Developing efficient diagnostic tools for assessing resistance to viral infection in oysters and abalone.

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Non-Technical Summary

Developing efficient diagnostic tools for assessing resistance to viral infection in oysters and abalone.

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PROJECT OBJECTIVES:

1. Identification of virus-resistant abalone family lines.
2. Identification of virus-resistant oyster family lines.
3. Identification of the antiviral compounds produced by abalone and oyster.
4. Correlate antiviral quantification to in vitro antiviral activity in the hemolymph of oysters and abalone.
5. Commence work to develop a chemical screening test for antiviral activity and extend this as a tool in abalone and oyster breeding programs.

Project Summary

The project examined the genetic basis of antiviral resistance in oysters and abalone in response to the significant disease threats posed by oyster and abalone herpesviruses. Laboratory-based systems for the growth and assay of such viruses are problematic. The project undertook to measure the antiviral activity of different breeding lines of oysters and abalone using as a proxy measure the antiviral activity against the human herpesvirus HSV-1. We demonstrated that oysters and abalone have antiviral activity against HSV-1, and that this trait is heritable. We identified a hemolymph compound providing antiviral activity. Our analysis of the oyster genome identified what is likely to be a key role for the protein viperin in resistance to viral infection. The project has provided outstanding value in three important areas. First, in adding to the knowledge base directing mollusc breeding programs. Second, in enhancing knowledge of molluscan immunity to viral infections. Third, in building research and development capacity in the Australian seafood industry.

OUTCOMES ACHIEVED

This project's outcomes will assist in ensuring the Australian oyster and abalone industry will have access to disease-resistant stock in the future. This project advised the oyster and abalone industry of family breeding lines that display heightened antiviral activity. It is envisioned that breeding companies will utilise these results to produce the next generation of disease resistant stock. A putative antiviral protein responsible for inhibiting virus infection and replication was identified in the hemolymph of oysters. Further characterisation of this protein will assist the oyster industry to implement marker-driven selection into their breeding programs. Several laboratory studies were undertaken to increase our knowledge of the anti-viral response of adult and juvenile oysters at different water temperatures. These studies provided insight into the molecular mechanisms that cause oyster lethality as a result of viral infection. Combining these observations with previous Australian research (FRDC Project No. 2011/053) suggests farmers could reduce oyster

mortalities by implementing farming strategies that are stressful to their stock. Studies conducted to increase our knowledge of the antiviral response of oysters also provided the foundation of a new concept to increase the antiviral resistance of oyster progeny using trans-generational immune priming. The key findings arising from this project were successfully communicated to industry at workshops and conferences.

LIST OF OUTPUTS PRODUCED

I. Identified Australian oyster family lines with high antiviral resistance.
II. Confirmed antiviral resistance is heritable in the Australian oyster population.
III. Identified a putative antiviral protein in the hemolymph of *C. gigas*.
IV. Identified Australian abalone family lines with high antiviral resistance.
V. Confirmed antiviral resistance is under genetic control in the Australian abalone population.
VI. Development of a concept to increase the disease resistance of family lines without the need for direct selection.
VII. Two scientific manuscripts published, another is in press, and another manuscript has been submitted for publication.

ACKNOWLEDGEMENTS

Dr Wayne O'Connor for oyster hemolymph samples.
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Nick Savva (ABTAS Pty Ltd) for abalone family line material.

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Drs Peter Kube, Peter Kirkland and Paul Hick for providing estimated breeding values for OsHV-1 resistance

1. Introduction and Background

Oysters and abalone in Australia are under threat from destructive herpesvirus infections: oyster herpesvirus 1 (OsHV-1) in the case of oysters, and abalone herpesvirus (AbHV) infecting abalone. Epidemics of infection with these viruses have caused widespread deaths of oysters and abalone in Australia. There is little knowledge regarding the immunity against viral infection in these economically important molluscs, and there is inadequate data to address the question of whether infection with these viruses alone is necessary and sufficient to cause lethality. The options available to counter these threats are limited. Because molluscs lack what we typically consider to be adaptive immunity, there is little or no prospect of a vaccine proving effective, however the phenomenon of "trained immunity" in which exposure to virus associated molecular patterns (e.g. dsRNA) leads to a temporarily heightened immune responses has been noted in other molluscs. There are no antiviral drugs available. A key aspect of reducing the risk from this threat is in identification of oyster and abalone family lines that resist infection with these viruses. Identification of such families can potentially be facilitated by determination of the nature of the endogenous antiviral compounds that within these oysters and abalone contribute to their ability to resist infections.

Our previous research (Australian Seafood CRC Project 2008/739) identified that the hemolymph of oysters and abalone possesses activity against common human viruses (Herpes simplex virus type 1, HSV). In this previous project, we developed a simple assay to quantify the antiviral activity of molluscan tissues (i.e. hemolymph, digestive gland). Subsequently, overseas research (Bivalife project, www.bivalife.eu) demonstrated that oysters resistant to OsHV-1 maintain low viral loads during infection. We observed the same phenomenon in abalone. These observations suggest resistant molluscs produce antiviral compounds that limit viral infection and control virus replication.

1.1 Need

Molluscan aquaculture industries in Australia are threatened by herpesviruses. AbHV has caused mass mortalities in Victorian and Tasmanian abalone farms since 2005 and is endemic off Victorian and possibly Tasmanian coasts. OsHV-1 has been detected in Pacific oysters in NSW, where it caused mass mortalities and threatens the entire oyster industry in the states of NSW, Tasmania and South Australia.

Research in France, where OsHV-1 is endemic, indicates that it is possible to select for higher resistance to this virus in oysters (i.e. there is a strong genetic effect on resistance). Developing Australian family lines of oysters resistant to OsHV-1 is been investigated in CRC project 2009/743.

Because molluscs lack acquired immunity, they cannot be vaccinated. The only feasible way to counter the threat of these viruses is to identify and breed from populations resistant to these viruses. Traditionally, breeding programs have relied on exposing oyster or abalone populations to field infections and then breeding from the survivors. This approach is very expensive and time consuming. It also has the limitation that field infections are variable and survivors may have avoided the pathogen by chance. Future breeding programs will rely on marker assisted selection to avoid these limitations. In our previous CRC project (2008/739), we developed an assay for screening antiviral activity in abalone hemolymph. We

utilised this assay to identify family lines of abalone and oysters with high antiviral activity and we performed genetic analysis to determine whether hemolymph antiviral activity is under genetic control, is positively correlated with survival after challenge, and whether there are any undesirable associated effects. We also utilised this assay to identify the antiviral agents in molluscan hemolymph. It will now be feasible to apply this knowledge to develop efficient diagnostic tests (marker assisted selection) to rapidly screen populations of oysters and abalone for antiviral activity.

