Responses to double stranded RNA in oysters:

developing a model for antiviral immunity

Muhammad Masood

MSc. (Hons) P.B.G., UAF Pakistan

Department of Biological Sciences

Macquarie University

North Ryde

NSW 2019

A thesis submitted to Macquarie University in partial fulfilment of the requirement of the degree of Doctor of Philosophy

March 2016

Declaration of Authorship and Originality

I certify that the work in this thesis, entitled' Responses to double stranded RNA in oysters: developing a model for antiviral immunity' has not previously been submitted for a degree nor has it been submitted as part of requirement for a degree to any other university or institute other than Macquarie University.

    I certify that work presented in this thesis contains only original work that has been written by me. In addition, I also certify that all information and literature sources used during the preparation of this thesis have been acknowledged.

……………………………………………..

Muhammad Masood

Acknowledgements

I would like to say thanks to my supervisor Dr. Sham Nair, a great teacher and my mentor. He provided extraordinary guidance and advice on professional and personal matters throughout my candidature or whenever needed. I have learned a lot from Sham's experience in teaching and research. I also thank Sham for his kindness, accessibility and support.

I am grateful to my other supervisor Professor Marie Herberstein for providing me guidance & support to keep me on track during my candidature. She is fantastic writer and her help during the writing of my manuscripts were extremely precious.

My co-supervisor Professor David Raftos has always provided an immense support and advice. It was a great pleasure and honour to learn from David's experience and knowledge.

I am so grateful to my parents my mom (Appa) Zubaida Maqbool and dad (Abee) Mr. Muhammad Maqbool who worked hard and sacrificed a lot in providing me with the best education in top education institutes in Pakistan. It's their love, kindness, guidance and support that made my life such a wonderful experience.

I would like to say big thanks to my wife Nadia Masood for her support through this journey. Her companionship has been great asset in my life. There were difficult times in life when I needed someone to talk to and she was always there to listen and support. My three kids Hurairrah, Eshal and Salba are amazing, their love and smiles provided me strength. I would like to say many

Summary

The aquaculture industry faces challenges and major economic losses from infectious disease outbreaks of farmed stocks. Ostreid herpes virus type 1 (OsHV-1 µvar) infection of larval and juvenile Pacific oysters is a worldwide phenomenon. This thesis investigates responses to exogenous double stranded RNA (dsRNA) in two oyster species that are farmed extensively in Australia - the Pacific oyster (*Crassostrea gigas*) and Sydney rock oyster (*Saccostrea glomerata*). dsRNAs are products of viral infection and in many biological systems, dsRNA provokes strong immune responses. While *C. gigas* is susceptible to viral infection, *S. glomerata* appears to be resistant. This study examines molecular basis for the differential susceptibility to OsHV-1 infection using state-of-the-art mass spectrometry (iTRAQ and SWATH) proteomics, transcriptomics and fluorescence microscopy techniques. This is the first comparative study of the immune systems of two oyster species that behave differently to OsHV-1 and also provides the proteome map of the Sydney rock and the Pacific oysters.

Both oyster species were capable of detecting exogenous dsRNA, but differed in the patterns of double stranded RNA uptake and processing, as well as in their proteomic responses to that perturbation. Gill tissues are an important site of dsRNA processing and response. In response to dsRNA, Sydney rock oysters activated cellular signalling pathways, as well as apoptotic (cell death) pathways, that are typical of antiviral responses in metazoans. These responses were absent in the Pacific oysters. The data provides a useful model for further studies into resistance to viral infection in these oysters, as well as unravelling the molecular mechanisms that underpin the biological differences in viral susceptibility.

Table of Contents

List of Common Abbreviations

μg	microgram
μl	microlitre
CTCF	Corrected total cell fluorescence
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded Ribonucleic acid
FISH	Fluorescence in situ hybridization
GFP	Green fluorescence protein
h	Hour
HSV-1 and -2	Herpes simplex virus-1 and -2
IFI44	Interferon induced 44
IFN	Interferon
IFN-α/β	Interferon Alpha/Beta
IMD	Immune deficiency
IRF3	Interferon Regulatory Factor 3
iTRAQ	Isobaric tags for relative and absolute quantitation
ml	millilitre
mRNA	Messenger Ribonucleic acid
NF-κB	Nuclear factor kappa B
NLR	Nod-like receptor
NSW	New South Wales
OsHV-1	Ostreid herpes virus type 1
OsHV-1 μvar	Ostreid herpesvirus-1 microvariant
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKR	Protein Kinase R
PO	Pacific Oyster
Poly (A:U)	Polyadenylic–polyuridylic acid
Poly (I:C)	Polyinosinic-polycytidylic acid
PRRs	Pattern Recognition Receptors
PTGS	Post transcriptional gene silencing

qPCR	quantitative Polymerase chain reaction
qRT-PCR	quantitative Reverse transcription Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RTPCR	Reverse transcription Polymerase chain reaction
SID	Systemic interference defective
siRNA	small interfering Ribonucleic acid
SOD	Superoxide dismutase
SR	scavenger receptor
SRO	Sydney Rock Oyster
ssDNA	Single stranded Deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
SWATH	Sequential Window Acquisition of all Theoretical fragment ion spectra
TLR	Toll like receptor

Introduction to thesis

Summary

The work described in this thesis outlines investigations into the proteomic responses of oysters (*Crassostrea gigas* and *Saccostrea glomerata*) to double stranded RNA (dsRNA). Metazoan immune systems possess receptor proteins that recognise dsRNA and subsequently invoke responses that induce an 'anti-viral' state in the host. Collectively, those responses are termed anti-viral responses or anti-viral immunity. This investigation is part of a larger project that examines immunity and anti-pathogen defenses in oysters.

Background

Molluscs are an important dietary component of many human societies consumption and parts of molluscs are used in a variety of industrial applications. It has been estimated that approximately 15 million tonnes of molluscs are produced in aquaculture farms internationally, of which oyster production constitutes more than 4 million tonnes [1].

The aquaculture industry faces challenges from infectious disease outbreaks of farmed stocks, which result in major economic losses. Studies have indicated that molluscs are subject to infection by various pathogens, including viruses and protozoan parasites. Haplosporidian and Cercozoan parasites are reported to infect the European flat oyster (*Ostrea edulis)* and Sydney Rock oyster [2-5]. Haplosporidian parasites also infect pearl oysters (*Pinctada maxima*), abalone (*Haliotis tuberculate*), mussel (*Dreissena polymorpha*), crab (*Carcinus maenus*) and gastropods (*Siphonaria* lessonii, *Siphonaria pectinata*) [6, 7].

In addition to parasites, viruses pose a significant threat to molluscs, causing disease and mortality in farmed abalone and oysters. The abalone

ganglioneuritis virus, first detected in 2005 in Victoria Australia, is a pathogen of abalone [8], while Iridovirosis infect Pacific oysters, European flat oysters and Portuguese oysters [9, 10]. There are several reports of herpes virus infections of Pacific oysters worldwide [11-15]. Ostreid herpes virus type 1 (OsHV-1) causes up to 100% mortality amongst larval and juvenile Pacific oysters [16]. OsHV-1 has been detected in three estuaries the Georges River-Port Botany, Port Jackson-Sydney Harbour and Hawkesbury River-Brisbane Water in NSW Australia. Thus far, this virus has been restricted to NSW and there have been no reports of its identification in the other parts of Australia.

Very little known about antiviral immunity in oysters. However, some studies indicate the presence of antiviral reactions in clams, Pacific oysters and pearl oysters [17-19]. Pacific and Sydney rock oysters are capable of detecting bacterial DNA, viral and synthetic double stranded RNA molecules [20-23]. Of the different families of PRRs that bind pathogen nucleic acids, the Toll like receptors (TLRs) are thought to play a central role in the activation of antiviral responses. Recent studies have identified various genes associated with TLR-mediated intracellular signalling pathways in oysters. Interferons, which are proteins that induce an 'anti-viral' state in hosts, is produced in many animals as a key response to viral infections. Infection by OsHV-1 evokes the production of interferon-stimulated genes Viperin, IFI44, PKR and ADAR in Pacific oysters [23, 24].

Proteins represent the functional end of the information flow in cells (i.e. the Central Dogma of molecular biology). Thus, proteins are the workhorses of cells and undertake most molecular functions that are central to life. Proteomics is the study of the total protein complement in a biological sample. Proteomics has

been used to provide snapshots of protein-level changes that occur in biological samples as a result of perturbed states (e.g. infected vs. non-infected samples). Such data can provide glimpses of molecular changes that underpin those changes in biological states. Mass spectrometry based proteomics is a powerful tool to identify and quantify thousands of proteins in complex samples [25]. This approach can be used to measure and characterise protein expression profiles in a given sample for potential biomarker discovery. Modern proteomics labelling and quantitation techniques such as isobaric tags for relative and absolute quantitation (iTRAQ) and sequential window acquisition of all theoretical fragment ion spectra (SWATH) can be employed to study quantitative changes in proteins.

Research aims and approach

This thesis investigates responses to exogenous dsRNA in two oyster species, Pacific oyster (*C. gigas*) and Sydney rock oyster (*S. glomerata*). The aim of this thesis is to establish the molecular basis for the differential susceptibility to OsHV-1 infection using a combination of proteomics, transcriptomics and fluorescence microscopy techniques. This is the comparative study of the immune systems of two oyster species that behave differently to OsHV-1, even though they are cultured in the same estuaries and river systems. The data presented in this thesis also provides proteome map of the Sydney rock oyster and the Pacific oyster. Thus far, our understanding has been limited to the use of genomic and transcriptomic methods. This study, however, focuses on proteins involved in responses to dsRNA. All chapters (except chapter 5) are self-contained manuscripts and formatted according to the specific guidelines of the journals that they have been/will be submitted to.

Layout of the thesis

Chapter 1 is a detailed review of the current literature in which I discuss various immuno-defense mechanisms that may protect invertebrates (especially molluscs) against viral infections including viral RNA silencing. Mechanisms that silence the expression of exogenous genes (e.g. genes that of viral origin) are critical to antiviral defenses. The aim of this review is to collate information on viral diseases in invertebrates of interest to aquaculture, as well as, invertebrate immunity, particularly antiviral defences. I also highlight how dsRNA can be used as a tool for post transcriptional gene silencing.

Chapter 2 investigated the distribution of fluorescently-labelled exogenous double stranded RNA in oyster tissues. Using confocal fluorescence microscopy, the fluorescence emanating from the dsRNA was tracked in different oyster tissues at various times after injection. Furthermore, changes in the expression of several target genes (Immune response genes) in those tissues were also investigated using qPCR.

Chapter 3 provides the first insight in to the proteome map of the Pacific oyster and Sydney rock oyster by using isobaric tags for relative and absolute quantitation (iTRAQ) tandem mass spectrometry. This aim of the study described in this chapter was to look at the differences in total proteome expression for these two closely related species when they were challenged with non-gene specific (generic) dsRNA (poly I:C and poly A:U). In addition, the transcriptome of those oysters were also interrogated using qPCR to determine if the expression of the targets identified in the proteomic investigation (as well as those identified in the literature) show similar expression patterns.

Chapter 4 provides a more comprehensive analysis of proteome mapping using SWATH (sequential window acquisition of all theoretical fragment ion spectra) Mass spectrometry. This newly developed mass spectrometric assay permits a more sensitive and accurate interrogation of proteomic changes that result from biological perturbations. In the study described in this chapter, I dissected the effects of two different generic dsRNAs, as well as evaluated time-dependent proteomic responses to those dsRNAs.

Chapter 5. The final component of this thesis is a discussion of conclusions drawn from this study.

References

1. FAO, *The State of World Fisheries and Aquaculture*. 2014, Food and Agiculture Organisation of the United Nations: Roma.

2. Carrasco, N., et al., *Bonamia exitiosa (Haplosporidia) observed infecting the European flat oyster Ostrea edulis cultured on the Spanish Mediterranean coast.* J Invertebr Pathol, 2012. **110**(3): p. 307-13.

3. Carnegie, R.B., et al., *The haplosporidian Bonamia exitiosa is present in Australia, but the identity of the parasite described as Bonamia (formerly Mikrocytos) roughleyi is uncertain.* J Invertebr Pathol, 2014. **115**: p. 33-40.

4. Carrasco, N., T. Green, and N. Itoh, *Marteilia spp. parasites in bivalves: A revision of recent studies.* J Invertebr Pathol, 2015. **131**: p. 43-57.

5. Perkins, F.O. and P.H. Wolf, *Fine Structure of Marteilia sydneyi sp. n.: Haplosporidan Pathogen of Australian Oysters.* The Journal of Parasitology, 1976. **62**(4): p. 528-538.

6. Arzul, I. and R.B. Carnegie, *New perspective on the haplosporidian parasites of molluscs.* J Invertebr Pathol, 2015. **131**: p. 32-42.

7. Friedman, C.S., et al., *'Candidatus Xenohaliotis californiensis', a newly described pathogen of abalone, Haliotis spp., along the west coast of North America.* Int J Syst Evol Microbiol, 2000. **50 Pt 2**: p. 847-55.

8. Hooper, C., P. Hardy-Smith, and J. Handlinger, *Ganglioneuritis causing high mortalities in farmed Australian abalone (Haliotis laevigata and Haliotis rubra).* Aust Vet J, 2007. **85**(5): p. 188-93.

9. Elston, R., *Bivalve mollusc viruses.* 1997(4): p. 393-403.

10. Williams, T., *Natural invertebrate hosts of iridoviruses (Iridoviridae).* Neotropical Entomology, 2008. **37**: p. 615-632.

11. Farley, C.A., et al., *Oyster herpes-type virus.* Science, 1972. **178**(62): p. 759-60.

12. Jenkins, C., et al., *Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in Crassostrea gigas (Pacific oysters) in Australia.* Dis Aquat Organ, 2013. **105**(2): p. 109-26.

13. Segarra, A., et al., *Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, Crassostrea gigas, in France in 2008.* Virus Res, 2010. **153**(1): p. 92-9.

14. Friedman, C.S., et al., *Herpes virus in juvenile Pacific oysters Crassostrea gigas from Tomales Bay, California, coincides with summer mortality episodes.* Dis Aquat Organ, 2005. **63**(1): p. 33-41.

15. Renault, T., et al., *Concomitant herpes-like virus infections in hatchery-reared larvae and nursery-cultured spat Crassostrea gigas and Ostrea edulis.* Dis Aquat Organ, 2000. **42**(3): p. 173-83.

16. Government, A., *FINAL REPORT OsHV-1 µVar* in *International OsHV-1 µVar workshop* 2011, Fisheries Research and Development Corporation: Cairns, Queensland, Australia

17. Li, M.F. and G.S. Traxler, *Antiviral activity of aqueous clam (Mya arenaria) extract on amphibian virus (LT-1).* Can J Microbiol, 1972. **18**(4): p. 397-402.

18. Olicard, C., et al., *Putative antiviral activity in hemolymph from adult Pacific oysters, Crassostrea gigas.* Antiviral Res, 2005. **66**(2-3): p. 147-52.

19. Tan, T.L., et al., *Resistance of Black-lip learl oyster, Pinctada margaritifera, to infection by Ostreid herpes virus 1muvar under experimental challenge*

may be mediated by humoral antiviral activity. Fish Shellfish Immunol, 2015. **44**(1): p. 232-40.

20. Aladaileh, S., S.V. Nair, and D.A. Raftos, *Induction of phenoloxidase and other immunological activities in Sydney rock oysters challenged with microbial pathogen-associate molecular patterns.* Fish Shellfish Immunol, 2007. **23**(6): p. 1196-208.

21. Zhang, Y., et al., *Characteristic and functional analysis of toll-like receptors (TLRs) in the lophotrocozoan, Crassostrea gigas, reveals ancient origin of TLR-mediated innate immunity.* PLoS One, 2013. **8**(10): p. e76464.

22. Green, T.J. and A.C. Barnes, *Inhibitor of REL/NF-KappaB is regulated in Sydney rock oysters in response to specific double-stranded RNA and Vibrio alginolyticus, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible.* Fish Shellfish Immunol, 2009. **27**(2): p. 260-5.

23. Green, T.J. and C. Montagnani, *Poly I:C induces a protective antiviral immune response in the Pacific oyster (Crassostrea gigas) against subsequent challenge with Ostreid herpesvirus (OsHV-1 muvar).* Fish Shellfish Immunol, 2013. **35**(2): p. 382-8.

24. Green, T.J., et al., *OsHV-1 countermeasures to the Pacific oyster's anti-viral response.* Fish Shellfish Immunol, 2015. **47**(1): p. 435-43.

25. Aebersold, R. and M. Mann, *Mass spectrometry-based proteomics.* Nature, 2003. **422**(6928): p. 198-207.

Chapter 1

General Introduction

This chapter will be submitted as review article to *Journal of Fish Diseases*. It has been modified to include additional information on proteomics for this thesis.

Author contributions:

Muhammad Masood – Concept development, literature search and writing of chapter.

Marie E. Herberstein – Technical advice and feedback

David A. Raftos – Project supervision and feedback

Sham V. Nair – Project supervision and feedback

1. Abstract

Aquaculture is an important component of many international economies. According to Food and Agriculture Organisation of the United Nations in 2012 the total world aquaculture production was 66.6 million tonnes and the global production of molluscs was over 15.1 million tons with a commercial value of US $15.85 million. With high intensity aquaculture, diseases from virus infections remain a constant threat to the aquaculture industry. Viral diseases in animals used in aquaculture have had disastrous consequences on affected economies worldwide, but control of viral disease remains a serious global challenge. Invertebrates have evolved various immuno-defense mechanisms that may protect them against viral pathogens. These mechanisms include systems that involve viral RNA silencing. Mechanisms that silence exogenous gene expression are critical antiviral defenses. The aim of this review is to collate the known viral diseases in invertebrates that are of interest to aquaculture. We discuss innate responses, how the defense mechanism works against these viruses and how RNA silencing or RNA interference (RNAi) may be a potent mechanism against viral infections. We also highlighted how dsRNA can be used as a tool for post transcriptional gene silencing (PTGS).

Key words: RNAi, invertebrates, dsRNA, delivery methods, PAMPs, virus, immunity.

2. Viruses of invertebrates

It is estimated that there are 10^{30} virioplankton in the world's oceans and the majority of these viruses are phage viruses that infect bacteria (Parsons et al., 2012). Animal viruses have caused mass mortalities in a number of species that important to the aquaculture industry (Fuhrman, 1999). Bivalves are filter feeders that digest single celled algae and other benthic debris. Consequently, they may bio-accumulate infectious viruses, thus resulting in morbidity and mortality (Paul-Pont et al., 2013b). Virus infections can cause significant losses of stocks and income in wild and farmed environments (Renault and Novoa, 2004). There are no global data available about the loss due to viral diseases, but it has been estimated that in 2010, China's aquaculture industry suffered production loss of around 295,000 tonnes (FAO, 2014). Despite the critical threat to aquaculture, little is known about the viruses that affect molluscs.

3. Viral pathogens of crustaceans and molluscs

Viral pathogens of crustaceans include white spot syndrome virus, yellow head virus and Taura syndrome virus. White spot syndrome virus causes enlarged nuclei and histopathological changes in the cells of gill, stomach, adipose, connective, hematopoietic tissues, as well as haemocytes. Yellow head virus infections show necrosis in the lymphoid organ, gills, connective tissues, haemocytes, and hematopoietic organs and Taura syndrome virus has been reported to target the cuticular epithelia of the foregut, hindgut, appendages, gills as well as general body cuticle (Liu et al., 2009).

Irido-like virus, Herpes-like virus and Papova-like viruses are reported to infect some oyster species. Irido-like viruses contain a single copy linear dsDNA genomes that range in size from 150 to 280 kbp, depending upon the viral species

involved. They are known to infect Portuguese and Pacific oysters. Irido-like viruses such as gill necrosis virus, haemocyte infection virus and oyster velar virus cause infection of branchial epithelial cells, an acute haemocyte inflammation and mortality. Oyster velar virus disease results in inflammation and swamping of ciliated velar epithelial cells forming blisters and a loss of cilia in larvae (Elston, 1997).

Herpes-like viruses were identified in adult oysters (*C. gigas*) from the USA, and New Zealand and European flat oysters (*Ostrea edulis* L) from France. All members of Herpesviridae have a dsDNA linear genome. Herpes virus infection in American oysters was reported to be more prevalent when the latter were grown at elevated temperatures of 28-30ºC, resulting oyster death (Farley et al., 1972). These viruses and virus like particles damage organelles such as nuclei (enlargement), swollen mitochondria and marginated chromatin. Ultra-structural examinations have revealed swollen mitochondria, condensed nuclei and abnormal accumulations of the granular endoplasmic reticulum in infected cells (Farley et al., 1972, Nicolas J. L., 1992, Comps and Cochennec, 1993, P. M. Hine, 1998, Yodmuang et al., 2006, Segarra et al., 2010). Herpes-like viruses have also been reported to infect abalone (*Haliotis* spp.) and were subsequently termed abalone viral ganglioneuritis as it causes inflammation of the nerve ganglia (Ellard K., 2009).

Papova-like virus infections were reported in golden-lipped pearl oysters *Pinctada maxima* from Torres Strait, Australia. This virus affects epithelial cells whose nuclei consequently enlarge up to seven times compared to non-infected nuclei (Norton et al., 1993).

Roni-like viruses, which have been identified in Chinese mitten crab, are enveloped, bacilliform-shaped and about 150-200 nm x 40-60 nm in size. Gills of

the infected animals were dark grey, partly black or dark black depending upon the severity of the infection. Light microscopy shows signs of tissue degeneration & abnormal nuclei (Zhang and Bonami, 2007). A reovirus was also reported from Chinese mitten crab *Eriocheir sinensis*. This virus infects the connective tissue of the gills, gut and hepatopancreas (Zhang et al., 2004).

It is obvious that infection by these viruses can cause poor health, infection, disease and death of their hosts. Factors such as stress, high density, reduced water quality, translocation and introduction of pathogens and transmission of pathogens through inter-specific or other closely related species are known to contribute to the incidence of viral infections in aquaculture (Kent, 2000, Walker and Winton, 2010,). There are few direct demonstrations of how the viruses actually infect their molluscan hosts. In abalone, the virus infects the animals through direct contact with infected animals or their mucus. Infection of the Pacific oyster, *Crassostrea gigas,* and the European flat oyster, *Ostrea edulis,* by *Ostreid Herpesvirus* microvariant (OsHV-1) is temperature dependent and usually occurs when water temperature exceeds 16°C. Animals are infected when they are cultivated in water that is contaminated with virus and diseased animals. There are compelling scientific and economic imperatives to study virus-host interactions in farmed molluscs, as well as the host immune responses towards viral infections.

4. Antiviral defenses

Invertebrates have evolved a number of immune-defence mechanisms to identify and fight against these infections. Viral nucleic acids and other structural components function as Pathogen-Associated Molecular Patterns (PAMPs), which are recognized by Pattern Recognition Receptors of the host immune systems (PRRs) (Medzhitov, 2007, Rajamuthiah and Mylonakis, 2014, Eigenbrod et al.,

2015). In metazoans, such recognition triggers effective and appropriate antiviral responses, including the production of various cytokines and the induction of inflammatory and, adaptive immune reactions (in case of mammals) (Liu et al., 2009). Bivalve's innate immune system consists of humoral components such as agglutinins, lysosomal enzymes, opsonizing molecules and antimicrobial peptides. Cellular immunity is also evident in many invertebrate species performed by phagocytes mediating the efficient removal of particulate microbes, while reactions such as encapsulation sequester invading pathogens. Apoptosis or programmed cell death is considered a vital component of various processes including proper development and functioning of the immune system. Infection by most viruses triggers apoptosis or programmed cell death of the infected cell. Some viruses seem to use apoptosis as a mechanism of cell killing and virus spread for successful virus replication. Apoptosis is also delayed in certain cases until sufficient viral progeny have been produced.

Host immune systems have evolved mechanisms to detect and interfere with viral infections at all levels of the viral life cycle, such as cell entry, replication, packaging and exit. There are three families of PRRs are known to recognize viral PAMPS. These are (RIG-I)-like DExD/H box RNA helicases are cytoplasmic viral sensors, transmembrane Toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors, which are intracellular sensors that recognize viral PAMPs (Liu et al., 2012, Fullam and Schroder, 2013).

The best studied of these receptors are the TLRs, a family of transmembrane glycoproteins that recognizes viral nucleic acid, such as ssRNA, dsRNA, glycoprotein and CpG (Cytosine-phosphate-guanine) nucleotides (Teleshova et al., 2006). TLR3 recognizes dsRNA whereas TLR7 & TLR9 recognizes ssRNA and are localized on the endoplasmic reticulum and or at the

cell surface (Thompson and Locarnini, 2007, Kawai and Akira, 2007, Akira, 2009). Other than viral dsRNA, TLR3 is also activated by endogenous dsRNA that may be released by dying cells (Kariko et al., 2004). Large numbers of TLR genes have been identified in the sequenced genomes of invertebrates such as the purple sea urchin, honey bee, flour beetle, silk worm, mosquito and fruit fly (Luna et al., 2002, Hibino et al., 2006, Evans et al., 2006, Cheng et al., 2008, Leulier and Lemaitre, 2008, Zhong et al., 2013).

Viral genomes may be composed of ssRNA, dsRNA, ssDNA and dsDNA (Ishii and Akira, 2005, Speir and Johnson, 2012). Viruses produce double stranded RNA (dsRNA) during replication, which are recognized by TLR3 and TLR7 in both vertebrates and invertebrates (Alexopoulou et al., 2001, Weber et al., 2006). dsRNA is also recognized by the RNA helicases RIG I and melanoma differentiation-associated gene 5 (MDA-5), as well as the NLR pyrin domain (NLRP) 3 protein (Jin et al.,2010). In mammals, TLR7 and TLR9 are involved in the recognition of ssRNA and dsRNA viruses such as Vesicular stomatitis virus (VSV), influenza, mouse cytomegalovirus (MCMV) and herpes simplex virus (HSV-1 and -2) (Schroder and Bowie, 2005, Kawai and Akira, 2006, Diebold, 2008). The binding of vertebrate TLRs to viral dsRNA or ssRNA leads to the activation of the transcription factor NF-κB. One class of genes that is stimulated by NF-κB are the cytokine genes, such as interferon (IFN-α/β). IFN-α/β is then secreted into the infected cell's immediate extracellular environment and binds to IFN receptors on the surfaces of adjacent cells. The activation of the IFN receptors in those cells in turn activates the JAK/STAT signal transduction pathway, which lead to the expression of numerous proteins that confer an 'antiviral' state. These proteins include virus-binding proteins, such as RLRs and NLRs, as well as components of the RNA interference (RNAi) system.

Although the details of these pathways have not been worked out in most invertebrates, a number of the proteins mentioned above have been identified in several invertebrates, suggesting that the latter are capable of mediating similar responses to viral infection. TLR3 leads to the activation of Nuclear Factor-kappa B (NF-κB) & IRF3. Interferon regulatory factor 3 (IRF3) regulates the expression of IFNβ, which activates several genes contributing to antiviral state by inhibiting protein synthesis and viral replication (Vercammen et al., 2008, Brownell et al., 2014). In giant tiger shrimps Toll like receptors have been identified that are closely related to Toll1 and Toll5 that may be involved in immune defence and there is a possibility that TLR/NFκB cassette is involved in dsRNA-induced response (Robalino et al., 2004, Arts et al., 2007). Similarly, TLR7 recognizes endosomal ssRNA to detect viral infection by RNA viruses and trigger the production of Interferons (IFNs) (Diebold et al., 2004, Kawai and Akira, 2007). Insect innate immune responses are largely composed of three signalling pathways: The Toll, IMD and JAK-STAT. Honey bee, mosquito and Drosophila have 71, 209 & 196 genes respectively implicated in insect immunity (Evans et al., 2006). In mosquito, suppression of the JAK-STAT pathway through RNAi-mediated depletion of the Domeless receptor (Dome) and Janus kinase (Hop) increases its susceptibility to dengue virus. Conversely, silencing of PIAS, which is a negative regulator of the JAK-STAT pathway, confers resistance to viral infection (Souza-Neto et al., 2009). Presence of Rel/NF-κB signalling pathway components have been reported in the Pacific cupped oysters, pearl oysters, abalone and squids.

Various cell receptors and signalling pathways operate in a coordinated way to combat viral infections. In cells that have been activated by interferon signalling, RNA interference plays a vital role in resisting viral infection. RNA

interference (RNAi) is a highly conserved defence mechanism that targets and destroys viral dsRNA.

4.1.   RNA interference

Post transcription gene silencing (PTGS) was originally called co-suppression, RNA interference or and RNA quelling by researchers working with plants, flies and fungi respectively. RNA interference is a sequence specific gene silencing mechanism triggered by exogenous dsRNA. Genetic and biochemical data suggest that RNAi is a two-step mechanism in which the enzyme ribonuclease (Dicer) and a protein complex (Ribosomal Induced Silencing Complex, RISC) play a central role in the destruction of invading viral RNA. Viral dsRNA is recognized and cleaved by RNase III enzyme Dicer, forming small interfering RNAs (siRNA). Virus-derived siRNAs (viRNAs) are produced in cells upon viral infection (Aliyari and Ding, 2009). Dicer is evolutionarily conserved, with homologs identified in fungi, plants, vertebrates and invertebrates (*C.elegans, Drosophila*). Dicer-2 contains an amino-terminal DExD/H box helicase domain. These DExD-box helicases comprise largest family of RNA helicases found in almost all organisms including invertebrates. These RNA helicases participates in all aspects of RNA metabolism from transcription to RNA degradation. In *Drosophila*, Dicer-2, which senses and cleaves dsRNA into siRNA, contains the DExD-box helicase domain (Deddouche et al., 2008, Kemp and Imler, 2009, Ulvila et al., 2009,). It is presumed that this domain is not directly involved in sensing of viral dsRNA but mediates a conformational change of molecule, which is essential for Dicer activity. These siRNAs are then incorporated into the RISC complex: when mRNAs are bound to the siRNAs, the RISC cleaves the target mRNA, thus inhibiting their translation (Keene et al., 2004). PTGS is thought to be an ancient self defense mechanism evolved to combat virus infections and transposons. By

exploiting this system specific genes can be switched off in a variety of organisms. In recent years' researchers have successfully used exogenous dsRNA to trigger RNAi and demonstrated that exogenous dsRNA is capable of triggering sequence-specific gene silencing. Studies have indicated that many invertebrates are capable of RNAi and such mechanisms have been found in organisms like *Caenorhabditis elegans, Drosophila, Neurospora crassa* and *Dictyostelium* (Ketting et al., 2001, Olenkina et al., 2012, Chang et al., 2012, Xue and Hou, 2012). Delivery and uptake of dsRNA are important in any successful RNAi experiment. These methods are employed depending upon the type of experiment and required results.

4.2.   Uptake of exogenous dsRNA

Exogenous dsRNA may enter cells via a number of distinct pathways, such as transmembrane channels and receptor-mediated endocytosis (Saleh et al., 2006). In *C. elegans,* extracellular dsRNA is believed to be imported into cells by transmembrane channels, which are created by membrane attack complex. Systemic interference defective (SID-1 & SID-2) genes encode multispan transmembrane proteins, which are expressed not only in *C. elegans*, but also in the intestinal tissues of arthropods (Winston et al., 2007, Xu and Han, 2008, McEwan et al., 2012). SID-1 proteins have an extracellular N-terminal region and an intracellular C-terminal segment. dsRNA that are internalized in this fashion are then acted upon by the RNAi machinery in the cytoplasm.

Scavenger receptors are a class of membrane-bound receptors that bind polyanionic molecules such as dsRNA. In *Drosophila* and *C. elegans* scavenger receptor homologues (e.g. SR-C1 and Eater) bind to dsRNA and trigger their uptake by receptor mediated endocytosis (Huvenne and Smagghe, 2010). Double stranded RNA uptake by endocytosis-mediated mechanism has been

demonstrated in S2 cells, as exogenous dsRNA in the growth medium induced RNAi in the host cells (Huvenne and Smagghe, 2010). Macrophage-like S2 cells initiated RNAi responses when they were exposed to high concentrations of dsRNA in serum-free media, followed by cultivation in serum-supplemented media for 72 hours. In another study a gene specific dsRNA encoding firefly luciferase was co-transfected with S2 cells displayed luciferase activity and dsRNA dose-dependent silencing response was detected (Feinberg and Hunter, 2003). In 2006 Ulvila et al. reported that scavenger receptors, SR-C1 and Eater accounted for more than 90% of the dsRNA uptake into *Drosophila* S2 cells (Ulvila et al., 2009).
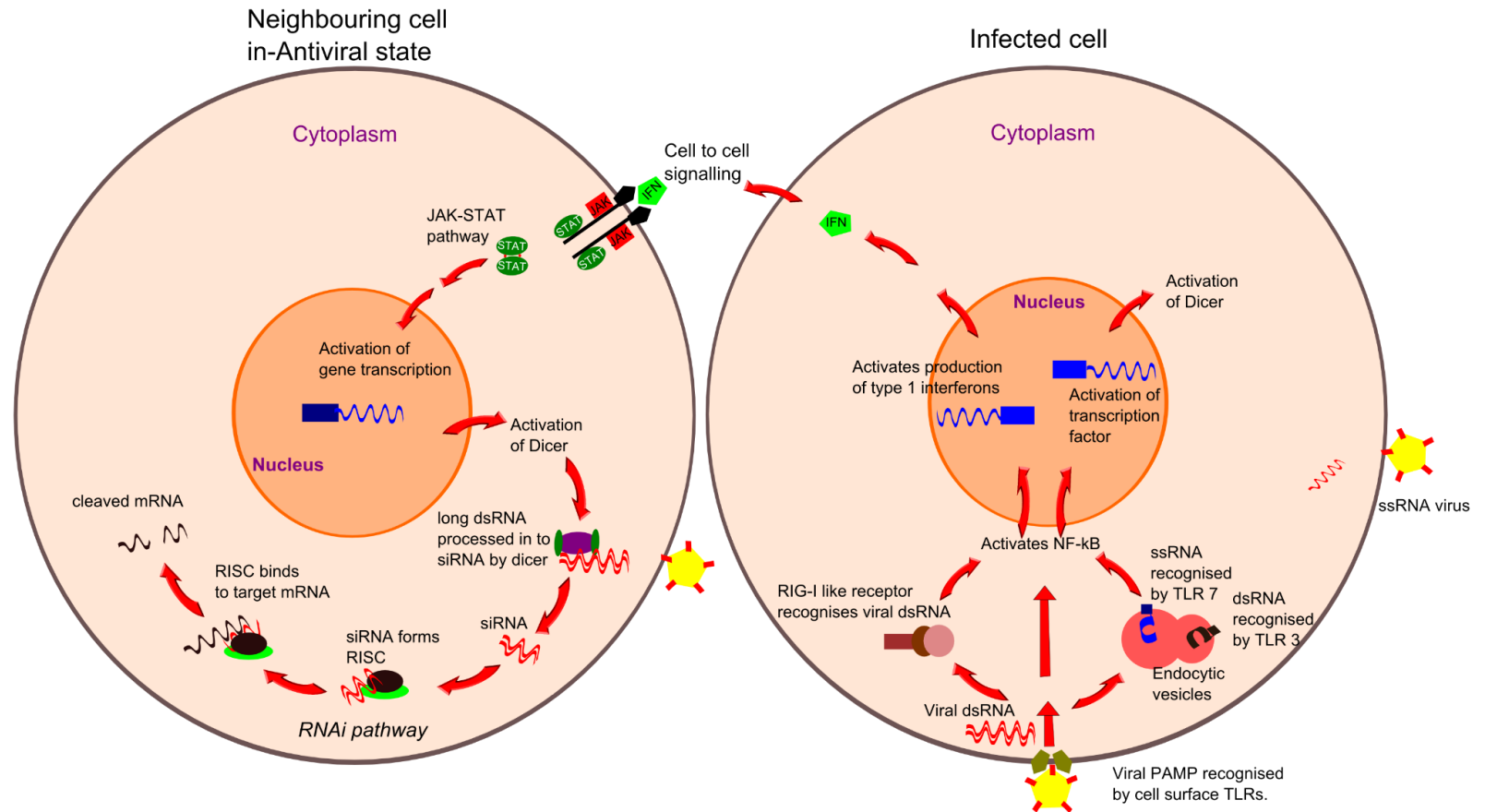
Figure 1: Viral ssRNA is recognized by TLR7 whereas dsRNAs are recognized by TLR7 & RIG-I like receptors, which induces the synthesis of interferon (IFN) with in host cell. IFN can activate JAK-STAT pathway leading to activation of antiviral genes. These genes can bring changes to cellular function such as degradation of viral nucleic acid through RNAi pathway.

4.3. Delivery of exogenous dsRNA

Successful RNAi-mediated abrogation of gene expression requires efficient delivery of dsRNA into host organisms. The success of any RNAi experiment depends upon good transfection. Not surprisingly, a number of different techniques have been employed to deliver RNA to invertebrates. These include oral delivery (e.g. feeding insects with transgenic plants expressing dsRNA, feeding dsRNA-laced nanoparticles to shrimp) or direct injection into animals.

In an effort to induce larval deaths, dsRNA against a number of targets, which are involved in cellular metabolism were applied to the agar diets of *Diabrotica virgifera* (western corn root worms, WCR) at concentrations ranging from 520 ng/cm^2 to 780 ng/cm^2. Larvae that were fed the dsRNA in this manner suffered from stunting and succumbed to death. Total RNA from dsRNA-fed and untreated larvae were analysed using northern blot to confirm down-regulation of specific targeted genes. Northern blot analysis showed complete suppression of targeted transcripts in dsRNA-fed WCR larvae. This degree of suppression was interpreted by the authors to indicate that the RNAi effect was not confined to gut epithelial cells, but also involved other somatic tissues. Transgenic corn plants that were engineered to express dsRNA to WCR-specific V-ATPase showed significant reduction in the damage resulting from WCR feeding (Baum et al., 2007). Oral delivery of white spot syndrome virus (WSSV) VP28 gene dsRNA-coated chitosan nanoparticles to tiger shrimps induced protective antiviral immunity in the animals (Sarathi et al., 2008). In those experiments, shrimp were fed with either bacterium that expressed VP28 dsRNA coupled to chitosan or VP28 dsRNA coupled directly to chitosan continuously for 5 days. Subsequent oral challenge with WSSV-infected meat indicated that the shrimp that were fed

the VP28dsRNA were protected from infection and that they did not contain VP28 transcripts in their tissues (indicating successful RNAi-mediated inhibition of WSSV).

The ability to deliver dsRNA via non-invasive methods is considerably more advantageous than delivery via invasive methods, such as injections.   For example, when dsRNA was delivered in blood meal to tsetse flies (*Glossina morsitans morsitans)*, the insect mortality rate was 1.8%, compared to 37.9% when the dsRNA was injected into the insects (Walshe et al., 2009). However, this contrasts with the effectiveness of the delivery procedure to induce RNAi effect in these animals.   The same study indicated that *TsetseEP* dsRNA delivered in blood meal failed to knockdown the transferrin gene (2A192) expressed in fat bodies, although injections of the same dsRNA induced effective RNAi-mediated abrogation of the transferring gene expression (Walshe et al., 2009).

In *Drosophila* dsRNA was delivered by injections using a dose of 50 nl at a concentration of 5 mg/ml of naked dsRNA corresponding to two different regions of Sindbis virus genome (dsSin1 and dsSin2) and *Drosophila* C virus (DCV) was injected in thorax using a nanoinjector. Two days' later flies were infected with recombinant Sindbis virus expressing GFP (Sindbis-GFP virus) diluted in 10mM Tris-Cl (pH 7.5) on the opposite side of thorax. Other studies have shown the effectiveness of injecting dsRNA into haemocoelic space or into specific tissues. These include the Sydney rock oyster *Saccostrea glomerata* (Green and Barnes, 2009), shrimp (Robalino et al., 2004), mosquito, abalone and Chinese mitten crab.

