a more uniform appearance. Following germinal vesicle breakdown, two polar bodies are released consecutively (Figure 9b). The time to polar body release can vary and largely depends on water temperature, salinity and egg quality. At 25°C, embryos have developed to a free-swimming trochophore in approximately 6 h and the first hinged D-veligers appear 16 h post-fertilisation (Figure 9c). The general stages of larval development are displayed in Figures 9a to 9e and Figure 10.


a
Nucleus
Germinal vesicle
b
Polar bodies

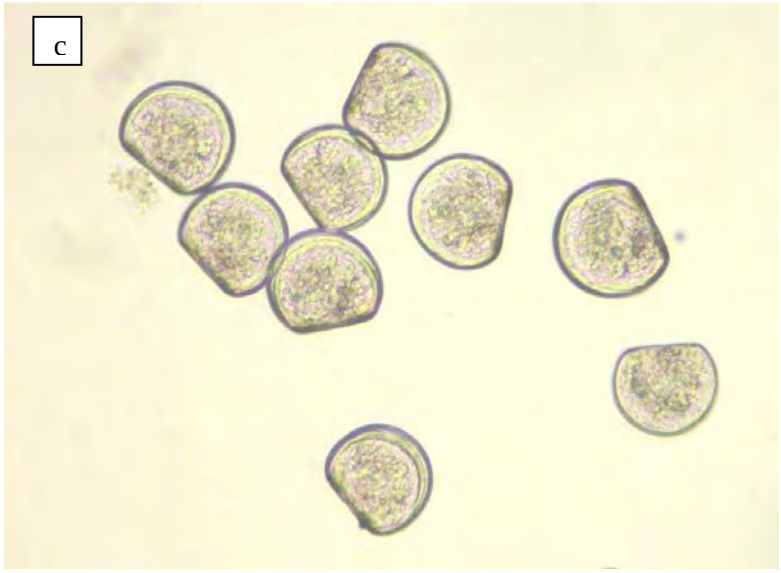

c

Figure 9 Stages of *Saccostrea glomerata* larval development: a) An egg with nucleus bounded by the germinal vesicle, b) polar body extrusion following fertilisation, c). D-veliger larvae, d) umbonate veliger larva, e) Pediveliger larva.

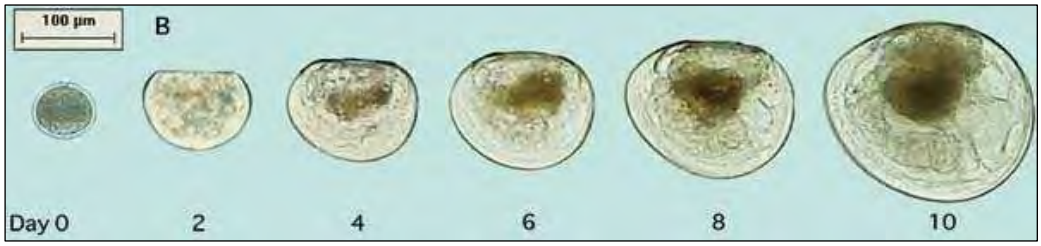


Figure 10 Developmental stages of bivalve larvae. (Source: Helm *et al.* 2004, page 101)

Table 1. Larval screen sizes used at the PSFC and their diagonal measurements.

Nominal Size	Diagonal (µm)	Nominal Size	Diagonal (µm)
35	40.00	142	200.82
45	63.64	150	212.13
55	74.95	170	240.42
63	89.00	180	254.56
85	120.21	190	268.70
90	123.00	200	283.00
100	141.00	212	299.81
108	152.00	236	333.75
118	166.88	250	354.00
124	175.36	265	374.77
132	179.00	292	412.95

Larval sampling, measuring and feeding

Equipment used to sample, observe and measure larvae are displayed in Figure 11. At each water change, larvae are rinsed into a 20 L bucket and gently resuspended uniformly into the water column using a perforated plunger. Two replicate 1 mL samples are taken and are placed on separate Sedgewick-Rafter slides for counting the number of larvae using a miroscope (Figure 12). A microscope that is fitted with an ocular micrometer allows measurement of the the antero-posterior shell lengths (Figure 12) of larvae. At least 30 larvae should be measured to assess larval growth. Both the number of larvae and larval size are needed to calculate re-stocking density and the daily algal ration. Comparing daily larval size measurements to a long-term average size calculation for that species can help identify health issues in a batch before they are visibly evident.

It is more problematic assessing growth and feeding in pair mated lines due to the numbers of tanks that are used and time constraints to collect all of the batch information: Further details about this are listed below:

- The large number of larval cultures produced at one time necessitates that half the larval cultures are water changed on alternate days. This can be achieved by strip spawning over a two day period to offset certain families by one day.
- In the early stages of development it is not possible to enumerate larvae at every water change. Depending on the number of cultures produced it may only be possible to count larvae from every third or fourth family. Efficient and accurate record keeping is required so information is available to make decisions related to retaining or disposing of a family. It is best to make this decision within the first five days of cultivation so that alternate action can be taken, for example create more family crosses if family numbers are too low. It is important to note that not all D-veligers will develop and successfully metamorphose into spat. Generally, less than 20% of the initial D-veligers stocked will develop into spat.

A sample of larvae should be examined live to assess their general health then fixed with a drop of 10% formalin and seawater to allow accurate counting and sizing. The number and size of larvae are used to determine feeding rates and adjust larval densities when required.

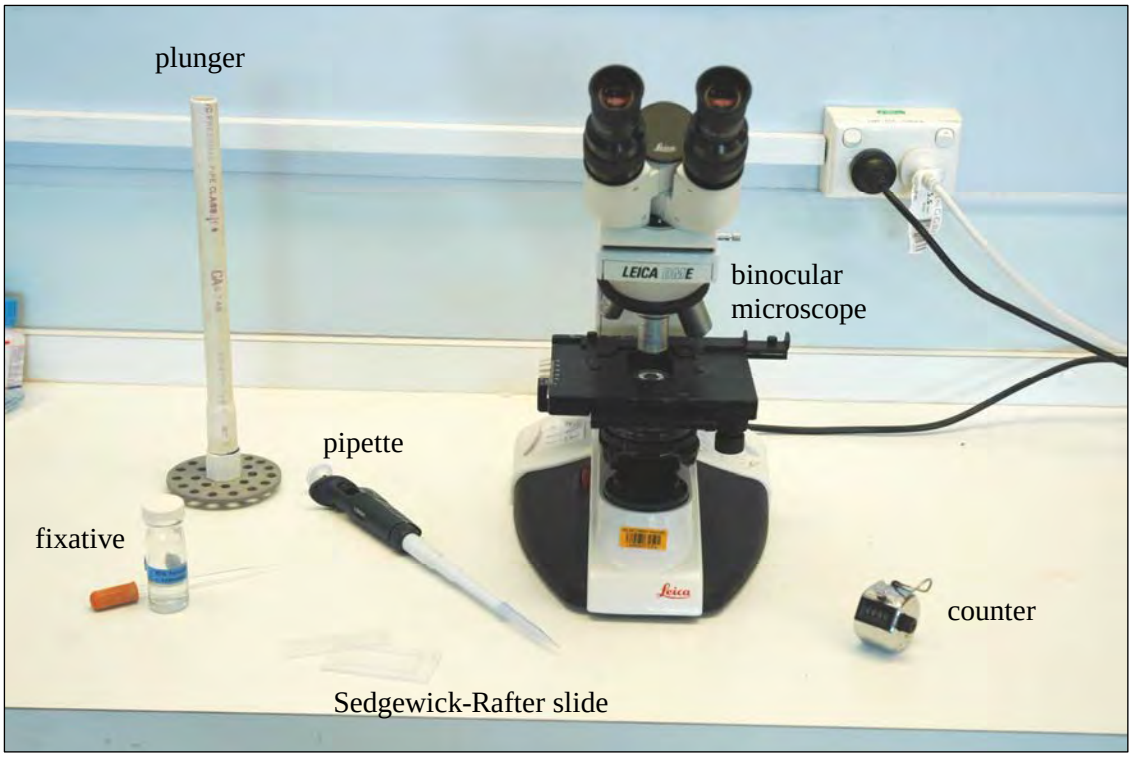


Figure 11. Equipment used to sample, observe and measure larvae

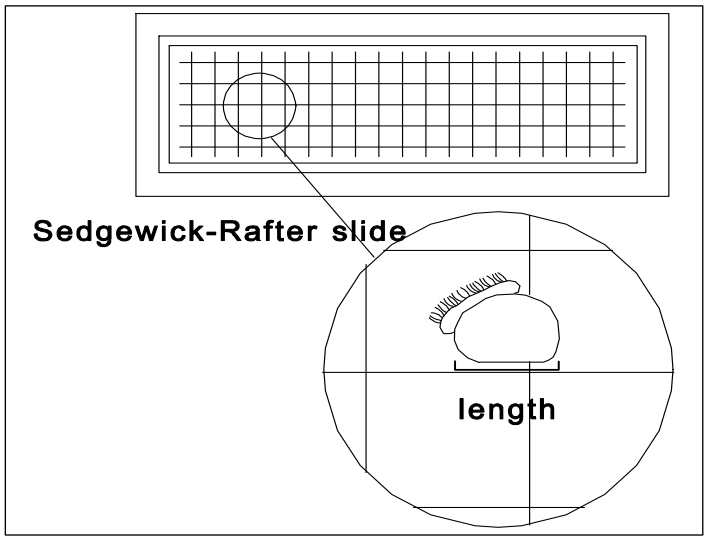


Figure 12. Sedgewick-Rafter slide

Larval feeds

Larval feeding commences when the first D-veligers are observed, usually after 16-18 h post fertilisation. Knowing the total number of larvae and their average shell size enables the algal ration to be determined. Figure 13 shows a feed curve developed for *Saccostrea glomerata*. This feed curve is used as a guide only and is altered in accordance with daily observations of the larvae. If excess food is observed in the culture water the feeding rate may be reduced, conversely if larvae have a light gut colouration the feeding rate may be increased. Inexperience can sometimes introduce a tendency to over-feed but this should be avoided as the consequences can be equally as damaging to larval performance as under-feeding.

The daily larval ration is always divided over a number of feeds with at least a minimum of a morning and afternoon feed. Four species, T. *Isochrysis* (T. iso), *P. lutheri* (Pav), *C. calcitrans* (Cal) and *C. muelleri* (Muel), are fed to SRO larvae. Both T. *Isochrysis* and *P. lutheri* are fed continuously throughout the larval run and both individually comprise 25% of the total daily algal ration. *C. calcitrans* is fed to the larvae (in conjunction with T. *Isochrysis* and *P. lutheri*) for the first seven days at a rate of 50% of the total daily feed ration. After Day 7, *C. calcitrans* is phased out of the diet and *C. muelleri* is introduced (approx 10% day^{-1}) until *C. muelleri* forms 50% of the total daily feed ration. Where possible during the larval run, larvae are fed only algae cultured in autoclaved seawater.

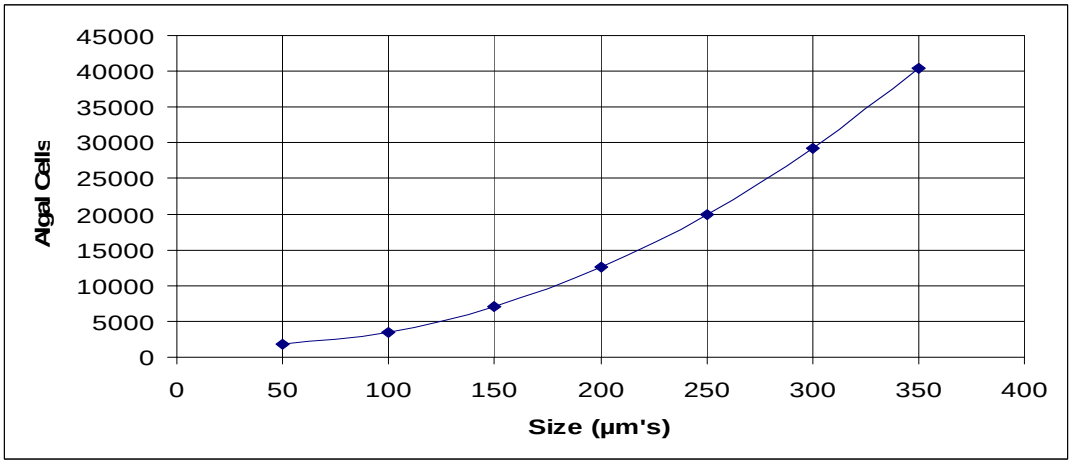


Figure 13. Sydney rock oyster larval feeding curve.

