Fisheries Research Report No. 230, 2012

Aquatic Animal Health Subprogram: Development of a DNA microarray to identify markers of disease in pearl oysters *(Pinctada maxima)* and to assess overall oyster health

Final FRDC Report – Project 2008/030

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Correct citation:

Raftos, D., Nair, S., Crockford, M. and Jones, B. (2012). Aquatic animal health subprogram: Development of a DNA microarray to identify markers of disease in pearl oysters *(Pinctada maxima)* and to assess overall oyster health. Final Report FRDC Project No. 2008/030. Fisheries Research Report No. 230. Department of Fisheries, Western Australia. 72p.

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ISSN: 1035 - 4549 ISBN: 978-1-921845-40-6

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1.0 Non Technical Summary

2008/030 Aquatic animal health program: Development of a DNA microarray to identify markers of disease in pearl oysters (Pinctada maxima) and to assess overall oyster health.

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Objectives:

1. To construct a cDNA library using healthy and stressed oysters.
2. To design and print DNA microarray slides for the analysis of diseased states in pearl oysters (*P. maxima*).
3. To use the DNA microarray to identify molecular markers that differentiate between pearl oysters that are stressed from those that appear to be healthy.

To use the DNA microarray to test for markers of adverse health in pearl oysters that appear to be affected by environmental stressors other than OOD.

Outcomes achieved to date

The project outputs have contributed to or will lead to the following outcomes

- The development of a cDNA microarray that can be used by industry to test the effects of a range of environmental variables in the field.
- The generation of sequence data for clones obtained from the cDNA library including *P. maxima* and non *P. maxima* data has added to the current limited sequence data available for *P. maxima* oysters. The new sequence data will be added to Genbank database.
- Identification of a suite of gene biomarkers that can be used to assess the relative impacts of different processes within farming practices, with the intention of ameliorating or mitigating particularly harmful processes.
- The study has provided early evidence that, based on comparative cDNA microarray analysis of healthy and stressed oysters, some environmental stressors, particularly exposure to air, may be relatively more harmful than other factors, and showed very substantial differences in gene expression between controls and "stressed" oysters. This provides management with the opportunity to adjust management practices to mitigate effects of stress on oysters.
- There are three ways in which these genes might be used in a routine assay of stress. The first is the quantitative real time PCR method that we used in the project. A second, less expensive option is to use normal semi-quantitative PCR. The level of difference in expression for some of the genes that identified is probably great enough that semi-quantitative PCR could identify simple on/off differences in expression of those genes. Both of these PCR methods require samples to be rapidly frozen in the field and then transported to a laboratory for analysis. The most realistic and cheapest option would require more work to develop antibodies against proteins encoded by the differentially expressed genes. That way the concentration of proteins from the corresponding genes could be measured in relatively simple colorimetric assays, potentially on site. The advantage of this is that proteins are more stable than mRNA and so sample collection and storage would not be so problematic.
- The project results have been communicated to industry and were also communicated through the FRDC Australasian Aquatic Animal Health Scientific Conference 5-8 July 2011.

This project used DNA-based technology to study the effects of environmental stress on pearl oysters. During the oyster farming process, oysters are subjected to a range of changes in their environment, such as fluctuating temperature and salinity. These changes are exacerbated by farming practices, during which oysters are exposed to additional stresses, such as routine antifouling and nucleation. All of these factors have the potential to stress oysters, potentially affecting their growth, susceptibility to disease and other important characteristics that decrease productivity. At its most basic level, stress causes changes in the activity of genes in oysters, switching some on and turning others off. It is these changes in gene activity that alter characteristics such as growth rate and susceptibility to disease.

The goal of our project was to identify the genes in oysters that are affected by environmental stress. This had two significant benefits. It allowed the activities of stress-response genes to be monitored in the farming process in an effort to identify and modify particularly stressful components of the farming practice. The project also identified a set of genes that can be used

as sensitive monitors of stress in the future to identify the onset of stressful events before they affect productivity.