1.2 Objectives

The overall objective of our project was to assist the molluscan aquaculture industry with the ability to identify oysters and abalone with high antiviral activity.

The specific objectives of our project were to

1) Screen a large number of abalone family lines for antiviral activity and assess genetic parameters.
2) Compare antiviral activity in Pacific oyster breeding lines with known resistance and susceptibility.
3) Identify antiviral agent(s) from the hemolymph of oysters and abalone.
4) Correlate antiviral quantification to in vitro antiviral activity in the haemolymph of oysters and abalone.
5) Commence work to develop a chemical screening test for antiviral activity and extend this tool in abalone and oyster breeding programs.

2. Methods

2.1 Oysters, family lines and hemolymph sampling.

The oyster family lines used in this study were derived from single pair matings such that all individual were full sibs. The foundation parents of this population were obtained from the three commercial hatcheries in Tasmania together with some animals from feral Tasmanian populations and were first spawned in the summer of 1997/98 (31) to establish an Australian oyster industry owned commercial breeding program (Australian Seafood Industries Pty Ltd). Successive selection and crosses of these family lines has since occurred with the emphasis primarily on weight, condition and shell characteristics. Matings were constructed to avoid inbreeding.

Single seed spat (diploids) from nineteen family lines of *C. gigas* were transferred from Tasmania to Port Stephens, New South Wales. These family lines were originally transferred to evaluate their performance in NSW using standard commercial grow-out trials. These trials were completed in November 2010 when the oysters had reached commercial size (2-years) and coincided with the first detection of OsHV-1 µvar causing mass mortalities of wild and farmed *C. gigas* in the Georges and Parramatta Rivers, New South Wales (21). NSW Department of Primary Industries transferred representatives from each family line to the Georges River as an opportunistic approach to quickly assess the resistance of Australian *C. gigas* to OsHV-1 µvar. Prior to deployment to the Georges River, hemolymph was withdrawn from the adductor muscle sinus from ~20 individuals per family using a 3-ml syringe equipped with a 19-gauge needle. Hemolymph samples were centrifuged at 1000 x g for 5 minutes before cell-free hemolymph was frozen at -20^{o}C. Hemolymph samples were transported on dry ice to Flinders University of South Australia using a commercial courier. Resistance of each family line to OsHV-1 was

estimated by measuring the viral load of 15 individual oysters per family using DNA extraction and qPCR protocols outlined in Jenkins et al. (21).

Adult oysters were provided by Zippel Enterprises Pty Ltd (Smoky Bay, South Australia) for characterisation of the anti-HSV-1 protein (see below). These oysters were housed in a marine aquarium (salinity 35 ppt, 17^{o}C) at Flinders University of South Australia (FUSA) and hemolymph was collected from these oysters as required.

2.2 Abalone family lines and sampling

During the project, significant difficulties were encountered in obtaining access to abalone samples. Parties within industry in Victoria, who had earlier undertaken to provide access to abalone samples, became averse to send material. South Australian Government agency PIRSA also expressed strong reluctance to allowing the import of certain abalone tissue samples into SA.

Abalone hemolymph samples was kindly provided by Mr Nick Savva of ABTAS Pty Ltd and originate from their in-house breeding program. Abalone were only sampled from Tasmania due to import restrictions imposed by PIRSA.

2.3 Cell culture, HSV-1 & plaque reduction assay

African green monkey kidney (Vero) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma #D6429) supplemented with 10 % newborn calf serum (Sigma #N4762) and 1 % antibiotics (+10 000 IU penicillin ml^{-1}, +10 000 µg streptomycin ml^{-1}; Gibco #15140) at 37^{o}C in a humidified atmosphere of 5% CO_2. A well-characterised strain, SC16 (30), of wild-type HSV-1 was propagated in Vero cells and supernatants from infected cell cultures were clarified by centrifugation 1500 x g for 10 min and stored in aliquots at -80^{o}C. HSV-1 titre was calculated from plaque numbers according to the method of Reed & Muench (26).

The antiviral activity of *C. gigas* hemolymph against HSV-1 was determined by a plaque reduction assay following the methods of Dang et al., (8). Vero cell monolayers were obtained in a 24-well plate (Corning® #3524), and 45 µl (8 %) of oyster hemolymph was added to the cells in duplicate. Cells and oyster hemolymph were incubated for 15 min on a bidirectional rotator (Thermo Scientific #4631) before monolayers were infected with ~50 p.f.u of HSV-1. Cells and virus were incubated for 2 days at 37^{o}C before monolayers were fixed with 10 % formaldehyde and stained with 2 % toluidine blue. Plaques were counted using an inverted light microscope and antiviral activity of the hemolymph was expressed as the percentage reduction in plaque number compared to controls (DMEM).

2.4 Estimate of anti-HSV-1 heritability

An animal model, that accommodated data from replicated HSV-1 plaque reduction assays on the same individual, was applied to estimate genetic parameters and explore non-genetic influences on phenotype. A Markov chain Monte Carlo (MCMC) method using a multi-trait generalized linear mixed effect model (glmm) in a Bayesian estimation framework, with animal breeding value fitted as a random effect, was used for the analysis (R Package MCMCglmm, Hadfield, 2010, http://www.cran.r-project.org). Our main interest was to determine whether hemolymph anti-HSV-1 activity was under genetic control and the following model was fitted as

P% = mu+trial+animal+ID

Where, *P%* was the percent protection measured by the plague reduction assay, *animal* and *ID* are random animal effects (ID was the same as animal factor, but was used by MCMCglmm to dissociate individual records from the pedigree and give an indication of between individual variance) and *mu* represented unknown random residual effects. Duplicated measurements of HSV-1 protection were treated as different trials (so trial was fitted as a fixed effect in the model).