Calculating larval feed rates

Algal rations are determined using the feed equation (Figure 14) and the feed curve (Figure 13) which is based on the equivalent number of T. *Isochryisis* cells to be fed to each larva day^{-1}. To determine the total daily ration for a batch of larvae it is necessary to know the number of larvae, average larval size, algal species required and the number of algal cells mL^{-1} of each algal species to be fed. To compensate for the different cell sizes of each different algal species a correction factor to account for smaller or larger species is applied (see below). These correction factors have been determined on the basis of the individual dry weights of each alga.

Dry weight correction factors: T. *Iso* = 1, *C. muelleri* = 1, *P. lutheri* = 0.8, *C. calc* = 1.2.

Total algal feed = Total No. of larvae x cells/larvae/day (from feed curve)

$$\textbf{Algal species} = \frac{\textbf{Dry weight correction fac. x Total algal feed}}{\textbf{No. of diets (*) x Algal cell count}}$$

Figure 14. Formula for calculating algal ration for Sydney rock larvae.

Hatchery preparation and timing SRO production

- 8-10 weeks before spawning

Generally, any attempt to spawn selected line broodstock is timed to coincide when the required oysters have attained, or are close to, their peak reproductive condition. Alternatively broodstock can be collected and "conditioned" for up to 10 weeks in the hatchery to allow gametogenesis to occur. The conditioning process involves raising the water temperature and daily feeding to satiation on mixed algal diet.

- 1-2 weeks before spawning

Preparation within the hatchery starts 1-2 weeks before the designated spawning date.
Hygiene protocols ensure a complete dry-out of the hatchery for a period of at least one week. During this time, all seawater delivery lines and valves are dismantled and cleaned, water storage tanks are cleaned, larval rearing vessels and valves are dismantled and cleaned, and hatchery floors are disinfected with chlorine solution (0.25 mL of chlorine L^{-1} of freshwater).

The inoculation of algal cultures, to be used for larval food, commences about one week before the spawning date, but this may vary depending on algal species and the growth rates obtained at a particular hatchery.

The temperature controlled rooms at PSFI are set to 26.5°C two days before spawning. This alleviates the need to use immersion heaters in the larval rearing vessels once the run starts as the room temperature can maintain the water temperature in tanks at a constant 25°C.

At PSFI the water storage tanks are filled and settled for a minimum of 48 h prior to use.

- Before spawning

The day before the spawning, the seawater delivery system lines are reconnected and fresh seawater is pumped through the lines to flush the system. The old set of 1µm filter bags are replaced with a new set and the incubation vessels are filled with 1µm filtered seawater from storage tanks. Immersion heaters are placed into the incubation vessel and the water is heated to 26°C. The vessel is gently aerated and the airlines and ceramic weights (used to hold the airlines down in the water) are autoclaved before use.

Broodstock are taken out of the conditioning system and cleaned using a povidone-iodine or dilute chlorine solution and left emersed to dry overnight.

- Spawning

Spawning is conducted following the protocols described previously for mass selection or pair mating (refer to Chapters 5 and 6). A half daily ration of mixed algal species (Pav, T. Iso, C. cal) is added after 16 h so that larvae can commence feeding as soon as they are capable.

Filter bags from the seawater delivery line are removed at the end of each day and placed into a chlorine bath (0.25 mL of chlorine L^{-1} of freshwater) for a 24 h period. It is recommended that the hatchery have at least two sets of filter bags so that one set can be used while the other is chlorinated or in the case that a filter bag fails.

- Day 1

Larval rearing vessels are filled with 1µm filtered seawater as close to the time of water change as possible. The time taken to do this will depend on the incoming water temperature. To minimise stress to the larvae, when being transferred to a new rearing vessel, the water temperature is matched to within 1^{o}C of the larval rearing temperature. Where heating is required, heaters are not left in the vessel with the larvae and should be removed as soon as the desired temperature is reached. For the pair mated lines several 1,000 lt tanks are filled and heated and the water is then pumped into the 200 L rearing vessels as required.

Note: Depending on the number of breeding lines produced concurrently it may not be possible to inspect every larval culture. It is important to maintain precise larval records to insure:

- During the 1st water change the larval cultures are screened in the order of fertilisation timing.
- larval cultures are inspected as frequently as possible and inspection is not duplicated.

Larvae are dropped onto a 35µm screen, rinsed and then washed into a known volume of 25ºC seawater (usually a 20 L bucket) and 2 x 1mL samples are taken. The larval samples are used to determine the total number of larvae present as well as larval size and percentage development. Once the number of the larvae has been determined, the new larval rearing vessel is stocked at a density of approximately 8 larvae mL^{-1}. The daily algal ration is determined (Figure 13 and 14) and divided into a morning and evening feed. The morning feed is added to the new larval rearing vessel before larvae are stocked.

The empty rearing vessel and tap are dismantled and rinsed with freshwater, scrubbed using Virkon S solution (5 g L^{-1}), rinsed again in freshwater and then left to dry for at least 24 h. Air lines and air stones from the incubation vessel are placed into a chlorine bath (0.25 mL of chlorine L^{-1} of freshwater) for 24 h, washed with freshwater and hung up to dry for a further 24 h. Before

leaving, the evening feed ration is fed to the larvae, the water temperature is checked and a larval sample from the top of the water column is taken to ensure that the larvae are healthy.

Note: Given the low fertilisation and survival rates of stripped spawned gametes, if the quantity of larvae produced is to low to give a viable number at metamorphosis the larval culture will be disposed of under quarantine protocols listed in Chapter 3.

- Day 2

Larval water changes are carried out every second day. On arrival in the morning the larvae are fed immediately using the same quantity of food supplied to the larvae the previous evening. If a considerable number of larval cultures are reared concurrently, half of the larval cultures will be water changed on alternate days. Where possible, collect larval samples and inspect live larvae before they are fixed for measurement. Calculate the required quantity of algae and make any adjustments to the morning feeds that are required.

Feed the balance of the daily feed ration as late in the afternoon as possible and take a small sample of larvae to inspect their health. In practice, feeding usually occurs at 7:00-8:00 am and 5:00-6:00 pm.

- Day 3

A new larval rearing vessel is filled, heated and aerated (as Day 1). Add 10% of the previous evenings feed ration to the larval rearing vessel (containing larvae) before starting the water change. The larvae are rinsed thoroughly and then washed from the screen into a 20 L bucket and sampled to establish total numbers, gut content, development, motility and general health. A known number of larvae are stocked into the new rearing vessel to which 50% of their daily feed ration has been added. The balance of the daily feed ration is added in the evening in accordance with the feed curve. The used larval rearing vessel is washed out and all equipment is cleaned as described previously.

- Days 4-6

Days 4-6 are as per the protocols described above in Days 2-3. As larval growth rates differ from batch to batch, the screen sizes selected for each water change should be chosen in accordance with the size of the larvae. It is important to note the diagonal measurement for each screen mesh (refer to Table 1) as this determines the size of larvae that can pass through the screen.

Stocking densities during this period are maintained between 5-7 larvae mL^{-1}. By Day 7 stocking densities are reduced to approximately 4 larvae mL^{-1}.

- Days 7-18

The above pattern regarding water changes, feeds, daily larval samples and hygiene protocols are continued throughout the larval cycle until settlement. From Day 7 (or when larvae exceed 100 µm in size) to Day 10 the proportion of C. calc in the diet is incrementally reduced (10% per day) and replaced with Muel. Usually at Day 7, when the larvae are approximately 120-130 µm in size, the first umbonate larvae are present (Figure 9d).

- Day 18-19

As larvae grow, they advanced though several identifiable morphological stages of development. By Day 17 to 19 larvae should have developed two eye spots (Figure 15). Around Day 19 to Day

20, larvae develop to the pediveliger stage and as the name suggests develop the ability to "crawl" (Figure 23). The extension of the foot can be observed whilst the larvae are swimming or stationary. It is very difficult to identify pediveligers using a sample that has been fixed using 10% formalin and seawater. Therefore it is suggested that a fixed sample and a live sample of the larvae are collected to clearly identify such behavioural characteristics.

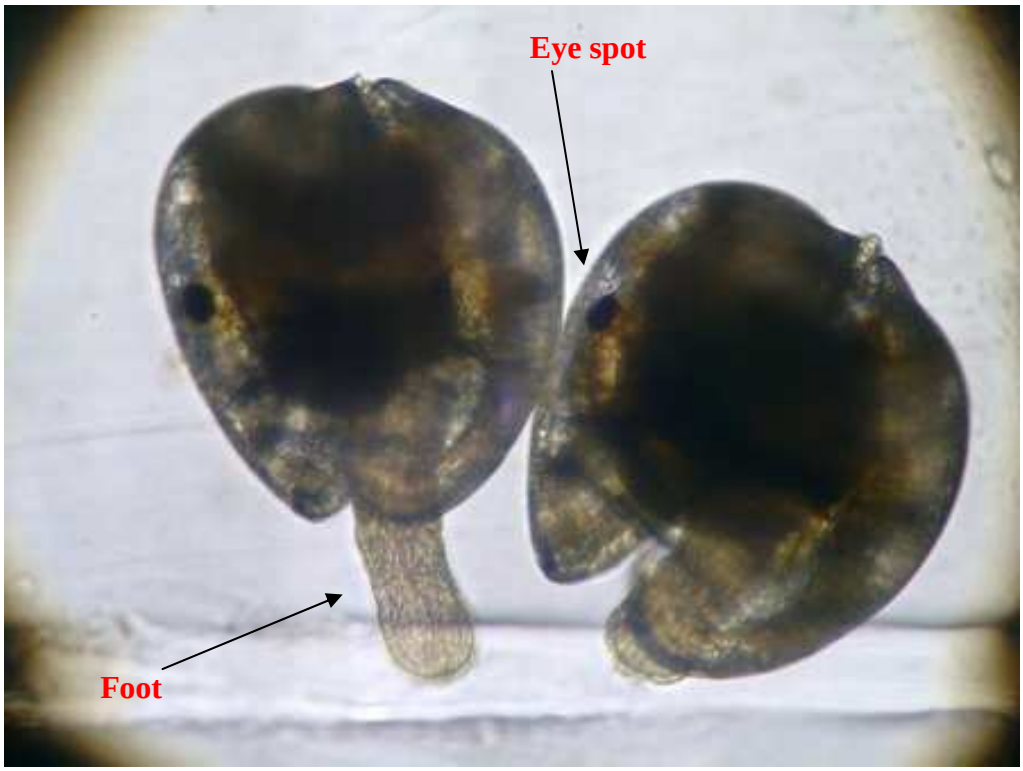


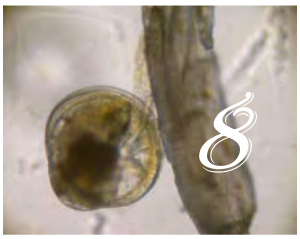
Figure 15. Pediveliger *Saccostrea glomerata* larvae showing foot protruding and eyespot.

- Days 20-22

Water changes are made using 212 µm primary screen and a 180 µm backup screen. Larvae retained on the 212 µm screen are washed into a 20 L bucket and sampled to establish total numbers, development and general health. It is essential during these later stages that close attention is given to larval development. Strong indicators that the larvae are ready to settle are when more than 35% of larvae are crawling pediveligers and when there are between 3-5 gill buds (Chapter 7) present on 50% of the pediveligers. Larvae retained on the 212µm screen are then put to set using epinephrine bitartrate (refer to Chapter 7).

If a significant number of larvae are retained on 180µm back-up screen, they are restocked into a new larval rearing vessel until they are large enough to be retained on a 212µm screen and the pediveliger larvae show signs that they are ready to metamorphose.

Settlement



Introduction

Settlement and metamorphosis marks a distinct morphological change from the free swimming larval stage to the sedentary benthic existence of the adult oyster. A number of physical characteristics are evident in SRO larvae indicating when metamorphosis is imminent. The larvae have eyespots and have reached a shell size of approximately 300 µm. The foot has developed and a percentage of the larvae can be observed both swimming with their foot protruding and have begun to crawl. At this stage it is also important to monitor the development of gill buds in the larvae (Figure 16). At least three (preferably 4-5) rudimentary gill buds on the gill arch should be observed under the microscope at 100 x magnification before settlement protocols are commenced. Gill buds are quite difficult to observe in a fixed sample, therefore it is recommended to observe gill bud development in live samples.

