Towards Breeding Better Oysters: A Proteomic Investigation of Disease Resistance in Sydney Rock Oysters

By

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गुरुर्ब्रह्मा गुरुर्विष्णुः गुरुर्देवो महेश्वरः ।

गुरुः साक्षात् परं ब्रह्म तस्मै श्री गुरवे नमः ॥

Gurus (teachers) are the creator, preserver, and highest lord. I bow my head in respect.

Dedicated to all my teachers, first of whom were my parents.

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Thesis Declaration

I certify that this thesis titled '***Towards Breeding Better Oysters: A Proteomic Investigation of Disease Resistance in Sydney Rock oysters***' is composed of my original work and has not been previously published or submitted as part of degree requirement.

I have clearly stated the contribution of other people in this project, including project design, field works, data analysis, manuscript preparation, professional advice and due acknowledgement has been given to the original works I have reported in my thesis. I have also declared the contribution of other researchers in the co-authored work that I have published and included in this thesis.

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Thesis Summary

Sydney Rock oysters (*Saccostrea glomerata*) are a native Australian oyster species present mainly along the eastern coast of New South Wales. These oysters are economically very important as they are consumed as food across Australia. Sydney Rock oysters are known in particular for their distinctive taste, making them one of the major aquaculture products of Australia. During the early 20th century, the introduction of modern farming methods resulted in rapid growth in Sydney Rock oyster production. However, during the 1970s production started to decline as a result of mass mortalities caused by different diseases such as Queensland Unknown (QX) and Winter Mortality (WM). These diseases, in addition to the changing environmental conditions such as water pollution, further diminished the production of oysters. While there has been some previous research on environmental and disease stress in oysters, the causes and impacts of different diseases on the Sydney Rock industry are relatively underexplored. This thesis makes an attempt to enhance our fundamental understanding of two diseases of Sydney Rock oysters - QX and WM - using quantitative shotgun proteomics.

Winter mortality was previously believed to be caused by a protozoan – *Bonamia roughleyi*, however the exact aetiology is still not clear. Whereas, QX disease is known to be caused by a paramyxean protozoan, *Marteilia sydneyi*, and is the major cause of the mass mortality of Sydney Rock oysters. The current mode of mitigating the impact of diseases relies on the farming of selected line of Sydney Rock oysters. The selective breeding programme of Sydney Rock oysters was established by NSW DPI using the survivors of mass mortality as a pioneering parent population. However, this mode of selection lacked the biological information of selection mechanisms and therefore it is feared that inbreeding depression might affect the future generations. Therefore, for sustainable growth of the industry it is very important to understand the biological basis of selection.

This thesis aims to understand the underlying molecular processes of better adaptability of selected lines of oysters against diseases, by identifying the proteomics differences in selected and unselected populations. Chapter 1 of this thesis deals with the proteomic investigation of oysters selected for WM. Using 2DE in association with LC-MS/MS, we

identified differential proteomic expression of proteins involved in many different biological processes.

The major focus of this thesis is on QX disease and the following three experimental chapters of the thesis addresses different aspects of QX disease of Sydney Rock oysters. Chapter 3 reports a time dependent proteomic response of Sydney Rock oyster populations. We have identified a pattern of cytoskeletal breakdown in this study which shows a stepwise breakdown of cytoskeletal proteins with the disease period progression. Chapter 4 investigated the differences and similarities between selected Sydney Rock oysters from the wild Sydney Rock oysters grown in the same estuary. This work showed that in spite of being grown very close to each other the proteomic differences between the two populations was significant, suggesting a lack of genetic exchange or cross breeding. Chapter 5 used digestive gut tissue of Sydney Rock oyster for studying the comparative proteomics using a cutting edge proteomic analysis technique known as SWATH-MS. For the peptide to spectrum matching we used a newly available, but still unannotated, genome sequence of Sydney Rock oysters, and we identified a much greater number of proteins as compared to previous studies using the Pacific oyster genome sequence as a reference. This study reiterates the importance of the availability of functionally annotated genome sequence information for proteomics studies. The findings of this study are the first step towards generation of an identified protein library of Sydney Rock oyster which will be highly valuable for future studies.

Considered together, the body of work described in this thesis represents a significant advancement in our fundamental understanding of the molecular mechanisms and biology of disease resistance and progression in Sydney Rock oysters.

Acknowledgements

This is the most emotional section of my thesis. This PhD thesis work is not only three and half years in making, it goes way back in time. For me, to be able to express my gratitude to everyone who has been a part of my evolution as a researcher requires more than just a few words. This PhD thesis is just a humble testimony to all those selfless acts of kindness and love towards me. To all the teachers who touched my life, made me the person I am today; I thank you all from the bottom of my heart. Life during my PhD journey has been a roller coaster ride and I am thankful to have concluded it to the best of my abilities.

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Publications arising from this thesis

1. **Vaibhav, V**., Thompson, E., Raftos, D.A., Haynes, P.A., 2017. Proteomic Analysis of Disease in Sydney Rock Oysters in: Colgrave, M.L. (Ed.), Proteomics in Food Science. Academic Press, pp. 343-357.
2. **Vaibhav, V**., Lepretre, M., Thompson, E.L., Raftos, D.A., Haynes, P.A., 2016. Biomarkers of Winter Mortality resistance in selectively bred Sydney Rock oysters (*Saccostrea glomerata*). Aquaculture. 465, 323-329.
3. **Vaibhav, V**., Thompson, E., Raftos, D.A., Haynes, P.A., 2017. Temporal comparison of Sydney Rock oysters selectively bred for QX disease resistance reveals biomarkers of the disease progression response. Submitted to Aquaculture in August 2017.

Conference presentations

1. **Vaibhav, V.**, Lepretre, M., Thompson, E., Raftos, D.A., Haynes, P.A., 2015. Selective breeding of Sydney Rock Oysters- Brief overview. 2nd Proteomics and Beyond Symposium-2014, Sydney, Australia. (Poster)

1. **Vaibhav, V**., Lepretre, M., Thompson, E., O'Connor, W., Raftos, D.A., Haynes, P.A., 2015. Sydney Rock Oysters: Disease resistance biomarker discovery. The Malacological Society of Australasia. Molluscs- 2015. Coffs Harbour. (Talk)

2. **Vaibhav, V**., Thompson, E., O'Connor, W., Raftos, D.A., Haynes, P.A., 2015. Identification of disease resistance genes in Sydney Rock oysters using quantitative label free shotgun proteomics. 20th Lorne Proteomics Symposium-2015, Lorne, Australia. (Poster)

3. **Vaibhav, V**., Lepretre, M., Thompson, E.L., O'Connor, W., Raftos, D.A., Haynes, P.A., Queensland Unknown (QX) and Winter Mortality (WM) disease of Sydney Rock Oysters: A comparative proteomic study. 21st Lorne Proteomics Symposium-2015, Lorne, Australia. (Poster)

4. **Vaibhav, V**., Thompson, E., O'Connor, W., Raftos, D.A., Haynes, P.A. From Down under: A comparative proteomic analysis of Sydney Rock oysters. EMBO-2016, Mannheim, Germany. (Poster)

5. **Vaibhav, V**., Thompson, E., O'Connor, W., Raftos, D.A., Haynes, P.A. Identification of disease resistance genes I Sydney Rock oysters using quantitative label free shotgun proteomics. ANZSMS-2016, Brisbane, Australia. (Poster).

6. **Vaibhav, V**., Lepretre, M., Thompson, E.L., O'Connor, W., Raftos, D.A., Haynes, P.A., Diseases of Sydney Rock Oysters: A Proteomic Investigation. 21st Lorne Proteomics Symposium-2017, Lorne, Australia. (Talk and Poster)

Abbreviations

1-DE	One Dimensional Gel Electrophoresis
2-DE	Two-Dimensional Gel Electrophoresis
ACN	Acetonitrile
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
cDNA	complementary Deoxyribonucleic Acid
DNA - AFLP	Deoxyribonucleic Acid - Amplified Fragment Length Polymorphism
DPI NSW	Department of Primary Industries, NSW
DTT	Dithiolthreitol
DIA	Data-Independent Acquisition
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionisation
FDR	False Discovery Rate
GO	Gene Ontology
GPM	Global Proteome Machine
HC	Hatchery Control
HPLC	High Performance Liquid Chromatography
HSP	Heat Shock Protein
IAA	Iodoacetamide
IDA	Information dependent acquisition
IEF	Iso-Electric Focussing
iTRAQ	Isobaric Tags for Relative and Absolute Quantitation
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LTQ	Linear Trap Quadrapole
MALDI	Matrix Assisted Laser Desorption/Ionisation
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MSX	Minchinia nelson
MVP	Major Vault Protein

m/z	Mass to Charge ratio
NanoLC-MS/MS	Nanoflow Liquid Chromatography Tandem Mass Spectrometry
NSAF	Normalised Spectral Abundance Factor
OA	Ocean Acidification
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
QIT	Quadrupole Ion Trap
qPCR	Quantitative Polymerase Chain Reaction
QX	Queensland Unknown disease
QXr	Queensland Unknown disease resistant line
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-qPCR	Quantitative Reverse Transcription PCR
SAM	Significance Analysis of Microarrays
SDS	Sodium Dodecyl Sulphate
SILAC	Stable Isotope Labels with Amino Acid in Culture
SOD	Superoxide dismutase
SRM	Selected Reaction Monitoring
SRO	Sydney Rock oyster
SSH	Suppression Subtractive Hybridization
SWATH-MS	Sequential Window Acquisition of all Theoretical Mass Spectra
TMT	Tandem-Mass-Tag
TOF	Time of Flight
TRI	Trizol reagent
UniProt	The Universal Protein Resource
WB	Western Blot
WM	Winter Mortality
XIC	Extracted Ion Chromatogram

Chapter 1

General Introduction

Chapter 1. General Introduction

1.1 History of oyster farming in Australia

The Australian continent is surrounded by water on all sides and ranks 6th in the world in terms of length of its coastline (Harvey and Caton, 2010). As is always the case, water bodies are the culminating points of human civilisation as they provide support to communities in different ways, including providing food in the form of fish and other marine food products including oysters. The presence of shell middens on different archaeological sites in Australia suggests that oysters have been consumed for food by the native populations for at least 3000 years (Colley, 2005).

Captain Cook sailed to Australia before European settlement and reported encounters with the natives at Botany Bay, mentioning a camp where oysters and mussels were consumed for food (Bell, 1994). When European settlers arrived in the early 1800s they started settling colonies on the coastal areas, mainly on the eastern coast, which is now reflected by the population distribution. With population distributed densely along the coastline, the water bodies came under pressure because of the ever-increasing population and demands associated with that, such as food, drinking water and water for industrial uses. The earliest account of oyster farming by European settlers is reports of a nascent industry started in the 1870s, and hence it is the oldest aquaculture industry of Australia (Nell, 2001b). The oysters were used not only as a food source but also as source of lime, which was required in great quantities for construction of buildings and roads. This unchecked consumption resulted in depletion of the naturally occurring resources, and subsequently the government put a control on consumption of natural spats. One of the earliest mentioned instances of farming oysters includes intertidal harvesting of oysters by placing different kinds of flat surfaces under water so as to allow oyster growth, followed by dredging. This mode continued until an invasion by a mud worm in 1895 which resulted in heavy loss to the industry. Afterwards this mode was replaced by the stick and tray method. As we will see later in this chapter, different pathogens in different oyster species have historically affected the farming and various measures have been taken to counteract those outbreaks.

1.2 Modern improvements in farming

Around 1970 the oyster production increased by about 40% which was mainly attributed to a newly adopted method of farming known colloquially as “highway farming”. This was

named after the fact that farmers regularly moved their oysters from one estuary to another in order to make the most of different estuarine conditions for favourable growth outcomes. This resulted in faster growth of oysters and the time it would have otherwise taken to get to market size was reduced. On one hand this method was very fruitful for the farmers, but on the other hand it contributed to the presence of Pacific oysters in the northern estuaries of NSW, which otherwise were restricted to the southern estuaries and Tasmanian estuaries (**Figure 1.1**). Pacific oysters are relatively faster growing oyster species and co-existence of Pacific oysters with Sydney Rock oysters (SRO) usually has a negative impact on SRO population growth.

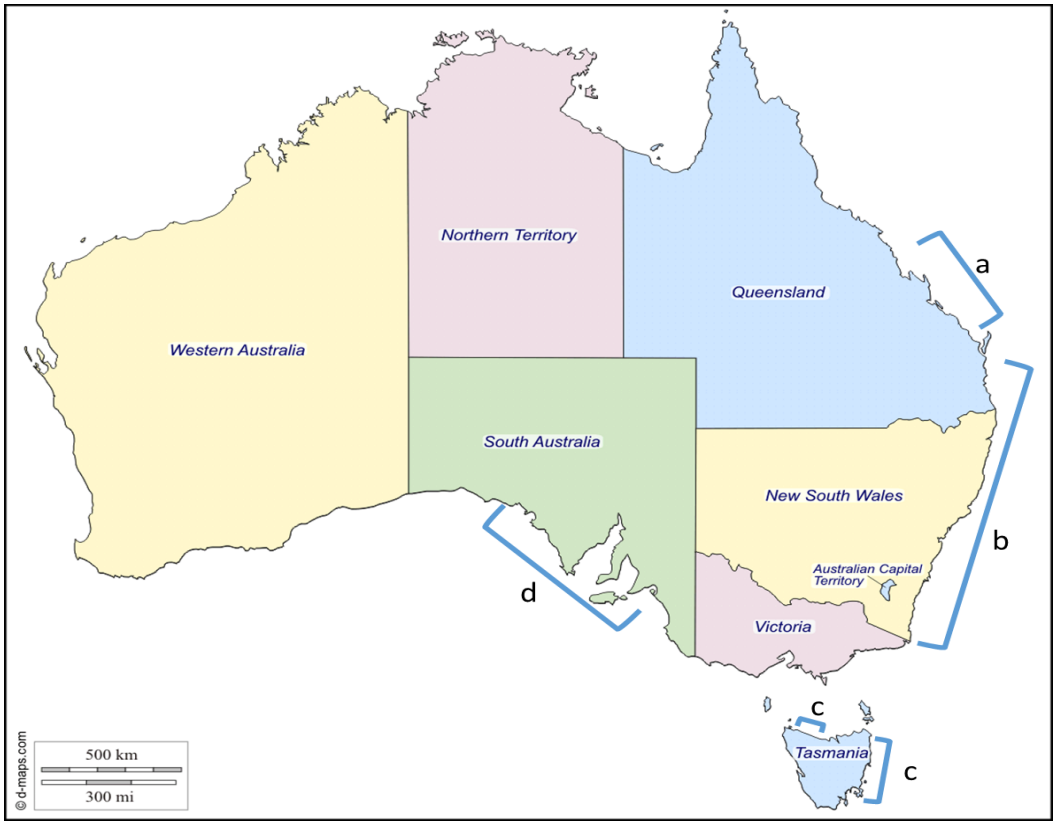

Figure 1.1: Oyster growing areas in Australia. a. *Saccostrea mytiloides*, b. *Saccostrea glomerata*, c. *Crassostrea gigas*, d. *Crassostrea gigas*. (Nell et al., 2000)

1.3 A national aquaculture Industry

It was evident from the earlier success and failures of the oyster farming in NSW that there was a need to establish a governing body to define a set of rules to ensure sustainable development of the industry. Aquaculture is a major responsibility of the Department of Agriculture and Water Resources of the Australian Government. This department is committed to the economic development of the industry in a sustainable manner. The

industry is comprised of different types of production which includes crustaceans, molluscs, and freshwater fishes. In NSW, oysters are the main aquaculture species with a production value of more than $44 million during the year 2015/16 (Livingstone, 2016). Putting this in perspective of the total Australian aquaculture production of $64 million, oysters are clearly very important. Oysters in NSW are very important for the economy and as can be seen in **Figure 1.2**, $36 million of the $44 million annual production value is contributed by SRO. The remainder comes from Pacific and other oysters, and oyster spats. **Figure 1.3** shows the production trend of SRO in NSW, depicting the dramatic decrease in production following its apex in the 1970s.

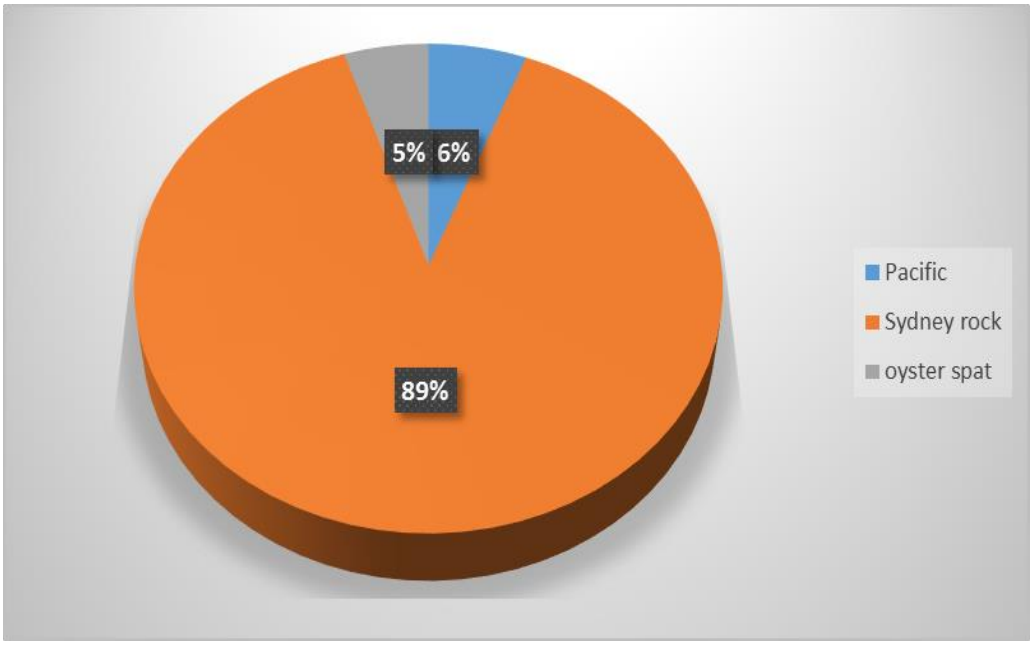

Figure 1.2: Percentage contribution of different oyster species types in total production value for the year 2015/2016 in NSW (Livingstone, 2016).

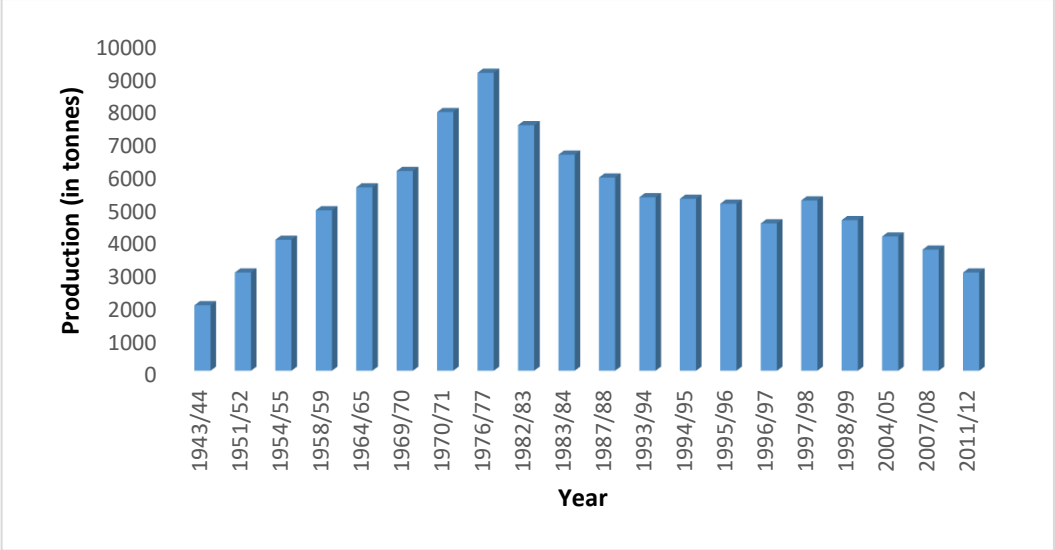

Figure 1.3: Production trend of SRO in NSW showing a gradual decline in production after the 1970s. The oyster industry reached its peak in the years 1976-77 during which the production was estimated to be more than 9300 tonnes. In the years that followed the production started to go down, which was mainly attributed to different oyster diseases and other anthropogenic induced changes in the water quality of the estuaries. Modified from (Livingstone, 2016).

Production of oysters in NSW is overseen by Department of Primary Industries (DPI), and like other state government organisations the DPI ensures the sustainable aquaculture production in estuaries of NSW. As far as oyster production is concerned, there are 41 estuaries where commercial production of oysters is carried out. The major estuaries in terms of production are shown in Figure 1.4.

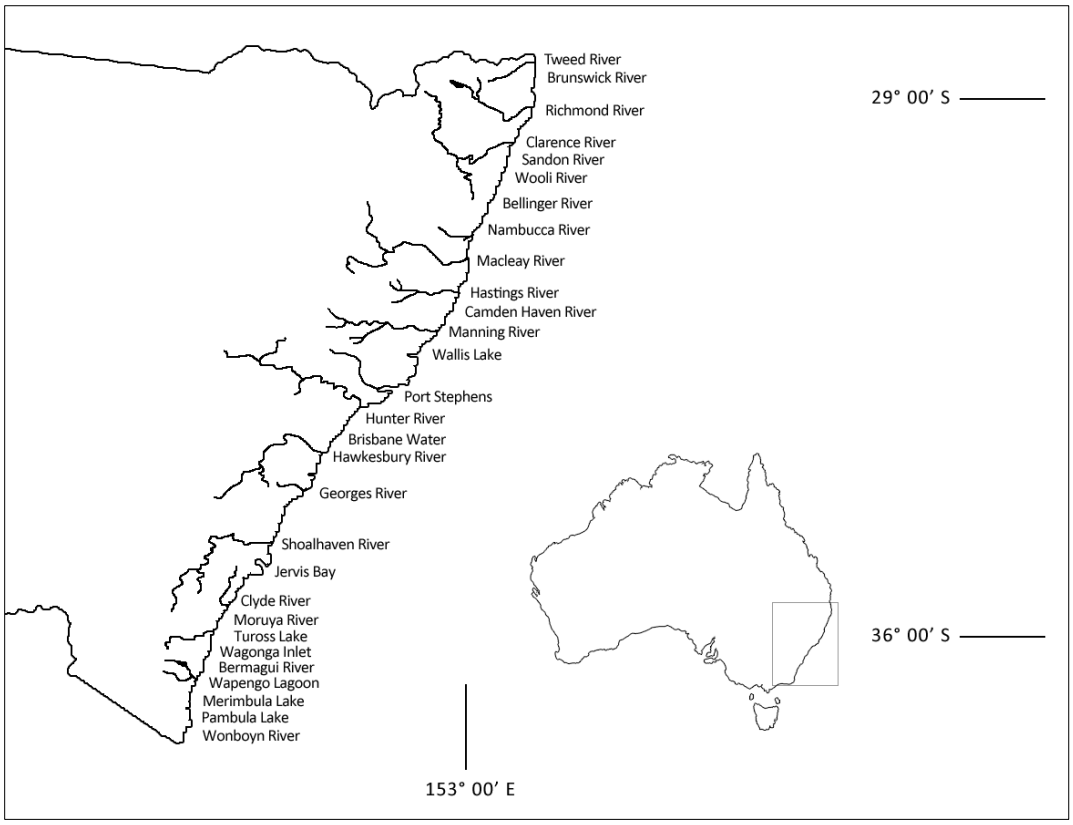

Figure 1.4: Major oyster farming estuaries in NSW along the coastline start from the Tweed River on the NSW - Queensland border in the north to Wonboyn Lake on the NSW - Victoria border in South (Vaibhav et al., 2017).

1.4 The Sydney Rock oyster (SRO)

1.4.1 Background

Sydney Rock oysters (*Saccostrea glomerata*) are an edible species which are endemic to Australia. This species of oysters is known for its distinct taste as compared to its counterpart *Crassostrea gigas* and hence is known popularly as an Australian delicacy. While the freshly shucked oysters taste best, another standout feature of SRO is that they have very good shelf life – approximately two to four weeks at cool room temperature.

Geographically, SRO are distributed mainly along the eastern coast in New South Wales (Lamprell, 1998), and the major estuaries dealing with commercial production of SRO are shown above in **Figure 1.4**. SRO are relatively slow growing; it takes approximately 3.5 years to reach marketable weight (~50g) while Pacific oysters take 1.5-2 years (Troup et al., 2005). Breeding efforts have produced faster growth of SRO, hence decreasing the time it takes to get the desired plate size and weight. Scientists have come up with different ways

to improve the production of oysters. The motivating factors in order to do so were either to meet the production demands, competition with other exporters, making them less susceptible to detrimental effects of environmental changes, or to protect them from disease outbreaks. **Figure 1.5** identifies major anatomical features of a mature SRO freshly shucked.

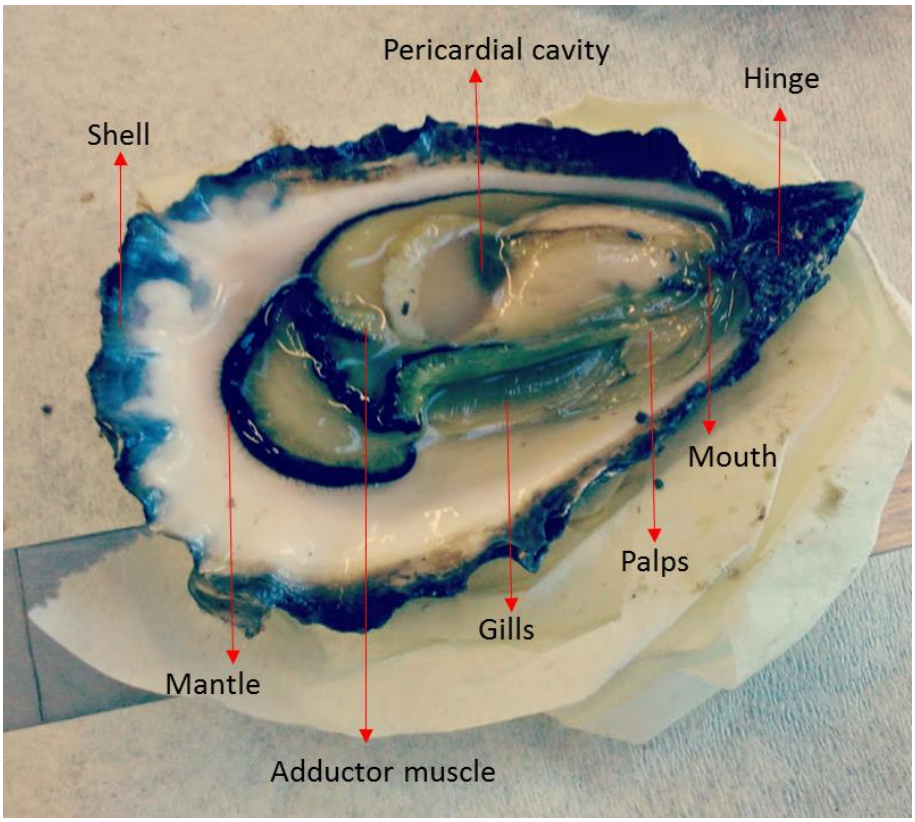

Figure 1.5: Anatomy of a mature Sydney Rock oyster (*Saccostrea glomerata*) freshly shucked open. The Pericardial cavity was poked by a pipette tip to release the haemolymph. (Photo credit: Vineet Vaibhav)

1.4.2 Triploid SRO

Triploid oysters are genetically manipulated oysters which have an extra set of chromosomes i.e. instead of the normal two sets they have three sets of chromosomes. It has been shown that triploid SRO are faster growing as compared to typical diploid SRO (Troup et al., 2005) The probable reason for this is that the predominantly sterile triploids use their cellular resources mainly for somatic growth rather than gamete formation and hence grow relatively fast as compared to the more fertile diploids (Beaumont and Fairbrother, 1991). In the same study it was also suggested that growth of oysters is affected by the location in the estuary, for example, oysters in the intertidal areas grow faster.

1.4.3 Selective Breeding of the SRO

In another attempt to enhance the growth of SRO, NSW DPI established a selective breeding programme in 1990 (Nell et al., 2000). The parent lines to start the selection were selected based on field observations for fast growth, or were the survivors of disease outbreaks, or shown to thrive better than wild oysters under disease stress. The programme was started with four different breeding lines at three different locations in Port Stephens; these lines were selected for fast growth. Along with this, four more breeding lines at three locations in Georges River were also started, aimed at breeding SRO resistant for winter mortality (WM) disease. The disease resistance selection of oysters requires exposure to the disease-causing conditions on a regular basis, hence ensuring sure the survivors remain resistant to the disease.

The breeding program for fast growth was successful and after selection it was found that the growth time was reduced by 10 months and oysters were ready to go to market after 2.5 years of growth. However, in 1994, the WM disease resistance selective breeding at Georges River was terribly affected by an outbreak of another disease known as Queensland Unknown (QX) (Anderson et al., 1995). This outbreak disrupted the ongoing selective breeding and forced a halt to the breeding at two sites in the Georges River, while the third site (Quibray Bay) was fortunately untouched by the QX outbreak (Nell et al., 2000). QX disease, as we will see later in the introduction, is one of the most devastating diseases of SRO. In this instance, it almost wiped out the entire population of SRO at the two locations in Georges River, with a mortality rate of >85%.

1.4.4 Selective Breeding version 2.0

Following the QX outbreak in Georges River, the selective breeding programme was re-organised in 1997 and started afresh with selection of three different lines at three different locations (**Figure 1.6**). These lines included (i) fast growth and resistance for both WM and QX at Woolooware Bay, (ii) fast growth and resistance for WM at Quibray Bay, and (iii) fast growth and QX resistance at Lime Kiln Bar. The pioneering oysters for QX selection programme were randomly selected from populations surviving the disease outbreaks (Nell, 2003).

The parent oysters represented the population of oysters which survived the QX mass mortality in 1994; therefore, there was very little information about how many oysters were used to start the line, their genetic makeup, and especially the reasons behind their survival.

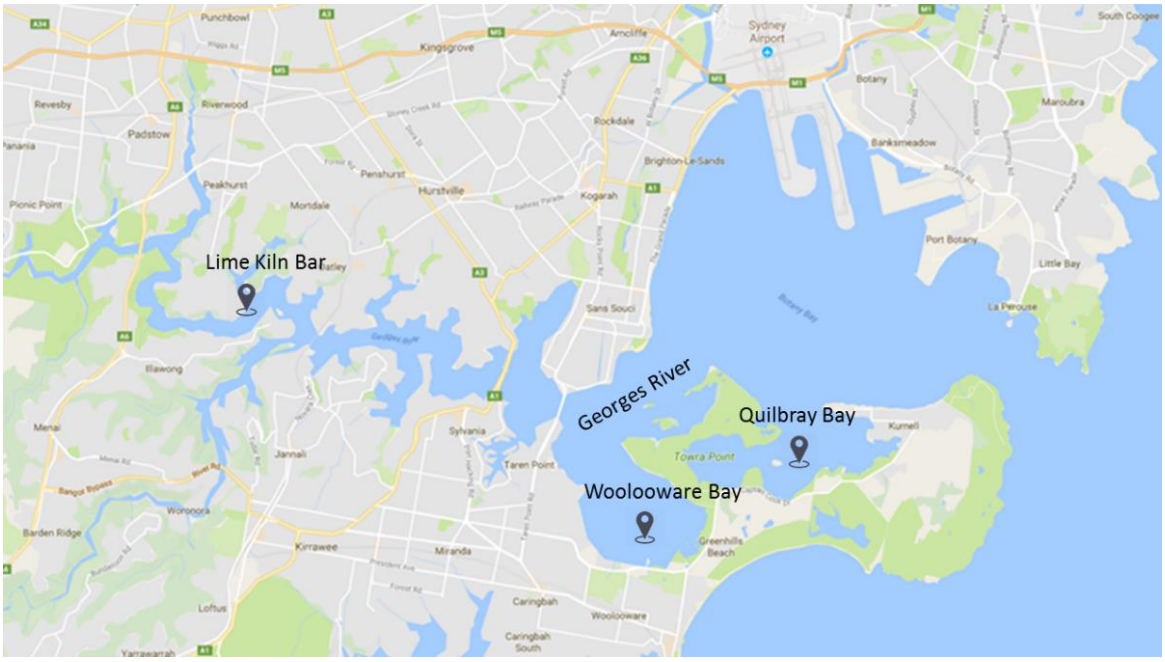

Figure 1.6: Location of estuaries in Georges River selected for selective breeding following the QX outbreak in 1994. Modified from (Nell, 2003).

1.4.5 History of selective breeding internationally

Selective breeding of oysters has mainly emphasised the selection of commercially important factors such as: growth rate, to reduce the time it takes to reach the market; disease resistance, to increase the resistance to a particular disease; or selection for aesthetics such as shell shape and size. Over the years, the international oyster farming industry has seen many disease outbreaks, a few of which are discussed here in the context of the history of selective breeding around the word.