4.4.   Effect of dsRNA dose on the induction of RNAi

Apart from various delivery methods, the dose of dsRNA used is also important in any RNAi experiment. An excessive dose of dsRNA can cause toxicity in cells whereas too low a dose   may   not   trigger   detectable   RNAi

24

response. To-date, there is no unequivocal data that indicate the amount of dsRNA that is required to induce RNAi in invertebrates. Upon entry in to cells, a single molecule of dsRNA can induce production of interferon (Marcus and Sekellick, 1977). Factors that may determine the amount of dsRNA required to elicit RNAi may include (i) method of delivery, (ii) age and life cycle stage of the host, (iii) level of expression of the target genes, (iv) toxicity of dsRNA and (v) the nature of the RNAi response (i.e. specific vs. non-specific). Accumulating evidence suggest that many animals are able to tolerate a wide range of dsRNA without exhibiting apparent, harmful side-effects. Work with shrimp (*L. vanamei*) has shown that while 1 µg of dsRNA was sufficient to evoke antiviral responses, injection of 100 µg of dsRNA did not show any obvious deleterious effects (Robalino et al., 2004). Studies have shown that 1 µg of dsRNA, which elicited antiviral responses in honeybees, was not toxic to the hosts (Maori et al., 2009). Experiments in zebrafish have shown that approximately 60 pg of dsRNA (to the *Pou-II* gene) per embryo (pg/e) is required for induction of RNAi. When the animals were injected with 150 pg/e control dsRNA to GFP (dsRNA-*GFP),* similar types and intensities of defects were observed in the embryos. As the GFP gene is not endogenous to zebrafish, the authors concluded that the defective phenotypes that were observed were not due to gene silencing, but to the toxicity of the injected dsRNA. This toxicity was dependent on the dose of the dsRNA that was injected into the embryos (Zhao et al., 2001). Further analysis of transcripts in dsRNA-injected embryos using Fluorescence *In Situ* Hybridisation (FISH) indicated that the dsRNA reduced the stability of mRNA in a sequence-independent manner. The indiscriminate destruction of mRNAs in the embryo at 150 pg/e of dsRNA may be the underlying reason for the toxic effects of dsRNA.

In honeybees, the RNAi pathway was triggered when those insects were injected with 1μg of dsRNA per animal. This dose was sufficient to induce immunity to infection by the viral pathogen, IAPV (Israeli acute paralysis virus). Bees were fed inoculated with 10 ng/μl of IAPV followed by three treatments. IAPV inoculums containing no dsRNA, dsRNA-GFP, dsRNA corresponding to IAPV and untreated hives as control. Results showed that bees treated with dsRNA homologous to IAPV sequences were protected from subsequent infection whereas dsRNA-GFP treated bees did not show any protection by IAPV infection (Maori et al., 2009).

In *Schistosoma mansoni* (a human parasite) was maintained in *Biomphalaria glabrata* and *Mesocricetus auratus*. Eleven genes encoding diverse proteins (normally expressed in gut and tegument tissues) were selected for screening. It is demonstrated that the gut is an important route through which RNAi was triggered. It was achieved by co-incubating the parasite and dsRNA for a period of 6 days. It was enough to register maximum RNAi of transcriptional suppression of cannabinoid receptor 1(CB1), methionine amino-peptidase (MetAP) and protein phosphatase-2a (PP2a). It was demonstrated that Cy5-labeled dsRNA to CBI, CC or mCherry was taken up by gut tissues within minutes of its administration. Toxicity due to dsRNA was also tested and there were large number of abnormal parasites which showed rounding, darkening, slowed mortality and death. Such careful titrations of the variables led the authors to conclude that lower doses of dsRNA could achieve the intended outcomes without inducing toxicity to the host. (Stefanic et al., 2010). A detailed study on the toxicity of dsRNA was also carried out by Baum and his co-workers on Western Corn Root (WCR). When they compared the LD50 values of 67 distinct dsRNA sequences, they discovered a wide range of values, from $1.82 ng/cm^2$ (the area

refers to the surface are of the agar blocks used in the feed) to 5.2 ng/cm^2 (Baum et al., 2007). In shrimps, although intramuscular injections of dsRNA (at a dose of 100 μg /injection) against WSSV was not toxic to the host, injections of 7 to 15 µg of dsRNA was sufficient to induce antiviral immunity (Robalino et al., 2004). A survey of the literature has revealed a wide range of conditions used in RNAi assays. A summary of dose and type of dsRNA used with regard to species, its delivery method, effect and its quantified are presented in table 1.

Species	ssRNA dsRNA	Concentration	Quantity	Quantification	Results	Reference
S. glomerata (Sydney Rock Oyster)	Poly (I:C) Poly (G:C) dsDNA *Vibrio alginolyticus*	5mg/ml in PBS	100µlin abductor muscle	qRT-PCR for EcSOD,Prx6, Ik & I_kB genes.	Upregulation of I_kB with Poly (I:C) & *vibrio alginolyticus* Upregulation of IK with Poly (I:C) No effect on EcSOD & Prx6 gene expression.	(Green and Barnes, 2009)
L. vanamei (Shrimp)	Poly (C:G) Poly (I:C) Poly C		7-15 µg intramuscular	mortality	dsRNA induces innate antiviral response.	(Robalino et al., 2004)
Haliotis discus discus (Abalone)	Poly (I:C) (Sigma, USA)	10 µg/ml in PBS	100 µl intramuscular	RT-PCR for Mx gene.	Poly (I:C) induction was more prominent in gill & digestive tissues. No Mx expression in mantle & foot tissues.	(De Zoysa et al., 2007)
Eriocheir sinensis (Chinese mitten crab)	GFP-dsRNA & NoPSD-dsRNA	1 µg/ µl	150 µl in the base of last walking foot	qRT-PCR for *EsALF* gene. Enzyme activity assay.	Upregulation of *EsALF* gene expression. Increased PO, ACP & Increased PO, ACP & SOD activity with stable MDA contents. Broad spectrum immune response can be induced by dsRNA.	(Dong et al., 2009)

Species	ssRNA dsRNA	Concentration	Quantity	Quantification	Results	Reference
Penaeus monodon (Shrimps)	YHVprotease-dsRNA dsRNA-GFP	1 µg/ µl	25 µg	RT-RCR for YHV gene & Western blot for YHV antigen	Sequence specific pathway by RNAi mechanism is a prominent antiviral defense that exerts both preventive & curative modes.	(Tirasophon et al., 2007)
Penaeus monodon (Shrimps)	dsRNA(YHV) dsRNA(GFP) dsRNA(TSV-pol)	25 µg/50 µl in 150mM NaCl	50 µl	RT-PCR for YHV gene & Western blot for YHV antigen	dsRNA induction of antiviral immunity in shrimps goes through two pathways (sequence dependent & sequence independent).	(Yodmuang et al., 2006)
Drosophila melanogaster	dsRNA (dsSin1 & dsSin2) Sindbis- GFP Virus		Injection in thorax	Sindbis-GFP virus replication Fluorescence imaging Western blot with GFP antibody	Upon infection, infected cells spread systematically silencing signal that draw protective RNAi-dependent immunity & insufficient cell-autonomous response.	(Saleh et al., 2009)

Species	ssRNA dsRNA	Concentration	Quantity	Quantification	Results	Reference
Drosophila melanogaster	DCV-ORF$_2$ dsRNA Non-specific dsRNA	400ng/ µl 200ng/ µl	100nl in abdomina l cavity	qRT-PCR for *CG12780, vir-1* & *CG9080* genes. Survival percentage	Upregulation of reporter gene with DCV-ORF$_2$ dsRNA injection but dsRNA & non-infectious DCV particles were not sufficient to stimulate upregulation of three reported genes.	(Hedges and Johnson, 2008)
Anopheles gambiae (Mosquito)	ONNV-eGFP Co injection of ONNV-eGFP + viral genomic dsRNA (dsnsP3,dsβgal,dsAg Ago1, dsAgAgo2, dsAgAgo3,dsAgAgo 4 & dsAgAgo5)	1 µg/ µl in PBS	0.5 µl intrathora cic	eGFP expression under UV. Immunofluorescence assay for ONNVE2 protein. Northern blot ONNVE2 gene.	RNAi acts as an antiviral response to ONNV-eGFP infections & AGO2 & possibly AGO3 proteins are critical component of RNAi pathway in *A. gambiae*	(Keene et al., 2004)
Pacific oyster (*C.gigas*)	Poly (I:C) OsHV-1 µvar *Vibrio splendidus*	5mg/ml in PBS	50µl in abductor muscle	qRT-PCR for TLR, MyD88, IRF, PKR,	TLR, MyD88, IRF and PKR genes were upregulated as a result of poly I:C challenge. MyD88, IRF and PKR genes were upregulated as a result of OsHV-1µvar infection.	(Green and Montagnani, 2013)

Table 1: Range of RNAi assays that have been used in invertebrates.

4.5. Use of specific and/or generic dsRNA for RNAi

Studies in vertebrates have indicated that generic dsRNA such as (poly (I:C)), poly (A:U), poly(I:C12U), polyinosinic-polycytidylic acid (poly (ICLC)), are ligands of TLR3 and elicit RNAi responses (Jin et al., 2010). ssRNA poly U has also shown to elicit the release of high levels of IFN-α from plasmacytoid dendritic cells (PDC) (Diebold et al., 2004). In the vertebrates, the injection of both dsRNA and ssRNA induced the production of interferons. The production of interferons via dsRNA applies to both sequence-specific and sequence-independent inhibition pathways. For example, injections of poly (I:C) in mice stimulated the production of type I IFN and conferred immunity against influenza virus infection (Jin et al., 2010, Vercammen et al., 2008). However, there appears to be significant differences in abilities of the different dsRNAs to evoke antiviral immunity. When mouse macrophages were treated with poly (I:C) and poly (A:U), the expression of the NF-κB reporter gene (a key step in the production of interferons) was observed. However, synthetic ssRNA (poly(C) and poly (dI:dC) had no effect (Alexopoulou et al., 2001).

RNAi-mediated antiviral immunity following the use of generic dsRNA has also been observed in invertebrates. Synthetic RNA analogues that induce antiviral responses in the vertebrates may not function in the same manner in the invertebrates. For example, poly (I:C), which is a potent inducer of the innate antiviral response in vertebrates (Vercammen et al., 2008, Alexopoulou et al., 2001, Jin et al., 2010) failed to induce protection against WSSV infection in shrimps (Robalino et al., 2004). Similarly, poly (I:C) & ssRNA poly C failed to induce antiviral responses in shrimp against White spot syndrome virus, whereas poly (C:G) dsRNA provided protection against WSSV (Robalino et al., 2004). Poly

(I:C) also induces resistance against Myxovirus infection in the abalone *Haliotis discus discus*. Myxovirus resistance (*Mx*) mRNA was expressed in gill, digestive tissue, digestive gland, mantle and foot tissue after poly (I:C) injection. Mx is an antiviral protein that is induced by Type I interferon (IFNα/β) in both vertebrates and invertebrates (De Zoysa et al., 2007). Poly (I:C) has stimulated expression of inhibitor of Rel/NF-κB (*IκB*) when injected into Sydney rock oysters, whereas poly (G:C) failed to do so. Rel or NF-κB is a signalling pathway that induces transcription of numerous genes involved in immune function and inflammation (Green and Barnes, 2009).

Other than commercially available dsRNAs (such as poly I:C and A:U), researchers have also used employed other non-specific dsRNAs. For example, shrimps injected with dsRNA derived from duck immunoglobulin Ig v heavy chain gene (duck Ig dsRNA)) showed protection against both TSV and WSSV. The sequences of the dsRNAs used in that study have no similarity to any known shrimp gene or to the genomes of WSSV or TSV. Shrimps injected with dsRNA corresponding to a noncoding sequence from the catfish IgH locus (dsRNA IgH), pig IgG cDNA (dsRNA IgG) or a 1,184-bp fragment of the vector pBeloBAC11 (pBeloBAC11 dsRNA) also showed resistance to WSSV infection (Robalino et al., 2004).

DsRNA derived from virus genomes are capable to inducing specific antiviral immunity. Shrimps injected with dsRNA corresponding to the protease motif of yellow head virus (YHV) genome showed complete inhibition of YHV replication whereas shrimps injected with sequence unrelated dsRNAs showed lower extent of YHV replication inhibition (Yodmuang et al., 2006). Comparable results have also been demonstrated in the black tiger shrimp (*Penaeus monodon)* (Rajeshkumar et al., 2009).

5. Pacific oyster and Sydney rock oyster comparison in relation to OsHV-1 µvar infection

Oysters are infected by viral and protozoan pathogens (Elston, 1997, Williams, 2008, da Silva et al., 2014). Since Farley first reported Herpes type virus infection in *Crassostrea virginica* in 1972*,* there have been several reports of herpes virus infections worldwide (Farley et al., 1972, Renault et al., 2000, Friedman et al., 2005, Segarra et al., 2010, Jenkins et al., 2013). Ostreid herpes virus type 1 (OsHV-1) cause up to 100% mortality in larvae and juvenile Pacific oysters (FRDC, 2011). Symptoms of this infection include a reduction in feeding and swimming activity of oyster larvae and abnormal nuclei in gill, mantle, labial palps and digestive glands of infected adult oyster (FRDC, 2011). Pacific oysters (*C. gigas*) are native to Japan and have been introduced to many countries, including Australia. This is the most widely cultured shellfish species, due to its fast growth and high production rates. According to the NSW Department of Primary Industry in 2011 total production of Pacific oyster was worth $ 60.52 million in New South Wales, South Australia and Tasmania. This species often displaces native oyster species including Sydney rock oyster (*S. glomerata*) and has been declared a Class 2 Noxious species by NSW Fisheries Management Act 1994. Sydney rock oyster (*S. glomerata*) is endemic to Australia. It occurs in estuaries mostly on east coast of Australia, from Harvey Bay to Victoria (D.A. and P.A., 1990). In recent years Ostreid herpes virus type 1(OsHV-1 µvar) has been the reported to cause infection in Pacific oyster, killing both juvenile and adult oysters (Paul-Pont et al., 2013a). According to the World Organisation of Animal Health, the Pacific oyster (*C. gigas*) and Portuguese cupped oyster (*C. angulata*) are the only species known to develop clinical disease due to OsHV-1 micro-variant disease (OIE., 2014). So far OsHV-1 µvar has been detected in three estuaries the Georges

River-Port Botany, Port Jackson-Sydney Harbour and Hawkesbury River-Brisbane water in NSW Australia. This virus has been restricted to NSW and there have been no reports of infection from this strain in other parts of Australia.

According to trials conducted by the Department of Primary Industry, it appears that the Sydney rock oyster (*S. glomerata*) is not susceptible to infection by OsHV-1 μvar (Jenkins et al., 2013, Whittington et al 2015). OsHV-1 micro-variant infection is temperature dependent. Normally it occurs when the water temperature rises above 16°C (Renault et al., 2014). After infection, surviving oysters that carry this pathogen can die if they are exposed to further stress, which also increase the chances of disease transmission. It is interesting that Sydney rock oyster is not susceptible to OsHV-1 micro-variant infection, even though both of these species Sydney rock oyster and Pacific oyster are cultured in the same river system estuaries (Jenkins et al., 2013). Recent studies have identified various immune system genes and intracellular signalling pathways in oyster species. These include NFκB, TLR, MyD88, PKR, SOD and IFI44, which play an important role in the host defense system. (Green and Barnes, 2009, Schikorski et al., 2011, Zhang et al., 2013). Finally, OsHV-1 virus has been reported to stimulate the production of interferons in Pacific oyster species (Green and Montagnani, 2013, Green et al., 2015).

6. Molecular biomarkers and proteomics

The use of biomarkers as indicators of biological phenomena is prevalent in many areas of research. Molecular markers such as allozymes, DNA, PCR and proteins based markers have been used in numerous biological systems. To investigate impact of pathogens on Sydney rock oyster immune system, a range of enzymatic assays including phenoloxidase, superoxide dismutase, and

peroxidase were used (Aladaileh et al., 2007). Most recently analytic techniques such as suppression subtractive hybridisation (SSH), DNA microarrays, RT-PCR and or proteomics are also used to investigate host virus interaction in oyster species such as Pacific oyster (Renault et al., 2011, Schikorski et al., 2011, Corporeau et al., 2014, Green et al., 2015). These techniques can be used simultaneously to analyse the expression of genes/proteins expressed by an organism at any given time. Combining these techniques with bioinformatics can help understand various metabolic pathways at cellular level and to investigate various biological functions. Hence, transcriptomics and proteomics can identify hundreds or thousands of genes and proteins simultaneously that are expressed in response to viral infection.

Mass spectrometry based proteomics is a powerful tool to identify and quantify thousands of proteins in complex samples (Aebersold and Mann, 2003). This approach can be used to measure and characterise protein expression profiles in a given sample for potential biomarker discovery. Modern proteomics labelling and quantitation techniques such as isobaric tags for relative and absolute quantitation (iTRAQ) and sequential window acquisition of all theoretical fragment ion spectra (SWATH) can be employed to study quantitative changes in proteins. In the iTRAQ technique, isobaric reagents are used to label amines of peptides and proteins, whereas SWATH is label free technique based on data-independent acquisition strategy. The iTRAQ and SWATH work flow is described in figure 1 and 2 respectively. There is high degree of correlation between iTRAQ and SWATH techniques in term of quantitation (Bourassa et al., 2015). iTRAQ method identifies more proteins in a biological sample, whereas SWATH technique provides quantitatively accurate measurements of a large fraction of a proteome

across multiple samples and more sensitive in detection of small differential ratios than iTRAQ (Zhang et al., 2014, Bourassa et al., 2015,).
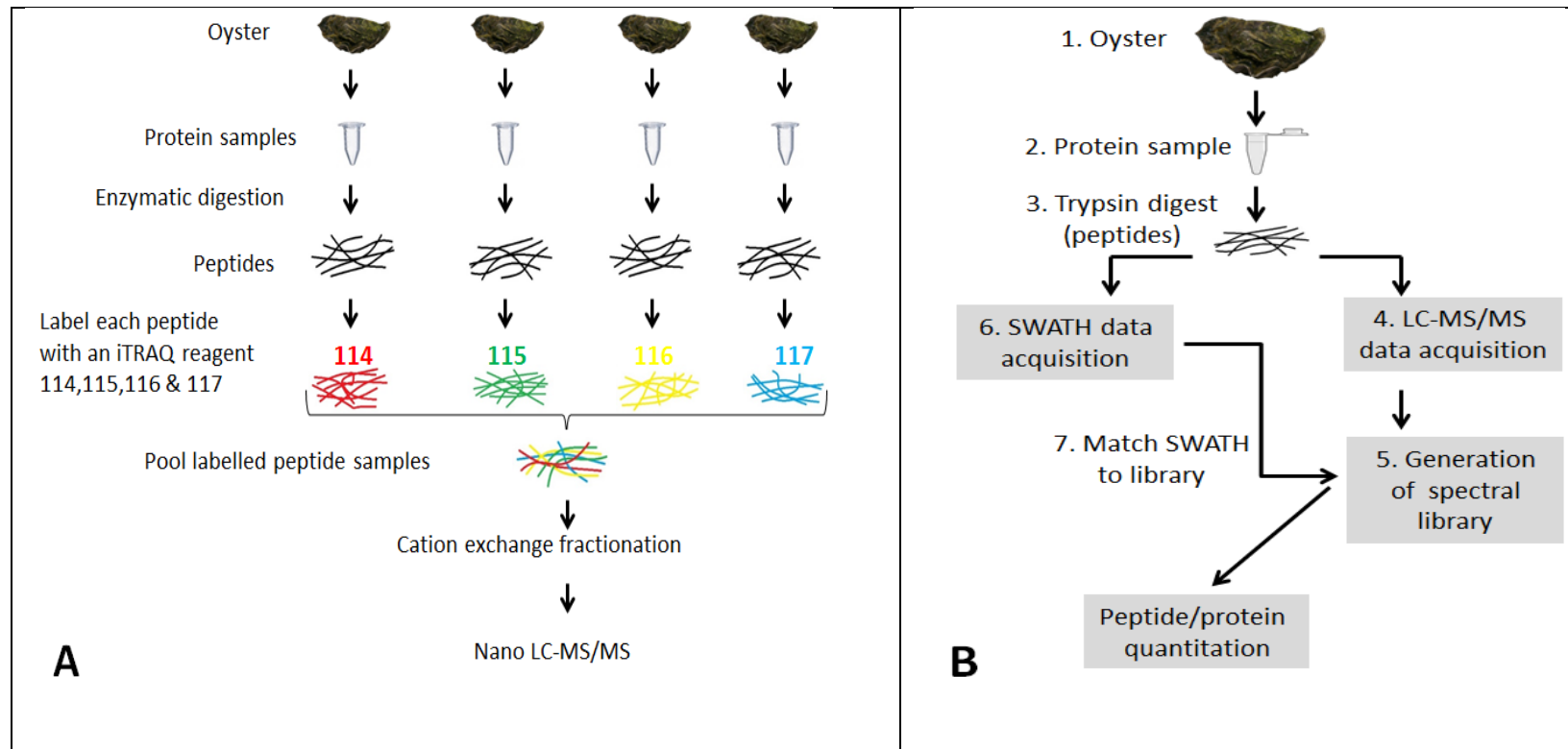
Figure 2: A). iTRAQ mass spectrometry flow chart; B). SWATH mass spectrometry flow chart.

Although iTRAQ-labelling has been widely applied, there are some limitations to the accuracy of this technique in identified proteins quantitation particularly among highly complex sample mixtures. Whereas SWATH is a nice tool for quantification of large number of proteins from a complex mixture.

The reliance on 'omics technologies, such as proteomics. Despite the significant advances that have occurred in recent times, the data generated from such investigations must be validated using other platforms. However, the lack of reagents that may be used to interrogate oyster systems (e.g. suitable antibodies) prevented the use of validation assays, such as Western blots and ELISAs. A number of other cellular assays (e.g. apoptotic and cytokine assays) that are currently available have proven to be unreliable when it comes to oyster systems and do not produce robust results. They need to be further optimised for use with non-model organisms. Although generic dsRNA is a powerful tools to mimic viral infection but the use of virus-specific reagents (e.g. OsHV-1-specific dsRNA, including shRNAs and siRNAs) can be used to validate the responses of oyster tissues to dsRNA challenge. Furthermore, the use of such reagents in combination with infections by live, purified viruses or viral DNAs will delineate virus-specific immune responses.

7. Conclusion and research directions

At the commencement of this research project, reports from oyster farmers indicated that the Pacific oysters were succumbing to infection that were symptomatic of herpesvirus infection described elsewhere. Confirmation of the etiological agent of the disease in Pacific oysters came from both electron microscope investigations and PCR assays. Sydney Rock oysters did not manifest symptoms of herpesvirus infections, although viral DNA was present in

those animals. It was suspected that differences in the biological responses of the animals to viral PAMPs underpinned the differential susceptibility of the hosts to the viruses. Thus, the overall goals of the research project were to:

i.   Investigate the RNAi pathways of both oyster species, and

ii. Identify possible protein targets in Sydney Rock oysters that mediated immunity to herpesviruses (which may be used in marker-assisted selected programs to identify disease-resistant Pacific oysters).

In the initial stages of the project, there was no information available on the sequence of the Ostreid herpesvirus (var) genome. Thus, generic dsRNAs were employed in the studies. Subsequently, this approach was substantiated with work performed by other research groups (Green and Barnes, 2009, Green and Montagnani, 2013) who showed that the generic dsRNA, poly (I:C), was capable of inducing protective immunity in Pacific oysters against herpesvirus infections. As oyster-specific dsRNA assays were not available when this project was started, the initial part of the project was dedicated to developing suitable assays for the delivery of dsRNA, as well as determining their fates in oysters. It was decided that the oysters' responses to dsRNA challenge would be determined using proteomic assays. This was based on the following considerations:

- As proteins represent the 'functional' end of biological information flow, the analysis of proteomic perturbations resulting from dsRNA injections maybe a good indicator of biological responses to those challenges.

- The cutting-edge proteomic technologies and expertise available at Macquarie University (Australian Proteome Analysis Facility) allowed for the development of robust proteomic assays, as well as the subsequent bioinformatics

The specific aims of the project were:

1. To determine an optimal route of dsRNA delivery that does not result in significant injury to the animals

2. To determine the *in vivo* destination of the exogenous dsRNAs (i.e. the tissues that take up the dsRNAs)

3. To determine the proteomic responses of the two oyster species to dsRNA, including a comparison of the similarities and differences of the proteomic responses.

4. To determine if there are temporal differences, as well as differences in the hosts' responses to different exogenous dsRNAs.

8. References

Acton, R.T. & Evans, E.E. (1968) Bacteriophage clearance in the oyster (Crassotrea virginica). *J Bacteriol,* **95,** 1260-1266.

Aebersold, R. & Mann, M. (2003) Mass spectrometry-based proteomics. *Nature,* **422,** 198-207.

Akira, S. (2009) Innate immunity to pathogens: diversity in receptors for microbial recognition. *Immunol Rev,* **227,** 5-8.

Aladaileh, S., Nair, S.V. & Raftos, D.A. (2007) Induction of phenoloxidase and other immunological activities in Sydney rock oysters challenged with microbial pathogen-associate molecular patterns. *Fish Shellfish Immunol,* **23,** 1196-1208.

Alexopoulou, L., Holt, A.C., Medzhitov, R. & Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature,* **413,** 732-738.

Aliyari, R. & Ding, S.W. (2009) RNA-based viral immunity initiated by the Dicer family of host immune receptors. *Immunol Rev,* **227,** 176-188.

Arts, J.A., Cornelissen, F.H., Cijsouw, T., Hermsen, T., Savelkoul, H.F. & Stet, R.J. (2007) Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, Penaeus monodon. *Fish Shellfish Immunol,* **23,** 504-513.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. & Roberts, J. (2007) Control of coleopteran insect pests through RNA interference. *Nat Biotechnol,* **25,** 1322-1326.

Bourassa, S., Fournier, F., Nehme, B., Kelly, I., Tremblay, A., Lemelin, V., Lamarche, B., Couture, P. & Droit, A. (2015) Evaluation of iTRAQ and SWATH-MS for the Quantification of Proteins Associated with Insulin

Resistance in Human Duodenal Biopsy Samples. *PLoS One,* **10,** e0125934.

Brownell, J., Bruckner, J., Wagoner, J., Thomas, E., Loo, Y.M., Gale, M., Jr., Liang, T.J. & Polyak, S.J. (2014) Direct, interferon-independent activation of the CXCL10 promoter by NF-kappaB and interferon regulatory factor 3 during hepatitis C virus infection. *J Virol,* **88,** 1582-1590.

Chang, S.S., Zhang, Z. & Liu, Y. (2012) RNA interference pathways in fungi: mechanisms and functions. *Annu Rev Microbiol,* **66,** 305-323.

Cheng, T.C., Zhang, Y.L., Liu, C., Xu, P.Z., Gao, Z.H., Xia, Q.Y. & Xiang, Z.H. (2008) Identification and analysis of Toll-related genes in the domesticated silkworm, Bombyx mori. *Dev Comp Immunol,* **32,** 464-475.

Comps, M. & Cochennec, N. (1993) A Herpes-like Virus from the European Oyster Ostrea edulis L. *Journal of Invertebrate Pathology,* **62,** 201-203.

Corporeau, C., Tamayo, D., Pernet, F., Quere, C. & Madec, S. (2014) Proteomic signatures of the oyster metabolic response to herpesvirus OsHV-1 muVar infection. *J Proteomics,* **109,** 176-187.

D.A., P. & P.A., H. (1990) A review of exotic marine organisms introduced to the Australian region. 2. Invertebrates and Algae. *Asian Fisheries Science,* **3,** 223-250.

Da Silva, P.M., Scardua, M.P., Vianna, R.T., Mendonca, R.C., Vieira, C.B., Dungan, C.F., Scott, G.P. & Reece, K.S. (2014) Two Perkinsus spp. infect Crassostrea gasar oysters from cultured and wild populations of the Rio Sao Francisco estuary, Sergipe, northeastern Brazil. *J Invertebr Pathol,* **119,** 62-71.

De Zoysa, M., Kang, H.S., Song, Y.B., Jee, Y., Lee, Y.D. & Lee, J. (2007) First report of invertebrate Mx: cloning, characterization and expression analysis

of Mx cDNA in disk abalone (Haliotis discus discus). *Fish Shellfish Immunol,* **23,** 86-96.

Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., Galiana-Arnoux, D., Dostert, C., Antoniewski, C., Hoffmann, J.A. & Imler, J.L. (2008) The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in drosophila. *Nat Immunol,* **9,** 1425-1432.

Diebold, S.S. (2008) Recognition of viral single-stranded RNA by Toll-like receptors. *Adv Drug Deliv Rev,* **60,** 813-823.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. & Reis E Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science,* **303,** 1529-1531.

Dong, C., Zhao, J., Song, L., Wang, L., Qiu, L., Zheng, P., Li, L., Gai, Y. & Yang, G. (2009) The immune responses in Chinese mitten crab Eriocheir sinensis challenged with double-stranded RNA. *Fish Shellfish Immunol,* **26,** 438-442.

Eigenbrod, T., Keller, P., Kaiser, S., Rimbach, K., Dalpke, A.H. & Helm, M. (2015) Recognition of Specified RNA Modifications by the Innate Immune System. *Methods Enzymol,* **560,** 73-89.

Ellard K., P.S., Handlinger J. & Andrewartha R. (2009) Findings of disease investigations following the recent detection of AVG in Tasmania. In, Cairna, Australia.

Elston, R. (1997) Bivalve mollusc viruses. 393-403.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z. & Hultmark, D. (2006) Immune pathways and defence mechanisms in honey bees Apis mellifera. *Insect Mol Biol,* **15,** 645-656.

FAO (2014) The State of World Fisheries and Aquaculture. In. Food and Agiculture Organisation of the United Nations, Roma.

Farley, C.A., Banfield, W.G., Kasnic, G., Jr. & Foster, W.S. (1972) Oyster herpes-type virus. *Science,* **178,** 759-760.

Feinberg, E.H. & Hunter, C.P. (2003) Transport of dsRNA into cells by the transmembrane protein SID-1. *Science,* **301,** 1545-1547.

FRDC. (2011) FINAL REPORT OsHV-1 μVar In: *International OsHV-1 μVar workshop* Fisheries Research and Development Corporation, Cairns, Queensland, Australia

Friedman, C.S., Estes, R.M., Stokes, N.A., Burge, C.A., Hargove, J.S., Barber, B.J., Elston, R.A., Burreson, E.M. & Reece, K.S. (2005) Herpes virus in juvenile Pacific oysters Crassostrea gigas from Tomales Bay, California, coincides with summer mortality episodes. *Dis Aquat Organ,* **63,** 33-41.

Fuhrman, J.A. (1999) Marine viruses and their biogeochemical and ecological effects. *Nature,* **399,** 541-548.

Fullam, A. & Schroder, M. (2013) DExD/H-box RNA helicases as mediators of anti-viral innate immunity and essential host factors for viral replication. *Biochim Biophys Acta,* **1829,** 854-865.

Green, T.J. & Barnes, A.C. (2009) Inhibitor of REL/NF-KappaB is regulated in Sydney rock oysters in response to specific double-stranded RNA and Vibrio alginolyticus, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible. *Fish Shellfish Immunol,* **27,** 260-265.

Green, T.J. & Montagnani, C. (2013) Poly I:C induces a protective antiviral immune response in the Pacific oyster (Crassostrea gigas) against subsequent challenge with Ostreid herpesvirus (OsHV-1 muvar). *Fish Shellfish Immunol,* **35,** 382-388.

Green, T.J., Rolland, J.L., Vergnes, A., Raftos, D. & Montagnani, C. (2015) OsHV-1 countermeasures to the Pacific oyster's anti-viral response. *Fish Shellfish Immunol,* **47,** 435-443.

Hedges, L.M. & Johnson, K.N. (2008) Induction of host defence responses by Drosophila C virus. *J Gen Virol,* **89,** 1497-1501.

Hibino, T., Loza-Coll, M., Messier, C., Majeske, A.J., Cohen, A.H., Terwilliger, D.P., Buckley, K.M., Brockton, V., Nair, S.V., Berney, K., Fugmann, S.D., Anderson, M.K., Pancer, Z., Cameron, R.A., Smith, L.C. & Rast, J.P. (2006) The immune gene repertoire encoded in the purple sea urchin genome. *Dev Biol,* **300,** 349-365.

Huvenne, H. & Smagghe, G. (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J Insect Physiol,* **56,** 227-235.

Ishii, K.J. & Akira, S. (2005) Innate immune recognition of nucleic acids: beyond toll-like receptors. *Int J Cancer,* **117,** 517-523.

Jenkins, C., Hick, P., Gabor, M., Spiers, Z., Fell, S.A., Gu, X., Read, A., Go, J., Dove, M., O'connor, W., Kirkland, P.D. & Frances, J. (2013) Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in Crassostrea gigas (Pacific oysters) in Australia. *Dis Aquat Organ,* **105,** 109-126.

Jin, B., Sun, T., Yu, X.H., Liu, C.Q., Yang, Y.X., Lu, P., Fu, S.F., Qiu, H.B. & Yeo, A.E. Immunomodulatory effects of dsRNA and its potential as vaccine adjuvant. *J Biomed Biotechnol,* **2010,** 690438.

Kariko, K., Ni, H., Capodici, J., Lamphier, M. & Weissman, D. (2004) mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem,* **279,** 12542-12550.

Kawai, T. & Akira, S. (2006) [Role of IPS-1 in type I IFN induction]. *Nihon Rinsho,* **64,** 1231-1235.

Kawai, T. & Akira, S. (2007) Antiviral signaling through pattern recognition receptors. *J Biochem,* **141,** 137-145.

Keene, K.M., Foy, B.D., Sanchez-Vargas, I., Beaty, B.J., Blair, C.D. & Olson, K.E. (2004) RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of Anopheles gambiae. *Proc Natl Acad Sci U S A,* **101,** 17240-17245.

Kemp, C. & Imler, J.L. (2009) Antiviral immunity in drosophila. *Curr Opin Immunol,* **21,** 3-9.

Kent, M.L. (2000) Marine netpen farming leads to infections with some unusual parasites. *Int J Parasitol,* **30,** 321-326.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J. & Plasterk, R.H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev,* **15,** 2654-2659.

Leulier, F. & Lemaitre, B. (2008) Toll-like receptors--taking an evolutionary approach. *Nat Rev Genet,* **9,** 165-178.

Liu, H., Soderhall, K. & Jiravanichpaisal, P. (2009) Antiviral immunity in crustaceans. *Fish Shellfish Immunol,* **27,** 79-88.

Liu, Y., Chen, H., Sun, Y. & Chen, F. (2012) Antiviral role of Toll-like receptors and cytokines against the new 2009 H1N1 virus infection. *Mol Biol Rep,* **39,** 1163-1172.

Luna, C., Wang, X., Huang, Y., Zhang, J. & Zheng, L. (2002) Characterization of four Toll related genes during development and immune responses in Anopheles gambiae. *Insect Biochem Mol Biol,* **32,** 1171-1179.

Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E. & Sela, I. (2009) IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. *Insect Mol Biol,* **18,** 55-60.

Marcus, P.I. & Sekellick, M.J. (1977) Defective interfering particles with covalently linked [+/-]RNA induce interferon. *Nature,* **266,** 815-819.

Mcewan, D.L., Weisman, A.S. & Hunter, C.P. (2012) Uptake of extracellular double-stranded RNA by SID-2. *Mol Cell,* **47,** 746-754.

Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. *Nature,* **449,** 819-826.

Nicolas J. L., C.M., Cochennec N. (1992) Herpes-like virus infecting Pacific oyster larvae, Crassostrea gigas. *Bulletin European Association of Fish Pathologists,* **12,** 11-13.

Norton, J.H., Shepherd, M.A. & Prior, H.C. (1993) Papovavirus-like Infection of the Golden-Lipped Pearl Oyster, Pinctada maxima, from the Torres Strait, Australia. *Journal of Invertebrate Pathology,* **62,** 198-200.

OIE (2014) 'Manual of Diagonostic Tests for Aquatic Animals 2014'. In. World organisation for Animal Health, Paris.

Olenkina, O.M., Egorova, K.S., Aravin, A.A., Naumova, N.M., Gvozdev, V.A. & Olenina, L.V. (2012) Mapping of cis-regulatory sites in the promoter of testis-specific stellate genes of Drosophila melanogaster. *Biochemistry (Mosc),* **77,** 1285-1293.

P. M. Hine, B.W., P. Besant (1998) Replication of a herpes-like virus in larvae of the flat oyster Tiostrea chilensis at ambient temperatures *Dis Aquat Organ,* **32,** 161-171.

Parsons, R.J., Breitbart, M., Lomas, M.W. & Carlson, C.A. (2012) Ocean time-series reveals recurring seasonal patterns of virioplankton dynamics in the northwestern Sargasso Sea. *ISME J,* **6,** 273-284.

Paul-Pont, I., Dhand, N.K. & Whittington, R.J. (2013a) Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters Crassostrea gigas. *Aquaculture,* **412–413,** 202-214.

Paul-Pont, I., Dhand, N.K. & Whittington, R.J. (2013b) Spatial distribution of mortality in Pacific oysters Crassostrea gigas: reflection on mechanisms of OsHV-1 transmission. *Dis Aquat Organ,* **105,** 127-138.

Rajamuthiah, R. & Mylonakis, E. (2014) Effector triggered immunity. *Virulence,* **5,** 697-702.

Rajeshkumar, S., Venkatesan, C., Sarathi, M., Sarathbabu, V., Thomas, J., Anver Basha, K. & Sahul Hameed, A.S. (2009) Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). *Fish Shellfish Immunol,* **26,** 429-437.

Renault, T., Bouquet, A.L., Maurice, J.T., Lupo, C. & Blachier, P. (2014) Ostreid herpesvirus 1 infection among Pacific oyster (Crassostrea gigas) Spat: relevance of water temperature to virus replication and circulation prior to the onset of mortality. *Appl Environ Microbiol,* **80,** 5419-5426.

Renault, T., Faury, N., Barbosa-Solomieu, V. & Moreau, K. (2011) Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, Crassostrea gigas, challenged with Ostreid herpesvirus 1. *Dev Comp Immunol,* **35,** 725-735.

Renault, T., Le Deuff, R.M., Chollet, B., Cochennec, N. & Gerard, A. (2000) Concomitant herpes-like virus infections in hatchery-reared larvae and

nursery-cultured spat Crassostrea gigas and Ostrea edulis. *Dis Aquat Organ,* **42,** 173-183.

Renault, T. & Novoa, B. (2004) Viruses infecting bivalve molluscs. *Aquatic Living Resources,* **17,** 397-409.

Robalino, J., Browdy, C.L., Prior, S., Metz, A., Parnell, P., Gross, P. & Warr, G. (2004) Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. *J Virol,* **78,** 10442-10448.

Saleh, M.C., Tassetto, M., Van Rij, R.P., Goic, B., Gausson, V., Berry, B., Jacquier, C., Antoniewski, C. & Andino, R. (2009) Antiviral immunity in Drosophila requires systemic RNA interference spread. *Nature,* **458,** 346-350.