Figure 16. Pediveliger *S. glomerata* larvae showing well developed gill buds

Epinephrine bitartrate treatment

The treatment process conducted for the breeding lines is intended to allow consecutive treatment of a series of separate breeding lines using the same epinephrine bitartrate solution. To insure that no cross contamination of larvae occurs during the epinephrine treatment process, labelled 150 µm screens are used as settlement screens. Larvae are treated *in situ* on the settlement screens while immersed in an epinephrine bitartrate solution contained in an appropriate sized tub or bucket. This process allows the re-use of the epinephrine solution several times.

Figure 17. Individual set tanks and screens for pair mated breeding lines

Figure 18. Down-welling system used for mass selected spat production

Figure 19. Spat retained on a settlement screen

Because larvae within batches develop at different rates and are never uniformly ready to set at the one time, multiple treatments of epinephrine are necessary to obtain maximum yields. The frequency of the epinephrine treatments varies from run-to-run in accordance with the response of the larvae to the treatment.

Before beginning epinephrine treatment, check that the epinephrine is within its “use by date” and has been stored according to the manufacturer’s instructions. Epinephrine bitartrate is generally purchased before each larval run so that it is still active when used. Old batches of epinephrine that are discoloured (brownish hue) are less effective in inducing settlement. Epinephrine bitartrate solution is made up freshly as required.

It is important to note here that epinephrine bitartrate is not freely available to hatcheries in Australia and guidance is required for its use. Hatcheries wishing to use epinephrine bitartrate are required to join the National Aquaculture Council (NAC) and meet a series of guidelines and protocols for its use.

The protocol used here, for the treatment with epinephrine bitartrate of several separate breeding lines, differs slightly from the protocol used for the normal treatment of SRO larvae. Larvae are immersed for 1 h in a solution of 2 x 10^{-4} molar epinephrine bitartrate while retained on the labelled setting screen. The quantity of solution used depends on the screen size and overall number of larvae to be treated in three or four consecutive treatments. Using the same formula, the required quantity of epinephrine bitartrate is dissolved in 25^{o}C FFSW and placed in the appropriate size tub or bucket. All treatments are conducted in a temperature controlled room in complete darkness. The setting screens are simply immersed in the solution for 1 h, removed rinsed thoroughly in FFSW and returned to the appropriate labelled down-welling unit. To insure no cross contamination of larval cultures can occur the epinephrine bitartrate solution is passed through a 30µm screen before re-use. Again, all screens and down-welling units are labelled clearly, designating family origin and a unique indentifying number (Figure 17 and 18) to ensure screens are returned to the correct system.

To maintain the genetic diversity of the mass selected lines, the larvae are repeatedly treated with epinephrine bitartrate until most of the larvae have metamorphosed. Given the genetic homogeneity of the selected family lines treatment with epinephrine bitartrate need only be conducted until the required quantity of larvae have metamorphosed. Following treatment, spat and larvae are retained in separate, labelled down-welling units until they are opf a size that they can be removed from the hatchery. Different sized down-welling units are used for the mass selected lines (Figure 18) compared to the individual units used fro the selected family lines (Figure 17).

Procedures for the use of epinephrine

Chemical: Epinephrine Bitartrate Salt
Sigma E-4375

General use of epinephrine: 1.2g of epinephrine per 50g of larvae in 20L of seawater in a conical tank for 60 minutes treatment.

First Epinephrine Treatment

The method for epinephrine bitartrate treatment used here is a modified from that used for commercial production at the PSFI.

1. A fresh solution of 2 x 10^{-4} molar epinephrine bitartrate is made up as required. As indicated previously, if a large number of larval cultures are produced the exact number of larvae to be treated may not be known. 15 L of epinephrine bitartrate solution is place in an appropriate size tub or bucket and the larvae immersed for 1 hr in the dark while retained on the screen. It is not necessary to aerate larvae during this treatment process.
2. The screen and larvae are removed for the epinephrine bitartrate rinsed and returned to appropriate set system. The solution is passed though a 30 µm screen before re-use. Depending on the number of larvae treated, the epinephrine bitartrate solution can be re-used up to five times before disposal.
3. Before retreatment gently brush off any adherent larvae from the screen and follow steps one and two above.

4. The mode of genetic selection, either mass selection or pair mated, will determine the number of treatments required. To maintain genetic diversity of the mass selected lines, larvae will need to be treated for several days to ensure as many larvae metamorphose as possible.

Spat - down-welling systems

Only larvae from the same mass selected or selected family lines are retained in the same down-welling unit. All down-welling units and screens are clearly labelled with a unique description identifying parentage and a stock number. Spat from a mass selected line are retained on screens in a 750 L tub (Figure 18), where spat from family lines are retained on screens in a separate 200 L down-welling unit (Figure 17).

A spray bar provides flow to the spat by way of a submersible pump located in the bottom of the unit. This unit is aerated and an immersion heater maintains the water temperature at 24-25ºC. The screens are rinsed daily and every second day the screens are transferred into a new system with fresh pumps, hoses, airlines and spray bars. The used tank and fittings are cleaned using Virkon S solution (5 g L^{-1}), before being rinsed with freshwater and allowed to dry.

The feeding rates for larvae/spat held in down-welling systems start at approximately 40,000 - 50,000 cells $larva^{-1}day^{-1}$ and are adjusted according to the rate of algal consumption (refer Chapter 8.4). Larvae are retained in the down-welling units until they have reached a suitable size for direct transfer to a farm based nursery location.

Spat - diet composition and calculating food ration

It is not uncommon for algal consumption to stabilise or reduce slightly in the down-welling units during larval metamorphosis. Due to the large number of down-welling units used at this stage of hatchery rearing, it is not possible to count the number of algal cells present in the down-welling unit to calculate the required daily ration. With experience it is possible to determine the need for increased or decreased feed rations by the colour and clarity of the culture water in the down-welling units. If the number of down-welling units in use is small enough to allow counting residual algae from the previous feed, algal cell density in the culture water is maintained between 100,000 to 150,000 cells mL^{-1}.

In the first few days after settlement the spat diet is the same as that used for the latter stages of the larval cycle. After this time period, it is recommended that the percentage of Muel in the diet is increased, percentage of *T. iso* and Pav decreased and other algal species are introduced into the diet (eg. 10% *T. chuii*). Spat are fed in the morning and evening of each day. Algal consumption can increase markedly at this stage of the juvenile life cycle. Hence, algal production needs constant attention and a dietary supplement such as M1 (Nosan Corporation) can be stored as a back up in the case of algal production problems.

Farm based nursery systems

The nursery process departs slightly from the normal SRO nursery process. Larvae are retained in the hatchery until a mean shell length of 3 mm and can be retained on 2mm mesh. The mass selected lines, one line per tray, are retained in plastic meshed pine timber trays (Figure 20 and 21) and family lines are placed in mesh bags (1100 µm) secured within a section of a 8 mm mesh plastic tray. All trays are labelled using numbered plastic tags (Figure 21) allowing identification of the line or family and only one tray is opened and worked through at any time to reduce the risk of cross contamination.

Both types of nursery systems require inspection, cleaning, grading, culling and restocking every two to four months. The frequency of inspection is dependent on environmental conditions

governing: oyster growth; siltation;, and, biofouling of the trays. A set of new risks arise once spat are placed into the estuary, therefore careful consideration is needed to select sites used to for spat grow-out. Sites that are readily accessible, secure and are visible from a land base are ideal. Furthermore, it is advisable to split families or lines over separate trays and sites in case of a tray loss or impact to a site.

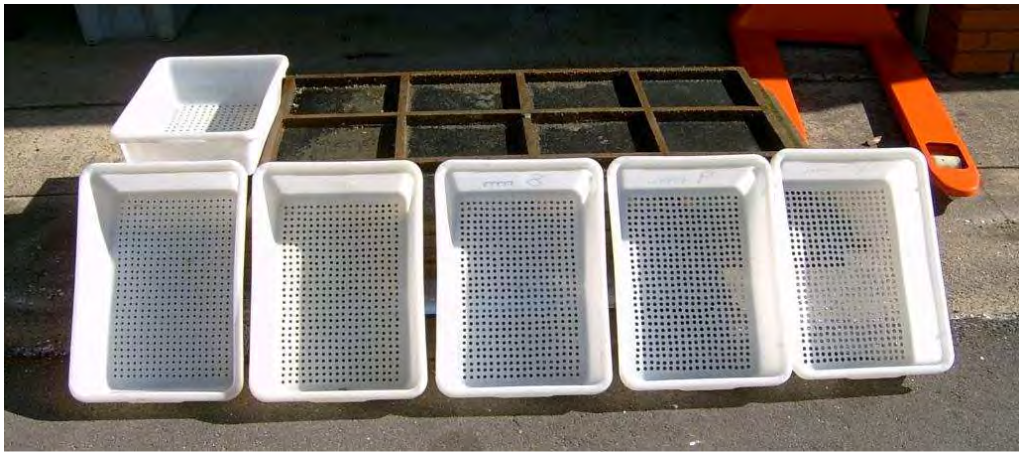


Figure 19. Grading screens

Figure 20. Nursery rearing trays for mass selected lines. Note tag on trays for easy identification.

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CRYOPRESERVATION TECHNIQUES FOR SYDNEY ROCK OYSTERS

Report Prepared for:
The Select Oyster Company

July 2007

Industry & Investment NSW, Fisheries
Port Stephens Fisheries Institute
Taylors Beach Road
Taylors Beach NSW 2316
Phone: 02 4982 1232
Fax: 02 4982 1107

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EXECUTIVE SUMMARY

As part of Seafood CRC/FRDC program 2006/226, cryopreservation techniques suitable for Sydney rock oysters (SRO) have been investigated by NSW DPI in collaboration with Dr Serean Adams (Cawthron Institute, New Zealand). This work demonstrated that cryopreservation of SRO sperm and eggs is possible and shows significant potential for integration into future SRO breeding programs. Reliable techniques for sperm have been developed and cryopreserved eggs have been successfully fertilised. A major outcome from this work is a 'standard operating procedure' (SOP) for cryopreservation of SRO sperm based on the most successful method developed to date. The most effective cryoprotectant (CPA) was 1.0 M dimethyl sulphoxide combined with 0.08-0.51 M trehalose and sperm is frozen by holding 3 cm above liquid nitrogen for 10 minutes followed by plunging directly into liquid nitrogen. At least 1,000 cryopreserved sperm should be added for each egg to ensure maximum rates of fertilisation. Cryopreserved sperm from a single male has been used to produce approximately 100,000 D-veliger stage larvae. Fertilisation success of cryopreserved eggs has ranged between five and nine percent. However, future research direction has been identified to improve cryopreservation techniques for SRO eggs which is expected to increase fertilisation rates. This research will investigate the freezing and cooling method along with different CPAs, CPA concentrations and diluents. The progress in cryopreservation techniques for SRO so far is significant, particularly for use in future SRO breeding programs. Irrespective of further developments in cryopreservation of SRO eggs, the methods developed to date will deliver a number of safeguards for future breeding programs and allow breeding and hatchery procedures to be operated more efficiently.

1.0 INTRODUCTION

Cryopreservation of Sydney rock oyster (SRO, *Saccostrea glomerata*) gametes could be a valuable tool for SRO breeding programs. Among the potential benefits are that cryopreserved gametes can:

- be used to improve hatchery operations by providing eggs or sperm on demand and simplifying the timing of induced spawning;
- streamline hatchery operations by eliminating the need to maintain live broodstock;
- Protect valuable genetic lineages, research models or improved farmed strains, by storing frozen gametes. This could be critical for oysters, where potentially extremely valuable broodstock must be stored in natural waters;
- Store gametes well beyond the natural life span of a particular oyster to allow its contribution to future breeding exercises;
- be used in breeding programs to create new, improved lines and shape the genetic resources available for aquaculture operations;
- alleviate some of the biosecurity risks associated broodstock movements, and
- cryopreserved sperm from successive generations can be used to fertilise eggs from a single female or pool of females to positively demonstrate genetic gains.

NSW DPI in collaboration with Dr Serean Adams from the Cawthron institute, New Zealand, investigated cryopreservation techniques for Sydney rock oysters during two periods of intensive experimentation held at the Port Stephens Fisheries Centre (PSFC). Cryopreservation techniques tested during these workshops were based on successful protocols developed for Pacific oysters (*Crassostrea gigas*). The results thus far have clearly demonstrated that cryopreservation of SRO gametes is possible. Reliable techniques have been developed for sperm and further research is expected to improve the cryopreservation techniques for SRO eggs, thereby increasing the utility of cryopreservation to future breeding programs.