1.4.5a European Flat oysters

European Flat oysters *(Ostrea edulis*) were affected by two different pathogens at different times that resulted in mortality of the oysters in large numbers. In 1967, the protozoan *Marteilia refringens* was responsible for confining the production of flat oysters to the sub-tidal areas in France (Alderman, 1979). To make things worse, in 1979 another protozoan parasite, *Bonamia ostrea*, wreaked havoc on the oyster farming in sub-tidal areas, as a result of which the oysters number dwindled drastically (Naciri-Graven et al., 1998). Efforts were

made to re-establish the oyster cultivation and a selection programme was established which was concentrated mainly on resistance to *B. ostrea* (Hervio et al., 1995).

The selection was performed by manual infection of oysters with the purified parasite, *B. ostrea,* thereby imposing selection pressure on the organism (Hervio et al., 1995). Following the selection, two different strains of *O. edulis* were developed in 1985 and 1989, known as S85 and S89, respectively. The resistance displayed by these two strains was significantly improved as compared to the unselected oysters (Martin et al., 1992). The selection programme was further improved by crossing within and amongst different strains of the selected lines (Baud et al., 1997). However, because of high selection pressure in addition to the small pioneering parent population, the breeders observed a decrease in survival rate after several generations which was believed to be linked to inbreeding depression.

1.4.5b American Oysters

Another example of selective breeding occurred in American oysters (*Crassostrea virginica*) reared at Delaware Bay which were affected by a Haplosporidian parasite, *Minchinia nelsoni* (MSX) (Haskin et al., 1966). In 1958-59 more than 90% of the native oysters were killed by the parasite in the high salinity regions, and 50-70 % in the lower salinity regions (Haskin and Ford, 1979). Along similar lines as discussed previously, oysters which survived the disease outbreak were selected and reared in laboratory conditions followed by field trials over generations. It was observed that the rate of survival in the selected oysters improved over the generations as compared with wild oysters, and hence it was concluded that the resistance to MSX is heritable (Haskin and Ford, 1979).

These studies seem to show that offspring inherit an enhanced suite of resistance genes as compared to the susceptible oysters, and hence they display disease resistance. In an extrapolation of the previous study over several generations, the same authors studied the impact and mortality pattern in native American oysters selected for resistance against MSX (Ford and Haskin, 1987). This study was conducted over six generations and it was expected from the previous study that the rate of survival would increase gradually with the inherited set of resistance genes. However, in generations four to six the survival rate dwindled unexpectedly (Vrijenhoek et al., 1990). This can possibly be attributed to the fact that selection of a single trait imposed genetic pressure on the organism and hence a

reduction in viability was observed. Also, because of inbreeding there is a continuous loss in heterozygosity as a result of which growth and fertility may be affected (Falconer, 1996; Vrijenhoek et al., 1990). Certain approaches to maintain the heterozygosity, or of minimising the effect of inbreeding, are discussed by (Sheridan, 1997). Emphasis has been given to maintaining variability in the parent population from which the selection is commenced, i.e. the parent population needs to be widely sampled. Also, inclusion of triploid oysters, although problematic due to their much lower fertility levels, should be considered, as the triploid oysters reach market size sooner than their diploid counterparts and selection can be performed using reciprocal crosses of either diploid or triploid oysters.

1.5 Proteomics in Biomarker discovery

Proteomics is the comprehensive study of all the proteins encoded by the genome of an organism, which corresponds to their expression level after translation, cellular localization, protein-protein interactions, and post-translational modifications. An extensive proteomic analysis gives a comprehensive view of cellular processes including those which are affected by any disease or stress (Anderson and Anderson, 1998; Blackstock and Weir, 1999).

The expressed proteome of an organism reflects the genome sequence, but the protein expression is not solely dependent on genomic information (Anderson and Seilhamer, 1997), it may vary depending on many molecular or cellular conditions. Hence, the genome and proteome share a complex relationship (Rogers et al., 2008), which is one reason why studying proteins is vital, especially when we know that the proteins have such wide application as biomarkers for the identification of disease. Also, proteomic investigation has broad applications in biomonitoring of stress response of organisms, as changes in the level of protein present in body fluids in response to stress can be readily monitored (Blackstock and Weir, 1999).

1.5.1 Tools of proteomics

Recent advancements in proteomic technologies have offered us several options by which the proteome of an organism can be studied in detail. A typical proteomics protocol includes: (a) sample procurement - which could be a piece of tissue, body fluid or cultured cells; (b) protein extraction, depending on the sample type, as different samples require specific treatments to keep the protein functionality intact; (c) protein estimation to

determine the quantity of proteins to be used for further analysis; (d) enzymatic digestion depending on the subsequent technique to be used; (e) further protein analysis steps which may be performed, which again depends on the type of sample, and question(s) to be answered. One of the first tools established for studying the whole cell proteome was Two Dimensional gel Electrophoresis (2DE) (O'Farrell, 1975). This technique is still a popular technique to visualise the differential protein expression across two sample cohorts. In this technique, proteins are not digested enzymatically and are separated in the first dimension based on their isoelectric point (pI), and then further separated on the basis of an orthogonal property, their molecular weight, thereby giving the user a two-dimensional separation. The protein spots on the gel can be manually excised for protein identification by mass spectrometry based techniques (Delahunty and Yates, 2005).

However, 2DE falls short in identifying relatively low abundance proteins and also suffers problems associated with reproducibility attributed to separation inconsistencies. Moreover, identification of relatively small number of proteins is a major drawback. Therefore, in recent times, other high throughput techniques such as shotgun proteomics have become popular (Wolters et al., 2001). Shotgun proteomics functions on the bottom up proteomics principle, as it involves first digesting complex mixtures of proteins into even more complex mixtures of peptides, and then identifying the resulting peptide fragments using mass spectrometry and computerised peptide-to-spectrum matching software (Aebersold and Mann, 2003). It is important to note that proteins are not formally identified in this approach; rather, the presence of proteins is inferred from the identification of proteolytic peptides. Shotgun proteomics employs high performance liquid chromatography in conjunction with tandem mass spectrometry (LC-MS/MS) for peptide fragments separation (Wu and MacCoss, 2002). A typical shotgun mass spectrometry based workflow outline is shown in **Figure 1.7**.

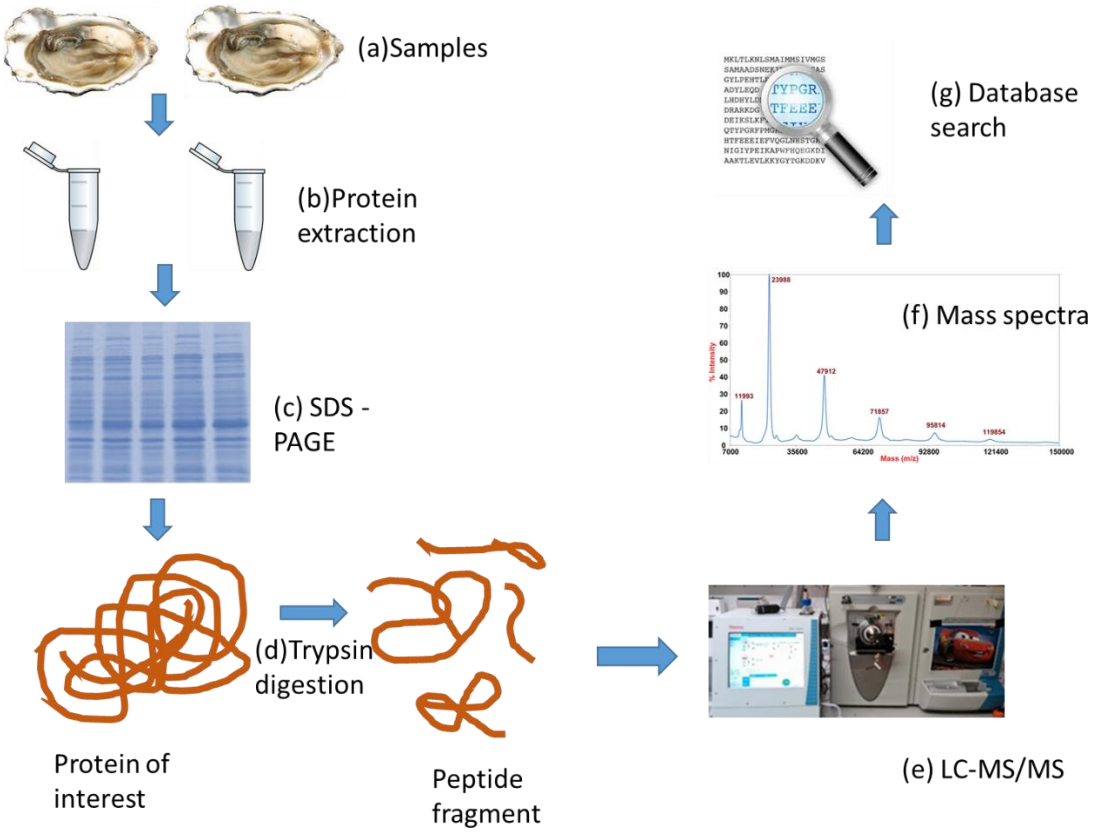

Figure 1.7: A schematic of a typical mass spectrometry based proteomic experimental workflow. Depending on the design of the experiments this could be modified to accommodate different types of samples and objectives.

In addition to shotgun proteomics designed for identifying large numbers of proteins, many methods have been developed which allow for simultaneous identification *and* quantitation of thousands of peptides in a single experiment. These can be divided into two main groups, those which are based on isotopic labelling and those which are label-free. The principle of isotopic labelling is that relative quantitation can be performed by measuring the ratios of peptides present in different isotopically labelled samples (Treumann and Thiede, 2010). These labels can be incorporated metabolically in cultured cells using either 15-N labelling (McClatchy and Yates, 2014), or SILAC - Stable isotope labelling by amino acids in cell culture (Ong et al., 2002). In some cases, for example oysters used in this study, metabolic labelling is not an option. Therefore, other approaches haven been developed in which amino acids are chemically labelled following the protein extraction and isotopically different labels can be applied to different samples. Examples of such approaches include Isotope-Coded Affinity Tags (ICAT) and, more recently, Tandem Mass Tag (TMT) labelling, which allows for simultaneous multiplexed labelling and analysis of thousands

of peptides from up to ten samples or more in a single experiment (Dayon et al., 2008; Gygi et al., 1999). All these approaches allow the user to analyse a mixture of samples labelled with different isotopes in a single LC-MS/MS run. This eliminates instrument related variation during individual sample processing and hence enhances the reliability of the quantitation in the datasets.

Label-free quantitation in shotgun proteomics is lower throughput as the samples are not generally run in such a multiplexed fashion. There are two main types of label-free quantitation, based on either integrated area under the curve (AUC) quantitation in MS1 data (McIlwain et al., 2012; Zhang et al., 2010), or spectral counting, such as the use of Normalized Spectral Abundance Factor (NSAF) values (Neilson et al., 2013). Label-free quantitation has the advantage, however, of not requiring any expensive labelling reagents, and has been shown to produce reliable quantitative data which can be translated into important biological knowledge (Neilson et al., 2011).

All of these techniques can be used to study the fold changes of a protein across two or more cohorts, thus making them very popular workflows in biomarker discovery based research (Sandberg et al., 2014). The following section deals with a general overview of the contribution of proteomics in biomarker discovery across varied biological systems, along with its pertinence to bivalve research.

1.5.2 Applications of proteomics in Biomarker Discovery

Proteomics in recent years has contributed to the discovery of biomarkers for different types of cellular states, such as disease, environmental effects, and stress response, in a wide variety of biological systems.

A biomarker can be defined as a quantifiable unit, which is representative of a particular biological state concerning the cellular system under consideration (Rifai et al., 2006). Proteomic approaches have provided a means to study the immediate cellular response of the organism. The biological system under study can be essentially any species. Different stressors affecting the biological system causes perturbation of the transcriptome and hence proteome differently. These changes can be monitored by different approaches, for example LC-MS/MS for proteome study and microarray assays for transcriptomic studies.

1.5.2a Medical proteomics

In diseases like cancer, early diagnosis is very important for one to be sure that cells are not metastasized throughout the body. Once the cells have metastasized, it is mostly beyond the reach of therapeutic control. Therefore, detection of cancer at a very early stage is crucial, i.e. while it is benign or in the premalignant stage. Under such circumstances the likeliness of successfully treating the disease increases. One such case which requires early diagnosis is ovarian cancer which is usually detected only when the cancerous cells have metastasized to the peritoneal cavity (Petricoin et al., 2002a). In cases such as these, the treatment is usually delayed, and the five year survival rate of ovarian cancer patients fluctuates around one third of the cases reported (Petricoin et al., 2002a).

As discussed earlier, serum, along with some other easily accessible body fluids, is the ideal study material for biomarker discovery. Serum, in particular, reflects the body's immune response to the change in systemic conditions as the body responds to an immunological challenge (Petricoin et al., 2002b; Ray et al., 2011). Some proteins may be up-regulated in the defence response, to limit the growth of the disease, while some may be down-regulated. The protein response to induction of the disease state may involve the synthesis of novel proteins, or the triggering of new pathways. The sum of these responses can be considered as the proteomic response of an organism. Therefore, identification of a protein expression pattern which is particular for a diseased state will help to diagnose the disease in the early stages. One major drawback of working with serum is that it contains very high levels of a small number of proteins, which makes it difficult to analyse subtle changes other proteins which may be very important biologically but are expressed at a much lower level (Bellei et al., 2011).

1.5.2b Proteomics in other biological systems

Proteomic studies conducted in our laboratory have examined changes in the expressed proteome of numerous different biological systems exposed to many different conditions. In a study comparing two rice varieties exposed to drought stress, major changes in photosynthetic machinery were reported which contributed towards better understanding of drought tolerance in rice (Wu et al., 2016). In another work on grape vine (George et al., 2015), changes in sugar and phenylpropanoid metabolism were observed as a result of thermal stress. With regards to marine organisms, proteomics is a relatively new tool to study the effect of environment and disease on the cellular expression. A proteomic

investigation of mussels in response to heat stress identified differentially expressed proteasome subunits and heat shock proteins (Tomanek and Zuzow, 2010). With regards to fisheries industries, proteome analysis of Atlantic salmon exposed to anthropogenic pollutants revealed significant proteome changes (Hampel et al., 2015). These studies not only helped in better understanding of the biological system under consideration but also opened up new lines of thought in the field. Proteomics as an analytical tool has been an indispensable part of biomarker discovery in recent years. However, proteome studies rely heavily on the presence of a well annotated genome sequence to identify the proteins following LC-MS/MS analysis. Above-mentioned studies were made possible by the availability of relatively well annotated genome sequence data.

1.6 Proteomics in oysters and bivalves

Bivalves are an important component of marine ecosystems, because their involvement in biodeposition, which is a result of their filter feeding mechanisms, is crucial for the ecological maintenance of marine environments (Dame and Olenin, 2003). Bivalves are also involved in nutrient cycling, further substantiating the importance of bivalves in marine ecology. However, in recent years changes in water temperature and acidity imposed by human activities are affecting the observed longevity of bivalves, including oysters, in a variety of different marine environments. The expressed proteome of oysters is not yet the subject of a large number of research studies. This can be attributed at least in part to the unavailability of a fully annotated genome sequence. The first oyster genome sequence that was made publicly available was that of *C. gigas* which appeared in October 2012 (Zhang et al., 2012) and opened up the path for oyster proteome research. An immediate surge in proteome-based research in *C. gigas* was observed. One of the earliest works using the shotgun proteomics approach showed the importance of the discovery (Timmins-Schiffman et al., 2013) in addressing different biological questions, such as studying the effect of ocean acidification. (Timmins-Schiffman et al., 2014)

Prior to that, oyster proteome research relied on identification of proteins performed by homology searches using genome sequence information from related species. In many cases proteins were not identified (Simonian et al., 2009b) or there was ambiguity associated with the protein identifications (Simonian et al., 2009a). In the absence of an annotated oyster genome, Muralidharan et al (Muralidharan et al., 2012) used a database consisting of 14000 peptide sequences from different bivalve molluscs. This approach

provided an adequate but temporary workaround for the problem of protein identification, and as a result of this several proteomics studies conducted on SRO were published soon after (Muralidharan et al., 2012; Thompson et al., 2012b; Thompson et al., 2012c). However, this database composed from different bivalves had some issues caused by the presence of the same proteins from different bivalves but with slightly different peptide sequences, so significant manual curation of search results and further statistical filtering was required in order to produce data of sufficiently high statistical confidence.

1.6.1 Survey of published literature on proteomics and transcriptomics in oysters

Table 1.1 lists a selection of proteomic and transcriptomic based studies in *C. gigas* and *Saccostrea glomerata* during the last decade pertaining to different stress response. As can be seen, proteomic studies on oysters are mostly based on 2DE and protein spot identification using LC-MS/MS or MALDI. The lack of large scale proteome information is mainly attributed to the dependency of proteome discovery work on a well annotated genome sequence.

The work described later in this thesis mainly utilises the annotated genome sequence of *C. gigas* for proteomic study of *Saccostrea glomerata*, using both 2DE coupled with LC-MS/MS, and quantitative shotgun proteomics, to identify disease biomarkers. This thesis also reports a SWATH-MS based study of the proteome of SRO which is, to the best of our knowledge, the first time this has been performed.

Table 1.1: A summary of recent research work in stress studies of oysters (*Saccostrea glomerata* and *C. gigas*) using proteomics and transcriptomics approaches. Literature is presented in chronological order.

Oyster species	Stress	Approach	Reference
C. gigas and C. virginica	Protozoan	Transcriptomics (SSH)	(Tanguy et al., 2004)
C. gigas	Radioactive	Transcriptomics (RT-PCR)	(Farcy et al., 2007)
C. gigas	Temperature	Transcriptomics (RT-PCR)	(Meistertzheim et al., 2007)
C. gigas	Pesticide and Bacterial	Transcriptomics (RT-PCR)	(Gagnaire et al., 2007b)
C. gigas	domestic sewage (pollution)	Transcriptomics (SSH)	(Medeiros et al., 2008)
Saccostrea glomerata	Disease (QX)	Proteomics (2DE)	(Simonian et al., 2009a)

Saccostrea glomerata	Disease (QX)	Proteomics (2DE)	(Simonian et al., 2009b)
C. gigas	Bacterial	Transcriptomics (cDNA-AFLP)	(Taris et al., 2009)
Saccostrea glomerata	Metal contamination	Proteomics (2DE, LC-MS/MS)	(Thompson et al., 2011)
C. gigas	Temperature	Transcriptomics (Next gen sequencing)	(Zhang et al., 2012)
Saccostrea glomerata	Metal contamination	Proteomics (2DE, LC-MS/MS)	(Thompson et al., 2012c)
Saccostrea glomerata	metal contamination	Proteomics (LC-MS/MS)	(Muralidharan et al., 2012)
C. gigas	Salinity	Transcriptomics (RNA seq)	(Zhao et al., 2012)
Saccostrea glomerata	Disease and Anthropogenic	Transcriptomics (RNA seq)	(Hook et al., 2014)
C. gigas	Ocean acidification	Proteomics (LC-MS/MS	(Timmins-Schiffman et al., 2014)
C. giags	Temperature and Salinity	Proteomics (iTRAQ, 2DE, LC-MS/MS)	(Zhang et al., 2015)
Saccostrea glomerata	Ocean acidification	Proteomics (2DE, LC-MS/MS)	(Thompson et al., 2015)
Saccostrea glomerata	Metal contamination	Transcriptomics (RT-qPCR)	(Taylor et al., 2015)
C. gigas	Temperature	Transcriptomics (Q-RT-PCR)	(Zhu et al., 2016)
C. gigas	Temperature	Transcriptomics (RNA seq)	(Lim et al., 2016)
C. gigas	Temperature	Transcriptomics (RT-PCR)	(Huang et al., 2016)
Saccostrea glomerata	Ocean acidification	Transcriptomics (qPCR)	(Goncalves et al., 2016)
Saccostrea glomerata, C. gigas	Virus infection	Proteomics (iTRAQ)	(Masood et al., 2016)
C. gigas	Virus infection	Proteomics (2DE, LC-MS/MS)	(Green et al., 2016)
Saccostrea glomerata	Disease (WM)	Proteomics (2DE, LC-MS/MS)	(Vaibhav et al., 2016)
Saccostrea glomerata	Pollution	Proteomics (2DE, LC-MS/MS)	(Melwani et al., 2016)
Saccostrea glomerata	Ocean acidification	Proteomics (2DE, LC-MS/MS) , Transcriptomics (qPCR)	(Goncalves et al., 2017)

1.6.2 Proteomics in Sydney Rock oysters

The availability of the *C. gigas* genome sequence expedited the advancement of proteome research in oysters. Even though it is not the ideal database for SRO proteomics study, it is the best which is publicly available at the time of writing, since the two species are related.

Recent proteomic studies involving SRO have contributed in different ways towards understanding the biology of the organism. Effects of environmental factors have been studied thoroughly using proteomics and other approaches. Elevated levels of CO_2 are considered to be one of the major controlling factors of oysters distribution as studies have revealed that distribution of oysters may be affected negatively when CO_2 level is higher (Scanes et al., 2017). Increased CO_2 levels affect the oysters indirectly by causing ocean acidification resulting in lowering of the pH of the ocean by formation of carbonic acid. The historic increase in CO_2 level as a result of the industrial revolution has resulted in decline of pH by more than 0.1 units, and it is predicted to continue to decline with a further reduction of 0.3-0.5 pH units expected by the end of the 21st century (Caldeira and Wickett, 2005). Organisms with shells made up of calcium carbonate are mainly affected by this change in acidity (Caldeira and Wickett, 2003; Gazeau et al., 2013), so understanding the effect of CO_2 on oysters is also very important for sustainable farming of oysters.

In one of the earlier studies in this area, it was shown using 2DE based proteomics that the larval growth of SRO selectively bred for faster growth and disease resistance is less affected by elevated CO_2 as compared to the larvae of unselected SRO (Parker et al., 2011). In a similar comparative proteomics study using 2DE and LC-MS/MS, a differential proteomic response was obtained for selected and unselected populations of SRO in response to elevated CO_2 level. This study revealed differential regulation of proteins involved in five different functional categories including energy metabolism, cell signalling, cellular stress, protein synthesis and cytoskeleton (Thompson et al., 2015; Thompson et al., 2016). Another proteomic study investigated the effect of prolonged environmental stress on the proteome of SRO collected from different bays in Sydney harbour. The study identified proteins of only two functional categories differentially expressed between selected and wild populations – energy metabolism and cytoskeletal proteins (Melwani et al., 2016).

A recent transcriptomic investigation suggested that successive generations of a selected line of SRO were better adapted to the increased CO_2 level as compared to the unselected SRO. This study reiterates the heritability of the genes of selection, as over successive generations these genes are imparting better adaptability to the oysters against different stressors (Goncalves et al., 2017).

The effects of CO_2 in the presence and absence of other stressors have also been studied

and it was found that in absence of other stressors, increased levels of CO_2 had a positive effect on the growth of the larvae, however, in presence of other stressors it was maladaptive (Parker et al., 2017). This observation was explained with reference to the common phenomenon observed in bivalves exposed to different stressors – an increase in metabolic rate to meet the energy requirement generated by adaptive processes (Melwani et al., 2016; Parker et al., 2015; Parker et al., 2012; Raftos et al., 2016).

The schematic diagram in **Figure 1.8** summarises the hypothesis developed from findings of numerous studies in our laboratory and others in recent years. As the oyster is exposed to changed environmental conditions challenging its survival, its intracellular system responds by increasing ATP production through the electron transport chain in the mitochondria. This sudden surge in the ATP production results in abnormally high production of oxygen free radicals such as superoxides. This results in an imbalance between the production and consumption of ROS, which is also known as oxidative stress (Betteridge, 2000). If not checked, oxidative stress inevitably leads to damage of proteins, DNA, and RNA (Vendemiale et al., 1999). In oysters, as a result of oxidative stress, cytoskeleton damage has been widely observed. Hence, increased abundance of cytoskeleton associated proteins, attributed to cytoskeletal remodelling, has been observed in various studies involving effects of increased CO2, effects of metal contamination, and disease stress (Melwani et al., 2016; Muralidharan et al., 2012; Thompson et al., 2015; Vaibhav et al., 2016).

1.7 Diseases of Sydney Rock oysters

In this chapter we are mainly concerned about two diseases of SRO - WM and QX. As we will discuss, these two diseases are major factors in contributing to the mortality of SRO. The following section deals with the disease background and literature concerning these two diseases.

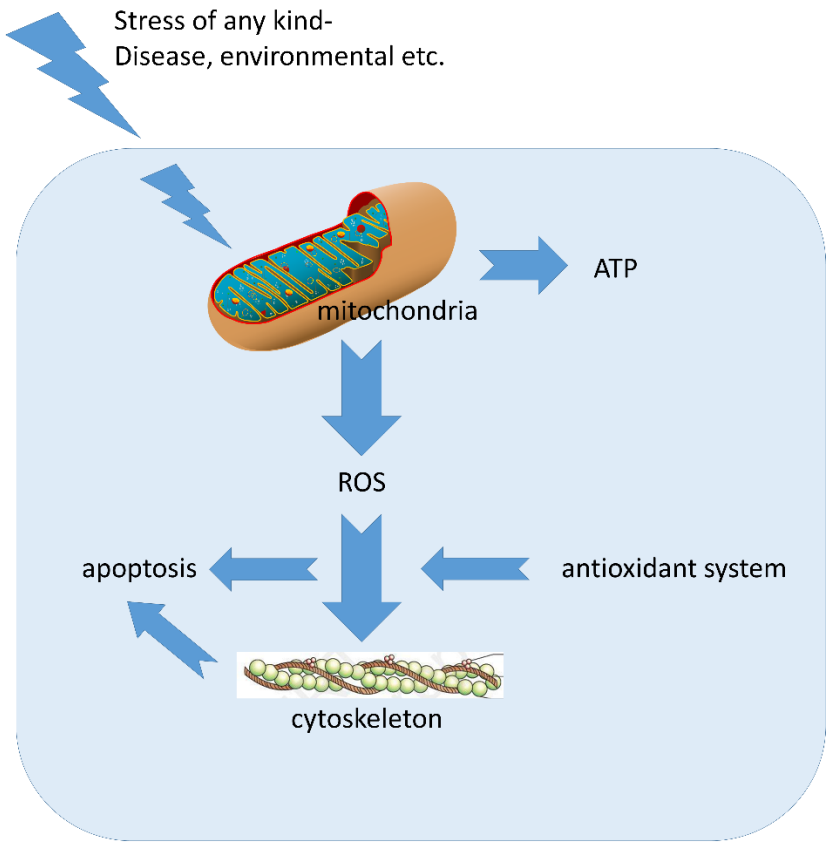

Figure 1.8: A general schematic of the hypothesis proposed by (Anderson et al., 2015b; Raftos et al., 2016) which shows a common intracellular response of an oyster exposed to different types of stressors, both biotic and abiotic.

1.7.1 Winter Mortality

Winter Mortality is a relatively less well known and studied disease of SRO, and as the name suggests this disease occurs during the winter season (during the months April-September). Oysters of the southern estuaries are the most affected. Winter Mortality (WM) affects the oysters in their second or third year of growth so the SRO crop which is close to the market size is damaged and consequently the industry may suffer heavy losses.

Although the first report of WM outbreak goes back to the early 1920s the exact cause of this disease is still uncertain (Spiers et al., 2014).The first inquiry, following the outbreak in 1923 (Nell, 2001a), suggested low temperature as one of the causative agents along with increased salinity of the water. However, later in 1988 the aetiological agent of WM was identified in work by (Farley et al., 1988) as *Mikrocytos roughleyi* (later classified as *Bonamia roughleyi* (Carnegie and Cochennec-Laureau, 2004)). Gross symptoms of WM disease include the presence of lesions associated with the shell, abscesses in focal tissue,

and the formation of pustules in gills, gonads and the digestive tract (Farley et al., 1988; Spiers et al., 2014).

In recent studies it has been found that disease severity could also be exacerbated by relative unavailability of oyster nutrition such as phytoplankton (Spiers et al., 2014). However, the presence of *Bonamia roughleyi* has not been observed in all cases of oysters suffering from WM. The inconsistency in this observation makes this disease all the more puzzling. In recent work it was found that a small percentage of another *Bonamia* species (*B. exitiosa*) was detected in DNA based PCR tests, and their inability to identify presence of *Bonamia roughleyi* in the lesions further escalated the controversy regarding WM disease aetiology (Spiers et al., 2014).

Salinity of the water bodies plays an important role in exacerbating WM disease severity (Nell and Perkins, 2006). As a temporary solution to avoid WM losses and prevent the oysters from being exposed to higher salinity, some farmers transferred their crop upstream before the onset of winter (Nell et al., 2000; Smith et al., 2000). In another approach to address WM losses, scientists tested the triploid oysters exposed to similar conditions as diploids; it was observed that the survival rate of triploid oysters was greater as compared to diploid (Hand et al., 1998). WM has previously been known to affect the yield of SRO greatly (mortality rate >80%), so it is very important to address the underlying biological phenomenon. There are still major gaps in our understanding of the WM disease aetiology, which are exacerbated by the relative lack of recent scientific literature on the subject. This thesis reports important findings in WM disease research and represents one step towards a better knowledge of the effects of selective breeding on oysters affected by WM disease. Chapter 2 in this thesis presents the findings of the first comparative proteomic analysis of control oysters against oysters selectively bred for resistance to WM.

1.7.2 Queensland Unknown (QX) disease

Queensland Unknown (QX) disease is caused by a haplosporidian parasite, a protozoan named *Marteilia sydneyi*. This parasite was identified as the causative organism in 1972 , from oysters collected from Moreton Bay (Wolf, 1972). In 1976 the microscopic structure of *M. sydneyi* was elaborated and it was found to involve various sporulation stages passing through a chain of internal cleavage events, resulting in the formation of sporangiospores which consists of sporangia and spores (Perkins and Wolf, 1976). These observations

provided evidence for why it should be considered in this genus. An oyster infected by this parasite displays pale yellow coloured gut when compared against an uninfected oyster. However, it should also be noted that this symptom does not necessarily confirm the presence of QX parasites. This condition could just be a case of an unhealthy oyster and therefore to confirm the presence of this parasite in oyster further tests are required.

1.7.2a Parasitic development: Life cycle - In and Out

As noted above, *M. sydneyi* is responsible for QX disease in Sydney Rock oysters (*Saccostrea glomerata*) (Grizel et al., 1974). The pathogen *M. sydneyi* infects while it is in the free floating parasitic stage and the oyster, being a filter feeder organism, takes in the parasites through gills and palps (Roubal et al., 1989a).

The parasite undergoes extrasporogonic development in the gills and palps epithelia, which is preceded by complicated cell-within-cell multiplication of the parasite. Following extrasporogonic development, the parasite spreads into connective tissue and haemolymph spaces. In the study conducted on initial infective stages of parasites (Kleeman et al., 2002), a large number of parasites in extrasporogoinc stages were marked on the floor of epithelial cells before the occurrence of subepithelial infection. It was suggested that the number of extrasporogonic parasites present might assist in weakening the basal membrane and thereby help establish the sub-epithelial infection. A schematic of progression cycle of *Marteilia sydneyi* in Sydney Rock oyster is shown in **Figure 1.9**.

From gills and palps, the parasite further sporulates in the digestive gland of the oyster (Anderson et al., 1995; Kleeman et al., 2002; Roubal et al., 1989a). It is in the digestive epithelium where the formation of sporont takes place (Kleeman et al., 2002). It was observed that an increase in parasite numbers takes place while it is in the digestive tubules. As the infection progresses, the parasite invades all available places in the epithelium (Kleeman et al., 2002). Before the oyster dies, the parasite passes out into the environment through the alimentary canal, ready for the next cycle of infection as sporonts (Roubal et al., 1989a). Death of the oysters occurs because of starvation, which is a direct implication of destruction of the digestive gland tissues (Roubal et al., 1989a).