Saleh, M.C., Van Rij, R.P., Hekele, A., Gillis, A., Foley, E., O'farrell, P.H. & Andino, R. (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat Cell Biol,* **8,** 793-802.

Sarathi, M., Simon, M.C., Venkatesan, C. & Hameed, A.S. (2008) Oral administration of bacterially expressed VP28dsRNA to protect Penaeus monodon from white spot syndrome virus. *Mar Biotechnol (NY),* **10,** 242-249.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P. & Pepin, J.F. (2011) Experimental infection of Pacific oyster Crassostrea gigas spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. *Vet Res,* **42,** 27.

Schroder, M. & Bowie, A.G. (2005) TLR3 in antiviral immunity: key player or bystander? *Trends Immunol,* **26,** 462-468.

Segarra, A., Pepin, J.F., Arzul, I., Morga, B., Faury, N. & Renault, T. (2010) Detection and description of a particular Ostreid herpesvirus 1 genotype

associated with massive mortality outbreaks of Pacific oysters, Crassostrea gigas, in France in 2008. *Virus Res,* **153,** 92-99.

Souza-Neto, J.A., Sim, S. & Dimopoulos, G. (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc Natl Acad Sci U S A,* **106,** 17841-17846.

Speir, J.A. & Johnson, J.E. (2012) Nucleic acid packaging in viruses. *Curr Opin Struct Biol,* **22,** 65-71.

Stefanic, S., Dvorak, J., Horn, M., Braschi, S., Sojka, D., Ruelas, D.S., Suzuki, B., Lim, K.C., Hopkins, S.D., Mckerrow, J.H. & Caffrey, C.R. (2010) RNA interference in Schistosoma mansoni schistosomula: selectivity, sensitivity and operation for larger-scale screening. *PLoS Negl Trop Dis,* **4,** e850.

Teleshova, N., Kenney, J., Williams, V., Van Nest, G., Marshall, J., Lifson, J.D., Sivin, I., Dufour, J., Bohm, R., Gettie, A. & Pope, M. (2006) CpG-C ISS-ODN activation of blood-derived B cells from healthy and chronic immunodeficiency virus-infected macaques. *J Leukoc Biol,* **79,** 257-267.

Thompson, A.J. & Locarnini, S.A. (2007) Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. *Immunol Cell Biol,* **85,** 435-445.

Tirasophon, W., Yodmuang, S., Chinnirunvong, W., Plongthongkum, N. & Panyim, S. (2007) Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. *Antiviral Res,* **74,** 150-155.

Ulvila, J., Hultmark, D. & Ramet, M. (2009) RNA silencing in the antiviral innate immune defence--role of DEAD-box RNA helicases. *Scand J Immunol,* **71,** 146-158.

Vercammen, E., Staal, J. & Beyaert, R. (2008) Sensing of viral infection and activation of innate immunity by toll-like receptor 3. *Clin Microbiol Rev,* **21,** 13-25.

Walker, P.J. & Winton, J.R. (2010) Emerging viral diseases of fish and shrimp. *Vet Res,* **41,** 51.

Walshe, D.P., Lehane, S.M., Lehane, M.J. & Haines, L.R. (2009) Prolonged gene knockdown in the tsetse fly Glossina by feeding double stranded RNA. *Insect Mol Biol,* **18,** 11-19.

Weber, F., Wagner, V., Rasmussen, S.B., Hartmann, R. & Paludan, S.R. (2006) Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J Virol,* **80,** 5059-5064.

Whittington, R., Hick, P., Rubio, A., Dhand, N., Evans, O. (2015) Aquatic Animal Health Sub program: Pacific oyster mortality syndrome (POMS) – risk mitigation, epidemiology and OsHV-1 biology. Sydney.

Williams, T. (2008) Natural invertebrate hosts of iridoviruses (Iridoviridae). *Neotropical Entomology,* **37,** 615-632.

Winston, W.M., Sutherlin, M., Wright, A.J., Feinberg, E.H. & Hunter, C.P. (2007) Caenorhabditis elegans SID-2 is required for environmental RNA interference. *Proc Natl Acad Sci U S A,* **104,** 10565-10570.

Xu, W. & Han, Z. (2008) Cloning and phylogenetic analysis of sid-1-like genes from aphids. *J Insect Sci,* **8,** 1-6.

Xue, D.M. & Hou, L.S. (2012) Shortening of the cell cycle and developmental interruption in a Dictyostelium discoideum cell line due to RNAi-silenced expression of allantoicase. *Genet Mol Res,* **11,** 1923-1933.

Yodmuang, S., Tirasophon, W., Roshorm, Y., Chinnirunvong, W. & Panyim, S. (2006) YHV-protease dsRNA inhibits YHV replication in Penaeus monodon and prevents mortality. *Biochem Biophys Res Commun,* **341,** 351-356.

Zhang, F., Lin, H., Gu, A., Li, J., Liu, L., Yu, T., Cui, Y., Deng, W., Yan, M., Li, J. & Yao, M. (2014) SWATH- and iTRAQ-based quantitative proteomic analyses reveal an overexpression and biological relevance of CD109 in advanced NSCLC. *J Proteomics,* **102,** 125-136.

Zhang, S. & Bonami, J.R. (2007) A roni-like virus associated with mortalities of the freshwater crab, Eriocheir sinensis Milne Edwards, cultured in China, exhibiting 'sighs disease' and black gill syndrome. *J Fish Dis,* **30,** 181-186.

Zhang, S., Shi, Z., Zhang, J. & Bonami, J.R. (2004) Purification and characterization of a new reovirus from the Chinese mitten crab, Eriocheir sinensis. *J Fish Dis,* **27,** 687-692.

Zhang, Y., He, X., Yu, F., Xiang, Z., Li, J., Thorpe, K.L. & Yu, Z. (2013) Characteristic and functional analysis of toll-like receptors (TLRs) in the lophotrocozoan, Crassostrea gigas, reveals ancient origin of TLR-mediated innate immunity. *PLoS One,* **8,** e76464.

Zhao, Z., Cao, Y., Li, M. & Meng, A. (2001) Double-stranded RNA injection produces nonspecific defects in zebrafish. *Dev Biol,* **229,** 215-223.

Zhong, D., Pai, A., Wang, M.H., Keech, N. & Yan, G. (2013) Fine-scale analysis of parasite resistance genes in the red flour beetle, Tribolium castaneum. *Genetics,* **195,** 253-261.

Chapter 2

Double stranded RNA is processed differently in two oyster species.

Formatted for Submission to:

Cell and Tissue Research

Author contributions:

Muhammad Masood – Concept, experimental design, laboratory work, data analysis, interpretation of results and writing of manuscript.

Marie E. Herberstein – Technical advice.

David A. Raftos – Project supervision.

Sham V. Nair – Technical advice and Project supervision.

Abstract

Ostreid herpes virus causes serious disease in the Pacific oyster (*Crassostrea gigas*), but not in the Sydney Rock Oyster (*Saccostrea glomerata*). To investigate differences in disease progression, we injected oysters with double stranded RNA. dsRNA is known to mimic viral infection, and can evoke immune responses when Toll-like receptors detect the dsRNA, leading to the production of type 1 interferon and inflammation cytokines. The uptake and processing of dsRNA was tracked in gill and mantle tissue of *Crassostrea gigas* and *Saccostrea glomerata* after injection of fluorochrome labelled poly (I:C) dsRNA. The two species showed significant differences in tissue uptake and clearance, and differences in immune responses confirmed by real time PCR. These results showed that *S. glomerata* was more efficient in processing dsRNA than *C. gigas*, and that the gill tissue is an important site of dsRNA processing and response.

Key words: Invertebrate; immune response; virus; oyster; poly (I:C)

Introduction

The world's hydrosphere may harbour 10^{30} million virus particles (Parsons, et al., 2012). Because oysters are filter feeders, they have the potential to bio-accumulate viruses, bacteria and other microbes, some of which may cause infections (Paul-Pont, et al., 2013a). Viruses that belong to the irido, herpes and papova-like families are known pathogens of oysters (Elston, 1997, Norton, et al., 1993, Segarra, et al., 2010), causing morbidity and mortality, including cytopathic effects such as enlarged nuclei, marginated chromatin, swollen mitochondria and abnormal accumulation of granular endoplasmic reticulum. Other abnormalities include infection of branchial epithelial cells, haemocyte inflammation, inflammation and swamping of ciliated velar epithelial cells to form blisters (Comps and Cochennec, 1993, Farley, et al., 1972, Nicolas J. L., 1992).

Host immune systems can detect and interfere with viral infections at all stages of the viral life cycle, including cell entry, replication, packaging and cellular release. Although epithelial cells act as barriers to viral entry, some viruses are able to breach this barrier by interacting with cell surface proteins (Grove and Marsh, 2011). Viruses usually enter the host cells through receptor mediated endocytosis (Chinchar, et al., 2011). For example, the membrane glycoproteins of herpes simplex virus interact with cell surface receptors. This interaction is well coordinated and leads to the fusion of viral and cellular membranes, resulting in the successful entry of the viruses into host cells (Krummenacher, et al., 2013, Sedy, et al., 2008).

Innate immunity plays an important role in protection against invading pathogens such as viruses. Viral nucleic acids and other structural components are recognized as pathogen-associated molecular patterns (PAMPs). The pattern

recognition receptors (PRRs) of the host immune system recognize these PAMPS and trigger specific signalling pathways that lead to appropriate and coordinated antiviral responses (Medzhitov, 2007, Takeuchi and Akira, 2010). Intracellular antiviral responses include activation of transcriptional factors and production of various cytokines and induction of inflammatory and adaptive immune reactions that can eliminate pathogens and infected cells (Kawai and Akira, 2007, Liu, et al., 2009, Takeuchi and Akira, 2010). There are three families of PRRs known to recognize viral PAMPS. These are the RIG-I-like DExD/Hbox RNA helicases, transmembrane Toll-like receptors (TLR) and nucleotide oligomerization domain-like receptors.

RNA viruses can produce double stranded RNA (dsRNA) during replication, which act as PAMPS and are recognized by Toll-like receptors in both vertebrates and invertebrates (Alexopoulou, et al., 2001, Brencicova and Diebold, 2013, Ishii and Akira, 2005, Kawai and Akira, 2010). Binding of vertebrate TLRs to viral dsRNA leads to activation of the transcription factor NFκB that stimulates cytokine genes resulting in the production of interferons. The activation of the interferon receptors in those cells activates the JAK/STAT signal transduction pathway, which lead to the expression of numerous proteins that confer an 'antiviral' state.

Pacific oyster (*C. gigas*) and Portuguese cupped oyster (*C. angulata*) are the only species known to develop clinical disease due to OsHV-1 micro-variant (here after OsHV-1µVar is used specifically to reflect the variant reported by Segarra et al 2010) disease (OIE., 2014). Ostreid herpesvirus microvariant-1 (OsHV-1 µvar) infection of Pacific oysters (*Crassostrea gigas*) has been reported in France, New Zealand and Australia (Dundon, et al., 2011, Paul-Pont, Dhand

and Whittington, 2013a, Renault, et al., 2014, Roque, et al., 2012, Segarra, Pepin, Arzul, Morga, Faury and Renault, 2010). Infection mostly occurs when water temperature is above 16^{0}C, and juvenile oysters suffer the highest levels of mortality, although adult oysters are also affected (Renault, Bouquet, Maurice, Lupo and Blachier, 2014). OsHV-1 is transmitted from host to host and or through vector particles present in water column (Paul-Pont, Dhand and Whittington, 2013a, Paul-Pont, et al., 2013b, R.M., et al., 1994, Schikorski, et al., 2011). There is considerable damage to the cytoskeleton of mitochondria and rough endoplasmic reticulum of OsHV-1 infected cells (Hwang, et al., 2013), perhaps explaining its effects on oyster survival. The virus appears to infect only Pacific oysters and no other oyster species, such as the Sydney rock oyster (*Saccostrea glomerata*), despite the fact that these are often co-cultivated with *C. gigas*.

Synthetic double stranded RNA, such as Poly (I:C), has been used successfully *in vivo* and *in vitro* to mimic infection and evoke immune responses in both vertebrate and invertebrate species (Ye, et al., 2013). Poly (I:C) can be labelled with fluorochromes to track its entry and survival in cells (Mian, et al., 2013, Singh, et al., 2013, Watanabe, Tatematsu, Saeki, Shibata, Shime, Yoshimura, Obuse, Seya and Matsumoto, 2011). The length of the double stranded *RNA* is an important factor in eliciting an immune response as demonstrated by Mian et al (2013) when they used Poly (I:C) molecules with a length of 1-1.5 kb and >5kb. The longer Poly (I:C) molecules induced strong antiviral response and elicit higher cytokine in mammalian cells (Mian, Ahmed, Rad, Babaian, Bowdish and Ashkar, 2013). High molecular weight poly (I:C) is also more effective in inducing the expression of immune related genes such as *TL3, MDA5,* and *RIG-I* when compared with their low molecular weight homologues (Zhou, et al., 2013). We used high molecular weight poly (I:C) for this

experiment, with an average size of 1.5-8 kb. Here we undertook a cytological study to investigate the cellular fate of dsRNA in Sydney Rock and Pacific oysters. Alexa Fluor 645-labelled dsRNA (poly I:C) was injected into the pericardial cavities of oysters and confocal microscopy was used to visualise the uptake and processing of dsRNA in various tissues. Gene expression of selected dsRNA response genes was studied using qPCR.

Material and Methods

Oyster collection and maintenance

Saccostrea glomerata and *Crassostrea gigas* were purchased from aquaculture farms in Pambula and Tathra NSW, respectively. These oysters were approximately two years of age. PO were hatchery triploid produced for commercial farming. SRO were wild type oyster caught in hatchery where they were farmed. Spat for both species of oysters were reared by standard farming practices involving either oyster bags or racks suspended intertidal. The SROs were free of all known oyster diseases, and the POs were certified to be free of OsHV-1, Marteiliosis, Mikrosporidiosis, Perkinsiosis, Bonamiasis and Iridovirosis according to tests conducted by the Tasmanian Department of Primary Industries, Parks, Water and Environment. Oysters were housed at 25°C in a closed recirculating seawater system at Macquarie University. They were acclimatized to aquarium conditions for a period of 10 days prior to experimentation. During the acclimatization period, oysters were fed with M1 shellfish feed (Aquasonic Pty Ltd).

Preparation of dsRNA

Poly (I:C) (Invivogen) was prepared at a final concentration of 2 mg/mL in sterile phosphate buffered saline (PBS). Poly (I:C) was labelled with Ulysis Alexa Fluor 647 Nucleic Acid Labelling Kit Catalogue number: U-21660 (Life Technologies) according to the manufacturers' instructions. The labelled poly (I:C) was then purified using Micro Bio-Spin RNAse-free columns packed with Bio-Gel P-30 polyacrylamide gel matrix (Bio-Rad, Hercules, CA).

60

Experimental setup

The following experimental design was used for both SRO and PO. Two groups of oysters were tagged and placed in separate aquaria. Each group consisted of 9 oysters, three of which were sampled for analysis at different time points (1, 24 and 48 hours) after dsRNA or PBS injection. One microgram of Alexa Fluor 645 labelled poly (I:C) was injected into animals in the treatment group (the injected volume was 100 µL per animal). Animals in the control group received 100 µl of PBS each. The dsRNA and PBS solutions were injected into the pericardial cavity through the hinge using a 23-gauge needle attached to a 1 ml syringe. Each group of animals was kept in separate aquaria. At each time point gill and mantle tissues were excised for microscopy analysis and RNA extracted for qPCR.

Sample preparation

Gill and mantle tissue were excised from three oysters 1, 24 and 48 hours post injection of Alexa Fluor labelled dsRNA poly (I:C). These pre-treated tissues were used for either RNA extraction or vibratome sectioning for microscopy. The detailed procedures for vibratome sectioning, nuclear counter staining with Hoechst 33342 and slide preparation are described in the Supplementary Methods. Total RNA was extracted from dsRNA injected and PBS injected (controls) oysters using Tri-Reagent. cDNA was synthesized using Superscript III reverse transcriptase system (Invitrogen), in accordance with the manufacturer's protocol. qPCR was performed using the Sybr qPCR master mix from Kappa Biosystems (KAPA SYBR FAST qPCR master mix). Briefly, various dilutions (undiluted and five-fold dilutions (1/5 to 1/125) of *TLR, PIWI, IFI44* and *PRDX* genes (Supplementary table 2) of the reverse transcription reaction were used as templates for the qPCR (each amplification reaction was performed in triplicate).

61

Actin was used as reference gene. The templates were mixed with the qPCR master mix and the forward and reverse primers (Table). The amplification conditions were as follows: initial denaturation (95°C, 10min) followed by 45 cycles of denaturation (95°C, 10s), annealing and elongation (60°C, 60s). A subsequent melt temperature curve of the amplicon was performed. Changes in the expression of target sequences were determined using the efficiency-corrected $^{\Delta\Delta}$Ct method (Pfaffl, 2001). Significance of changes in the abundance of transcripts was determined using the REST software package (Pfaffl, et al., 2002).

Image analysis

Tissue sections were visualized by Olympus FluoView FV1000 confocal microscope. Alexa Fluor 647 and Hoechst 33342 associated fluorescence in these cells was visualised using an Olympus UplanSApo 60X/1.35 Oil immersion objective and quantified using Fluoview version 4 image processing software (see Table 1). The emitted fluorescence of cells in 15 randomly-selected fields of view (FOVs) was determined. We ensured the settings for Fluoview were consistent at all times to ensure the data were not biased. Images in figure 2 were enhanced using GIMP 2 software (www.gimp.org).

Dye	Laser type	Laser setting HV	Offset
Alexa Fluor 645	635	635	35%
Hoechst 33342	405	590	34%
Lambda scan start 530 nm and end 630 nm			

Table 1 Confocal microscopy laser settings.

3-D micrographs were taken for each tissue sample, and for each sample fifteen FOVs were inspected. Ten 1μm planes were then taken at each FOV,

representing a total thickness of 10µm. Cell fluorescence was measured using Fiji software (Schindelin, et al., 2012). In order to analyse the sample an image was loaded in grey scale colour mode and all 10 slices of the image were stacked as one image using the Z-project command. The fluorescence area of interest was then selected using a grid of known standard area to maintain consistency in all images. The selected area was then analysed using the 'area integrated intensity' and 'mean grey value'. Similarly, areas that were found to have no fluorescence (outside fluorescence) were selected to calculate the background. The data were acquired and fluorescence was corrected using the following formula:

Corrected total cell fluorescence (CTCF) = Integrated Density – [(Area of selected cell) X (Mean fluorescence-of background readings)]

Statistical analysis

For each time point, the average fluorescence of the tissues was determined. A one-way ANOVA and Tukey's HSD tests ($\alpha = 0.05$) were used to compare fluorescence intensity (CTCF) responses between species, tissues and time points. All single effects and interactions between species, tissues and time were analysed using full factorial analysis.

Results and discussion

1.1. Delivery and uptake of dsRNA

To determine if auto-fluorescence was evident, examined gill and mantle tissues from untreated animals using confocal microscopy. No auto fluorescence was observed between 600-633nm (data not shown), indicating that all observed signals at these wavelengths were due to dsRNA-associated fluorophores. Moreover, no auto-fluorescence was detected with the reagents used for the processing of the dsRNA (e.g. phosphate buffer saline) or for tissue processing. RNAi is a cellular response that requires the intracellular localization of dsRNA. In *in-vitro* systems, intracellular delivery of exogenous *dsRNA* is achieved by processes such as transfection, electropermeabilization or direct injection of nucleic acid into cells (Green and Barnes, 2009, Paganin-Gioanni, et al., 2011). However, *in-vivo* cells can internalise extracellular exogenous *dsRNA* that is delivered into animals by feeding, soaking or injection in body cavity (Mian, Ahmed, Rad, Babaian, Bowdish and Ashkar, 2013, Ulvila, et al., 2009, Walshe, et al., 2009). We demonstrated that fluorescently-labelled dsRNA (poly I:C) that was injected into the Sydney rock oyster's pericardial cavity was taken up by circulating blood cells (haemocytes) within 30 minutes of treatment (Chapter 3). Furthermore, in response to the dsRNA, those haemocytes expressed a number of markers associated with anti-viral immune responses. This immunisation route also minimises damage to the tissues of the animal. Hence, for the currently study, we employed a similar immunisation protocol.

1.2. Mantle and gill tissues

The mantle is an organ in oysters that forms a cavity bounded by two sheets of tissues and encloses all organs including open circulatory system. The mantle has the ability to store nutrients, has sensory capacity and plays an important role in bio-mineralisation (Gong, et al., 2008, Saucedo and Gómez-Robles, 2008). Mantle tissues are composed of internal and external epithelia. The internal epithelium is exposed to the open circulatory system and is in direct contact with haemolymph (Supplementary figure 1). The external epithelium layer is in direct contact with the shell of the oyster. These two tissue layers of epithelial cell sheets are connected by a complex network of cells that comprise connective tissues. The gills are one of the largest organs in oysters. This consists of two layers of tissues. The gills are ctenidia like structure with two rows of flattened filaments comprising a single gill. The main function of this organ is gaseous exchange and feeding. Coelomic fluid circulates in these filaments and lamellae where gases are exchanged. On each end gill tissue fuses with mantle and at the base (Supplementary figure 1) it attaches to the visceral mass (Galtsoff, 1964).

2. Fate of fluorescence-labelled dsRNA

Both species samples did not showed any auto fluorescence and fluorescence difference developed only after challenging with labelled dsRNA. During the course of the investigation, there was an increase in uptake of labelled poly (I:C) in the mantle tissues of both oyster species. Maximum fluorescence was detected at 48 hours post injection. However, we observed a different pattern in gill tissues of both species as fluorescence increased at 24-hours post injection but decreased after 48 hours. At each time point, the total fluorescence intensity was greater in PO tissues (Least mean square CTCF: 6297590.1) than in SRO tissues (Least mean square CTCF: 2774595.2). Statistical analyses of fluorescence

intensity data indicated that the distribution of the injected poly (I:C) differed significantly between the two oyster species (P<0.0001). Within each species, differences in the time-dependent changes in fluorescence intensity was also observed (P<0.0001). Least mean square CTCF values was 4625161.5 for gill tissue and 4447023.8 for mantle tissue. Overall there was no significant difference between tissues (P>0.05). Our statistical data indicate that processing of dsRNA is efficient in SRO when compared to PO and it is dependent on time and tissue. With progression of time gill tissue clears fluorescence labelled dsRNA in both species. This data indicates that there are significant interactions between 1) species and time, 2) species and tissue and 3) time and tissue P=0.0008, <0.0001 and <0.0001 respectively (Supplementary table 1A). There was also a significant three-way interaction between species, time and tissue with (P = 0.0002). A detailed statistical analysis is presented in supplementary table 1.
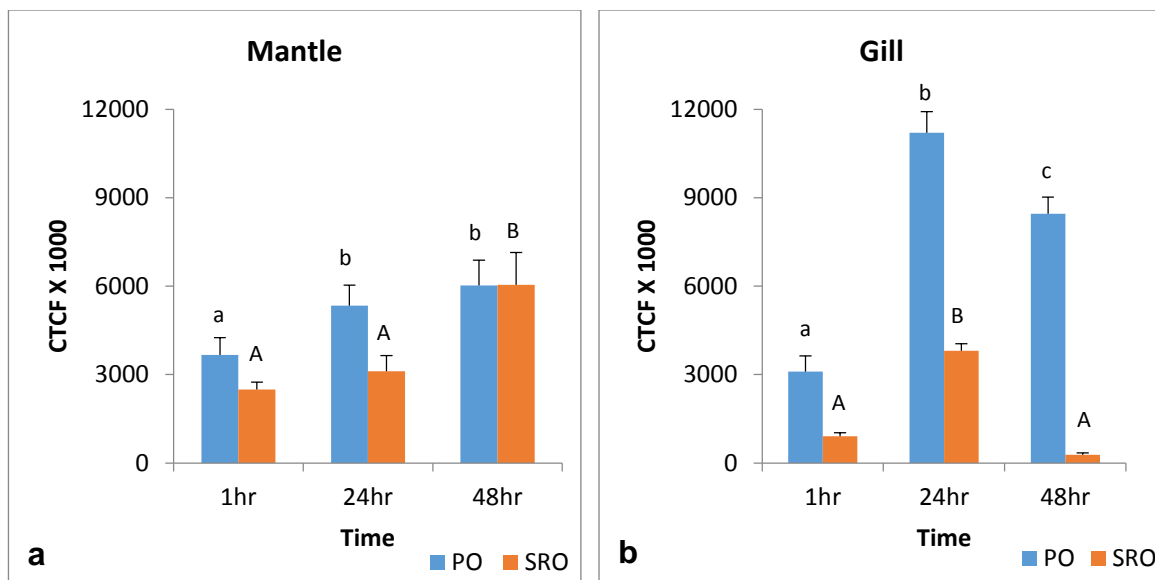
Fig 1 Corrected total cell fluorescence (CTCF) data for mantle and gill tissue for both species PO and SRO. **a**). Mantle tissue CTCF at various time points. **b**). Gill tissue CTCF at various time points. There is no significant difference between time points when sharing the same lower case letter in PO, and upper case letter for SRO within each tissue of that particular species.

Student's t-test results showed that the fluorescence was much greater in Pacific oyster compared to Sydney rock oyster species (least square mean: 6297590 and 2774595 respectively with standard error 244606). Within an hour of injection, fluorescence associated with the poly (I:C) was evident in the mantle tissues of both species (Fig 2c, d, e, f, m, n and o). Double stranded RNA was internalised into internal epithelial cells as shown in figure 2 (d, j & m). There was no difference between the fluorescence intensity data of the mantle tissues of the Pacific and Sydney rock oysters ($P>0.05$). Fluorescence intensity tended to increase with time (i.e. post-injection) and we found signals in other parts of mantle tissues such as connective tissue and the cells of the lower epithelium (Fig 2c). However, we found differences in fluorescence intensities of mantle tissue between two species at various time points (supplementary table 1G).

These observations suggest that the epithelial tissues of the mantle are capable of actively taking up exogenous dsRNA. The dsRNA also appears to be transported to the underlying tissues (as suggested by the gradient of fluorescence intensity from the external epithelium to the internal cells).

However, over the time course of the investigation, the relative fluorescence intensities remained fairly constant. This suggests that the mantle tissues did not process the dsRNA. The gill tissues of the oysters showed significantly different fluorescence intensities between the two species ($P<0.0001$) as well as between the time steps ($P<0.0001$) and there was a significant interaction between species and time ($P<0.0001$). Uptake of poly (I:C) was observed in the epithelial cells of gill tissues of Pacific oysters within an hour of injection, whereas the gill tissues of Sydney rock oyster showed minimal levels of poly (I:C) associated fluorescence (Figure 2p).
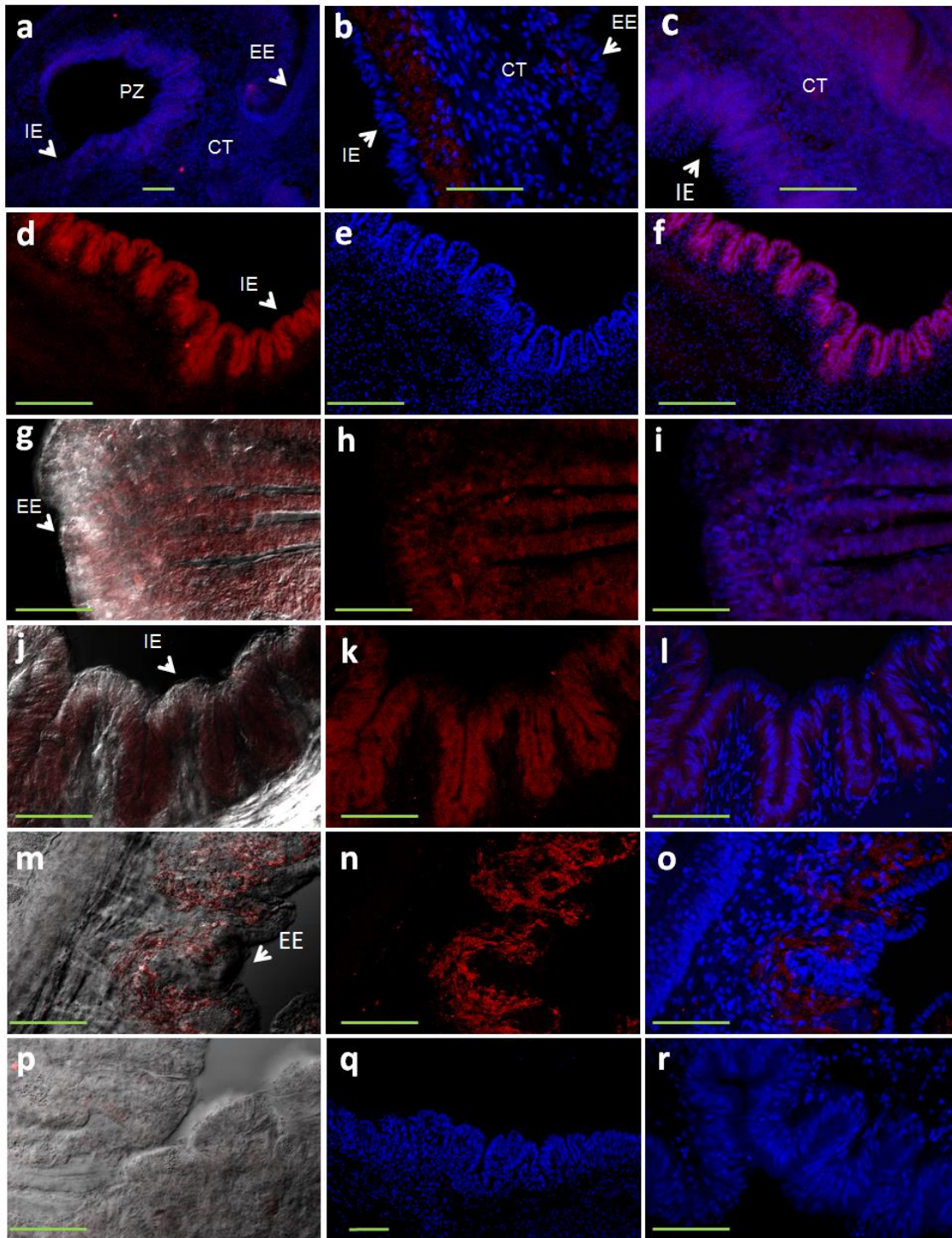
Figure 2 Longitudinal section of mantle and gill tissues. a). SRO mantle tissue (20X); b). SRO Mantle tissue; c). PO mantle tissue 1hour; d) PO mantle 1hr Alexa Fluor labelled poly (I:C); e). PO mantle 1hr Hoechst 33342 stained; f). Merged image d and e; g).PO gill tissue 24hr DIC image showing poly (I:C); h). PO gill tissue 24hr Alexa Fluor poly (I:C); i). Merged image g and h; j). SRO mantle 24hr DIC image showing poly (I:C) uptake; k). SRO mantle 24hr Alexa Fluor labelled poly (I:C); l). Merged image J and K; m). SRO Mantle 1hr DIC image showing poly (I:C) uptake; n). SRO mantle 1hr Alexa Fluor labelled poly (I:C); o). Merged image m and n; p). SRO gill 1hr DIC. q). PO mantle control showing only Hoechst 33342 stain. r). SRO mantle showing only Hoechst 33342 stain. (Key: IE= Internal Epithelium; EE= External Epithelium; CT= Connective Tissue; PZ= Pallial Zone. Each scale bar is 50 microns.)

After 24 hours post injection, gill tissue of Pacific oyster showed significant amount of fluorescence (mean fluorescence: 11202387) but Sydney rock oyster had much less uptake (mean fluorescence: 3809619) P<0.0001 (Fig 1 and Fig 2g, h, i). At 48 hours' post-injection, differences in the fluorescence intensities between the two species became apparent: Pacific oysters continued to express high levels of fluorescence (mean fluorescence: 8453130), while Sydney Rock oyster tissues showed minimal fluorescence (mean fluorescence: 274002) showing significant difference (P<0.0001).

In the gill tissues of both species, fluorescence intensity decreased with time (post-injection), with maximum fluorescence detected at 24 hours (Fig 1b). Overall our results suggest that dsRNA is internalised and processed at a much faster rate by the gill tissues in Sydney rock oysters than by the corresponding tissues of the Pacific oysters.

Gene expression analysis

We selected four genes *Toll Like Receptor (TLR), Piwi, interferon-induced 44 (IFI44) and Peroxyredoxin (PRDX)* transcript sequences, as targets for evaluating transcriptional responses to dsRNA injection. We selected these genes as viral and non-gene specific dsRNA such as poly (I:C) are known to be detected by TLR receptors resulting in the production of interferon (Alexopoulou, et al., 2001, Brencicova and Diebold, 2013, Ishii and Akira, 2005, Kawai and Akira, 2010). *Piwi* is one of the key gene involved in RNAi pathway whereas, *PRDX* are family of antioxidants that plays an important role in immune modulation (Hock and Meister, 2008). Our qPCR analysis (table 2) showed that relative abundance of several transcripts was altered in response to dsRNA challenge. Detailed statistical analyses are presented in supplementary table 2. In gill tissue of Pacific

oyster (PO), at 1 and 48 hours post dsRNA injection, *IFI44* abundance increased significantly (P<0.05), while the relative abundance of all other targets remained unchanged at these time points. In the gill tissues of Sydney rock oyster (SRO), the relative abundance of *TLR* transcripts was decreased at 1hour post-injection (P<0.05). However, at 24-hour post-injection, the relative abundance of *TLR, Piwi* and *IFI44* transcripts was significantly increased (P<0.05).

Table 2 Gene expression of target genes.

Species/tissue	Time/hour	*TLR*	*PIWI*	*IFI44*	*PRDX*
SRO-Gill	1	↓	↔	↔	↔
	24	↑	↑	↑	↔
	48	↑	↔	↔	↑
SRO-Mantle	1	↓	↑	↓	↔
	24	↓	↔	↓	↔
	48	↔	↑	↓	↑
PO-Gill	1	↔	↔	↑	↔
	24	↔	↔	↔	↔
	48	↔	↔	↑	↔
PO-Mantle	1	↓	↓	↓	↓
	24	↑	↔	↔	↓
	48	↓	↓	↓	↓

Comparison of qPCR analyses of gill and mantle tissues in SRO and PO oyster samples at 1, 24 and 48 hours.

Key: ↑ indicates increased abundance; ↓ indicates decreased abundance; ↔ indicated that the relative abundance was unchanged.

 Abbreviations used: *TLR: Toll-like Receptor;* **PIWI***: Piwi;* **PRDX:** *Peroxyredoxin;* *IFI44: interferon-induced protein 44-like.*

TLR and *PRDX* transcript levels were significantly increased (P<0.005) after 48 hours. The increased abundance of the *Piwi* transcripts in SRO suggests that

RNAi pathway is activated in those tissues. *Piwi* is RNA processing enzyme that participates of the RNA interference pathway (a component of the Dicer-Argonaut-Piwi complex). This observation corroborates our previous analysis of proteomic responses to dsRNA challenge in oysters, which indicated that Piwi proteins were detected only in SRO tissues, but not PO tissues. This data suggests that in SRO, RNAi pathway is operating not only at transcriptomic level but also at proteomic level.

In mantle tissue of PO, at 24-hour post-injection, the relative abundance of *TLR* and *Piwi* was increased, relative to controls (P<0.05). The abundance of all other transcripts that were tested at 1, 24 and 48 hours were decreased relative to controls, except *IFII44* at 24 hours, which remained unchanged. Similarly, in SRO, *Piwi* gene was up regulated but only at 1 and 48 hours. *Peroxyredoxin* mRNA expression level was also upregulated (P<0.05). *Peroxyredoxin* at (1hour and 24hour), *Piwi* at (1hour) and TLR at (48hour) remained unchanged. The relative abundance of all other transcripts were decreased relative to controls. Detailed statistical analyses of gene expression are presented in supplementary table 3.

Conclusion and perspectives

Taken together, our investigations suggest that both species of oysters are capable of internalising exogenous poly (I:C). The molecular mechanisms that are involved in the transport of exogenous poly (I:C) to intracellular destinations are not known. Microscopic analyses of the tissues indicated that although the poly (I:C) molecules were present in the mantle tissues of both species, it is unlikely that those tissues are involved in the processing of the dsRNA. However, the gill tissues of the two oyster species showed different responses to the poly (I:C). We interpret the decrease fluorescence intensity to be a consequence of dsRNA

processing in these tissues. Between 24 to 48 hours, the fluorescence in SRO gills decreased to the levels found at 1hour post injection, while the decrease in PO tissues was smaller. Concomitantly, the relative increase in the abundance of targets associated with RNA processing in SRO tissues suggest that those tissues may be responding to the poly (I:C) by initiating RNAi pathways. *TLRs* are known to detect viral ligands such as ssRNA, dsRNA and glycoproteins (Fullam and Schroder, 2013, Green and Montagnani, 2013, Liu, et al., 2012, Teleshova, et al., 2006), while *Piwi* targets mRNA molecules for silencing and destruction (Hock and Meister, 2008). *Piwi* was upregulated at 24 hours in gill tissue of SRO but remained unchanged in gill tissue of PO. *Piwi* was down regulated at 1 and 48 hours in mantle tissue of PO but remained unchanged at 24hours. Although the mantle tissues of both species showed high levels of fluorescence for the duration of the study, the relative abundance of many of the transcripts we tested was decreased. It is possible that while the mantle tissues may not be involved in RNAi responses, they may respond in other ways to the presence of dsRNA.

It is of interest to extrapolate these finding in the wake of field data that suggest differential susceptibility of the two oyster species to OsHV-1 infection. Segarra et al (2014) suggested that Pacific oysters are genetically diverse in terms of their susceptibility to OsHV-1 infection. However, our robust statistical analysis shows that there was no variability in our oyster samples. Pacific oyster, which is susceptible to OsHV-1 infection, does not appear to trigger an RNAi response to poly (I:C), while less susceptible species, SRO, appears to do so. Further studies using virus-specific dsRNA or siRNA may shed further light on the antiviral mechanisms that may underpin the biological responses to infection.