The initial workshop was a preliminary evaluation of a range of techniques for cryopreservation of sperm and eggs that tested different cryoprotectants (CPAs) and concentrations, different cooling regimes and optimal storage volumes. Three sperm freezes and four egg freezes were done and short-term storage of eggs and sperm at 4°C and its effect on fertility was investigated. The methods and results of all experiments conducted for the first cryopreservation workshop are contained in Section 2.0 of this report. The main outcomes from this first workshop were optimal cryopreserved sperm concentration for fertilisation of fresh eggs and successful fertilisation of cryopreserved eggs

The November 2006 workshop focussed on SRO sperm cryopreservation and used information gathered from the first workshop as a platform for further experiments. The sperm freezing method and storage volume remained fixed while different CPAs, CPA concentrations, diluents and dilution ratios were investigated. More specifically, the experiments conducted for sperm for this workshop involved comparing:

- Four CPAs (dimethyl sulphoxide, ethylene glycol, propylene glycol and glycerol) at a concentration of 1.5 M.
- Dimethyl sulphoxide as a CPA at a concentration of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 M.
- Milli-Q water, seawater and trehalose made with Milli-Q water as diluents for sperm preservation. and,
- The effect of different sperm : diluent ratios (1:1, 1:5 and 1:10) using 1 M DMSO, 0.34 M trehalose in 50% seawater (final concentrations).

To examine the applicability of cryopreservation for the breeding program, 250,000 eggs were fertilised using cryopreserved sperm and the proportion embryos to develop to the D-veliger larval stage was determined after 20 hours of incubation. Twenty to forty percent of eggs fertilised with cryopreserved sperm developed to the D-veliger larval stage. The egg freeze experiments investigated different freezing regimes and different CPAs. Detailed methods, results and conclusions for of all experiments conducted for the second workshop are presented in Section 3.0 of this report.

2.0 CAWTHRON REPORT (No 1128) FROM WORKSHOP ONE: SYDNEY ROCK OYSTER CRYOPRESERVATION WORKSHOP

2.1. Introduction

Preliminary cryopreservation experiments were conducted on Sydney Rock oyster (*Saccostrea glomerata*) sperm and eggs. A number of critical factors were evaluated including: (i) different cryoprotectants (CPAs) and concentrations, (ii) different cooling regimes and (iii) storage volumes. Protocols that had been found to be successful for Pacific oyster (*Crassostrea gigas*) sperm and eggs were also evaluated.

Sperm typically showed a 10-100 fold reduction in fertility following cryopreservation. High fertilization success was recorded using thawed sperm at 10^5 sperm/mL after some freezing treatments. The standard freezing method for Pacific oyster eggs was unsuccessful for Sydney rock oyster eggs. However, using a variation of the method, 9% post-thaw fertilization was achieved. In other treatments, eggs were typically lysed upon thawing. These results are very encouraging for a first attempt and it is likely that further work would enable higher fertilization rates to be achieved with both cryopreserved eggs and sperm and robust protocols could be established.

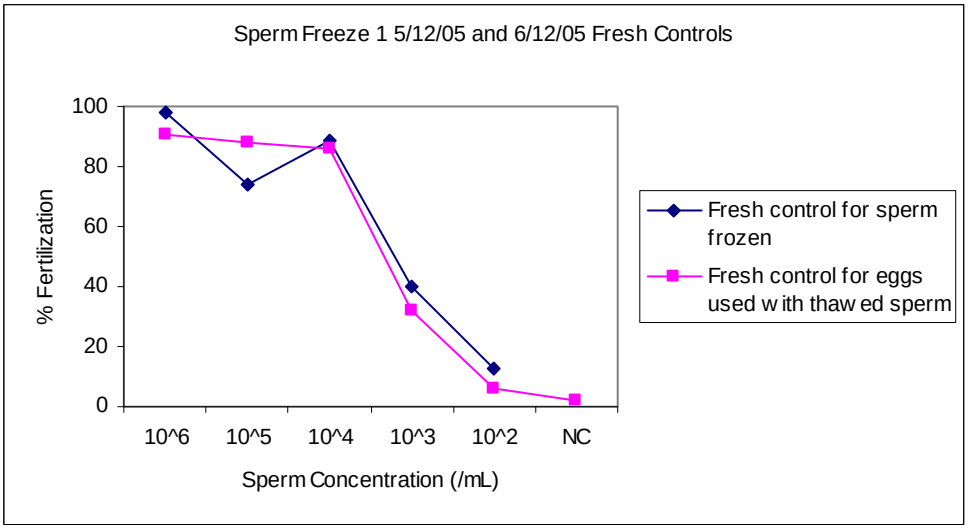
2.2. Sperm freeze experiments

2.2.1. Sperm Freeze 1

Objective:
Experiment compared three freezing methods, three CPAs and two storage volumes using stripped sperm. The experiment included treatments that were the same as those developed for cryopreservation of Pacific oyster (*Crassostrea gigas*) sperm.

- Freezing methods: ethanol/dry ice bath, floating rack over liquid nitrogen and programmable freezer (P2 program: 0 to -75°C at 5°C/min)
- CPAs: 10% dimethyl sulphoxide (DMSO) (final concentration) in either milli-Q, 0.5 M Trehalose (TRE) or seawater (SW). CPAs were cooled on ice and added 1:9 to stripped sperm.
- Storage volumes: 4.5 mL cryovials (CVs) and 0.5 mL straws.
- Pacific oyster sperm protocol: ethanol/dry ice bath or floating rack over liquid nitrogen, 10% DMSO in 0.5 M TRE, 4.5 mL CV.

Results:



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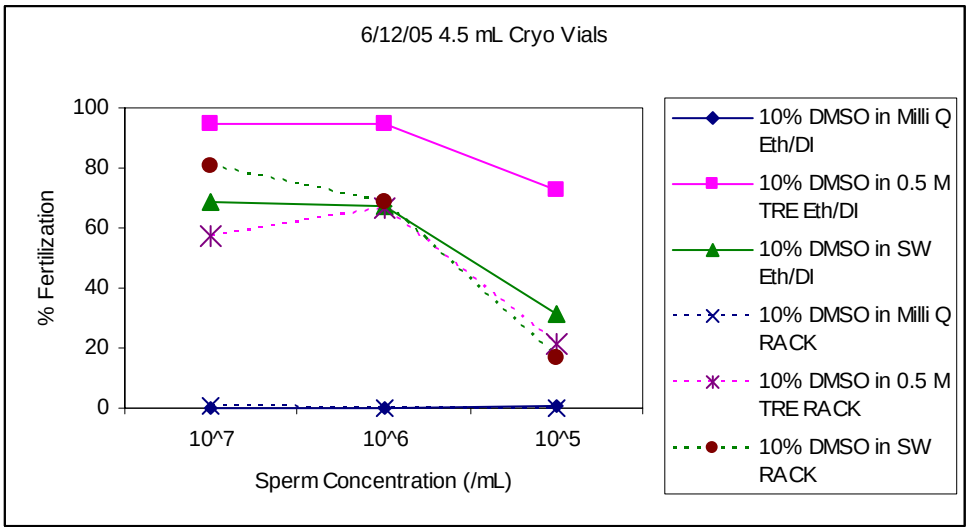

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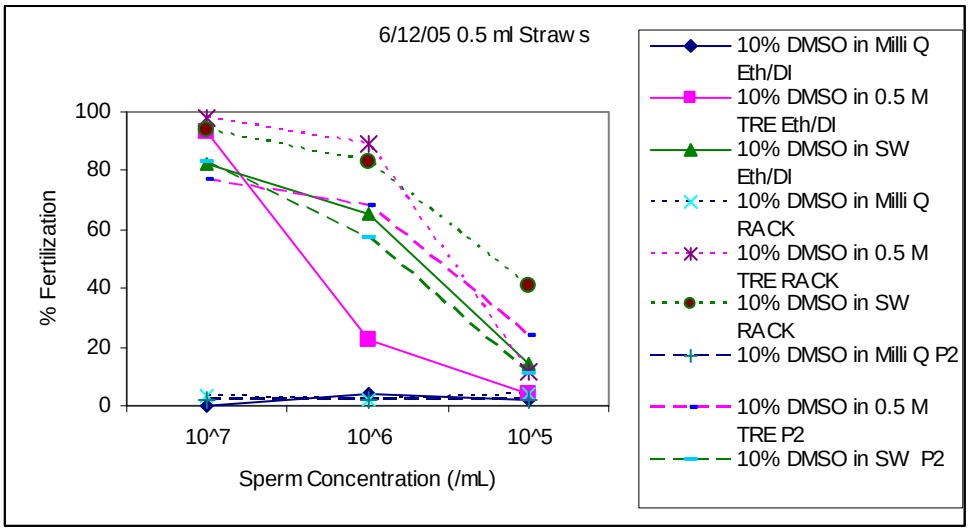


Figure 2.3.

Summary of Results:

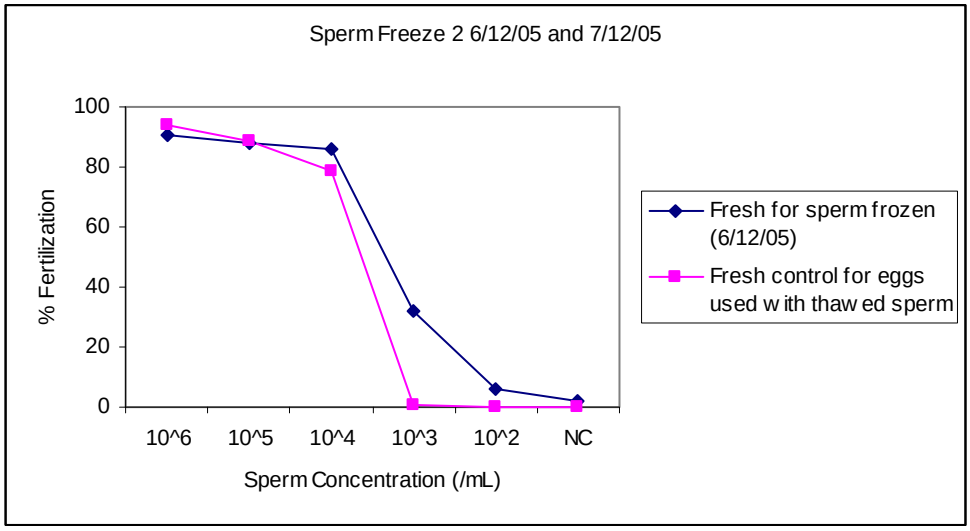
- Sperm frozen in 10% DMSO in 0.5 M TRE in 4.5 mL CVs using ethanol/dry ice bath had highest post-thaw fertilization rate.
- Post-thaw fertilization for sperm frozen in 10% DMSO in Milli-Q was poor, irrespective of cooling regime or straw/CV, probably due to 1:9 dilution rate, which made osmolality low.
- For 0.5 ml straws, there was some indication that post-thaw fertilization was slightly higher using the rack than the ethanol/dry ice bath or P2 program.

2.2.2. Sperm Freeze 2

Objectives:

- Used same diluents as freeze 1 but sperm diluted 1:1 instead of 1+9.
- Used spawned sperm.
- Compared 3 cryovial sizes and 0.5 ml straws on the rack freeze.