The project used DNA microarrays to identify stress response genes. In microarrays, the DNA from thousands of oyster genes is printed as microdots onto the surface of glass slides. Our pearl oyster microarrays carried 3,000 spots of DNA. Those spotted microarrays were analysed to see which particular genes were turned on or off under particular environmental conditions. The end result was a list of stress-response genes that are affected by particular types of environmental stress that can be used to gauge the effects of environmental stress during routine farming practices. The final stage of the project tested the effect of a common management practice used in pearl oyster farming (routine de-fouling of oysters) on the "stress response" genes identified in laboratory-based experiments. The data showed that stress associated with de-fouling has a substantial impact on the expression of many of the "stress response" genes originally identified by microarray analysis. In some cases, the expression of "stress response" genes changed by up to 28 fold in response to de-fouling treatment. This suggests that many of the stress response genes that we have identified by microarray analysis may be extremely useful markers of stress in the field, and that simultaneous (multivariate) analysis of sets of stress response genes provide an even more robust distinction between "stressed" and healthy oysters.

In terms of immediate advice to industry, our analysis suggests that exposing oysters to air is the most stressful factor in the current farming practice. The microarrays also provide a valuable resource for future research that will be freely available to the broader research community.

Despite this, one significant conclusion from this study is that, without a complete genome sequence for pearl oysters, the utility of the microarray that we have developed is limited because it does not allow us to identify some of the genes associated with stress responses in oysters. Hence, one of our key recommendations at the end of this project is that industry and the relevant government authorities should support a nationwide effort to sequence the pearl oyster genome.

Keywords: pearl oyster, microarray, gene expression, environmental stress, oyster health

2.0 Acknowledgements

The project was funded by the FRDC together with the Pearl Producers Association through the then ministerial Pearling Industry Advisory Committee, and with contributions from Maquarie University and the Deapthment of Fisheries, Government of Western Australia. The Pearling Industry Advisory Committee was disestablished in 2009. Strong in-kind support was provided by Paspaley Pearls, Cygnet Bay Pearls, the Darwin Aquaculture Centre and the Kimberley Marine Research Station.

As part of the project agreement with the FRDC and the Pearl Producers Association, a scientific steering committee was set up to oversee progress. Members of the Steering Committee were: Mt Brett McCallum (Pearl Producers Association), Mr. David Mills (Paspaley Pearls), Mr. Sam Buchanan (Blue Seas Pearling Company). Leigh Taylor (Department of Fisheries). As with any project of this size, a number of staff contributed to the project. These included:

Mr. Adam Wilkins, PhD student, Macquarie University. Adam worked on various aspects of the project, particularly microarray analysis and real time PCR. He undertook his Honours degree in A/Prof Raftos' laboratory working on oyster microarrays, the same technology that was used in the current project.

Ms. Camille LeCroix, Masters student/intern, Macquarie University. Camille joined A/Prof. Raftos' laboratory as an intern from l'Université Paris-Sorbonne. She contributed to the later stages of the project, specifically real-time PCR analyses.

Ms. Druime Nolan, Technical Officer, Fisheries WA. Druime was directly involved in the preparation of samples for cDNA library construction.

Dr. Meike Berger, Scientific Officer, Fisheries WA. Dr. Berger was also involved in the construction of the cDNA library.

3.0 Background

In October 2006, unexplained mortalities of farmed pearl oysters (*Pinctada maxima*) were reported in Western Australia. The disease associated with these mortalities has been termed Oyster Oedema Disease (OOD). It is likely that OOD is caused by an infectious agent. The disease spread rapidly and there are no known control measures, no knowledge of a causative agent, no understanding of how widespread the disease is, and no way to test for it. However, experience in other oyster species indicates that environmental stress is a major contributing factor to such disease outbreaks.