Acknowledgements

References

Akira S (2009) Innate immunity to pathogens: diversity in receptors for microbial recognition. Immunol Rev 227:5-8

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732-738

Arts JA, Cornelissen FH, Cijsouw T, Hermsen T, Savelkoul HF, Stet RJ (2007) Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, Penaeus monodon. Fish Shellfish Immunol 23:504-513

Brencicova E, Diebold SS (2013) Nucleic acids and endosomal pattern recognition: how to tell friend from foe? Frontiers in cellular and infection microbiology 3:37

Chinchar VG, Yu KH, Jancovich JK (2011) The molecular biology of frog virus 3 and other iridoviruses infecting cold-blooded vertebrates. Viruses 3:1959-1985

Comps M, Cochennec N (1993) A Herpes-like Virus from the European Oyster Ostrea edulis L. Journal of Invertebrate Pathology 62:201-203

Dundon WG, Arzul I, Omnes E, Robert M, Magnabosco C, Zambon M, Gennari L, Toffan A, Terregino C, Capua I, Arcangeli G (2011) Detection of Type 1 Ostreid Herpes variant (OsHV-1 µvar) with no associated mortality in French-origin Pacific cupped oyster Crassostrea gigas farmed in Italy. Aquaculture 314:49-52

Elston R (1997) Bivalve mollusc viruses. 393-403

Farley CA, Banfield WG, Kasnic G, Jr., Foster WS (1972) Oyster herpes-type virus. Science 178:759-760

Fullam A, Schroder M (2013) DExD/H-box RNA helicases as mediators of anti-viral innate immunity and essential host factors for viral replication. Biochim Biophys Acta 1829:854-865

Galtsoff PS (1964) The American oyster, *Crassostrea virginica* Gmelin. In: Fisheries Service NFSC, NOAA (ed), pp 121-122

Gong N, Li Q, Huang J, Fang Z, Zhang G, Xie L, Zhang R (2008) Culture of outer epithelial cells from mantle tissue to study shell matrix protein secretion for biomineralization. Cell and tissue research 333:493-501

Green TJ, Barnes AC (2009) Inhibitor of REL/NF-KappaB is regulated in Sydney rock oysters in response to specific double-stranded RNA and Vibrio alginolyticus, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible. Fish Shellfish Immunol 27:260-265

Green TJ, Montagnani C (2013) Poly I:C induces a protective antiviral immune response in the Pacific oyster (Crassostrea gigas) against subsequent challenge with Ostreid herpesvirus (OsHV-1 muvar). Fish Shellfish Immunol 35:382-388

Grove J, Marsh M (2011) The cell biology of receptor-mediated virus entry. The Journal of cell biology 195:1071-1082

Hock J, Meister G (2008) The Argonaute protein family. Genome biology 9:210

Hwang JY, Park JJ, Yu HJ, Hur YB, Arzul I, Couraleau Y, Park MA (2013) Ostreid herpesvirus 1 infection in farmed Pacific oyster larvae Crassostrea gigas (Thunberg) in Korea. Journal of fish diseases 36:969-972

Ishii KJ, Akira S (2005) Innate immune recognition of nucleic acids: beyond toll-like receptors. Int J Cancer 117:517-523

Kawai T, Akira S (2007) Antiviral signaling through pattern recognition receptors. Journal of biochemistry 141:137-145

Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology 11:373-384

Krummenacher C, Carfi A, Eisenberg RJ, Cohen GH (2013) Entry of herpesviruses into cells: the enigma variations. Advances in experimental medicine and biology 790:178-195

Liu H, Soderhall K, Jiravanichpaisal P (2009) Antiviral immunity in crustaceans. Fish Shellfish Immunol 27:79-88

Liu Y, Chen H, Sun Y, Chen F (2012) Antiviral role of Toll-like receptors and cytokines against the new 2009 H1N1 virus infection. Molecular biology reports 39:1163-1172

Medzhitov R (2007) Recognition of microorganisms and activation of the immune response. Nature 449:819-826

Mian MF, Ahmed AN, Rad M, Babaian A, Bowdish D, Ashkar AA (2013) Length of dsRNA (poly I:C) drives distinct innate immune responses, depending on the cell type. Journal of leukocyte biology 94:1025-1036

Nicolas J. L. CM, Cochennec N. (1992) Herpes-like virus infecting Pacific oyster larvae, Crassostrea gigas. Bulletin European Association of Fish Pathologists 12:11-13

Norton JH, Shepherd MA, Prior HC (1993) Papovavirus-like Infection of the Golden-Lipped Pearl Oyster, Pinctada maxima, from the Torres Strait, Australia. Journal of Invertebrate Pathology 62:198-200

Paganin-Gioanni A, Bellard E, Escoffre JM, Rols MP, Teissie J, Golzio M (2011) Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells. Proceedings of the National Academy of Sciences of the United States of America 108:10443-10447

Parsons RJ, Breitbart M, Lomas MW, Carlson CA (2012) Ocean time-series reveals recurring seasonal patterns of virioplankton dynamics in the northwestern Sargasso Sea. The ISME journal 6:273-284

Paul-Pont I, Dhand NK, Whittington RJ (2013a) Influence of husbandry practices on OsHV-1 associated mortality of Pacific oysters Crassostrea gigas. Aquaculture 412–413:202-214

Paul-Pont I, Dhand NK, Whittington RJ (2013b) Spatial distribution of mortality in Pacific oysters Crassostrea gigas: reflection on mechanisms of OsHV-1 transmission. Diseases of aquatic organisms 105:127-138

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 29:e45

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research 30:e36

OIE (2014) 'Manual of Diagonostic Tests for Aquatic Animals 2014'. World organisation for Animal Health, Paris.

R.M. LD, L. NI, T. RT, Cochennec N (1994) Experimental transmission of a herpes-like virus to axenic larvae of Pacific oyster, *Crassostrea gigas* Bulletin European Association of Fish Pathologists 14(2):

Renault T, Bouquet AL, Maurice JT, Lupo C, Blachier P (2014) Ostreid herpesvirus 1 infection among Pacific oyster (Crassostrea gigas) Spat: relevance of water temperature to virus replication and circulation prior to the onset of mortality. Applied and environmental microbiology 80:5419-5426

Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, Warr G (2004) Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol 78:10442-10448

Roque A, Carrasco N, Andree KB, Lacuesta B, Elandaloussi L, Gairin I, Rodgers CJ, Furones MD (2012) First report of OsHV-1 microvar in Pacific oyster (Crassostrea gigas) cultured in Spain. Aquaculture 324–325:303-306

Saucedo PE, Gómez-Robles E (2008) Does gametogenesis occur naturally in pearl oyster mantle tissue? SPC Pearl Oyster Information Bulletin #18, pp 15-17

Schikorski D, Faury N, Pepin JF, Saulnier D, Tourbiez D, Renault T (2011) Experimental ostreid herpesvirus 1 infection of the Pacific oyster Crassostrea gigas: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. Virus research 155:28-34

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nature methods 9:676-682

Sedy JR, Spear PG, Ware CF (2008) Cross-regulation between herpesviruses and the TNF superfamily members. Nature reviews Immunology 8:861-873

Segarra A, Pepin JF, Arzul I, Morga B, Faury N, Renault T (2010) Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, Crassostrea gigas, in France in 2008. Virus research 153:92-99

Segarra A, Mauduit F, Faury N, Trancart S, Degremont L, Tourbiez D (2014) Dual transcriptomics of virus-host interactions: comparing two Pacific oyster

families presenting contrasted susceptibility to ostreid herpesvirus 1. BMC genomics.15:580.

Singh D, Qi R, Jordan JL, San Mateo L, Kao CC (2013) The human antimicrobial peptide LL-37, but not the mouse ortholog, mCRAMP, can stimulate signaling by poly(I:C) through a FPRL1-dependent pathway. The Journal of biological chemistry 288:8258-8268

Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. Cell 140:805-820

Teleshova N, Kenney J, Williams V, Van Nest G, Marshall J, Lifson JD, Sivin I, Dufour J, Bohm R, Gettie A, Pope M (2006) CpG-C ISS-ODN activation of blood-derived B cells from healthy and chronic immunodeficiency virus-infected macaques. J Leukoc Biol 79:257-267

Thompson AJ, Locarnini SA (2007) Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. Immunol Cell Biol 85:435-445

Ulvila J, Hultmark D, Ramet M (2009) RNA silencing in the antiviral innate immune defence--role of DEAD-box RNA helicases. Scand J Immunol 71:146-158

Walshe DP, Lehane SM, Lehane MJ, Haines LR (2009) Prolonged gene knockdown in the tsetse fly Glossina by feeding double stranded RNA. Insect molecular biology 18:11-19

Watanabe A, Tatematsu M, Saeki K, Shibata S, Shime H, Yoshimura A, Obuse C, Seya T, Matsumoto M (2011) Raftlin is involved in the nucleocapture complex to induce poly(I:C)-mediated TLR3 activation. The Journal of biological chemistry 286:10702-10711

Ye S, Wu L, Luo M, Xu T, Wu X (2013) Characterization and function of an E2-17 kDa (UBE2D) in an invertebrate Haliotis diversicolor supertexta. Fish Shellfish Immunol 34:1496-1504

Zhang L, Li L, Guo X, Litman GW, Dishaw LJ, Zhang G (2015) Massive expansion and functional divergence of innate immune genes in a protostome. Scientific reports 5:8693

Zhou Y, Guo M, Wang X, Li J, Wang Y, Ye L, Dai M, Zhou L, Persidsky Y, Ho W (2013) TLR3 activation efficiency by high or low molecular mass poly I:C. Innate immunity 19:184-192.

Appendix to chapter 2

Supplementary table 1 Detailed statistical analysis of corrected total cell fluorescence.

A: Fixed Effect Tests (Species, Tissue and Time).

Source	Nparm	DF	DFDen	F Ratio	Prob > F
Species	1	1	168	103.7188	<.0001*
Time	2	2	168	34.4373	<.0001*
tissue	1	1	168	0.2652	0.6073
Species*time	2	2	168	7.4468	0.0008*
Species*tissue	1	1	168	48.1753	<.0001*
time*tissue	2	2	168	20.3921	<.0001*
Species*time*tissue	2	2	168	9.0185	0.0002*

B: Least Square mean and standard error of Species.

Level	Least Sq Mean	Std Error
Pacific oyster	6297590.1	244606.64
Sydney rock oyster	2774595.2	244606.64

C: Least Square mean and standard error of Tissues.

Level	Least Sq Mean	Std Error
Gill	4625161.5	244606.64
Mantle	4447023.8	244606.64

D: Least Square mean and standard error of Time steps.

Level	Least Sq Mean	Std Error
1 hour	2542531.3	299580.73
24 hour	5864516.4	299580.73
48 hour	5201230.3	299580.73

E: Least Square mean and standard error of Species and Tissues.

Level	Least Sq Mean	Std Error
Pacific oyster, Gill	7587168.0	345926.03
Pacific oyster, Mantle	5008012.2	345926.03
Sydney rock oyster, Gill	1663155.0	345926.03
Sydney rock oyster, Mantle	3886035.4	345926.03

F: Least Square mean and standard error of Species and Time.

Level	Least Sq Mean	Std Error
Pacific oyster 1 hour	3383973.2	423671.14
Pacific oyster 24 hour	8268983.6	423671.14
Pacific oyster 48 hour	7239813.5	423671.14
Sydney rock oyster 1 hour	1701089.4	423671.14
Sydney rock oyster 24 hour	3460049.2	423671.14
Sydney rock oyster 48 hour	3162647.0	423671.14

G: Least Square mean and standard error of Tissue and Time.

Level	Least Sq Mean	Std Error
Gill 1 hour	2005914.3	423671.14
Mantle 1 hour	3079148.4	423671.14
Gill 24 hour	7506003.4	423671.14
Mantle 24 hour	4223029.3	423671.14
Gill 48 hour	4363566.8	423671.14
Mantle 48 hour	6038893.7	423671.14

H: Least Square Means Differences Tukey HSD (honest significant difference) test showing comparison of fluorescence between species, tissue and time.

Level								Least Sq Mean
PO,1, Mantle	A							3661961
PO,24,Mantle		B	C					5335579
PO,48,Mantle			C					6026496
SRO,1,Mantle	A			D				2496336
SRO,24,Mantle	A							3110479
SRO,48,Mantle			C					6051291
PO,1,Gill	A							3105986
PO,24,Gill					E			11202388
PO,48,Gill						F		8453131
SRO,1,Gill				D			G	905843
SRO,24,Gill	A	B						3809619
SRO,48,Gill							G	274003

Levels not connected by same letter are significantly different

Abbreviations used:

Nparm: nonparametric; **DF**: Degree of freedom; **DFden:** Denominator degrees of freedom; **Std Error**: Standard Error; **Least Sq Mean**: Least square mean (the average of observations associated with a treatment, as these means are estimated from the linear model).

Supplementary table 2 Primers list.

Gene	Genbank or reference	Primer	Sequence
TLR	Ref Green and Montagnani 2013	Forward	GCAGGACTCCACTTTCTCAC
		Reverse	GTTGGCACCCAGGTAAAGG
PIWI	EKC35279	Forward	AGAGCTCTGGAGGGTTGACA
		Reverse	ACACCATCCCGGTACACAAT
IFI44	Fj440108	Forward	AAGATCCAACGATGAAAGAC
		Reverse	TTGTCGACATCACTACAAAC
PRDX	Green and Barnes 2009	Forward	GAAGGATGGAAGGACGGTGAT
		Reverse	CACCTGTGGAAACACCTTCTC
ACTIN	Green and Barnes 2009	Forward	GTATTGCTGACCGTATGCAGAAG
		Reverse	GGTGGAGCAATGACCTTGATC

Abbreviations used: *TLR: Toll-like Receptor; **PIWI**: Piwi; **PRDX:***

*Peroxyredoxin; **IFI44**: interferon-induced protein 44-like*

Supplementary table 3 Detailed statistical analysis of gene expression. Comparison of qPCR analyses of gill and mantle tissues in SRO and PO oyster samples at 1, 24 and 48 hours.

Species/Tissue/Time	Gene	Type	Expression	Std. Error	95% C.I.	P-value	Result
PO Gill 1 hour	TLR	TRG	1.134	0.766 - 1.607	0.501 - 2.277	0.381	↔
	PIWI	TRG	1.229	0.921 - 1.630	0.727 - 2.342	0.067	↔
	IFI44	TRG	1.714	1.219 - 2.525	1.038 - 3.837	0.000	↑
	PRDX	TRG	0.944	0.795 - 1.116	0.660 - 1.325	0.377	↔
	Actin	REF	1.000				
PO Gill 24 hours	TLR	TRG	1.679	0.894 - 4.101	0.483 - 5.621	0.052	↔
	PIWI	TRG	0.723	0.275 - 1.214	0.193 - 5.266	0.287	↔
	IFI44	TRG	1.727	0.587 - 7.158	0.485 - 13.679	0.197	↔
	PRDX	TRG	1.094	0.498 - 2.689	0.374 - 4.288	0.706	↔
	Actin	REF	1.000				
PO Gill 48 hours	TLR	TRG	1.238	0.880 - 1.844	0.594 - 2.147	0.112	↔
	PIWI	TRG	0.983	0.844 - 1.158	0.777 - 1.351	0.738	↔
	IFI44	TRG	1.249	1.034 - 1.474	0.897 - 1.969	0.003	↑
	PRDX	TRG	0.905	0.781 - 1.055	0.712 - 1.256	0.077	↔
	Actin	REF	1.000				

Species/Tissue/Time	Gene	Type	Expression	Std. Error	95% C.I.	P-value	Result
PO Mantle 1 hour	TLR	TRG	0.482	0.314 - 0.740	0.239 - 1.033	0.001	↓
	PIWI	TRG	0.641	0.425 - 0.985	0.364 - 1.271	0.007	↓
	IFI44	TRG	0.669	0.461 - 1.051	0.394 - 1.338	0.008	↓
	PRDX	TRG	0.191	0.143 - 0.279	0.119 - 0.330	0.000	↓
	Actin	REF	1.000				
PO Mantle 24 hours	TLR	TRG	3.451	1.895 - 5.450	1.560 - 9.214	0.000	↑
	PIWI	TRG	0.913	0.791 - 1.064	0.724 - 1.236	0.073	↔
	IFI44	TRG	1.079	0.736 - 1.495	0.558 - 2.291	0.545	↔
	PRDX	TRG	0.750	0.568 - 0.987	0.472 - 1.320	0.009	↓
	Actin	REF	1.000				
PO Mantle 48 hours	TLR	TRG	0.000	0.000 - 0.002	0.000 - 0.005	0.000	↓
	PIWI	TRG	0.000	0.000 - 0.001	0.000 - 0.005	0.000	↓
	IFI44	TRG	0.001	0.000 - 0.003	0.000 - 0.011	0.000	↓
	PRDX	TRG	0.001	0.000 - 0.004	0.000 - 0.011	0.000	↓
	Actin	REF	1.000				

Species/Tissue/Time	Gene	Type	Expression	Std. Error	95% C.I.	P-value	Result
SRO Gill 1 hour	TLR	TRG	0.748	0.667 - 0.844	0.593 - 0.945	0.000	↓
	PIWI	TRG	0.880	0.564 - 1.493	0.444 - 1.746	0.372	↔
	IFI44	TRG	0.941	0.685 - 1.286	0.572 - 1.538	0.529	↔
	PRDX	TRG	0.969	0.542 - 1.666	0.476 - 2.197	0.795	↔
	Actin	REF	1.000				
SRO Gill 24 hours	TLR	TRG	1.289	1.132 - 1.509	0.980 - 1.695	0.000	↑
	PIWI	TRG	1.331	0.980 - 1.833	0.850 - 2.693	0.014	↑
	IFI44	TRG	1.839	1.595 - 2.198	1.484 - 2.518	0.000	↑
	PRDX	TRG	1.227	0.919 - 1.782	0.735 - 2.456	0.092	↔
	Actin	REF	1.000				
SRO Gill 48 hours	TLR	TRG	1.456	1.111 - 1.851	0.900 - 2.258	0.001	↑
	PIWI	TRG	1.111	0.785 - 1.530	0.602 - 1.798	0.323	↔
	IFI44	TRG	0.706	0.218 - 1.424	0.170 - 1.638	0.221	↔
	PRDX	TRG	1.967	1.303 - 3.112	1.009 - 4.341	0.000	↑
	Actin	REF	1.000				

Species/Tissue/Time	Gene	Type	Expression	Std. Error	95% C.I.	P-value	Result
SRO Mantle 1 hour	TLR	TRG	0.152	0.074 - 0.278	0.065 - 0.350	0.000	↓
	PIWI	TRG	4.241	1.671 - 8.635	1.213 - 12.380	0.000	↑
	IFI44	TRG	0.578	0.335 - 1.089	0.214 - 1.461	0.011	↓
	PRDX	TRG	1.251	0.781 - 2.205	0.479 - 2.786	0.226	↔
	Actin	REF	1.000				
SRO Mantle 24 hours	TLR	TRG	0.624	0.504 - 0.790	0.414 - 0.960	0.000	↓
	PIWI	TRG	1.156	0.829 - 1.668	0.678 - 1.983	0.216	↔
	IFI44	TRG	0.738	0.599 - 0.940	0.506 - 1.057	0.001	↓
	PRDX	TRG	1.450	0.621 - 7.883	0.511 - 11.554	0.398	↔
	Actin	REF	1.000				
SRO Mantle 48 hours	TLR	TRG	1.034	0.801 - 1.296	0.703 - 1.535	0.660	↔
	PIWI	TRG	13.467	5.111 - 38.300	3.831 - 76.567	0.000	↑
	IFI44	TRG	0.730	0.498 - 1.116	0.346 - 1.429	0.032	↓
	PRDX	TRG	2.843	1.720 - 4.753	1.173 - 7.941	0.000	↑
	Actin	REF	1.000				

Key: ↑ indicates increased abundance; ↓ indicates decreased abundance; ↔ indicated that the relative abundance was unchanged. **Abbreviations used**: *TLR: Toll-like Receptor; PIWI : Piwi; PRDX: Peroxyredoxin; IFI44: interferon-induced protein 44-like;* **TGR:** Target gene; **REF:** Reference gene.

Supplementary Methods Detailed procedures for the preparation of tissues for microscopy.

1. *Tissue harvest*

Tissues were harvested and preserved for either RNA extraction or vibratome sectioning. Each tissue type (gill and mantle) was excised from the animal and immediately placed in an Eppendorf tube and transferred to liquid nitrogen. These snap frozen tissues were then stored at $-80^{\circ}C$ and later used to extract RNA. The remaining tissues were roughly cut into 10 mm x 10 mm cubes a sharp razor blade. These tissue sections were immediately transferred into a container with 4% (v/v) paraformaldehyde and incubated at $4^{\circ}C$ for 24 hours. The tissue sections were then washed three times in PBS for 30 min each wash to remove the fixative. The dissected tissues were stored in PBS at $4^{\circ}C$ in the dark until further use.

2. *Vibratome sectioning*

After trimming the down edges, tissue samples were cast into the agarose moulds using 2% DNA grade agarose in 1% PBS and placed in a boiling water bath (in 50ml falcon tubes), using the following method; the gels were half set by placing the moulds on ice. Warm agarose was poured into the cooled mould to the half-way point and allowed to harden. The tissue was then placed on-top and the remainder of the agarose was poured over the top and allowed to set. The set cast was removed from the mould and trimmed using a razor blade. Filter paper was used to dry the bottom and side of the set gel. Superglue was then used to adhere the set gel block onto a pin stub. Thin sections were then cut using a LEICA VT1000s vibratome using the following setting; frequency: 4, speed: 4,

thickness: 70μm). A number of sections were cut and the two best were selected for tissue culture. The tissue was then placed in a tissue culture plate containing 1%PBS and stored at 4°C in dark until further use.

3.   *Slide preparation*

After fixing the tissue samples, Hoechst 33342 was used as a counter stain to identify dsDNA by emitting blue fluorescence. Each tissue sample was placed into a tissue culture well and overlaid with 500 μl of 2 μg/ml Hoechst 33342 solution. After 15 min of incubation the tissue was removed and washed twice with 1% PBS for 30 minutes. The tissue was placed onto an acid washed slide and overlaid with 10 μl of Fluoro-gel water based mounting media (ProSciTech; product # IM030). A coverslip was then placed on top and edges were sealed using clear nail polish. Tissues and slides were kept at 4°C in the dark at all times until they were analysed. The tissues and cell samples were housed in the dark in order to avoid photo-bleaching.

Supplementary Figure 1: Oyster anatomy
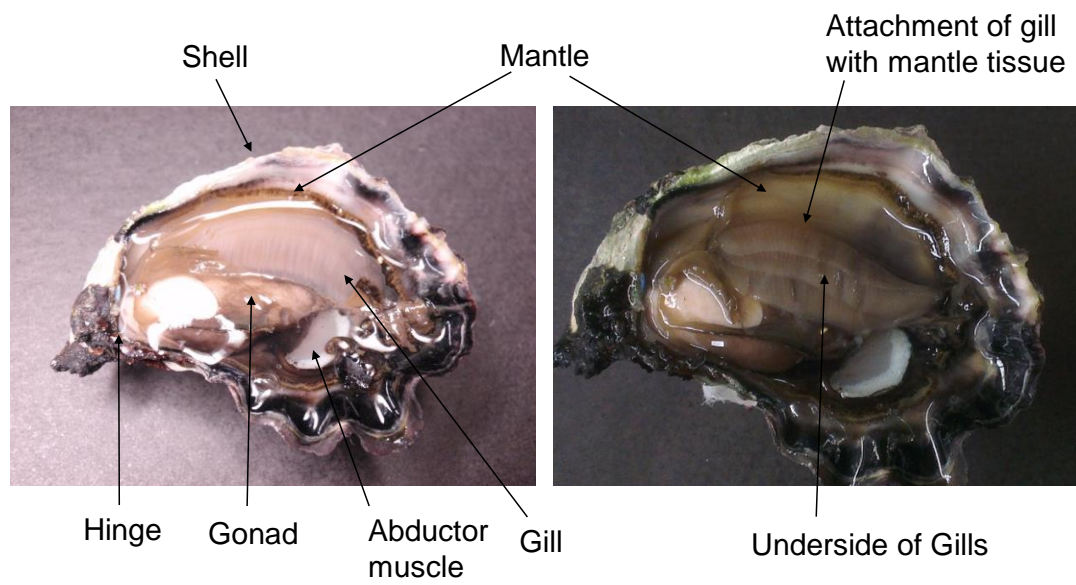
Chapter 3

Two oyster species that show differential susceptibility to virus infection also show differential proteomic responses to generic dsRNA.

Published

Journal of Proteome Research

Copy of published manuscript at the end of the thesis.

Author contributions:

Muhammad Masood - Concept, experimental design, laboratory work, data analysis, interpretation of results and writing of manuscript.

David A. Raftos – Project supervision.

Sham V. Nair – Technical advice and Project supervision.

Preface

Chapter 2 provided fundamental data for experiments described in this chapter. Collectively chapter 2 results demonstrated that *S. glomerata* is more efficient in processing dsRNA than *C. gigas* and gill tissue plays an important role in antiviral responses.

This chapter provides the insight in to the proteome map of the Pacific oyster and Sydney rock oyster by using isobaric tags for relative and absolute quantitation (iTRAQ) tandem mass spectrometry. This aim of this chapter was to investigate total proteome when challenged with non-gene specific dsRNA (poly I:C and poly A:U). Molecular targets in response to OsHV-1 infection using Real time PCR were investigated and compared with proteomic data.

The work in this chapter has been submitted to *Journal of Proteome Research* and is in press. This chapter is presented in the manuscript format of this journal. Supplementary data is presented in the appendix for additional information.

Abstract

Viral diseases are a significant cause of mortality and morbidity in oysters, resulting in significant economic losses. We investigated the proteomic responses of these two species of oysters to generic double stranded RNAs (poly I:C and poly A:U). Analysis of proteomic data (iTRAQ) indicated that there were significant differences in the proteomic responses of the two oyster species resulting from this treatment. Gene ontology analysis showed that several biological processes, cellular component and molecular function were unique to the different datasets. For example, a number of proteins implicated in the TLR signalling pathway were associated with the *S. glomerata* dataset, but were absent in the *C. gigas* dataset. These results suggest that the differences in the proteomic responses to dsRNA may underpin the biological differences in viral susceptibility. Molecular targets previously shown to be expressed in *C. gigas* in response to OsHV1 infections were not present in our proteomic datasets, although they were present in the RNA extracted from the very same tissues. Taken together, our data indicate that there are substantial disparities between transcriptomic and proteomic responses to dsRNA challenge and a comprehensive account of the oysters' biological responses to these treatments must take into account that disparity.

Introduction

Oysters are filter feeders that ingest plankton and other benthic debris. Consequently, they may bio-accumulate pathogenic viruses, bacteria and other microbes, resulting in significant rates of morbidity and mortality amongst wild-type and farmed oysters.[1-4] Recent studies shows that herpes virus that infect oysters enter the host cell by binding to the membrane. HSV-1 capsid entry to the cells is mediated by pH-dependent endocytosis or cortical actin-dependent.[5] This, in turn, translates into devastating economic losses to aquaculture industries worldwide.[6-8] Edible oysters are a significant produce of the aquaculture industry in Australia. Of late, this industry has been plagued by production losses resulting from large-scale infections of oysters by a variety of microbial pathogens, viruses and parasites. While selective breeding techniques have ameliorated the impact of certain types of infectious diseases amongst farmed oysters, little is known about the molecular mechanisms that underpin molecular and immunological mechanisms that may be involved in disease resistance.

The Pacific oyster, *Crassostrea gigas* is susceptible to infection by the herpesvirus *Ostreid Herpesvirus* microvariant (OsHV-1).[9-12] Stock losses arising from OsHV-1 (particularly OsHV-1 μVar) infections has been reported in several countries.[13-20] Viral nucleic acids (e.g. viral ssRNA, dsRNA, ssDNA and dsDNA) and other structural components function as Pathogen-Associated Molecular Patterns (PAMPs), which are recognized by Pattern Recognition Receptors of the host immune systems (PRRs).[21-23] Although little is known about antiviral responses in the invertebrates, in mammalian systems, the detection of viral PAMPS trigger antiviral responses including production of various cytokines and induction of inflammatory and adaptive immune reactions in case of mammals.[24]

Many components of these antiviral systems have also been identified in a number of invertebrates.[25-28]

The RNA interference (RNAi) response to double stranded RNA (dsRNA) is a major defence against viral infection in many organisms. Viral infections often result in the production of dsRNA, which are recognised and processed by components of the RNAi pathway. This leads to the production of cytokines and other signalling molecules, which induce an 'anti-viral' state in neighbouring cells. Such responses have been observed with sequence-specific dsRNA, as well as with generic dsRNA.[29-33] Indeed, RNAi-mediated host immune responses against generic dsRNA such as poly (I:C), poly (A:U) and poly (G:C) has been demonstrated in a number of vertebrates and invertebrates.[34-35]

It has been observed that although the Pacific oysters were susceptible to OsHV-1 infection, the Sydney Rock oysters (*Saccostrea glomerata*) were apparently not susceptible to infection by the same pathogen. Sydney Rock oysters collected from river systems that contained virus-infected Pacific oysters did not show pathologies that were consistent with virus infection. We hypothesised that either the Sydney Rock oysters were immunologically resistant to the virus or that the virus was unable to productively infect the host. In order to evaluate the differences in the OsHV-1 susceptibility phenotypes between these oyster groups, we examined the proteomic changes associated with dsRNA injection.

In the present study, we employed combination of genomic and proteomic approaches to detect the effect of synthetic dsRNA in oysters. Changes to the proteomes of Sydney Rock oyster and Pacific oyster tissues after synthetic dsRNA (poly (I:C) and poly (A:U)) injection were determined using Isobaric Tags

for Relative and Absolute Quantitation (iTRAQ). Since previous studies have reported on changes in the relative abundance of certain PO transcripts after dsRNA injection, we explored those transcriptomic perturbations in our tissue samples using quantitative real-time PCR. Our data indicated that there were substantial differences in the proteomic responses of the two oyster species to dsRNA injection. While there was a good correlation between our real time PCR data and the data that have been published in the literature, those transcript-level changes were not reflected in proteomic alterations.

Materials and Methods

Oyster collection and maintenance

The Sydney rock oysters (SRO), *Saccostrea glomerata*, and Pacific oysters (PO), *Crassostrea gigas*, were purchased from farm in Pambula and Tathra NSW respectively. These oysters were approximately two years of age. PO were hatchery triploid produced for commercial farming. SRO were wild type oyster caught in hatchery where they were farmed. Spat for both species of oysters were reared by standard farming practices involving either oyster bags or racks suspended intertidal. SRO were deemed to be free of known oyster diseases, while the PO were certified to be free of disease (Marteiliosis, Mikrosporidiosis, Perkinsiosis, Bonamiasis and Iridovirosis) according to tests conducted by the Tasmanian Department of Primary Industries, Parks, Water and Environment. The oysters were also found not to harbour OsHV-1 μvar DNA (formally certified through histological examination and RT-PCR assay), which was carried out by the Department of Primary Industries as part of their routine screening procedures). Specimens were housed at 25°C in a closed seawater recirculating system at Macquarie University Sea Water Facility. They were held for a period of 10 days prior to experimentation. During the acclimatization period, animals were fed with M1 shellfish feed (Aquasonic Pty Ltd).

Preparation of double stranded RNA

Poly (I:C) and poly (A:U) (Invivogen) were solubilised in physiological water (NaCl 0.9%) as per manufacturer's instructions and diluted to final concentrations of 2.5 and 5 mg/mL in sterile phosphate buffer saline (PBS). Rhodamine labelled poly I:C (Invivogen) was solubilised in endotoxin-free water as per manufacturer instructions and diluted to a final concentration of 6 ng/µL in PBS.

Experimental setup

The following experimental design was carried out for both SRO and PO. Six groups of oysters were tagged and placed in separate aquaria. Each group consisted of 12 oysters, which were harvested at different time points (3, 8, 24 and 48 hours) after dsRNA or PBS injection. The doses of dsRNA used for the injections are shown in table 1 below (the injected volume was 100 μL per animal). Animals in the control group received 100 µL of PBS each. The dsRNA and PBS solutions were injected in to the animals' mantle cavity through the hinge. Each group of animals was kept in separate aquaria. At each time point (3, 8, 24 and 48 hours post injection), gill tissues were excised for RNA and protein extractions. Previous studies have shown that gill tissues are sites of OsHV-1 infections.[36-37]

Table 1: Experimental deign

Group	Treatment	Notes
1	250 μg poly I:C	
2	250 μg poly A:U	
3	500 μg poly I:C	At 3, 8, 24 and 48 hours after injection, gill tissues from three animals in each treatment group were harvested.
4	500 μg poly A:U	
5	250 μg poly I:C + 250 μg poly A:U	
6	Control (PBS)	

RNA and protein extractions

Gill tissues were excised and ground to a fine power in liquid nitrogen. RNA and proteins was extracted using TRI Reagent (Sigma Aldrich, Castle Hills, NSW, Australia) as per manufacturer's instructions. For iTRAQ analysis, the protein precipitates were washed thrice with ice-cold acetone. The protein precipitates were solubilised in 400uL of 0.25M Triethyl ammonium bicarbonate buffer containing 0.1% SDS. The protein content of those samples was determined using the BCA assay (Bicinchoninic acid protein assay).

iTRAQ 4 plex analysis

iTRAQ 4 plex analysis was performed at the Australian Proteome Analysis Facility (APAF) on a fee-for-service basis. All protein samples from the dsRNA-injected animals were pooled into a single sample, as were the proteins from the PBS-injected animals. The pooling was carried out such that each sample contributed the same amount of protein to the pooled sample. This approach to sample pooling is well-established and is similar to the work of Sylvie et al (2015) and Angel P Diz (2009).[38-39] One hundred micrograms of each pooled sample was reduced and alkylated, before being digested with trypsin. The digested samples were labelled with iTRAQ reagents (isotope labels). After labelling, the samples were cleaned and fractionated by strong cation exchange high performance liquid chromatography (SCX HPLC). Subsequently, reversed phase nanoLC eluent was subject to positive ion nanoflow electrospray analysis in an information dependant acquisition mode (IDA). In IDA mode a Time-of-flight mass spectrometry (TOFMS) survey scan was acquired (m/z 400 - 1500, 0.25 second), with the ten most intense multiply charged ions (counts >150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 200

milliseconds in the mass range m/z 100 – 1500 with the total cycle time 2.3 seconds.

Mass Spectrometry Data processing

A custom amino acid sequence database was created for protein identification. The sequences in this database consisted of those from *Crassostrea gigas* (57,202 sequences) and Bivalvia (55,852 sequences). The PO sequences were translated from open reading frames identified in the PO genome (OysterDB), while Bivalvia sequences were downloaded from NCBI. Both sequence sets were current as of October 2012. iTRAQ quantification of peptides was performed with ProteinPilot 4.0 (AB Sciex, Mt Waverley, Victoria, Australia) using the Paragon algorithm. All reported data were based on 95% confidence interval for protein identification as determined as recommended in the manual of ProteinPilot (unused score 1.3). Geometric means of the ratios were calculated (corresponding to "mean in log space") as per recommendation Protein pilot.[40] The search parameters took into consideration cysteine modification by methyl methanethiosulfonate, digestion of peptides with trypsin (with one missed cleavage permitted) and default biological modification settings. Data were normalized for loading error by bias corrections calculated using ProteinPilot. For the iTRAQ ratio of a peptide to be reported by ProteinPilot, the peptide has to be unique (not shared by other proteins) with good identification confidence (>95%) and the iTRAQ reporting ion intensity in the MS/MS spectrum must be sufficiently high (S/N > 10). A further requirement included a protein p-value, which ensured protein identification and quantitation was not based on a single peptide hit. For detailed methods, refer to Supplementary file_MS.

Live cell analysis

Two groups of nine SRO individuals were injected with 100 μL rhodamine-labelled poly I:C (6 ng/μL in PBS) each. At 3, 15 and 24 h after injection, haemocytes were harvested by shucking the oysters and withdrawing hemolymph directly from the pericardial cavity using 22-guage needles fitted to 5-mL syringes (three oysters were shucked at each time point). Hemolymph was mixed immediately with an equal volume of marine anticoagulant (MAC1; 0.1M glucose, 15mM trisodium citrate, 13mM citric acid, 10mM EDTA, 0.45M NaCl, pH 7.0). The hemolymph suspensions were centrifuged at 800 rpm for 2 min and the supernatants were discarded. The cell pellets were suspended in 200μL PBS. Acid washed microscope slides were coated with PEI (polyethylenimine) for improved adhesion of the cells. Wells were formed on the slides using a PAP pen (ABCAM, Australia). One hundred microliter haemocyte suspensions were added to each well. Cells were allowed to adhere for 30 minutes in a darkened, humidified chamber. After 30 minutes, excess liquid was removed and the adhered cells were washed twice with 100μL PBS (each). Finally, the cells were overlaid with 50 μL of the PBS and covered with a 20 x 20mm coverslip and sealed with nail polish. Cells were visualized using Olympus Fluoview 300 confocal fluorescence equipped with TX71 inverted microscope using Helium neon lasers (excitation wavelength was 543nm and emission at 546nm). Rhodamine-associated fluorescence in these cells were visualised using 40X objective UPLAPO 40XOU NA 0.85 and quantified using image processing software (Fluoview version 4). The fluorescence in cells in 10 fields of view were determined, as well as the areas of the cells in those fields. For each time point, the average fluorescence per μm^2 of cell area was determined and these values were then log transformed. We compared the average fluorescence data

between the various time points using One-way ANOVA (Minitab; (α) = 0.05). This was followed with Tukey's post hoc test for pairwise comparison to determine the significance of differences (95% confidence interval).

Reverse transcription and quantitative PCR

Total RNA (10 ng each) from dsRNA injected and PBS injected (controls) oysters were reverse transcribed using Superscript III reverse transcriptase system (Invitrogen), in accordance with the manufacturer's protocol. qPCR was performed using the Sybr qPCR master mix from Kappa Biosystems (KAPA SYBR FAST qPCR master mix). Briefly, various dilutions (undiluted and five-fold dilutions (1/5 to 1/125)) of the reverse transcription reaction were used as templates for the qPCR (each amplification reaction was performed in triplicate). The templates were mixed with the qPCR reaction master mix and the forward and reverse primers (Table 2). The amplification conditions were as follows: initial denaturation (95°C, 10min) followed by 45 cycles of denaturation (95°C, 10s), annealing (60°C, 20s) and extension (72°C, 25s). A subsequent melt temperature curve of the amplicon was performed. Changes in the expression of target sequences were determined using the efficiency-corrected $^{\Delta\Delta}$Ct method.[41] Significance of changes in the abundance of transcripts was determined using the REST software package.[42]

Gene Ontology Analysis

The cellular component, molecular function, biological process ontologies of the proteins identified in this study were annotated through web-accessible software Blast2Go version 2.8 (www.blast2go.com). When required, functional annotations were also curated manually, using Blast searches against the Uniprot database (http://www.uniprot.org) and KEGG database. Gene ontology and

mapping step were performed. The enrichments of specific protein datasets in our samples (control and treatments) were analysed using the Fisher Exact Test facility that was accessed via Blast2go. This test employs the FATIGO algorithm to determine if specific GO terms are enriched in the different datasets.