Results:



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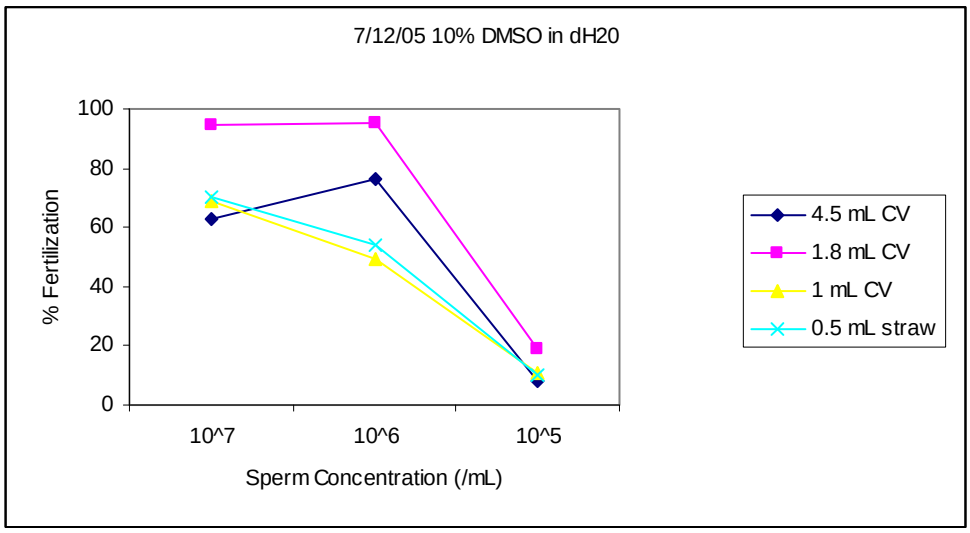

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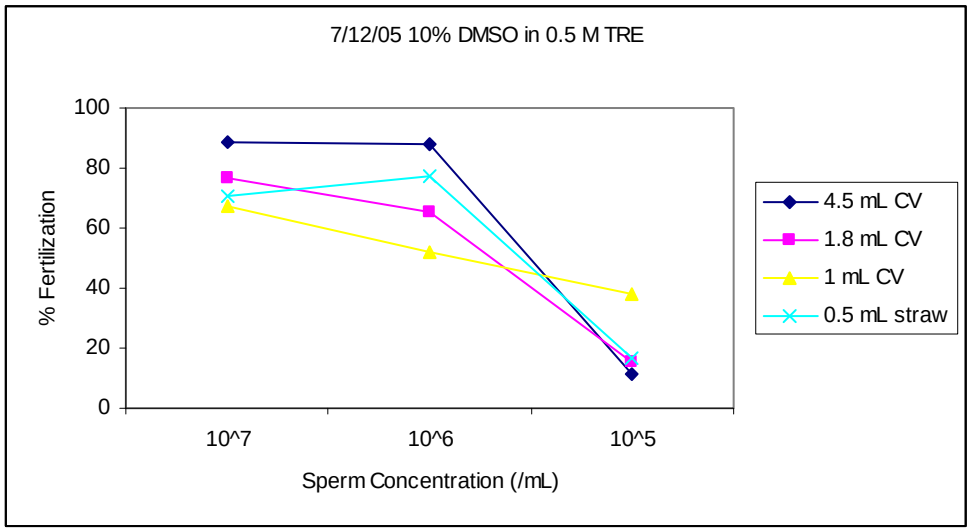

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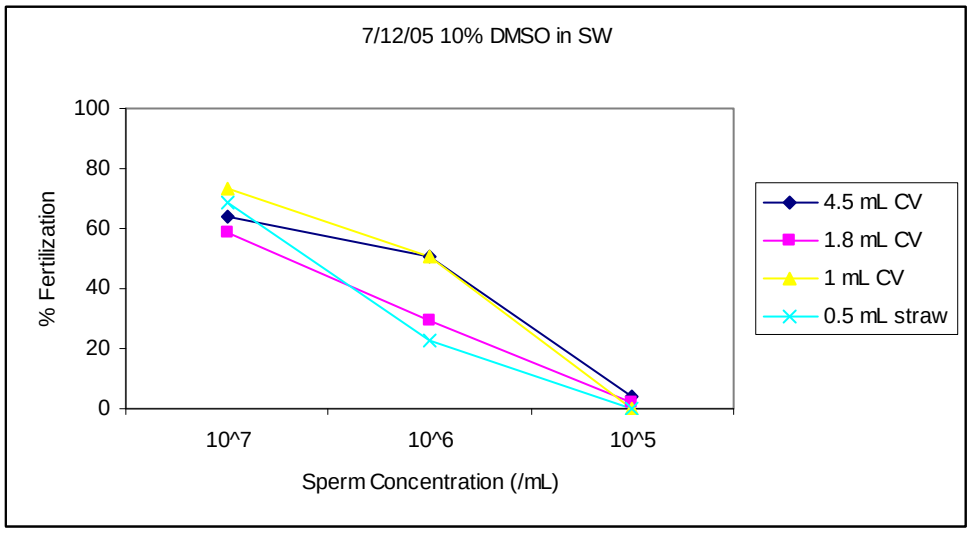


Figure 2.7.

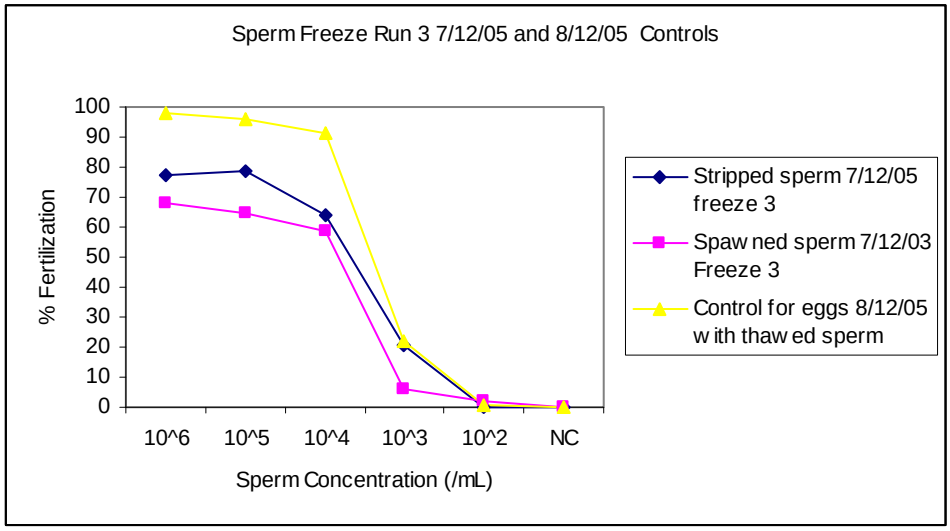
Summary of Results:

- In general, sperm performed better when frozen in milli-Q or 0.5 M TRE than in SW. There was no clear trend between the cryovials and straw volumes tested.

2.2.3. Sperm Freeze 3

Objective:
Experiment compared three DMSO concentrations, three diluents and spawned vs stripped sperm using the rack method.

- 5, 10, 15% DMSO
- in SW, 0.5 M Trehalose, 0.5 M sucrose (SUC)
- NB: for stripped sperm 10% DMSO and 15% DMSO in SW treatments were lost.



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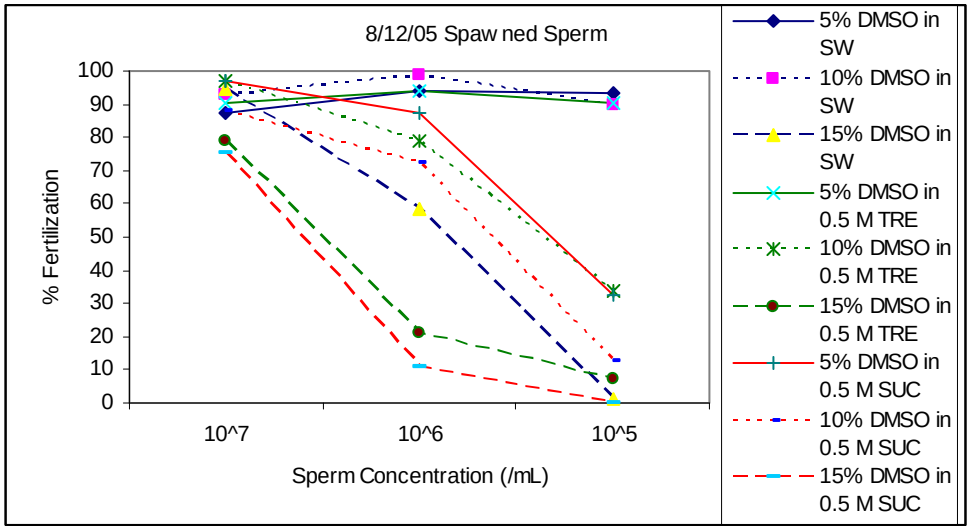

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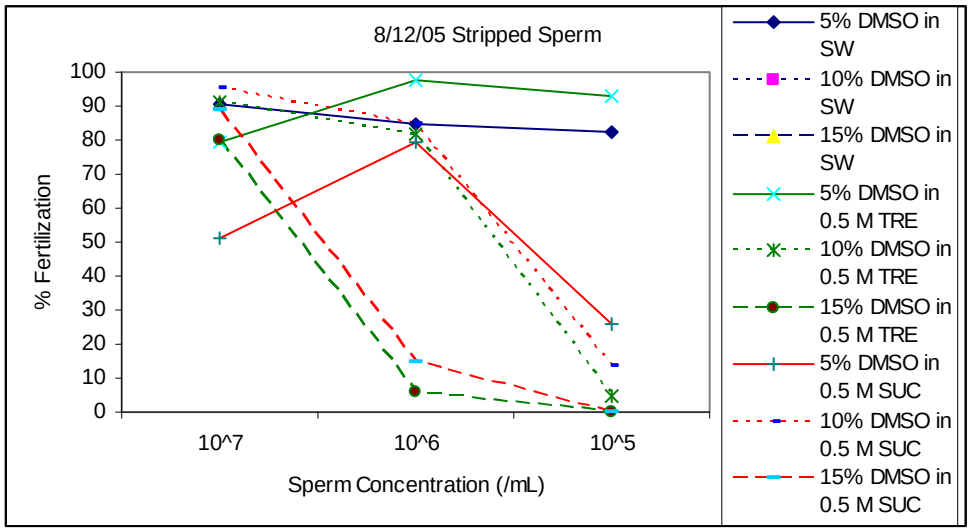


Figure 2.10.

Summary of Results:

- Spawned and stripped sperm appeared to have similar fertilization rates.
- 5 or 10% DMSO gave higher post-thaw fertilization than 15% DMSO. 5% or 10% DMSO in SW or 5% DMSO in 0.5 M Trehalose gave highest fertilization rates.

2.3. egg freeze experiments

2.3.1. Egg Freeze 1

Objective:

- Three CPAs (DMSO, EG and PG) at 10% (final concentration) in milli-Q or SW were compared. The experiment included the standard protocol developed for cryopreservation of Pacific oyster (*Crassostrea gigas*) eggs (10% EG in milli-Q)
- Eggs were frozen using Pacific oyster egg cooling program (0°C for 5 min, then cool at 1°C/min to -10°C, hold for 5 min (check ice seeding), then 0.3°C/min to -35°C, then plunge).

Summary of Results:

- Eggs were completely lysed upon thawing with the odd intact egg appearing in some treatments

2.3.2. Egg Freeze 2

Objective:

- Compared various diluents: 10% EG used as permeating CPA made up in Milli-Q, 0.25 M TRE, 0.5 M TRE, 0.75 M TRE, 1% polyvinylpyrrolidone (PVP) or 1%PVP +0.5 M TRE.
- Eggs were frozen using Pacific oyster egg cooling program.

Summary of Results:

- The odd intact egg in some treatments but most eggs blasted apart.

2.3.3. Egg Freeze 3

Objective:

- Compared the same diluents as Egg Freeze 2 but a different cooling program was used.
- 0°C for 5 min, then cool at 1°C/min to -10°C, hold for 5 min (check ice seeding), then 1°C/min to -35°C, then plunge.

Summary of Results:

- The odd intact eggs in some treatments but most eggs ruptured.

2.3.4. Egg Freeze 4

Objective:

- Compared 10% EG and 10% DMSO as CPAs in milli-Q, 0.5 M TRE, 1% PVP and 1% PVP + 0.5 M TRE using a very slow cooling rate post-hold.
- 0°C for 5 min, then cool at 1°C/min to -10°C, hold for 5 min (check ice seeding), then 0.1°C/min to -35°C, then plunge.
- At -35°C, a straw of each treatment was removed and the morphological integrity of the eggs was examined.

Summary of Results:

- After cooling to -35°C, 30% of eggs were found to be intact in 10% EG and 44% in 10% EG +1% PVP. In other treatments, the eggs were lysed.
- Some eggs thawed after LN plunge from these two treatments fertilized!