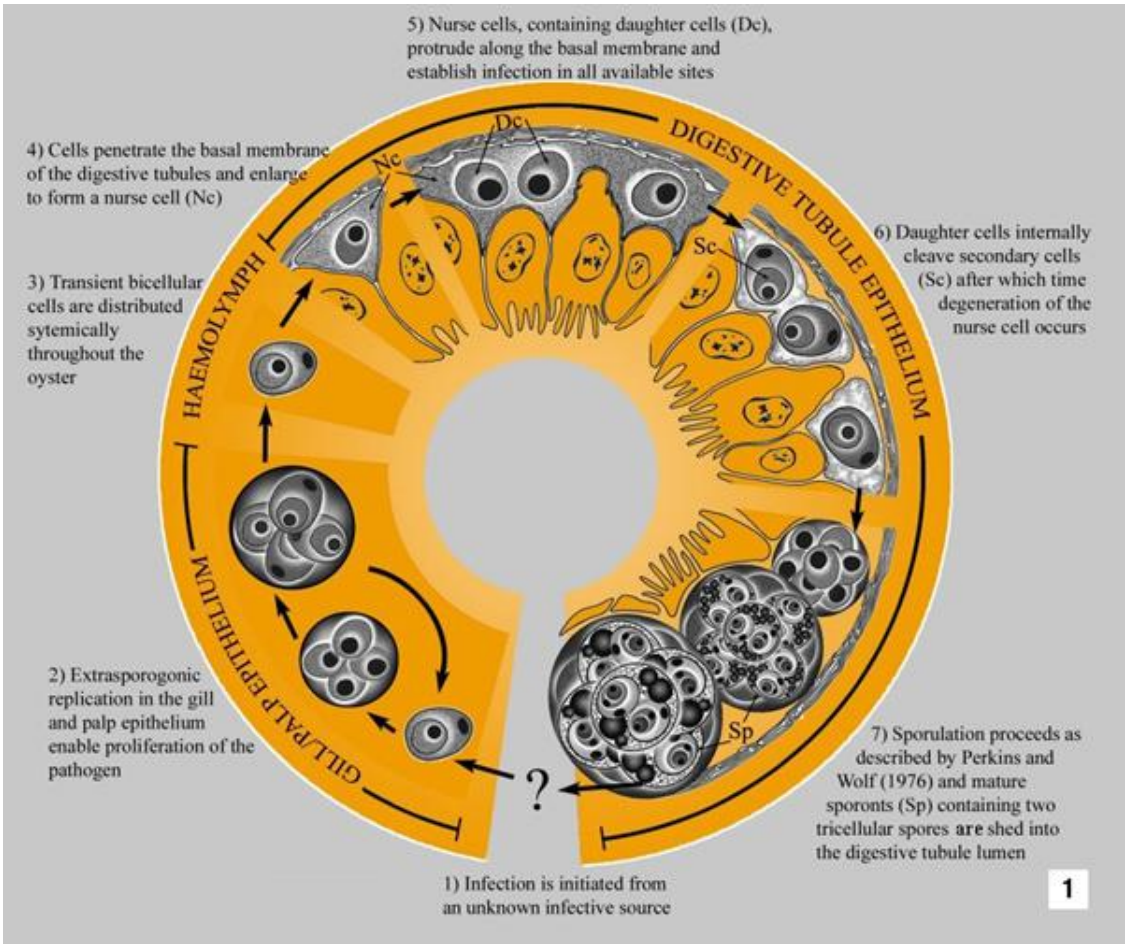

Figure 1.9: Progression cycle of *Marteilia sydneyi* in Sydney Rock oyster *(Saccostrea glomerata*) prior to the knowledge of secondary host. (Kleeman et al., 2002).

However, very little is known about the rest of the life cycle of the pathogen and there is ongoing disagreement regarding the stage of parasite which marks the beginning of infection. There is a possibility of the involvement of more than one secondary host since no infection from one oyster to other has been observed, and attempts to transfer infection in the laboratory from infected to uninfected oysters were also unsuccessful (Lester and Healy, 1986). Involvement of a filter feeding fish or polychete worm was suggested in an early investigation by (Roubal et al., 1989a). Very recently, it has been confirmed that the secondary host of QX disease parasite is a polychete worm *Nephtys australiensis* (Adlard and Nolan, 2015b).

1.7.3 Host Response and Defence Mechanism

Two major enzymes have been reported in regard to *Saccostrea glomerata* immune response against the pathogen *M. sydneyi*. The first is Phenoloxidase and the second is Superoxide dismutase (Green et al., 2011; Holmblad and Söderhäll, 1999).

1.7.3a Phenoloxidase

Phenoloxidase was initially characterized as a host defence protein in insects and arthropods (Söderhäll and Cerenius, 1998; Sugumaran, 2002). However, recent studies have demonstrated its significance in host defence mechanism of molluscs and bivalves (Butt and Raftos, 2008b; Deaton et al., 1999; Hellio et al., 2007) .

The immune system of invertebrates differs from that of vertebrates in that they lack clonal selection mechanisms and antibodies. Therefore, in the absence of acquired or antibody mediated immunity, invertebrates are dependent on their innate immune system (Mendoza and Faye, 1999). One way in which the invertebrates demonstrate innate immunity is via the production of antimicrobial peptides when presented with a foreign agent, displaying recognition molecules such as those present on bacterial walls (Beutler et al., 2006).

Phenoloxidase enzyme is a component of a complex proteinase system of PRPs (Pattern recognition proteins) and proteinase inhibitors, and together they constitute a prophenoloxidase-activating system. In invertebrates like bivalves, phenoloxidase is present as an inactive form of prophenoloxidase in the haemolymph. Conversion of prophenoloxidase is mediated by serine proteinases when the PRPs recognize the presence of a pathogen (e.g. lipopolysaccharide, β-1,3-glucan, and peptidoglycans). Phenoloxidase is a copper dependent enzyme which catalyses the conversion of monophenols to O-diphenols, which undergo further dehydrogenation into O-quinones (Kong et al., 1998). Without any enzymatic interference, the O-phenols polymerize to produce melanin. An outline of the prophenoloxidase activation scheme is shown in **Figure 1.10**.

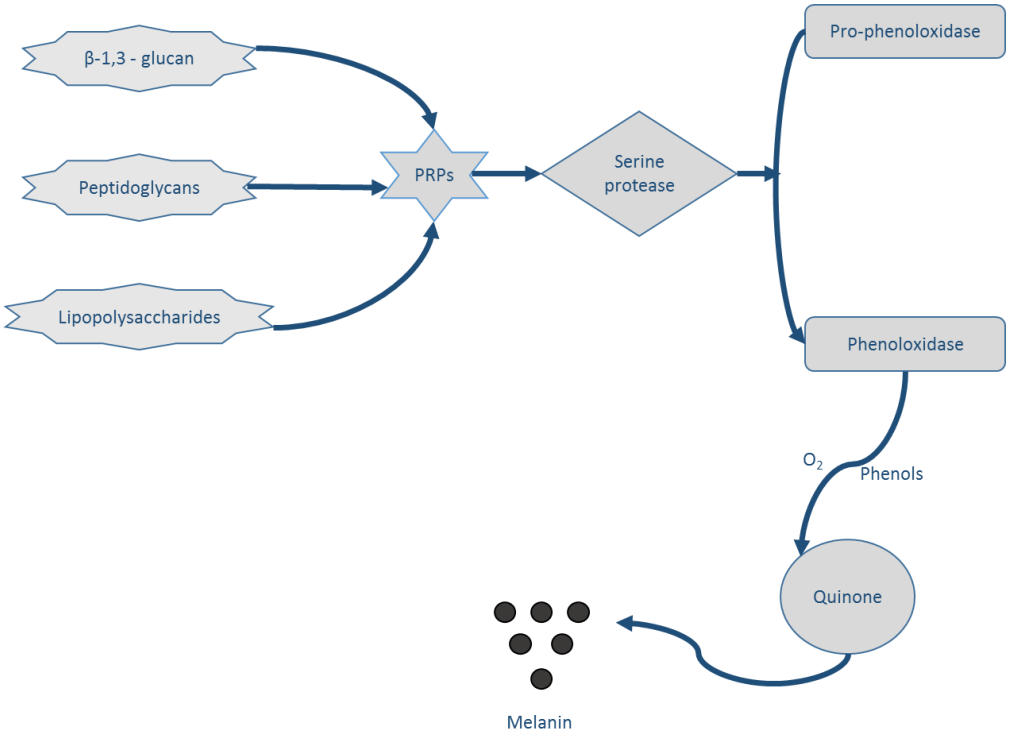

Figure 1.10: Prophenoloxidase activation scheme, modified from (Söderhäll and Cerenius, 1998).

Primarily, phenoloxidase functions to deposit the pigment melanin with the assistance of tyrosine-based substrates, thereby facilitating the parasite killing mechanism. Phenoloxidase activity can also be very important when measuring the impact of environment or while evaluating the health of the oyster, because the activation of the prophenoloxidase system is dependent on foreign agents or environmental factors. Hence, it can be used a tool for rapid and inexpensive testing for environmental stress, or disease conditions the organism is experiencing (Hellio et al., 2007; Tujula et al., 2001).

With regard to QX disease, it has been observed previously that as phenoloxidase activity decreased, the susceptibility of oysters to infection from *M. sydneyi* increased (Butt and Raftos, 2008b). Therefore, it can be inferred that a high level of phenoloxidase activity is associated with increased resistance against *M. sydneyi* infection (Butt and Raftos, 2008b). In another instance, in the oysters bred in the selective breeding programme of New South Wales Department of Primary Industries, it was noted that the QX resistant oyster lines had significantly elevated phenoloxidase activity as compared to those wild oysters which were never exposed to infectious elements or disease conditions (Newton et al., 2004).

1.7.3b Superoxide dismutase (SOD)

Superoxide dismutases are a very important set of Cu, Zn, Fe and Ni containing enzymes which play an important role in scavenging the free radicals such as superoxides ($O_2^{\cdot -}$), which are generated as a side product of aerobic respiration (Miller, 2012). As shown in **Figure 1.11**, conversion of peroxide free radicals to hydrogen peroxides is mediated by SODs. Different classes of organism have different prosthetic groups associated with SOD. Humans contain SOD1 which is Cu and Zn containing. Prokaryotes have been found to contain mainly FeSOD, while plants have MnSOD in their mitochondria and FeSOD in the chloroplast (Miller, 2012). On the other hand, molluscs have been reported to contain Cu and Zn associated SOD (Geret et al., 2004).

These free radicals generated are highly reactive species and pose immediate threats by affecting different biological processes such as cellular growth, gene expression and apoptosis (Fukai and Ushio-Fukai, 2011). In bivalves, as a result of oxidative stress, the free radicals generated cause damage to the cytoskeleton (McDonagh and Sheehan, 2007) which has also been observed in previous studies in SRO (Thompson et al., 2015; Vaibhav et al., 2016).

The presence of SOD in the organisms ensures better adaptability to oxidative stress by consuming the superoxide free radicals that are produced. In a comparative study of SRO selected for disease resistance with unselected SRO, it was found that genes of different antioxidant enzymes which included SOD were more highly expressed in selected lines of oysters, suggesting the importance of SOD in prevention of oxidative stress (Green et al., 2009).

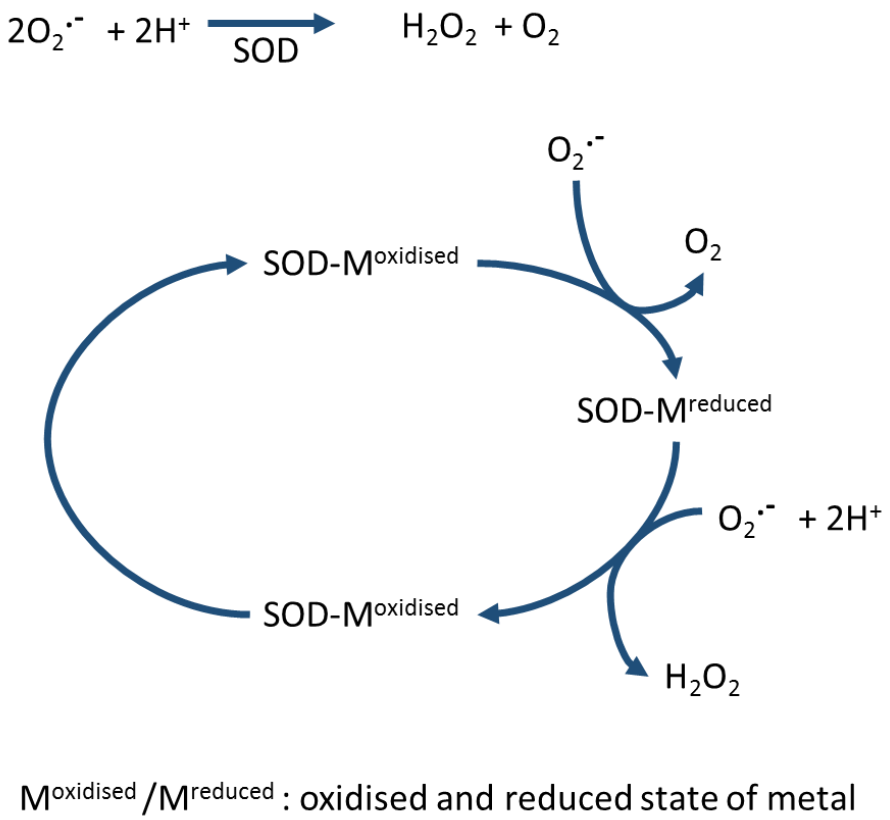

Figure 1.11: Schematic diagram showing the involvement of SOD in conversion of superoxide free radicals to hydrogen peroxides. Modified from (Fukai and Ushio-Fukai, 2011).

1.8 Specific aims of the work presented in this thesis

This thesis aims to better our understanding of diseases of Sydney Rock oysters, as well as identify protein biomarkers for the two diseases - WM and QX. Comparative quantitative proteomics is a relatively low throughput, data-rich technique which enables us to study the expressed proteome of an organism present under different conditions. In this case the comparisons are made between selectively bred lines of SRO and unselected hatchery control or wild shoreline control oysters.

Previous research aimed at understanding the molecular basis of diseases of SRO were limited by the absence of a sequenced genome. Transcriptomics studies have been undertaken, aimed at understanding QX disease and other stressors affecting SRO (Ertl et al., 2016a; Ertl et al., 2016b; Hook et al., 2014). Other studies in a similar vein include immunological studies which have helped in understanding aspects of QX disease stress, which have identified successfully some disease response related genes.

However, very little has previously been done towards acquiring more detailed knowledge of the expressed proteome of oysters selected for resistance to QX and WM. Until the first shotgun proteomics work published on oysters in 2012 (Muralidharan et al., 2012), proteomics studies were mostly 2DE based. The work described in this thesis employs both 2DE and shotgun proteomics approaches to study the expressed proteome of the gills and gut tissue of SRO. In addition to that we have also performed a SWATH-MS study for the first time in SRO. The work described herein presents a significant step towards a better understanding of WM, and identification of the first protein biomarkers known for QX disease response. The primary objectives of this thesis are:

1. Comparative proteomics analysis of WM selected Sydney Rock oysters and identification of disease biomarkers.

2. Temporal comparison of QX selected Sydney Rock oysters to study time dependent proteome response.

3. Proteomic investigation of QX disease resistant oysters collected from different estuaries – Woolooware Bay and the Hawkesbury River.

4. Investigation of the application of SWATH-MS analysis for studies in Sydney Rock oysters.

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Chapter 2

Biomarkers of Winter Mortality resistance in selectively bred Sydney Rock oysters (*Saccostrea glomerata*)

Chapter 2. Biomarkers of Winter Mortality resistance in selectively bred Sydney Rock oysters (*Saccostrea glomerata*)

2.1 Biological Context

Winter mortality (WM) is a disease affecting Sydney Rock oysters (*Saccostrea glomerata*), which results in mass mortality of more than 80% of oysters, with the oyster industry suffering heavy losses as a consequence. A protozoan parasite was originally thought to be the causative organism, but recent studies have shown the disease aetiology to be much more complicated. A selectively bred line of oysters was maintained by inbreeding naturally resistant oysters to decrease the mortality cause by WM. This study reports the first comparative proteomic analysis of oysters selectively bred for WM with unselected oysters. Employing two-dimensional protein gel electrophoresis in conjunction with nanoflow LC-MS/MS, we investigated differential proteome responses of two different oyster populations with the aim of identifying potential disease biomarkers. This study provided the first insight into proteomic differences between the two populations, which reflected different adaptive measures to combat the disease.

The study is presented in the form of a manuscript published in the journal Aquaculture as - *Biomarkers of Winter Mortality resistance in selectively bred Sydney Rock oysters (Saccostrea glomerata).* ***Vaibhav, V****., Lepretre, M., Thompson, E., Raftos, D., Haynes, P. A. Aquaculture, Vol. 465, (2016), p.323-329. 10.1016/j.aquaculture.2016.09.006.*

2.2 Contributions

This experimental part of the work described in this chapter was shared between Mr. Maxime Lepretre and myself. I estimate I performed 70% of the experimental work involved. Protein extractions and the first set of 2DE experiments and spot volume image analysis were performed by Mr. Lepetre. I performed necessary replicates of the two-dimensional electrophoresis experiments for identification of protein spot features from the gels, protein spot excision, in-gel digestion using trypsin, nanoflow LC-MS/MS analysis of the digested peptides, database searching for peptide to spectra matching, and protein functional annotation. The oysters were kindly provided by Wayne O'Connor at DPI, Port Stephens. I contributed 80% of the interpretation of the results and writing the manuscript,

with assistance from Mr. Lepetre, Prof. Paul A. Haynes, Prof. David A. Raftos and Dr Emma Thompson.

2.3 Manuscript information

Biomarkers of Winter Mortality resistance in selectively bred Sydney Rock oysters (*Saccostrea glomerata*)

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2.4 Abstract

Sydney Rock oysters (SRO, *Saccostrea glomerata*) are an important part of Australian cuisine and are considered to be a gourmet delicacy for their distinctive flavor. The species is native to Australian estuaries where it faces challenges from environmental stressors and human impacts. In addition, the Sydney Rock oyster aquaculture industry has been periodically affected by outbreaks of diseases such as Winter Mortality and QX disease. These diseases limit the production of SRO but do not affect other major oyster species

farmed in New South Wales. Over the past few decades, breeding lines of SRO have been selected for resistance to Winter Mortality and QX disease. In this study, we report a proteomic comparison between the Winter Mortality selective breeding line and hatchery control oysters. We identified 22 protein features showing differential intensities between the two proteomes. The identities of 19 of these features were established using nanoflow liquid chromatography - tandem mass spectrometry. The identified proteins were categorized into five functional categories - energy metabolism, stress response, cytoskeleton, protein processes, and cellular communication. The differential proteome identified in the current study coincides well with previous analyses of stress responses in oysters, suggesting commonalities between intracellular reactions to disease and stress. For instance, cytoskeletal effects found in the current study to be associated with Winter Mortality resistance have been previously linked to intracellular stress responses. In addition, we identified novel proteins such as proteasome subunit alpha type-6 and calcium-dependent protein kinase 31, both of which are reported for the first time in disease resistant SRO. Results of this study will add to our understanding of stress responses and pioneer the proteomic survey of Winter Mortality resistance in Sydney Rock oysters.

Key words

Sydney Rock oyster, *Saccostrea glomerata*, Winter Mortality, oyster, proteomics, protein biomarkers

Abbreviations

2DE, two-dimensional (IEF/SDS-PAGE) protein gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; CDPK, calcium dependent protein kinase; DPI, department of primary industries; DTT, dithiothreitol; xg, gravity; FC, fold change; HC, hatchery control; IEF, Isoelectric focusing; IPG, immobilized pH gradient; LC-MS/MS, liquid chromatography – tandem mass spectrometry; QX , Queensland unknown disease; ROS, reactive oxygen species; SAM, significance analysis of microarrays; SDS-PAGE, sodium dodecyl sulphate – polyacrylamide gel electrophoresis; SOD, superoxide dismutase; SRO, Sydney Rock oyster; WM, winter mortality disease.

Highlights

- We compared the proteome of oysters selected for resistance to Winter Mortality disease with that of hatchery control oysters.
- We used two-dimensional protein gel electrophoresis to highlight 22 protein features that show differential intensity between the two proteomes.
- We identified and categorised proteins responsible for these differences, which represent potential biomarkers of Winter Mortality resistance.

2.5 Introduction

Sydney Rock oysters (*Saccostrea glomerata,* Gould 1850, previously known as *Saccostrea commercialis,* (Buroker et al., 1979)) are endemic to Australia. They are the focus of a major aquaculture industry in the eastern states of New South Wales (NSW) and Queensland (QLD). Sydney Rock oyster farming is the largest aquaculture industry in NSW (Simonian et al., 2009b). However, there has been a continuous decline in Sydney Rock oyster production since the 1970s (Nell, 2001a). This decline has been attributed, at least in part, to the impacts of two infectious diseases; Winter Mortality (WM) and QX disease, in conjunction with environmental factors such as anthropogenic pollution (Simonian et al., 2009b).

Both Winter Mortality and QX disease can result in > 80% mortality of farmed Sydney Rock oysters in particular areas (Nell, 2001a; b). Winter Mortality is more pronounced in the southern range of the species, closer to the NSW-Victorian border. Initially WM was thought to be caused by a protozoan, *Bonamia roughleyi* (previously known as *Mikrocytos roughleyi*) (Carnegie et al., 2003; Cochennec et al., 2003; Farley et al., 1988; Nell, 2001a). However, the aetiology of WM is currently under review and new evidence suggests that it represents a complex syndrome (Spiers et al., 2014). The disease is now thought to be associated with environmental factors, such as temperature and salinity, in addition to the presence of pathogenic agents. A recent study showed that there is no direct correlation with the severity of WM and the presence of *B. roughleyi* (Spiers et al., 2014). The authors concluded that the disease state might be attributed to some other aetiological agent and/or environmental conditions. Regardless of its aetiology, WM becomes epidemic during autumn and winter (Lauckner, 1983). Affected oysters are typified by superfluous

haemocyte infiltrations in the gills, palps, gonads and mantle, which makes those tissues appear ulcerated. The soft tissue exhibit the formation of yellow pustules and the wall of the digestive diverticulum shows obvious signs of inflammation associated with the formation of pustules (Lauckner, 1983).

Historically, oyster farmers have managed WM by transferring the oysters to upstream leases before the potential onset of the disease at the beginning of winter, as low salinity was thought to decrease the impacts of WM (Smith et al., 2000). It had also been demonstrated that the severity of the disease could be reduced by raising the level in the water column at which oysters were grown (Smith, 1991; Smith et al., 2000; Wolf, 1967). However, these management practices are labour intensive and have had limited efficacy.

In 1990, the NSW Department of Primary Industries (DPI) initiated a selective breeding program to produce WM resistant oysters, and so provide a more reliable and effective response to disease management. This effort was based on a previous program in the USA to breed Eastern oysters (*Crassostrea virginica*) that were resistant to the parasite *Minchinia nelsoni* (Ford and Haskin, 1987). The NSW DPI program used mass selection of brood stock originally sourced from four major NSW growing estuaries (Wallis Lake, Port Stephens, Hawkesbury River and Georges River) (Nell et al., 2000). Successive generations of the WM breeding line were reared in the Georges River, NSW, which suffered recurrent annual outbreaks of WM. Survivors of these outbreaks were used as broodstock of each successive generation. Nell and Perkins first reported on the performance of oysters selected for WM resistance in 2006 after three generations of selection. Their work suggested that breeding for resistance to WM reduced losses from disease by 46% in the WM resistant line compared to hatchery controls (HC) (Nell and Perkins, 2006). Subsequent analyses have reported continuing improvements in the survival of WM selected oysters to the extent that they are now functionally resistant to the disease (Dove et al., 2013).

Despite the observed improvements in survival of the WM breeding line, the molecular and genetic basis of disease resistance is still poorly understood. The current study uses proteomics to investigate differences at the molecular level between the WM breeding line and HC oysters to provide preliminary information on the molecular basis of disease resistance. Several recent studies have used a similar proteomic approach to better

understand the molecular mechanisms underlying the response of oysters to a variety of environmental factors. For example, Thompson et al. and Muralidharan et al. (Muralidharan et al., 2012; Thompson et al., 2015) used two dimensional electrophoresis (2DE), mass spectrometry and shotgun proteomics to identify an intracellular stress response that involves differential regulation of several biological processes including antioxidant systems, cytoskeletal activities, protein synthesis, cell signaling and energy metabolism. Comparable proteomic studies have tested the effect of stressors such as metal contamination (Thompson et al., 2011) and the combination of elevated temperature and ocean acidification (Parker et al., 2009) on oysters. In each of these studies observations of changes to the proteome of Sydney Rock oysters revealed a generic response to stressors (i.e. protein responses that are common to all) as well as responses that were unique to each type of stressor. The current study takes a similar approach to identify the molecular processes involved in resistance to Winter Mortality in the WM breeding line. Its overall goal is to identify a suite of proteins that might act potential biomarkers for WM disease resistance in marker assisted breeding programs.

Several proteins associated with QX disease resistance of Sydney Rock oysters have already been identified (Thompson et al., 2015). Additional analyses suggested that disease resistance is associated with a decrease in the heterogeneity amongst the genes encoding disease resistance proteins (Green et al., 2009). Such a decrease in heterogeneity is possibly a result of inbreeding as observed in selective breeding of American oysters against MSX (Ford and Haskin, 1987). These previous studies provide a reliable benchmark for comparison with the current study. The current work is also supported by the availability of a complete genome sequence for the Pacific oyster (*Crassostrea gigas*), which is closely related to *Saccostrea glomerata* (Zhang et al., 2012). Analyses of the *C. gigas* genome revealed that oysters are highly polymorphic with a combined (wild and inbred) polymorphism level of 2.3%. The *C. gigas* genome also revealed the involvement of genes in several biological pathways which include defense, protein folding, oxidation, immune response, apoptosis, and shell formation.

2.6 Materials and Methods

2.6.1 Oyster collection and acclimation

Twenty selectively bred (WM) and twenty HC Sydney Rock oysters were provided by the Port Stephens Fisheries Institute (PSFI) of NSW DPI. Oysters were collected on 27th February 2014. WM oysters were from the sixth generation of selective breeding and had been reared in Port Stephen, NSW, which does not suffer recurrent outbreaks of Winter Mortality. However, the WM line had previously been selected for resistance to Winter Mortality in the Georges River, NSW, which is severely affected by the disease.

The oysters were transferred from PSFI to the aquarium facility at the Sydney Institute of Marine Science, Mosman. They were allowed to acclimatize to the aquaria conditions for 10 days in 50 L aquaria filled with filtered flow through oceanic seawater (Thompson et al., 2012c). The oysters were fed every three days with Aquasonic invertebrate food supplement (5mL/ 200 L water) and exposed to identical growing conditions.

2.6.2 Protein extraction

Proteins were extracted from the gills tissue of oysters. Gills were chosen for this study as they are the first point of contact with the external environment and in addition to that they are submerged in hemolymph which is the carrier of bivalve's immune cells. After acclimation, *Saccostrea glomerata* were shucked and their gills were excised. The gill tissues were immediately mixed within 1.5 ml of Tri-reagent (Sigma–Aldrich) and homogenized using microcentrifuge tube pestles to lyse the cells. An initial clean up step was performed by centrifugation at 12 000×g for 2 min. Proteins in the supernatant were extracted following published procedures (Thompson et al., 2011). RNA was removed by adding 150 µl of bromochloropropane for 15 min followed by centrifugation for 15 min at 12000×g (4 ^{o}C) and removal of the colourless aqueous phase. DNA was then extracted by adding 450 µl of 100% ethanol for 3 min followed by centrifugation at 2000×g for 5min (4 ^{o}C). Finally, proteins were precipitated by adding 3 volumes of ice cold acetone, standing samples at room temperature for 10 min and centrifugation for 10 min at 12000×g (4 ^{o}C). The remaining protein pellet was washed four times for 10 min per wash in 1ml of 0.3M guanidine hydrochloride in 95% ethanol (V:V) followed by centrifugation at 8000×g for 5 min (4 ^{o}C) and removal of the supernatant. A final wash was performed in 1ml of 95% ethanol before protein pellets were dried at room temperature and re-suspended in 50 µl of

sample re-hydration buffer (7M urea, 2M thiourea, 4% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate (CHAPS), 50mM DT dithiothreitol (DTT)).

2.6.3 Protein quantification and pooling of samples

Protein concentrations were measured by Bradford assay (Bradford, 1976). A calibration curve was generated using a range of standard bovine serum albumin (BSA) concentrations. After quantification, protein samples from five randomly selected oysters were pooled to give a total of three replicates per treatment (3 × WM and 3 × HC). Each pooled gill sample contained 30 µg of protein per oyster in 125 µl of sample re-hydration buffer. This pooling of specimens from different oysters to produce aggregate samples is common procedure in proteomic studies (Thompson et al., 2015). It is employed to increase throughput and enhance the capacity to detect differences between experimental treatments by reducing the weight of individual variability (Diz et al., 2009).

2.6.4 Two-dimensional protein gel electrophoresis

Isoelectric focusing (IEF) was performed using an IPGphor IEF system (GE Healthcare). Immobilized pH linear gradient (IPG) gel strips (7 cm, pH 4–7; GE Healthcare) were re-hydrated overnight with 150 µg of extracted proteins (as described above). IEF was performed at 100V for 2 h, 500V for 20 min, a gradient up to 5000V for 2 h and 5000V for 2 h to give a total of 15,000–16,000 voltage hours. The IPG strips were reduced (1% DTT, 20 min) and alkylated (2.5% iodoacetamide (IAA), 20 min) before second dimension separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12% Tris– HCl polyacrylamide gels (1.5M Tris–HCl: 10% SDS: 12% acrylamide) in a Mini PROTEAN system (Bio-Rad, CA, USA). Gels were stained using Coomassie blue and visualized using a Chemidoc scanner (Bio-Rad, CA, USA).

2.6.5 Two-dimensional protein gel image and statistical analysis

Two-dimensional protein gel electrophoresis (2DE) proteome maps were analysed to identify protein spots that differed significantly in fluorescence intensity between HC and WM (selectively bred) oysters using PD Quest analysis software (Non-Linear Dynamics, Newcastle-upon-Tyne, UK). The three 2DE gels of WM oysters (each representing pooled protein samples from 5 oysters) were compared against the three gels of HC oysters. Coefficients of variation were calculated for protein spot intensities between replicate gels in the WM and HC populations. The statistical significance of differences (WM vs HC) in

fluorescent intensities for each spot were determined using Significance Analysis of Microarray (SAM) tests in the "R" statistical software (Meunier et al., 2005; Tusher et al., 2001). This statistical method, derived from the standard t-test, uses a fold change (FC) and a data permutation technique to determine the significance of changes in gene expression or protein abundance between different biological treatments. We adopted a FC > 2 as a threshold value, and 500 permutation sets were used for the analyses. Fold differences for each spot were calculated from the mean normalized volumes (average spot intensity from each gel normalized to background intensity of the gel) of WM oysters compared to the HC oysters. A positive fold value represents a higher spot intensity in the WM population compared to HC oysters, while a negative fold value represents a lower spot intensity in the WM compared to HC oysters. Molecular weights (Mr, kDa) and isoelectric points (pI) for the proteins spots were estimated with MWt standards and a pI scale adapted to the gel dimensions.

2.6.6 In-gel digestion of statistically significant protein spot features

Differentially expressed protein spots (WM vs HC) were manually extracted from a fresh set of 2DE gels. The excised gel pieces were then destained three times for ten minutes in freshly made 100mM ammonium bicarbonate (NH_4HCO_3). This was followed by simultaneous destaining and dehydration, twice in 200 µl 50% acetonitrile / 50% 50mM NH_4HCO_3, and then dehydration for 5 minutes in 100% acetonitrile prior to air drying. Once the gel pieces were dry, they were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for one hour at 37oC. After reduction, the gel pieces were alkylated with 50mM IAA in NH_4HCO_3 for one hour at room temperature. Washing and dehydration was then repeated before the gel pieces were covered with trypsin solution (12.5 ng/µL trypsin in 50mM NH_4HCO_3, freshly reconstituted), incubated on a rocker at 4^{o}C for one hour, and digested overnight at 37^{o}C. Peptides were extracted from the digested gel pieces twice in 50% acetonitrile and 2% formic acid in distilled water. 100µl of the extracted peptides were evaporated to dryness in a vacuum centrifuge and then reconstituted in 2% formic acid solution.

2.6.7 Nanoflow liquid chromatography- tandem mass spectrometry

Peptide extracts of the 22 differential protein spots were analyzed by nanoflow liquid chromatography-tandem mass spectrometry on an LTQ-XL linear ion trap mass spectrometer (Thermo Velos Pro). Samples were applied to a fused silica column (100 µm

ID) packed with 10cm of Magic C18AQ (200 Å, 5μm diameter, Michrom Bioresources) with an integrated electrospray tip. This was coupled with a precolumn containing PS-DVB resin (3cm, 100μm ID, Agilent Technologies). A 1.8kV electrospray voltage was applied though a liquid junction upstream of the electrospray column. The sample injection and chromatographic separation were performed using an Easy nLC II system (Thermo). This was followed by washing of peptides with buffer A (2% v/v ACN, 0.1% v/v formic acid) for two minutes at 550nl/min. Peptides were eluted from the column with 0-20% buffer B (99.9% v/v ACN, 0.1% v/v formic acid) at 500nl/min for 38 minutes, followed by a wash step with 95% buffer B at 800nl/min for 10 minutes. The spectra acquisition time was 50 minutes in positive ion mode for the scan range of 400m/z to 1500 m/z, using Xcalibur software (v2.06, Thermo) to perform dynamic exclusion, automated peak recognition and MS/MS of the top 9 most intense precursor ions at normalized collision energy of 35%.

2.6.8 Peptide to spectrum matching for protein identification

The raw data from the LC-MS/MS analysis were converted to mzXML format. Global Proteome Machine (GPM) software v2.1.1 (http://www.thegpm.org) and the X!Tandem algorithm (Craig and Beavis, 2003b) were then used to search the mzXML files against a database containing 29,000 predicted peptide sequences from the *Crassostrea gigas* genome (Zhang et al., 2012). A reversed sequence database search was also performed to allow calculation of the False Discovery Rate (FDR). To ensure very high stringency results, we applied a minimum peptide match count of seven. This refined list of protein sequence entries was then searched against the UniProt protein database for information on predicted biological functions. The identified proteins were categorized into five functional categories - energy metabolism, stress response, cytoskeleton, protein processes, and cellular communication based on the functional classifications described in Anderson et al. (2015).