Results and Discussion

Injected dsRNA is localised in cytoplasmic vesicles

When SRO were injected with rhodamine-labelled poly (I:C) into the mantle cavity, the labels were rapidly localised to cytoplasmic vesicles within the haemocytes (Figure 1). Most cells contained numerous fluorescent endosomal vesicles. Cell-associated fluorescence was evident one hour after the introduction of the label (data not shown), although the intensity was maximal at around 3 hours after dsRNA injection (Figure 2). After this period, the level of fluorescence within the haemocytes decreased significantly, with minimum total fluorescence occurring at 15 hr post-injection (3 hr vs 15 hr: $t = -3.59$, $df = 2$, $P<0.001$). The fluorescence intensity value rose between 15 to 24 hr (15 hr vs 24 hr: $t = 2.96$, $P =0.005$) and the fluorescence intensity values at 24 hr post injection were similar to those at 3 hr post injection (3 hr vs 24 hr: $t = -1.18$, $P =0.24$). Micrographs of haemocytes at 24 hr post injection showed that the fluorescent signals became delocalised. The signals did not appear to be contained within endosomal vesicles, but were broadly distributed within the cytoplasm (data not shown).

dsRNA injection modulates the expression of target genes in Sydney Rock oysters haemocytes

We examined the transcriptional responses of oyster haemocytes to dsRNA. Based on the work by Green and Barnes (2009) [35] we selected a number of transcriptomic targets for qPCR analysis. Our data (figure 3) showed that

relative abundance of several transcripts were altered in response to the treatment.
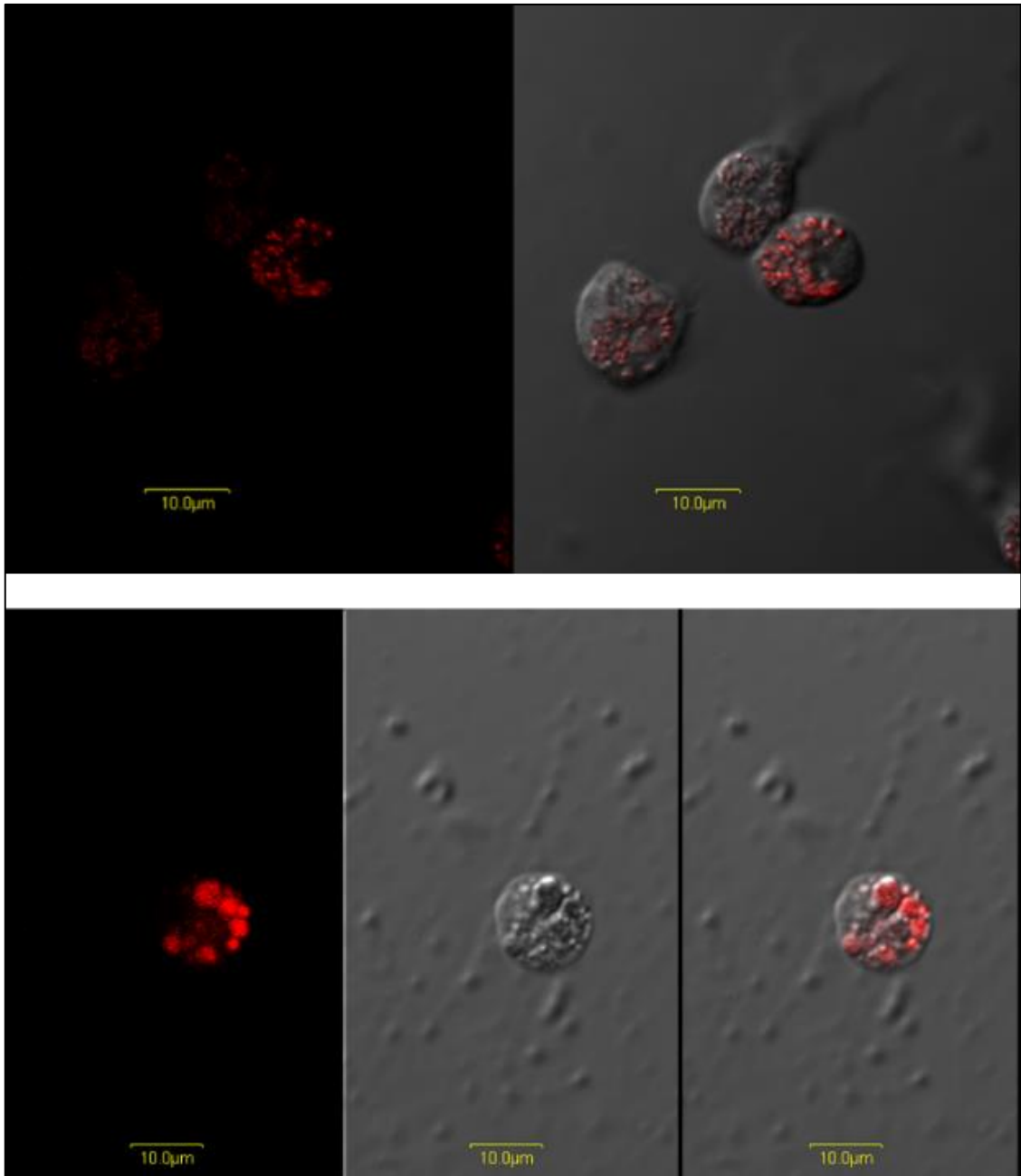
Figure 1: Confocal fluorescence micrographs of Sydney Rock oyster's haemocytes. Haemocytes were harvested from oysters that had been injected with rhodamine-labelled dsRNA into their mantle cavities. This image shows haemocytes from two distinct fields of view that have taken up the labelled dsRNA (3 hr post injection). The figures show both fluorescence micrographs, as well as the corresponding phase contrast images. The fluorescently-labelled dsRNA appears to be contained in cytoplasmic vesicles.
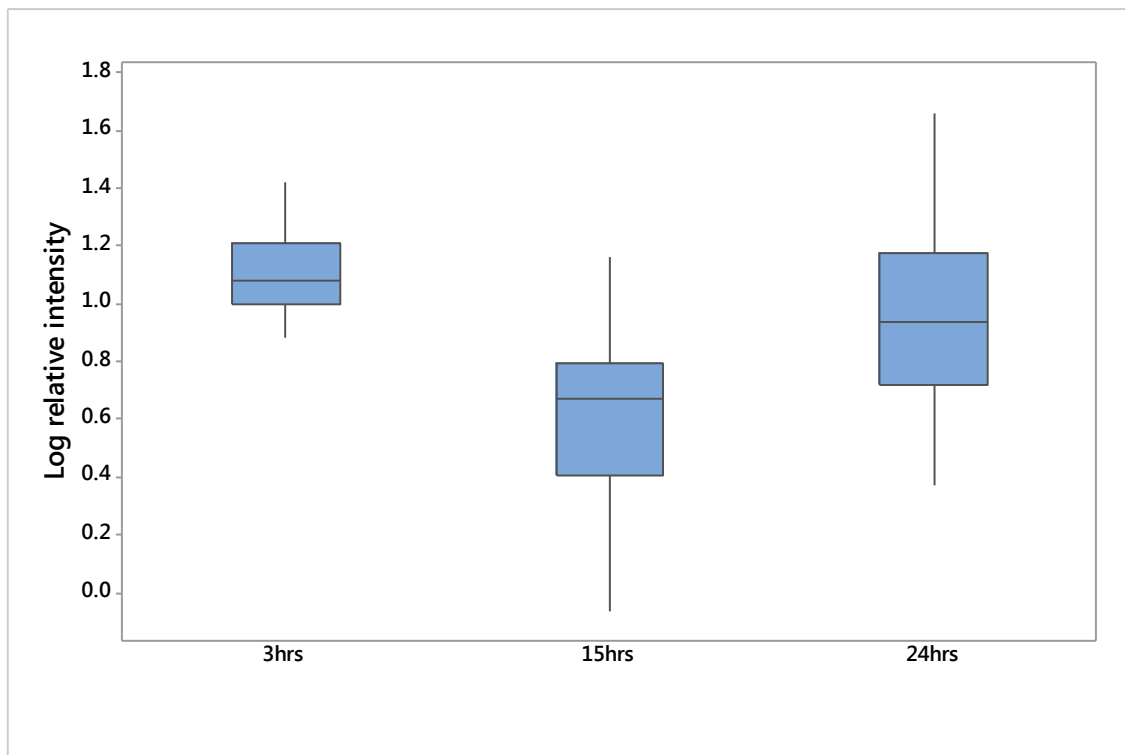
Figure 2: Relative fluorescence intensity (per μm^2 of cell area) of Sydney Rock oysters haemocytes harvested at various time points after rhodamine-labelled poly I:C was injected into the oysters. Haemocytes from three animals (n=3) were analysed at each time point. Statistical analysis (ANOVA) of the data indicated that the differences between all time points, except the 3 - 24 hr comparison, were significant (3 hr vs 15 hr: t = -3.59, df = 2, P<0.001; 15 hr vs 24 hr: t = 2.96, P=0.005; 3 hr vs 24 hr: t = -1.18, P=0.24).

Compared to the 3h time point, the relative abundance of *IkB* (Inhibitor Kappa B) transcripts was elevated at 15 hr and remained elevated at 24h (p<0.05). The transcripts for *IK Cytokine* also showed a similar trend, except that at 24 hr, the relative abundance of the transcripts was the same as that at 3 hr (p>0.05). For both extracellular *superoxide dismutase* (SOD) and *Peroxyredoxin* (PRO), the transcript abundance at 15 h was similar to that at 3h. However, at 24h, the relative abundance of PRO transcripts had increased significantly (P<0.05), while that of the SOD transcripts was significantly lower than that at both 3 and 15h (Figure 3).
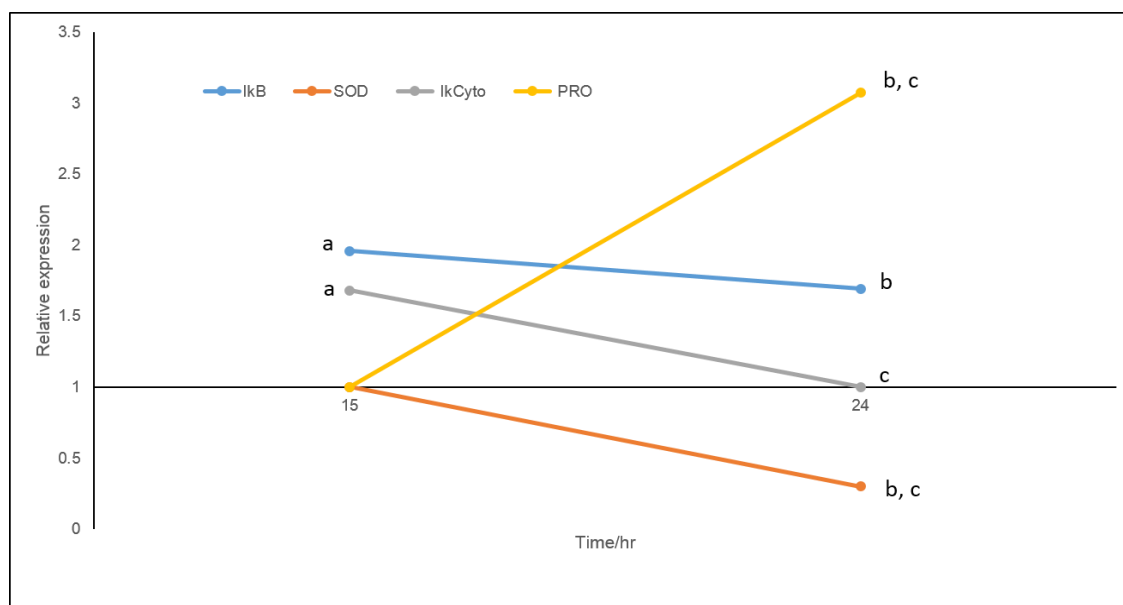
Figure 3: Relative expression of target genes in oyster haemocytes that were injected with poly I:C dsRNA. At 15 hr post-injection, the expression of IkB and IKCyto was increased relative to 3 hr post-injection, while the expression of SOD and PRO were unchanged at the same time point. At 24 hours post-injection, All genes, except for PRO showed a decreased relative abundance. For each time point shown in this figure, the expression of the target genes in dsRNA-injected (treatment) and PBS-injected (control) was measured by quantitative PCR. Actin was used as the reference gene. Statistical analyses of qPCR data were performed using REST software package. a: 15 hr vs 3 hr, $P<0.05$; b: 24 hr vs 3 hr, $P<0.05$; c: 24 hr vs 15 hr, $P<0.05$. Key: IkB – Inhibitor kappa B; SOD – superoxide dismutase; IkCyto – Ik cytokine; PRO – Peroxyredoxin.

In their work, Green and Barnes[35] also showed that the expression of *IkB* and *IK Cytokine* genes were elevated in response to poly I:C injection. They reported that the expression of those targets was elevated at 6h post-injection and remained unchanged at 24 hr post-injection. However, our data showed that while the relative abundance of the *IkB* transcripts were unchanged between 15 and 24h, the relative abundance of *IK Cytokine* transcripts decreased significantly during the same period. A subtractive suppression hybridisation analysis of Pacific oysters indicated that IK cytokine was produced in response to OsHV1 infection.[43]

While Green and Branes[35] reported that the expression of the SOD and PRO genes were unchanged in response to poly I:C injection, our data indicated otherwise. The significant decrease in SOD transcripts, together with the

109

increase in PRO transcripts between 15-24h post-injection suggest that oxidative metabolic pathways may be modulated in response to the dsRNA treatment.

Taken together, our data suggest that the oyster haemocytes modulate the expression of several genes in response to poly I:C injection. Both *IkB* and *IK Cytokine* genes are known to be expressed after dsRNA challenge.[44-45] IkB is a component of TLR signalling, while Peroxyredoxin regulates cytokine-induced peroxidase activity in cells.[46-48] These observations support a model wherein the poly I:C dsRNA that was injected into the oysters was probably internalised via endosomal TLR systems and processed via the RNA interference pathway, leading to the production of cytokines.

Analysis of proteomic changes in response to dsRNA injection in Sydney Rock oysters and Pacific oysters

In many biological systems, numerous cellular processes are activated when dsRNA is internalised. Such process have been observed when generic dsRNA, such as poly (I:C) and poly (A:U), are internalised. In addition to the activation of gene expression, changes to the proteomes of cells responding to such dsRNA has also been documented.[49]

We proceeded to analyse proteomic changes in oysters that were injected with dsRNA using iTRAQ (see Materials and Methods). For each oyster species, protein samples were analysed in duplicate, with reciprocal isobaric reagent labelling. The abundance of the identified proteins in dsRNA-injected and in PBS-injected oyster samples were determined, and their relative abundance in those samples were used as a measure of proteomic changes that occurred in response to dsRNA injection.

iTRAQ analysis of dsRNA-injected SRO samples identified a total of 669 proteins (Figure 4, Supplementary tables S1, S2, Supplementary file_Raw Data). Of these, 256 proteins (38.3%) increased in abundance (fold change > 1.2, combined p-value < 0.05), while 243 proteins (36.3%) proteins decreased in abundance (fold change <0.83, P-value < 0.05) as a result of the treatment. One hundred and seventy proteins in the SRO proteome remained unchanged in response to the treatment.

For PO, a total of 1745 proteins were identified (Figure 4 and Supplementary tables S3, S4), of which 47 proteins (2.6%) had no peptide-level P>0.05. Of the remaining proteins, 399 (23.5%) were in greater abundance in dsRNA-injected samples, while the relative abundance of 321 (18.9%) proteins were significantly decreased in those samples. The relative abundance of 1025 proteins was similar in treated and control samples (i.e. unchanged). A list of significant proteins is provided in Supplementary Tables S6 and S7.

Three hundred and eighty seven proteins showed similar trends in both species. These proteins, which are common to both proteomes, represent 57.8% of the SRO proteome and 22.8% of the PO proteome. Of the 387 proteins, 168 proteins showed increased abundance after dsRNA injection, while the relative abundance of 135 proteins decreased in response to the treatment. In both species, 84 proteins were unaffected by the treatment.
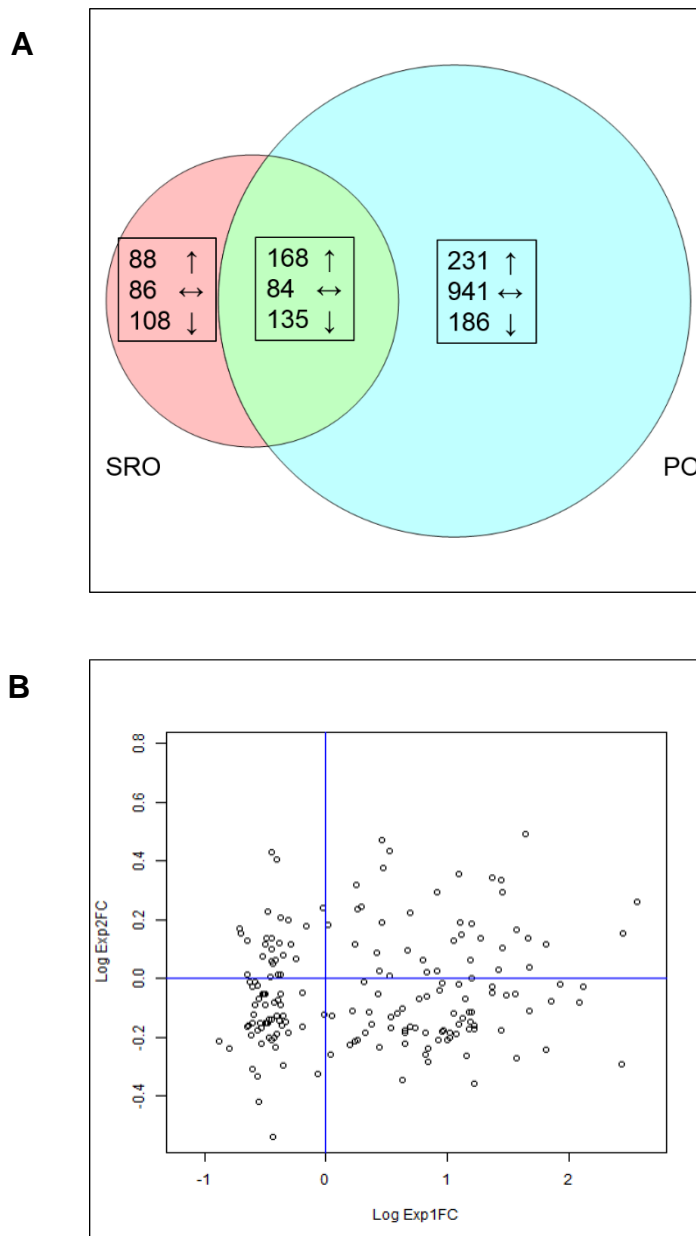
Figure 4: Analysis of proteome-level alterations (iTRAQ) in Sydney Rock oysters and Pacific oyster in response to dsRNA injection. The numbers in this Venn diagram indicate the numbers of proteins whose relative abundance had increased (↑), decreased (↓) or remained unchanged (↔) as a result of the treatment ($P<0.05$). **B**. Comparison of proteins whose relative abundances (expressed as log fold change) were altered in response to dsRNA injection in both oyster species. The vertical axis indicates relative abundance in Pacific oyster (LogExp2FC), while the horizontal axis indicates data from Sydney Rock oysters (LogExp1FC). The blue lines indicate a fold change value of one (unchanged in treated and control samples). Dots in the upper right and lower left quadrants have similar relative abundance profiles in both species, while those in the upper left and lower right quadrants have opposing profiles.

In summary, a large proportion of the proteins identified in the PO proteome (77.2%) were not identified in the SRO dataset. Conversely, 42.2% of the proteins in the SRO dataset were not identified in the PO dataset. These differences may, in part, reflect differences in biological responses of the two species of oysters to dsRNA treatment. We proceeded to evaluate the functional attributes of the proteins in the two datasets using Gene Ontology.

Gene Ontologies

We analysed the proteomic datasets for the putative biological processes that those proteins may be involved in. To do this, we obtained protein sets that had either changed in their relative abundances or those that remained unchanged as a result of dsRNA injection. Each of these datasets were then analysed using the Blast2GO platform. Proteins that had changed in their relative abundance in response to dsRNA injections in either species were classified into 39 biological process gene ontology categories (Figure 5a). In contrast, proteins that displayed similar patterns of relative abundances in both species were classified into 40 biological process gene ontology categories (Figure 5b). Of these, 15 biological process categories were unique to PO (i.e., not represented in the SRO dataset), while nine were unique to SRO (Table 3). For cellular component ontologies, 13 categories were identified (two unique to PO and 2 unique to SRO), while 21 molecular function ontology categories were identified (nine unique to PO and four unique to SRO) (data not shown).

Both of the iTRAQ datasets and the gene ontology analyses indicated that there were substantial differences in the two species' proteomic responses to dsRNA injection. In order to gain insights into the biological processes that may be represented by these ontologies, we investigated if specific gene ontology

terms were enriched in the two iTRAQ datasets using the Fisher exact test. This was performed using the FatiGO package, accessed via Blast2GO.[50-53] The enrichment test (Fisher exact test) calculates the relative abundance of Gene Ontology terms in the two datasets, after correcting for False Discovery Rates (Multiple Testing for False Discovery Rates by the method of Benjamini and Hockberg).

Figure 5A
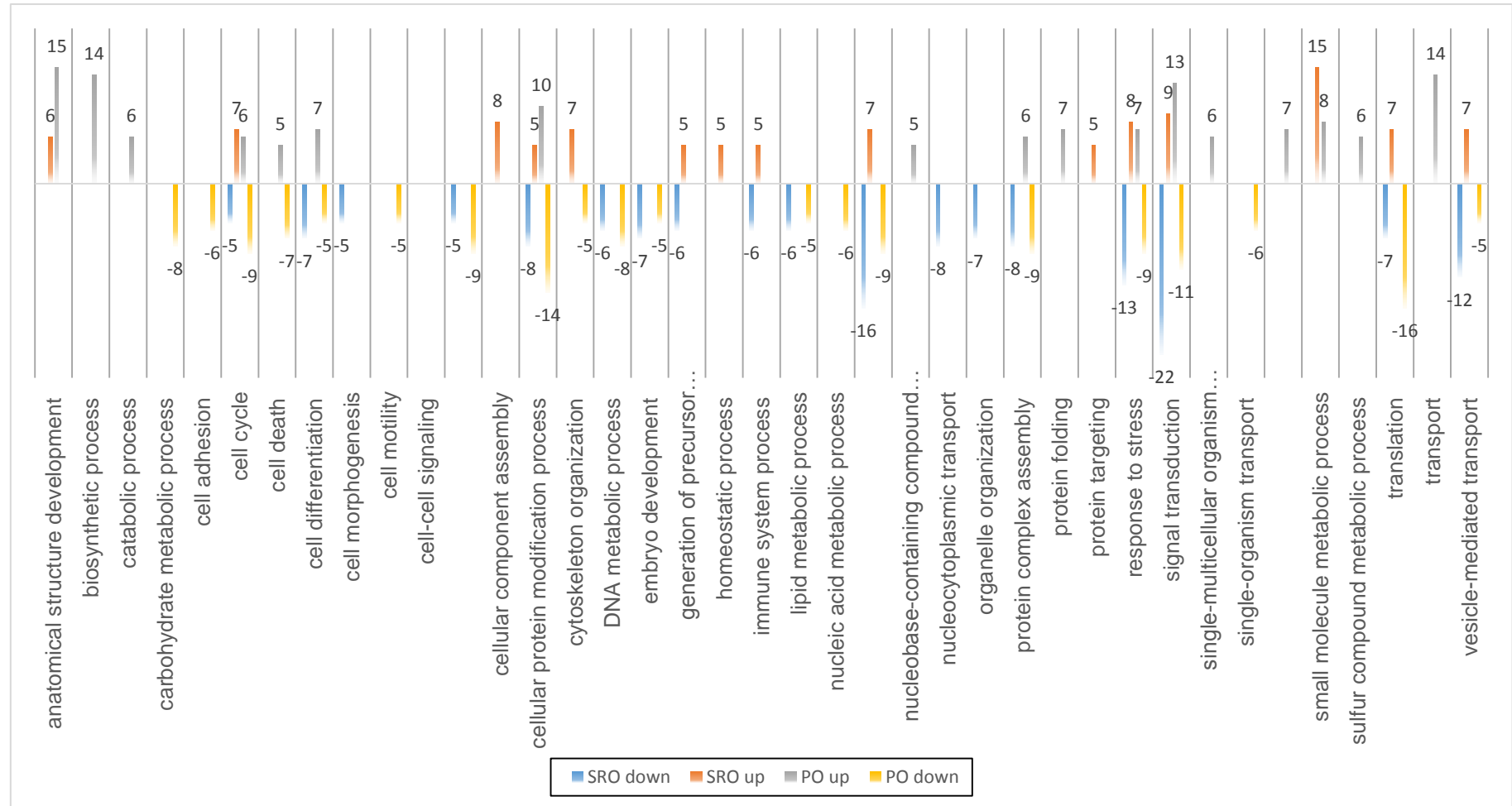
Figure 5B
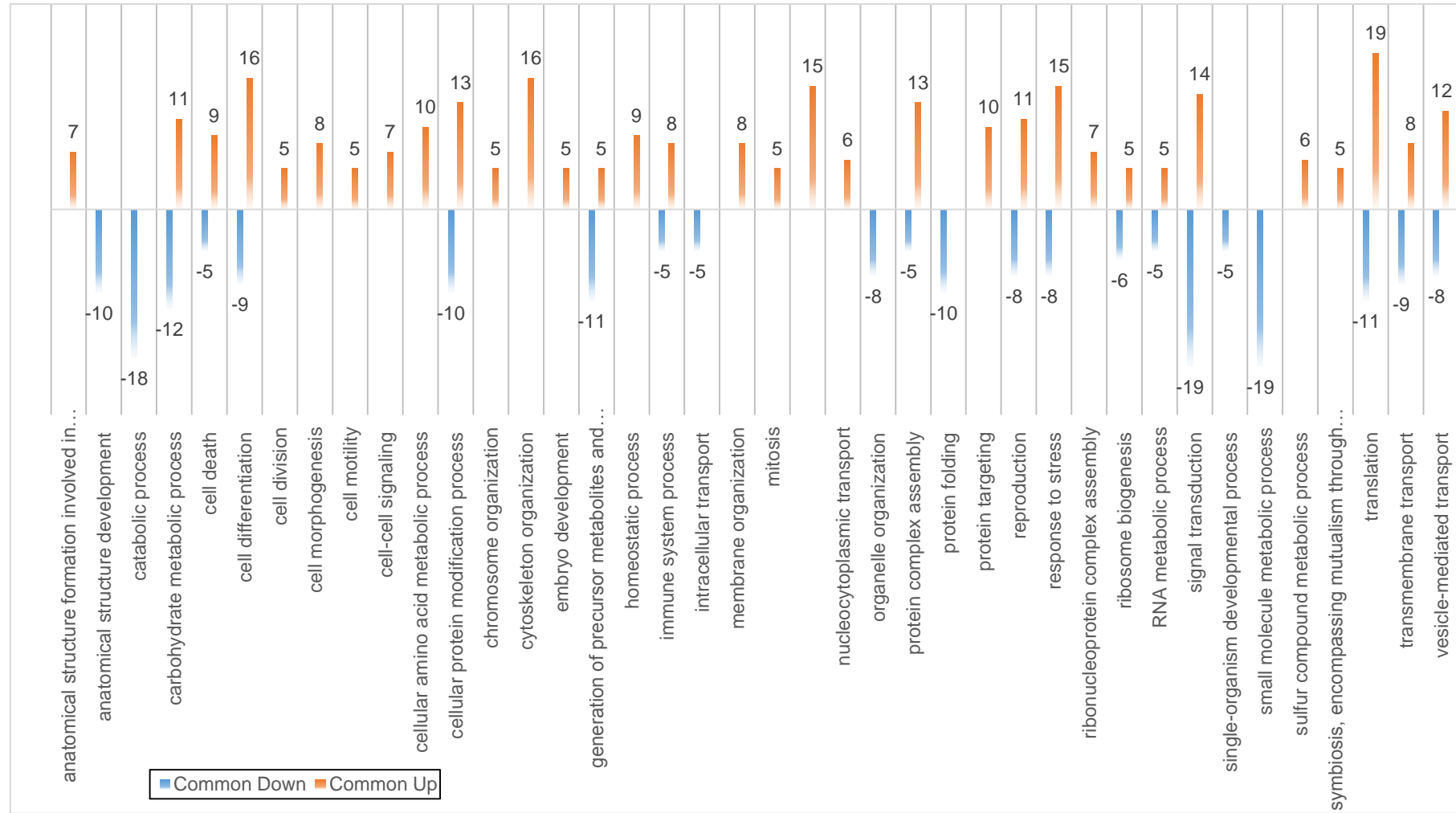
Figure 5 Gene ontology classification of proteins that were expressed in both Sydney Rock oysters and Pacific oyster samples. Negative numbers in each category indicate the number of sequences that were reduced in abundance (dsRNA vs control), while positive numbers indicate the numbers of sequences that had increased in abundance. A. Gene ontology assignments for sequences that were unique to Sydney Rock oysters or Pacific oyster. B. Gene ontology assignments for sequences that was common to both Sydney Rock oyster and Pacific oyster.

The results (Supplementary Table S5a) indicated that several gene ontology categories (Biological Process, Cellular Component and Molecular Function) were differentially represented in the two iTRAQ datasets. Gene ontology results for the significant proteins for both species SRO and PO are presented in supplementary tables S6 and S7 respectively. Interestingly, all of those categories were more highly represented in the SRO proteome dataset, compared to the PO proteome dataset.

Since the enrichment test requires that each gene ontology term be found in both datasets, terms that are found in only one dataset cannot be analysed using this method. We have listed (Supplementary Table S5b) gene ontology terms that are unique to each dataset. There were seven Biological Process categories each that were unique to the PO and SRO datasets. It has not escaped our notice that the 'immune process' Biological Process category was uniquely associated with the SRO dataset. A significant number of the proteins within this category are known to be associated with the TLR signalling pathway (gi|46359622; Polyubiquitin-C isoform x1, gi|405954301; MAP kinase-activated protein kinase 2, gi|405967516; ubiquitin-conjugating enzyme e2n). Indeed, the endosomal TLR pathway has been implicated in dsRNA processing. Endosomal TLRs, such as TLR4 bind to dsRNA within endosomal vesicles and subsequently activate the TLR signalling pathway, which includes regulators such as NFκB and IkB. According to our data these proteins were activated (treatment relative

117

to control) only in the SRO. The lack of response of TLR signalling pathway genes in *C.gigas* to the two types of dsRNA that were used may not be indicative of the response to all types of dsRNA. Potentially, they may respond to other types of dsRNA, including viral, particularly those that are not low complexity.

Another protein of interest that was identified in the course of our investigation is Piwi. Piwi is an RNA processing enzyme that participates of the RNA interference pathway (a component of the Dicer-Argonaut-Piwi complex).[54] This protein was found only in dsRNA-injected SRO samples, but not in PBS-injected Sydney Rock oysters samples (hence, the Stouffer p-value could not be determined). The Piwi protein was not identified dsRNA-injected or control PO samples.

Taken together, these observations imply that SRO may respond to the dsRNA that were employed in this study, while the PO may not do so. We believe that such observations about the differences in the proteomic responses of the two oyster species may underpin susceptibility to viral infections (as the PO susceptibility, but not the SRO's, to OsHV1 infections in the Sydney basin has been documented).

Proteomic changes do not correlate with transcriptomic responses to dsRNA challenge

During the course of this project, manuscripts by Green & Barnes[35] and Green & Montagnani[37] described the relative abundance of various transcripts (using qRT-PCR) in PO haemocytes responding to poly I:C injection. Curiously, none of those gene products were identified in our SRO and PO iTRAQ datasets. In order to address this discrepancy, we performed a qRT-PCR analysis on RNA extracted from the same PO samples that we used for the iTRAQ experiment.

118

In the first part of our study, we attempted to validate the findings of the iTRAQ study using qPCR. The results, shown in Table 4, show the degree of correspondence between qPCR and iTRAQ datasets. For the qPCR assay, *EF1a* (Elongation Factor 1 alpha) was used as the control. As previous analysis shown that among a range of genes identified *EF1a* was the most stable housekeeping gene determined by geNORM. The targets that were selected showed different expression patterns in the iTRAQ analysis (i.e., targets that had increased or decreased their relative abundances, as well as those that were unchanged) and the statistical significance of the transcript abundances were analysed using the REST software (see Materials and Methods). For the targets whose protein abundances were unchanged (iTRAQ), their transcript abundances were also unchanged. Of the seven protein targets whose abundance was not changed as a result of the treatment, six targets showed similar trends in the qPCR studies (85.7% correspondence; Table 4). Of the seven protein targets whose abundances had either increased or decreased, only one target showed a correspondence between the iTRAQ and qPCR analyses (14.3% correspondence; Table 4). Overall, the correspondence between the iTRAQ and qPCR data was 50%.

In the second part of this study, we employed the same primers (10 targets in total) that Green & Barnes[35] and Green & Montagnani[37] used in their experiments on the transcriptional responses to poly I:C injection. As shown in Table 5, there was a close correlation between our qPCR results and those reported by Green & Barnes[35] and Renault *et al.*[43] Of the 10 targets we analysed, nine targets (90%) showed similar expression patterns between our studies and those that have been published. When we examined our iTRAQ dataset, only one of those 10 qPCR targets was identified.

On the whole, our findings indicate that there was a poor correlation between proteomic and transcriptomic analyses of tissue samples. This was particularly true of transcriptomic targets whose relative abundances were altered by the treatment. There was a greater correlation between transcriptomics and proteomics when target abundances were unchanged. We believe that this has significant implications for previous studies that have reported only transcriptomic responses to poly (I:C) treatment.

Concluding perspectives

In this study, we investigated proteomic responses to dsRNA injections in two species of oysters that are key cultivated species in Australia. Susceptibility to Ostreid herpesvirus infections is a key differentiating phenotype between these species. In an effort to understand the biological basis of this difference, we examined their responses to generic dsRNA, which is often used to examine antiviral responses in many biological systems. Our work focussed on 2-year-old oysters. There is some data that suggest that juvenile Pacific oysters are more susceptible to OsHV-1 infections than the adults.[55-56] Pacific oysters that are farmed in Australia are not specifically bred for resistance to OsHV-1 infection. Data from Australia indicates that mortality rates arising from OsHV-1 infections are approximately 100%.[57] This indicates that resistance to infection, if present, is an extremely rare and isolated trait. [58] We believe that our observations of the proteomic responses are broadly applicable to all current brood stocks. Our data indicated that haemocytes in the SRO actively take up exogenous dsRNA and induce changes in the expression of target genes. At the proteomic level, we observed differences in tissue-specific responses to dsRNA injection between SRO and PO. While many of those differences could not be attributed to know biological pathways or processes, we noticed that the TLR signalling pathway

appears to be activated in the SRO only. We believe that this provides a crucial insight into the observed differences in infection phenotype between the two species.

Our data also highlighted the significant disparity between proteomic and transcriptomic datasets. While our transcriptomic data (qPCR) correlated well with target gene expression patterns that have been published in the literature, our proteomic data did not. However, we acknowledge that the lack of correlation may also suggest that iTRAQ is insensitive compare to qPCR. We suggest that this disparity must be taken into account when conclusions are drawn about biological processes using transcriptomic data. Despite this, our investigations have identified a number of molecular targets that may function as biomarkers of biological responses to dsRNA, which may have important implication for the analysis of virus disease susceptibility and antivirus immunity in these oysters.

performed at Australian Proteome Analysis Facility, Macquarie University, North

Ryde, NSW, Australia.

References

1.   Wendling, C.C., F.M. Batista, and K.M. Wegner, *Persistence, seasonal dynamics and pathogenic potential of Vibrio communities from Pacific oyster hemolymph.* PLoS One, **2014**. 9, e94256.

2.   Hughes, F.M., et al., *Apoptosis as a host defense mechanism in Crassostrea virginica and its modulation by Perkinsus marinus.* Fish Shellfish Immunol, **2010**. 29, 247-57.

3.   Kuchel, R.P., et al., *Phagocytosis of the protozoan parasite, Marteilia sydneyi, by Sydney rock oyster (Saccostrea glomerata) hemocytes.* J Invertebr Pathol, **2010.** 104, 97-104.

4.   Paul-Pont, I.; Dhand, N. K.; Whittington, R. J., Spatial distribution of mortality in Pacific oysters Crassostrea gigas: reflection on mechanisms of OsHV-1 transmission. *Dis Aquat Organ* **2013,** 105, 127-38.

5.   Jouaux, A.; Lafont, M.; Blin, J. L.; Houssin, M.; Mathieu, M.; Lelong, C., Physiological change under OsHV-1 contamination in Pacific oyster Crassostrea gigas through massive mortality events on fields. *BMC Genomics* **2013,** 14, 590.

6.   Bartley, D.; Naeve, H.; Subasinghe, R. *World inventory of fisheries. Impact of aquaculture on biodiversity*; Rome, 2005.

7.   AQUAVETPLAN, Disease strategy infection with osteid herpesvirus-1 microvariant. In Australian Government, Department of Agriculture., Ed. National Biosecurity Committee: Canberra ACT 2601 Australia, 2015; Vol. 1.

8.   Voorhees, D. V., Fisheries of the United States. In National Marine Fisheries Services, Fisheries Statistical Division, Ed. Silver Spring, MD 20910-3282 USA, 2013.

9.   Renault, T.; Bouquet, A. L.; Maurice, J. T.; Lupo, C.; Blachier, P., Ostreid herpesvirus 1 infection among Pacific oyster (Crassostrea gigas) Spat: relevance of water temperature to virus replication and circulation prior to the onset of mortality. *Appl Environ Microbiol* **2014,** 80, 5419-26.

10.   Davison, A. J.; Trus, B. L.; Cheng, N.; Steven, A. C.; Watson, M. S.; Cunningham, C.; Le Deuff, R. M.; Renault, T., A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **2005,** 86, 41-53.

11.   Jenkins, C.; Hick, P.; Gabor, M.; Spiers, Z.; Fell, S. A.; Gu, X.; Read, A.; Go, J.; Dove, M.; O'Connor, W.; Kirkland, P. D.; Frances, J., Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 micro-var) in Crassostrea gigas (Pacific oysters) in Australia. *Dis Aquat Organ* **2013,** 105, 109-26.

12.   Segarra, A.; Pepin, J. F.; Arzul, I.; Morga, B.; Faury, N.; Renault, T., Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, Crassostrea gigas, in France in 2008. *Virus Res* **2010,** 153, 92-9.

13.   Authority, E. F. S. *Scientific opinion on the increased mortality events in Pacific oysters, Crassostrea gigas*; 2010; pp 1-59.

14.   Dundon, W. G.; Arzul, I.; Omnes, E.; Robert, M.; Magnabosco, C.; Zambon, M.; Gennari, L.; Toffan, A.; Terregino, C.; Capua, I.; Arcangeli, G., Detection of Type 1 Ostreid Herpes variant (OsHV-1 μvar) with no associated

mortality in French-origin Pacific cupped oyster Crassostrea gigas farmed in Italy. *Aquaculture* **2011,** 314, 49-52.

15.	Lynch, S. A.; Carlsson, J.; Reilly, A. O.; Cotter, E.; Culloty, S. C., A previously undescribed ostreid herpes virus 1 (OsHV-1) genotype detected in the pacific oyster, Crassostrea gigas, in Ireland. *Parasitology* **2012,** 139, 1526-1532.

16.	Martenot, C.; Oden, E.; Travaille, E.; Malas, J. P.; Houssin, M., Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster Crassostrea gigas. *J Virol Methods* **2010,** 170, 86-9.

17.	Martenot, C.; Oden, E.; Travaille, E.; Malas, J. P.; Houssin, M., Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, Crassostrea gigas between 2008 and 2010. *Virus Res* **2011,** 160, 25-31.

18.	Peeler, E. J.; Reese, R. A.; Cheslett, D. L.; Geoghegan, F.; Power, A.; Thrush, M. A., Investigation of mortality in Pacific oysters associated with Ostreid herpesvirus-1 muVar in the Republic of Ireland in 2009. *Prev Vet Med* **2012,** 105, 136-43.

19.	Renault, T.; Moreau, P.; Faury, N.; Pepin, J. F.; Segarra, A.; Webb, S., Analysis of clinical ostreid herpesvirus 1 (Malacoherpesviridae) specimens by sequencing amplified fragments from three virus genome areas. *J Virol* **2012,** 86, 5942-7.

20.	Roque, A.; Carrasco, N.; Andree, K. B.; Lacuesta, B.; Elandaloussi, L.; Gairin, I.; Rodgers, C. J.; Furones, M. D., First report of OsHV-1 microvar in Pacific oyster (Crassostrea gigas) cultured in Spain. *Aquaculture* **2012,** 324–325, 303-306.

21.   Aliyari, R.; Ding, S. W., RNA-based viral immunity initiated by the Dicer family of host immune receptors. *Immunol Rev* **2009,** 227, 176-88.