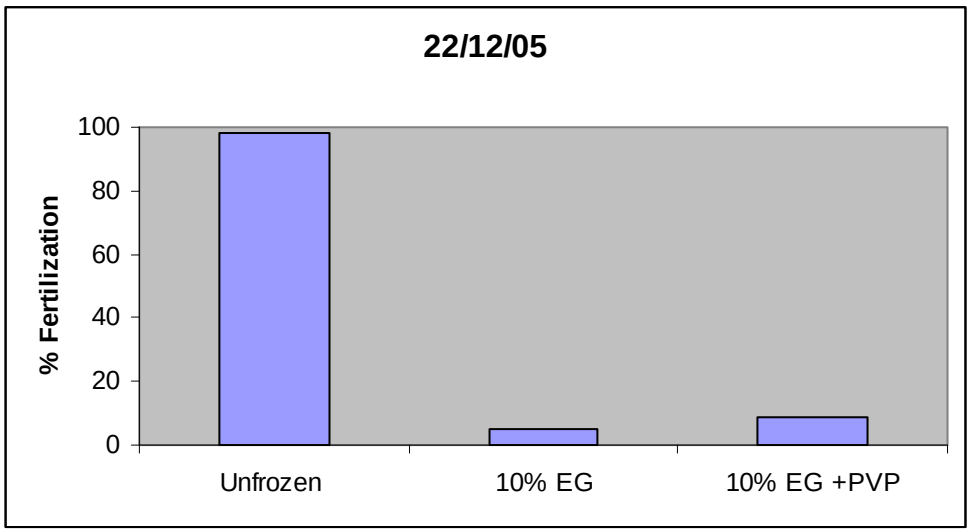
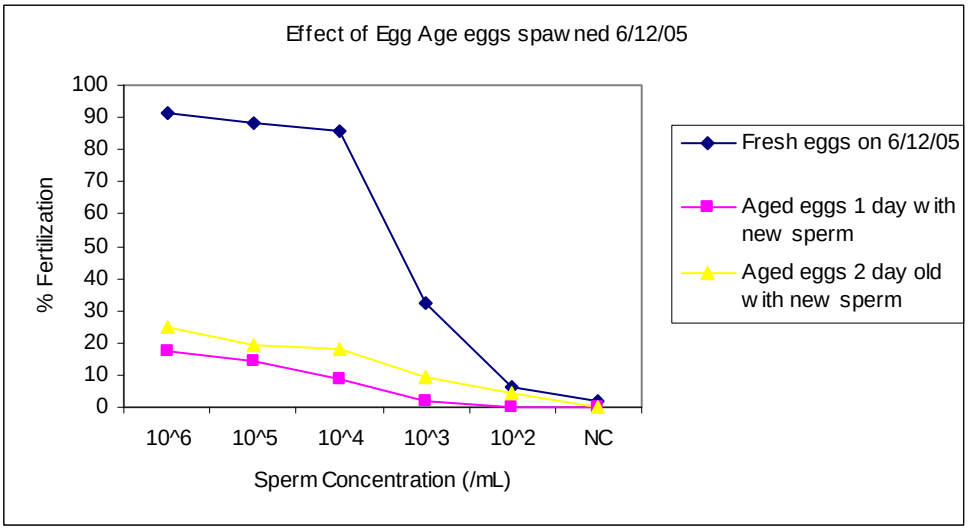


Figure 2.11.

2.4. Egg and sperm age experiments

Objective:

- Sperm and eggs were maintained either in the fridge or on ice and their change in fertility over time was evaluated.
- NB: spawned and stripped sperm collected on 7/12/05 were left overnight on ice which had mostly melted after 24 h.



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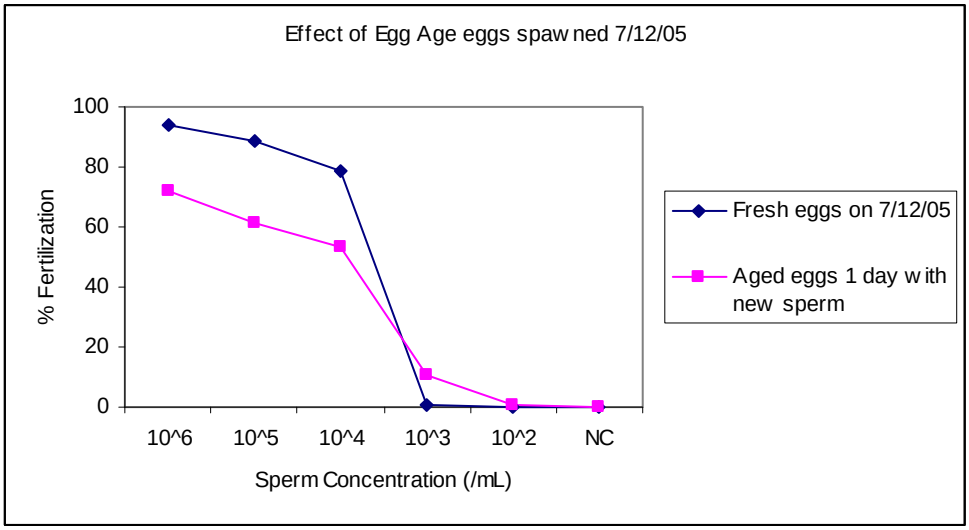

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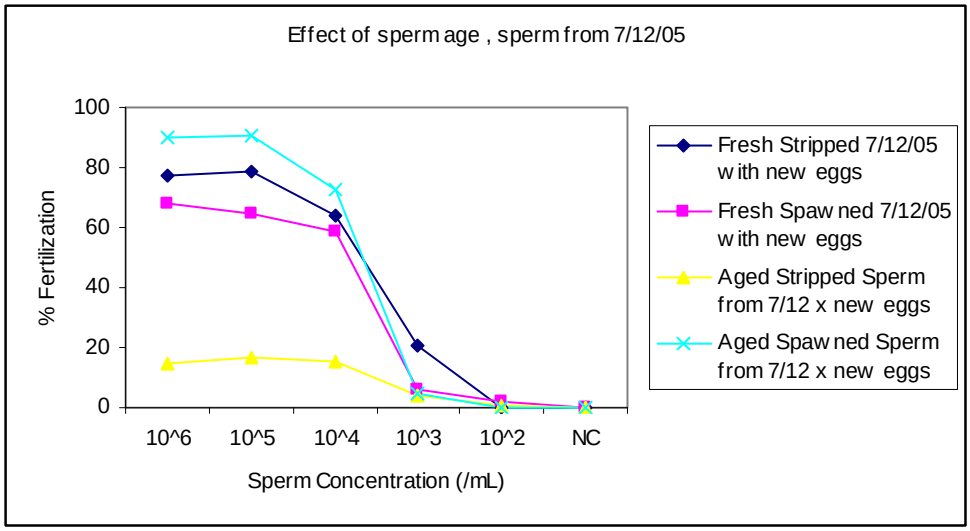

Figure 2.14.

Summary of Results:

- Egg viability declined over the first 24 h post-spawning. The effect of aging on sperm is less clear and results need repeating.

2.5. CONCLUSIONS

2.5.1. Sperm Cryopreservation

Both stripped and spawned sperm can be readily cryopreserved although spawned sperm is difficult to collect. The lack of difference in the fresh fertilization suggests that future work could be carried out using just stripped sperm.

5 – 10 (v/v) % DMSO is an effective CPA for SRO sperm and it appears that a range of freezing conditions can be used. Further replication is needed to sort out the best diluent, freeze method, dilution ratio and volume size. Other permeable CPAs could also be tested and additional trehalose concentrations. The effect of additives such as antioxidants, motility stimulants, membrane stabilizers, etc. also warrant investigation.

2.5.2. Egg Cryopreservation

SRO egg cryopreservation is feasible and further research will likely improve the fertilization rates that can be attained. In future work, the fertilised cryopreserved eggs should be allowed to develop further to ensure that they can develop into normal larvae / spat. Further work should investigate other cooling regimes (eg. slower cooling rates, hold temperatures, hold times and plunge temperatures), other CPA concentrations and combinations, different diluents (eg. varying seawater concentration). The effect of various additives could also be investigated (eg. cholesterol, antioxidants etc.)

2.5.3. Gamete Aging

Egg fertility appears to decline in the first 24 h. Further work should quantify the effect of egg age at times < 24 h and ambient versus fridge as storage temperatures. The effect of sperm age is less clear with different results for the stripped and spawned sperm that were evaluated. Sperm were

left on ice which had mostly melted after 24 h rather than in the fridge. In future work, the sperm should be aged in the fridge to ensure that a constant low temperature is maintained.

3.0. CAWTHRON REPORT (No 1310) FROM WORKSHOP TWO: SYDNEY ROCK OYSTER (*SACCOSTREA GLOMERATA*) CRYOPRESERVATION WORKSHOP – NOV 2006

3.1. Introduction

This workshop expanded on the Sydney rock oyster (SRO) cryopreservation workshop carried out in November 2005. The main goal was to collect publishable data sufficient for a paper on SRO sperm cryopreservation. Different cryoprotectants (CPAs), CPA concentrations, diluents and dilution ratios were investigated using a single freezing method and volume (selected based on November 2005 results). The use of replicate batches of sperm enabled us to test the robustness of the selected CPA solutions.

Further egg cryopreservation experiments were also carried out. A small percentage of eggs fertilised post-thawing (5% at best). Further research is required to increase this percentage.

3.2. Sperm Freeze 4

3.2.1. Experiment 1

Objective: This experiment compared four cryoprotectants (dimethyl sulphoxide (DMSO), ethylene glycol (EG), propylene glycol (PG) and glycerol (GLYC)) at one concentration (1.5 M) on three batches of stripped sperm pooled from at least three males in approximately equal volumes.

- 3 M solutions of DMSO, EG, PG and GLYC were made up in seawater (SW).
- CPAs were added 1:1 on ice in 10 steps over 12 minutes then loaded into 0.5 ml straws.
- Freezing Method = Rack 3 cm above liquid nitrogen (LN) for 10 minutes then plunge into LN.
- For thawing, straws were placed in a water bath at 23.5°C.

3.2.2. Experiment 2

Objective: This experiment used the same three batches of sperm as above but compared DMSO at a range of final concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 M).

- DMSO was made up in SW to give varying concentrations that were double the final concentration required (range: 0.0 – 5.0 M).
- CPAs were added 1:1 on ice in 10 fixed volume steps over 10 minutes then loaded into 0.5 ml straws.
- Freezing Method = Rack 3 cm above LN for 10 minutes then plunge into LN.
- For thawing, straws were placed in a water bath at 23.5°C.

3.2.3. Results

Males were very difficult to strip because animals had been out of water for 4-5 days. The sperm collected was very clumpy. Not all fertilisation assays were counted for all treatments. Fertilisations were quite poor, even in fresh controls. For fresh sperm, fertilisation only recorded sperm at 10^5 and 10^6 sperm/ml. For cryopreserved sperm, between 22-60% fertilisation was recorded at 10^7 sperm/ml but otherwise fertilisation was low.

3.3. Sperm Freeze 5

Experiment 1 and 2 above were repeated on new oysters.

3.3.1. Experiment 3

Objective: Compared 1.0 M DMSO in either Milli-Q water, SW or in varying levels of trehalose (TRE) (0.08-0.51 M) made up with Milli-Q water.

- CPAs were made up at twice the final concentration required and placed on ice.
- CPAs were added 1:1 on ice in 10 fixed volume steps over 10 minutes then loaded into 0.5 ml straws.
- Freezing Method = Rack 3 cm above LN for 10 minutes then plunge into LN.
- Because eggs collected on the same day as sperm frozen were mostly fertilised (~30% polar bodies observed), fertilisation assays were carried out one day later. Sperm from each batch was kept in the fridge and an unfrozen assay carried on the day of thawing. A pool of spawned sperm from different males collected on the day of thawing was also used to test the fertility of the newly collected eggs.

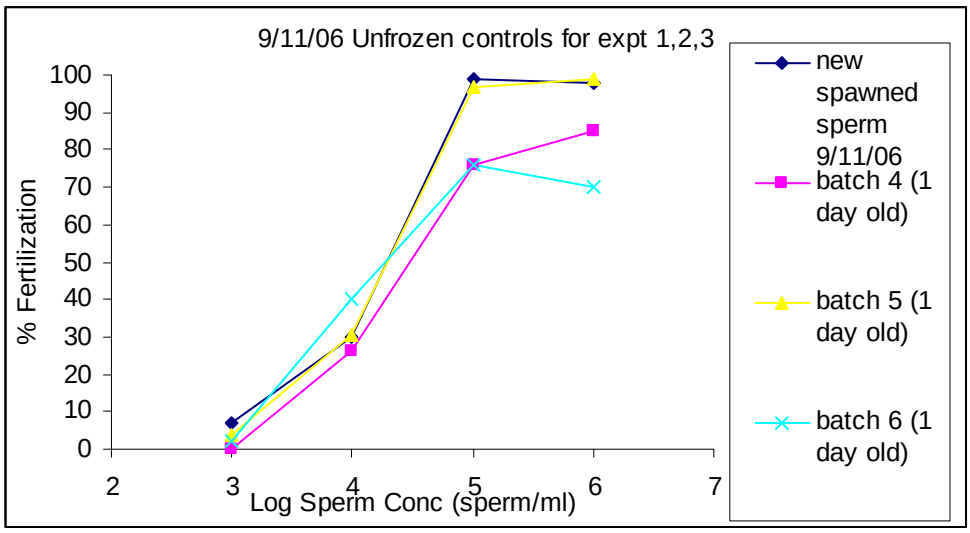

Figure 3.1.