2.7 Results

2.7.1 Protein expression patterns in WM disease resistant oysters

Three proteome maps (each a composite of five oysters) of total gill proteins from the WM selective breeding line were compared with three maps from HC oysters. A representative proteome map is shown in **Figure 2.1**. A total of 419 distinct protein spots were identified by PDQuest software across all of the maps. These spots were distributed in the pI range

of 4–7 and a molecular weight range of 14-220 kDa. Only 4% of protein spots exhibited a coefficient of variation greater than 1% in WM and 3% in HC oysters. SAM analysis revealed significant differences in intensity (WM vs HC) for 22 proteins spots with a fold change > 2.

Of the 22 spots that differed significantly in intensity between WM and HC oysters, seven (spots P1 to P7) appeared at higher intensity in the WM population relative to HC oysters. These seven proteins had estimated molecular weights ranging from 14 to 48 kDa and pIs of 4.5 to 6 (**Table 2.1**). Five of the more highly expressed proteins had fold changes of approximately 2 in WM relative to HC oysters, whilst the remaining two proteins had fold differences greater than 5. The greatest differential spot intensity was found for spot P5 (11.52 fold greater in WM than HC).

The remaining 15 differential protein spots had lower relative intensities in WM oysters compared to the HC population. Among these proteins, the lowest fold change was observed for spot P19, which was two times less abundant in WM oysters compared to HC oysters. Three spots exhibited fold changes greater than -5, whilst the intensities of P14 and P21 were more than 7 times lower in WM compared to HC oysters.

2.7.2 Putative protein characterization

Out of the total 22 significant spots detected by PD quest analysis, the identity of 19 was putatively established using LC-MS/MS by searching against the *Crassostrea gigas* sequence database (**Table 2.1**). Eight of the nineteen identified protein spots were found to contain more than one protein match and the remaining eleven corresponded to one protein.

The identified proteins were categorized into five classes based on their involvement in biological functions - energy metabolism, stress response, cytoskeleton, protein processes, and cellular communication (**Figure 2.2** and **Table 2.1**).

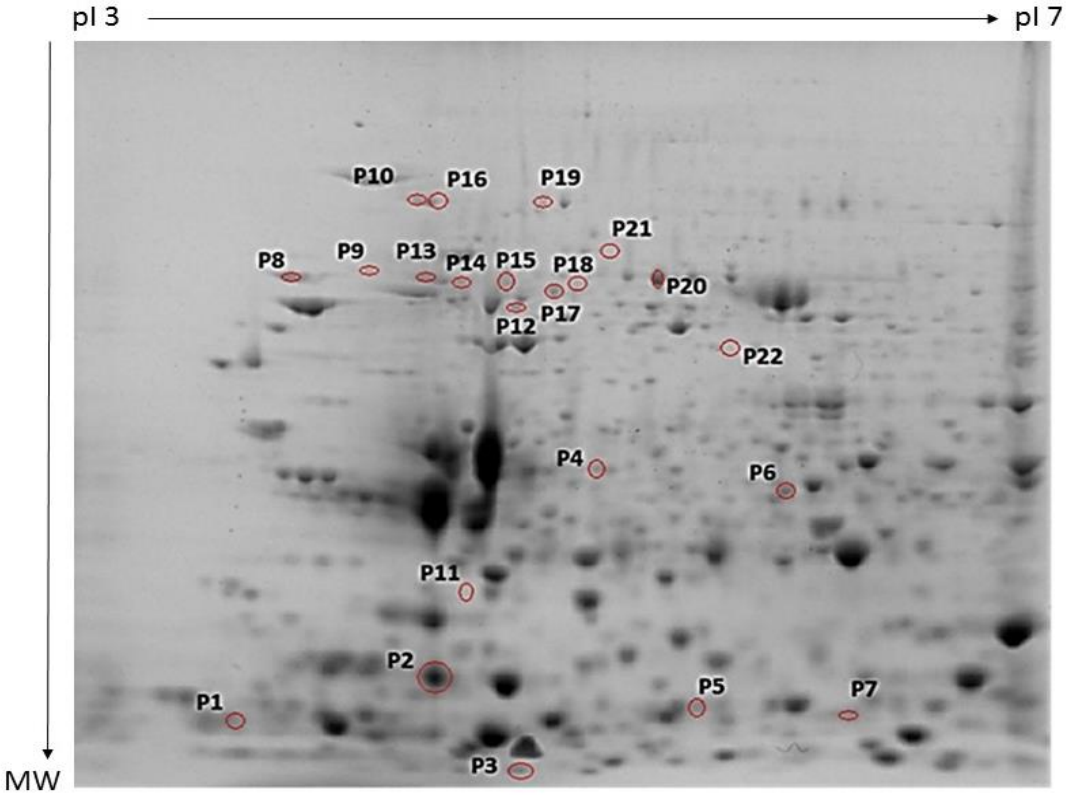

Figure 2.1: A representative 2DE gel image of the selected Sydney Rock oysters' gills protein. The protein spots identified as significant are marked in the gel picture (both upregulated and downregulated). The MS identities of these spots are listed in **Table 2.1**.

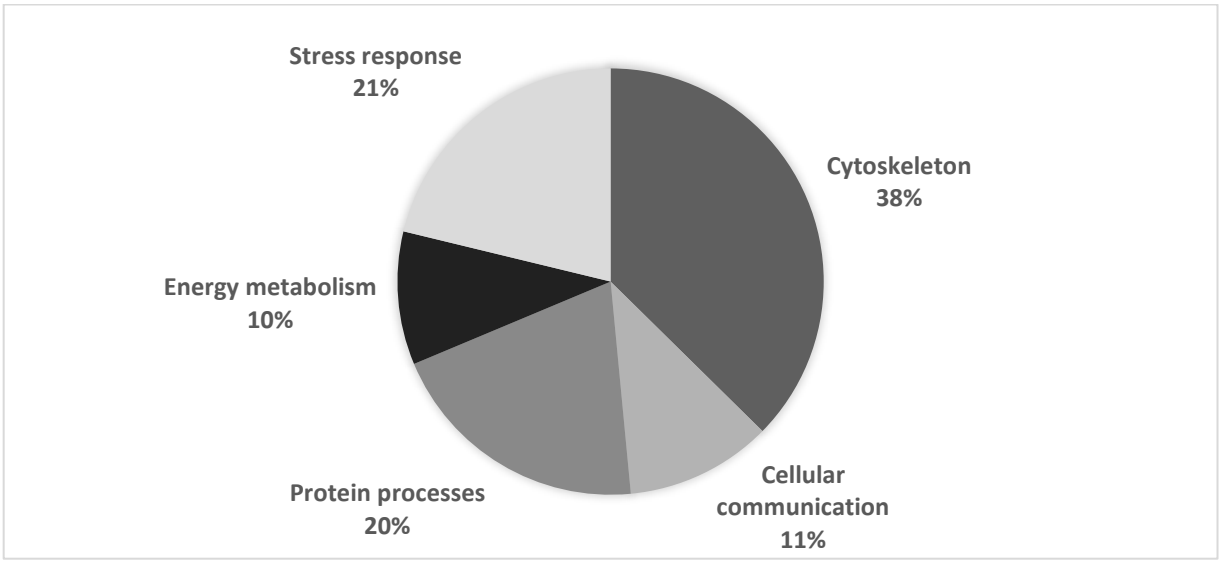

Figure 2.2: Percentage distribution of identified proteins based on functional categories.

Table 2.1: Identities of protein spots that differ significantly in intensity using LS-MS/MS Mr (theory): Molecular weight obtained by mass spectrometry. Mr (Exp): Molecular weight predicted using 2DE.

Spot no.	*Putative Protein*	*Peptide count*	*Fold change*	*Mr (theory)*	*Mr (Exp)*	*Biological function*
1	*no ID*		*2.2* ⇑		*21*	
2	*Calcium-dependent protein kinase31*	*9*	*2.6* ⇑	*20.3*	*24*	*Energy metabolism*
3	*Histone H4 gpmDB homologues protein*	*13*	*2.4* ⇑	*11.4*	*14*	*Stress response*
4	*Tumor protein D54 gpmDB homologues protein*	*7*	*5.7* ⇑	*31*	*48*	*Stress response*
5	*no ID*		*11.5* ⇑	*21.5*		
6	*Proteasome subunit alpha type-6*	*12*	*2.3* ⇑	*25.1*	*48*	*Protein Processes*
7	*Ubiquitin-conjugating enzyme E2*	*7*	*2.7* ⇑	*24.8*	*21.5*	*Protein Processes*
8	*Protein disulfide-isomerase*	*27*	*3.1* ⇓	*55.5*	*180*	*Protein Processes*
9	*Radial spoke head protein 3-like protein*	*59*	*2.3* ⇓	*45*	*180*	*Cytoskeleton*
	Na(+)/H(+) exchange regulatory cofactor_NHE-RF1	*7*		*45.3*		*Cytoskeleton*
10	*Radial spoke head protein 3-like protein*	*7*	*2.7* ⇓	*45*	*>200*	*Cytoskeleton*
11	*Ras-related protein Rab-11B*	*7*	*5.7* ⇓	*19.3*	*31*	*Cell communication*
12	*Actin-2*	*77*	*3.4* ⇓	*41.8*	*130*	*Cytoskeleton*
	Severin	*18*		*43.2*		*Cytoskeleton*
13	*Tubulin beta chain*	*129*	*3.5* ⇓	*49.9*	*180*	*Cytoskeleton*
14	*Actin*		*7.2* ⇓	*41.8*	*180*	*Cytoskeleton*
	ATP synthase subunit beta, mitochondrial	*36*		*44.9*		*Energy metabolism*
15	*no ID*		*2.5* ⇓			
16	*78 kDa glucose-regulated protein*	*87*	*4* ⇓	*73*	*>200*	*Stress response*
	Tubulin beta chain	*38*		*49.9*		*Cytoskeleton*
	heat shock protein 70	*23*		*72*		*Stress response*
	Tubulin alpha-1C chain	*10*		*50.1*		*Cytoskeleton*
	Coiled-coil domain-containing protein 63	*7*		*67*		
17	*Tektin-4*	*65*	*2.4* ⇓	*52.5*	*170*	*Cytoskeleton*
	Tubulin beta chain	*63*		*49.9*		*Cytoskeleton*
	actin	*29*		*41.8*		*Cytoskeleton*
	Non-neuronal cytoplasmic intermediate filament protein.	*10*		*69.2*		*Cytoskeleton*
	[Protein ADP-ribosylarginine] hydrolase	*7*		*87.6*		*Protein Processes*
18	*Tubulin beta chain*	*84*	*3.9* ⇓	*49.9*	*170*	*Cytoskeleton*
	Tektin-4	*37*		*52.5*		*Cytoskeleton*
	Tubulin alpha-1C chain	*33*		*50.1*		*Cytoskeleton*
	Non-neuronal cytoplasmic intermediate filament protein.	*8*		*69.2*		*Cytoskeleton*
	Protein disulfide-isomerase A3	*7*		*55.5*		*Protein Processes*
	Putative WAS protein family-like protein 4	*7*		*45.1*		*Cytoskeleton*
19	*heat shock protein 70*	*51*	*2* ⇓	*72*	*>200*	*Stress response*
	Stress-70 protein, mitochondrial	*34*		*76.3*		*Stress response*
	Non-neuronal cytoplasmic intermediate filament protein	*9*		*69.2*		*Cytoskeleton*
20	*Splicing factor U2AF 50 kDa subunit*	*8*	*2.2* ⇓	*47*	*170*	*Protein Processes*
	Alpha-aminoadipic semialdehyde dehydrogenase	*8*		*55.2*		*Stress response*
21	*Plasma alpha-L-fucosidase*	*17*	*8.1* ⇓	*62.8*	*200*	*Cell communication*
	Tubulin beta chain	*11*		*49.9*		*Cytoskeleton*
22	*Lysosomal aspartic protease*	*21*	*3.3* ⇓	*47.6*	*100*	*Stress response*

2.8 Discussion

This study identified substantial differences between the proteomes of Sydney Rock oysters that have been selected for resistance to Winter Mortality and the HC breeding line. Twenty-two protein spots had significantly different relative intensities on 2DE proteome maps of WM oysters when compared to the HC population. Of these, ~32% were expressed at greater abundance in the WM selective breeding line and the remainder were expressed at lower abundance relative to control HC oysters. Fold differences among the differential proteins ranged from 2 to 11.5.

These data complement previous analyses of QX disease resistance in SRO. In an attempt to identify biomarkers of QX disease, (Simonian et al., 2009a) reported differences in protein expression amongst QX selected and HC populations. However, that study was unable to identify the proteins involved. Follow up work identified two proteins (cavortin and dominin), which were homologous with superoxide dismutase (SOD) (Simonian et al., 2009a). SOD is an important protein in bivalve immunological defence and plays a role in antioxidant mechanisms during intracellular stress responses (Epelboin et al., 2015; Gonzalez et al., 2005; Itoh et al., 2011).

A similar involvement of intracellular stress responses is evident in the current study of WM resistance in SRO. 86% (19/22) of the differential proteins detected in 2DE comparisons of WM and HC oysters were identified using nanoflow LC-MS/MS. The identified proteins fell into five different categories of intracellular function: energy metabolism, stress response, protein processes, cytoskeletal, and cell communication. Nine differential protein spots (37%) were found to be associated with the cytoskeleton, six (21%) were putatively involved in intracellular stress responses, five with protein processes, and two each were associated with energy metabolism and cell communication related processes. In a meta-analysis studying the effect of environmental stress on oysters, 12 functional gene categories were assigned, which included all of those identified in this study (Anderson et al., 2015c).

Amongst the protein spots that were more intense in selected oysters, we identified calcium dependent protein kinase 31, CDPK31 (spot 2). This protein has been reported to be associated with the nervous system of the marine mollusk *Aplysia californica* (Schulman et al., 1985). It is also reported that the CDPK levels increase in response to hypo-osmotic

stress or defense elicitation (Romeis et al., 2001). The current study is the first time this protein has been putatively identified with reference to oyster disease resistance. Spot 3 was identified as histone H4. Histones are key components of chromatin and are involved in antimicrobial activities (Kawasaki and Iwamuro, 2008). Histone H4 has been previously identified in the American oyster (*Crassostrea virginica*) in response to various pathogens and environmental stress. It was suggested that histone H4 could potentially be used as a biomarker to determine if the oyster is susceptible to disease (Nikapitiya et al., 2014a). In a study on *C. virginica*, different isoforms of histones were found to be upregulated in response to the protozoan *Perkinsus marinus* (Dorrington et al., 2011; Wang et al., 2010).

Spot number 6 was identified as proteasome subunit alpha type-6, a member of the peptidase T1A protease family. In a study on two different species of mussels, one heat sensitive and the other heat tolerant, a differential response was observed for proteasome components when the mussels were exposed to acute heat stress (Tomanek and Zuzow, 2010). Differential expression of the proteasome is also reported in another study conducted on *Mytilus galloprovincialis*, exposed to metal stress (Varotto et al., 2013).

Ubiquitin-conjugating enzyme E2 was identified in spot number 7. This protein has previously been reported in a microarray-based gametogenesis study on *Crassostrea gigas*. The authors suggested that it may be involved in apoptotic processes (Dheilly et al., 2012). It is not certain at this stage what role it plays in the selective breeding of oysters. However, its higher expression in the current study parallels with the spermatogenesis suggests that it might help in breeding in some way which is yet to be elucidated. Upregulation of ubiquitin has been associated with proteasome upregulation, which is reported in a previous study in flat oysters (Martin-Gomez et al., 2012). In eukaryotes, degradation of proteasomes mediated by ubiquitin has been identified as a major proteolytic pathway, which is reported to be affected by many diseases (Roos-Mattjus and Sistonen, 2004).

Cytoskeletal proteins were also a substantial component of the differential proteome in the comparison between WM and HC oysters. We identified differential abundance of core cytoskeletal proteins such as actin, tubulin and tektin. In previous studies, changes in abundance of these cytoskeletal proteins have been associated with different types of stress, including ocean acidification and heavy metal contamination (Muralidharan et al., 2012; Thompson et al., 2011; Thompson et al., 2015). In these previous studies, changes in the

expression of cytoskeleton proteins as a result of stress were accompanied by increased expression of proteins associated with energy production, such as ATP synthase. ATP synthase was also found to be differentially expressed in our current comparison of WM and HC oysters. This fits a working hypothesis based on the available literature, which is that an increase in energy production is triggered when SRO are exposed to stress, and that Winter Mortality can be seen as a form of stress. In studying the effect of temperature on *Crassostrea virginica*, Lannig et al. (2006) observed that with the increase in water temperature from 20 to 28^{o}C the standard metabolic rate doubled. A similar observation was made in a study of Pacific oysters exposed to the stress of ocean acidification (Lannig et al., 2010). Such increases in standard metabolic rates may be used to meet the energy requirements of adaptive responses to stress.

Four other proteins identified in this study (proteins 16, 19, 20, 22) provide further evidence for a differentiation of the intracellular stress response pathway in WM and HC oysters. The proteins were HSP 70, Stress 70 protein, and 78kDa glucose regulated protein, all of which are molecular chaperones that are routinely associated with intracellular stress in many species. Several studies have reported the upregulated expression of HSP70 in oysters is undergoing stress (Chapman et al., 2011). Increased abundance of HSP70 has been associated with thermal stress (Hamdoun et al., 2003), metal contamination (Thompson et al., 2012a), and increased CO_2 levels (Thompson et al., 2015). The presence of HSP70 in different studies of stress in oysters signifies that this protein plays an important role in helping oysters combat the intracellular consequences of stress. This protective mechanism may be triggered in response to increased energy production, which results in the production of reactive oxygen species (ROS) (!!! INVALID CITATION !!! (Thompson et al., 2015)). Heat shock proteins and other stress responsive proteins (Alpha-aminoadipic semi aldehyde dehydrogenase (Brocker et al., 2010), 78 kDa glucose-regulated protein (Dana et al., 1990)) play an important role in reducing the damage caused by ROS.

Even though we did find that numerous proteins involved in intracellular stress responses were differentially expressed between WM and HC oysters, these did not include proteins directly involved in protection against oxidative damage, such as SOD. Even though SOD and peroxiredoxin have been implicated in QX disease resistance by Simonian et al (2009a) and Green et al (2009), they were not found to be differentially expressed in the current comparison of WM and HC oysters. This may be a discriminator between resistance to

QX and WM, or it could reflect the different tissues used for analysis (hemocytes in previous studies vs gills used here).

Regardless, the current study has identified substantial differences in the proteomes of WM and HC oysters. There are a number of potential explanations for the differences observed. Firstly, the environmental conditions in which the two populations were reared may have led to differences in their proteomes. However, this can be largely discounted due to the acclimation period to which both populations were subjected prior to sampling. Secondly, the individual proteomes of the two populations may reflected inherent genetic differences due to the different brood stock used to generate the WM and HC lines. Even though different sets of parents were used as founders of the two lines, large numbers of wild oysters from multiple estuaries were used as the initial broodstock in both cases, decreasing the possibility that the HC and WM lines began with substantially different genetic provenance. Having discounted these possibilities, the most likely explanation for the observed proteomic differences between WM and HC oysters are adaptive changes resulting from the intense selection pressure for disease ressitance that has been imposed on the WM population.

2.9 Conclusion

We have undertaken the first proteome study of Sydney Rock oysters selected for resistance to Winter Mortality (WM), and have been successful in identifying numerous proteins that differ in intensity between WM and control HC oysters. However, there remains much to be discovered about WM resistance. The application of 2DE and mass spectrometry in combination was beneficial in this study, but higher samples sizes would have been advantageous to decrease the impacts of inter-individual variability. We also used the genome of the Pacific oyster, *C. gigas*, to identify differential proteins from mass spectrometric data. Although *Saccostrea glomerata and C. gigas* are very closely related, we cannot rule out the possibility of misidentifying some proteins due to the absence of specific peptide to spectrum matches. Despite these limitations, this study has identified a number of biomarkers for WM disease resistance, such as histone H4 and tumor protein D54, both of which displayed significant upregulation in WM selected oysters. As such, the study provides a strong foundation for future work, and supports additional experiments at both the genome and proteome level in order to fully understand the molecular basis of disease resistance.

2.10 Acknowledgements

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Chapter 3

Temporal comparison of Sydney Rock oysters selectively bred for QX disease resistance reveals biomarkers of the disease progression response

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Chapter 3. Temporal comparison of Sydney Rock oysters selectively bred for QX disease resistance reveals biomarkers of the disease progression response

3.1 Biological Context

This chapter deals with a temporal comparison of selectively bred Sydney Rock oysters with unselected hatchery control oysters, with the aim of characterising the disease progression response. This section is presented as a manuscript, which was submitted to the journal *Aquaculture* in August 2017. This study reports the proteomic changes in the selected and unselected Sydney Rock oyster populations collected at two time-points during which QX disease affected the oysters. The first time-point oyster samples were collected during the middle of disease progression, whereas the second time-point were collected towards the end of the disease infection period. The two time-point collections were separated by four weeks. This work reports how the protein expression changes as the disease progresses, and also highlights the differences in protein expression between the selectively bred and hatchery control oysters. Important findings concern proteins such as HMGB1, which has been reported to be associated with the innate immune systems of oysters, Major Vault Protein (MVP), Rab11b, and proteasome subunits. In addition to identifying a unique disease response, we have also identified another instance of apparent cytoskeletal remodeling of bivalves in response to stress. The time-point comparison gave us an insight into the stepwise breakdown of cytoskeletal proteins as a result of the stress imposed. The primary objective of this work was to identify the underlying biological phenomenon of disease resistance, and the findings of this work are an important step towards achieving that goal.

3.2 Contributions

I performed 90% of the experimental work in this study, which included sample collection from the Hawkesbury River, sample processing steps including isolation of gills, extraction and quantification of protein, shotgun proteomic sample preparation, SDS-PAGE protein separation and in gel digestion, nanoflow LC-MS/MS analysis, database searching for peptide to spectrum matching, and statistical analysis of results. Samples were collected from the Hawkesbury River with the permission of oyster farmer Mr Robert Moxham, to whom I would like to express my gratitude.

Prof. Paul A. Haynes supervised this work as well as assisted in experimental design, statistical analysis and manuscript preparation. Inputs from Prof. David A. Raftos and Dr Emma Thompson were also instrumental in the manuscript preparation.

3.3 Manuscript information

Temporal comparison of Sydney Rock oysters selectively bred for QX disease resistance reveals biomarkers of the disease progression response

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3.4 Abstract

The Sydney Rock oyster (SRO) industry of Australia has been challenged, time and again, by threats both anthropogenic and natural. One of the important factors that has caused great loss to the industry is a protozoan born disease of SRO, known as QX disease. To ensure sustainable production of SRO, a selective breeding programme has been employed to develop a line of QX disease resistant oysters. In this study, we have performed a time-dependent, label-free shotgun proteomics analysis of control and selectively bred SRO to identify biomarkers of disease resistance. We extracted proteins from the gills of two different oyster populations (QX resistant and non-selected wild type, n=20 per population)

sampled at two different time-points, to gain insights into disease progression. The current study identifies a pattern of protein changes over two time-points, coinciding with some previous stress studies, as well as unveiling new information, both general and specific. We have identified 21 differentially expressed proteins between the two lines of oysters at time-point one, and 22 differentially expressed proteins at time-point two. These include proteins involved in cytoskeleton remodelling, immune response, energy metabolism, and numerous other functions. In particular, we report the presence of HMGB1, which has been reported to play an important role in the oyster immune response. In addition, we have identified major vault protein, Histone H2A, Dscam, Ras related Rab11B, proteasome subunits, and other proteins that have previously been reported to be relevant in the context of molluscan stress responses. These proteins could be used as disease biomarkers, and also help to understand the underlying biological phenomenon of selection for disease resistance.

Key words

Sydney Rock oyster, *Saccostrea glomerata*, Queensland unknown (QX), proteomics, protein biomarkers, aquaculture

Highlights

- We have performed a comparative proteomic analysis of two populations of Sydney Rock oysters collected at two time-points to study disease progression response.

- We have employed shotgun proteomics to identify the differential proteomic expression and have successfully identified proteins associated with various biological processes.

- We have identified proteins common to both time-points (e.g. MVP), which could be potential disease biomarkers, as well as unique to one time-point (e.g. HMGB1), which suggest a time dependent response.

Abbreviations

CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; CDPK, calcium dependent protein kinase; DPI, department of primary industries; DTT, dithiothreitol; xg, gravity; FC, fold change; HC, hatchery control; LC-MS/MS, liquid chromatography – tandem mass spectrometry; NSAF, Normalised Spectral Abundance Factors; POMS, Pacific Oyster Mortality Syndrome; PSDVB, Poly(styrene-co-divinylbenzene); QX,

Queensland Unknown disease; QXr, QX resistant line; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate – polyacrylamide gel electrophoresis; SOD, superoxide dismutase; SRO, Sydney Rock oyster; WM, Winter Mortality disease

3.5 Introduction

Australia is a country blessed with ample resources for both terrestrial farming, such as rice, wheat and barley, as well as aquaculture of fish, shrimps and molluscs. The climatic conditions in coastal areas favour the production of land-based food crops, and the surrounding marine environment provides ideal conditions for aquaculture farming. Aquaculture is an important part of the Australian lifestyle; from small scale local farming to large scale industrial farming, it affects most of the population directly or indirectly. In this context, Sydney Rock oysters (SRO), which have been present in New South Wales (NSW) waterways since before European settlement, are a good exemplar of the aquaculture industry of Australia.

The farming of oysters in Australia is part of a broader aquaculture industry, which also includes crustaceans, fishes (freshwater and marine), and other molluscs. According to the 2014/2015 aquaculture production report (Livingstone, 2016), the total annual value of all aquaculture products in the state of New South Wales (NSW) was worth more than $60 million. The contribution of molluscs alone is more than $40 million, of which Sydney Rock oysters are responsible for $34 million (Livingstone, 2016). In the 1970s the oyster production was at its peak but there has been a steady decline since then (Adlard and Ernst, 1995; Nell, 2001b). This decline is mainly attributed to outbreaks of diseases such as Queensland Unknown (QX) and Winter mortality (WM) in conjunction with other factors such as changes in water quality and the introduction of faster growing Pacific oysters (Nell, 2001a).

QX is an epizootic disease specific to SRO which was reported for the first time in SRO obtained from Moreton Bay, Queensland (Wolf, 1972). QX is also known as summer mortality, as the period of infection is during the summer months in Australia (December-April). During the worst outbreaks it has caused the death of more than 97% of SRO in certain populations. In a single outbreak in the Georges River estuary in 1994 (Adlard and Ernst, 1995), QX disease decimated the local oyster farming industry. Similarly, in the

autumn of 2004, oysters in the Hawkesbury River estuary experienced mass mortality, which was reported to be caused by QX disease.

QX disease is believed to be caused by a protozoan, *Marteilia sydneyi* (Perkins and Wolf, 1976), which belongs to the phylum Paramyxea (Anderson et al., 1995). This protozoan is taken up by the oyster during the filter feeding process, and once ingested it begins sporulation. During this process it consumes available nutrients and the oyster essentially dies of starvation. The parasite typically undergoes a complete life-cycle during the Australian summer season, with oysters showing signs of infection at the start of summer, and widespread mortality by the end of summer, approximately 3 months later (Adlard and Nolan, 2006).

M. sydneyi does not affect Pacific oysters, which is why it poses an immediate existential threat only to SRO (Kleeman et al., 2002; Roubal et al., 1989b). It is believed that a secondary host or reservoir is also involved, as no cross infection between oyster species has been reported and attempts to transfer infection in the laboratory were also unsuccessful (Roubal et al., 1989b). More recently, it has been suggested that the secondary host may be the polychaete *Aglaophamus australiensis* (Adlard and Nolan, 2015a). Pacific oysters, on the other hand, are affected exclusively by different diseases, such as Pacific Oyster Mortality Syndrome (POMS) which is caused by a microvariant of Ostreid herpesvirus 1 (OsHV-1) (Green et al., 2015). The most recent outbreak of POMS in Tasmania in January 2016 caused major losses to the industry (Whittington et al., 2016).

The selective breeding programme for SRO was established in 1990, and aimed at breeding a line of oysters resistant for WM, as well as selecting for faster growth (Nell et al., 2000). This program was inspired by earlier efforts in North America and Europe. During 1958-59 in Delaware Bay (USA), American oysters were severely affected by a Haplosporidian parasite, *Minchinia nelsoni* (MSX), which killed more than 90% of the population during serious outbreaks (Haskin and Ford, 1979). A selective breeding program was initiated, and local breeders observed a reduction in mortality after several generations of selection (Ford and Haskin, 1987). In 1979 a similar selection programme was introduced to prevent European flat oysters against the parasites *Marteilia refringens* and *Bonamia ostrea* following a mass mortality outbreak event in France (Hervio et al., 1995; Naciri-Graven et al., 1998).

The NSW selective breeding program for fast growth and resistance to winter mortality enjoyed initial success. This program relied on mass selection, whereby new generations of oysters were produced by interbreeding the fastest growing survivors of disease epizootics. However, an outbreak of QX disease at the Georges River in 1994 where selectively bred oysters were reared not only destroyed the harvest but also wasted years of effort that had been invested thus far in selective breeding (Adlard and Ernst, 1995). As a result, in 1997 the NSW Department of Primary Industries (DPI) reorganised the breeding programme to include selection of QX resistant (QXr) lines in addition to lines aimed at faster growth and WM resistance (Nell et al., 2000). The selective breeding program has produced substantially beneficial results. In a published comparison of 4th generation QXr oysters with unselected oysters, it was found that the cumulative mortality of QXr oysters was 21.7% as against 80.1% for unselected oysters. In addition, the whole weight and shell height was greater for the QXr oysters. This demonstrates that the selection has helped to select oysters that survive better when compared to unselected oysters (Dove et al., 2013). However, similar results were not obtained for the 5th generation oysters when assessed for growth and mortality. Although the selectively bred oysters reached the market weight standard earlier than the unselected oysters, there was no notable difference in mortality of the two populations (Dove and O'Connor, 2009). A similar observation was made in the American Eastern oysters when it was observed that after the sixth generation of selection, the oysters' decrease in mortality rate ceased to improve, and in addition the breeders observed reduced fecundity. The reason for this in Eastern oysters was thought to be inbreeding depression which was a result of the imposed selection pressure of fewer traits on a small founding population (Ford and Haskin, 1987). This was later explained to be the result of continuous loss of heterozygosity, which resulted in inbreeding depression (Falconer and Mackay, 1996; Vrijenhoek et al., 1990).

Previous studies on SRO suggest that there is substantial heritability of beneficial traits from one generation to the next. Hence, sufficient understanding of the genes associated with disease resistance should make it possible to progress further the breeding program using marker assisted selection rather than mass selection of the survivors of disease outbreaks. Previous studies found that selective breeding had affected the phenoloxidase (PO)-based defence mechanism of oysters, such that the level of PO activity was greater in the 3rd generation of QX resistant oysters when compared to non-selected controls (Newton et al., 2004). It was later discovered that the loss of one particular isoform of PO is

correlated with disease resistance (Bezemer et al., 2006). This was one of the first breakthroughs in understanding the disease resistance, and that beneficial traits are inherited from one generation to the next.

Earlier attempts to understand the selection phenomenon also provided preliminary information about differential protein expression in disease resistant oysters. Studies by Simonian et al provided a starting point for proteome based discovery (Simonian et al., 2009a; Simonian et al., 2009b). However, these studies were limited by the absence of a complete genome sequence for SRO, or the sequenced genome of a closely related species, which is a basic requirement for mass spectrometry based protein identification. A study on the effect of metal contamination on the proteome of SRO also suffered from a similar disadvantage in terms of genome sequence unavailability, and to bypass this problem a manually curated bivalve database to identify proteins was employed (Muralidharan et al., 2012).

The oyster research community has seen considerable growth following the release of the complete *C. gigas* genome sequence in 2012 (Zhang et al., 2012). A comparative proteomic study of WT and selectively bred SRO exposed to CO_2 stress was performed and the authors identified stress responsive proteins, and proteins involved in cytoskeletal breakdown, cell signalling and energy metabolism (Thompson et al., 2015). In another comparative proteomics study on WM selected oysters versus non-selected oysters, similar observations were made, along with unique findings such as identification of proteins that had not been identified before in the context of disease resistance (Vaibhav et al., 2016).

These studies build our knowledge of the molecular basis for selective breeding in SROs, and the data generated have begun to address the issue of inbreeding depression and limited understanding of parental bloodstock. This is important in order to find a longer-term sustainable strategy for breeding oysters that incorporates marker assisted selection. Recent advancements in biotechnology have helped greatly in development of disease resistant lines of plants and animals. Marker assisted selection has also allowed selection for faster growth of commercial plants and animals to reach market in a shorter time frame and has allowed farmers to select for many commercially beneficial traits.

In this study, we focussed on the effect of QX disease on the SRO population using a time-course experiment to study disease progression. We performed a label-free quantitative shotgun proteomics analysis of control and selectively bred SRO sampled at two different time-points during a typical QX disease cycle. The first sampling time-point corresponds to the early stages of well-established infection, while the second sampling time-point represents the late stage of infection that is accompanied by onset of widespread oyster mortality. Proteins were extracted from gill tissues and resulting peptides were analysed using nanoflow liquid chromatography – tandem mass spectrometry. The proteins identified provide valuable information in terms of understanding the cellular response to stress, assessing how that differs between selectively bred and non-selected oysters, and determining the potential of proteins differentially expressed between the two populations as biomarkers of disease progression.