22.   Ishii, K. J.; Akira, S., Innate immune recognition of nucleic acids: beyond toll-like receptors. *Int J Cancer* **2005,** 117, 517-23.

23.   Medzhitov, R., Recognition of microorganisms and activation of the immune response. *Nature* **2007,** 449, 819-26.

24.   Liu, Y.; Chen, H.; Sun, Y.; Chen, F., Antiviral role of Toll-like receptors and cytokines against the new 2009 H1N1 virus infection. *Mol Biol Rep* **2012,** 39, 1163-72.

25.   Dostert, C.; Jouanguy, E.; Irving, P.; Troxler, L.; Galiana-Arnoux, D.; Hetru, C.; Hoffmann, J. A.; Imler, J. L., The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. *Nat Immunol* **2005,** 6, 946-53.

26.   Huang, X. D.; Liu, W. G.; Wang, Q.; Zhao, M.; Wu, S. Z.; Guan, Y. Y.; Shi, Y.; He, M. X., Molecular characterization of interferon regulatory factor 2 (IRF-2) homolog in pearl oyster Pinctada fucata. *Fish Shellfish Immunol* **2013,** 34, 1279-86.

27.   Souza-Neto, J. A.; Sim, S.; Dimopoulos, G., An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc Natl Acad Sci U S A* **2009,** 106, 17841-6.

28.   He, Y.; Jouaux, A.; Ford, S. E.; Lelong, C.; Sourdaine, P.; Mathieu, M.; Guo, X., Transcriptome analysis reveals strong and complex antiviral response in a mollusc. *Fish Shellfish Immunol* **2015,** 46, 131-44.

29.	Paro, S.; Imler, J. L.; Meignin, C., Sensing viral RNAs by Dicer/RIG-I like ATPases across species. *Curr Opin Immunol* **2015,** 32C, 106-113.

30.	Sidahmed, A.; Abdalla, S.; Mahmud, S.; Wilkie, B., Antiviral innate immune response of RNA interference. *J Infect Dev Ctries* **2014,** 8, 804-10.

31.	Wang, P. H.; Weng, S. P.; He, J. G., Nucleic acid-induced antiviral immunity in invertebrates: an evolutionary perspective. *Dev Comp Immunol* **2015,** 48, 291-6.

32.	Yodmuang, S.; Tirasophon, W.; Roshorm, Y.; Chinnirunvong, W.; Panyim, S., YHV-protease dsRNA inhibits YHV replication in Penaeus monodon and prevents mortality. *Biochem Biophys Res Commun* **2006,** 341, 351-6.

33.	Zambon, R. A.; Vakharia, V. N.; Wu, L. P., RNAi is an antiviral immune response against a dsRNA virus in Drosophila melanogaster. *Cell Microbiol* **2006,** 8, 880-9.

34.	De Zoysa, M.; Kang, H. S.; Song, Y. B.; Jee, Y.; Lee, Y. D.; Lee, J., First report of invertebrate Mx: cloning, characterization and expression analysis of Mx cDNA in disk abalone (Haliotis discus discus). *Fish Shellfish Immunol* **2007,** 23, 86-96.

35.	Green, T. J.; Barnes, A. C., Inhibitor of REL/NF-KappaB is regulated in Sydney rock oysters in response to specific double-stranded RNA and Vibrio alginolyticus, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible. *Fish Shellfish Immunol* **2009,** 27, 260-5.

36.	Zhang, R.; Liu, R.; Wang, W.; Xin, L.; Wang, L.; Li, C.; Song, L., Identification and functional analysis of a novel IFN-like protein (CgIFNLP) in Crassostrea gigas. *Fish Shellfish Immunol* **2015,** 44, 547-54.

37.   Green, T. J.; Montagnani, C., Poly I:C induces a protective antiviral immune response in the Pacific oyster (Crassostrea gigas) against subsequent challenge with Ostreid herpesvirus (OsHV-1 muvar). *Fish Shellfish Immunol* **2013,** 35, 382-8.

38.   Diz, A. P.; Truebano, M.; Skibinski, D. O., The consequences of sample pooling in proteomics: an empirical study. *Electrophoresis* **2009,** 30, 2967-75.

39.   Bourassa, S.; Fournier, F.; Nehme, B.; Kelly, I.; Tremblay, A.; Lemelin, V.; Lamarche, B.; Couture, P.; Droit, A., Evaluation of iTRAQ and SWATH-MS for the Quantification of Proteins Associated with Insulin Resistance in Human Duodenal Biopsy Samples. *PLoS One* **2015,** 10, e0125934.

40.   Pascovici, D.; Song, X.; Solomon, P. S.; Winterberg, B.; Mirzaei, M.; Goodchild, A.; Stanley, W. C.; Liu, J.; Molloy, M. P., Combining protein ratio p-values as a pragmatic approach to the analysis of multirun iTRAQ experiments. *J Proteome Res* **2015,** 14, 738-46.

41.   Pfaffl, M. W., A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **2001,** 29, e45.

42.   Pfaffl, M. W.; Horgan, G. W.; Dempfle, L., Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **2002,** 30, e36.

43.   Renault, T.; Faury, N.; Barbosa-Solomieu, V.; Moreau, K., Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, Crassostrea gigas, challenged with Ostreid herpesvirus 1. *Dev Comp Immunol* **2011,** 35, 725-35.

44.   Alexopoulou, L.; Holt, A. C.; Medzhitov, R.; Flavell, R. A., Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **2001,** 413, 732-8.

45.   Matsumoto, M.; Seya, T., TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev* **2008,** 60, 805-12.

46.   Kawai, T.; Akira, S., TLR signaling. *Cell Death Differ* **2006,** 13, 816-25.

47.   Paula, F. M. M.; Ferreira, S. M.; Boschero, A. C.; Souza, K. L. A., Modulation of the peroxiredoxin system by cytokines in insulin-producing RINm5F cells: Down-regulation of PRDX6 increases susceptibility of beta cells to oxidative stress. *Molecular and Cellular Endocrinology* **2013,** 374, 56-64.

48.   Wood, Z. A.; Schroder, E.; Robin Harris, J.; Poole, L. B., Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* **2003,** 28, 32-40.

49.   Verillaud, B.; Gressette, M.; Morel, Y.; Paturel, C.; Herman, P.; Lo, K. W.; Tsao, S. W.; Wassef, M.; Jimenez-Pailhes, A. S.; Busson, P., Toll-like receptor 3 in Epstein-Barr virus-associated nasopharyngeal carcinomas: consistent expression and cytotoxic effects of its synthetic ligand poly(A:U) combined to a Smac-mimetic. *Infect Agent Cancer* **2012,** 7, 36.

50.   Conesa, A.; Gotz, S., Blast2GO: A comprehensive suite for functional analysis in plant genomics. *Int J Plant Genomics* **2008,** 61, 9832.

51.   Conesa, A.; Gotz, S.; Garcia-Gomez, J. M.; Terol, J.; Talon, M.; Robles, M., Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **2005,** 21, 3674-6.

52.   Gotz, S.; Arnold, R.; Sebastian-Leon, P.; Martin-Rodriguez, S.; Tischler, P.; Jehl, M. A.; Dopazo, J.; Rattei, T.; Conesa, A., B2G-FAR, a species-centered GO annotation repository. *Bioinformatics* **2011,** 27, 919-24.

53.   Gotz, S.; Garcia-Gomez, J. M.; Terol, J.; Williams, T. D.; Nagaraj, S. H.; Nueda, M. J.; Robles, M.; Talon, M.; Dopazo, J.; Conesa, A., High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res* **2008,** 36, 3420-35.

54.   Hutvagner, G.; Simard, M. J., Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol* **2008,** 9, 22-32.

55.   Paul-Pont, I.; Evans, O.; Dhand, N. K.; Rubio, A.; Coad, P.; Whittington, R. J., Descriptive epidemiology of mass mortality due to Ostreid herpesvirus-1 (OsHV-1) in commercially farmed Pacific oysters (Crassostrea gigas) in the Hawkesbury River estuary, Australia. *Aquaculture* **2014,** 422–423, 146-159.

56.   OIE *Infection with ostreid herpesvirus 1*; Department of Agriculture 33: World Organisation for Animal Health, Paris, 2012 a.

57.   Services, A. A. H. *'Final report OsHV-1 µ Var International Workshop'*; AusVet Animal Health Services: Canberra, 2011.

58.   Segarra, A.; Mauduit, F.; Faury, N.; Trancart, S.; Degremont, L.; Tourbiez, D.; Haffner, P.; Barbosa-Solomieu, V.; Pepin, J. F.; Travers, M. A.; Renault, T., Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. *BMC Genomics* **2014,** 15, 580*.*

Appendix to chapter 3

Table 2 List of PCR primers used in this study.

Accession number	Type	Targets	Primer Sequence
FJ626709	N	SOD	F: AACTCTACCACGGCGAGCAT R: CCACGGTCGTCATCATGAAG
FJ626708	N	PRO6	F: GAAGGATGGAAGGACGGTG R: CACCTGTGGAAACACCTTCTC
GH612255	N	IKC	F: AACTGTTTTTACCGGGACGTATG R: GGTACAGGGCACGTCATTCTC
GH612349	N	I_kB	F: TCGCGATTCTCGGTGTTTC R: GGACACGGCACTTCATATTGC
* Reference 28		Actin β	F: GTATTGCTGACCGTATGCAGAA R: GGTGGAGCAATGACCTTGATC
EKC21335	P	OAS	F: CACCCATAGCGTGGACATAC R: GCAGTGGAGCGAGAGCTG
EU678310	N	Bcl2	F: CTCACACAACGGGGGATGGGTAAGTG R: GCACAAGCCCCTAACCATGCAAAAC
AB122066	N	EF1α	F: CAAGAACGGAGATGCTGGTATGG R: TTTCACTCTTTCCACCGGCTTT
EU678311	N	Glypican	F: CGAGTTCTCCCTGGGCAGGTACC R: GGCTCGTTGAGTTCCGAGTGGTAGGC
EF627976	N	IKC	F: GGAGCGCGAGGAAGAGGAGATAATGG R: ATCCGTCCCGGCAGAAACAGCTC
EU678312	N	Ig Domain CAM	F: CATTCTGCCCGGAGACAATCAGATG R: TCCCTCCAGCGATCACTAGATCCAC
DQ250326	N	IkB	F: GAAAAAGTGGCAAGAGTGTC R: GAAGAGTCATCGAAAGCAAC
EKC43155	P	IRF2	F: CGAAACGCAGAAACTGTTC R: ATTTGCCTTCCATCTTTTGG
FJ440108	N	IFI44	F: AAGATCCAACGATGAAAGAC R: TTGTCGACATCACTACAAAC
EKC34807	P	PKR	F: GAGCATCAGCAAAGTGTTGAG R: GTAGCACCAGGAGATGGTTC
EF627979	N	MEP1	F: GCCACCGAAAGCCGGAGAAGATGTC R: ACCGAGACCGAGTTTCAGGGGGTAG
EU678320	N	MCO	F: GATGGGTGGGAGGCGAAACGACTTG R: CATCCATGTAAGGGGTCCCCGTCTG
DQ530619	N	MyD88 adaptor	F: AGGTACCGGCTGTGATACGA R: TTCAAACGCCACCAAGACTG
EU678316	N	PI-4-Kβ	F: CGAGTGCTGGTCTCAGGAGGATGATG R: GCTTTTGGGAGCTGTGAAGTGCTCAG
EU678317	N	PTPase	F: TTGCGTCCCAAGGCCCTAAGTCAAAC R: GTTGGCTTTGGTTTGACTTCCGGTG
EU678314	N	S/Tpk38	F: AAATTGCCCATGTACGAGCCGAGAG R: GTCATCATGTCGCCTCCTGGTAAAAAC
AAK72690	P	Rel1	F: GCTGAACCAGAACCTCATGA R: CGAAGGACATGTTCTGATCC
HM034838	N	Melatonin receptor	F: GGCGATTTCCGTCATTGTGATCCTC R: GCCGTCAGCCACAAGCGTTACATAC
*		TLR	F: GCAGGACTCCACTTTCTCAC R: GTTGGCACCCAGGTAAAGG
EKC43150	P	RPL18	F: GTTGTTGGCACTGTGACTGA R: CTGCTTGCTCTCCTTCCTCT

Accession	Type	Targets	Primer Sequence

132

number			
EKC27095	P	PEPCK	F: CGCCGAGCACATGTTGATTA R: CTGTCCCAGCCAGTTAGTGA
EKC24882	P	ArgK	F: GGGAGAGCTGAAGGGGAAAT R: GCGTTTACTGTAGGCGAAGG
EKC20023	P	RPS8	F: GATTGATGCCACCCCATTCC R: AGCTAGAACTCGTCCAGCTG
EKC37694	P	Plastin3	F: ACGACATAATCAGACCTGGGG R: CCTCATCAGCTGCCATACCA
EKC36004	P	VDASCP2	F: TACCACAACAGGCAGTCACA R: AGTCGCTGGCTTCATAACCT
EKC39411	P	ATP syn β	F: GTGCTGGACCCTTCTCTCTT R: GAAAACGAACACCACACCGA
ACT35639 & EKC39086	P	SAHC	F: TGTGGTTTTCTCTCCCTGGT R: CGAGATATCCGGACTGATCGT
EKC40827	P	SNRPD3	F: GTGCCGCCTTACAAATCGAT R: GTTTTCCTGCTTCCTGGGTG
AEF33422	P	Cdc42-like protein	F: CCGAACACTCCAAGTACCCA R: AGCCCCGACAGTTTAGAAAA
EKC32302	P	RPL13	F: AGTGGCCACAGTCGGATTAA R: GAAAACGAACACCACACCGA
EKC26497	P	BCAAT	F: CGCCGAACTTAGCAAGACTC R: GAACAATCGCTGGAATCCCC
EKC18743	P	SIP	F: GCGGTTTGTCCTCGATACAC R: ACTGGGCGATTGTTCTGGTA
EKC19808	P	Tub	F: CAGCCAACCAACTCGTCAAA R: TTCACGGGCCTCAGAGAATT
EKC38521	P	Enkurin	F: TGCCACGACCATCTAACCTT R: ACAATGCCCTCTAAACCGGA
EKC30468	P	GIPh	F: AAAATCGGGGAGGAATGGGT R: TACACCACCTTGAGACGGTC

* The sequence was obtained from Green and Barnes (2009).

Type: the sequences were obtained from NCBI nucleotide (N) or protein (P) databases.

Abbreviations used: **SOD**: Superoxide Dismutase; **PRO6**: Peroxiredoxin6; **IKC**: IK Cytokine; **OAS**: 2'-5'-oligoadenylate synthetase 3; **EF1α**: Elongation Factor 1 alpha; **Ig domain CAM**: Immunoglobulin domain cell adhesion molecule subfamily protein; **IRF-2**: Interferon regulatory factor 2; **IFI44**: interferon-induced protein 44-like protein; **PKR**: Interferon-induced, double-stranded RNA-activated protein kinase; **MEP**: macrophage expressed protein 1-like protein; **MCO**: multicopper oxidase; **PI-4-kinase beta**: phosphoinositide 4-kinase beta; **PTPase**: protein tyrosine phosphatase; **S/Tpk38**: serine/threonine protein kinase 38; **Rel1**: transcription factor Rel 1; **Melatonin receptor**: Transmembrane receptor (putative melatonin receptor); **TLR**: Toll-like Receptor; **RPL18**: 60S ribosomal protein L18; **PEPCK**: Phosphoenolpyruvate carboxykinase; **ArgK**: Arginine kinase; **RPS8**: 40 s ribosomal protein S8; **VDASCP2**: Voltage-dependent anion-selective channel protein 2; **ATP syn β**: ATP synthase subunit beta, mitochondrial; **SAHC**: S-adenosylhomocysteine hydrolase; **SNRPD3**: Small nuclear ribonucleoprotein Sm D3; **RPL13**: 60S ribosomal protein L13; **BCAAT**: Branched-chain-amino-acid aminotransferase; **SIP**: Stress-induced-phosphoprotein 1; **Tub**: Tubulin alpha-1C chain; **GIPh**: Glycogen phosphorylase, muscle form.

Table 3

Unique to PO	Unique to SRO
Biosynthetic process	Cell morphogenesis
Catabolic process	Cellular component assembly
Carbohydrate metabolic process	DNA metabolic process
Cell adhesion	Generation of precursor metabolites and energy
Cell death	Homeostatic process
Cell motility	Immune system process
Cofactor metabolic process	Nucleocytoplasmic transport
Nucleic acid metabolic process	Organelle organization
Nucleobase-containing compound metabolic process	Protein targeting
Protein folding	
Single-multicellular organism process	
Single-organism transport	
Single-organism developmental process	
Sulphur compound metabolic process	
Transport	

Gene Ontology categories that were unique to Sydney Rock oysters and Pacific oyster.

Table 4

Gene	iTRAQ (PO)	qPCR (PO)
SIP	↑	↔
Enk	↑	↔
PEPCK	↓	↑
ATPsyn	↓	↑
Tub	↓	↔
GIPh	↓	↔
Plastin 3	↔	↑
SAHC	↓	↓
BCAAT	↔	↔
SNRPD3	↔	↔
Cdc42-like protein	↔	↔
ArgK	↔	↔
RPS8	↔	↔
VDASCP2	↔	↔

Correlation in the expression of target proteins/transcripts in Pacific oyster and Sydney Rock oyster ↑ indicates increased relative abundance (treatment vs. control), ↓ indicates decreased relative abundance (treatment vs. control), while ↔ indicates no difference in abundance. Shaded cells indicate that those targets changed in relative abundance in a similar manner in both proteomic and transcriptomic analyses.

Table 5

		iTRAQ	qPCR	
Accession number	Protein	Current study	Current study	Published results [b,c]
EKC21335	OAS	X	↔	↔[b]
EU678311	Glypican	X	↑	↑[c]
EF627976	IK cytokine	X	↑	↑[c]
EU678312	Ig domain CAM	X	↑	↑[c]
EKC43155	IRF-2	X	↑	↑[b]
FJ440108	IFI44	↔[a]	↑	↑[c]
EKC34807	PKR	X	↔	↑[b]
EU678320	MCO	X	↑	↑[c]
DQ530619	MyD88	X	↑	↑[bc]
	TLR	X	↑	↑[b]

Comparison of iTRAQ and qPCR analyses of oyster samples. A number of gene targets, whose relative abundance was **increased** in OsHV-1-infected Pacific oysters, were used to screen our iTRAQ datasets. None of those targets were identified in the Sydney Rock oyster or Pacific oyster samples (see note (a) below). PCR primers were then designed to those sequences and the relative abundance of the corresponding targets was determined in the Pacific oyster samples (used for the iTRAQ analysis) using qPCR. Key: **X** indicates that the targets were not identified in our proteomic analysis; ↑ indicates increased abundance; ↔ indicated that the relative abundance was unchanged.

[a] IFI44 was detected in Pacific oyster proteome, and its relative abundance was unchanged in the dsRNA-injected samples vs control samples.

[b] Green and Barnes (2009) Ref 32.

[c] Renault et al (2011) Ref 40.

Abbreviations used: SIP: Stress induced protein, **Enk**: Enkurin, **PEPCK**: Phosophoenolpyruvate carboxykinase, **ATPsyn**: ATP synthase subunits beta mitochondrial, **Tub**: Tubulin alpha-1C chain, **GlPh**: Glycogen phosphorylase**, SAHC**: S-adenosylhomocysteine hydrolase, **BCAAT**: Branched chain amino acid aminotransferase, **SNRPD3**: Small nuclear ribonucleoprotein Sm D3, **ArgK**: Arginine kinase, **RPS8**: Ribosomal protein S8, **VDASCP2**: Voltage dependent anion selective channel protein 2.

Associated content

Supplementary tables S1 to S7, Supplementary methods MS and Supplementary file (Peptide Raw Data) are presented as a soft copy in the provided CD.

Supplementary Table S1: All proteins identified in Sydney Rock oysters samples. Accession number with the 'RR' prefix indicates that those sequences were identified in reversed sequence database searches.

Supplementary Table S2: All proteins identified in Sydney Rock oysters samples. Accession number with the 'RR' prefix indicates that those sequences were identified in reversed sequence database searches. The Geomeans indicate the mean fold change values (treatment vs control). The Stouffers P values indicate the statistical significance of the identity.

Supplementary Table S3. All proteins identified in Pacific oyster samples. Accession numbers with the 'RR' prefix indicates that those sequences were identified in reversed sequence database searches.

Supplementary Table S4: All proteins identified in Pacific oyster samples. Accession number with the 'RR' prefix indicates that those sequences were identified in reversed sequence database searches. The Geomeans indicate the mean fold change values (treatment vs control). The Stouffers P values indicate the statistical significance of the identity.

Supplementary Table S5: A. Fisher enrichment analysis of GO terms in Sydney Rock oysters and PO iTRAQ data sets. This test calculates the Fisher test statistic for differences in the annotations of the sequences identified in the datasets. The p-vales have been corrected by the False Discovery Rate controls.

Note that for these comparisons to be made, the GO terms must appear in both datasets. By corollary, GO terms that appear in only one dataset are ignored.

B. GO terms that were unique to the Pacific oyster or to Sydney Rock oyster dataset. These categories cannot be evaluated for enrichment using the Fisher Exact test.

Supplementary Table S6: Sydney rock oyster significant proteins BLAST results.

Supplementary Table S7: Pacific oyster significant proteins BLAST results.

Supplementary methods_MS: Supplementary methods used in MS showing iTRAQ labelling pattern & Proteolytic digestion, Conditions used for SCX HPLC, HPLC separation for MS analysis & Protein pilot settings, Bias results for Pacific oyster proj15417 combined search and Bias results for Sydney Rock oyster proj.14202 combined search.

Supplementary file_Peptide Raw Data: Pacific oyster and Sydney rock oyster peptides and proteins raw data.

Chapter 4

SWATH mass spectrometry analysis of oyster tissues reveals the activation of TLR signalling, cytokine production and apoptotic pathways in response to dsRNA challenge: implications for host resistance to virus infections.

Formatted for submission in:

PLOS ONE

Author contributions:

Muhammad Masood – Design of the study, laboratory work, data analysis, interpretation of results and writing of manuscript.

David A. Raftos – Project supervision.

Sham V. Nair – Project supervision and feedback.

Preface

The data described in chapter 3 indicated that exposure to exogenous dsRNA induced significant changes in the oyster proteome. For that study, a mixture of dsRNAs (poly I:C and poly A:U) were used. The samples obtained at the different time points were pooled together, so that a direct comparison could be made between the treatment and control samples. Having established those basic parameters using the iTRAQ proteomics assay, the work described in this chapter extends those initial findings. As previous work described in the literature has indicated that biological responses to dsRNA challenge is often specific to the type of dsRNA used (including generic dsRNAs), treatments were designed to differentiate those effects. Furthermore, time-dependent changes to the proteomes were also investigated to determine if early and late responses to dsRNA could be distinguished. During the course of this project, a new mass spectrometry technique became available. SWATH-MS is more sensitive than iTRAQ and permits the detection of low abundance proteins in biological samples.

The work in this chapter has been formatted for PLOS ONE journal. This chapter is presented in the manuscript format of this journal. Supplementary data are presented in the appendix for additional information.

Abstract

Infection of Pacific oyster (*Crassostrea gigas*) by Ostreid herpes virus (OsHV-1 µvar) is a world-wide problem resulting in the morbidity and mortality of the host. Non gene specific dsRNA are known to mimic viral infection and has been used successfully to evoke immune responses in a number of animals including oysters. We examined the proteome responses to dsRNA challenge in two oyster species. In this current work we studied the effects of two different generic dsRNAs poly (I:C) and poly (A:U), as well as evaluate time-dependent proteomic responses to those dsRNAs. Our SWATH mass spectrometry analysis of oyster tissues reveals the activation of TLR signalling, cytokine production and apoptotic pathways in response to dsRNA challenge. There are substantial differences in the proteomic responses between these two species and they can discriminate between types of dsRNA based on differential expression of proteins. We also identified that Pacific oyster (*C.gigas*) proteins that regulate apoptosis had significantly decreased expression levels even after 24 hours of dsRNA challenge and anti-apoptotic protein with increased expression levels were identified. Whereas in Sydney rock oyster (*S. glomerata*) we found the opposite patterns. Within 3 hours apoptosis proteins were identified with increased expression levels. Hence providing vital information that Pacific oyster may have different mechanism from Sydney rock oyster in dealing with viral infections.

Key words: Invertebrate; dsRNA immune response; virus

Introduction

Most metazoans possess pattern recognition receptors (PRRs) that recognise pathogen associated molecular patterns (PAMPs) (1, 2). Double stranded RNA (dsRNA), which is often produced by replicating viral genomes, function as PAMPS and can activate host innate immunity (3). Receptors such as Toll like receptor are known to detect both viral and synthetic dsRNA. The binding of synthetic dsRNA such as Polyinosine–polycytidylic acid (poly I:C) and polyadenylic–polyuridylic acid (poly A:U) to TLR3 and RIG-1 receptors provoke antiviral immune responses, such as the production of type I interferon (IFN) (4-7). Poly (I:C) and poly (A:U) has been successfully used in various invertebrates and vertebrates systems to study antiviral and antitumor immunity (8-11). These two molecularly different dsRNAs may, under certain circumstances, evoke similar immune response, while at other times, produce distinct biological outcomes. Both poly (I:C) and poly (A:U) activated TLR (TLR3) signalling, although signalling via the RIG-like Receptor (RLR) pathway was activated only by poly (I:C) (4). It is also known that the lengths of the dsRNAs are important determinants of their efficacy as PAMPs (12). Infection of oysters by Ostreid herpesvirus type 1 (OsHV-1) has been a serious challenge to the aquaculture industry, causing morbidity and mortality of infected animals. According to OIE-World animal health, Pacific oyster (*Crassostrea gigas*) are reported to develop clinical symptoms of OsHV-1 disease, whereas Sydney rock oyster (*Saccostrea glomerata*) do not (13). Despite the absence of clinical symptoms associated with the infection, OsHV-1 DNA has been identified in the tissues of *S. glomerata*.

Previous research on Pacific and Sydney rock oysters has focused on transcriptomic studies (14-16). In chapter 3 we have used ITRAQ-MS to identify

142

differentially expressed proteins in Sydney rock oyster and Pacific oysters when they were challenged with poly (I:C) and poly (A:U). Our data indicated that these animals displayed unique proteomic signatures when responding to the dsRNA challenge. The present study employs a more sensitive proteomic approach to explore both time-dependent and dsRNA-dependent change in both oyster species. SWATH-MS (Serial Window Acquisition of Theoretical Spectra-Mass Spectrometry) technique in conjunction with bioinformatics tools using blast 2 go for functional annotation of proteins identified. By using iTRAQ and SWATH technique a similar number of proteins could be quantitated however; SWATH and had the advantage of quantifying a larger percentage of low abundance proteins (17). In addition, SWATH is a label free technique and is highly reproducible.

Material and methods

Oyster collection and maintenance

Hatchery reared Pacific oysters were purchased from an oyster farm in Wapengo NSW, while Sydney rock oysters were kindly donated by farm in Pambula NSW Australia. These oysters were between 18 to 24 months of age. PO were hatchery triploid produced for commercial farming. SRO were wild type oyster caught in hatchery where they were farmed. Spat for both species of oysters were reared by standard farming practices involving either oyster bags or racks suspended intertidal. Pacific oysters were certified to be free of disease for all diseases of shellfish prescribed within schedule 6B of the NSW Fisheries Management Act "Diseases Affecting Fish and Marine Vegetation". The oysters were also tested for the presence of OsHV-1 μvar at the Department of Primary Industries, Parks, Water and Environment Animal Health Laboratories Tasmania and results of this testing were negative. Upon arrival these oysters were housed at 22 ± 2°C for 10 days in a closed seawater recirculating system at Macquarie University Sea Water Facility prior to experimentation. During the acclimatization period, animals were fed with M1 shellfish feed (Aquasonic Pty Ltd, Wauchope NSW).

Preparation of double stranded RNA

Poly (I:C) high molecular weight (Cat# tlrl-pic) and poly (A:U) (Cat # tlrl-pau) were purchased from InvivoGen Australia. These two adjuvants (Poly I:C and poly A:U) were solubilised in physiological water (NaCl 0.9%) as per manufacturer's instructions and diluted to final concentration of 2.5mg/ml in sterile phosphate buffer saline (PBS) (Cat # P4417-50TAB Sigma-Aldrich Australia).

Experimental setup

Three groups of 6 oysters from both species (Pacific oyster and Sydney Rock oysters), were tagged and placed in separate aquaria. Oysters were either injected with 100 µl of PBS or 250 µg/100 µl of dsRNA (poly I:C and poly A:U). From each group 3 oysters were harvested at two time points, 3 and 24 hours post injection. Gill tissues were excised for protein extraction.

Protein extraction and sample preparation

Gill tissues were excised and ground to fine powder using liquid nitrogen. TRIzol Reagent (ThermoFisher Scientific Australia Cat # 10296010) was used for protein extraction as per manufacturer's instructions. For Mass spectrometry proteins were precipitated and washed three times using ice cold acetone. Protein pellets were resuspended in 500 µl of 1% sodium deoxycholate, 30 mM triethyl ammonium bicarbonate, using sonic probe. Proteins assay were performed using Direct Detect Infrared Spectrometer (Merick Millipore Australia). 100 µg of sample was used for digestion. Samples were reduced with dithiothreitol (10 mM DTT), alkylated with iodoacetamide (20 mM IAA) and then digested with 2 µg trypsin for 16 hours at 37°C. An equal portion of each digested sample was taken to make two pools, Pacific oyster (25 µL of each sample) and Sydney rock oyster (25uL of each sample). Fifty microliters of each pool was taken into separate tubes for one dimensional - *Information-dependent acquisition (*1D-IDA) runs. The remaining pools were dried for 2D-IDA runs by strong cation exchange. The digested samples and IDA pools were acidified, dried and resuspended in 40 µl of loading buffer (2% acetonitrile 0.1% formic acid). Ten microliters of each sample was transferred to vial for LC-MS/MS. A pool of all samples was prepared by taking the remaining 30 µL of each digested

sample and forming two pools (PO and SRO). High pH reverse phase fractionation was performed using Pierce™ High pH reversed-phase peptide fractionation kit (Cat # 86848) as per manufacturer's instructions. Eight fractions (F1-F8) were eluted from the column and dried. Fractions were re-suspended in 10 µL of loading buffer.

Mass spectrometry

1. *Strong cation exchange HPLC*

Pooled samples were fractionated by SCX HPLC. Buffer A was 5mM Phosphate 25% Acetonitrile, pH 2.7 and buffer B was 5mM Phosphate 350mM KCL 25% Acetonitrile, pH 2.7. The dried pooled sample was first desalted using a Waters Sep-Pak column, then dried and resuspended in loading buffer which was the same as the buffer A. After sample loading and washing with buffer A, buffer B concentration increased from 10% to 45% in 70 minutes and then increased quickly to 100% and stayed at 100% for 10 minutes at a flow rate of 300 µL/min. The eluent of SCX was collected every 2 minutes at the beginning of the gradient and at 4 minutes' interval later. Five pooled fractions from each plate were run using the IDA method.

2. Data acquisition

i). Information dependent acquisition (IDA)

Each pooled sample, SCX fraction and High pH fraction (10µL) was injected onto a peptide trap (Bruker peptide Captrap) for pre-concentration and desalted with 0.1% formic acid, 2% ACN, at 10 µL/min for 5 minutes. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using linear solvent gradients, with steps, from mobile phase A: mobile phase B (98:2) to mobile phase A: mobile phase B (90:10) for ten

146

minutes, then to (65:35) where mobile phase A is 0.1% formic acid and mobile phase B is 99.9% ACN/0.1% formic acid at 550 nL/min over a 98 min period. After peptide elution, the column was cleaned with 95% buffer B for 10 minutes and then equilibrated with 98% buffer A for 20 minutes before next sample injection. The reverse phase nanoLC eluent was subject to positive ion nanoflow electrospray analysis in an information dependant acquisition mode (IDA). In the IDA mode a TOFMS survey scan was acquired (m/z 350 - 1500, 0.25 second), with the ten most intense multiply charged ions (2+ - 5+; counts >150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 50 milliseconds in the mass range m/z 100 – 1500 with rolling collision energy.

ii). Data independent acquisition (SWATH)

The sample (10 uL) were injected onto a peptide trap (Bruker peptide Captrap) for pre-concentration and desalted with 0.1% formic acid, 2% ACN, at 10 µL/min for 5 minutes. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using linear solvent gradients, with steps, from mobile phase A: mobile phase B (98:2) to mobile phase A: mobile phase B (90:10) for ten minutes, then to (65:35) where mobile phase A is 0.1% formic acid and mobile phase B is 99.9% ACN/0.1% formic acid at 550 nL/min over a 98 min period. After peptide elution, the column was cleaned with 95% buffer B for 10 minutes and then equilibrated with 98% buffer A for 20 minutes before next sample injection. The reverse phase nanoLC eluent was subject to positive ion nanoflow electrospray analysis in a data independent acquisition mode (SWATH). For SWATH MS, m/z window sizes were determined based on precursor m/z frequencies (m/z 400 – 1250) in previous IDA data (SWATH variable window acquisition, 60 windows in total). In SWATH mode, first

a TOFMS 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 50 milliseconds in the mass range m/z 350-1500 with rolling collision energy optimised for lowed m/z in m/z window +10%. To minimize instrument condition caused bias, SWATH data were acquired in random order for the samples with one blank run between every duplicate sample injection.

3. Data processing and statistical analysis

i). IDA acquisition

The LC-MS/MS data of all the IDA runs of pooled samples (i.e. 1D and 2D runs) were searched using ProteinPilot (v5.0) (AB Sciex) in thorough mode and combined into a single search report file. The PO sequences were translated from open reading frames identified in the PO genome (OysterDB), while Bivalvia sequences were downloaded from NCBI. Both sequence sets were current as of September 2015. The search result was exported into protein summary and peptide summary text files. Listed protein and peptide unused scores of 2 or above indicate a better than 99% confidence for identification. The unused scores larger than 1.3 indicate a better than 95% confidence.

ii). SWATH quantitation

An extended library was constructed by merging IDA 1D data with IDA 2D data (i.e. SCX fractions and high pH fractions), and used to process the SWATH data. SWATH data were extracted using PeakView (v2.1) with the following parameters:

Top 6 most intense fragments of each peptide were extracted from the SWATH data sets (75 ppm mass tolerance, 10 min retention time window). Shared

peptides were excluded. After data processing, peptides (max 100 peptides per protein) with confidence $\geq$ 99% and FDR $\leq$ 1% (based on chromatographic feature after fragment extraction) were used for quantitation. The extracted SWATH protein peak areas were analysed by APAF bioinformatics group Macquarie University. The protein peaks were normalized to the total peak area for each run and subjected to T-Test to compare relative protein peak area between samples. Protein T-Test with P-value smaller than 0.05 and fold change larger than 1.5 were highlighted. Additionally, a similar method for determining differential expression was run at the peptide level, with the peptide level fold changes then averaged for each protein. The peptide level analysis is more conservative, as peptide level p-values are only generated when a protein was identified by at least two proteins.

Gene Ontology Analysis

The cellular component, molecular function, biological process ontologies of the proteins identified in this study were annotated through Blast2Go version 2.8 (www.blast2go.com). When required, functional annotations were also manually curated, using Blast searches against the Uniprot database ([http://www.uniprot.org) and KEGG database](http://www.uniprot.org).

Results and discussion

Overview of changes in the relative abundance of proteins in the oyster proteomes

In this study, we analysed the proteomes of Pacific and Sydney rock oysters using the SWATH MS technique. Initially a library of spectra was generated by analysing all samples using mass spectrometry. A complete recording of all fragment ions of the detectable peptide precursors present in samples were used to generate this spectral library. SWATH MS/MS data were searched against spectral library. A total of 1106 proteins were identified in the PO samples and 574 proteins were identified in the SRO samples. Of the proteins that were identified, 693 were unique to the PO and 164 were unique to the SRO, while 410 proteins common to both species (Table 4). Changes in the relative abundance of proteins were determined as fold changes (relative to controls). Proteins with fold change values <0.67 were considered to be decreased in abundance, while those with fold change values >1.50 were deemed to be increased in abundance. Proteins with fold change values within this range (1.5 > x > 0.67) were considered to be unchanged. Changes in the relative abundance of proteins were considered statistically significant if the P-values associated with their fold change values were less than 0.05.

Trends in changes to the Pacific oyster proteome

A detailed list of proteins identified in the PO proteome along with their relative abundance and statistically significant (P<0.05) proteins are presented in

Supplementary Table 1. Between 3 and 24 hours after injection with poly (I:C), the proportion of proteins with increased abundance (relative to controls) remained the similar (12-14%). However, the proportion of proteins with decreased abundance declined (from 39% to 21%) during the same period, with a concomitant increase (49% to 65%) in the proportion of proteins whose relative abundance remained unchanged. The trend in the changes to the PO proteome resulting from poly (A:U) injection was the same as for poly (I:C) injection (table 1).

A multi-dimensional Venn diagram analysis of proteins expressed in response to the treatments, as well as during the sampling times, indicated that only a few proteins were common to all datasets. For example, among the sets of proteins whose relative abundance had increased in response to poly (I:C) challenge, only 4 proteins were common to the 3 h and 24 h datasets. Amongst the sets of proteins with decreased abundance with the same treatment, 88 proteins were common to the 3 h and 24 h datasets. (Fig1A and 1B). Taken together, we conclude the following:

a. Poly (I:C) and poly (A:U) induced different sets of proteins and hence different biological responses in PO.
b. For each treatment, different biological processes were induced at the various time points after dsRNA injection.

Some of the key differences will be discussed in subsequent sections of this manuscript.

Table 1: Changes in protein abundance as a result of dsRNA challenge. Number of proteins abundance was increased, decreased and unchanged represented by fold change and p-values.

Species	Treatment	Time (h)	Increased		Decreased		Un-changed
			Total number changed	Number of proteins that changes significantly (P<0.05)	Total number changed	Number of proteins that changes significantly (P<0.05)	Total number changed
Pacific oyster	Poly I:C	3	127 (12%)	5	435 (39%)	74	544 (49%)
		24	159 (14%)	6	231 (21%)	16	716 (65%)
	Poly A:U	3	149 (13%)	10	287 (26%)	20	670 (61%)
		24	263 (24%)	30	143 (13%)	7	700 (63%)
Sydney rock oyster	Poly I:C	3	171 (30%)	13	90 (16%)	13	313 (54%)
		24	342 (60%)	10	84 (14%)	5	148 (26%)
	Poly A:U	3	176 (31%)	29	84 (14%)	5	314 (55%)
		24	193 (34%)	19	81 (14%)	6	300 (52%)

Blast2go analyses provided an overview of the biological processes that were predominant at the different time points. Gene ontology analysis showed that majority of proteins (either with increased or decreased abundance) identified in PO samples in response to both poly (I:C) and poly (A:U) challenges were clustered in to biological processes such as cellular component organization or biogenesis, localization, response to stimulus, signalling, single organism process, cellular and metabolic process (Fig 3 and 4). At 3 h after dsRNA injection (both poly I:C and poly A:U), more proteins in PO proteome showed a decreased abundance (relative to controls) than proteins that had increased in abundance. It is not clear if the decreased abundance was due to decreased mRNA translation or increased levels of protein degradation. Majority of the proteins with decreased abundance belonged to the 'localisation, single organism process, cellular and metabolic processes' gene ontology categories. However, this trend was reversed at 24 h post injection, suggesting a recovery from the dsRNA challenge (Fig 3 and 4). Microscopy analysis (chapter 2) also revealed clearing of fluorescence labelled dsRNA after 24 h in gill tissue. Similarly, in another study, PO has shown to reduce concentration of OsHV-1 DNA between 27 and 54 h post injection suggesting that *C. gigas* may have a mechanism to clear OsHV-1 from infected gill tissue but after 24 h (15). Although we used non-specific dsRNA but our proteomic and confocal microscopy data fits well with these finding reported by Green et al (14).