Changes in environmental conditions can lead to acute physiological stress in marine invertebrates, such as oysters. Many of these changes affect the immune systems of oysters, potentially increasing susceptibility to disease (Cheng, 1988; Aladaileh et al., 2008). Naturally occurring environmental stressors include changes in temperature, pH, dissolved oxygen content, food availability, and salinity (Le Moullac et al., 1998; Perazzolo et al., 2002). Practices associated with pearl oyster faming, such as handling, sorting, cleaning, and nucleation, are likely to exacerbate these physiological stressors. Pearl oysters are usually removed from the water every 3-6 months for de-fouling, during which byssal threads are broken and oysters are exposed to air and mechanical agitation for extended periods. Prior to seeding, oysters are starved, and they are induced to spawn by temperature shocks. For nucleation, they are forced to gape, often involving the use of mechanical wedges that can damage the adductor muscle, mantle margins and the hinge, whilst incisions are made in the gonad resulting in substantial loss of hemolymph (Lintilhac, 1987; Mills et al., 1997; FAO, 2007).

Oysters actively respond to stress in an effort to maintain homeostasis (Aladaileh et al., 2008). This physiological regulation is due, at least in part, to altered gene expression and is influenced by the endocrine system. Emerging evidence suggests that such changes often impair immune function. Recent studies of Sydney rock oysters by A/Prof Raftos' group have identified a clear link between environmental stress, suppression of the oyster immune system and susceptibility to infectious disease (Aladeileh et al., 2007b, 2008; Peters and Raftos, 2003; Butt and Raftos, 2007a,b; Butt et al., 2006, 2007a,b, 2008; Kuchel and Raftos, 2010; Kuchel et al., 2010, 2011). The immune system of bivalves comprises cellular and humoral reactions (Barracco et al., 1999). Although molluscs lack antibody-based humoral immune systems, they are believed to have defence molecules that are similar in function to antibodies (Arason, 1996; Muller et al., 1999).

As in many invertebrates, the phenoloxidase (PO) cascade of oysters represents a critical host defence response (Aladaileh et al., 2007a; Butt and Raftos, 2007b). Products of the PO pathway are involved in immunological mechanisms such as wound healing, cytotoxicity, phagocytosis and pathogen encapsulation (Butt and Raftos, 2007b; Kuchel et al., 2010; Soderhall & Cerenius, 1998; Newton et al., 2004).

Newton et al. (2004) found that the PO cascade is directly related to QX disease resistance and susceptibility in Sydney rock oysters. In QX disease-susceptible oysters, PO is suppressed prior to infection by *Marteilia sydneyi*, the aetiological agent of QX disease in Sydney rock oysters (Peters and Raftos, 2003; Butt and Raftos, 2007; Butt et al., 2006, 2007). Butt et al. (2006) have shown that *M. sydneyi* is not responsible for the inhibition of PO activity seen in QX disease. Instead, it appears that environmental stress causes the decrease in PO activity, and that opportunistic infection by *M. sydneyi* occurs as a result.

Other evidence suggests that the inhibition of immune function that results from environmental stress is due to a hormonal stress response in oysters (Aladeileh et al., 2007b, 2008; Butt et al., 2006). Lacoste et al. (2001a-d, 2002a,b) found that the release of the hormone, noradrenaline, during stress reactions in Pacific oysters inhibits immunological functions, such as phagocytosis, and results in programmed cell death. Similar results have been obtained by A/Prof Raftos' group working with Sydney rock oysters. They found that a range of common environmental stressors stimulate the release of noradrenaline, and that noradrenaline causes profound decreases in phenoloxidase activity and a number of other important cellular defence reactions, probably resulting from apoptosis (Aladaileh et al., 2007b; Kuchel et al., 2010a,b; 2011a,b).

4.0 Need

New evidence has identified a clear link between common practices in pearl oyster farming and disruption of the oyster immune system. Kuchel et al. (2010) found that mechanical agitation, hypo-saline conditions, and exposure to the air have substantial effects on the immune system of Akoya pearl oysters (*P. imbricata)*. All three stressors led to significant decreases in phagocytic activity and phenoloxidase activity (Kuchel et al., 2010).