3.6 Materials and Methods

3.6.1 Oysters sample collection

Sydney Rock oysters were collected at two different times from a breeding farm in the Hawkesbury River (Mooney Mooney, NSW). The first sampling time-point was February 25th, 2015 and the second sampling time-point was April 2nd, 2015. We collected twenty oysters per time-point from two distinct SRO populations; one selected for a combination of fast growth and QX disease resistance (B2 breeding line, designated here as QXr) and the other a non-selected hatchery control (HC) line. Both populations were from the 5th (F5) generation of breeding and were originally derived from the same sets of parents in the F0 generation. Both of these oyster populations were farmed under the same environmental conditions and in the same estuary (33°31'44.9"S, 151°12'11.7"E). The gills from oysters were harvested immediately after collection and were flash frozen in liquid nitrogen then stored at -80^{o}C for future proteomics study. For future reference, we will be referring the two populations as QXr and HC.

3.6.2 Gills protein extraction

The protein extraction protocol was as described in (Thompson et al., 2015). Thawed gill samples were homogenised with 1.5ml of TRI reagent® (Sigma-Aldrich) using a microfuge mortar and pestle. RNA and DNA extraction was preceded by a 2 minute centrifugation at 12,000 xg. 150µl of BCP (bromochloropropane) was added to the supernatant, followed

by centrifugation at 12,000 xg for 15 minutes 4°C, to separate RNA from protein. DNA was separated from protein by adding 450µl 100% ethanol and centrifuging at 2000 xg for 3 minutes at 4°C. Lastly, the protein supernatant from the previous step was precipitated by addition of ice cold acetone for 10 minutes followed by centrifugation at 12,000 xg, for 10 minutes, at 4°C.

3.6.3 Protein quantitation

The pellets obtained from the extraction were suspended in 8M urea in 100 mM Tris buffer (pH 8.5). The amount of protein in the samples were estimated by Bradford assay (Bradford, 1976) using a range of concentrations of BSA to generate a standard calibration curve. Following the quantitation, five protein solutions from different oysters gills were pooled together to constitute one sample. Hence, for one treatment population of 20 oysters we obtained 4 biological replicates per time-point. This was done to ensure sufficient quantity of protein was available for shotgun proteomics experiments, and also to reduce the inherent biological variability observed between individual oysters (Diz et al., 2009).

3.6.4 SDS-PAGE protein fractionation

After sample preparation and quantitation, 100 ug of extracted protein was taken in a separate tube for SDS-PAGE fractionation. To the solution, 4x Laemmli sample buffer (Bio-Rad, CA, USA) was added, and the mixture was boiled at 95°C for 5 minutes. SDS-PAGE fractionation was performed to separate proteins based on their molecular weight using a 10% Mini-PROTEAN® TGX™ precast protein gel (Bio-Rad, CA, USA) and Mini PROTEAN system (Bio-Rad, CA, USA).

3.6.5 In-gel Digestion

Each lane in the SDS-PAGE gel was cut into 16 approximately equal size pieces using a clean scalpel and each of the sixteen pieces was finely chopped into small pieces. These were transferred to V-bottom 96 well plates for further processing. Gel pieces were washed in freshly prepared 100mM ammonium bicarbonate (NH_4HCO_3) for 10 minutes at room temperature. The pieces were washed again, two times for 10 minutes, in 1:1 solution of 100% acetonitrile and 100mM ammonium bicarbonate. Following these washing steps, the pieces were dehydrated by addition of 100% acetonitrile and left at room temperature to air dry. Reduction was performed with 10mM DTT in 100mM ammonium bicarbonate for one hour at 37°C. Alkylation was then performed with 50mM IAA in 100mM ammonium

bicarbonate for one hour at room temperature. The washing and dehydration steps were repeated again following reduction and alkylation. Freshly prepared trypsin solution (12.5ng/µl in 50mM 100 mM ammonium bicarbonate) was added to the gel pieces at 4^{o}C and incubated at 4^{o}C on a rocker for one hour, and then transferred to 37^{o}C for overnight digestion. The next day, peptide extraction was performed twice with 50% acetonitrile and 2% formic acid. The extracted peptides were dried in a vacuum centrifuge and reconstituted in 10ul of 2% formic acid.

3.6.6 Nano LC-MS/MS

Analysis of peptide fragments was performed by nanoflow LC-tandem mass spectrometry on an LTQ Velos Pro linear ion trap mass spectrometer (Thermo). Samples were applied to a fused silica column (100µm ID x 8cm) with an integrated electrospray tip which was coupled to a precolumn. The analytical column was packed in-house with Magic C18AQ, 200 Å, 5µm particle size (Michrom bioresources), using a high-pressure column packing cell. The packing material in the precolumn was PLRP-S resin (300 Å, 5µm, Agilent technologies, 100µm ID x 5cm). Chromatographic separation and sample injection to the mass spectrometer was performed by an Easy nLC II system (Thermo). The liquid chromatographic separation consisted of (a) washing of peptides in buffer A (2%v/v acetonitrile, 0.1% v/v formic acid) for 2 minutes at a flow rate of 550nl/min (b) elution of peptides with 0-20% buffer B (99.9% v/v acetonitrile, 0.1% v/v formic acid) at a flow rate 500nl/min for 38 minutes and (c) column washing with 95% buffer B for 10 minutes at flow rate 800nl/min. Spectra acquisition was performed in positive ion mode over the scan range of 400m/z to 1500m/z using Xcalibur software (Thermo, v2.06). A normalized collision energy of 35% was used to perform MS/MS of the top 9 most intense precursor ions, with dynamic exclusion enabled for 90 seconds.

3.6.7 Protein identification

Peptide to spectrum matches, and concomitant protein identifications, were performed using the Global Proteome Machine software v2.1.1 and the X!Tandem algorithm (Craig and Beavis, 2003a). A database of 37,289 oyster peptide sequences from *Crassostrea gigas* was downloaded from NCBI and used in all searches. The criteria for the database search included carbamidomethylation as complete modification, oxidation as potential modification, with fully tryptic cleavage sites.

3.6.8 Data processing for protein quantitation

The criteria required for an initial protein identification in the XTandem searches was a protein – log (e) value of <2.5. The low stringency search data from individual replicates was transformed into high stringency data by combining the three biological replicates into a single list of reproducibly identified proteins, using the Scrappy program (Neilson et al., 2013; Pascovici et al., 2016a; Wu et al., 2016). The criteria for a reproducibly identified protein was that a protein must be present in all three replicates of at least one sampling point, with a minimum peptide spectral count criterion of six. Applying these criteria produced a final data set, with a peptide level false discovery rate calculated at <4%, containing 212 proteins. The Scrappy program was also used to perform label free protein quantitation, with the spectral counts from the lists of reproducibly identified proteins transformed into Normalised Spectral Abundance Factors (NSAFs) (Zybailov et al., 2006). Pairwise comparisons of QX versus HC oysters at each time-point was then performed using student's t-tests of the NSAF values. Proteins with a p-value less than 0.05 were considered to be differentially expressed, while those with a p-value of greater than 0.05 were considered to be unchanged.

3.6.9 Protein Ontology

We performed protein classification based on biological functions, using UniProt identifiers as inputs into the PloGO software to provide gene ontology information (Pascovici et al., 2012). Based on the information provided, the proteins were categorised into 26 different GO terms. We also performed manual annotation to categorise proteins broadly into 5 different functional categories - cytoskeleton, protein processes, energy metabolism, stress response, and cellular signalling.

3.7 Results and discussion

3.7.1 Numerical summary of protein identification results

To our knowledge this is the first shotgun proteomics study conducted on QX resistance selected SRO gill tissue proteins. Analysis was performed on two oyster populations sampled at two different time-points separated by five weeks. As expected, the proteome of the two populations differed in response to disease resistance selection, and also changed with sampling time. It is important to note that we have used the *C. gigas* genome sequence for the identification of proteins because of the unavailability of a complete *Saccostrea*

glomerata genome sequence at the time this study was completed. This means that there may be some missing proteins due to lack of spectral assignments, but previous studies indicate that this approach works well for protein identification (Thompson et al., 2015; Vaibhav et al., 2016)

Following protein extraction, there was a higher average protein concentration in the HC compared to QXr oyster population. This observation is consistent with previous studies showing that when a cell undergoing a stress it produces more protein. This has been reported in previous work on cells undergoing stress in a variety of biological systems – rice plants (Gammulla et al., 2011; George et al., 2015; Wu et al., 2016), human cells (Ray et al., 2012; Srivastava et al., 2012), and Sydney Rock oysters (Muralidharan et al., 2012; Thompson et al., 2015; Vaibhav et al., 2016).

In this study, we defined reproducibly identified proteins as those which were present in all three replicates of at least one sampling point, with a minimum peptide spectral count of six. Using these criteria, at time-point one there were a total of 117 reproducibly identified proteins, of which 96 were not statistically different between the QX and HC oysters. As presented in **Table 3.1**, there were 10 proteins which were significantly more abundant in QX oysters than in HC oysters, as evidenced by a p-value of less than 0.05 when comparing normalised spectral abundance factors, and 11 proteins which were more abundant in the HC oysters than in the QX oysters. For time-point two there were 95 reproducibly identified proteins, of which 73 did not differ between populations, 19 were more abundant in QX oysters, and three were more abundant in HC oysters (**Table 3.1**). The protein ontology as shown in **Figure 3.1** reveals the involvement of proteins in various biological function categories. It also demonstrates how the two time-points differ in protein expression. For example, as indicated by the colour code, it can be seen that more structural proteins are present in time-point two while the presence of more regulatory proteins in time-point one is suggested.

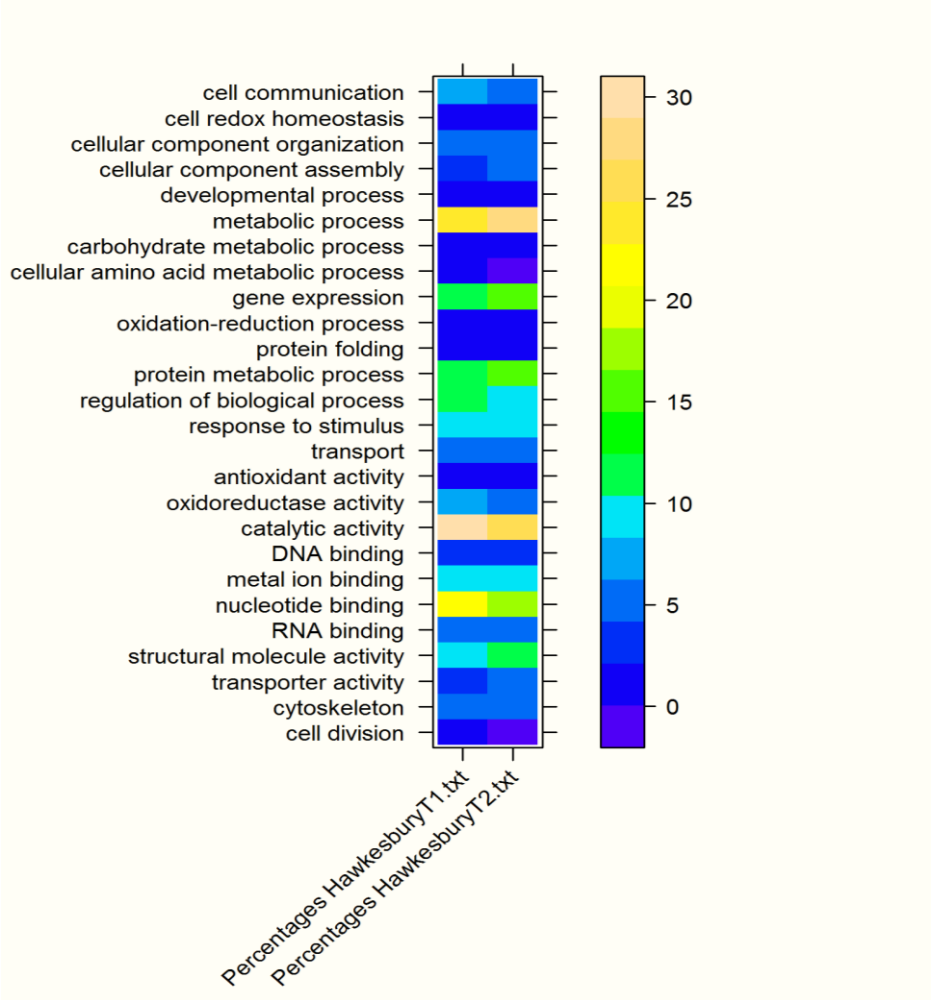

Figure 3.1: Protein distribution based on gene ontology terms (PloGo) showing the biological functions and relative intensities of the reproducibly identified proteins in the two populations at time-points one and two. The scale bar shows fold difference between QXr and HC.

Table 3.1: Time-point one - reproducibly identified proteins in Sydney Rock oyster gill proteins.

a. Lower abundance in QXr oysters relative to HC

Identifier	Protein	Fold change	Biological process
gi|405975243	unc-87	21.0	cytoskeletal
gi|405976016	Villin-1	17.0	cytoskeletal
gi|405978809	Serine/threonine-protein phosphatase 2A	13.0	cytoskeletal
gi|405977154	Exonuclease 3'-5' domain-containing protein 2	11.0	nucleic acid metabolism
gi|405974416	hypothetical protein CGI 10018541	10.0	predicted G protein
gi|405976274	hypothetical protein CGI 10021278	9.0	predicted calmodulin
gi|414081625	Dscam	7.0	immune response
gi|405978716	Alpha-actinin, sarcomeric	5.0	cytoskeletal
gi|405974681	Major vault protein	2.8	signalling
gi|405974703	ATP synthase subunit alpha, mitochondrial	2.4	energy metabolism
gi|405974703	ATP synthase subunit beta, mitochondrial	1.8	energy metabolism

b. Higher abundance in QXr oysters relative to HC

Identifier	Protein	Fold change	Biological process
gi|405975395	Chromodomain-helicase-DNA-binding protein	13.0	transcription related
gi|405977366	E3 ubiquitin-protein ligase MIB2	8.0	ubiquitination
gi|405977088	Band 4.1-like protein 5	8.0	cytoskeletal reorganisation
gi|405976727	Spermatogenesis-associated protein 13	8.0	cell migration
gi|405976185	Deleted in malignant brain tumors 1 protein	5.0	cellular transfer
gi|465828333	HMGB1 (High mobility group protein B3)	1.5	immune related
gi|405974545	Collagen alpha-1(IX) chain	1.4	stuctural/ cytoskeletal
gi|405978693	Uncharacterized protein yfeX	1.4	peroxidase activity
gi|405977799	S-phase kinase-associated protein 1	1.4	ubiquitination
gi|405974492	40S ribosomal protein S14	1.3	protein processes

Table 3.2: Time-point two - reproducibly identifiable proteins in Sydney Rock oysters gills proteins.

a. Lower abundance in QXr oysters relative to HC

Identifier	Protein	Fold change	Biological process
gi|405976084	Tubulin polymerization-promoting protein family member 3	13.0	cytoskeletal
gi|405977974	Proteasome assembly chaperone 1	12.0	proteolysis
gi|405978120	Putative helicase with zinc finger domain	9.0	protein processes
gi|405976031	hypothetical protein CGI 10025611	8.0	no identity
gi|405976463	Transketolase-like protein 2	8.0	energy processes
gi|405978555	Huntingtin	8.0	anti-apoptotic
gi|523430340	retinoic acid-inducible protein 1	8.0	stress
gi|405975796	hypothetical protein CGI 10018667	7.0	predicted Myosin
gi|405975869	Proteasome subunit alpha type-6	7.0	proteolysis
gi|405978716	Alpha-actinin,	6.6	cytoskeletal
gi|405976318	6-phosphogluconate dehydrogenase, decarboxylating	6.0	energy processes
gi|405977192	Putative RNA-binding protein 16	4.5	protein processes
gi|405975834	Isocitrate dehydrogenase NADP , mitochondrial	3.4	energy processes
gi|405974681	Major vault protein	2.0	signalling
gi|405976915	Proteasome subunit alpha type-7-like protein	1.8	proteolysis
gi|3182893	Actin	1.7	cytoskeletal
gi|405974541	Actin, cytoplasmic	1.7	cytoskeletal
gi|405974534	Actin	1.7	cytoskeletal
gi|405977263	Heterogeneous nuclear ribonucleoprotein A2-like protein 1	1.4	protein processes

b. Higher abundance in QXr oysters relative to HC

Identifier	Upregulated	Fold change	Biological process
gi|405976910	Basement membrane-specific heparan sulfate proteoglycan core protein	7.0	signalling
gi|405977015	Ras-related protein Rab-11B	2.3	signalling
gi|405975240	Histone H2A	1.5	stress

3.7.2 Proteins differentially expressed between QXr and HC oysters at time-point one

To understand the biology behind the disease resistance selection process, we sampled oysters at two different time-points during the disease progression season. Time-point one corresponds to the presence of a well-established infection in the oysters, while time-point two corresponds to the later stages of infection, when the oysters are severely distressed and showing more obvious anatomical symptoms of infection. The following section deals with the outcomes of the time-point one data set.

3.7.3 Proteins which were less abundant in QXr oysters at Time-point one

3.7.3a Cytoskeletal proteins

We observed several cytoskeletal proteins such as alpha actinin, villin and unc-87, which suggests that the cell may be starting the process of breaking down actin into its components. These three proteins all play a role in actin bundling. Sarcomeric alpha actinin helps actin anchor to different intracellular structures (Roulier et al., 1992) while villin is an actin binding protein that is regulated by calcium (Rossenu et al., 2003). Unc-87 is reported to be an actin bundling protein, which binds to F actin, and thereby provides structural stability to the actin filaments. It is thought to act as a cross-linking molecule that binds actin filaments together (Kranewitter et al., 2001). It has been observed that absence of unc-87 in *Caenorhabditis elegans* resulted in malformed cytoskeleton structures (Goetinck and Waterston, 1994a). These proteins are thought to be required for maintenance of the structural integrity of myofilaments in cells (Goetinck and Waterston, 1994b). To our knowledge, this is the first report of differential expression of unc-87 in molluscan stress research. Identification of higher abundance of actin bundling proteins in time-point one HC oysters suggests that the cytoskeletal remodelling has begun, and it is starting with the untangling of actin filaments as an initial step towards actin and tubulin reorganisation. This hypothesis is supported by the observation in time-point two where we actually see differential expression of actin and tubulin subunits.

3.7.3b Immune response proteins

We are also reporting significantly increased abundance of Down syndrome cell adhesion molecule (Dscam) in HC SRO. Dscam is an immunoglobulin superfamily (IgSF) member (Schmucker and Chen, 2009) and it represents the largest IgSF cell adhesion molecule

family. The gene *Dscam* was discovered in human chromosome band 21q22 which is known to play a key role in Down's syndrome (Yamakawa et al., 1998). However, it has also been reported that the Dscam gene is involved in many biological process of invertebrates. It can be seen from **Table 3.1** that this protein is only present in HC and there are no putative identifiers of this protein in the QX disease resistance selected oysters. It is also interesting that we did not identify Dscam in the time-point two HC oysters.

Oysters are filter feeders and are in direct interaction with their surroundings. Hence, they are at a risk of ingesting microorganisms which then poses risks of infection. It is worth noting that oysters, unlike vertebrates, do not have an adaptive immune system (Guo et al., 2015), which makes them completely dependent on the innate immune responses (Zhang et al., 2014). This may explain the presence of proteins such as Dscam, which is known to be a very important protein in the innate immunity system of invertebrates. It is reported to be involved in cell to cell communication, self- and non-self-recognition, and providing innate immunity defences (Wang et al., 2013). This has been reported in a range of invertebrates such as mosquitoes (Dong et al., 2012), crabs (Hauton et al., 2015), shrimps (Wang and Wang, 2013) and other arthropods (Armitage et al., 2012; Kurtz and Armitage, 2006). Transcripts of Dscam have been reported in flat oyster and Pacific oyster (Gorbushin and Iakovleva, 2013) and Sydney Rock oysters, where a transcriptomic study identified ORFs for Dscam suggesting it was important in immune response (Ertl et al., 2016b). The higher abundance of Dscam in HC oysters may be part of the initial phase of innate immune response, which occurs when the level of pathogens become sufficient that cells begin to respond. Dscam belongs to the family of pattern recognition receptors, which are generally induced in response to cell injury or stress, and trigger intracellular pathways leading to inflammatory immune responses (Ertl et al., 2016b).

3.7.3c Major vault protein

We also report, for the first time in context of oyster studies, a significant fold change in the level of major vault protein (MVP). MVP is a principal component of ribonucleoprotein particles that are associated with different tumour types (Suprenant et al., 2007). The exact function of MVP is not clear but it has been reported to be involved in different processes including cellular transport, signalling, and immune response (Berger et al., 2009). Changes in expression of MVP in mammalian cells have been reported in a range of different

conditions including increased abundance in hyperthermia (Petricoin et al., 2002b), ageing (Ryu et al., 2008), and tumour development (Berger et al., 2001; Meijer et al., 1999).

In molluscs, MVP has been previously reported to be involved in stress related pathways, which supports the presence of this protein in this study (Suprenant et al., 2007). In a study performed on *M. edulis*, the expression of MVP was found to be elevated in the digestive gland tissue during anaerobiosis, although no change in expression was observed in oysters exposed to osmotic and temperature stress. It was hypothesised that increased accumulation of MVP-like transporter proteins occur in response to an increase in overall metabolic rate, which leads to accumulation of the end products of metabolism (Luedeking and Koehler, 2004). In another proteomic study on mussel congeners, reduced expression of MVP was associated with heat tolerance, suggesting that higher levels of the protein were associated with lower tolerance to cellular stress (Tomanek and Zuzow, 2010).

We report here that MVP is more abundant in the HC oyster populations from time-points, by a factor of greater than two relative to QXr. The presence of this protein at both the time-points indicates that this is an important marker for distinguishing between the two oyster populations. Higher levels of MVP in HC oysters could be attributed to the fact that these animals suffer more acutely from disease stress than QXr oysters. QXr oysters are more resistant to disease hence the MVP may not be required to be present at such high levels. This is an interesting observation which warrants further investigation in follow up studies.

3.7.4 Proteins more abundant in QXr oysters at time-point one

3.7.4a High Mobility Group Box 1 protein

Amongst the proteins with a higher abundance in QXr oysters were High Mobility Group Box 1 protein (HMGB1). HMGB1 is known to play a role in many important biological processes including DNA repair (Lange and Vasquez, 2009), inflammation, and injury response (Lotze and Tracey, 2005). It is also reported to be implicated in Alzheimer's disease (Fujita et al., 2016), and has been reported as a signalling molecule in inflammation and tumour associated complications (Li et al., 2003; Sun and Chao, 2005; Tang et al., 2010). More specifically, in oysters this protein has previously been reported to play an important role in inflammatory response by enhancing the mRNA level of cytokines in *C. gigas* (Li et al., 2013b) and *C. ariakensis* (Xu et al., 2012). HMGB1 is known to be more

abundant in response to pathogen infections (Li et al., 2013b), and we have observed an increased abundance of HMGB1 in the QXr population. This is an especially interesting observation, as it suggests the possibility that this protein may have been more intense in the initial response to stress, and then remained at higher levels as part of an adaptive response.

3.7.4b Cell cycle proteins

Amongst other proteins found to be more abundant in QXr oysters, we have identified proteins involved in DNA replication, transcription, and translation, including chromodomain helicase DNA binding protein (Micucci et al., 2015; Nagarajan et al., 2009), and 40S ribosomal protein S14 (Antunez de Mayolo and Woolford, 2003; Fewell and Woolford, 1999). This suggests that the disease resistant oysters are expending more energy on growth than their disease susceptible counterparts. We also report increased abundance of E3 ubiquitin protein ligase and S phase kinase associated protein 1 in QXr oysters. These proteins perform ubiquitination of proteins involved in cell division, transcription, translation and cell signalling (Chiorazzi et al., 2013). Ubiquitination is also associated with carrying out targeted protein degradation and re-localisation (Berndsen and Wolberger, 2014). The increased abundance of ubiquitin ligase protein suggests that more proteins within the cells are being synthesised, modified and degraded, as a result of higher levels of cell cycle growth and maintenance (Ardley and Robinson, 2005; Berndsen and Wolberger, 2014; Choo and Zhang, 2009).

3.7.4c Deleted in malignant brain tumours 1

DMBT1 also had a higher abundance in QXr oysters. It is known from previous studies that this protein has multiple peptide and carbohydrate binding sites and is involved in pattern recognition, thereby it is an important part of innate immune reactions (Bikker et al., 2002; Ligtenberg et al., 2010). Multiple sequence alignment of DMBT1 of *Euprymna tasmanica* with *Homo sapiens* and *C. gigas* performed by (Salazar et al., 2015) indicated the presence of a scavenger receptor domain. Scavenger receptor domains are reported to be associated with bacterial infection and a study on *C. virginica* challenged by bacterial infection identified differential expression of DMBT1 protein (McDowell et al., 2014). Increased abundance of DMBT1 in response to parasitic infection hints at its involvement in innate immune response. However, more research needs to be performed to decipher its role in selective breeding.

3.7.5 Proteins differentially expressed between QXr and HC oysters at Time-point two

There were 22 differentially expressed proteins between the two oyster populations in the Hawkesbury River during time-point two (**Table 3.2**). Of those 22 proteins, 19 were more abundant in the HC population while three3 were more intense in the QXr selected population. Of the 19 proteins that were more intense in HC oysters, five were putatively identified as isoforms of actin (4) and tubulin (1). It has been previously observed in molluscs that as a result of different stresses, the cells undergo cytoskeletal remodelling, causing many changes in the level of actins and tubulins (Maria et al., 2013).

3.7.5a Cytoskeletal proteins

One hypothesis to explain the cytoskeletal breakdown and reorganisation observed in oyster tissues under stress is that it represents a side effect of increased energy production (Kultz, 2005; Tomanek, 2011). ATP synthesis by the electron transport chain generates various ROS byproducts which include free radicals such as hydroxyl radicals and superoxide radicals (Chandel and Schumacker, 2000; Murphy, 2009). Accumulated ROS have various deleterious properties in the cell. For example, they are known to actively participate in cytoskeletal reorganisation by regulation of RHO GTPase (Stanley et al., 2014; Thompson et al., 2015; Tomanek and Zuzow, 2010). In the context of molluscs, cytoskeletal damage caused by different stressors has been reported in various instances. This has been reported in studies in oysters involving ocean acidification caused by elevated carbon dioxide levels (Thompson et al., 2015), Winter Mortality disease (Vaibhav et al., 2016), and in heat stress in mussels (Fagotti et al., 1996; Gagnaire et al., 2009; Tomanek and Zuzow, 2010). It has also been suggested that, as a result of stress, cells in the oysters gills undergo a process of cytoskeletal reorganisation to form actomyosin (Burridge and Wennerberg, 2004).

In addition to finding major changes in actins and tubulins, we have observed higher intensity of proteins involved in oxidative phosphorylation, such as ATP synthase, at time-point one. We have also reported the increased intensity of proteins associated with energy production both at time-point one (ATP synthase) and time-point two (Isocitrate dehydrogenase, 6- phosphogluconate dehydrogenase). It has been discussed previously that increased expression of these proteins results in production of ROS (Sussarellu et al., 2010; Valavanidis et al., 2006). The increase in ROS can be partly attributed to the fact that

NADH produced as a result of energy metabolism is then consumed by the electron transport chain, the end product of which is ATP (Nelson et al., 2008). It has also been reported that the electron transport chain is responsible for generation of ROS as a side product, as it has many redox reaction steps along the reaction which may result in formation of excess superoxide anions (Chandel and Schumacker, 2000; Turrens, 2003).

We also report higher abundance of transketolase like protein-2 in the HC population study for time-point two. A previous study in mussels reports the differential expression of transketolase in response to heat stress (Tomanek and Zuzow, 2010). The rationale for higher abundance of this protein can be established on the basis that it is involved in the synthesis of glutathione (GSH), which acts as a scavenger of ROS. The process by which the mitochondria carries out the elimination of ROS is dependent on two enzymes, including GSH, which is in turn regulated indirectly by transketolase (Nelson et al., 2008).

These observations support the hypothesis of increased energy production leading to the cytoskeletal breakdown, as discussed above. Additionally, these results demonstrate that the QXr oysters are better adapted to stressful conditions compared to the HC oysters. Taken together, our results align well with the results of previous studies of molluscan stress responses. A series of events triggered by stressors such as infection may lead to a cytoskeletal cascade and affect other biological process as represented schematically in **Figure 3.2**.

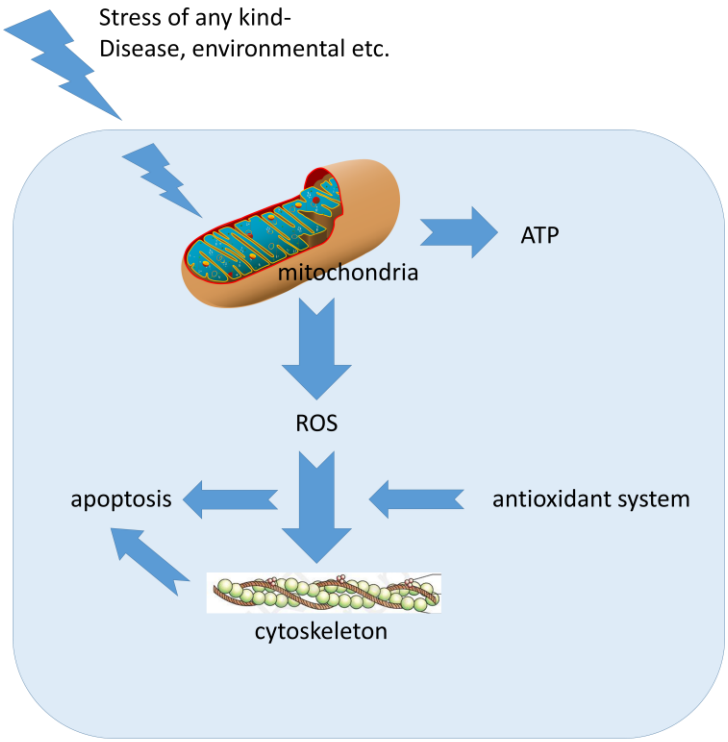

Figure 3.2: A schematic overview of the intracellular response of oyster to stress, based on the proteins identified, depicting the major biological processes affected. Modified from (Anderson et al., 2015a).

3.7.5b Proteolysis-related proteins

We also identified proteins associated with proteolysis, including proteasome subunits. We observe a clear increase in intensity of three proteasome subunits in the HC oyster population of time-point two samples. Proteasome subunit alpha type 6 and 7 are part of a multi-subunit enzyme complex which is reported to be a fundamental requirement for several different processes including degradation associated with ubiquitin (Ciechanover, 1994; Glickman and Ciechanover, 2002) and programmed cell death or apoptosis (Shah et al., 2001). Protein degradation mediated by ubiquitin has been reported previously to play an important role in innate (inflammation) immunity, as well as adaptive (antigen presentation) immunity response (Adams, 2003; Huber et al., 2012; Pickart, 2001) . Several studies in molluscs have reported changes in proteasome assembly; for example, as a result of heat stress in mussels (*Mytilus galloprovinciallis)* (Lockwood et al., 2010; Tomanek and Zuzow, 2010), heavy metal and carbon dioxide combined stress in *C. virginica* and *Mercenaria mercenaria* (Götze et al., 2014), carbon dioxide stress in *Saccostrea glomerata*, and hypoxia in *C. gigas* (David et al., 2005a).

3.7.5c Huntingtin

Another protein in time-point two HC oysters was identified as huntingtin, which has been previously reported in a thermal stress study in *C. gigas*. It is reported to be involved in cell proliferation and differentiation and it was found to be upregulated in gills and mantle as a result of increased temperature (Meistertzheim et al., 2007). It is also suggested that this protein is involved in anti-apoptotic processes. Hence, providing protection from inflammation is one possible functional role it performs in oysters (Raychaudhuri et al., 2008).

Differential expression of proteasomes impacts upon all the changes taking place in the proteome of the organism. We propose several plausible explanations regarding the observation that proteasome subunits are more intense in the HC oyster population. First, increased abundance could be attributed to the emergent energy requirement of the cell when it is placed under stress and is required to produce more energy in the form of ATP. This theory is supported by the concomitant increase in abundance of proteins involved in energy production via glycolysis, such as 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase, and electron transfer pathway – ATP synthase subunits. Second, as proteasomes are known to be involved in protein degradation, and cytoskeletal remodelling is a signature stress response in molluscs, the increased abundance of proteasomal subunits may be associated with the degradation of the cytoskeletal structural elements into other isoforms (Glickman and Ciechanover, 2002).

3.7.6 Perspectives

The work discussed above provides valuable information on the traits and genes contributing to the adaptation of oysters to environmental stressors such as infectious disease. The ultimate objective of the current study is to understand the underlying phenomenon of artificial selection. It is also to be noted, before we conclude this work, that the interaction of environment with hosts and pathogens also plays an important role in disease prevalence. It is already known that factors such as salinity (Bezemer et al., 2006; Butt and Raftos, 2008a) and chemical contaminations affect oyster health in ways that alter the proteome (Gagnaire et al., 2007a). This suggests that there are many levels at which QX disease of SRO is modulated. It is important to study the interplay of all aspects taken together in order to interpret the results conclusively and ultimately develop a sustainable strategy to combat the disease outbreaks.