Three hours after poly (I:C) injection, proteins belonging to gene ontologies such as signal transduction, transport and wound healing were abundant. At 24 h post-injection, we identified heat shock protein 27-like (gi|762131241). Heat shock proteins (HSP) elevation and re-localization occurs during replication of many viruses.
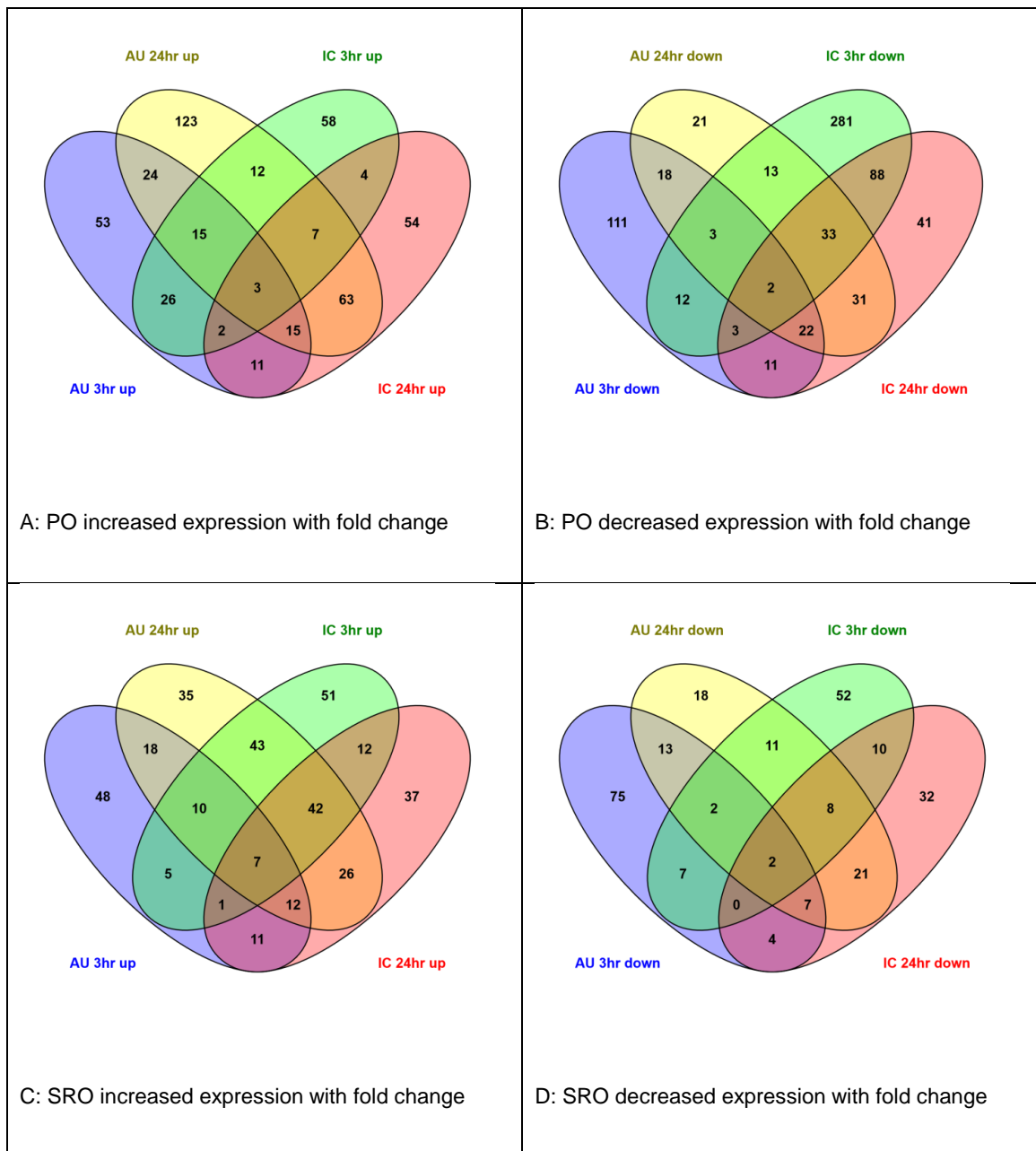
Figure 1: Protein comparison with fold change values in both species Pacific oyster (PO) and Sydney rock oyster (SRO).

HSP regulates apoptosis, exhibits antiviral activity and known to reduce viral replication (18, 19). HSP27 are considered anti-apoptotic proteins (20) but they have also shown to have pro-apoptotic functions (21). Among other functions of HSP is protein folding and protection of proteins from denaturing and are considered activators of innate immune system (22). Whereas calcium ion binding proteins are found to be involved in cellular immune response such as

production of chemokines and cytokines (23). Among other proteins identified with increased abundance after 24 h, are known to have biological functions such as protein folding, calcium ion binding was dominant.

Surprisingly, the biological processes occurring in oysters responding to poly (A:U) injections were different to those observed with poly (I:C) injections. At 3 h post-injection, proteins involved in biological processes such as proliferation, differentiation, motility, stress response, apoptosis, and survival were abundant. At 24 h, biological processes such as RNA binding and splicing, actin binding, lipid binding, transport, redox reactions, gluconeogenesis and oxidation-reduction process were predominant. Catalase-like isoform X4 protein was identified with increased abundance in response to dsRNA challenge. Catalase is known to maintain redox balance of immune system and essential for innate immunity. In clam (*Meretrix meretrix*) catalase showed increased abundance after infection with *Vibrio*, suggesting that it might be involved in immunity (24).

When the protein datasets for both treatments (poly A:U and I:C) and time point (3 and 24 h) were compared, there were only three proteins that were common (vascular endothelial growth factor A-like protein, a pro-proliferative and anti-apoptotic protein, collagen alpha-1(XII) chain-like protein, as well as an uncharacterized protein).

When analysed data after combining all treatments, our data suggest that challenging PO with poly (A:U) showed strong response in increased abundance of various proteins whereas a negative effect was observed when challenged with poly (I:C). Overall PO gill tissues have shown to process poly (I:C) and poly (A:U) differently by expressing different proteins at these two time points.

Trends in the changes in the Sydney rock oyster proteome

An overview of changes to SRO proteome in response to dsRNA injections for all treatments is presented in Supplementary table 2, which shows relative abundance of all proteins and statistically significant (P<0.05) proteins. The trends in the changes to the relative abundance of proteins significantly differed from that seen in the PO datasets. When challenged with poly (I:C), the proportion of proteins with increased relative abundance increased from 30% (3 h) to 60% (24 h). While the proportion of proteins with decreased abundance did not vary between these time points (16% (3 h) and 14% (24 h)), the proportion of proteins whose abundance was unchanged decreased from 54% (3 h) to 26% (24 h). Poly (A:U) challenge did not seem to affect the proportion of proteins with increased, decreased or unchanged abundance at all time points (table 1).

In the poly (I:C) treated sample, the relative abundance of 12 proteins remain elevated during the 3 and 24 h time point, while the abundance of 10 proteins were decreased during the same period. Similarly, in the poly (A:U) treated samples, 18 proteins showed elevated abundance at the same time points, while the abundance of 13 proteins was decreased. (Fig1C and 1D).

Gene ontology analysis shows that majority of proteins identified in SRO in response to both dsRNA challenge belonged to similar biological processes categories as in the PO datasets. However, relative abundance trends were opposite to the PO samples, as the majority of proteins had increased in abundance at 3 h post injection, suggesting that SRO respond to dsRNA challenge different from PO (Figure 5 and 6).

At three hours after injection with poly (I:C), proteins involved in biological processes such as endocytosis, RNA binding, gluconeogenesis, splicing and

translation control, DNA damage repair, apoptosis and immune responses to viruses increased in abundance. Whereas, after 24 h post injection, other biological functions, such as apoptosis, blood coagulation, muscle contraction, antigen processing, mRNA splicing, protein folding, ciliary process, proton transport, cell redox homeostasis, carbohydrate metabolic process and rRNA processing predominated the proteome.

Three hours after poly (A:U) challenge, the abundant proteins in the SRO proteome belong to gene ontology categories such as cell receptors, signalling pathways such as JAK/STAT and apoptosis were identified. Other proteins that are known to have functions such as antigen processing, cytoskeleton, RNA binding, RNA splicing, viral processes, metabolic process and cellular response to amino acid starvation were also identified. After 24 h of poly (A:U) challenge, protein involved in apoptosis and various signalling pathways such as TLR, MAPK and NFκB signalling were identified.

Comparison of proteomic responses of SRO and PO to dsRNA injection

SWATH mass spectrometry datasets and gene ontology analyses indicated that there were substantial differences in the responses of the two oyster species to dsRNA challenge. In both PO and SRO, Poly (I:C) and poly (A:U) induced different sets of proteins and hence different biological processes were induced at the various time points after dsRNA injection. In PO when compared proteins at 3 and 24 h, we found proteins involved in biological processes such as proliferation, differentiation, motility, stress response, apoptosis, and survival at 3 h. However, these trends change after 24 h and proteins that play an important role in immunity, gluconeogenesis and redox balance was identified with increased abundance. When compared with SRO, at

158

3h we found proteins responsible for endocytosis, RNA binding, cell receptors, signalling, gluconeogenesis, DNA damage repair, apoptosis, antigen processing, viral processes and immune responses to viruses. After 24 h in SRO apoptosis, blood coagulation, muscle contraction, antigen processing, protein folding, ciliary process, proton transport, cell redox homeostasis, carbohydrate metabolic process and rRNA processing and signalling were identified with increased abundance.

Both oyster species responded to dsRNA injection by increasing the expression of proteins that participate in TLR signalling pathway. This suggests that TLRs may be involved in binding to the dsRNA and activating subsequent signalling pathways. These findings are in agreement with similar findings in other organisms such as marine crustacean (*Litopenaeus vannamei*), flatfish (*Solea senegalensis Kaup*) and disk abalone (*Haliotis discus discus*) (8, 25-28). We also identified components of the MAPK signalling pathway. Studies have indicated that MAPK signalling pathway closely interacts with the TLR signalling pathway and is responsible for some of the biological responses of cells to various stimuli (29). Mitogen activate protein (MAPK) was also abundant relative to control at 3 h in Pacific oysters that were injected with either Poly (I:C) and Poly (A:U). MAPK can induce expression of multiple genes that play important roles in innate immunity (10, 30). 14-3-3 proteins, which were abundant in dsRNA-injected SRO, is known to modulate TLR-mediated innate immune response (18, 19, 30). 14-3-3 interacts in a variety of cellular processes, including metabolism, protein trafficking, apoptosis, signal transduction, and cell–cycle regulation (14). In SRO, Flotillin-1-like isoform X4 (gi|762105167) was found to be abundant in animals challenged with poly I:C (24 h) and poly (A:U) (3

& 24 h). Flotillin was activated as a result of exogenous dsRNA and among its function is to mediate dsRNA transport via endocytosis and signalling (31).

Virus-infected cells often commit suicide by activating apoptosis or programmed cell death, which is an important process to eliminate these infected cells (32). When Pacific oysters were injected with poly (I:C) or poly (A:U), the relative abundance of the apoptosis regulator, BAX-like isoform X2 (gi|762084026) protein, decreased and remained low for the duration of our study. Viruses that successfully infect host cells employ viral protein products to block or delay apoptosis until sufficient progeny have been produced (32). The decrease in the relative abundance of the BAX-like protein in the Pacific oysters suggests that apoptosis may be actively inhibited or delayed. Green *et al* have demonstrated that OsHV-1 genome can successfully inhibit apoptosis in gill tissue of *C. gigas* (14). The infection of the oyster, *C. virginica* by the parasite, *Perkinsus marinus*, also results in the suppression of apoptosis at the early stages of infection (33).

We have demonstrated that synthetic dsRNA (poly I:C and poly A:U) are capable of triggering anti-apoptosis proteins and PO have shown to delay apoptosis process up to 24 hours. This contrasts with the SRO, where the relative abundance of the BAX-like proteins remained elevated after dsRNA injection. In SRO we also identified Mo25-like proteins, along with Programmed cell death 10, both of which are pro-apoptotic proteins that play important role in innate immunity. They are direct regulator of Germinal Centre Kinases (GCKs). GCK-1kinases regulates NF-kB activation, PAMP signalling and inflammation (34). It may be speculated that the presence of dsRNA may have triggered anti-viral responses (i.e. apoptosis) in the SRO, a response that may not occur in the

160

Pacific oyster to dsRNA. Similarly, it has been demonstrated in cancer cells that poly (I:C) can induce apoptosis (35). The relative abundance of proteins involved in innate and anti-viral immunity was elevated in dsRNA-injected SRO. This was not evident in the proteomes of the dsRNA-injected PO. We identified Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein. The ATP synthase beta subunit protein was also abundant in dsRNA-injected SRO. The expression of ATP synthase beta subunit protein was significantly upregulated in shrimp that were infected with white spot syndrome virus and yellow head virus suggesting that this protein plays an important role in antiviral defense (36, 37).

Concluding perspectives

It is tempting to speculate that the differences that we observed in the oysters' response to dsRNA challenge may be an indicator of host responses to viral infections. Data from the literature suggest that there is a strong causal link between viral infections and cellular responses to dsRNA. The production of dsRNA during viral replication triggers host cellular responses that lead to death of the infected cell, as well as the induction of the anti-viral state in neighbouring cells. The data obtained in this study suggest that the Pacific and Sydney oysters respond in distinct ways to the presence of exogenous dsRNA. The proteome profile of the SRO is consistent with the data obtained in other studies that suggest that this organism activates anti-viral immune pathways, as well as RNA interference pathways. It also suggests that, on the other hand, the Pacific oysters do not respond in a similar fashion to dsRNA challenge and this may be the molecular basis for the organism's susceptibility to OsHV-1 infection. Conversely, it may also shed light on the SRO apparent ability to resist infection by this virus. These potential differences between species should be further assessed by investigating their responses to actual viral infection.

161

Acknowledgement

References

1.   Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.

2.   Eigenbrod T, Keller P, Kaiser S, Rimbach K, Dalpke AH, Helm M. Recognition of Specified RNA Modifications by the Innate Immune System. Methods in enzymology. 2015;560:73-89.

3.   Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology. 1996;219(2):339-49.

4.   Perrot I, Deauvieau F, Massacrier C, Hughes N, Garrone P, Durand I, et al. TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA. Journal of immunology. 2010;185(4):2080-8.

5.   Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology. 2010;11(5):373-84.

6.   Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature. 2006;441(7089):101-5.

7.   Sugiyama T, Hoshino K, Saito M, Yano T, Sasaki I, Yamazaki C, et al. Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7. International immunology. 2008;20(1):1-9.

8.   Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, et al. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol. 2004;78(19):10442-8.

9.   Gatti G, Nunez NG, Nocera DA, Dejager L, Libert C, Giraudo C, et al. Direct effect of dsRNA mimetics on cancer cells induces endogenous IFN-beta

production capable of improving dendritic cell function. European journal of immunology. 2013;43(7):1849-61.

10.   Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413(6857):732-8.

11.   Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. Cancer research. 2010;70(2):490-500.

12.   Mian MF, Ahmed AN, Rad M, Babaian A, Bowdish D, Ashkar AA. Length of dsRNA (poly I:C) drives distinct innate immune responses, depending on the cell type. Journal of leukocyte biology. 2013;94(5):1025-36.

13.   OIE. 'Manual of Diagonostic Tests for Aquatic Animals 2014'. World organisation for Animal Health, Paris., 2014.

14.   Green TJ, Rolland JL, Vergnes A, Raftos D, Montagnani C. OsHV-1 countermeasures to the Pacific oyster's anti-viral response. Fish & shellfish immunology. 2015;47(1):435-43.

15.   Green TJ, Barnes AC. Inhibitor of REL/NF-KappaB is regulated in Sydney rock oysters in response to specific double-stranded RNA and Vibrio alginolyticus, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible. Fish & shellfish immunology. 2009;27(2):260-5.

16.   Green TJ, Montagnani C. Poly I:C induces a protective antiviral immune response in the Pacific oyster (Crassostrea gigas) against subsequent challenge with Ostreid herpesvirus (OsHV-1 muvar). Fish & shellfish immunology. 2013;35(2):382-8.

17.   Basak T, Bhat A, Malakar D, Pillai M, Sengupta S. In-depth comparative proteomic analysis of yeast proteome using iTRAQ and SWATH based MS. Molecular bioSystems. 2015;11(8):2135-43.

18.   Rane MJ, Pan Y, Singh S, Powell DW, Wu R, Cummins T, et al. Heat shock protein 27 controls apoptosis by regulating Akt activation. The Journal of biological chemistry. 2003;278(30):27828-35.

19.   Liang D, Benko Z, Agbottah E, Bukrinsky M, Zhao RY. Anti-vpr activities of heat shock protein 27. Molecular medicine. 2007;13(5-6):229-39.

20.   Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, Kroemer G. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell cycle. 2006;5(22):2592-601.

21.   Gober MD, Wales SQ, Aurelian L. Herpes simplex virus type 2 encodes a heat shock protein homologue with apoptosis regulatory functions. Frontiers in bioscience : a journal and virtual library. 2005;10:2788-803.

22.   Wallin RP, Lundqvist A, More SH, von Bonin A, Kiessling R, Ljunggren HG. Heat-shock proteins as activators of the innate immune system. Trends in immunology. 2002;23(3):130-5.

23.   Chernov AV, Dolkas J, Hoang K, Angert M, Srikrishna G, Vogl T, et al. The calcium-binding proteins S100A8 and S100A9 initiate the early inflammatory program in injured peripheral nerves. The Journal of biological chemistry. 2015;290(18):11771-84.

24.   Wang C, Yue X, Lu X, Liu B. The role of catalase in the immune response to oxidative stress and pathogen challenge in the clam Meretrix meretrix. Fish & shellfish immunology. 2013;34(1):91-9.

25.   Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, et al. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science. 2008;320(5874):379-81.

26.   Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). Advanced drug delivery reviews. 2008;60(7):805-12.

27.   Fernandez-Trujillo A, Ferro P, Garcia-Rosado E, Infante C, Alonso MC, Bejar J, et al. Poly I:C induces Mx transcription and promotes an antiviral state against sole aquabirnavirus in the flatfish Senegalese sole (Solea senegalensis Kaup). Fish & shellfish immunology. 2008;24(3):279-85.

28.   De Zoysa M, Kang HS, Song YB, Jee Y, Lee YD, Lee J. First report of invertebrate Mx: cloning, characterization and expression analysis of Mx cDNA in disk abalone (Haliotis discus discus). Fish & shellfish immunology. 2007;23(1):86-96.

29.   Jo EK, Yang CS, Choi CH, Harding CV. Intracellular signalling cascades regulating innate immune responses to Mycobacteria: branching out from Toll-like receptors. Cellular microbiology. 2007;9(5):1087-98.

30.   Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. Nature reviews Immunology. 2013;13(9):679-92.

31.   Otto GP, Nichols BJ. The roles of flotillin microdomains--endocytosis and beyond. Journal of cell science. 2011;124(Pt 23):3933-40.

32.   Roulston A, Marcellus RC, Branton PE. Viruses and apoptosis. Annual review of microbiology. 1999;53:577-628.

33.   Hughes FM, Foster B, Grewal S, Sokolova IM. Apoptosis as a host defense mechanism in Crassostrea virginica and its modulation by Perkinsus marinus. Fish & shellfish immunology. 2010;29(2):247-57.

34.   Yin H, Shi Z, Jiao S, Chen C, Wang W, Greene MI, et al. Germinal center kinases in immune regulation. Cellular & molecular immunology. 2012;9(6):439-45.

35.   Palchetti S, Starace D, De Cesaris P, Filippini A, Ziparo E, Riccioli A. Transfected poly(I:C) activates different dsRNA receptors, leading to apoptosis or immunoadjuvant response in androgen-independent prostate cancer cells. The Journal of biological chemistry. 2015;290(9):5470-83.

36.   Bourchookarn A, Havanapan PO, Thongboonkerd V, Krittanai C. Proteomic analysis of altered proteins in lymphoid organ of yellow head virus infected Penaeus monodon. Biochimica et biophysica acta. 2008;1784(3):504-11.

37.   Wang HC, Wang HC, Leu JH, Kou GH, Wang AH, Lo CF. Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. Developmental and comparative immunology. 2007;31(7):672-86.

Appendix to chapter 4
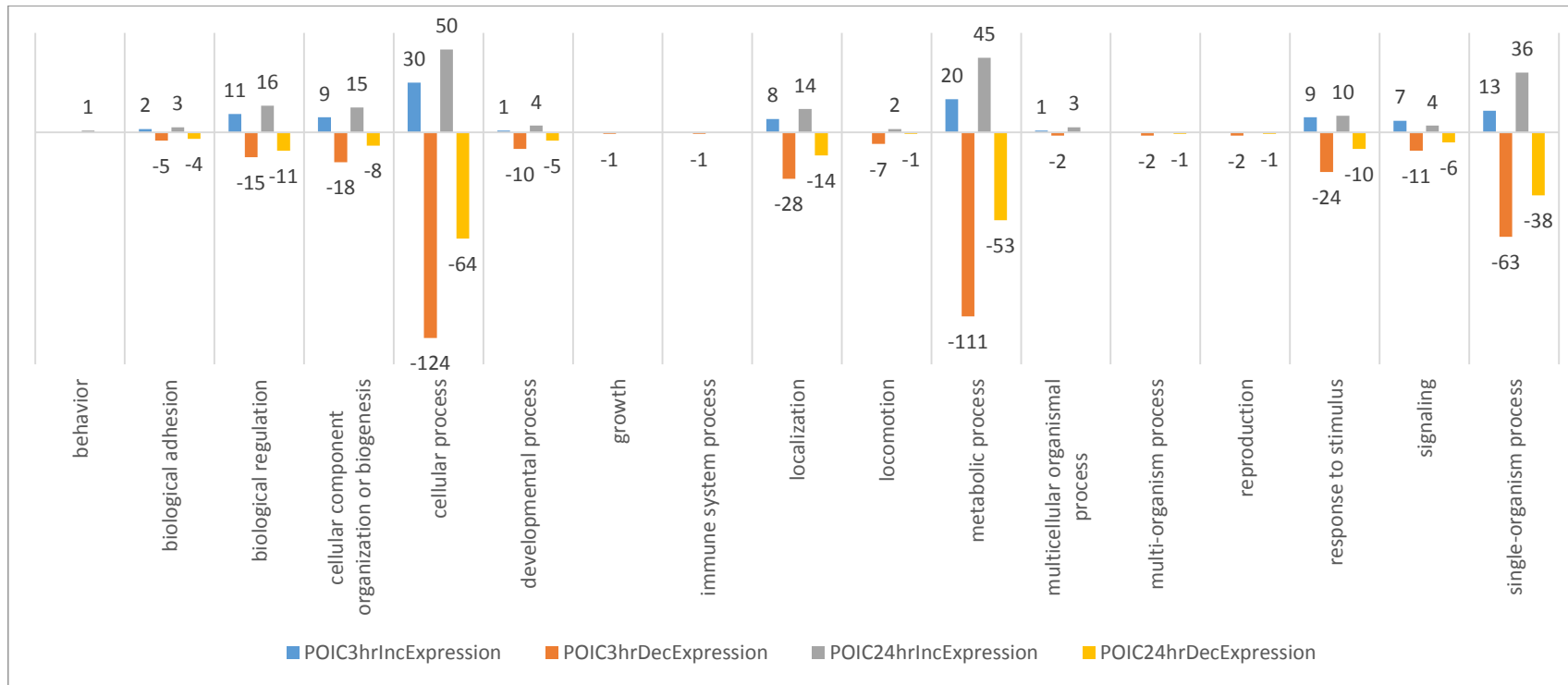
Figure 3: Pacific oyster poly (I:C) blast 2 go protein analysis with biological processes.
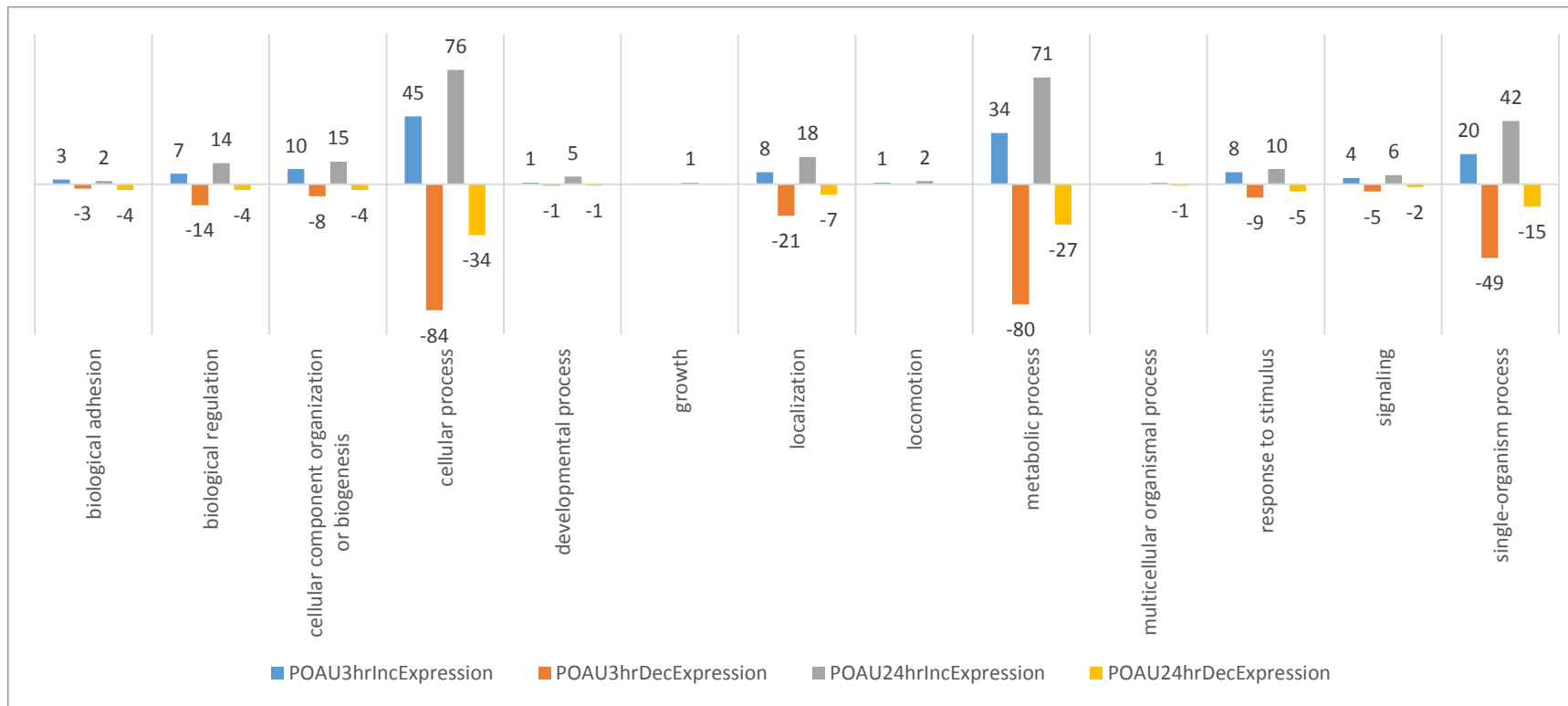
Figure 4: Pacific oyster poly (A:U) blast 2 go protein analysis with biological processes.
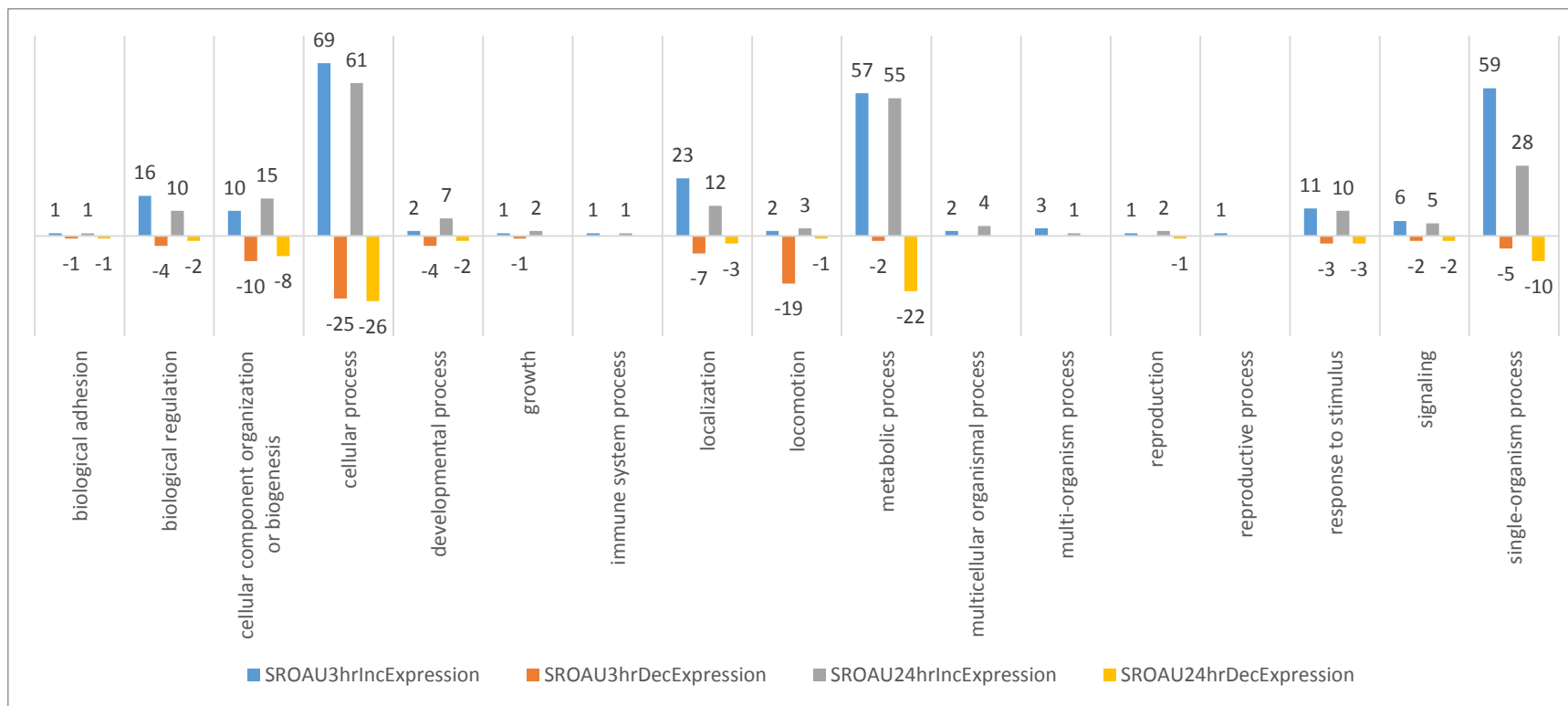
Figure 5: Sydney rock oyster poly (A:U) blast 2 go protein analysis with biological processes.
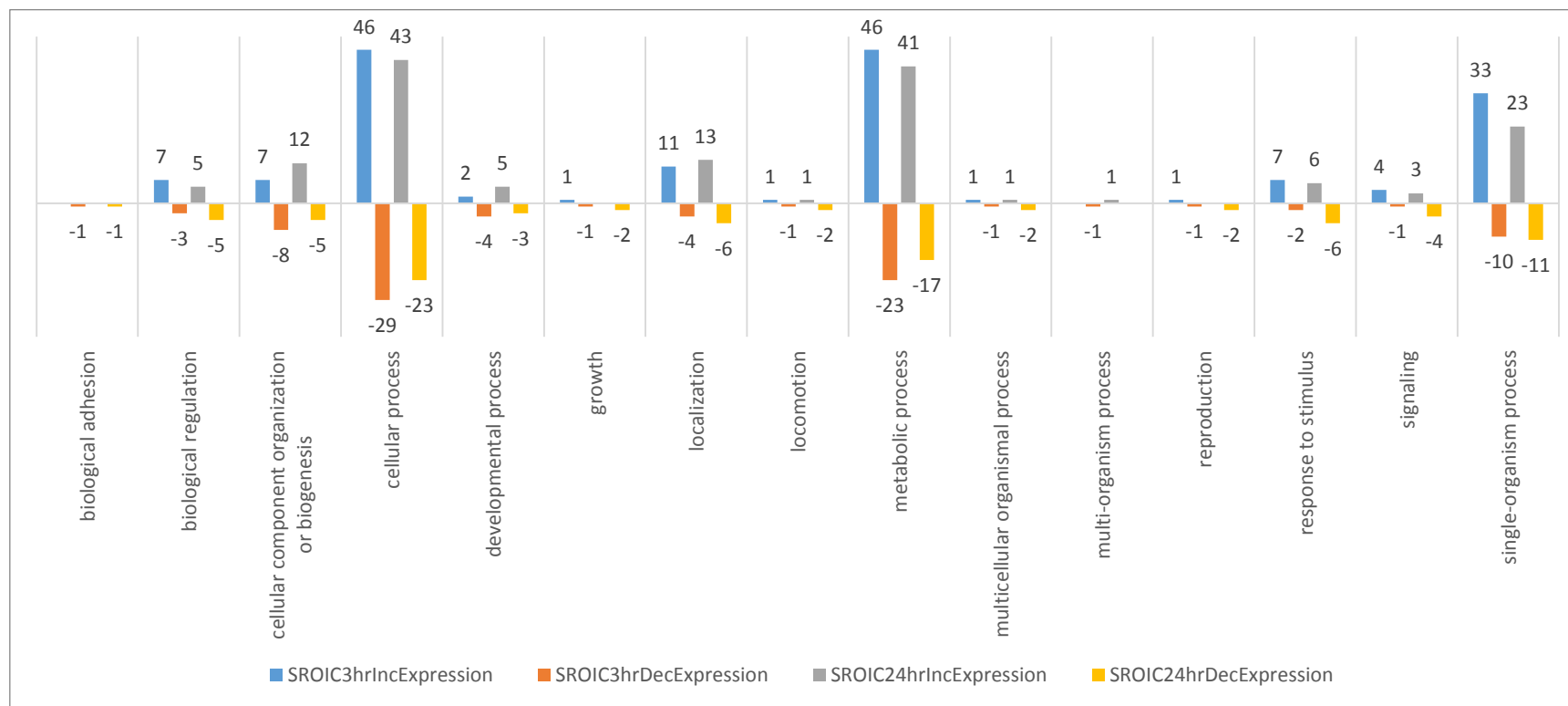
Figure 6: Sydney rock oyster poly (I:C) blast 2 go protein analysis with biological processes.

Table 2: Pacific oyster 3 h poly (I:C) significantly up and down regulated proteins with p-values and fold change.

Significantly upregulated proteins			
Protein	**Accession number**	**P-Value**	**Fold Change**
collagen alpha-1(II) chain-like [Crassostrea gigas]	gi|762119255	0.0150	2.1674
rhabdoid tumour deletion region protein 1-like [Crassostrea gigas]	gi|762074033	0.0458	3.5094
ras-related protein Rap-1b-like isoform X3 [Crassostrea gigas]	gi|762144545	0.0311	6.0560
TRAF3-interacting protein 1-like isoform X2 [Crassostrea gigas]	gi|762152860	0.0194	7.2820
40S ribosomal protein S14, partial [Crassostrea gigas]	gi|762165930	0.0183	9.3571

Significantly downregulated proteins			
Protein Name	**Accession number**	**P-Value**	**Fold Change**
ATP synthase F(0) complex subunit B1, mitochondrial-like [Crassostrea gigas]	gi|762167760	0.0035	0.0955
histone H3.3 [Crassostrea gigas]	gi|762164964	0.0152	0.1080
EF-hand calcium-binding domain-containing protein 6-like isoform X3 [Crassostrea gigas]	gi|762075042	0.0122	0.1106
bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]-like [Crassostrea gigas]	gi|762137593	0.0053	0.1143
adenylyl cyclase-associated protein 1-like isoform X1 [Crassostrea gigas]	gi|762090266	0.0292	0.1152
small nuclear ribonucleoprotein Sm D2 [Crassostrea gigas]	gi|762139183	0.0332	0.1277
sulfide:quinone oxidoreductase, mitochondrial-like [Crassostrea gigas]	gi|762128729	0.0326	0.1292
ras-related protein Rab-1A [Crassostrea gigas]	gi|762087977	0.0228	0.1336
neurofilament heavy polypeptide-like isoform X6 [Crassostrea gigas]	gi|762083904	0.0105	0.1461
myosin heavy chain, striated muscle-like isoform X4 [Crassostrea gigas]	gi|762119233	0.0240	0.1488
pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial-like isoform X1 [Crassostrea gigas]	gi|762101445	0.0006	0.1500
involucrin-like [Crassostrea gigas]	gi|762151344	0.0272	0.1512
alpha-aminoadipic semialdehyde dehydrogenase-like [Crassostrea gigas]	gi|762088534	0.0048	0.1534
hypothetical protein CGI_10010367 [Crassostrea gigas]	gi|405961556	0.0368	0.1645

Significantly downregulated proteins			
Protein Name	Accession number	P-Value	Fold Change
heat shock protein 78 [Hyriopsis cumingii]	gi|601125143	0.0395	0.1667
ADP-ribosylation factor 2 [Crassostrea gigas]	gi|762091898	0.0304	0.1865
cytochrome c oxidase subunit I (mitochondrion) [Crassostrea gigas]	gi|7212457	0.0390	0.1869
uncharacterized protein LOC105341577 [Crassostrea gigas]	gi|762076615	0.0019	0.1873
cytoplasmic actin, partial [Pinctada fucata]	gi|89255272	0.0277	0.1925
hypothetical protein CGI_10017851 [Crassostrea gigas]	gi|405968893	0.0367	0.2096
fumarylacetoacetase-like [Crassostrea gigas]	gi|762089599	0.0020	0.2115
UMP-CMP kinase-like [Crassostrea gigas]	gi|762124420	0.0313	0.2182
40S ribosomal protein S8-like [Crassostrea gigas]	gi|762070330	0.0371	0.2202
HSP70 [Azumapecten farreri]	gi|92430370	0.0208	0.2210
40S ribosomal protein S16 [Crassostrea gigas]	gi|762135008	0.0141	0.2231
60S ribosomal protein L3-like [Crassostrea gigas]	gi|762104754	0.0073	0.2350
40S ribosomal protein S15Aa [Crassostrea gigas]	gi|762098305	0.0149	0.2391
gastric intrinsic factor-like [Crassostrea gigas]	gi|762099376	0.0475	0.2453
nucleolin-like isoform X6 [Crassostrea gigas]	gi|762133599	0.0446	0.2453
uncharacterized protein LOC105343659 [Crassostrea gigas]	gi|762132423	0.0222	0.2535
ADP,ATP carrier protein-like [Crassostrea gigas]	gi|762108838	0.0259	0.4009
40S ribosomal protein S26-like, partial [Crassostrea gigas]	gi|762152715	0.0054	0.4192
adenylyl cyclase-associated protein 1-like isoform X3 [Crassostrea gigas]	gi|762090270	0.0165	0.4248
putative defense protein 3 isoform X2 [Crassostrea gigas]	gi|762153158	0.0132	0.2602
coronin-1B-like isoform X4 [Crassostrea gigas]	gi|762094011	0.0278	0.2657
Receptor-type tyrosine-protein phosphatase T [Crassostrea gigas]	gi|405968105	0.0124	0.2667
uncharacterized protein LOC105343414 [Crassostrea gigas]	gi|762070431	0.0004	0.2775
heat shock protein 83-like [Crassostrea gigas]	gi|765826487	0.0294	0.2944
fasciclin-2-like isoform X8 [Crassostrea gigas]	gi|762085334	0.0356	0.3004
tubulin beta chain [Crassostrea gigas]	gi|762130794	0.0026	0.3093
40S ribosomal protein S23 [Crassostrea gigas]	gi|765826531	0.0369	0.3195
uncharacterized protein LOC105344505 precursor [Crassostrea gigas]	gi|765826452	0.0275	0.3289
uncharacterized protein LOC105317356 isoform X12 [Crassostrea gigas]	gi|762148707	0.0300	0.3329
phosphoenolpyruvate carboxykinase [GTP]-like [Crassostrea gigas]	gi|762070888	0.0499	0.3351
40S ribosomal protein S4-like [Crassostrea gigas]	gi|762101882	0.0377	0.3408
aldehyde dehydrogenase, mitochondrial-like [Crassostrea gigas]	gi|762077372	0.0115	0.3450

Significantly downregulated proteins			
Protein Name	Accession number	P-Value	Fold Change
protein BRICK1-like [Crassostrea gigas]	gi|762088893	0.0308	0.4277
malate dehydrogenase, mitochondrial-like [Crassostrea gigas]	gi|762103501	0.0302	0.4316
ras-related protein Rab-8A-like [Crassostrea gigas]	gi|762093755	0.0230	0.4470
electron transfer flavoprotein subunit alpha, mitochondrial-like [Crassostrea gigas]	gi|762141795	0.0315	0.4614
synaptojanin-2-binding protein [Crassostrea ariakensis]	gi|333449479	0.0367	0.4708
40S ribosomal protein S25-like [Crassostrea gigas]	gi|762119804	0.0087	0.4813
tektin-3-like isoform X3 [Crassostrea gigas]	gi|762135472	0.0374	0.4983
coiled-coil domain-containing protein 124-like [Crassostrea gigas]	gi|762143449	0.0458	0.5110
annexin B9-like isoform X2 [Crassostrea gigas]	gi|762147718	0.0341	0.5122
tetratricopeptide repeat protein 25-like isoform X10 [Crassostrea gigas]	gi|762096452	0.0300	0.5266
probable ATP synthase subunit g 1, mitochondrial [Crassostrea gigas]	gi|762145153	0.0274	0.5331
60S acidic ribosomal protein P1-like [Crassostrea gigas]	gi|762094446	0.0111	0.5356
60S ribosomal protein L23 [Crassostrea gigas]	gi|762084493	0.0397	0.5448
transgelin-2-like isoform X5 [Crassostrea gigas]	gi|762134397	0.0237	0.5495
peptidyl-prolyl cis-trans isomerase-like [Crassostrea gigas]	gi|762122623	0.0274	0.5508
uncharacterized protein LOC105346629 isoform X3 [Crassostrea gigas]	gi|762140848	0.0373	0.5576
probable chaperone protein HSP31 [Crassostrea gigas]	gi|762142307	0.0311	0.5647
protein TBATA-like isoform X2 [Crassostrea gigas]	gi|762108569	0.0454	0.6036
proprotein convertase subtilisin/kexin type 5-like [Crassostrea gigas]	gi|762073407	0.0291	0.6312
calponin homolog OV9M-like isoform X4 [Crassostrea gigas]	gi|762134395	0.0025	0.6318
translationally-controlled tumor protein homolog	gi|762154913	0.0466	0.6601
plastin-3-like [Crassostrea gigas]	gi|762155362	0.0351	0.3486
40S ribosomal protein S18 [Crassostrea gigas]	gi|765826521	0.0324	0.3519
tubulin beta chain-like [Crassostrea gigas]	gi|762118370	0.0046	0.3560
cilia- and flagella-associated protein 20 [Crassostrea gigas]	gi|762102752	0.0469	0.3637
flotillin-1-like isoform X4 [Crassostrea gigas]	gi|762105167	0.0209	0.3728
sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1-like [Crassostrea gigas]	gi|762075110	0.0437	0.3781
actin-like [Crassostrea gigas]	gi|762109106	0.0373	0.4001

Table 3: Pacific oyster 24 h poly (I:C) significantly up and down regulated proteins with p-values and fold change.