Models were run using 300,000 burn-in iterations, followed by 1,000,000 iterations and a thinning rate of 500. Estimates of additive genetic variance and residual variance were calculated from the modes of the posterior distribution and a Bayesian equivalent of 95% confidence intervals was obtained by calculating the values of estimates that bound 95% of the posterior distributions. Narrow sense heritability (h^2), or the proportion of total phenotypic variation that is additive genetic in origin, was estimated under the animal model described above as $V_A(V_A + V_E + V_e)$, where V_A, V_E and V_e were variance attributed to additive genetic, permanent environmental effects unconstrained by pedigree and residual error effects, respectively. Heritability was considered significant when the 95% credible interval of the posterior distribution did not span zero. Phenotypic correlation between HSV-1 protection and OsHV-1 resistance in the field were tested using Pearson's correlation coefficient (*r*).

2.5 Identification of oyster anti-HSV-1 compound

Hemolymph proteins from 3 adult oysters (pooled) were separated using native-polyacrylamide gel electrophoresis (BIO-RAD, Mini-PROTEAN® II Cell). Discontinuous gels, 0.75 mm thick, were prepared using native (no sodium dodecyl sulphate, no reducing agent) Tris-glycine buffered gel with the resolving gels (10% acrylamide/bis) prepared with 1.5 M Tris-HCl pH 8.8, and the stacking gel (4% acrylamide/bis) prepared with 0.5 M Tris-HCl, pH 6.8. An aliquot of oyster hemolymph (0.3 ml) was resolved at 120 V for 2 hours. The resolving gel was then divided into five horizontal strips and hemolymph compounds eluted overnight in 1 mL of 0.01 M phosphate buffered saline (PBS). Hemolymph fractions were then assayed for antiviral activity using the HSV-1 and Vero cell plaque reduction assay described above. Estimation of molecular mass of protein bands in each hemolymph fraction was determined by electrophoresis on 14 % SDS-PAGE gels alongside unstained Precision Plus Protein™ Standard (BIO-RAD #161-0363). SDS-PAGE gels were stained with SYPRO® Ruby protein gel stain (Invitrogen #S-12000), Pierce® Silver Stain Kit (Thermo Scientific #24612) and periodic acid-Schiff (PAS) stain to detect of glycosylated proteins.

Glycoslyation analysis was performed on the protein fraction that corresponded with highest anti-HSV-1 activity. N-deglycosylation was done under native conditions using peptide N-glycosidase F (PNGase F) and the GlycoProfile™ II Enzymatic In-Solution N-Deglycosylation Kit (Sigma #PP0201) with RNase B as a control. O-deglycolaytion was done under reducing conditions using neuraminidase (New England Biolabs #P0720S) and O-glycosidase (New England Biolabs #P0733S) according to the manufacturers protocol with fetuin (New England Biolabs #P6042) as a control.

The protein fraction that corresponded with highest anti-HSV-1 activity was subjected to digestion by addition of 200ng of trypsin (100ng/ul in 10mM NH_4HCO_3 buffer, pH 7.8). After overnight digestion at 37°C, the sample was analysed with a Thermo Orbitrap XL Linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron, San Jos, CA). The samples were applied to a 300 mm id x 5 mm C18 PepMap 100 precolumn (Dionex, Sunnyvale, CA) and separated on a 75 mm x 150 mm C18 5 µm 100 Å column (Nikkyo Technos, Tokyo, Japan), using a Dionex Ultimate 3000 HPLC (Dionex) with a 55-min gradient from 2% CAN to 45%

CAN containing 0.1% formic acid at a flow rate of 200 nL/min followed by a step to 77% CAN for 9 min. The mass spectrometer was operated in positive ion mode with one FTMS scan of m/z 300-2000 at 60 000 resolution followed by ITMS or FTMS product ion scans of the six most intense ions with dynamic exclusion of 30 s with 10 ppm low and high mass width relative to the reference mass, an exclusion list of 500 and CID energy of 35 %. Only multiply charged ions were selected for MS/MS. The spectra were searched with Thermo Proteome Discoverer version 1.2 (Thermo Electron) using the SEQUEST algorithm against the oyster genome (34)

2.6 Characterisation of oyster hemolymph antiviral protein

A putative antiviral protein, termed cavortin, was identified in the section above (see Results). Cavortin was pelleted from oyster hemolymph by ultracentrifugation (286 000 x g for 3 hours in a Beckman NTV90 rotor) according to Scotti et al., (28) and the pellet resuspended in 0.2 µm filtered seawater. Purity of cavortin was assessed using SDS-PAGE in 14 % acrylamide/bis gel and antiviral activity confirmed by assaying whole hemolymph, cell-free hemolymph (1000 x g for 5 min), cavortin-free hemolymph (286 000 x g for 3 hours) and purified cavortin using the HSV-1 and Vero cell plaque reduction assay described above. Five-fold dilutions of purified cavortin were prepared using 0.2 µm filtered-seawater and tested in the plaque reduction assay to determine if the antiviral activity is titratable. The form of antiviral activity that cavortin exerts (virucidal, antiviral state, prevents virus infection or replication) was assessed by varying the addition and incubation times of cavortin relative to HSV-1/Vero cell incubation, similar to the previously described procedures (8, 24). Briefly, purified cavortin was included in the plaque reduction assay for one of the following times: (i) pre-incubated with Vero cells for 2 hours prior to addition of HSV-1; (ii) added simultaneously with HSV-1; (iii) added 2 hours after HSV-1 infection of Vero cells; and (iv) pre-incubated with HSV-1 at room temperature for 60 min prior to addition to Vero cells.

3. Results

3.1 Anti-HSV-1 of *C. gigas* family lines and heritability

Anti-HSV-1 activity of oyster hemolymph was variable between family lines of *C. gigas* (Figure 1A, ANOVA $p < 0.001$). The narrow-sense heritability of this trait (anti-HSV-1 activity) was calculated by MCMC-glmm and found to be significantly different from zero ($h^2 = 0.21$ with 95% confidence intervals at 0.11 and 0.43 for 307 animals tested in 19 full-sibling families). No genetic correlation was observed between the estimated breeding values for anti-HSV-1 activity of the hemolymph and resistance to OsHV-1 in the field (Figure 1B, $r^2 = 0.019$, $p > 0.05$).