At the same time that these data provided a clear link between the immune system of oysters and environmental stress, other studies have shown that stress increases susceptibility to disease (Hegaret et al., 2003; de Almeida et al., 2007). The collapse of the black abalone industry in California due to withered foot syndrome has been linked to a combination of stressors, including anthropogenic pollution and increased water temperature (Davis et al., 1992), whilst Butt et al. (2006) found that low salinity significantly inhibits immunological activity in Sydney rock oysters immediately prior to severe QX disease outbreaks. Similarly, high temperatures seem to play a role in the infection of *C. virginica* by herpes-like viruses (Farley et al., 1972). Baculovirus infections in the pink shrimp (*Penaeus duorarum*) have also been linked to overcrowding (Couch, 1974a, 1974b), and decreased water temperature inhibits inflammatory responses in *P. maxima*, increasing infection by *Vibrio harveyi* and *Psuedomonas putreficiens* (Dybdahl and Pass, 1985, Pass et al., 1987). Bacterial infections are also thought to be exacerbated by stress in both *P. fucata* (Subhash and Lipton, 2010) and *P. maxima* larvae (Humphrey and Norton, 2005), whilst extreme water temperatures have been associated with decreased growth and survival of *P. maxima* and *P. fucata* spat (Mills, 2002; Pouvreau and Prasil, 2001; Tomaru et al., 2002).

Despite information on some cellular responses, little is known about the immune and other physiological systems of oysters. This is particularly true of pearl oysters. Current methods for the analysis of pearl oyster health are limited to histology. Abnormalities observed by histology are investigated by electron microscopy, but currently there are no molecular methods available to assess pearl oyster health. It is clear that rapid, cheap generic methods of detecting oysters in poor health are required.

The lack of available data means that molecular markers of pearl oyster health that can be used in rapid diagnostic tests will only be found efficiently using new holistic analytical tools, such as DNA microarrays. DNA microarrays display thousands of genes (cDNAs) from a particular species in a microdot format that can be screened to determine which of those genes are active under particular environmental conditions. This provides a comprehensive snapshot of an organism's physiological status. The sensitivity of microarrays means that animals subjected to different stressors can be compared to identify distinct gene expression signatures associated with particular stressors. This gives microarrays a high throughput discriminatory capacity previously unavailable in monitoring animal health, and provides the perfect vehicle for identifying molecular markers that can be used in rapid diagnostic tests.

Microarrays, and their allied technologies (proteomics and subtractive transcriptomics) have already been used in other bivalve species to identify biomarkers of stressors, such as infection and chemical pollution (Apraiz et al., 2006.; Jenny, et al., 2007; Boutet, et al., 2004, Tanguy et al., 2005; Gueguen et al., 2003; Simonian et al., 2009a,b; Thompson et al., 2011a,b). All of these studies have shown that numerous genes respond to stress, that there are some generic stress-response genes that can be used to gauge the overall health of the animals, whilst other response genes are specific to different stressors. The existing data also suggest that gene expression analysis can provide early warning of harm, and can detect stress with far greater

sensitivity than existing diagnostic systems. For instance, Apraiz et al. (2006) found that the effects of chemical pollution on mussels could be detected earlier and at lower doses using gene expression analysis compared to traditional ecotoxicological tests. Similarly, A/Prof. Raftos' group has used proteomics to identify changes in the expression of more than 50 proteins in the hemocytes of oysters exposed to heavy metal contamination, both in the laboratory and in the field (Thompson et al., 2011a,b).

This project (FRDC 2008/030) uses DNA microarray technology to detect abnormal levels of stress response genes in pearl oysters as a way of identifying factors that might contribute to disease susceptibility. Our long term goal is to use these genes to develop new, rapid diagnostic tests for stressed pearl oysters. The requirement for such rapid diagnostic tests for the detection of stress and disease susceptibility is well acknowledged. The spread of disease could be limited if there were tests available to differentiate sick/stressed animals from healthy ones. Such assays would also have applications in routine translocation testing and the assessment of general oyster health. Translocation samples are treated as high priority and current methods report results after several days. With rapid tests developed for genes discovered by DNA microarray analysis it may be possible to report results within 24 hours. The attraction of molecular stress-response markers is that their expression levels change dramatically during times of stress. This can act as an indicator of physiological stress that may be associated with disease susceptibility. Hence, our main goal was to use the *P. maxima* microarray to identify key stress-response genes that could be used to develop a new generation of rapid, inexpensive tests of environmental stress and overall oyster health.