3.8 Conclusions

In this study, we have performed a shotgun proteomic analysis of gills from SRO collected at two different time-points from the same site in the Hawkesbury River. The results contribute towards the biological knowledge of QX disease selection and progression in SRO. We have identified proteins involved in various biological functions. Some of them were common to both time-points (e.g. MVP, actin), while others were unique to time-point one (e.g. HMGB1, Dscam) or time-point two (e.g. Huntingtin, isocitrate dehydrogenase). Based on the proteins identified, we hypothesise that the oyster proteome exhibits a time dependent response wherein differential expression of proteins is dependent on the disease progression status of oysters collected at two different time-points. Given that SRO are of great importance, both ecologically and economically, this study provides an important step forward in identifying a set of disease biomarkers. The success of this research also provides further information to support marker assisted selection in marine molluscs.

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Chapter 4

Proteomic comparison of shore caught wild oysters and disease-resistance selected Sydney Rock oysters from Woolooware Bay.

Chapter 4. Proteomic comparison of shore caught wild oysters and disease-resistance selected Sydney Rock oysters from Woolooware Bay

4.1 Biological Context

This chapter deals with a proteomic comparison of selectively bred Sydney Rock oysters with shoreline catch wild oysters, in order to identify proteomic differences despite the close proximity of the two populations. The oysters used were from a completely separate geographical location (Woolooware Bay) to those used in Chapter 3 (Hawkesbury). The Sydney Rock oyster breeding programme, started in 1990, has been used to selectively breed a line of oysters to improve the traits they carry for disease resistance. The selectively bred line of oysters represents a genetically conserved system because of continuous inbreeding among the founding populations of survivors of disease. On the other hand, the shoreline oysters are wild oysters which are found on the shores of estuaries. They are unselected and are grown in a natural environment, and hence they represent unforced genetic composition.

The wild oysters used in this study were collected from Woolooware Bay, which has been used as one of the sites for the selective breeding programme. To reduce the effect of spatial differences, the selected oysters were also collected from the same estuary. This study investigated the proteomic composition of these oyster populations in order to identify the impact of selective breeding on the genetic composition. Oysters reproduce by spawning i.e. by releasing eggs and sperm in the water body, therefore, close proximity of these two populations posed a question of whether potential for interbreeding may limit the proteomic differences between them. The results of this work provided evidence to the contrary, in the form of differential proteomic responses of the two populations. It was found that the expressed proteomes of the two oyster populations were significantly different; peptides of 24 out of 31 proteins identified in the wild oysters were absent in the selected oysters, suggestive of negative selection of genes encoding those proteins.

4.2 Contributions

Woolooware oyster samples were collected and provided by Macquarie University Ph.D. student Ms. Jessica Thompson. I performed the sample processing for proteomics experiments, statistical tests, data generation and results interpretations.

Prof. Paul A. Haynes supervised this work as well as assisted in experimental design, statistical analysis and manuscript preparation. Inputs from Prof. David A. Raftos were also instrumental in the manuscript preparation.

4.3 Abstract

Selective breeding of the Sydney Rock oyster has been performed since the early 1990s, mainly to improve the growth rate and resistance against Winter Mortality disease. Following a mass outbreak of QX disease in 1994 the NSW DPI selective breeding programme was reorganised to include a QX disease resistant selective breeding line. The oysters used in the selective breeding programme were grown at three different locations in the Georges River, Sydney - Woolooware Bay, Lime Kiln Bar, and Quibray Bay. In addition to the selectively bred oysters, wild or shoreline oysters were also growing in close proximity. Since oyster fertilisation is external, there are opportunities for cross-fertilisation which led us to investigate the proteomic differences between the two populations. We report that the expressed proteome complement of the two oyster populations seems to be very different from each other even though they belong to the same species and are collected from adjacent geographical locations. The findings of this study confirm recently reported evidence of a lack of genetic overlap between the two oyster populations.

4.4 Introduction

Woolooware bay (-34.036671, 151.141912) is an oyster farming site on the Georges River, south of Sydney. This was one of the locations chosen for the NSW DPI selective breeding programme in 1997 (Nell, 2003). Oysters in this estuary were selectively bred for resistance to both QX and WM diseases, and the line of oysters selectively bred for six generations is known as the B2 line (Nell et al., 2000). Two oyster populations were used in this study, one of which belonged to the B2 line, which were collected from the farming racks in the estuary, while the other population represented shoreline catch of wild oysters collected from the shore of Woolooware bay (-34.02142, 151.08515), at least 1km away from the commercial farming lease as shown in **Figure 4.1**. This study comprises a quantitative proteomics analysis of the gills of these Sydney Rock oysters using shotgun proteomics. The objectives of this study were to examine the impact of selection on the proteome of selected oysters, when compared with wild shoreline oysters, and to examine whether

observed proteome differences were consistent with those seen in similar populations from a distinct geographical location.

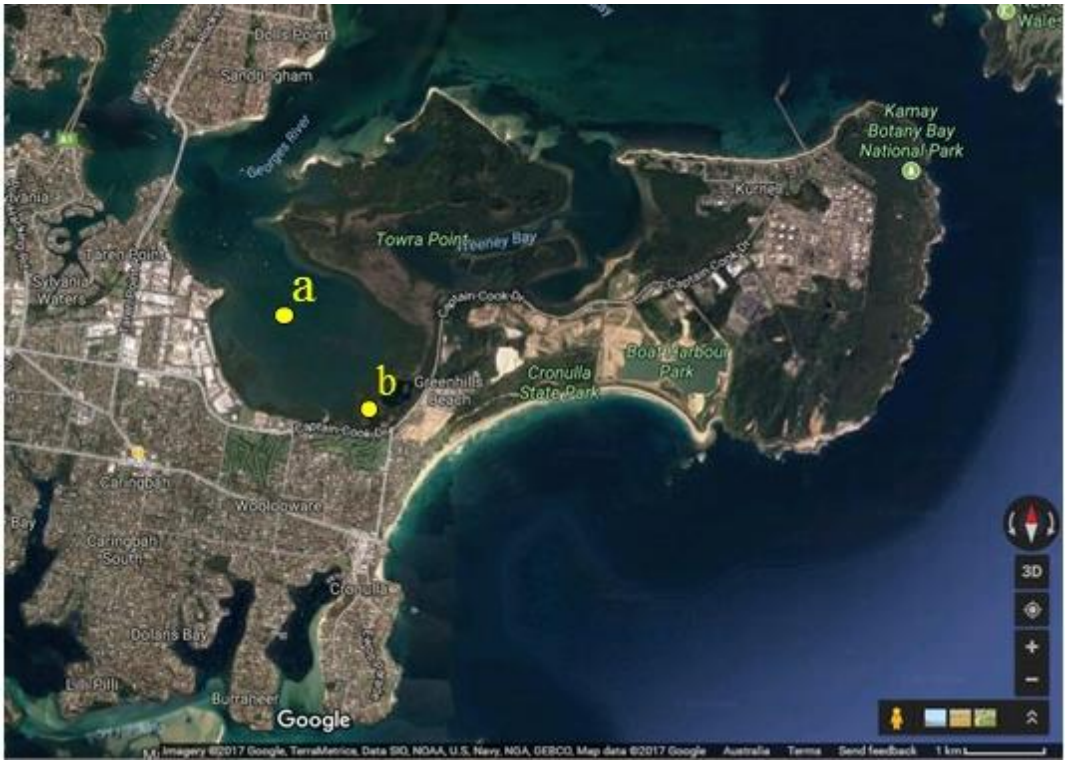

Figure 4.1: Collection sites for the two population of oysters in Woolooware Bay of Georges River. Yellow spot (a) represents the farming site for the selected line of oysters whereas (b) is the site of collection of shore caught or wild oysters. Picture courtesy of Google Maps.

Figure 4.2a: Selected oysters were farmed in the open bay as seen in the distant oyster farming leases. Spot (a) in **Figure 4.1** refers to this picture.

Figure 4.2b: The wild oysters were collected along the shore marked by mangrove trees as seen above. Spot (b) in **Figure 4.1** refers to this picture.

4.5 Materials and Methods

4.5.1 Sample collection

Oyster gills tissue samples were provided by Macquarie University Ph.D. student Jessica Thompson. The gills tissue used in this study were excised from oysters collected from two

sites in Woolooware bay, as shown in Figures 4.1 and 4.2. The two populations of oysters used were a selected line of oysters and wild, or shoreline catch, oysters. Toensure enough protein was present for further experiments, five oysters were pooled together to form one biological replicate, and three biological replicates from each oyster population were analysed.

4.5.2 Oyster gills tissue protein extraction

The protein extraction from the gills tissue was performed as described in (Thompson et al., 2015). The gills tissue samples were immersed in TRI reagent® (Sigma-Aldrich), and manually homogenised using a pellet pestle. This was followed by removal of DNA and RNA using 100% ethanol and bromochloropropane, respectively. The final step of protein purification was acetone mediated protein precipitation. The protein pellet was kept at room temperature to let the excess acetone evaporate, and it was then suspended in 5M urea in 100mM Tris buffer, pH 8.5.

4.5.3 Protein quantification

Protein quantification was performed using Pierce™ BCA protein assay kit as per the manufacturer's instructions using a range of different BSA concentrations as standard (Smith et al., 1985). 2 µl of each sample (diluted 1:1 in MilliQ water) was aliquoted in duplicates into wells of a 96 well microtitre plate. 180 µl BCA reagent (A and B mixed in 50:1 ratio) was added to each well containing unknown sample to be quantified, and incubated at 37^0C for 30 minutes. After incubation the protein absorbance was recorded at 562 nm. Protein concentrations of the unknown samples were estimated using the straight line equation obtained by the standard BSA solutions.

4.5.4 Shotgun Proteomics work flow

4.5.4a SDS-PAGE protein separation

Protein samples were separated on the basis of their molecular weight using SDS-PAGE. 100ug each in biological replicates of the oyster gills protein samples for the two populations was taken in a microfuge tube. To the protein sample, 4X Laemmli sample buffer (Bio-Rad, CA, USA) was added and the mixture was boiled at 95^0C for 5 minutes. Protein separation was performed initially at 70V and then at 150V using a 10% Mini-PROTEAN® TGX™ precast protein gel (Bio-Rad, CA, USA) and Mini PROTEAN system

(Bio-Rad, CA, USA). The gel containing proteins was stained for two hours using a solution of 0.25% coomassie brilliant blue in 10% glacial acetic acid, 40% methanol in distilled water. The gel was then de-stained overnight in solution of 10% glacial acetic acid, 40% methanol in distilled water.

4.5.4b In-gel digestion

The SDS-PAGE separated gill proteins were then prepared for in-gel digestion using trypsin (Shevchenko et al., 1996). Each lane of SDS-PAGE representing one replicate was cut into 16 equal size pieces, and further chopped into smaller pieces to improve the digestion efficiency of the enzyme. These pieces were washed in 100mM NH_4HCO_3, followed by another washing in 1:1 solution of acetonitrile in 100mM NH_4HCO_3. Washing was followed by dehydration of the gel pieces in 100% acetonitrile and then reduction in 10mM DTT in 100mM NH_4HCO_3 for one hour at 37^0C. After the proteins in the gel pieces were reduced, alkylation was performed using 50mM iodoacetamide (IAA) in 100mM NH_4HCO_3 in the dark for one hour. Washing and alkylation was repeated after alkylation to remove any remaining DTT or IAA before dehydration and subsequent trypsin (12.5 ng/µl) digestion overnight at 37^0C. Peptide extraction was performed using a formic acid (2%) and acetonitrile (50%) solution. The extracted peptide solution was dried using a vacuum centrifuge and peptides were reconstituted in 0.1% formic acid solution.

4.5.4c Sample clean-up

The reconstituted peptide solutions were cleaned up using Zip-Tips (Merck) packed with µ-C18 resin (Gundry et al., 2009). The first step was activation of the tip using pipetting in and out with 100% acetonitrile. The second step was loading of solutions containing peptides; to ensure maximum efficiency the solution was slowly pipetted in and out several times. The third step was washing which was performed using desalting solution (0.5% formic acid). Finally, the desalted peptides were eluted in two steps, with the first elution solution containing 0.5% formic acid in 1:1 (v/v) water:acetonitrile and the second containing 100% acetonitrile. The cleaned and extracted peptides were dried in a vacuum centrifuge and reconstituted in 0.1% formic acid, and stored at -20^0C until required.

4.5.4d Nano LC- MS/MS

Analysis of peptides and peptide fragments was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Q-Exactive hybrid Quadrupole Orbitrap

mass spectrometer (Thermo). An in-house packed trap (Halo® 2.7µm 160 Å ES-C18, 100µm x 3.5cm) and analytical column (Halo® 2.7µm 160 Å ES-C18, 75µm x 10cm) were used for sample application. Prior to the mass spectrometry, high performance liquid chromatography (HPLC) separation was performed using nanoflow liquid chromatography (EASY-nLC™ II, Thermo). A linear gradient of buffers A (2% v/v acetonitrile, 0.1% v/v formic acid) and B (99.9% v/v acetonitrile, 0.1% v/v formic acid) was used to elute of peptides. The starting gradient was 1-50% buffer B for 50 minutes followed by increase in buffer B concentration from 50 to 85% for two minutes and column washing at 85% of buffer B for 8 minutes. The flow rate was kept constant at 300nl/min during the gradient. The mass spectrometry spectral acquisition was performed in the scanning range of 350-2000 m/z in positive mode. For the tandem mass spectrometry analysis, HCD fragmentation of the top 10 most intense precursor ions was performed at normalised collision energy of 30% (Xcalibur, Thermo).

4.5.4e Protein identification

The LC-MS/MS generated files were converted to mzXML format to be used for identification of proteins by peptide to spectrum matching. The Global Proteome Machine software v2.1.1 and the X!Tandem algorithm were used for the peptide to spectrum matching and subsequent protein identifications, along with a manually assembled database of 37,289 oyster peptide sequences of *Crassostrea gigas*. The search criteria included carbamidomethylation of cysteine as a complete modification, N-terminal acetylation and oxidation of methionine as potential modifications, with fragment monoisotopic mass error of 0.4Da and parent monoisotopic mass error of +/- 100ppm. A reversed sequence search was also performed to allow calculation of false discovery rate.

4.5.4f Data processing and quantitation

The output files generated for three replicates of each population following the protein database search were further analysed quantitatively and statistically by converting to normalized spectral abundance factors using the Scrappy program (Neilson et al., 2013; Pascovici et al., 2016b). Filtering criteria included presence of at least six matched peptide spectra across the three replicates (and present in all three replicates), – log(e) value cut off 2.5 for each peptide, and p-value filter of 0.05 indicating statistical significance. The peptide FDR was also calculated for the total output by using the following equation: FDR=

total number of peptides identified for reversed proteins hits / total number of peptides identified for all proteins x100.

4.6 Results and Discussion

This study provides a different perspective on the comparative proteomics of Sydney Rock oysters. In contrast to other studies in the thesis (chapters 2,3 and 5), this section analyses the difference in proteome of selectively bred B2 SRO line and shore catch wild oysters. Shore catch oysters are a result of wild spats without any prior exposure to imposed selection pressure, whereas the selectively bred Sydney Rock oysters represented a genetically conserved population which has experienced severe and sustained selection pressure against QX disease.

The oyster proteins were subjected to SDS-PAGE fractionation in three biological replicates of each population followed by LC-MS/MS analysis. The proteins putatively identified using the X!Tandem database search were further quantitatively analysed using a two sample comparison in the Scrappy program. This generated a non-redundant total of 154 proteins with a minimum peptide count of six per protein, and –loge value of -2.5. The peptide false discovery rate was 3.4%.

Of a total of 154 proteins, 96 proteins were statistically unchanged according to Student's t-test analysis of normalized spectral abundance factor values (with p-values less than 0.05 indicating significance), whereas 12 proteins were expressed at significantly higher levels in the selected B2 line of oysters as compared to wild oysters, and 46 proteins were expressed at higher levels in wild oysters, as shown in **Figure 4.3**. It is to be noted here that calculation of fold changes based on NSAF values took into account the proteins for which no matching peptides were identified in one sample, by substituting zeroes with a mathematical construct of a fraction of a spectrum. As a consequence, the fold-change values reported for proteins which were absent in one sample were a good first approximation and require follow up experiments for further validation (Neilson et al., 2013).

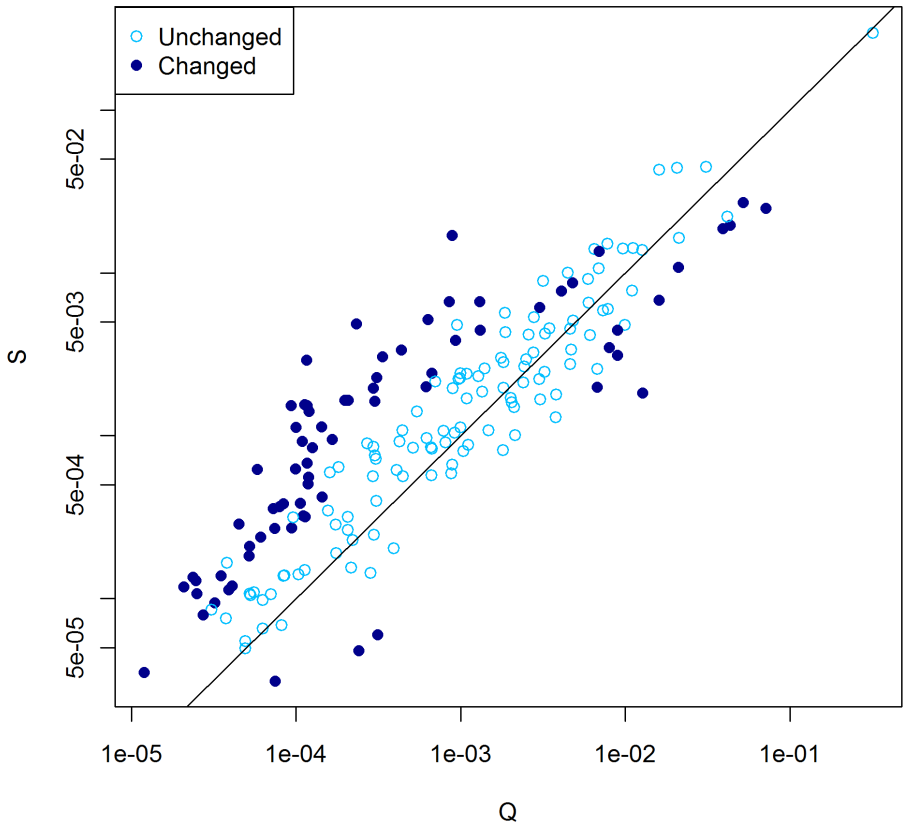

Figure 4.3: LogNSAF plot generated by the scrappy programme shows the distribution of proteins – changed and unchanged in expression level. Dark blue spots above the diagonal are the proteins with higher expression in wild oysters whereas the dark spots underneath the diagonal are expressed more in selected B2 line oysters. Light blue hollow spots are those proteins which are statistically unchanged.

Table 4.1: LC-MS/MS revealed identities of proteins differentially regulated in the two population so Sydney Rock oysters. The fold changes measured are based on logNSAF values.

a. Higher expression in selected B2 line of Sydney Rock oysters

Identifier	Protein name	Fold change
gi 126363566	lysozyme 1M	7.0
gi 405975234	Dual oxidase 2, partial	5.1
gi 405977755	Caprin-2	3.4
gi 405974470	40S ribosomal protein S30	2.9
gi 405976085	CDGSH iron sulfur domain-containing protein 2	2.9
gi 405974897	Peroxiredoxin-5, mitochondrial	2.4
gi 405976037	Charged multivesicular body protein 5	2.3
gi 405976932	Synaptobrevin, partial	2.2
gi 405978788	60S ribosomal protein L18, partial	2.1
gi 405978204	Tubulin-specific chaperone A	2.0

gi 405978738	hypothetical protein CGI 10022305	1.9
gi 405974492	40S ribosomal protein S14	1.9

b. Higher expression in wild caught Sydney Rock oysters

Identifier	Protein name	Fold change
gi 405976897	LSM14-like protein A	25.2
gi 405976260	Ras-related protein Rab-10	20.9
gi 405974790	ATP synthase subunit beta, mitochondrial	19.2
gi 405974726	U1 small nuclear ribonucleoprotein A	16.5
gi 405976470	Rab GDP dissociation inhibitor beta	13.7
gi 405978649	Laminin-like protein epi-1	11.8
gi 405974406	NipSnap-like protein 2	11.3
gi 405974545	Collagen alpha-1(IX) chain	10.7
gi 405976865	Translocon-associated protein subunit alpha	9.1
gi 405976128	Coiled-coil domain-containing protein 6	8.5
gi 597059637	Jumonji-C domain-containing protein Jumonji5	8.4
gi 405977380	Radial spoke head protein 3-like protein	8.1
gi 405977412	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	8.0
gi 405976936	Leucine-rich repeat-containing protein 23	7.9
gi 405974703	ATP synthase subunit alpha, mitochondrial	7.8
gi 405975834	Isocitrate dehydrogenase NADP , mitochondrial	7.7
gi 405974949	cAMP-dependent protein kinase regulatory subunit	6.7
gi 405976093	DPY30 domain-containing protein 1	6.7
gi 405975392	Filamin-A	6.4
gi 405975243	unc-87	6.3
gi 405975636	Tektin-1	5.8
gi 405974421	hypothetical protein CGI 10018546	5.7
gi 405976142	ATP-binding cassette sub-family A member 5	5.7
gi 405978506	M-phase phosphoprotein 6	5.4
gi 405975730	Cleavage and polyadenylation specificity factor subunit 5	5.1
gi 405978215	Myelin expression factor 2	4.7
gi 405978127	Angiotensin-converting enzyme	4.6
gi 405978132	Tetraspanin-7	4.6
gi 405975708	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	4.3
gi 405975580	Dedicator of cytokinesis protein 6	4.3
gi 405975424	Calcium-dependent protein kinase 31	4.1
gi 405974927	Multidrug resistance-associated protein 5	3.9
gi 405976588	Serine/threonine-protein kinase PAK 1	3.6
gi 405975466	hypothetical protein CGI 10010025	3.6
gi 405975453	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	3.4
gi 405978177	Polyadenylate-binding protein 2	3.2
gi 405974950	GTPase SLIP-GC	3.0
gi 405975383	tRNA selenocysteine 1-associated protein 1	2.9

gi 405976916	Tyrosine-protein phosphatase non-receptor type 1	2.9
gi 405978372	Protein WWC2	2.9
gi 405974984	Glutamate dehydrogenase 1, mitochondrial	2.9
gi 405976244	Neutral ceramidase B	2.8
gi 405977517	40S ribosomal protein S23	2.0
gi 405978841	Voltage-dependent anion-selective channel protein 2	2.0
gi 405976915	Proteasome subunit alpha type-7-like protein	1.9
gi 405974727	Transcriptional activator protein Pur-alpha	1.8

rows highlighted are the proteins which we observed only in wild oysters i.e. no corresponding peptides were found in selected oysters*.**

4.6.1 Proteins found expressed at higher levels in the B2 line

Twelve proteins were found to be upregulated in the proteome of selected B2 line oysters (**Table 4.1**a), which included proteins associated with protein processes (40S and 60S ribosomal subunits), suggesting that active protein synthesis was required for cellular growth and function. We also identified a relatively higher expression of peroxiredoxin, which has been reported in previous oyster stress research and which has been identified as an indicator of various types of environmental stress (David et al., 2007; Epelboin et al., 2015; Green et al., 2009). Peroxiredoxin plays an important role as a component of innate immunity in many invertebrates. It prevents cellular damage such as cytoskeletal remodelling, and nucleic acid degradation caused by the presence of reactive oxygen species (Knoops et al., 2016; Perkins et al., 2015). Identification of peroxiredoxin in the list of proteins expressed at a higher level in the selected B2 oyster line suggested that innate immunity in the selected line of SRO is improved as compared to the shore caught oysters. This is important when put in perspective with selection for QX resistance over many generations of the disease resistant B2 line. Not only these lines are resistant to the disease, and in addition to that they are also better performing under environmental stressors such as ocean acidification and increased temperature. Increased expression of perioxiredoxin in the B2 line appears to be one of the reasons why this is so, as it is consistent with previous literature.

Amongst other proteins, we also identified increased abundance of CDGSH iron-sulphur domain-containing protein 2 in the B2 line. This protein has been previously reported in ocean acidification stress in *C. gigas* and it is believed to be associated with autophagy (Timmins-Schiffman et al., 2014). In humans, CDGSH iron-sulphur domain-containing

protein 2 is reported to be involved in counteracting BECN1 mediated autophagy (Chang et al., 2010). However, the functional implications of the presence of this protein in bivalves are not clear.

4.6.2 Proteins expressed at lower levels in B2 line

We identified a total of 46 proteins which were found to be expressed at lower levels in B2 line oysters compared to wild caught oysters (**Table 4.1**b). It is important to emphasise that out of the total downregulated proteins, peptides for 30 proteins (highlighted in shading in **Table 4.1**b) were found only in the shore line oyster samples - no matching peptides were identified in B2 line oysters. This demonstrates that the expressed proteomes of the two oyster populations, despite belonging to the same species, are very different. This observation is in stark contrast to the results in previous chapters where both the studied cohorts (HC and selected) originated from the same parent population and resulted in relatively similar proteome profiles in contrast to the cohorts descridbed here (wild and selected) (Nell and Perkins, 2006). The wild oysters were not related in any way to the selected line of oysters, and therefore we have observed striking differences between the expressed proteomes, exemplified by the number of proteins identified only in the shoreline oysters.

The results of this study are in concurrence with a genetic investigation designed around similar populations from two estuaries in the Georges River - Woolooware Bay and Quibray Bay. The objective of that study was to identify the flow of genes from the selected line of oysters to the shore line oysters. It was revealed that there is a striking lack of genetic overlap between the two populations despite the fact that the oyster estuaries in Georges river have been used for breeding for more than two decades (Thompson et al., 2017). A principal component analysis based on selectively neutral single nucleotide polymorphisms, demonstrated that the selected oysters shared a close relationship to each other whereas they were very different from the wild oysters.

Differences in the proteome profiles of the two populations may be attributed to many factors, but the primary reason is likely to be the different founding populations. The B2 line oysters are the 6th generation of selectively bred oysters which were bred from a small number of mass mortality survivors (Dove et al., 2013). Therefore, the B2 line oysters are genetically conserved. In addition, as a result of continuous inbreeding, negative selection

of some genes such as an isoform of phenoloxidase, was observed in the disease resistant line (Bezemer et al., 2006; Butt and Raftos, 2008b). This directs our attention to the fact that with the pressure of a genetic bottleneck plus resistance to QX, the B2 line of oysters may be pushed continuously to maintain a close genetic relationship. Another possible explanation of the lack of genetic overlap could be the viability of the cross fertilised spat, so that even if there is cross fertilisation the spat may not survive to form a full grown adult. Also, the fact that the selected Sydney Rock oysters are conditioned over a number of generations under hatchery conditions means they have become less adept at spawning in wild conditions. At the same time, because of the large number of wild oysters as compared to the selected oysters in the estuary, it becomes statistically less probable for the two populations to cross fertilise.

It is very interesting how the findings of this study are different from the proteomic comparisons in chapters 2 and 3, especially given that we observe a very small percentage of proteins associated with the cytoskeleton in the B2 line vs shoreline oyster comparison. In the previous studies (chapters 3 and 4), cytoskeletal remodelling was one of the highlights. However, we did observe some similar findings such as identification of increased abundance of proteasome subunits, E3 ubiquitin, and 40 S ribosomal proteins, suggesting a shared response. In the following section we have discussed the functional implications of some of the findings of this work.

4.6.2a Stress response

NipSnap

We have identified proteins involved in stress response and host defence which are present at higher levels in wild oysters, which suggests the possible presence of some kind of stress during the sample collection. NipSnap protein structure and function has been reported to be associated with HSP60, however the exact physiological function of NipSnap is not entirely clear (Yamamoto et al., 2017). It is located on the inner mitochondrial membrane and is involved in maintaining mitochondrial function (Tummala et al., 2010). To our knowledge this is the first reports to identify NipSnap protein in oysters or bivalves, and we believe it is involved in the stress response pathway because of its association with HSPs. The absence of matching peptides of this protein, and several other related proteins, in the selected line of Sydney Rock oysters could be caused by loss of the genes, either over

the time scale of selective breeding or during the longer term process of evolutionary selection.

Another stress protein that we identified as upregulated in wild oysters is calcium dependent protein kinase. We have reported the presence of this protein in the winter mortality resistant vs. hatchery control protein expression study in Chapter 2 (Vaibhav et al., 2016). The observed level of expression of this protein was higher in the selected line of oysters, which at first glance is perplexing. However, to explain this difference, it is important to note that the populations were different, as the oysters in Chapter 2 were selected for WM disease resistance only, and compared with hatchery control rather than wild caught oysters.

Voltage dependent anion selective channel protein

We also identified voltage dependent anion selective channel protein 2, which has been previously reported to be an anti-apoptotic protein in Pacific oyster (Li et al., 2016b). Several studies have suggested its involvement in apoptotic processes (Premkumar and Simantov, 2002), however, other studies have argued that its involvement in apoptosis is debatable (Galluzzi and Kroemer, 2007). The higher abundance of this protein in this study in wild caught oysters could be a sign of elicitation of host defence in response to stress.

4.6.2b Cytoskeletal remodelling and energy production

In addition to the above, we have identified proteins involved in cytoskeletal pathways, such as filamin-A, laminin, and radial spoke protein. These are indicative of cytoskeletal remodelling as has been observed previously in different stress response studies in bivalves (Muralidharan et al., 2012; Thompson et al., 2015; Vaibhav et al., 2016). Upregulation of cytoskeletal proteins has been reported to be associated with proteins involved in energy production, which we have identified in this work as well. A relative upregulation of ATP synthase, ATP binding cassette protein, isocitrate dehydrogenase, and Rab-GDP was observed, suggesting a surge in production of energy to meet the enhanced energy demands as a part of stress response of the organism (Rabasa and Dickson, 2016; Segerstrom, 2007).

4.6.2c Sexual maturation and sex determination

An important finding of this study was the upregulation of proteins involved in sexual maturity and sexual determination of oysters - angiotensin converting enzyme, and

cleavage and polyadenylation specificity factor subunit 5. In mammals, angiotensin converting enzyme has two isoforms – somatic and germinal. While the somatic isoform is responsible for maintenance of blood pressure (Corvol et al., 1995), the germinal isoform is involved in fertility (Turner and Hooper, 2002). The implication of the presence of orthologues of this protein in a number of invertebrates, such as *D. melanogaster* (Cornell et al., 1995) and *C. elegans* (Brooks et al., 2003), has been reported. The presence of an orthologue of angiotensin converting enzyme in oyster was reported for the first time by (Corvol et al., 1995). They reported the involvement of this protein specifically in sexual maturation of male oysters. Increased expression of angiotensin converting enzyme was reported to be directly related to spermatogenesis, suggesting its significance in male sexual growth. In this study we did not identify a single matching peptide for this protein in the selected line of oysters, which is a very interesting observation. It is difficult to explain negative selection of a protein such as this, but it opens up the possibility of further research studies which will be required to understand this phenomenon.

On the other hand, to our knowledge this is the first time that cleavage and polyadenylation specificity factor subunit 5 has been reported in the context of oyster research. This protein is reported to be involved in RNA processing (Dickson et al., 1999), but little has been reported about this protein with reference to its biological function in bivalves. However, the gene responsible for this protein has been previously reported to be associated with sex determination and control of germline in scallops (Llera-Herrera et al., 2013). It is not certain what biological function it is associated with in oysters, but based on the study in scallops it is believed to be associated with sex determination.

4.7 Conclusions

This shotgun analysis of gills tissue of wild Sydney Rock oysters, compared with a selected line of Sydney Rock oysters, was performed to investigate the proteome makeup of the two similar species separated on the basis of continuous selection for QX resistance and different growth conditions, in a distinct geographical location from our previous studies (chapters 2 and 3). We have identified a common pattern of stress response in the wild oysters (cytoskeletal remodelling and increased expression of ATP synthase) and at the same time we also observed expression of proteins which were seen exclusively in the wild populations, such as angiotensin converting enzyme and NipSnap. The results agree with the idea established in previous studies of cytoskeletal breakdown occurring as a stress

response. However, one of the more interesting observations in this study was the identification of quite a number of proteins (65% of the total downregulated proteins) which were seen to be present only in the wild population, suggesting a striking difference in protein expression patterns. We have identified important proteins such as voltage-dependent anion channel 2 and angiotensin converting enzyme, which are involved in host defence and sexual maturation, respectively. These proteins have been reported here for the first time in the context of oyster research and hence their differential expression warrants further enquiry. It should be noted that the background information of the wild oysters was not known, in contrast to the selected line of oysters. However, despite the closeness of the populations, being of the same species and grown at the same location, separated by less than a kilometre, the expressed proteomes are qualitatively and quantitatively distinct from each other. The results of this study are sufficiently interesting to encourage us to undertake further research aimed at building greater understanding of the proteome of wild oysters. Such information may prove highly valuable in providing a different angle on the influence of the selective breeding programme on protein expression in Sydney Rock oysters.