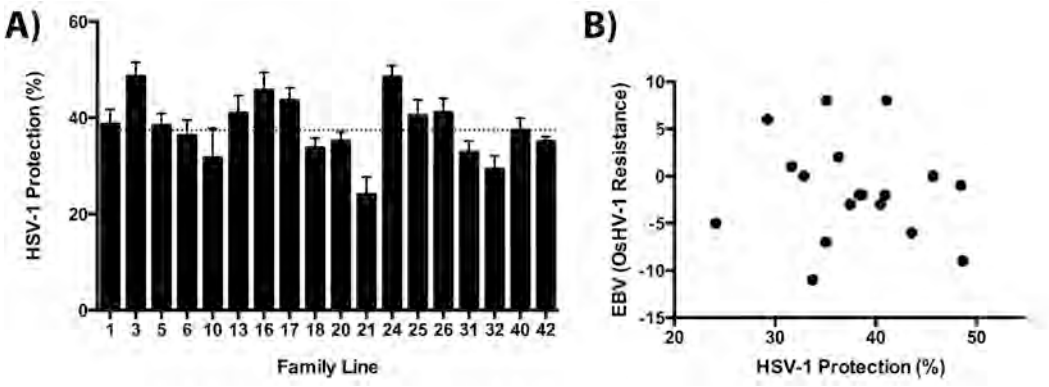

Figure 1: Antiviral activity against herpes simplex virus type 1 (HSV-1) of selected Australian family breeding lines of *Crassostrea gigas*. B) No genetic correlation was observed between pair-mated family lines for estimated breeding values (EBV) of anti-HSV-1 activity and resistance to OsHV-1 in the field.

3.2 Identification and characterization of hemolymph anti-HSV-1 activity

Anti-HSV-1 activity was observed in all proteins fractions resolved by native-PAGE (Figure 2B). The negative control (native-polyacrylamide gel soaked in PBS) also displayed antiviral activity against HSV-1, but was not cytotoxic to the Vero cells. Highest anti-HSV-1 activity was observed in fraction 2 (Figure 2B), which had similar anti-HSV-1 activity to the hemolymph control. SDS-PAGE revealed fraction 2 contained a single band with an estimated molecular mass of 21 kDa under non-reducing conditions (Figure 2A). However, following reduction, this band migrated with an estimated molecular mass of 30 kDa. This 21 kDa band was positively stained by SYPRO Ruby, silver and PAS stain, which suggests it is a glycoprotein. Deglycosylation of the 21 kDA protein band with PNGase F, but not O-glycosidase, confirmed this finding with a protein migrating faster after SDS-PAGE under reducing conditions (Figure 3). The antiviral activity of each hemolymph fraction was positively correlated to the abundance of this 21 kDA glycoprotein (Table 1, r = 0.89, $p < 0.05$). Antiviral activity did not correlate with any other band identified by SDS-PAGE (Table 1).

Table 1: Antiviral activity is correlated with a 21 kDa protein.

Protein (kDa)	Pearson r	*p* (two-tailed)
21	**0.8933**	**0.0411***
55	0.3777	0.5308
75	-0.0951	0.8791
142	-0.2663	0.6649
250	-0.5574	0.3289

LC/MS/MS analysis of this 21 kDa protein band revealed six peptides that matched the cavortin cDNA sequence (Genbank #AY551094) with high confidence. Sedimentation of CFH proteins, including cavortin, was performed by ultracentrifugation (Figure 4A) and this hemolymph fraction did not have anti-HSV-1 activity (< 3 % reduction in plaques, Figure 4B). CFH proteins resuspended in sterile seawater had similar anti-HSV-1 activity to the acellular hemolymph fraction. This crude purification of cavortin was used to assess its mode of antiviral action.

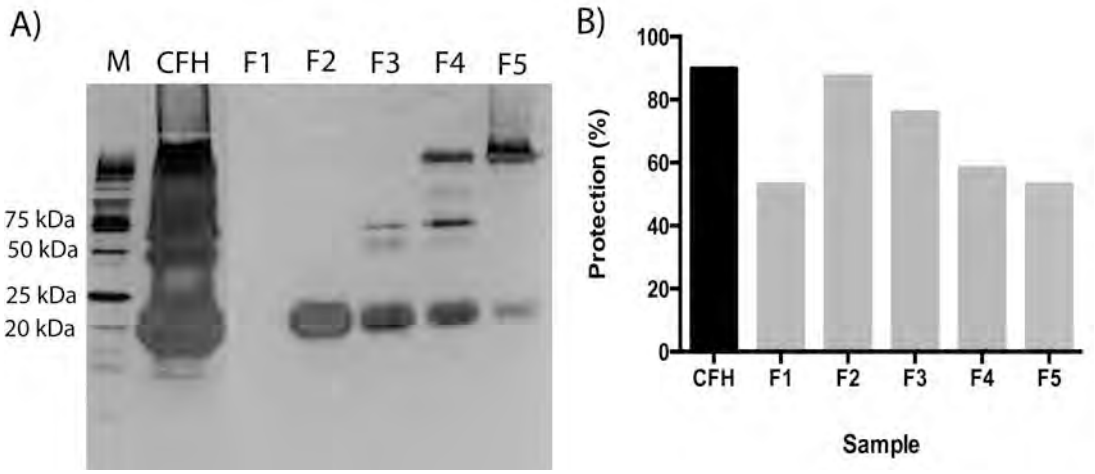

Figure 2: Identification of anti-HSV-1 protein in oyster hemolymph. **A**) Oyster hemolymph was fractioned based on native molecular weight and proteins in each fraction was visualised on a 14 % non-reducing SDS polyacrylamide gel followed by silver staining. **B**) Each protein fraction was then assessed for anti-HSV-1 activity using a Vero cell plaque assay. Highest anti-HSV-1 activity corresponded to F2. M = protein marker; CFH = cell free hemolymph.