In this project, we developed a DNA microarray that can be used to develop a new generation of generic tests for adverse health in pearl oysters. By the end of the project, we were able to identify the key stress-response genes in *P. maxima*. These key genes represent the basis to develop a new series of rapid, inexpensive diagnostic tests for a range of factors that affect *P. maxima* production, possibly including OOD. We envisage that these new tests could be licensed to veterinary pathology laboratories, or even undertaken on-farm.

The expertise of A/Prof. Raftos and Dr. Nair has been used to develop the cDNA library and DNA microarray, and this technology will be transferred to Dr. Crockford for implementation of the technology in WA. Dr. Crockford has experience in many other molecular biology techniques and has produced significant results to date in the investigation of OOD. By the completion of this project, the Department of Fisheries WA had received considerable training in microarray technology that will benefit the pearl oyster industry and also enhance, at a national level, the capabilities of Australian scientists in using advanced molecular techniques.

5.0 Objectives

1. Collect RNA samples from “stressed” and “unaffected” oysters for cDNA library construction
2. Prepare oyster RNA for microarray comparisons
3. Fabricate pearl oyster microarrays
4. Undertake trial microarray comparisons (to test the performance of microarrays)
5. Identify stress response genes by testing samples from initial stress experiments using the microarray to identify potential stress response genes
6. Sequence DNA to identify and characterise differentially expressed genes
7. Validate microarray data using real time PCR
8. Test field samples using validated markers to confirm the utility of the technology

6.0 Methods

6.1 Collection of RNA samples from "stressed" oysters for cDNA library construction

A/Prof. Raftos and Dr Nair (Macquarie University) met with Dr Crockford (Fisheries WA) in Sydney to discuss collaboration over the project in July 2008. A detailed plan was developed for initial stress exposure experiments to collect mRNA samples for library construction and microarray development. Following industry consultations, a decision was made to change both the location and facility at which the experiments would be conducted. The original design envisaged holding pearl oysters in Perth. However, the offer by Paspaley Pearls to assist with experiments at a tropical facility in Darwin (Darwin Aquaculture Centre) that was already set up to keep *Pinctada maxima*, has algal cultures established, and industry expertise readily available, was accepted (Figure 1). This resulted in a delay in beginning these experiments to late January 2009. However, the delay has proven very worthwhile in terms of the overall project. It allowed for the superior aquarium facilities at the Darwin Aquaculture Centre to be used for stress experiments, meaning that individual stressors were more easily isolated from other environmental conditions.

Figure 1. The Darwin Aquaculture Centre situated on Channel Island in Darwin Harbour some 50km from the city. The centre has specific areas dedicated to algal food production and environmental control work. A bank of self-cleaning sand filters maintains a supply of suitable sea water to maintain oysters in conditions akin to the field (http://www.nt.gov.au/d/Fisheries/index.cfm?header=Darwin%20Aquaculture%20Centre).

Two rounds of stress experiments were conducted by Dr. Crockford at the Darwin Aquaculture Centre with the assistance of Paspaley Pearls. In these experiments, replicated groups of oysters were exposed to five different forms of stress that are commonly associated with farming practices (in addition to non exposed controls). These were: altered salinity, mechanical agitation, “clipped open”, air exposure and starvation. In the first round of experiments, only hemolymph was collected from oyster exposed to these conditions. However, in the second round of experiments, samples for RNA extraction were taken from hemolymph, mantle and gills over a range of time points during the stress exposures. These samples were prepared in a variety of reagents to ensure the recovery of high quality mRNA. Analyses prior to library construction indicated that samples of gill tissue harvested and stored in RNAlater provided the most efficient yields of mRNA. Dr. Crockford reported that the stress experiments were extremely successful, and appropriate mRNA samples were collected for all treatments. These samples were shipped to Macquarie University for further processing.