4.8 References

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Chapter 5

SWATH – MS proteomic analysis of disease resistant Sydney Rock oysters

Chapter 5. SWATH – MS proteomic analysis of disease resistant Sydney Rock oysters

5.1 Biological context

This chapter reports the findings of first experiments performed on Sydney Rock oysters using a Sequential Windowed Acquisition of all Theoretical Mass Spectra (SWATH-MS). The oysters used in this work were collected from Hawkesbury River and we have used digestive gut tissue to study the whole cell proteome. These oysters belonged to two different populations - one population belonged to a selectively bred line of QX resistant oysters (QXr), whereas the other was unselected hatchery grown oysters (HC).

In addition to using a different technique, SWATH-MS, for the first time, we have also used a very recently available draft version of the Sydney Rock oyster genome sequence database in order to characterize the Sydney Rock oyster proteome. We identified a total of 444 statistically significant proteins, of which 45 were expressed more in QXr and 66 were expressed more in HC oysters. Due to the lack of annotated proteins, the identities of these proteins were putatively established using protein Blast searching homology results.

5.2 Contributions

I performed 80% of the experimental work in this study, which included sample collection from the Hawkesbury River, sample processing steps including isolation of gut tissue, extraction and quantification of protein, and protein sample processing and preparation. The SWATH-MS runs and data analysis were performed by Dr Matthew Mackay and myself. I would also like to acknowledge Macquarie University Masters students Ms. Flora Cheng and Mr. Folio Emilio for their help with protein Blast searches.

Prof. Paul A. Haynes supervised this work as well as assisted in experimental design, statistical analysis and manuscript preparation. Professor Abigail Elizur of the University of the Sunshine Coast, Australia, provided us with the first draft assembled version of the Sydney Rock oyster genome sequence on 2nd of August 2017. The genome sequence data were acquired and assembled in her laboratory.

5.3 Abstract

Oysters are an important marine species, both ecologically and economically. The Sydney Rock oyster is one such native Australian oyster species which is a major contributor to total aquaculture production in New South Wales, Australia. Oysters take nutrition by a filter feeding mechanism using gills and palps. The filter feeding mechanism, however, makes them prone to infection by different parasites present in the water. Queensland unknown (QX) disease is one such opportunistic protozoan borne disease which affects oysters and results in mass mortality of more than 90%. This work reports a comparative proteomic investigation of two populations of Sydney Rock oysters - one selected for QX disease resistance and one unselected. We have performed a Sequential Windowed Acquisition of all Theoretical Mass Spectra (SWATH-MS) analysis to study the proteomic expression changes across the two populations. This is the first SWATH-MS analysis of Sydney Rock oysters and therefore this work is an important contribution to improving our understanding of the relatively underexplored proteome of this oyster.

Key words

Proteomics, SWATH-MS, Aquaculture, Sydney Rock oyster, QX disease

5.4 Introduction

In the past, attempts have been made to identify the genes or proteins which help the selectively bred Sydney Rock oysters to be better adapted to withstand QX disease, as compared to unselected oysters. Earlier works were limited by absence of an oyster genome sequence database, however, the availability of the *C. gigas* database has helped the oyster research community greatly. In Sydney Rock oysters, the effect of different stressors such as metal contamination (Muralidharan et al., 2012), elevated carbon dioxide levels (Thompson et al., 2015) and WM disease (Vaibhav et al., 2016) have been studied by applying proteomics techniques, such as 2DE and shotgun proteomics, using the *C. gigas* genome sequence information as a reference.

This study investigates the gut proteome of two population of SRO collected from the Hawkesbury River – one selected for QX disease resistance and one unselected hatchery control population. The expressed proteome of an organism gives a very good indicator of the physiological changes in the cells, especially when undergoing different kinds of stresses (Dalle-Donne et al., 2005). Production of different molecular factors to overcome the adverse conditions varies in different organisms and species, which is the underlying principle behind this work. The two populations are raised in different conditions and hence expected to have different proteomic responses to an identical stress (Meistertzheim et al., 2007). While there could be a generic underlying response profile, what we aim to identify and analyse is the uniqueness in the proteomic responses across the two species which allows one of them to be resistant while the other to succumbs to the infection (Rifai et al., 2006).

In addition to being an important independent work, this chapter also provides a validation study for the previous chapters. Initially, we attempted to perform immunoassays to confirm the findings from the shotgun experiments in Chapters 3 and 4. However, due to unavailability of commercially (or otherwise) available antibodies for the differentially expressed proteins it was not feasible. We successfully performed western blotting experiments using antibodies with low homology percentage (74% for MVP and 80% for Rab11, identified in Chapter 3), and achieved inconclusive results. Therefore, we pursued SWATH - MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion - Mass Spectra), in part to serve as an orthogonal validation study for the previous shotgun proteomic analyses.

SWATH-MS analysis of the proteome of an organism is a novel technique to identify and quantify low abundance proteins, and in addition to that it has also proven to be instrumental in studying post-translational modifications in various biological systems. This technique is very different to the data-dependent shotgun proteomics analyses performed in the other chapters of this thesis. In this data-independent approach, an expanded mass isolation window is "stepped" across the entire mass range covering the mass distribution of peptides. A full scan tandem MS spectrum is collected at each step. Following acquisition, extracted ion chromatograms for specific fragment ions of peptides of interest can be integrated for quantitation. Acquisition is performed with a user defined retention time – m/z window. The peptide fragments are then subjected to database searching for identification of corresponding proteins. Therefore, SWATH-MS analysis requires extensive and specific peptide-level genome sequence information to identify successfully the corresponding proteins (Gillet et al., 2012). We believe that this study is the first proteomic analysis in Sydney Rock oysters using SWATH – MS, and hence presents many more opportunities for future follow-up proteomics experiments.

5.5 Materials and methods

5.5.1 Sample collection

Oyster samples were provided by a commercial oyster farming business in Mooney Mooney, Hawkesbury River (-33.529232, 151.203347). We received 20 oysters selectively bred for QX disease resistance and 20 unselected hatchery control oysters. These two populations of oysters belong to the NSW DPI selective breeding programme, which at the time of collection were in their 5^{th} generation. The two lines belonged to the same parent population, while one line was selectively bred for QX disease resistance, and the other was not selected for any disease resistance. The digestive gut tissue was carefully excised at the site of collection and was immediately flash frozen in liquid nitrogen and subsequently stored at -80^{o}C for future experiments. The oysters used in this study were collected on April 2nd, 2014 i.e. towards the end of infection period. As per the parasitic development, we believe gut tissues would be more affected than they were in initial stages of infection, making them of greater biological interest.

5.5.2 Digestive gut tissue protein extraction

The protein extraction from the gut tissue, thawed beforehand at 4^0C, was performed as in (Thompson et al., 2015). Homogenisation of tissue samples, completely immersed in TRI reagent® (Sigma-Aldrich), was manually performed using a pellet pestle. Removal of DNA and RNA was performed using 100% ethanol and bromochloropropane, respectively. Acetone was used to precipitate proteins, the protein pellet was kept at room temperature to evaporate excess acetone, and it was then suspended in 6M urea in 100mM Tris buffer, pH 8.5. To ensure that enough protein was present for further experiments, five oysters were pooled to form one biological replicate. The three hatchery control biological replicates were designated as W1-3 and the three QX disease resistant line biological replicates were designated as Q1-3.

5.5.3 Protein quantification

Protein quantification was performed using Pierce™ BCA protein assay kit as per the manufacturer's instructions using a range of different BSA concentration as standard (Smith et al., 1985). 2 µl of each sample (diluted 1:1 in MiliQ water) was taken in duplicate in a well of 96 well microtitre plate. 180 µl BCA reagents (A and B mixed in 50:1 ratio) were added to each well containing an unknown sample to be quantified, and BSA standard and the mixture was incubated at 37^0C for 30 minutes. After incubation, the protein absorbance was recorded at 562 nm. Protein concentration of the unknown samples were estimated using the straight line equation obtained by the standard BSA solutions.

5.5.4 SWATH-MS work flow

5.5.4a Sample preparation

The original protein solution in 6M urea in 100mM Tris buffer, pH 8.5, was diluted with distilled water 1:3 to obtain the final urea concentration of 1.5 M. To this solution, Lys-C was added and the digestion was carried out at 37ºC for 4 hours. The Lys-C digested protein sample was reduced with DTT and subsequently alkylated with IAA. Following this, the protein was digested overnight at 37ºC in trypsin solution, in enzyme to substrate ratio of 1:100. Formic acid was added to the digested solution, to bring the pH to the acidic range (3-4) and peptides were desalted and concentrated using C18 Omix tips. The peptides were eluted with 90% acetonitrile, and after drying in a vacuum centrifuge the samples were reconstituted in 100µl of 0.1% formic acid.

5.5.4b Data acquisition

Information dependent acquisition (IDA)

For initial protein/peptide library generation, a pooled sample of Q1-3 and W1-3 was analysed by information dependent acquisition. The sample (10µL) was injected onto a peptide trap column (5µL/min for 3 minutes) prior to elution onto a reverse phase cHiPLC column, and subsequent separation using a linear solvent gradient from 2% ACN and 0.1% formic acid to 40% of a 99.9% ACN and 0.1% formic acid buffer at 600nL/min over a 120 min period. The LC eluent was subject to positive ion nanoflow electrospray MS analysis. In IDA mode on a Sciex 6600 Triple-TOF, a TOF-MS survey scan was acquired (m/z 350-1500, 0.25 sec), then the 10 most intense multiply charged ions (2+ - 4+; counts >200) in the survey scan were sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 0.1 sec (m/z 100-1800) with rolling collision energy.

Data independent acquisition (SWATH)

Each sample (10uL) was injected onto a peptide trap for desalting with 2% ACN and 0.1% formic acid at 5µL/min for 3 minutes. Peptides were eluted from the column using a linear solvent gradient from 2% ACN and 0.1% formic acid to 40% of a 99.9% ACN and 0.1% formic acid buffer at 600nL/min over a 60 min period. The LC eluent was subject to positive ion nanoflow electrospray MS analysis in a data independent acquisition mode (SWATH).

For SWATH MS, m/z window sizes were determined based on precursor m/z densities (m/z 400 – 1250) as identified in the previous IDA data, to generate a SWATH variable window acquisition method with 60 windows. In SWATH mode, first a TOF-MS survey scan was acquired (m/z 350-1500, 0.05 sec) then the 60-predefined m/z ranges were sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 0.03 sec (m/z 350-1500) with rolling collision energy optimised for 2+ precursors with an m/z of lowest m/z in variable window +10%.

5.5.4c Sydney Rock oyster genome assembly

The Sydney Rock oyster genome was sequenced using the gills and digestive gland tissue, however the annotation of the proteins corresponding to the sequences is not currently available (Hook et al., 2014). We obtained a copy of the first draft version of the predicted

protein sequences interpreted from the raw genome sequence data of the Sydney Rock oyster, on 2nd of August 2017. This unannotated FASTA file contained 29,700 putative protein sequences, which was compatible with peptide to spectrum matching software. However, there was no functional or identifier annotation provided, just a reference number. Due to the very strict time constraints involved, rather than wait for functional annotations for all proteins in the database file to be provided, we chose to pursue a manual annotation approach for only those proteins which were identified in our final dataset. Manual Blast searching was performed against the NCBI nonredundant database, and results with expectation score of less than 10e-20 were examined and matching proteins were reported.

5.5.4d SWATH-MS quantitation and data analysis

For SWATH quantitation, the ProteinPilot IDA search file was imported into PeakView2.1 with SWATH2.0 MicroApp (SCIEX) and used as a spectral library. SWATH files for all biological replicates (3xQ and 3xW) were imported. For retention time calibration, linear regression was performed by selecting five endogenous peptides across the elution profile to adjust the retention time shift due to differences in gradient lengths in the IDA and SWATH acquisition modes. The top six most intense fragments of each peptide were extracted from the SWATH data sets (75 ppm mass tolerance, 5 min retention time window). After data processing, peptides with confidence of 99% and FDR of 1% were used for quantitation (max 100 peptides per protein). Cumulative protein areas from extracted ion chromatograms were normalized to the mean protein area for each biological replicate. A two-sample t-test was performed comparing median log2 normalized protein areas of each protein present in both sample groups (QXr and HC). A protein was considered differentially expressed if the protein was quantified in all three biological replicates with a difference of mean/fold change of +/-2.0 and a p-value <0.05.

5.6 Results and discussion

Proteomic studies of Sydney Rock oyster to this point in time have mainly been performed using *C. gigas* genome sequence data for peptide to spectrum matching, as it was apparently the closest organism with a well annotated genome sequence. Analysis of our SWATH data using the same *C.gigas* protein sequence database resulted in much fewer peptide identifications than was expected from the MS1 and extracted ion chromatogram data.

Table 5.2 shows the summary of different databases we used, and the percentage of protein identifications obtained. We used Pacific oyster (*C. gigas*) genome and Pearl oyster (*Pinctada fucata*) genome, in an effort to check the closeness of the species to Sydney Rock oyster (*Saccostrea glomerata*). The percentage of spectra identified using the pearl oyster sequence database was close to 2% whereas for the Pacific oyster sequence database it was 7%, suggesting, not surprisingly, that the Pacific oyster is considerably closer, in terms of peptide sequence identity, to the Sydney Rock oyster than the pearl oyster is.

Table 5.1: A comparative summary of database search results using three different oyster genome sequence databases for the SWATH-MS experiments.

Oyster	**# Proteins**	**# Peptides**	**%Spectra ID**
Crassostrea gigas	196	801	7.4
Saccostrea glomerata	444	2467	22.7
Pinctada fucata	44	231	1.9

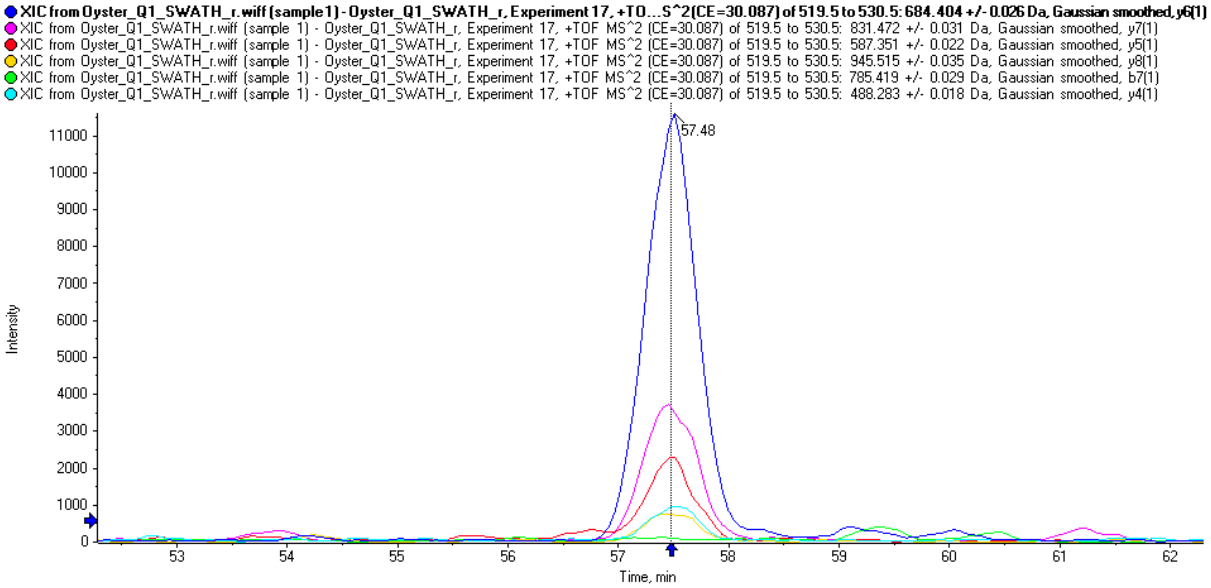
However, recently, we obtained a first draft version of a database of proteins predicted from the raw Sydney Rock oyster genome sequence data, and we formatted it for use in database searching. This sequence file has not been annotated and hence is of little use where protein functional annotation is required. However, in this case we were curious to see if we could increase the success rate of peptide identification by using this Sydney Rock oyster genome sequence information. We managed to increase the identification by three times when compared to searching the Pacific oyster genome sequence, which further reinforced the importance of the availability of a sequenced genome. it is important to emphasise that this is still a first draft version, and we have experienced numerous issues such as truncated protein sequences, which could be a reason for the still relatively low percentage of peptide identifications (23%), compared to other analyses reported in, for example, human cells. In samples where the genome is refined and well annotated, it is typical to find 60-70% of spectra identified successfully.

Using the Sydney Rock oyster genome sequence database as a reference, we identified a total of 444 proteins. Of those, 79 were more intense in QXr oysters whereas 94 were more intense in the HC oysters population. Upon applying the dual criteria of the Student's t test (p value ≤ 0.05) and a fold change cutoff of two or more, the number of differentially expressed proteins was reduced to 45 and 66 respectively. The number of proteins gave us some idea about the extent of differential regulation of proteins across the two different populations, however, to understand the functional importance of the proteins undergoing expression changes, the unannotated Sydney Rock oyster genome file was a limiting factor. Due to the lack of annotated proteins we performed a manual protein BLAST search for each of the sequences identified (unchanged and differentially regulated in expression) to establish the putative identity of the proteins.

Figure 5.1 (a) and (b) shows representative extracted ion chromatograms (XIC) of two peptides associated with the differentially regulated proteins. We have chosen one higher abundant protein (oxidoreductase) and one lower abundant proteins (HMGB1) in the QXr oyster gut tissue.

Figure 5.2 (a-f) shows an example of the relative quantitation based on integration of peak areas in extracted ion chromatograms for specific peptides. The six ion chromatograms shown represent three replicates each of QXr and HC oysters. The peaks shown correspond to a peptide from the protein Oxidoreductase, and by integration of peak areas it is clear that the peptide (and protein) are present at a higher level in the QXr oysters.

(a)



(b)

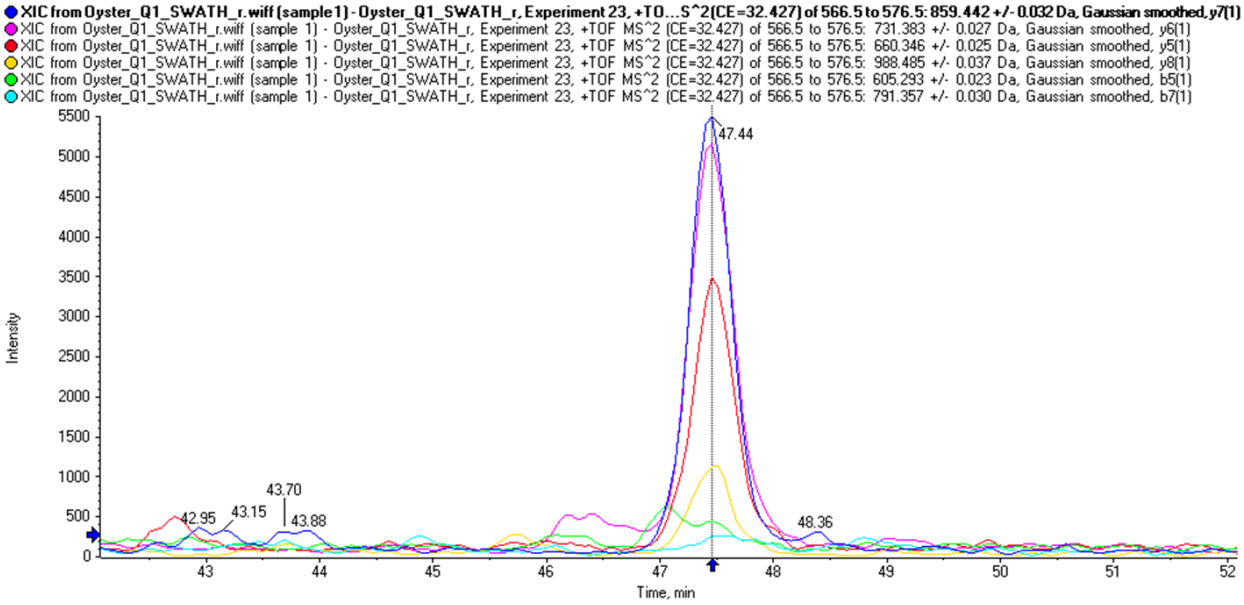
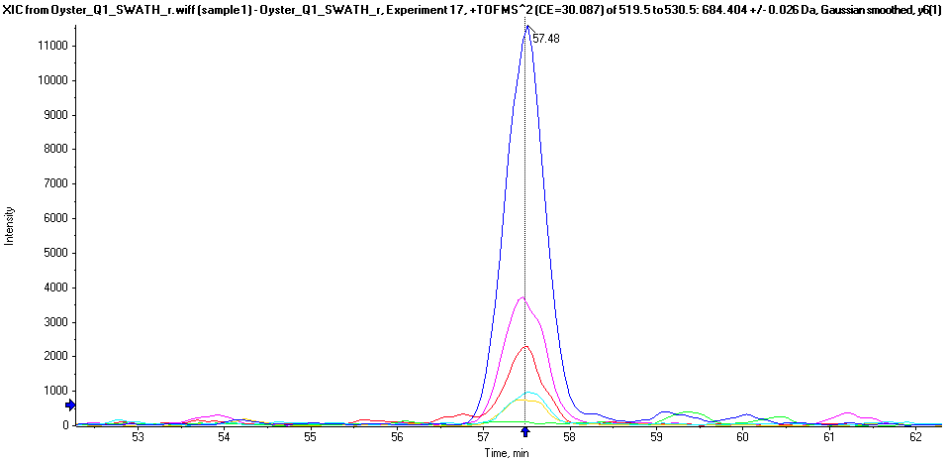
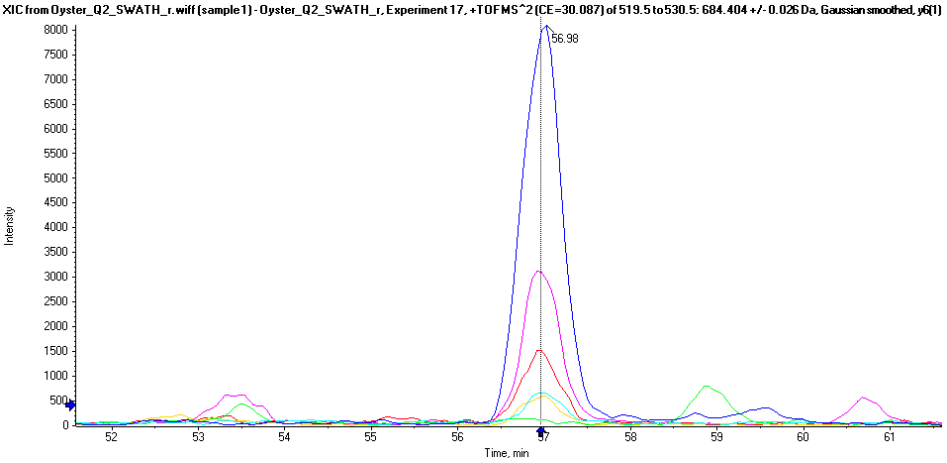


Figure 5.1: (a) Extracted ion chromatogram (XIC) generated from information dependent acquisition of peptide LNFPVVDR associated with the identification of the protein oxidoreductase is shown above where (b) shows (XIC) of peptide YEQALADWR associated with the identification of the protein HMGB1. Product ions for these peptides are represented by six different chromatograms as shown above. The product ion selection used in this algorithm prefers the ions with high specificity rather than sensitivity (Krisp and Molloy, 2017). In (a) the blue coloured, y6 daughter ion is the more specific and most intense, whereas in (b) the blue coloured, y7 daughter ion is the most specific and most intense.

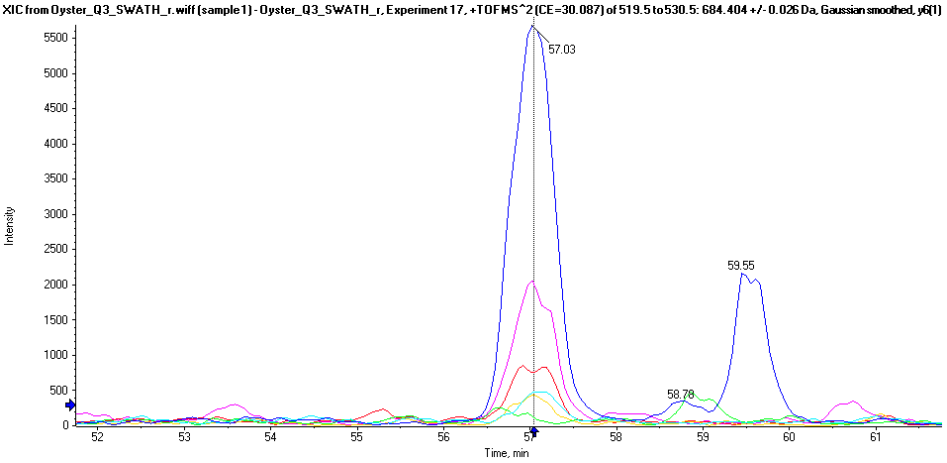
(a)


XIC from Oyster_Q1_SWATH_r.wiff (sample 1) - Oyster_Q1_SWATH_r, Experiment 17, +TOFMS^2 (CE=30.087) of 519.5 to 530.5: 684.404 +/- 0.026 Da, Gaussian smoothed, y6(1)
57.48
Intensity
Time, min

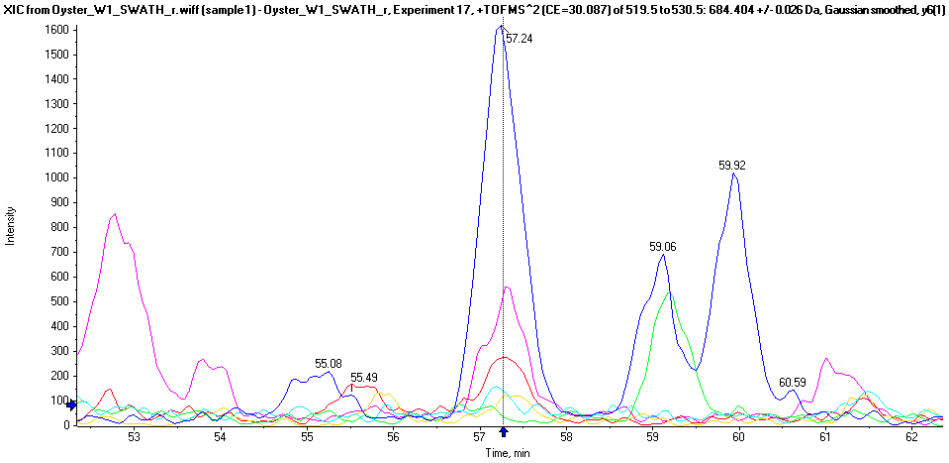
(b)


XIC from Oyster_Q2_SWATH_r.wiff (sample 1) - Oyster_Q2_SWATH_r, Experiment 17, +TOFMS^2 (CE=30.087) of 519.5 to 530.5: 684.404 +/- 0.026 Da, Gaussian smoothed, y6(1)
56.98
Intensity
Time, min

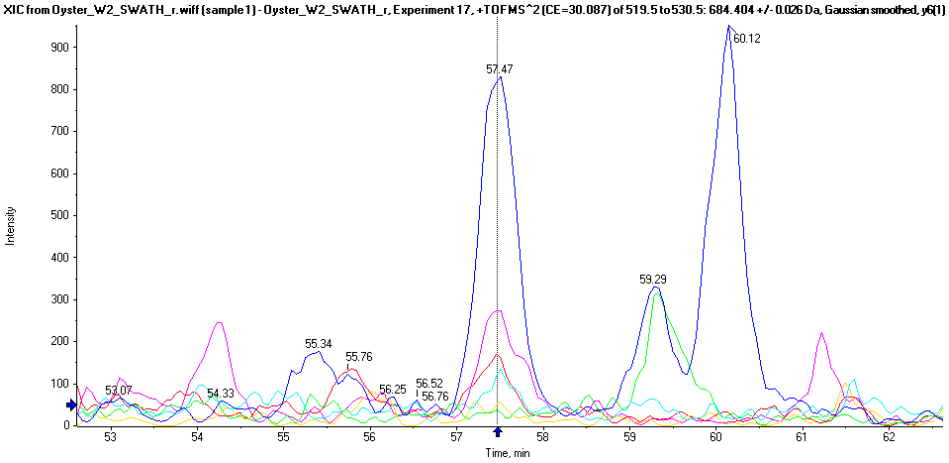
(c)


XIC from Oyster_Q3_SWATH_r.wiff (sample 1) - Oyster_Q3_SWATH_r, Experiment 17, +TOFMS^2 (CE=30.087) of 519.5 to 530.5: 684.404 +/- 0.026 Da, Gaussian smoothed, y6(1)
57.03
59.55
58.78
Intensity
Time, min

(d)



(e)



(f)

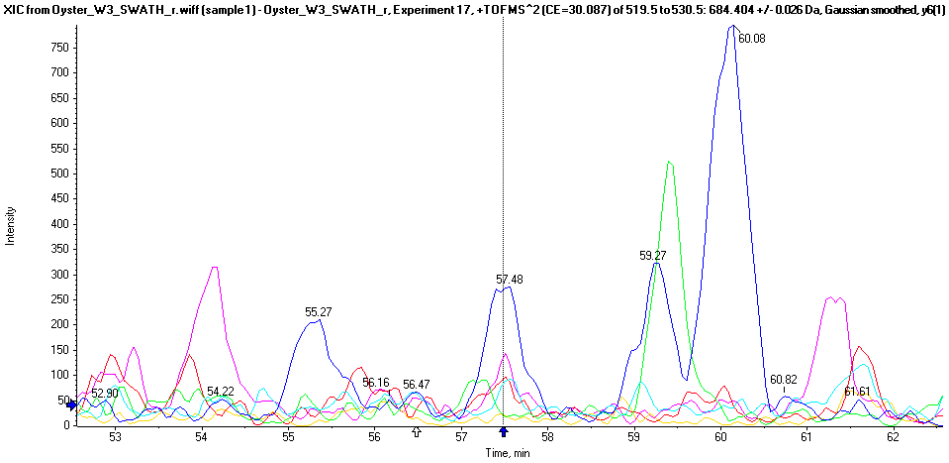


Figure 5.2: (a-c) XICs for LNFPVVDR associated with protein oxidoreductase for three replicates of QXr 1-3 and (d-f) for three replicates of HC (W1-3). Oxidoreductase is expressed at a higher level in the QXr oysters: as shown in the XIC above, the relative intensity corresponding to the QXr replicates ranges from 11,000-5500 whereas for HC replicates it is 1600-250.

We compared the findings of this work with the shotgun proteomics based findings in the previous chapters of this thesis and we observed some similarities. Comparing with findings of chapter 3 revealed that 12 proteins (25.6% of total proteins identified) were also reported in the SWATH-MS analysis whereas 10 proteins (25% of total proteins identified) reported in chapter 4 were common to the findings of this chapter. Proteins such as HMGB1, ubiquitin, isocitrate dehydrogenase, collagen alpha, and histone H2 were among the common proteins between chapter 3 and chapter 5 findings. On the other hand, lysozyme 1M, isocitrate dehydrogenase, translocon, ubiquitin, and filamin were common between chapters 4 and 5. Proteins such as HMGB1, histone H4 and H2, ubiquitin, Superoxide dismutase and lysozyme 1M are a few of the proteins which were major highlights of this work, and other works on Sydney Rock oysters or oysters in general, and thus need to be investigated more to increase our understanding of the stress response in oysters.

A relatively low percentage (25%) of common proteins between the LC-MS/MS approach used in chapters 3 and 4, and the SWATH-MS approach used in chapter 5, could be attributed to fundamental differences between the two approaches, and also the different protein sequence database used for peptide to spectrum matching. It is important to reiterate that the proteins identified in SWATH-MS study were as a result of only 23% of peptides being successfully identified, which means there are still a large number of unidentified peptides. Therefore, once the Sydney Rock oyster database is well annotated and curated, it will be well worth repeating the database search.

In addition to peptide to spectrum matching against the Sydney Rock oyster genome sequence database, we also performed several additional searches. This included searching against other related marine organisms, and also against a database of all bacterial proteins, and lastly against a global protein database. As the tissue we analysed was from the gut of the oyster, we wanted to see if we could identify any proteins from bacteria or other gut microbiome. Results showed that most of the protein identifications observed were restricted to other bivalves, presumably from homologous proteins. Our inability to identify proteins from microorganisms could be attributed to the fact that post proteins would be present at much higher abundance levels than those of the microorganism, or there may simply be no microorganisms present. Therefore, to pursue the microbiome

characterisation, either depletion of host proteins, or enrichment of microorganism proteins, is essential.

Table 5.2 summarises the findings of the SWATH-MS study.

Table 5.2a lists the proteins expressed at higher levels in QXr gut tissue samples, whereas

Table 5.2b lists the proteins expressed at higher levels in HC oyster gut tissues. The fold change for each protein was calculated from the ratio of mean values of $\log_e$ normalised peak area for each replicate.

Table 5.2a: List of proteins expressed at higher levels in QXr oysters. The list also identifies the organism to which the protein sequence was matched closely, along with the fold change ratio.