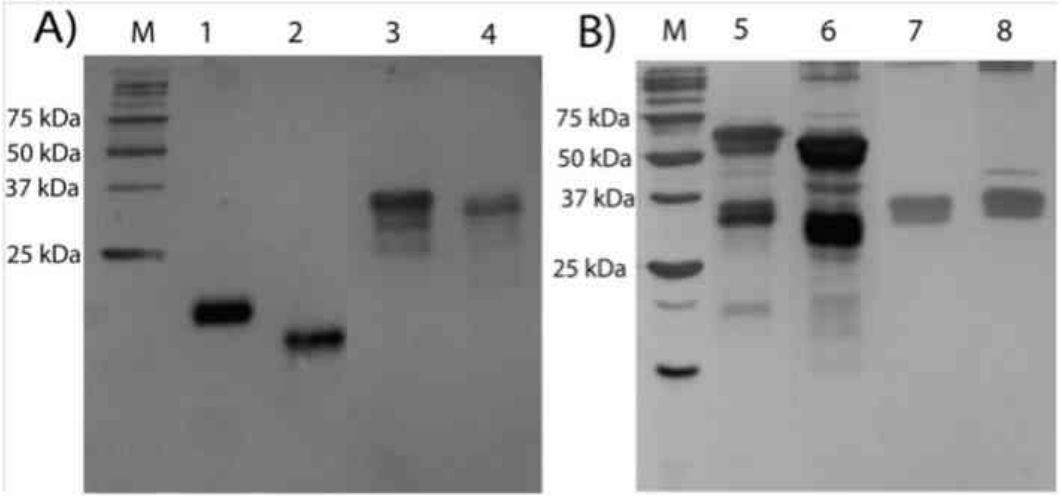

Figure 3: Oyster anti-HSV-1 protein subjected to N-linked (**3A**) and O-linked (**3B**) deglycosylation reactions. Oyster anti-HSV-1 is a N-linked glycoprotein. Electrophoresis was done in 14 % polyacrylamide gels followed by silver staining. Lane M = biorad marker; 1 = RNase B (control); 2 = RNase B + PNGase F; 3 = Oyster protein; 4 = Oyster protein + PNGase F; 5 = Fetuin (control); 6 = Fetuin + O-glycosidase; 7 = Oyster protein; 8 = Oyster protein + O-glyosidase.

3.3 Mode of antiviral action

Five-fold dilutions of purified cavortin revealed its antiviral activity is positively correlated with its abundance (R^2 = 0.895, p < 0.05). Cavortin exerts its antiviral activity by interfering with HSV-1 replication because pre-incubation of cavortin with Vero cells for 2 h prior to HSV-1 infection or the addition of cavortin at the time of infection did not reduce the number of plaques compared to addition of cavortin at 2 hours after HSV-1 was added to the Vero cells (ANOVA, p > 0.05). Likewise, cavortin did not have a virucidal effect (< 1 % reduction in plaque numbers, p > 0.05).

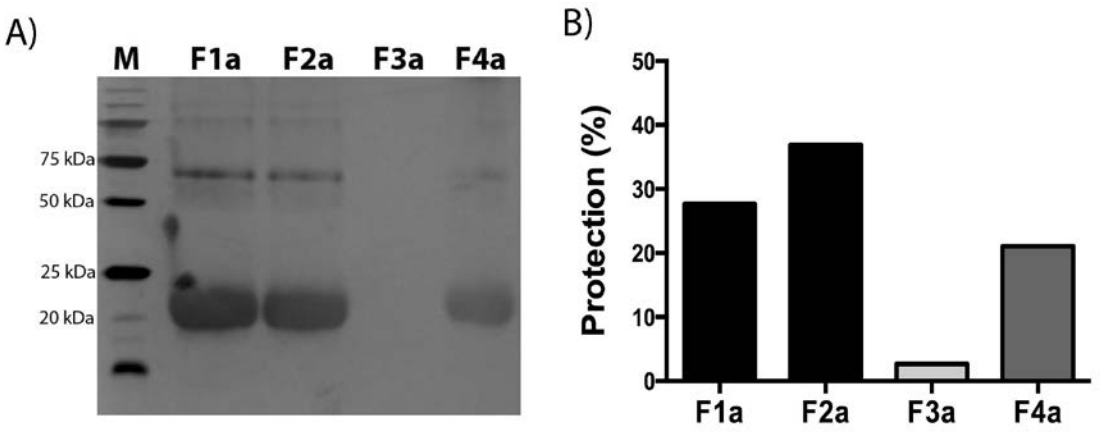

Figure 4: Ultracentrifugation of oyster hemolymph to purify cavortin. A) SDS-PAGE analysis of hemolymph (F1a), cell-free hemolymph (F2a), ultracentrifuged hemolymph (F3a) and hemolymph proteins resuspended in seawater. B) Hemolymph antiviral activity could be eliminated from hemolymph centrifuged at 286 000 x g (F3a) and restored when hemolymph proteins were resuspended in seawater (F4a).

3.4 Antiviral activity between abalone family lines.

Intra and inter-species differences in anti-HSV-1 activity was observed for abalone (Figure 5, ANOVA $p < 0.01$). A similar genetic parameter analysis was used for the abalone data (data from 129 animals in 14 full sibling families) as described in section 3.1. For abalone the following model was fitted:

P%=mu+species+settlement+animal+ID

where, *species* was either *H. laevigata* or *H. rubra*, *settlement* was the date the animal settled as larvae and other factors were as described above in section 3.3 for oysters.

As per oysters, the heritability of percent protection was found to be low but significant in abalone (0.76, with lower and upper 95% confidence limits of 0.47 and 0.94 respectively). Neither species nor date settled were significant factors in the model. However the number of animals/families tested was low and more data needs to be tested.

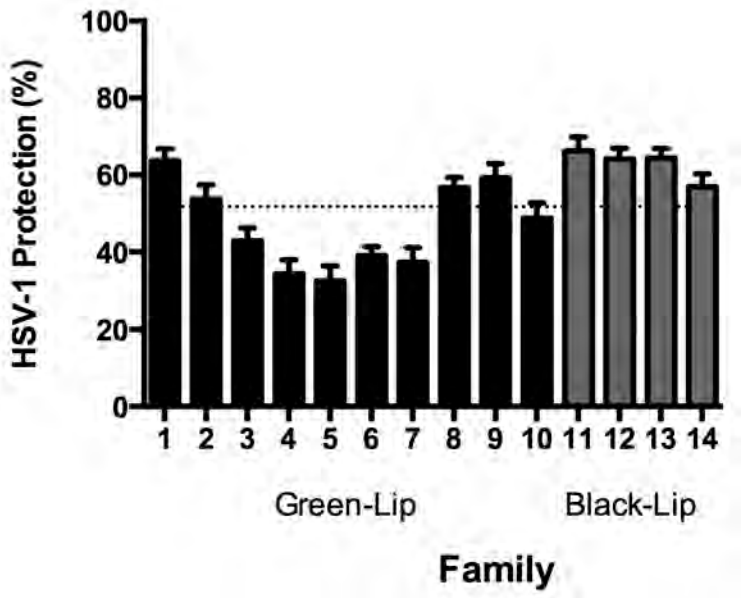

Figure 5: Inter- and intra-species differences in anti-HSV-1 activity of Australian abalone family lines.