At Macquarie University, preliminary experiments were conducted to optimise library construction protocols. Sufficient samples of hemolymph were collected from all of the different treatments in the first set of stress experiments to allow a first round of cDNA library construction. Having constructed that cDNA library, approximately 1000 clones were screened by PCR. The majority of clones had small (200-300bp) inserts that were not useful for microarray printing, suggesting that library construction had failed (Figure 2).

Figure 2. The results of a colony PCR screening of a random selection of clones from the original cDNA library. DNA sequencing of the amplicons indicated that they were the "stuffer" fragment contained in the original vector, and not oysters cDNAs.

As a result, the chief investigators (Dr Jones, Dr. Crockford, Dr. Nair and A/Prof Raftos, along with Dr Mark Crane, Director of the FRDC's Aquatic Animal Health Subprogram) met during the AAHS meeting in Cairns to devise a new strategy for sample collection and library construction. The group identified two problems with the original experimental protocol:

a. The yield of mRNA from the original hemolymph samples was low. From approximately 60 hemolymph samples we only obtained 2.8µg of cDNA. This was below the optimal 5µg of cDNA recommended for the Invitrogen Superscript Plasmid System for cDNA synthesis and cloning that we used for the original library construction. This yield of mRNA from pearl oyster samples is far below our experience with other oyster species (Pacific and Sydney rock oysters), and probably reflects the far lower number of hemocytes in the hemolymph of pearl oysters compared to other species.

b. Many of the clones contained "stuffer fragment" rather than oyster cDNA inserts. Stuffer fragment is a 200-300bp synthetic DNA fragment inserted into the multiple cloning site of the cDNA library plasmids during the production of the plasmids. Even though the technical specifications of the cDNA synthesis kit suggested that stuffer fragment was removed from the plasmids before shipment, there was obviously residual stuffer fragment contamination of the plasmids that we purchased. Combined with the low starting amount of cDNA that we obtained from the hemolymph samples, this meant that the insertion of oyster cDNAs into the plasmids was inefficient.

To rectify these problems we:

a. Decided to undertake a second round of cDNA library construction using mRNA purified from gill tissue rather than hemolymph. The literature suggests that gill tissue is a superior source of mRNA when compared to hemocytes, and that much of the mRNA in gill is derived from hemocytes that reside in the gill tissue. To confirm that gills are a superior source of mRNA, we undertook preliminary experiments using Akoya oysters, which can be obtained in NSW. In these experiments, we obtained 1.2 µg of mRNA from the gills of just 4 oysters, as opposed to 2.8µg of cDNA from the hemolmyph of approximately 60 oysters. This means that we were able to purify more than enough *P. maxima* mRNA from gill tissue for at least one more round of cDNA library construction.

b. Modified the cDNA library construction protocol by using a different vector system (pGEM-T Easy) to eliminate problems with contaminating DNA (stuffer fragment) being ligated.

Having addressed these technical issues, a second round of stress experiments and cDNA library construction was performed. In late June, 2009, Dr. Crockford conducted this second set of stress experiments at the Darwin Aquaculture Centre with the help of Paspaley Pearls. Dr. Crockford collected a large number of gill and mantle tissue samples. A total of 159 tissue samples of gill and mantle were collected from stressed animals and placed separately into Eppendorf tubes containing RNAlater, 80% ethanol or Trizol. In other words, per animal, there were 3 samples of gill and 3 samples of mantle, for approximately 90 animals in total. Stress experiments consisted of 5 treatments, including starvation, clipped open, air exposure, salinity and agitation. No animals died during the experiments, except when being sampled. Approximately half of these samples were then shipped to Macquarie University for mRNA isolation and cDNA library construction.