Significantly upregulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
uncharacterized protein LOC105340138 isoform X2 [Crassostrea gigas]	gi|762122762	0.0211	1.5509
60S ribosomal protein L12-like [Crassostrea gigas]	gi|762166234	0.0023	2.0859
serine protease inhibitor dipetalogastin-like [Crassostrea gigas]	gi|762151096	0.0389	2.5138
sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1-like [Crassostrea gigas]	gi|762075110	0.0016	4.4120
heat shock protein 27-like [Crassostrea gigas]	gi|762131241	0.0064	8.4516
mitochondrial intermembrane space import and assembly protein 40-like [Crassostrea gigas]	gi|762138179	0.0066	9.7441

Significantly downregulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
uncharacterized protein LOC105317175 [Crassostrea gigas]	gi|762148231	0.0044	0.1473
protein-glutamine gamma-glutamyltransferase Z-like [Crassostrea gigas]	gi|762088850	0.0357	0.1879
endoplasmic reticulum resident protein 44-like [Crassostrea gigas]	gi|762144171	0.0170	0.1981
uncharacterized protein LOC105344468 [Crassostrea gigas]	gi|762134647	0.0422	0.2212
calmodulin-beta-like [Crassostrea gigas]	gi|762168976	0.0041	0.2411
septin-2-like isoform X9 [Crassostrea gigas]	gi|762159760	0.0047	0.2488
deoxyuridine 5'-triphosphate nucleotidohydrolase-like [Crassostrea gigas]	gi|762083277	0.0149	0.3162
cytochrome b-c1 complex subunit Rieske, mitochondrial-like [Crassostrea gigas]	gi|765826145	0.0251	0.3448
mucin-17-like [Crassostrea gigas]	gi|762085926	0.0125	0.3588
actin, partial [Mytilus edulis]	gi|5714751	0.0377	0.4056
40S ribosomal protein S7-like [Crassostrea gigas]	gi|762155366	0.0445	0.4747
dominin precursor [Crassostrea virginica]	gi|113928362	0.0396	0.4964
UPF0691 protein C9orf116 homolog isoform X1 [Crassostrea gigas]	gi|762080266	0.0472	0.5503
uncharacterized protein LOC105343180 isoform X2 [Crassostrea gigas]	gi|762077057	0.0187	0.5586
dihydropyrimidinase-like isoform X1 [Crassostrea gigas]	gi|762124046	0.0217	0.6052
ran-specific GTPase-activating protein-like [Crassostrea gigas]	gi|762105818	0.0363	0.6303

Table 4: Pacific oyster 3 hours poly (A:U) significantly up and down regulated proteins with p-values and fold change.

Significantly up regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
calmodulin-like protein 4 [Crassostrea gigas]	gi|762169240	0.0497	1.7499
3-hydroxyanthranilate 3,4-dioxygenase-like [Crassostrea gigas]	gi|762154170	0.0319	1.9453
dual specificity mitogen-activated protein kinase kinase 1-like isoform X2 [Crassostrea gigas]	gi|762075653	0.0388	2.7347
laminin subunit beta-3-like [Crassostrea gigas]	gi|762078233	0.0059	4.1241
sex-determining region Y protein-like isoform X1 [Crassostrea gigas]	gi|762167577	0.0189	5.3587
beta-thymosin [Crassostrea gigas]	gi|806644466	0.0047	5.4069
uncharacterized protein LOC105327754 [Crassostrea gigas]	gi|762088711	0.0053	6.2988
trichohyalin-like [Crassostrea gigas]	gi|762092072	0.0128	9.8438
deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial-like [Crassostrea gigas]	gi|762132082	0.0238	11.0458
40S ribosomal protein S14, partial [Crassostrea gigas]	gi|762165930	0.0266	11.1988

Significantly down regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
hypothetical protein CGI_10010367 [Crassostrea gigas]	gi|405961556	0.0305	0.1415
Receptor-type tyrosine-protein phosphatase T [Crassostrea gigas]	gi|405968105	0.0117	0.1421
6-phosphogluconolactonase-like [Crassostrea gigas]	gi|762156115	0.0283	0.1431
neurofilament heavy polypeptide-like isoform X2 [Crassostrea gigas]	gi|762073188	0.0006	0.1861
ependymin-related protein 1-like [Crassostrea gigas]	gi|762168226	0.0381	0.1866
putative defense protein 3 isoform X2 [Crassostrea gigas]	gi|762153158	0.0077	0.2092
outer dense fiber protein 3-like [Crassostrea gigas]	gi|762077414	0.0156	0.2875
fumarylacetoacetase-like [Crassostrea gigas]	gi|762089599	0.0108	0.3147
magnesium/proton exchanger-like isoform X8 [Crassostrea gigas]	gi|762122669	0.0394	0.3413
heat shock 70 kDa protein 12B-like [Crassostrea gigas]	gi|762084439	0.0489	0.3666
uncharacterized protein LOC105340920 isoform X3 [Crassostrea gigas]	gi|762124935	0.0340	0.3893
transgelin-2-like isoform X5 [Crassostrea gigas]	gi|762134397	0.0147	0.4851
proprotein convertase subtilisin/kexin type 5-like [Crassostrea gigas]	gi|762073407	0.0197	0.5365
nucleoredoxin-like [Crassostrea gigas]	gi|762118232	0.0458	0.5795
splicing factor U2AF 50 kDa subunit-like [Crassostrea gigas]	gi|762110927	0.0046	0.6250
Calmodulin-5/6/7/8 [Crassostrea gigas]	gi|405952922	0.0056	0.6271
uncharacterized protein LOC105334990 [Crassostrea gigas]	gi|762108631	0.0195	0.6330
phospholipid scramblase 1-like isoform X3 [Crassostrea gigas]	gi|762102324	0.0480	0.6495
pedal retractor muscle myosin heavy chain, partial [Mytilus galloprovincialis]	gi|6682321	0.0419	0.6553
60S ribosomal protein L12-like [Crassostrea gigas]	gi|762166234	0.0167	0.6628

Table 5: Pacific oyster 24 hours poly (A:U) significantly up and down regulated proteins with p-values and fold change.

Significantly up regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
annexin B9-like isoform X2 [Crassostrea gigas]	gi|762147718	0.0185	1.5543
drebrin-like protein B isoform X5 [Crassostrea gigas]	gi|762155524	0.0486	1.6736
uncharacterized protein LOC105324699 [Crassostrea gigas]	gi|762167631	0.0083	1.7172
ATP synthase subunit alpha, mitochondrial-like [Crassostrea gigas]	gi|762126219	0.0278	1.7795
fasciclin-2-like isoform X8 [Crassostrea gigas]	gi|762085334	0.0186	1.7988
perilipin-2-like isoform X2 [Crassostrea gigas]	gi|762146054	0.0052	1.9642
deoxyuridine 5'-triphosphate nucleotidohydrolase-like [Crassostrea gigas]	gi|762083277	0.0190	1.9752
uncharacterized protein LOC105345190 [Crassostrea gigas]	gi|762136577	0.0358	2.0409
NAD(P) transhydrogenase, mitochondrial-like [Crassostrea gigas]	gi|762131751	0.0045	2.1069
uncharacterized protein LOC105343391 [Crassostrea gigas]	gi|765826464	0.0499	2.1617
uncharacterized protein LOC105343133 [Crassostrea gigas]	gi|762130987	0.0263	2.1749
radixin-like isoform X6 [Crassostrea gigas]	gi|762078280	0.0270	2.1889
60S ribosomal protein L27a-like isoform X2 [Crassostrea gigas]	gi|762070778	0.0458	2.8594
malate dehydrogenase, mitochondrial-like, partial [Crassostrea gigas]	gi|762141955	0.0477	2.8985
malate dehydrogenase, cytoplasmic-like [Crassostrea gigas]	gi|762133002	0.0103	3.2768
catalase-like isoform X4 [Crassostrea gigas]	gi|762122079	0.0275	3.4013
uncharacterized protein LOC105327754 [Crassostrea gigas]	gi|762088711	0.0120	3.4021
phosphate carrier protein, mitochondrial-like isoform X1 [Crassostrea gigas]	gi|762099556	0.0326	3.6546
cAMP-dependent protein kinase regulatory subunit-like isoform X5 [Crassostrea gigas]	gi|762095740	0.0115	3.6790
phosphoenolpyruvate carboxykinase [GTP]-like [Crassostrea gigas]	gi|762096225	0.0254	3.7025
thioredoxin [Crassostrea ariakensis]	gi|338815381	0.0297	4.8243
tryptophan--tRNA ligase, cytoplasmic-like [Crassostrea gigas]	gi|762152306	0.0129	4.8303
cilia- and flagella-associated protein 20 [Crassostrea gigas]	gi|762102752	0.0016	5.1399
60S ribosomal protein L37a-like, partial [Crassostrea gigas]	gi|762165355	0.0049	5.7821
uncharacterized protein LOC105334920 [Crassostrea gigas]	gi|762074019	0.0265	5.7874
uncharacterized protein LOC105335200 [Crassostrea gigas]	gi|762074115	0.0084	6.4931
cleavage and polyadenylation specificity factor subunit CG7185-like [Crassostrea gigas]	gi|762155489	0.0129	6.6246
trifunctional enzyme subunit beta, mitochondrial-like [Crassostrea gigas]	gi|762128137	0.0068	11.2505
proteoglycan 4-like isoform X2 [Crassostrea gigas]	gi|762078513	0.0383	15.9242

178

Protein	Accession number	P-Value	Fold change
uncharacterized protein LOC105329712 isoform X5 [Crassostrea gigas]	gi|762093807	0.0141	43.9059

Significantly down regulated proteins			
Protein	Accession number	P-Value	Fold change
uncharacterized protein LOC105346438 isoform X1 [Crassostrea gigas]	gi|762140257	0.0265	0.0690
frataxin, mitochondrial-like isoform X2 [Crassostrea gigas]	gi|762101136	0.0071	0.2089
N-acylethanolamine-hydrolyzing acid amidase-like [Crassostrea gigas]	gi|762138281	0.0325	0.3531
mucin-17-like [Crassostrea gigas]	gi|762085926	0.0271	0.4104
hypothetical protein CGI_10003636 [Crassostrea gigas]	gi|405954063	0.0184	0.5043
glutathione S-transferase-like [Crassostrea gigas]	gi|762123718	0.0065	0.5404
uncharacterized protein LOC105317989 [Crassostrea gigas]	gi|762150351	0.0176	0.6305

Table 6: Sydney rock oyster 3 hours poly (I:C) significantly up and down regulated proteins with p-values and fold change.

Significantly upregulated proteins			
Protein	**Accession number**	**P-Value**	**Fold Change**
adenylyl cyclase-associated protein 1-like isoform X3 [Crassostrea gigas]	gi|762090270	0.0166	1.7456
transgelin-3-like isoform X3 [Crassostrea gigas]	gi|762119391	0.0397	1.7557
transitional endoplasmic reticulum ATPase-like [Crassostrea gigas]	gi|762132070	0.0049	2.4307
40S ribosomal protein S18 [Crassostrea gigas]	gi|765826521	0.0298	2.8861
apoptosis regulator BAX-like isoform X2 [Crassostrea gigas]	gi|762084026	0.0200	3.3849
actin-related protein 3 isoform X2 [Crassostrea gigas]	gi|762150078	0.0372	5.3556
nucleolysin TIAR-like isoform X9 [Crassostrea gigas]	gi|762157790	0.0203	5.5389
ribosomal protein S2, partial [Mytilus trossulus]	gi|675304789	0.0392	6.5280
protein canopy homolog 2-like [Crassostrea gigas]	gi|762107602	0.0076	6.5481
peptidase inhibitor 15-A-like [Crassostrea gigas]	gi|762142939	0.0490	6.9686
malate dehydrogenase, cytoplasmic-like [Crassostrea gigas]	gi|762133002	0.0003	6.9895
ornithine aminotransferase, mitochondrial-like [Crassostrea gigas]	gi|762102898	0.0129	7.2005
COMM domain-containing protein 1-like [Crassostrea gigas]	gi|762110512	0.0005	37.7470

Significantly downregulated proteins			
Protein	**Accession number**	**P-Value**	**Fold Change**
paramyosin-1, partial [Pinctada fucata]	gi|472824657	0.0245	0.0380
protein Dr1-like [Crassostrea gigas]	gi|762145782	0.0014	0.0419
T-complex protein 1 subunit gamma-like [Crassostrea gigas]	gi|762129500	0.0109	0.0603
WW domain-binding protein 11-like [Crassostrea gigas]	gi|762124442	0.0390	0.1404
syntaxin-12-like isoform X6 [Crassostrea gigas]	gi|762167519	0.0084	0.2084
15 kDa selenoprotein-like [Crassostrea gigas]	gi|762084409	0.0290	0.2267
sterile alpha motif domain-containing protein 15-like [Crassostrea gigas]	gi|762097141	0.0068	0.2424
dihydropyrimidinase-like isoform X7 [Crassostrea gigas]	gi|762124058	0.0424	0.2930
mammalian ependymin-related protein 1-like [Crassostrea gigas]	gi|762153564	0.0114	0.3558
granulin epithelin precursor variant 3 [Pinctada fucata]	gi|746873486	0.0341	0.4920
bolA-like protein DDB_G0274169 [Crassostrea gigas]	gi|762155631	0.0141	0.53547
uncharacterized protein LOC105340758 [Crassostrea gigas]	gi|762124495	0.0305	0.5918
fibril-forming collagen alpha chain-like [Crassostrea gigas]	gi|762169167	0.0222	0.6243

Table 7: Sydney rock oyster 24 h poly (I:C) significantly up and down regulated proteins with p-values and fold change.

Significantly up regulated proteins			
Protein	Accession number	P-Value	Fold change
calreticulin precursor [Crassostrea gigas]	gi|765826537	0.0145	1.5724
flotillin-1-like isoform X4 [Crassostrea gigas]	gi|762105167	0.0451	1.5780
DPY30 domain-containing protein 1-like [Crassostrea gigas]	gi|762110949	0.0026	1.5841
titin-like [Crassostrea gigas]	gi|762119741	0.0106	1.7404
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial-like [Crassostrea gigas]	gi|762098613	0.0001	1.8589
uncharacterized protein LOC105330077 [Crassostrea gigas]	gi|762094920	0.0164	1.8595
probable splicing factor 3B subunit 5 [Crassostrea gigas]	gi|762158359	0.0015	2.0392
prefoldin subunit 6-like, partial [Crassostrea gigas]	gi|762165026	0.0071	2.0698
Cathepsin Z-like [Crassostrea gigas]	gi|762086974	0.0004	2.1089
protein CFAP45, mitochondrial-like isoform X2 [Crassostrea gigas]	gi|762164101	0.0213	3.1486
intraflagellar transport protein 20 homolog [Crassostrea gigas]	gi|762091171	0.0322	3.4997
uncharacterized protein LOC105327754 [Crassostrea gigas]	gi|762088711	0.0106	3.8340
V-type proton ATPase subunit G-like [Crassostrea gigas]	gi|762137893	0.0250	4.6716
NAD(P) transhydrogenase, mitochondrial-like [Crassostrea gigas]	gi|762131751	0.0171	4.8991
small nuclear ribonucleoprotein Sm D3-like [Crassostrea gigas]	gi|762136889	0.0278	4.9482
phosphoenolpyruvate carboxykinase, cytosolic [GTP]-like isoform X3 [Crassostrea gigas]	gi|762070871	0.0332	6.6115
synaptojanin-2-binding protein-like isoform X2 [Crassostrea gigas]	gi|762078178	0.0010	8.4134
ribosomal protein S2, partial [Mytilus trossulus]	gi|675304789	0.0091	8.8264
glutaredoxin-C3-like [Crassostrea gigas]	gi|762126087	0.0272	9.7351
N-terminal EF-hand calcium-binding protein 1-like [Crassostrea gigas]	gi|762096221	0.0250	13.4724

Significantly down regulated proteins			
Protein	Accession number	P-Value	Fold change
transport protein Sec16A [Crassostrea gigas]	gi|405952751	0.0007	0.0091
myosin essential light chain, striated adductor muscle-like [Crassostrea gigas]	gi|762147953	0.0006	0.0736
LDLR chaperone MESD-like [Crassostrea gigas]	gi|762134263	0.0032	0.2492
splicing factor 3B subunit 4-like [Crassostrea gigas]	gi|762089656	0.0225	0.3812
nucleoside diphosphate kinase homolog 5-like [Crassostrea gigas]	gi|762132773	0.0153	0.4247

Table 8: Sydney rock oyster 3 h poly (A:U) significantly up and down regulated proteins with p-values and fold change.

Significantly up regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
myosin heavy chain, striated muscle-like [Crassostrea gigas]	gi|762094064	0.0390	1.5001
transcriptional coactivator YAP1-like isoform X5 [Crassostrea gigas]	gi|762102951	0.0217	1.5087
actin-like [Crassostrea gigas]	gi|762109104	0.0172	1.5250
14-3-3-like protein 2 isoform X1 [Crassostrea gigas]	gi|762115032	0.0174	1.5291
actin-2-like [Crassostrea gigas]	gi|762123657	0.0252	1.6010
actin-like [Crassostrea gigas]	gi|762109106	0.0378	1.6517
glycine-rich cell wall structural protein 1-like isoform X6 [Crassostrea gigas]	gi|762141705	0.0469	1.6575
glyceraldehyde 3-phosphate dehydrogenase [Ostrea edulis]	gi|290463452	0.0156	1.7228
actin, adductor muscle [Crassostrea gigas]	gi|821595387	0.0178	1.7293
60S ribosomal protein L12-like [Crassostrea gigas]	gi|762166234	0.0382	1.7590
protein disulphide-isomerase A3-like [Crassostrea gigas]	gi|762140057	0.0050	1.7648
40S ribosomal protein S23 [Crassostrea gigas]	gi|765826531	0.0267	1.8615
tubulin beta chain-like [Crassostrea gigas]	gi|765826404	0.0486	1.8871
malate dehydrogenase, cytoplasmic-like [Crassostrea gigas]	gi|762133002	0.0309	1.8874
alpha-actinin, sarcomeric-like isoform X2 [Crassostrea gigas]	gi|762069966	0.0273	2.2752
gelsolin-like protein 2 [Crassostrea gigas]	gi|762089412	0.0425	2.4535
elongation factor 1-alpha [Crassostrea gigas]	gi|765826406	0.0004	2.4618
uncharacterized protein LOC105341613 [Crassostrea gigas]	gi|762076623	0.0415	2.8559
probable RING finger protein 207 homolog [Crassostrea gigas]	gi|762125148	0.0355	3.1812
ornithine aminotransferase, mitochondrial-like [Crassostrea gigas]	gi|762102898	0.0324	3.2795
60S ribosomal protein L23 [Crassostrea gigas]	gi|762084493	0.0087	3.2964
small nuclear ribonucleoprotein Sm D3-like [Crassostrea gigas]	gi|762136889	0.0415	3.4470
major vault protein-like [Crassostrea gigas]	gi|762100597	0.0183	3.4501
actin [Pinctada fucata]	gi|189473617	0.0456	3.8195
40S ribosomal protein S13 [Crassostrea gigas]	gi|762117040	0.0004	4.7066
uncharacterized protein LOC105343391 [Crassostrea gigas]	gi|765826464	0.0032	6.5917
40S ribosomal protein S7-like [Crassostrea gigas]	gi|762155366	0.0262	6.6796
calumenin-like isoform X2 [Crassostrea gigas]	gi|762104889	0.0403	7.3422
flotillin-1-like isoform X4 [Crassostrea gigas]	gi|762105167	0.0001	12.1338

Significantly down regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
paramyosin-1, partial [Pinctada fucata]	gi|472824657	0.0326	0.0446
fatty acid-binding protein homolog 6-like isoform X2 [Crassostrea gigas]	gi|762154717	0.0330	0.2453
septin-7-like [Crassostrea gigas]	gi|762153483	0.0229	0.3441
hypothetical protein CGI_10000297 [Crassostrea gigas]	gi|405946832	0.0040	0.4690
calcium-regulated heat stable protein 1-like [Crassostrea gigas]	gi|762107653	0.0187	0.6521

Table 9: Sydney rock oyster 24 h poly (A:U) significantly up and down regulated proteins with p-values and fold change.

Significantly up regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
voltage-dependent anion-selective channel protein 2-like [Crassostrea gigas]	gi|765826517	0.0385	1.6694
LIM and SH3 domain protein F42H10.3-like isoform X15 [Crassostrea gigas]	gi|762084523	0.0396	1.6827
cathepsin Z-like [Crassostrea gigas]	gi|762086974	0.0015	1.7374
calmodulin [Meretrix meretrix]	gi|529161415	0.0097	1.7638
uncharacterized protein LOC105330077 [Crassostrea gigas]	gi|762094920	0.0346	2.1755
serine/arginine-rich splicing factor 1A-like [Crassostrea gigas]	gi|762088953	0.0398	2.2542
protein CFAP45, mitochondrial-like isoform X2 [Crassostrea gigas]	gi|762164101	0.0343	2.5670
NAD(P) transhydrogenase, mitochondrial-like [Crassostrea gigas]	gi|762131751	0.0071	2.6346
ubiquitin-conjugating enzyme E2 2-like [Crassostrea gigas]	gi|762148837	0.0334	3.5187
cystathionine gamma-lyase-like isoform X3 [Crassostrea gigas]	gi|762089031	0.0228	4.7505
40S ribosomal protein S25-like [Crassostrea gigas]	gi|762119804	0.0044	5.0784
ATP synthase subunit alpha, mitochondrial [Crassostrea gigas]	gi|405974703	0.0199	5.2768
60S ribosomal protein L30 [Crassostrea gigas]	gi|762096308	0.0245	6.7449
translocon-associated protein subunit alpha-like [Crassostrea gigas]	gi|762090864	0.0084	7.3367
flotillin-1-like isoform X4 [Crassostrea gigas]	gi|762105167	0.0024	7.5049
N-terminal EF-hand calcium-binding protein 1-like [Crassostrea gigas]	gi|762096221	0.0337	7.8481
uncharacterized protein LOC105345408 [Crassostrea gigas]	gi|762137101	0.0071	11.7642
60S ribosomal protein L4-like, partial [Crassostrea gigas]	gi|762165392	0.0222	17.6505
small nuclear ribonucleoprotein Sm D3-like [Crassostrea gigas]	gi|762136889	0.0070	18.2267

Significantly down regulated proteins			
Protein	**Accession number**	**P-Value**	**Fold change**
myosin essential light chain, striated adductor muscle-like [Crassostrea gigas]	gi|762147953	0.0029	0.0707
obg-like ATPase 1 [Crassostrea gigas]	gi|762108397	0.0240	0.2082
calumenin-like [Crassostrea gigas]	gi|762165536	0.0162	0.2570
14 kDa phosphohistidine phosphatase-like [Crassostrea gigas]	gi|762090034	0.0404	0.2864
nucleoside diphosphate kinase homolog 5-like [Crassostrea gigas]	gi|762132773	0.0110	0.3685
transaldolase-like isoform X3 [Crassostrea gigas]	gi|762109222	0.0469	0.3734

Associated content

Supplementary tables S1 to S3, Supplementary methods MS and Supplementary file (Peptide Raw Data) are presented as a soft copy in the provided CD.

Supplementary table 1: Proteins unique to PO, SRO and common to both species.

Supplementary table 2: Pacific oyster proteins with Fold change and p-values when challenged with dsRNA (poly A:U and poly I:C) after 3 and 24 hours.

Supplementary table 3: Sydney rock oyster proteins with Fold change and p-values when challenged with dsRNA (poly A:U and poly I:C) after 3 and 24 hours.

Chapter 5

General discussion

General discussion

The studies reported in this thesis were undertaken to provide a better understanding of the response to exogenous generic dsRNA in two oyster species, Pacific oyster (PO) and Sydney rock oyster (SRO). The approaches adopted in this research project were premised on the well-accepted concept that cellular responses to dsRNA form the basis of antiviral immunity. This concept has been demonstrated in a number of metazoans, where clear parallels exist between cellular and molecular responses to dsRNA and viral infections. This thesis also provides a detailed analysis of oyster gill tissue proteomes. To date there has been limited comparable proteomic work undertaken on these two commercially important bivalve species. State-of-the-art proteomics techniques coupled with transcriptional, confocal microscopy and bioinformatics tools were used to evaluate cellular and molecular responses to dsRNA.

Responses to exogenous dsRNA

The host pathogen interactions between oyster species (PO and SRO) and Ostreid herpesvirus type 1 (OsHV-1) are poorly characterised. Non-specific dsRNA such as poly (I:C) and poly (A:U) are potent adjuvants known to mimic viral infection in various vertebrate and invertebrate species (1-4). The aim of the investigation described in Chapter 2 was to determine the fate of dsRNA that had been injected into oysters. Fluorescently-labelled (poly I:C) was injected into the oysters' pericardial cavities. Analysis of micrographs suggested that while the mantle tissues of both oyster species were capable of internalising exogenous dsRNA from the extracellular media within an hour, those tissues did not appear to process the dsRNA. On the other hand in SRO, the data suggested that gill

tissues appeared to process the dsRNA, as their fluorescence values had returned to control levels 24 hours after dsRNA injection. Similarly, in another study, apoptosis was not observed in PO gill tissue when challenged with OsHV-1 DNA, suggesting that PO gill tissue may have a different mechanism to deal with OsHV-1 infection (5). Data shown in chapter 3 also revealed that exogenous dsRNA was taken up by circulating haemocytes and were rapidly located within their cytoplasmic vesicles.

Real time PCR analysis on the relative abundance of a set of immune response genes indicated that oyster tissues responded to the dsRNA challenge by stimulating the expression of genes that are known to be involved in dsRNA and antiviral responses, such as *IkB, IK Cytokine*, *TLR*, *Piwi*, *Peroxyredoxin* and *IFI 44-like protein* genes (6-8). Although both oyster species detected and responded to dsRNA, the differences in tissue-level distributions, time-dependent changes in the fluorescence intensity data and the relative abundance of transcripts from immune response genes pointed to substantial differences in the biological responses of these two species to dsRNA (and hence, to viral infections).

Proteomic analyses (chapters 3 and 4) provided a global and holistic view of molecular changes that occurred in these animals as a result of the perturbations that were examined. iTRAQ data (chapter 3) revealed that 77.2% of proteins in PO tissues were unique to PO, while 42.2% of protein in the SRO dataset were unique to that species. A deeper analysis of these differences using SWATH mass spectrometry (chapter 4) revealed that the proteomic responses that were elicited were specific to the type of dsRNA used. Furthermore, the alterations to the proteomes changed during the 48-hour period after dsRNA injection, indicating a temporal component to the response. These differences

188

may, in part, reflect differences in biological responses of the two species of oysters to dsRNA treatment. Gene ontology analyses of the differentially-expressed proteins indicated that cellular pathways known to be involved in dsRNA metabolism, RNA interference, antiviral responses, cellular component and signalling were responding differently in the two oyster species to the perturbation. Those pathways appeared to be functional in dsRNA-injected SRO, but not in similarly-treated PO. For example, Piwi, which is a component of the Dicer-Argonaut-Piwi complex ((8) and is part of RNAi pathway, was only observed in gill tissues of dsRNA-injected SRO. SRO tissues that have taken up dsRNA also appear to trigger apoptotic pathways and protein associated with these pathways were noticeably absent in dsRNA-injected PO tissues.

Taken together, the data presented in chapters 2-4 point to a robust response to dsRNA challenge. A speculative model of the biological response is as follows: in the SRO, exogenous dsRNA is rapidly internalised into tissues such as the gills and are localised to endosomal vesicles. TLRs in the endosomes bind to the dsRNA and activate relevant signalling pathways. Some of the dsRNA are transported to the cytoplasm, when they become bound to cytoplasmic RNA receptors (e.g. RIG-1 proteins). The activation of the signalling pathways appears to stimulate two specific responses: the production of cytokines (interferons) and the activation of apoptosis.

It may be extrapolated that similar systems may be involved during OsHV-1 (μvar) infection of the SRO. dsRNA produced during viral infection will activate similar pathways in the oysters. The secretion of cytokines from infected cells will act in a paracrine manner to induce antiviral states (i.e. activation of the RNA interference machinery) in neighbouring cells. Furthermore, apoptosis of the infected cells prevents further propagation of viruses.

In accordance with this model, PO appear not to activate similar responses to dsRNA injections. The absence of key proteins involved in RNA interference and cytokine responses suggests the tissues of this organism fails to induce an antiviral state. Furthermore, the presence of anti-apoptotic proteins indicates that there may be the intracellular conditions may be favourable for viral replication and propagation. A published study has reported that OsHV-1 genome can successfully inhibit apoptosis in gill tissue of *C. gigas (5)*. Similarly, the infection of the oyster, *C. virginica* by the parasite, *Perkinsus marinus*, also results in the suppression of apoptosis at the early stages of infection (9). Although many aspects of this model remain to be validated in future experiments, it forms a relevant platform for evaluating the observed differences in the susceptibility of the two species to OsHV-1 (μvar) infection.

Key findings:

1. Both PO and SRO are capable of taking up exogenous dsRNA within few hours and mount an immune response. This uptake is likely to be receptor mediated endocytosis as we found increased expression of TLRs at transcriptional and proteomic levels. There are substantial differences in the proteomic responses between these two species and they can discriminate between types of dsRNA based on differential expression of proteins

2. Although gill tissues, mantle tissues and haemocytes were all capable of taking up exogenous dsRNA, it appears that only the gill tissues and haemocytes responded to the challenge by invoking relevant signalling and cytokine production pathways.

3. The RNAi-related protein, Piwi, was identified only in SRO (at both proteomic and transcriptional levels) after dsRNA injections.

4. PO proteins that regulate apoptosis had significantly decreased abundance levels, even after 24 hours of dsRNA challenge, while anti-apoptotic proteins were relatively more abundant. However, in SRO, the patterns of relative abundance of these proteins were reversed. Within 3 hours after dsRNA injection, apoptosis proteins were abundant.

5. Various immune related genes with increased expression were identified in transcriptional and proteomic data sets. However, there were disparities between our proteomic data and transcriptional data, suggesting that these disparities must be taken in to account when conclusions are drawn about biological processes using transcriptional data.

Perspective:

Although the project has generated a substantial volume of data, including clues to biological processes that occur in the two oyster species when they are challenged with dsRNAs, there are several limitations to the findings of the study. These include:

(i)   The reliance on 'omics technologies, such as proteomics. Despite the significant advances that have occurred in recent times, the data generated from such investigations must be validated using other platforms. However, the lack of reagents that may be used to interrogate oyster systems (e.g. suitable antibodies) prevented the use of validation assays, such as Western blots and ELISAs. As

several research groups are working on this currently, it is like that this hurdle will be overcome in the near future.

(ii) A number of other cellular assays (e.g. apoptotic and cytokine assays) that are currently available have proven to be unreliable when it comes to oyster systems and do not produce robust results. They need to be further optimised for use with non-model organisms and such development is currently underway in our laboratory. Some of those assays will be used to corroborate the findings of this research project. The recent publication of an apoptotic assay on oysters is an example of this development pipeline (11).

(iii) The experiments in this project have relied on the use of generic dsRNAs. Although they are powerful tools, the use of virus-specific reagents (e.g. OsHV-1-specific dsRNA, including shRNAs and siRNAs) will validate the responses of oyster tissues to dsRNA challenge. Furthermore, the use of such reagents in combination with infections by live, purified viruses or viral DNAs will delineate virus-specific immune responses.

Future directions:

Results obtained from this work extend the current understanding of the molecular mechanisms involved in resistance to disease in these economically important bivalve species. To date, there has been limited comparable proteomic work undertaken on these two commercially important species. By employing advanced proteomic techniques (iTRAQ and SWATH Mass Spectrometry), coupled with transcriptional and confocal microscopy, the data obtained in this project has improved our understanding of immune traits associated with dsRNA

challenge. This, in turn, may deepen our understanding of viral immune responses in oysters. The large protein datasets may be used to identify potential markers of virus resistance in Pacific oysters. Once validated, those protein markers that may be used marker-assisted selection programs in the oyster industry to identify brood stock that possess resistance traits, thus potentially mitigating economic losses due to OsHV-1 infection. For future study it will be interesting to look at the expression levels of dicer 1 and dicer 2 genes as well to show proper RNAi response, but these sequences are not yet identified in SRO for a comparative study. There is no study as such to determine the amount of dsRNA to be injected in oyster in order to evoke an immune response. It will be interesting to look in to a comprehensive dose response analysis in understanding the interacting dynamics of virus replication and proteome response may be a productive field of further research. Finally, the mass spectrometry datasets will be used in a future project to develop fine-scale mapping and annotations of the oyster genome assembly. There are significant ambiguities in the current assembly and protein-level data will lead to the refinement and resolution of those uncertainties. This will contribute to our understanding of genome level changes that have occurred during the evolution of this important group of molluscs.

References

1.   Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413(6857):732-8.

2.   Green TJ, Montagnani C. Poly I:C induces a protective antiviral immune response in the Pacific oyster (Crassostrea gigas) against subsequent challenge with Ostreid herpesvirus (OsHV-1 muvar). Fish & shellfish immunology. 2013;35(2):382-8.

3.   Dong C, Zhao J, Song L, Wang L, Qiu L, Zheng P, et al. The immune responses in Chinese mitten crab Eriocheir sinensis challenged with double-stranded RNA. Fish & shellfish immunology. 2009;26(3):438-42.

4.   Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, et al. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol. 2004;78(19):10442-8.

5.   Green TJ, Rolland JL, Vergnes A, Raftos D, Montagnani C. OsHV-1 countermeasures to the Pacific oyster's anti-viral response. Fish & shellfish immunology. 2015;47(1):435-43.

6.   Green TJ, Barnes AC. Inhibitor of REL/NF-KappaB is regulated in Sydney rock oysters in response to specific double-stranded RNA and Vibrio alginolyticus, but the major immune anti-oxidants EcSOD and Prx6 are non-inducible. Fish & shellfish immunology. 2009;27(2):260-5.

7.   Renault T, Faury N, Barbosa-Solomieu V, Moreau K. Suppression substractive hybridisation (SSH) and real time PCR reveal differential gene expression in the Pacific cupped oyster, Crassostrea gigas, challenged with

Ostreid herpesvirus 1. Developmental and comparative immunology. 2011;35(7):725-35.

8.   Hock J, Meister G. The Argonaute protein family. Genome biology. 2008;9(2):210.

9.   Hughes FM, Foster B, Grewal S, Sokolova IM. Apoptosis as a host defense mechanism in Crassostrea virginica and its modulation by Perkinsus marinus. Fish & shellfish immunology. 2010;29(2):247-57.

10.   Segarra A, Mauduit F, Faury N, Trancart S, Degremont L, Tourbiez D, et al. Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. BMC genomics. 2014;15:580.

11.   Rolland JL, Medhioub W, Vergnes A, Abi-Khalil C, Savar V, Abadie E, Masseret E, Amzil Z, Laabir M. A feedback mechanism to control apoptosis occurs in the digestive gland of the oyster *crassostrea gigas* exposed to the paralytic shellfish toxins producer *Alexandrium catenella*.   Marine Drugs . 2014; 12   (9):   5035-45.