4. Discussion

This study indicates that the major hemolymph protein of *C. gigas* has antiviral activity against herpesviruses and oysters can be selectively bred for increased hemolymph antiviral activity. Likewise, intra- and inter-species differences in anti-HSV-1 activity of abalone hemolymph were observed and this trait also appears to be heritable. We assessed hemolymph antiviral activity using a heterologous model because cell lines from marine molluscs do not currently exist (4) and OsHV-1 cannot be cultured in primary bivalve cell cultures (15). We choose to evaluate the antiviral activity of oyster and abalone hemolymph using a mammalian fibroblastic cell line (Vero cells) infected with HSV-1 because this model has been successfully employed in other studies (8, 18, 23, 24). These previous studies identified antiviral activity in *C. gigas* hemolymph (23, 24, 33), but were unable to identify the compound responsible for antiviral activity.

Research suggests a genetic component exists for OsHV-1 resistance and that genetic selection could improve this trait (9, 10). In France, genetic gains were easily obtained by breeding from oysters that had survived field exposure to OsHV-1. Genetic selection will be more complicated in Australia and New Zealand because oyster hatcheries that provide spat to industry are located outside of the jurisdiction of where OsHV-1 occurs (21). The risk of horizontal or vertical transmission of OsHV-1 in the hatchery is real (1-3) and could potentially aid the escape of OsHV-1 into uninfected jurisdictions. Therefore, hatcheries in the Oceania region require an alternative method to select progenitors with increased antiviral resistance. We chose to investigate if hemolymph antiviral activity against HSV-1 could be utilised as an indirect marker of OsHV-1 resistance. Our results provide evidence that the hemolymph anti-HSV-1 activity is variable between pair-mated family lines of *C. gigas* (Figure 1A) and this trait is heritability (h^2 = 0.21, 95% confidence intervals of 0.11 and 0.43). Narrow sense heritability theoretically ranges from one (variation is 100 % explained by additive genetic effects) to zero (no additive genetic component). It appears our heritability estimate of hemolymph antiviral activity (h^2 = 0.21) is low

compared to other traits, such as shell pigmentation (h^2 = 0.50) (12), body weight (h^2 = 0.313) (13) and survival (0.36 – 0.71) (14). However, we observed no genetic correlation between our laboratory test (anti-HSV-1 activity) and resistance of oysters to OsHV-1 in the field (Figure 1B). This is presumably because our approach only measured one aspect of the oyster's immune system. Oysters rely on humoral (*i.e.* antiviral substances), cellular (*i.e.* apoptosis) and physical/chemical barriers (*i.e.* epithelium and mucosal) to prevent infections (5) and other aspects of oyster physiology, such as age, also contribute to OsHV-1 resistance (25, 27).

Our study also provides preliminary data to suggest the hemolymph antiviral activity of *C. gigas* corresponds with a 21 kDa N-linked glycoprotein, termed cavortin. We identified cavortin by fractionating oyster hemolymph using native-PAGE and assaying each fraction using the HSV-1 and Vero cell plaque reduction assay. Anti-HSV-1 activity in each fraction was positively correlated to the abundance of cavortin (Table 1). Cavortin appears to exert its antiviral activity at a post-entry stage of infection, possibly by inhibiting HSV-1 replication.

Cavortin was first characterised as an immune protein, which comprised of a single Cu/Zn superoxide dismutase domain and was capable of binding to lipopolysaccharide via a RGD motif (16). The function of cavortin has subsequently been disputed within the literature with several studies finding no compelling evidence to suggest cavortin has superoxide dismutase activity (17, 20, 28). It has been suggested the superoxide dismutase domain had been altered during the course of evolution resulting in a protein that primarily functions as a metal ion chaperone (28). Regardless of its primary function, cavortin must have an important immune role because it is regularly identified to be differentially expressed in oyster family lines selected for increased resistance to parasitic and viral diseases (17, 19, 22, 29, 32). Indeed, Normand and colleagues (22) recently concluded that resistance or susceptibility of *C. gigas* to OsHV-1 maybe partly explained by mRNA expression levels of cavortin. The major hemolymph proteins of other marine invertebrates also have multiple functions, including those involved in immunity (6, 11). Proteolysis of hemocyanin, the respiratory pigment of marine shrimp, produces polypeptides with potent antifungal activity (11). Experimental infections of shrimp result in elevated levels of hemocyanin-derived antimicrobial peptides constituting an important innate immune response to infection (11). Hemocyanins isolated from other arthropods have antiviral activity against a wide range of viruses that impact aquaculture (reviewed by 6). Antiviral activity is also present in gastropod (*Haliotis laevigata*) hemocyanin with activity against HSV-1 (7).

This work has provided significant benefits to industry and to the body of scientific knowledge about molluscan immunity. The data showing heritability of resistance to viral infection in oysters and abalone supports breeding programs to develop resistant family lines. It is difficult to gauge the adoption level because the decision of which family lines are chosen for selection relies on multiple sources of information (disease resistance, growth rate, shell shape, condition, avoiding inbreeding). Significant work towards developing a laboratory test for quantifying antiviral resistance in oysters (objective 5) was undertaken. Attempts to determine whether different isoforms of cavortin have different antiviral activity were undertaken and we can currently quantify the expression level of cavortin in oysters using currently published assays (RT-qPCR). Comparison of laboratory and field data did not show a strong correlation between hemolymph cavortin levels as measured in the lab and resistance to viral infection seen in the field. Multiple genes are likely to control resistance to OsHV-1 in the field. We therefore undertook in-silico analysis of the Pacific oyster genome DNA sequence to identify other antiviral proteins. We determined that the important antiviral protein viperin is almost 100% conserved between oysters and humans. Such high conservation argues strongly for a critical

role for viperin in countering viral threats. This observation has given rise to a collaboration with A/Prof. Michael Beard, of the University of Adelaide, who is the foremost global authority on the role of viperin in viral immunity. Experiments to elucidate the contribution that viperin makes to oyster immunity will commence shortly. Data so generated will represent an additional legacy of this project.

Commercial Pacific oyster growers (principally in South Australia) and commercial Pacific oyster hatcheries (all states – Tasmania, South Australia, New South Wales) were kept informed of results throughout the project.

5. Further Development

At completion of this project there remain a number of key knowledge gaps, closure of which will provide greater understanding of the means by which economically important molluscs resist viral threats.