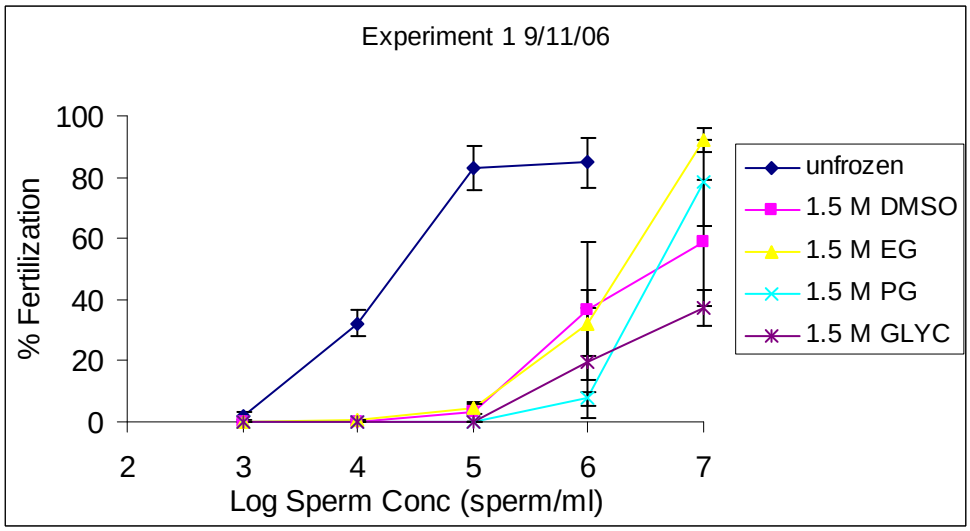

Figure 3.2.
Abbreviations: dimethyl sulphoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (GLYC)).

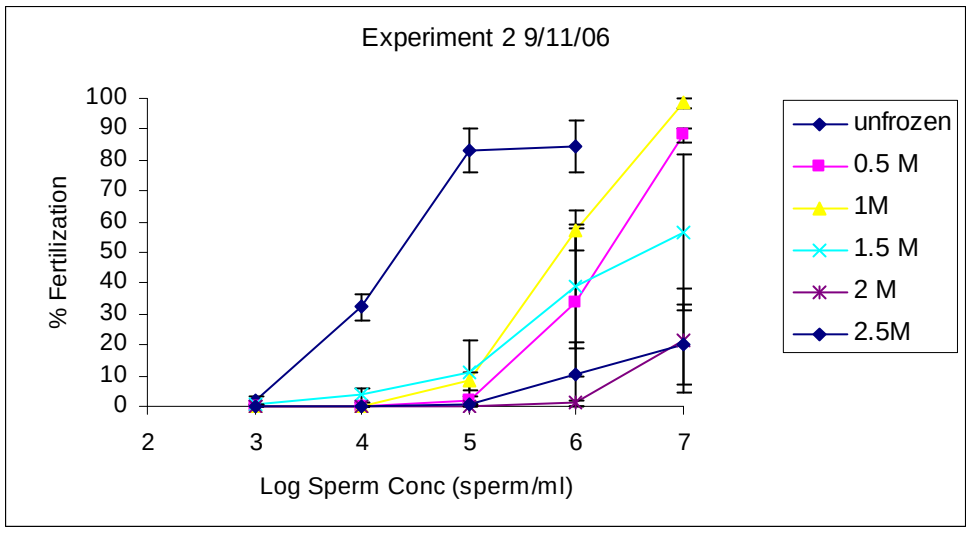

Figure 3.3.

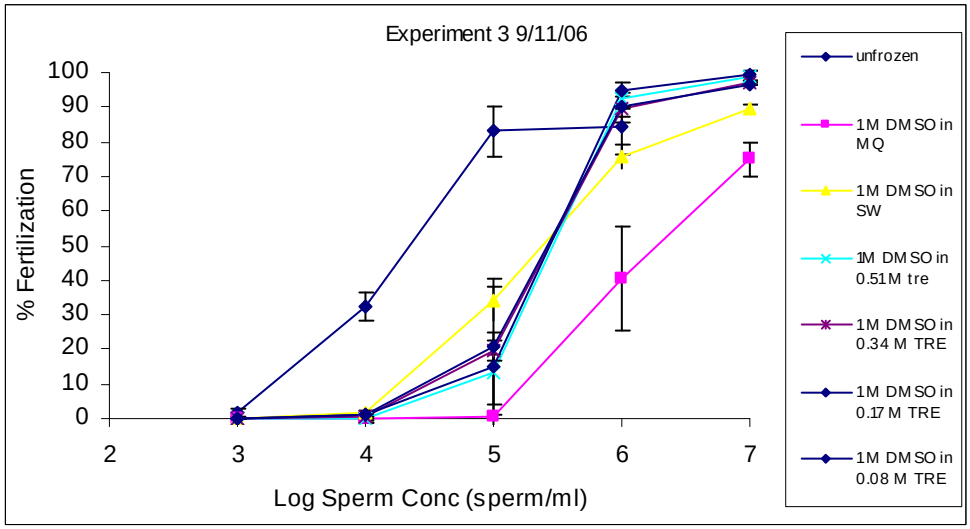

Figure 3.4.
Abbreviations: trehalose (TRE), dimethyl sulphoxide (DMSO).

3.3.2. Summary of Results

- Controls: Two of the day old batches (batches 4 and 6) gave slightly lower fertilisation than the newly spawned sperm at the highest concentrations.
- Sperm showed a 100 fold reduction in fertility following cryopreservation when only a permeating CPA was used. Surprisingly, of the permeating CPAs evaluated, EG was the most effective followed by PG and DMSO.
- DMSO was most effective at 0.5 and 1.0 M. At higher concentrations fertilisation was reduced, probably due to toxicity.
- Post-thaw fertilisation increased when 1 M DMSO was used in combination with 0.08 –0.51 M. In this experiment, the fertility of cryopreserved sperm was approximately 10% that of fresh sperm.

3.4. Sperm Freeze 6

3.4.1. Experiment 4

Objective: This experiment aimed to compare the effect of dilution ratios of 1:1, 1:5, 1:10 and 1:20. The same CPA concentration and same final salt concentration ratio were used (1M DMSO, 0.34 M TRE in 50% SW final). As in Experiments 1-3, sperm were frozen by placing on a rack floating 3 cm over liquid nitrogen. Due to an error in adding the CPA to the solution, the 1:20 data were not valid (the actual dilution of the stock solution was 1:10 and the final CPA concs for this treatment therefore 0.95 M DMSO and 0.32 M TRE).

3.4.2. Results

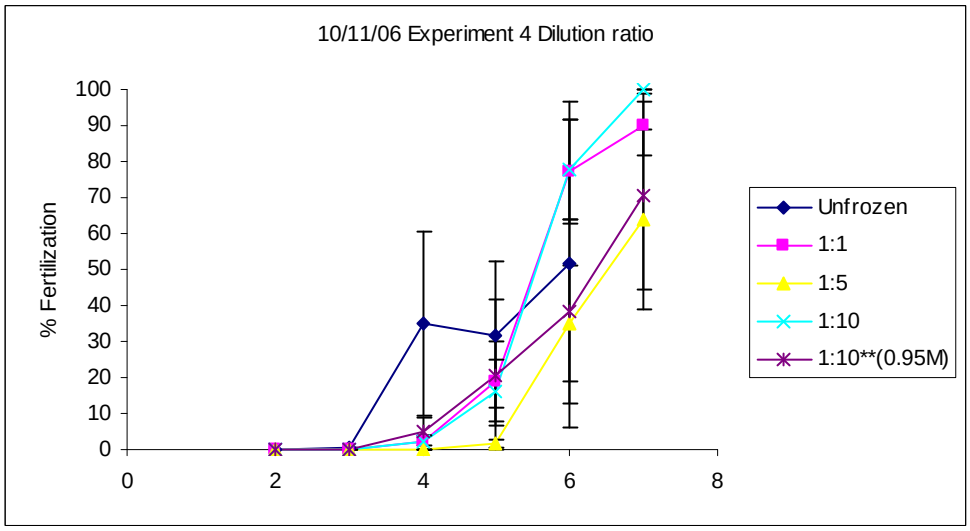

Figure 3.5.
** This treatment is the 1:20 treatment. The CPA was accidentally added 1:10 which gave final concentrations of 1 M DMSO and 0.32 M TRE

3.4.3. Summary of Results

- The fertility of unfrozen sperm was poor at higher concentrations. For one batch it appeared no sperm had been added at 10^6 and for another, poor fertilisation was recorded.
- The effect of dilution ratio was unclear with 1:1 and 1:10 giving higher post-thaw fertilisation than 1:5.

3.4.4. Experiment 5

Objective: This experiment investigated the effect of varying egg density and sperm:egg ratio. The experiment was carried out by Mike Dove on 25 May 2007 using a pool of eggs and sperm from batches 7, 8 and 9 (used in Experiment 4) that had been cryopreserved by diluting sperm 1:1 to give final CPA concentrations of 1 M DMSO and 0.34 M TRE in 50% SW final as previously described.

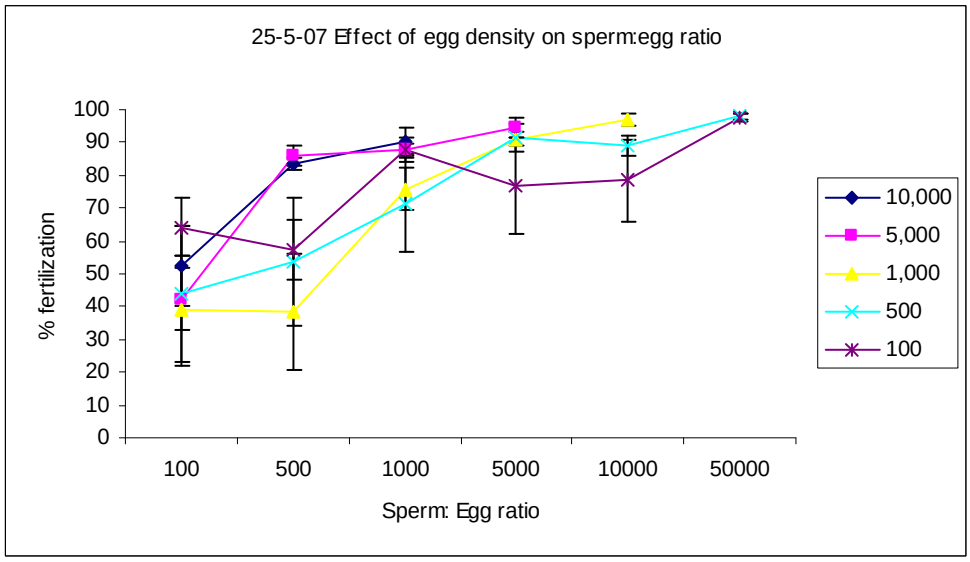

Figure 3.6.

3.4.5. Summary of Results

There appeared to be no clear effect of egg density on fertilisation success. Sperm:egg ratios of 1,000 sperm per egg or higher were required to achieve maximum fertilisation success from cryopreserved sperm.

3.4.6. Bulk Fertilisation Experiment using Cryopreserved Sperm

Objective: To confirm that cryopreserved sperm were capable of producing viable Ds, bulk fertilisations were performed using unfrozen and cryopreserved sperm from the same pool (Pool 7) and fertilizing aliquots of 250,000 eggs collected from a pool of females. Eggs at a density of 200 eggs/ml were fertilised at sperm:egg ratios of 500 sperm per egg for unfrozen sperm and ~4,500 sperm per egg for cryopreserved sperm. After approx 15-20 minutes, eggs were transferred to 200 L larval rearing tanks. Duplicate tanks containing 250,000 eggs were set up for each treatment.

3.4.7. Results

In the unfrozen sperm treatments, 64 and 76% of eggs developed to D-larval stage. In the cryopreserved sperm treatment, 20 and 40% of eggs developed to D-larval stage. The percentage of eggs fertilising in each treatment was not recorded.

3.5. Egg Freeze 5

Objective: Four CPA solutions were evaluated using one cooling programme.

- 10% EG in MQ, 10% EG + 1% PVP, 10% EG in SW, 10% EG+5% PVP (in milliQ water).
- CPA added in 10 fixed volume steps, 1 minute apart. Egg density = 571,666/ml.
- Freezing programme: Hold at 0°C for 5 minutes, then cool to -10°C at 1°C/min, hold for 5 minutes, then cool at 0.1°C/min to -35°C, then plunge into LN.
- Eggs were thawed by Mike Dove on or around 25 May 2007 in a waterbath at ambient temperature (25°C). Eggs were fertilised at 10^6 sperm/ml with fresh sperm.