NCBI -protein Blast (non-redundant)	Fold change
sjoegren syndrome nuclear autoantigen 1 [*Crassostrea gigas*]	46.41
histone H4 [*Larimichthys crocea*]	37.81
uncharacterized protein LOC105324594 [*Crassostrea gigas*]	35.78
sperm-specific protein PHI-2B/PHI-3-like [*Crassostrea gigas*]	25.06
mesencephalic astrocyte-derived neurotrophic factor homolog [*Crassostrea gigas*]	21.88
charged multivesicular body protein 4b [*Crassostrea gigas*]	20.99
calmodulin [*Crassostrea gigas*]	16.06
prefoldin subunit 2-like [*Crassostrea gigas*]	13.79
cytochrome c oxidase subunit 5A, mitochondrial [*Crassostrea gigas*]	13.50
transcription factor BTF3 homolog 4 [*Crassostrea gigas*]	12.93
tubulin polymerization-promoting protein family member 3-like [*Crassostrea gigas*]	12.39
16 kDa calcium-binding protein [*Crassostrea gigas*]	11.48
myosin essential light chain, striated adductor muscle [*Crassostrea gigas*]	10.97
histone H2A isoform 2 [*Haliotis discus discus*]	10.38
histone H2A [*Crassostrea gigas*]	9.80
vesicle-associated membrane protein [*Ostrea edulis*]	9.08
transgelin-3 isoform X1 [*Crassostrea gigas*]	8.22
carboxypeptidase inhibitor SmCI-like [*Crassostrea gigas*]	6.65
hypothetical protein CGI_10014271 [*Crassostrea gigas*]	6.45
ADAM family mig-17 [*Crassostrea gigas*]	5.83
dynein light chain roadblock-type 2 isoform X1 [*Crassostrea gigas*]	5.41
Kyphoscoliosis peptidase [*Crassostrea gigas*]	4.95
uncharacterized protein LOC105343211 [*Crassostrea gigas*]	4.88
enkurin isoform X1 [*Crassostrea gigas*]	4.76
14-3-3 protein epsilon [*Crassostrea gigas*]	4.60
40S ribosomal protein S18 [*Crassostrea gigas*]	4.01
annexin A6 [*Crassostrea gigas*]	3.90
uncharacterized protein LOC105319638 [*Crassostrea gigas*]	3.88
MICOS complex subunit Mic19-like [*Crassostrea gigas*]	3.84
troponin C isoform X1 [*Crassostrea gigas*]	3.70
calmodulin-A [*Crassostrea gigas*]	3.65
kunitz-type serine protease inhibitor C [*Crassostrea gigas*]	3.65
myotrophin homolog [*Crassostrea gigas*]	3.62
fatty acid-binding protein, adipocyte [*Crassostrea gigas*]	3.60
putative uncharacterized oxidoreductase YDR541C [*Crassostrea gigas*]	3.14
S-crystallin SL11 isoform X1 [*Crassostrea gigas*]	3.13
C-Myc-binding protein [*Crassostrea gigas*]	2.99
cytochrome c [*Crassostrea gigas*]	2.96

40S ribosomal protein S19 isoform X2 [*Crassostrea gigas*]	2.90
14-3-3-like protein 2 isoform X1 [*Crassostrea gigas*]	2.87
periostin [*Crassostrea gigas*]	2.67
cytochrome c oxidase subunit 4 isoform 1, mitochondrial-like [*Crassostrea gigas*]	2.65
synaptojanin-2-binding protein [*Crassostrea ariakensis*]	2.60
60S ribosomal protein L18 [*Crassostrea gigas*]	2.32
uncharacterized protein LOC105327554 [*Crassostrea gigas*]	2.24

Table 5.2b: List of proteins expressed at higher levels in HC oysters. The list also identifies the organism to which the sequence was matched closely along with the fold change ratio.

NCBI -protein Blast (non-redundant)	Fold change
actin [*Caenorhabditis brenneri*]	2.02
galaxin-like [*Acropora digitifera*]	2.03
S-adenosylhomocysteine hydrolase [*Crassostrea ariakensis*]	2.07
uncharacterized protein LOC105325994 [*Crassostrea gigas*]	2.12
uncharacterized protein LOC105318934 [*Crassostrea gigas*]	2.13
Radixin [*Crassostrea gigas*]	2.14
nascent polypeptide-associated complex subunit alpha-like [*Crassostrea gigas*]	2.14
ependymin-related protein 1-like [*Saccoglossus kowalevskii*]	2.15
uncharacterized protein LOC105324922 [*Crassostrea gigas*]	2.15
EF-hand calcium-binding domain-containing protein 1 [*Crassostrea gigas*]	2.22
78 kDa glucose-regulated protein precursor [*Crassostrea gigas*]	2.24
Retinal dehydrogenase 1 [*Crassostrea gigas*]	2.26
adhesion G-protein coupled receptor D1-like [*Crassostrea gigas*]	2.32
chromobox protein homolog 1 isoform X1 [*Crassostrea gigas*]	2.33
tyrosine-protein phosphatase non-receptor type 11 isoform X2 [*Crassostrea gigas*]	2.38
protein CDV3 homolog isoform X1 [*Crassostrea gigas*]	2.48
uncharacterized protein LOC105330607 isoform X7 [*Crassostrea gigas*]	2.51
retrograde protein of 51 kDa isoform X4 [*Crassostrea gigas*]	2.51
Zinc finger protein 706 [*Mizuhopecten yessoensis*]	2.53
28 kDa heat- and acid-stable phosphoprotein [*Crassostrea gigas*]	2.55
Isocitrate dehydrogenase [NADP], mitochondrial [*Crassostrea gigas*]	2.56
hypothetical protein CGI_10007081 [*Crassostrea gigas*]	2.59
uncharacterized protein LOC105326485 [*Crassostrea gigas*]	2.61
uncharacterized protein LOC105324691 [*Crassostrea gigas*]	2.61
beta-arrestin-1 isoform X3 [*Crassostrea gigas*]	2.63
high mobility group box 1 protein [*Saccostrea kegaki*]	2.63
cysteine-rich protein 1 [*Crassostrea gigas*]	2.64
elongation factor 1 alpha [*Crassostrea ariakensis*]	2.66
lysozyme 1M [*Crassostrea gigas*]	2.70
60S ribosomal protein L22-like [*Crassostrea gigas*]	2.73
uncharacterized protein LOC105324691 [*Crassostrea gigas*]	2.80
high mobility group protein DSP1 [Crassostrea hongkongensis]	2.83
apoptosis-inducing factor 3 isoform X2 [*Crassostrea gigas*]	2.89
metallothionein [*Hyriopsis cumingii*]	3.00
epididymal secretory protein E1-like [*Crassostrea gigas*]	3.02
Translocon-associated protein subunit beta [*Crassostrea gigas*]	3.02
L-rhamnose-binding lectin CSL3 isoform X2 [*Crassostrea gigas*]	3.06
protein phosphatase inhibitor 2 [*Crassostrea gigas*]	3.07

hypothetical protein CGI_10023805 [*Crassostrea gigas*]	3.40
uncharacterized protein LOC105335795 [*Crassostrea gigas*]	3.49
X-box binding protein-like protein [*Crassostrea ariakensis*]	3.49
extracellular superoxide dismutase [*Saccostrea glomerata*]	3.58
metallothionein [*Crassostrea ariakensis*]	3.65
protein disulfide-isomerase [*Crassostrea gigas*]	3.68
segon [*Crassostrea brasiliana*]	3.73
ubiquitin [*Crassostrea gigas*]	3.86
hypothetical protein CGI_10004229 [*Crassostrea gigas*]	3.99
hypothetical protein CGI_10016853 [*Crassostrea gigas*]	4.01
probable 60S ribosomal protein L37-A [*Crassostrea gigas*]	4.01
phospholipase D3-like isoform X1 [*Crassostrea gigas*]	4.08
translocon-associated protein subunit alpha [*Crassostrea gigas*]	4.11
uncharacterized protein LOC105341396 [*Crassostrea gigas*]	4.19
uncharacterized protein LOC105347441 [*Crassostrea gigas*]	4.26
hypothetical protein CGI_10023566 [*Crassostrea gigas*]	4.53
CD109 antigen [*Crassostrea gigas*]	4.68
uncharacterized protein LOC105340550 [*Crassostrea gigas*]	4.73
uncharacterized protein LOC105317369 [*Crassostrea gigas*]	4.77
uncharacterized protein LOC105344949 [*Crassostrea gigas*]	5.00
Tyrosine-protein phosphatase non-receptor type 6 [*Crassostrea gigas*]	5.60
uncharacterized protein LOC105346119 isoform X2 [*Crassostrea gigas*]	5.80
hypothetical protein CGI_10020609 [*Crassostrea gigas*]	6.08
uncharacterized protein LOC105318934 [*Crassostrea gigas*]	6.35
metallothionein [*Crassostrea angulata*]	6.67
hypothetical protein CGI_10013060 [*Crassostrea gigas*]	9.68
transmembrane protein 2-like [*Crassostrea gigas*]	10.06
uncharacterized protein LOC105342750 [*Crassostrea gigas*]	232.58

The proteins putatively identified in SWATH-MS study are involved with various biological processes such as sexual maturation, signalling, protein processes, shell formation, metabolism and immunity. We have discussed a few of them in the following section, along with their possible functional roles.

5.6.1a 3.2.1 Proteins expressed more in QXr oysters

Histone subunits

Three identified protein sequences matched closely with histone subunits H4, H2A and H2A isoform. H4 was matched with the marine fish *Larimichthys crocea*, whereas H2A isoform was matched with a disk abalone *Haliotis discus discus* and H2A with *C. gigas,*

reiterating the diversity in the genome identifications. Histone subunits have been implicated previously with reference to oyster response to bacterial infection. Studies performed in the past have identified various subunits such as H4 (Dorrington et al., 2011; Nikapitiya et al., 2014b; Vaibhav et al., 2016) and H2B (Seo et al., 2011). Furthermore, the histone subunit H2A from the mussel *Mytilus galloprovincialis* suggested it plays a role in chromatin dynamics and cell division (González-Romero et al., 2012). Increased expression of histone subunits might be related to stress response to the DNA damage. The presence of histone subunits in diverse molluscan studies emphasises the importance of this protein as a potential biomarker for cellular stress.

Calcium binding proteins

Calmodulin is a calcium binding protein and in mammals it is involved in for contraction of smooth muscle (Walsh, 1994). However, with reference to molluscs, this protein is implicated in biomineralisation (Yan et al., 2007). Overexpression of calmodulin may enhance the process of shell formation (Fang et al., 2008). Although the composition of shell in the pearl oyster is different from Pacific oyster, calmodulin is a one common factor, which may be involved in shell formation of Pacific oyster (Li et al., 2016a). Identification of this protein in this study suggests the same mechanism of biomineralisation may occur in Sydney Rock oysters as well. We also identified the proteins enkurin and transgelin, which are calcium binding proteins and share functional similarities with calmodulin. Enkurin has been reported to play a role in calcium cell-cell signalling by controlling calcium ion influx and efflux (Sutton et al., 2004), whereas transgelin is associated with smooth muscle fibres (Dheilly et al., 2012). In addition to calmodulin, we also identified a subunit of calcium binding protein which may act in association with calmodulin, or it may also be associated with signalling processes (Tomsig et al., 2003), or stress related immune response as reported in previous studies pertaining to molluscs (Bettencourt et al., 2009; Malagoli et al., 2007).

Cell growth and metabolism

We have identified nine proteins associated with cellular growth and metabolism, which we have identified in the previous studies and show increased expression in case of QXr oysters. This could be explained in terms of allocation of resources towards the growth and development of the organism, whereas in the case of unselected hatchery control oysters the resources are distributed more towards fighting for survival.

Kyphoscoliosis peptidase and periostin are found to be related to growth of *C. gigas* and the clam *Meretrix meretrix*, respectively. Other proteins associated with metabolism processes and cell division include MICOS complex subunit Mic19 (Ott et al., 2015), S-crystallin SL11 isoform X (Pales Espinosa et al., 2016), C-Myc-binding protein (David et al., 2005b), cytochrome c oxidase subunit 4 (Cooper et al., 1991; Lockyer et al., 2007), and 14-3-3-like protein (Pignocchi and Doonan, 2011).

ADAM and oxidoreductase

A disintegrin and metalloproteinase with thrombospondin motifs protein, or ADAM, and an oxidoreductase were identified as proteins that were more highly expressed in QXr oysters with functions associated with innate immunity. ADAM is a metalloproteinase that is involved in innate immunity in *Mytilus galloprovincialis (Moreira et al., 2015).* Little has been reported about this protein in the molluscan context, particularly with reference to oysters. However, like other members of this family, ADAM, in addition to microfibril formation, that it has some role in providing immunity to molluscs (Kelwick et al., 2015). Oxidoreductase, on the other hand, is a macrophage migration inhibitory factor (MIF) which is a very important component of innate immunity system (Li et al., 2011; Wang et al., 2009). Originally MIF was believed to control macrophage migration but it is also an important cytokine, which is associated with the inflammatory response of the organism (Kasama et al., 2010) in pearl oysters in response to bacterial infection (Cui et al., 2011). The reason for increased expression of this protein in selected oysters is unclear, however it is possible that there was an unaccounted bacterial, or other, infection in one or more oysters. We could also assert that increased expression of this gene is positive selection because of selective breeding. Further experimental validation, may be able to correlate this finding to disease resistance.

5.6.1b Proteins expressed more highly in HC oysters

Sixty-six proteins were expressed more highly in the HC oysters, of which we could putatively identify 43 proteins, while the rest were either uncharacterized or hypothetical proteins.

Cytoskeletal proteins

We have observed in a number of previous reports of the stress response in bivalves that it is almost always associated with cytoskeletal remodelling (Thompson et al., 2012c). We

have reported similar findings in (Vaibhav et al., 2016) as well as in chapter 3 and 4. In response to the environmental stress (in these cases disease), the cytoskeleton of the organism undergoes remodelling and as a result of which we observe higher expression of proteins associated with the cytoskeleton.

The result of this SWATH-MS study reiterates the same observation by identifying proteins such as actin, radixin, and retrograde protein of 51 kDa isoform X4, which are essential components of the cytoskeleton (Dominguez and Holmes, 2011; Hoeflich and Ikura, 2004).

Host defence

We have also identified proteins associated with host defence such as HMGB1, extracellular SOD, segon, lysozyme 1M, cysteine-rich protein 1, and CD109 antigen. Increased expression of proteins associated with innate immunity in the HC oysters suggests that they are fighting the adverse conditions caused by disease stress or environmental stress. The protein HMGB1 has been implicated as an important protein in oyster innate immunity (Li et al., 2013a; Xu et al., 2012), including in chapter 3 of this thesis. It behaves as a cytokine which initiates the inflammatory response in presence of a pathogen and therefore is an important component of the innate immune response.

Another important finding of this work is the identification of a metal binding protein known as segon. This protein has been identified in the hemolymph of *Crassostrea virginica* where it is a major component of hemolymph proteins and represented about 17% of the total protein (Xue et al., 2012). It is associated with detoxification and anti-oxidation processes, which we know is an important host defence mechanism in bivalves. Furthermore, in *Crassostrea brasiliana*, this protein is highly abundant in the hemolymph of oyster in response to environmental stress (Trevisan et al., 2017). Despite their abundance, we believe this is the first report of identification of segon in Sydney Rock oyster.

We also report findings concerning superoxide dismutase (SOD) and CD109 proteins. Superoxide dismutase in particular has been identified as an important component of bivalves defence mechanism against oxidative stress (Mruk et al., 2002). SOD has been implicated in prevention of cellular apoptosis from oxidative stress in many marine invertebrates (Abele and Puntarulo, 2004) as well as in other organisms such as plants

(Gupta et al., 1993), and it has been identified previously in Sydney Rock oyster as well (Green et al., 2009). As a result of a surge in energy production, to meet the demands of adaptive cellular processes, oxygen free radicals are generated which are detrimental to the organism (Lobo et al., 2010). SODs are a component of the anti-oxidant defence system. They scavenge the free radicals generated and hence attenuate the side effects of increased energy demands (Fukai and Ushio-Fukai, 2011). On the other hand, CD109 in *C. gigas* is associated with immune related processes (Dheilly et al., 2012). This antigen is a cell surface receptor antigen, and identification of this protein is interesting as it shows some sequence similarity to a component of innate immunity to the adaptive immunity.

5.7 Conclusions

This SWATH-MS study contributes greatly towards our understanding of the proteome of the Sydney Rock oyster, as with the help of protein Blast searching, we have provided a putative functional annotation for the proteins identified. Even after many attempts to increase the peptide match percentage, we were only able to identify 23% of the total peptides, which corresponds to 444 proteins, so we are still missing a large number of proteins. It will be interesting to re-examine these results once the genome sequence is well annotated and available.

We have managed to identify proteins which have not been widely mentioned previously in the context of Sydney Rock oyster research, including proteins such as ADAM, calmodulin, MICOS complex subunit, and lysozyme 1M. Furthermore, we have also reported finding SODs and HMGB1 protein, which are well known as host defence proteins in oysters, and consistent identification of histone subunits in this and other previous studies also warrants further investigation.

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Chapter 6

Conclusions

&

Future Directions

Chapter 6. Conclusions and General Discussion

The primary objectives of this thesis were to understand the underlying biological processes of disease resistance in selectively bred Sydney Rock oysters (*Saccostrea glomerata*). The Sydney Rock oyster industry is distributed along the coast of NSW and is one of the major aquaculture industries of the state. Two major diseases which affect the Sydney Rock oyster industry are Queensland unknown disease (QX) and Winter Mortality (WM). These two diseases together are responsible for mortality of more than 90% of the Sydney Rock oyster population during a disease outbreak, resulting in huge economic losses to the oyster industry in NSW. To reduce the mortality caused by these two diseases, a selective breeding programme was established to create a line of naturally resistant Sydney Rock oysters by inbreeding through many generations. However, the molecular level biology driving this selective breeding was not clear and hence the breeding programme has been under threat of reaching a genetic bottleneck. For the continued viability of this industry, it was important to shift from a mass selection towards a marker assisted breeding program for Sydney Rock oysters. Marker assisted selection relies on the identification of genes involved in disease resistance, understanding their implications in biological processes, and using this information to breed oysters. Therefore, as opposed to the conventional selective breeding where prior information about the genetics of the parents is not available, marker assisted selection is a much-improved scientific approach backed up with genetic information regarding which factors are driving the selection of oysters.

With regards to the aim of identifying markers for sustainable and robust oyster lines, this thesis provides whole cell proteome information for selectively bred and unselected Sydney Rock oysters, which can be used to identify disease biomarkers, thereby filling the knowledge gap of underlying biological selection. The following sections summarise the findings of this project which extended the knowledge about QX and WM disease resistance in Sydney Rock oysters.

6.1 Winter Mortality

Chapter 2 reported and discussed the outcomes of two-dimensional gel electrophoresis (2DE) based proteomics analysis of WM selected Sydney Rock oysters with unselected hatchery controls (HC). This study, to the best of our knowledge was the first proteomic profiling study initiated concerning WM in oysters. The disease was previously believed to be a protozoan (*Bonamis sp*.) borne disease but a recent study suggested that *Bonamia*

might not be the only aetiological agent (Spiers et al., 2014), and it is now believed that environmental factors play an important role as well.

Two-dimensional protein gel electrophoresis analysis, in conjunction with LC-MS/MS of specific protein features, of Sydney Rock oysters selected for winter mortality disease resistance and unselected hatchery control oysters revealed that there is a general stress response as exhibited by cytoskeletal remodelling, as well as unique responses that we have reported for the first time through this work. Cytoskeletal remodelling has been observed in a number of studies concerning stress response in bivalves, as observed in the study of QX disease in Sydney Rock oysters (**Chapter 3** of this thesis), where we have reported a time dependent proteome response (Thompson et al., 2012c).

Amongst the unique findings, we have identified proteins such as CDPK31 associated with winter mortality, which has not been reported previously with reference to oyster disease resistance. This protein demands further research to understand its involvement in facilitating disease resistance. Other general stress response proteins such as HSP70, histone H4 and proteasome subunits have been identified in this study, which accentuates the importance of this study in understanding the stress response. The proteome coverage of 2DE gels is not as good as shotgun proteomics but the findings of this study provide a very good starting point for future work. The lack of previous research in WM disease of oysters, and shortage of oyster samples resistant to WM, was an acknowledged limiting factor in this work. Further work employing shotgun proteomics (both isotopically labelled and unlabelled) could provide further information substantiating the findings of this study.

6.2 Queensland Unknown disease

QX disease, also known as summer mortality, affects Sydney Rock oysters during the summer season in Australia (December – April). This disease is caused by the protozoan *Marteilia sydneyi*, however, it is reported that the severity of disease is also affected by salinity and temperature. Previous studies have identified a few relevant genes which are involved, for example, an isoform of phenoloxidase which contributes to disease resistance and is heritable from generation to the next. This idea led us to believe that there are factors driving the selection about which we do not know, and this thesis makes an effort in the direction of identifying those factors as well as understanding their biological implications.

Chapters 3, 4 and 5 in this thesis dealt with the study of the Sydney Rock oysters selected for QX disease resistance.

In **Chapter 3** we performed a time dependent disease progression study of the selected and hatchery control unselected Sydney Rock oysters. The oysters were collected at two time-points; the first time-point being the end of February and second time-point being the first week of April. Sample collection at more than one time-point is very important when studying the relationship of host and pathogen, as it helps to identify the time dependent response of the organism with the parasitic development inside of the organism, as well as with the prevailing environmental conditions. A possible disease outbreak was observed two weeks prior to the first time-point oyster sample collection from the Hawkesbury River, although the extent of the disease outbreak was not recorded and no mass mortalities were observed. Therefore, it was assumed that some disease stress was imposed on both, the HC and QXr oyster populations.

As has been seen previously in other stress response studies in bivalves, we observed cytoskeleton proteins are more abundantly expressed in unselected populations at both time-points. However, the cytoskeletal proteins observed at both time-points differed in the expression pattern. The cytoskeletal proteins of time-point one were associated with actin bundling. Proteins such as actinin, villin and unc-87 are all reported to play roles in maintaining the actin structure. Comparing the QXr and HC population, QXr oysters show a lower abundance of proteins associated with energy production (e.g. ATP synthase), which is directly related to increased adaptive cellular processes as a result of disease stress (Thompson et al., 2015). Whereas, on the other hand, proteins associated with immunity (e.g. HMGB) were significantly more abundant.

At time-point two, we observed a clear progression in terms of cellular response to stress, in the form of cytoskeletal breakdown as exhibited by upregulation of actin proteins in unselected HC oysters (i.e. downregulation in QX). We also observed lower abundance of proteasome subunit proteins in the QXr population; proteasome proteins are responsible for proteolysis which we believe might be causing cytoskeleton breakdown. In addition to that we also observed that the cell still appears to keep up with the increased demand for energy production, such as the increased production of proteins associated with glycolysis – Isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase.

In **Chapter 4** we reported on a comparative proteomic investigation of shoreline catch wild Sydney Rock oysters with a hatchery bred selected line of Sydney Rock oysters. This study was performed to identify the difference in proteome of the selected line of oysters as a result of continuous selection, compared with that of wild oysters distributed along the nearby shore of Woolooware bay. The wild oysters represented the population which were exposed to different environmental stresses such as variations in water temperature and salinity, struggles for food and substratum, and the presence of parasites. Therefore, the proteome changes reflect the gross change in proteome as a result of all of the above stressors. Our results indicated the difference in proteome was very clear, and amongst the proteins upregulated in wild oysters, 65% were exclusively expressed in the wild oyster whole cell proteome. This could be attributed to the fact that because of continuous inbreeding amongst the small number of the founding population over many generations, the selected line of oysters shares a very similar genetic makeup. On the other hand, the wild population represented a much more varied genetic spectrum. Our results indicate a lack of exchange of genome sequence between the two populations, despite the fact that the site has been used for selective breeding alongside wild oysters for almost three decades.

In **Chapter 5**, we analysed the proteome of the digestive gut tissue of Sydney Rock oysters collected from Hawkesbury River. These oysters were collected towards the end of summer in Australia, which corresponds to the end of the usual infection period of QX disease. We performed SWATH-MS analysis of the digestive gut tissue of the oysters. This study was initially limited by unavailability of annotated genome sequence information for the Sydney Rock oyster and hence, during the initial phase it looked to be impossible to identify peptides at a satisfactory level. Peptide to spectrum matching using the *C. gigas* genome sequence as a reference produced peptide identifications just touching 7% of the total peptide spectra acquired, whereas with the *P. fucata* genome sequence as a reference we only identified peptides from 2% of the acquired spectra. For comparison, most experiments performed using this approach in other biological systems achieve peptide to spectrum matching success rates of 60-70%. This pushed us to think about using the Sydney Rock oyster genome sequence information, which no one had used previously for proteomics study. The use of a recently obtained draft version of the Sydney Rock oyster genome sequence increased the success rate for peptide to spectrum matching to 23%, and we managed to identify a total of 444 significant proteins. The proteins however, had no

functional annotations so we were required to perform manual protein blast searches for each of them, which enabled us to report putative identities for many of them.

6.3 Future directions

This comparative proteomic investigation of diseases of Sydney Rock oysters has allowed us to gain a better understanding of disease resistance. Other biochemical and ecotoxicological approaches in Sydney Rock oysters have also added to our understanding in different ways. Being an important part of the marine ecosystem, oyster studies really need to be backed up with ecological and physiological studies as well. Detailed knowledge of aspects such as water temperature, pH, salinity levels and other contaminants could add value to the work and help us to understand the findings better. It is worth mentioning that NSW DPI have recently launched a pilot program installing real time salinity sensors in a number of estuaries along the New South Wales coastline. It would be very interesting in future to examine changes in the proteome of Sydney Rock oysters and how this varies in response to specific changes in environmental salinity levels.

The work in this thesis has added strong proteomics based information to the already existing knowledge as well as provided new insights into the disease prevention strategies of Sydney Rock oysters. Molluscs are relatively understudied organisms and the proteomics aspect of the research in many cases is still virgin territory mainly because of the lack of a sequenced and annotated genome.

This is the first shotgun proteomics investigation of gills and gut tissue of Sydney Rock oysters. This study of Sydney Rock oysters, moreover, is the first to employ recently available genome sequence data of *Saccostrea glomerata* for a shotgun proteomics study. This makes this study novel, and since proteomics is a continuously expanding field there will always be new additions. The first, and one of the most important, requirements for expedited development in Sydney Rock oysters proteomics study is availability of a fully annotated genome sequence of *Saccostrea glomerata*. This will have significant influence on the future work, both in proteomics and transcriptomics.

Our work has provided evidence of the importance of comparative proteomics in two different oyster populations and we firmly believe that with recent technological advancements, this field will expand further. Tandem mass spectrometry has been widely

used, but of late the improvements in data acquisition have improved the proteome coverage of the biological samples examined. We analysed one set of samples with a quadrupole ion trap mass spectrometer (Schwartz et al., 1991), and another set with a hybrid quadrupole - orbitrap mass spectrometer. Orbitraps are advanced mass spectrometry devices with greatly improved mass accuracy accompanied with higher resolving power (Zubarev and Makarov, 2013).

Similarly, there are other advanced mass spectrometry techniques which could be employed to great effect in future studies, such as single reaction monitoring (SRM) mass spectrometry, which employs the peptide information for specific proteins that are quantified in a targeted experiment. Using that information, it quantifies selectively even low abundant proteins. In contrast to the shotgun mass spectrometry, SRM does not aim to explore the complete proteome, it only reads specific sets of peptides which are determined beforehand. SRM could be particularly useful in validation study of less studied organisms, including oysters, where very few commercial antibodies are available. With the peptide spectra information of desired proteins from shotgun identification experiments, we can monitor the expression change of that particular protein in different populations.

Furthermore, an emerging new technique called SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra) is also available for studying the whole cell proteome (Gillet et al., 2012). We have provided in this thesis the first proof-of-concept data which shows that SWATH can indeed be used on oyster tissues. This technique addressed some of the issues concerning the accuracy and reproducibility associated with conventional shotgun proteomics approaches, such as the inability to cover the whole proteome as a single large fraction.

SWATH-MS relies on data independent acquisition (DIA), which means that no prior information about the precursor ion to be fragmented is required, unlike SRM. Quantitation can be performed on peaks representing peptide features, which is completely decoupled from the identification steps. Using sequential windows based on different m/z values the acquisition is performed covering both low and high abundant peptides during the retention time of LC for a single large fraction. However, like any other mass spectrometry based proteomic study of a biological sample, SWATH – MS is not an exception when it comes to the need for a reliable annotated database of genome sequence information in order to

perform successful peptide to spectrum matching. In **Chapter 5** we have reported a preliminary SWATH-MS analysis, wherein we have used an unannotated Sydney Rock oyster genome sequence database to increase the percentage of peptide identifications. The Sydney Rock oyster gene sequences were unannotated, which can be a hurdle for high throughout proteomics analysis where protein identity and function are of importance.

Upon using the Sydney Rock oyster genome sequence information, we succeeded in identifying a larger percentage of peptides compared to when we used the *C. gigas* genome sequence information for peptide identifications. Therefore, it would be a good idea to reanalyse thoroughly the database searching results in **Chapters 2,3 and 4** using this new gene sequence information. In comparison to our studies using the *C. gigas* genome sequence database, it will be very interesting to see how the outcome improves. This SWATH-MS proteomics study contributes towards the Sydney Rock oyster genome assembly efforts and we believe it is a good starting point for future work.

In addition to this, isotopically labelled mass spectrometry studies such as TMT (Tandem Mass Tags) labelling and iTRAQ (Isobaric Tag for Relative and Absolute Quantitation) could also be beneficial in oyster research. TMT labelling allows the user to quantify similar peptides from different replicates much more precisely compared to unlabelled shotgun proteomics (Thompson et al., 2003). This feature is beneficial in biomarker research as the fold changes are defined more accurately. In a similar fashion, iTRAQ is another labelling based quantitative mass spectrometry technique, which employs isobaric reagents to quantify proteins (Ross et al., 2004).

Proteomics studies often require validation study to confirm the findings, in this regards immunoassays, typically western blots are a reliable and easy experiment to perform. Immunoassays rely on very specific protein-antibody interactions and therefore it is crucial for the success of experiment that the theoretical homology of the protein being examined and the protein used for antibody production is as close to 100% as possible. Immunoassays for molluscan study and oysters in general have been greatly limited by unavailability of antibodies. Very little work has been done in oysters with respect to immunoassays such as western blotting and ELISA, primarily because of low specificity of the antibodies that are commercially available. Therefore, for additional follow up work in future it would be very interesting to look at the western blot profiles of certain proteins which have been identified

in this study. This, of course, relies on appropriately specific antibodies being developed and produced. One potential alternative for validation experiments would be to use the SRM capability of high resolution mass spectrometry, and design follow-up experiments based on quantitative analysis of specific peptides, rather than a general shot gun approach. In this thesis, we have produced some information of this nature as part of the SWATH-MS data acquisition in **Chapter 5**, and the quality and specificity of the results are highly encouraging, especially for a first attempt.

6.4 Closing Statement

Taken together, this thesis describes a substantial body of work which represents a significant advancement in our fundamental understanding of the molecular biology and cellular mechanisms of disease progression and resistance in Sydney Rock oysters. It provides high value information for feeding back into the ongoing selective breeding program, and lays a strong foundation for future research in the field.

6.5 References

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Appendices

Appendix I Supplementary files

Chapter 2

Supplementary Table S1: Details of 2DE spots and their LC-MS/MS identifications with corresponding matched peptides (minimum 5) and experimental molecular weight (Mr). Spots 1-7 (shaded red) are more intense in winter mortality selected oysters whereas spots 8-22 are less intense. The filtering criteria of minimum 5 matching peptides resulted in more than one proteins per spot, on many occasions. Tabs 1-22 shows the LC-MS/MS details corresponding to the spots.

Chapter 3

Supplementary Table S2: The summary tab of this excel sheet shows the list of the significant proteins identified in the Hawkesbury River time-point one study. Other tabs provide for each set of proteins (upregulated, downregulated and unchanged), the details of LC-MS/MS runs. The NSAF values and p values for each replicate are shown and subsequent calculation of fold changes are represented in the table.

Supplementary Table S3: The summary tab of this excel spreadsheet shows the list of the significant proteins identified in the Hawkesbury River time-point two study. Other tabs provide for each set of proteins (upregulated, downregulated and unchanged), the details of LC-MS/MS runs. The NSAF values and p values for each replicate are shown and subsequent calculation of fold changes are represented in the table.

Supplementary Table S4: This excel spreadsheet provides the PloGo annotation of significant proteins obtained from both time-point studies. The table identifies the gene ontology terms in tab "GOSummary" as well as the distribution of proteins which is used to obtain the heat map.

Chapter 4

Supplementary Table S5-7:

The excel spreadsheet consists of the scrappy output for Upregulated, downregulated and unchanged proteins in separate excel sheets.

Chapter 5

Supplementary Table S8: This table provides the details of SWATH-MS run. Tab "Area-Proteins" shows the proteins identified using the unannotated Sydney Rock oyster database. Whereas the column NCBI-protein blast lists the corresponding putative protein identifications. This table also shows the protein areas (normalised and un-normalised) used for calculation for fold changes. Tab "Area-ions" shows the peptide details of all peptides identified in the study.

Appendix II of this thesis have been removed as they may contain sensitive/confidential content