Future developments that will arise out of this work include:

i. Studies of the role of the viperin gene.
ii. Studies of the role of cavortin in responding to viral threats.
ii. Complete characterization of a test for cavortin in pinpointing families resisting viral infection.

6. Planned Outcomes

Public Benefit Outcomes

This project's outcomes will assist in ensuring the Australian oyster and abalone industry gains access to disease-resistant stock in the future. This project advised the oyster and abalone industry of family breeding lines that display heightened antiviral activity. It is envisioned that breeding companies will utilise these results to produce the next generation of disease resistant stock. A putative antiviral protein responsible for inhibiting virus infection and replication was identified in the hemolymph of oysters. Further characterisation of this protein is expected to enable the oyster industry to implement marker-assisted selection into their breeding programs. Several laboratory studies were undertaken to increase our knowledge of the anti-viral response of adult and juvenile oysters at different water temperatures. These studies provided insight into the molecular mechanisms that cause oyster lethality as a result of viral infection. Combining these observations with previous Australian research (FRDC Project No. 2011/053) suggests farmers could reduce oyster mortalities by implementing farming strategies that are stressful to their stock. Studies conducted to increase our knowledge of the antiviral response of oysters also provided the foundation of a new concept to increase the antiviral resistance of oyster progeny using trans-generational immune priming. The key findings arising from this project were successfully communicated to industry at workshops and conferences.

Private Benefit Outcomes

Project legacy at Macquarie University/Sydney Institute of Marine Science.

The project provided the means to bring Dr Green back to Australia from his employment in France where he was working on OsHV-1 in the premier oyster research laboratory globally. Dr Green has now accepted an offer from Macquarie University, working at the Sydney Institute of Marine Science (SIMS). The immediate success that Dr Green generated on joining this project was pivotal to Macquarie University making the funds available for another 3 years research which will form an extension of this work.

Dr Green's position at Macquarie in all likelihood will result in him establishing himself as an independent investigator at SIMS. Dr Green's future at SIMS will provide a knowledge hub that will provide research and training into the future. This will be of inestimable value to the Australian seafood industry for the next generation.

Dr Green is an immensely talented and passionate researcher. His commitment to the Australian oyster industry is extraordinary. His ongoing involvement in this industry is possibly the most significant spin-off of this CRC project and will lead to greatly enhanced Australian research capacity in molluscan immunology.

Linkages with CRC Milestone Outcomes

Enhancement of Australian seafood industry productivity: The project outcomes will bolster the resilience of the oyster industry to disease threats which has considerable potential to boost productivity. While quantifying this increase in productivity is problematic because of the difficulty in predicting disease outbreaks, the disease threats are notable and can lead to crop devastation.

Because much aquaculture is based in rural, regional and remote areas, the improvement in viability of these enterprises that flows from this project will provide benefits targeted at these regions.

To a notable extent the success of this project can be traced to the response to an FRDC report (2005/641: Aquatic animal health subprogram: Current and future needs for aquatic animal health training and for systems for merit-based accreditation and competency assessments, by Dr Matt Landos, of University of Sydney) which highlights deficiencies in Australian aquatic animal health scientific training.

7. Conclusion

CRC grant 2011.758 has given outstanding value for money to the Australian seafood industry and to our broader understanding of disease and immunology in molluscs. Pragmatic adoption by industry of the outcomes is highly likely. Breeding programs aimed at developing molluscs that resist viral infections will be greatly supported by the work done in this project. A significant and enduring enhancement of the Australian molluscan research capacity will be a major legacy of this grant.

8. References

1. **Arzul, I., J.-L. Nicolas, A. J. Davison, and T. Renault.** 2001. French scallops: A new host for ostreid herpesvirus-1. Virol J **290:**342-349.
2. **Arzul, I., T. Renault, A. Thebault, and A. Gerard.** 2002. Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. Virus Res. **84:**151-160.
3. **Barbosa-Solomieu, V., L. D´egremont, R. V´azquez-Ju´arez, F. Ascencio-Valle, P. Boudry, and T. Renault.** 2005. Ostreid herpesvirus 1 (OsHV-1) detection among three successive generations of Pacific oysters (*Crassostrea gigas*). Virus Res. **107:**47-56.
4. **Bickham, U., and C. J. Bayne.** 2013. Molluscan cells in culture: primary cell cultures and cell lines. Can. J. Zool. **91:**391-404.
5. **Canesi, L., G. Gallo, M. Gavioli, and C. Pruzzo.** 2002. Bacteria-hemocyte Interactions and Phagocytosis in Marine Bivalves. Microsc. Res. Tech. **57:**469-476.
6. **Coates, C. J., and J. Nairn.** 2014. Diverse immune functions of hemocyanins. Dev. Comp. Immunol. **45:**43-45.
7. **Cuthbertson, A.** 2009. Anti-viral nutraceutical. Australia.
8. **Dang, V. T., K. Benkendorff, and P. Speck.** 2011. In vitro antiviral activity against herpes simplex virus in the abalone *Haliotis laevigata*. J. Gen. Virol. **92:**627-637.
9. **Degremont, L.** 2013. Size and genotype affect resistance to mortality caused by OsHV-1 in *Crassostrea gigas*. Aquaculture **416:**129-134.
10. **Dégremont, L.** 2011. Evidence of herpesvirus (OsHV-1) resistance in juvenile *Crassostrea gigas* selected for high resistance to the summer mortality phenomenon. Aquaculture **317:**94-98.
11. **Destoumieux-Garzon, D., D. Saulnier, C. Jouffrey, P. Bulet, and E. Bachere.** 2001. Crustacean Immunity: Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. J. Biol. Chem. **276:**47070-47077.
12. **Evans, S., M. D. Camara, and C. J. Langdon.** 2009. Heritability of shell pigmentation in the Pacific oyster, *Crassostrea gigas*. Aquaculture **286:**211-216.
13. **Evans, S., and C. Langdon.** 2006. Direct and indirect responses to selection on individual body weight in the Pacific oyster (*Crassostrea gigas*). Aquaculture **261:**546-555.
14. **Evans, S., and C. Langdon.** 2006. Effects of genotype x environment interactions on the selection of broadly adapted Pacific oysters (*Crassostrea gigas*). Aquaculture **261:**522-534.
15. **Garcia, D., A. Thebault, L. Degremont, I. Arzul, L. Miossec, M. Robert, B. Chollet, C. Francois, J.-P. Joly, S. Ferrand, N. Kerdudou, and T. Renault.** 2011. Ostreid herpesvirus 1 detection and relationship with *Crassostrea gigas* spat mortality in France between 1998 and 2006. Vet. Res. **42:**73-84.
16. **Gonzalez, M., B. Romestand, J. Fievet, A. Huvet, M. C. Lebart, Y. Gueguen, and E. Bachere.** 2005. Evidence in oyster of a plasma extracellular superoxide dismutase which binds LPS. Biochem. Bioph. Res. Co. **338:**1089-1097.
17. **Green, T. J., T. J. Dixon, E. Devic, R. D. Adlard, and A. C. Barnes.** 2009. Differential gene expression of antioxidant enzymes in Sydney rock oysters,

Saccostrea glomerata (Gould) selected for disease resistance. Fish Shellfish Immunol. **26:**799–810.