3.5.1. Results

No fertilisation was observed in any treatment. Most treatments contained lysed eggs. This result was disappointing since two of the treatments (10% EG in MQ and 10% EG +1% PVP) were repeats of the two successful treatments from the December 2005 workshop.

3.6. Egg Freeze 6

Objective: Eight CPA solutions and two freezing regimes were evaluated

- 1. 10% EG in SW
2. 15% EG in SW
3. 10% EG in MQ
4. 15% EG in MQ
5. 10% EG in 1% PVP
6. 15% EG in 1% PVP
7. 10% EG in 5% PVP
8. 15% EG in 5% PVP

- Freeze method 1: Hold at 0°C for 5 minutes, then cool to -10°C at 1°C/min, hold for 5 minutes, then 0.1°C/min to -35°C, then plunge into LN.
- Freeze method 2: Hold at 0°C for 5 minutes, then cool to -10°C at 1°C/min, hold for 20 minutes, then 0.1°C/min to -35°C, then plunge into LN.
- CPA added in 10 fixed volume steps, 1 minute apart. Egg density = 10^6 /ml.
- Eggs were thawed by Mike on or around 25 May 2007 in a water bath at ambient temperature (25°C). Eggs were fertilised at 10^6 sperm/ml with fresh sperm.

3.6.1. Results

At best, 5% fertilisation was observed in Treatment 6 frozen using Freeze Method 1. Treatments 4 and 5 also had some viable eggs for Freeze Method 1. In general, eggs in 15% EG treatments were more intact that in treatments containing only 10% EG, however this did not translate to a higher percentage fertilised.

3.7. Conclusions

3.7.1. Sperm

Overall, the use of 1.0 M DMSO in combination with trehalose at 0.08 – 0.51 M is effective as a CPA solution for SRO sperm. The optimal dilution ratio, however, is unclear and this experiment should be repeated. The effect of egg density on fertilisation is also unclear but a minimum sperm:egg ratio of 1,000 sperm per egg is required to achieve maximum fertilisation with cryopreserved sperm. D-larvae were produced using cryopreserved sperm, demonstrating that the method is useful for selective breeding. Further research on bulk fertilisation strategies for D-larvae should be carried out to confirm that the method is sufficiently robust for selective breeding. To avoid handling issues with DMSO, the use of EG as an alternative permeating CPA or trehalose alone could also be investigated.

3.7.2. Eggs

Although only a few eggs were able to survive and fertilise post-thawing, further research in this area is warranted. Further work should look at different combinations of cooling regimes (*e.g.* slower cooling rates, hold temperatures, hold times and plunge temperatures), other CPAs and

CPA concentrations, different diluents. Removing a sample of eggs at each step in the freezing process will help determine exactly where eggs are being lost.

4.0 OUTCOMES

Reliable methods for cryopreservation of sperm have been developed. Cryopreservation of sperm decreases sperm fertility. Based on this reduction, a standard operating procedure (SOP) was developed for SRO sperm cryopreservation. The title of this SOP is 'Methods for cryopreserving Sydney Rock oyster sperm and for fertilizing eggs with cryopreserved sperm' and is included as an appendix. Further testing of this SOP is proposed to validate this method and ensure it will yield sufficient numbers of viable larvae.

Further experiments that aim to identify: a) the best dilution ratio for freezing sperm samples; and b) the optimal sperm to egg density on fertilisation are suggested. Using the information from the workshops conducted to date, at least 1,000 sperm egg^{-1} should be used to maximise fertilisation with cryopreserved sperm.

Cryopreservation of eggs has provided mixed results to date. Nine percent of eggs were fertilised after freezing during the first workshop, whereas only five percent of eggs were fertilised from experiments conducted during the second workshop. Further research is required for egg cryopreservation that investigates the freezing and cooling method along with different CPAs, CPA concentrations and diluents. Identifying the point in the cryopreservation process at which the eggs are damaged or destroyed will further assist in the development of a suitable method for SRO eggs.

Cryopreservation of SRO gametes is possible and shows great potential as a research tool in future SRO breeding programs. Additional work on cryopreservation techniques for SROs is expected to: elevate fertilisation rates, particularly with respect to eggs; and, develop a robust method that can be incorporated into prospective SRO breeding programs. Research direction that will improve cryopreservation techniques for SRO gametes has been identified. The progress made to date is exciting and the methods already developed for sperm represent a significant achievement in being able to utilise the benefits of cryopreservation for future SRO breeding.

5.0 APPENDIX

Methods for cryopreserving Sydney Rock oyster sperm and for fertilizing eggs with cryopreserved sperm

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1 PURPOSE

To describe the procedure for cryopreserving Sydney rock oyster sperm and how to fertilize eggs using frozen sperm.

2 ACTIONS

NB: Staff should be fully familiar with liquid nitrogen handling and safety precautions before undertaking this work.

2.1 PREPARATION

- D(+) Trehalose (Sigma Product:T5251)
- Dimethyl sulphoxide (DMSO) (Sigma Product: D8779)
- Milli-Q or deionized water
- Lugol's iodine
- 50 ml falcon tubes
- Lab Serv 70 ml pottles
- Haemocytometer
- Steel straw rack attached to 3 cm thick polystyrene
- Polystyrene chilli bin
- Liquid Nitrogen
- Long forceps
- Safety gloves and glasses
- Storage goblets
- 0.5 ml straws
- Sealing powder
- Plastic Comb and Boat
- Storage Dewar
- Container for thawing
- Scissors
- Ethanol (70%)
- Tissues

- Prepare a trehalose solution (Sigma T5251) by adding 15.2 g trehalose to 20 mL distilled water in a 50 ml falcon.
- Then combine 15 ml of the trehalose solution with 4.3 ml of DMSO and 10.7 ml of Milli-Q water.
- Place solution on ice to cool before use (nb The addition of DMSO will give cause an exothermic reaction when added to the trehalose).
- Prepare the polystyrene chilli-bin for freezing by adding liquid nitrogen so that the chilli-bin is approximately 2/3 full. Be aware that the liquid nitrogen will bubble and splash initially as it cools the container. Gently float the straw rack attached to polystyrene on the liquid nitrogen. To obtain a more stable environment for freezing, loosely place the lid on the chilli-bin.

2.2 OBTAIN SPERM

- Strip sperm from males into a plastic "pottle" using a minimal amount of seawater and hold "dry" on ice.

2.3 DETERMINE SPERM DENSITY

- Set up Kimble glass test-tubes for diluting and counting the sperm by placing 900 μL of sea water into the first 2 test tubes and 800 μL of sea water into a third test tube. Ensure sperm is evenly mixed in suspension. Take a 100 μL sample of the sperm suspension and place in the first tube. Mix well using the pipette. Change pipette tip and transfer 100 μL from the first tube to the second tube. Mix well. Again change tip and transfer 100 μL from tube two to tube three. Mix well. Change tip and add 100 μL of Lugol's iodine. Mix well then take a 100 μL sample of the suspension. This is a 1000 fold dilution of the original stock. Take a haemocytometer slide and place the cover slip over the grid. Place the pipette tip near the edge of the coverslip and expel sample, allowing it to be drawn under the coverslip by capillary action. Place the haemocytometer slide under the microscope. If there are insufficient sperm to count, the dilutions should be repeated but with only a 200 fold dilution of the original stock (i.e. take 900 μL of sea water + 100 μL of sperm into first test tube, mix well, then into second test tube take 850 μL seawater and add 50 μL of sperm solution from first test tube plus 100 μL of Lugol's iodine).

- Count the number of sperm in a 1mm x 1mm square grid of the haemocytometer. Do not count cells on all of the boundaries. Only count those sperm touching the top and left that are touching the middle lines. Do not count cells touching the bottom and right middle lines. To speed up the count only 5 of the smaller squares and multiply the average by 25 to determine the number in the 1 mm x 1mm square.

- Determine the sperm concentration using the formula:

Sperm concentration (per ml) = no. in a 1mm x 1mm square x dilution factor (either 1000 or 200) x 10^4

2.4 DILUTING SPERM WITH CRYOPROTECTANT SPERM

- Set up the required number of Kimble disposable glass culture tubes. Mix sperm well then add 1 ml of sperm to each test tube. Keep on ice.
- Add the cryoprotectant solution to the sperm in 100 μL aliquots in 10 one minute steps to each Kimble tube. Final volume = 2 ml/test tube. Gently mix each test tube after each addition. After addition 8 and 10 the tubes should be capped and inverted several times to ensure good mixing of the sperm and the cryoprotectant solution.

2.5 LOADING THE STRAWS

- Load 0.5 ml straws with sperm-cryoprotectant mixture, by sucking the mixture up the straws. The flow will stop once the top plug becomes wet. This will seal this end of the straw. One test tube of the mixture will be sufficient to fill 3 straws. Place the straws onto the comb to form an air space in the straw and to allow the coloured sealing powder plug to be formed. It is important to do this as the air space allows room for the mixture to expand without blowing the plug from the straw. Tap in coloured sealing powder to plug the end. Wipe excess powder from the straws and place coloured plug end down into a 70 ml plastic pottle (Lab serve catalogue number LBS 30002) containing 20 mls of water to seal the plug. Leave straws in the water for approximately 10 seconds then remove, wipe

dry with a tissue and lie flat until 10 minutes has elapsed from the completion of the addition of the cryoprotectant solution.

2.6 FREEZING

- Gently place straws across the rack floating on liquid nitrogen in the polystyrene chilli-bin prepared earlier. Place the lid loosely back on the chilli-bin and wait **10 min**. After this time, plunge the straws into the liquid nitrogen bath. Place a plastic goblet in the liquid nitrogen bath and pack straws into the goblet for storage. Transfer the goblet to a liquid nitrogen dewar with canisters until ready for thawing.

2.7 OBTAIN EGGS

- Obtain eggs by inducing spawning in Sydney Rock oysters using standard procedures of temperature and salinity shock. It is important to ensure that eggs collected remain unfertilized.
- Separate spawning females into individual beakers and transfer several times into fresh seawater to remove sperm. Keep each lot of eggs collected from each female separate. Take a sample (approximately 200 µL) of the egg solution at least 30 min after spawning and check that it contains only unfertilised eggs – i.e. that there are no sperm present and/or polar bodies.
- For each female, pool up eggs that are unfertilised into one container to determine egg density.

2.8 DETERMINE EGG DENSITY

- Use a plastic transfer pipette to pick up settled eggs from the bottom of the beaker and transfer to a 15 ml Nunc disposable conical tube.
- Place the tube in a rack in the fridge and leave for up to 60 minutes to allow the eggs to settle and concentrate.
- Remove the excess sea water (leave a small amount, approximately 500 µL, of sea water above the eggs) from the surface with a plastic transfer pipette. Take care not to remove the eggs.
- Set up Kimble glass test-tubes for diluting and counting the eggs by placing 900 µL of sea water into the first 3 tubes. Re-suspend the eggs in the Nunc conical tube by inverting several times. Make sure that the eggs are well mixed and the concentrated eggs from the tip of the tube have been re-suspended. Take a 100 µL sample of the egg suspension and place in the first tube. Mix well using the pipette. Change pipette tip and transfer 100 µL from the first tube to the second tube. Mix well. Again change tip and transfer 100 µL from tube two to tube three. Mix well. Change tip and take 20 µL aliquots and make 4 drops in a well of a multi-well plate lid.
- Count the number of eggs in each drop. Take the mean of the four counts and multiply by 50,000. This is the number of eggs per ml.

2.9 THAWING

- Set up water bath for thawing. Use a thermos or other suitable container filled with tap water and adjust the temperature to 25°C. Use a thermocouple or thermometer to monitor the temperature. Increase the temperature with warm water when it drops to 22°C.
- Fill a plastic thermos with liquid nitrogen for holding straws. Wash one pair of scissors with 70% Ethanol.
- Select straw from liquid nitrogen. Check details. Plunge into water bath and hold until the ice melts. Remove and dry with tissue. Cut off cotton plug with sterile scissors. Place the straw into a test tube and cut off the PVP powder plug. Allow the contents of the straw to slowly run out. Blow the last drop out. The sperm are now ready to be used for fertilization.

2.8 BULK SCALE FERTILIZATION

- Suspend eggs at a density of approximately 1000 to 5000 mL^{-1}. Add sperm at a ratio of approximately 1000 to 5000 sperm per egg (nb the sperm concentration will be half of what was determined earlier allowing for a 1:1 dilution with the cryoprotectant solution). After a contact time of 15 - 20 min with continuos mixing, transfer the eggs to larval rearing tanks for incubation.