18. **Green, T. J., C. Montagnani, K. Benkendorff, N. Robinson, and P. Speck.** 2014. Ontogeny and water temperature influences the antiviral response of the Pacific oyster, *Crassostrea gigas*. Fish Shellfish Immunol. **36:**151-157.
19. **Huvet, A., A. Herpin, L. De´gremont, Y. Labreuche, J.-F. Samain, and C. Cunningham.** 2004. The identification of genes from the oyster *Crassostrea gigas* that are differentially expressed in progeny exhibiting opposed susceptibility to summer mortality. Gene **343:**211-220.
20. **Itoh, N., Q.-G. Xue, K. L. Schey, Y. Li, R. K. Cooper, and J. F. La Peyre.** 2011. Characterization of the major plasma protein of the eastern oyster, Crassostrea virginica, and a proposed role in host defense. Comparative Biochemistry and Physiology, Part B **158:**9-22.
21. **Jenkins, C., P. Hick, M. Gabor, Z. Spiers, S. A. Fell, X. Gu, A. Read, J. Go, M. Dove, W. O'Connor, P. D. Kirkland, and J. Frances.** 2013. Identification and characterisation of an ostreid herpesvirus-1 microvariant (OsHV-1 u-var) in *Crassostrea gigas* (Pacific oysters) in Australia. Dis. Aquat. Org. **105:**109-126.
22. **Normand, J., R. Li, V. Quillien, J.-L. Nicolas, P. Boudry, F. Pernet, and A. Huvet.** 2014. Contrasted survival under field or controlled conditions display associations between mRNA levels of candidate genes and response to OsHV-1 infection in the Pacific oyster, *Crassostrea gigas*. Mar. Genom.
23. **Olicard, C., Y. Didier, C. Marty, N. Bourgougnon, and T. Renault.** 2005. *In vitro* research of anti-HSV-1 activity in different extracts from Pacific oysters *Crassostrea gigas*. Dis. Aquat. Org. **67:**141-147.
24. **Olicard, C., T. Renault, C. Torhy, A. Benmansour, and N. Bourgougnon.** 2005. Putative antiviral activity in hemolymph from adult Pacific oysters, *Crassostrea gigas*. Antiviral Res **66:**147-152.
25. **Paul-Pont, I., O. Evans, N. K. Dhand, A. Rubio, P. Coad, and R. Whittington.** 2014. 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 **422:**146-159.
26. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fify per cen endpoints. Am. J. Hyg. **27:**493-497.
27. **Samain, J. F., L. Degremont, P. Soletchnik, J. Haure, E. Bedier, M. Ropert, J. Moal, A. Huvet, H. Bacca, A. Van Wormhoudt, M. Delaporte, K. Costil, S. Pouvreau, C. Lambert, V. Boulo, P. Soudant, J.-L. Nicolas, F. Le Roux, T. Renault, B. Gagnaire, F. Geret, I. Boutet, T. Burgeot, and P. Boudry.** 2007. Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. Aquaculture **268:**227-243.
28. **Scotti, P. D., S. C. Dearing, and D. R. Greenwood.** 2007. Characterisation of cavortin, the major haemolymph protein of the Pacific oyster (*Crassostrea gigas*). New Zeal. J. Mar. Fresh. **41:**91-101.
29. **Simonian, M., S. V. Nair, J. A. Nell, and D. A. Raftos.** 2009. Proteomic clues to the identification of QX disease-resistance biomarkers in selectively bred Sydney rock oysters, *Saccostrea glomerata*. J. Prot. **73:**209-217.
30. **Speck, P., and A. Simmons.** 1991. Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1. Journal of Virology **65:**4001-4005.
31. **Swan, A. A., P. A. Thompson, and R. D. Ward.** 2007. Genotype x environment interactions for weight in Pacific oysters (*Crassostrea gigas*) on five Australian farms. Aquaculture **265:**91-101.

32. **Taris, N., R. P. Lang, P. W. Reno, and M. D. Camara.** 2009. Transcriptome response of the Pacific oyster (*Crassostrea gigas*) to infection with *Vibrio tubiashii* using cDNA AFLP differential display. Animal Genetics **40:**663-677.
33. **Zeng, M., W. CUI, Y. Zhao, Z. Liu, S. Dong, and Y. Guo.** 2008. Antiviral active peptide from oyster. Chinese Journal of Oceanology and Limnology **26:**307-312.
34. **Zhang, G., X. Fang, X. Guo, and e. al.** 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature **490:**49-54.

9. Appendices

Copies of the following papers will be provided as a separate appendix.

Papers published:

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

Ontogeny and water temperature influences the antiviral response of the Pacific oyster, *Crassostrea gigas*. Timothy J. Green, Caroline Montagnani, Kirsten Benkendorff, Nick Robinson, Peter Speck. 2014. Fish and Shellfish Immunology 36:151-157.

Manuscript in press:

Anti-viral gene induction is absent upon secondary challenge with double-stranded RNA in the Pacific oyster, *Crassostrea gigas.* Timothy J Green, Kirsten Benkendorff, Nick Robinson, David Raftos, & Peter Speck.
-accepted by Fish and Shellfish Immunology.

Manuscript submitted:

Evidence that the major hemolymph protein of the Pacific oyster, *Crassostrea gigas*, has antiviral activity against herpesviruses. Timothy J. Green, Nick Robinson, Tim Chataway, Kirsten Benkendorff, Wayne O'Connor, Peter Speck.
-submitted to Fish and Shellfish Immunology.