Preparation of oyster RNA for microarray construction

mRNA isolation and cDNA library construction was conducted by Sham Nair, Adam Wilkins and Druime Nolan, who worked at Macquarie University during November, 2009. mRNA from gill tissue stored in either RNAlater or Trizol was isolated from total RNA using polyATract mRNA isolation system (Promgea, Wisconsin, USA). A total of 3.8 µg of mRNA was isolated from all tissue samples. cDNA synthesis yielded approximately 2.7 µg of cDNA. Prior to library construction, cDNAs were size fractionated to remove small RNA (which often co-purifies with mRNAs) and truncated cDNAs. Most of the large cDNAs eluted in a single high concentration fraction. The purified cDNAs were then used for library construction. They were ligated into pGEM-T Easy vector, and transformed into DH10B cells by either electroporation or chemical transformation. An aliquot of the transformed bacteria was plated out to determine whether transformation had been successful and to calculate the plating density of clones in the transformed samples. This analysis suggested that the transformed samples would yield approximately 50-100 clones per plate, which in our experience is the perfect density for plating out the library so that individual clones could be PCR screened to confirm that they have useful inserts. PCR screening showed that a very high proportion (>60%) of clones had large (>250bp) inserts and that there was a broad range of insert sizes among the clones (see Figure 3). This indicated that large numbers of unique cDNAs had been cloned. We then sequenced 10 randomly selected clones from the library to confirm that the cDNAs were from pearl oysters. BLAST searches of sequence from those 10 clones, showed that four of the clones were unequivocally from *Pinctada* species. The remainder did not yield significant hits against NCBI databases, suggesting that they were *Pinctada* genes for which there are no sequences in the available databases.

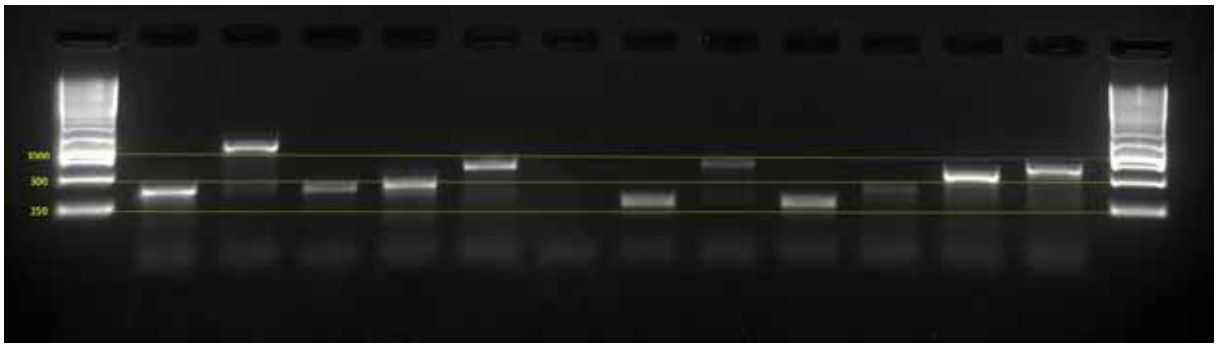

Figure 3. PCR products from 12 randomly selected clones from the cDNA library used for microarray construction. Note that the vast majority of the products have different sized, large (300bp – 1,500bp) inserts. The yields of the total RNA from this second experiment ranged from 35-67 µg. mRNA was purified from these samples and used to construct a cDNA library containing clones from all of the stress treatments and non-stressed controls.

Fabrication of pearl oyster microarrays

After the successful development of the pearl oyster cDNA library, the cloned inserts were amplified by PCR (using M13 forward and reverse primers). The cDNA library contained pooled cDNAs from all of the "stress" treatments plus controls to ensure that genes that were up- and down- regulated in the stress treatments were incorporated into the library. Amplicons were purified using the SV Miniprep kit (Promega) according to the manufacturer's protocols. The purified amplicons were quantified and then transferred to 384-well plates. The DNA solutions were air-dried and the plates were air freighted to the Adelaide Microarray Centre for array fabrication. The amplicons were arrayed onto glass slides in duplicate. To ensure adequate technical replication, each slide had two identical sectors, each sector incorporated approximately 3,000 clones from the cDNA library with each clone printed onto 2 spots in each sector (Figure 4). Two grids were spotted onto each slide and a total of 40 slides were printed. The slides were subjected to quality control assays, after which they were shipped to Macquarie University.

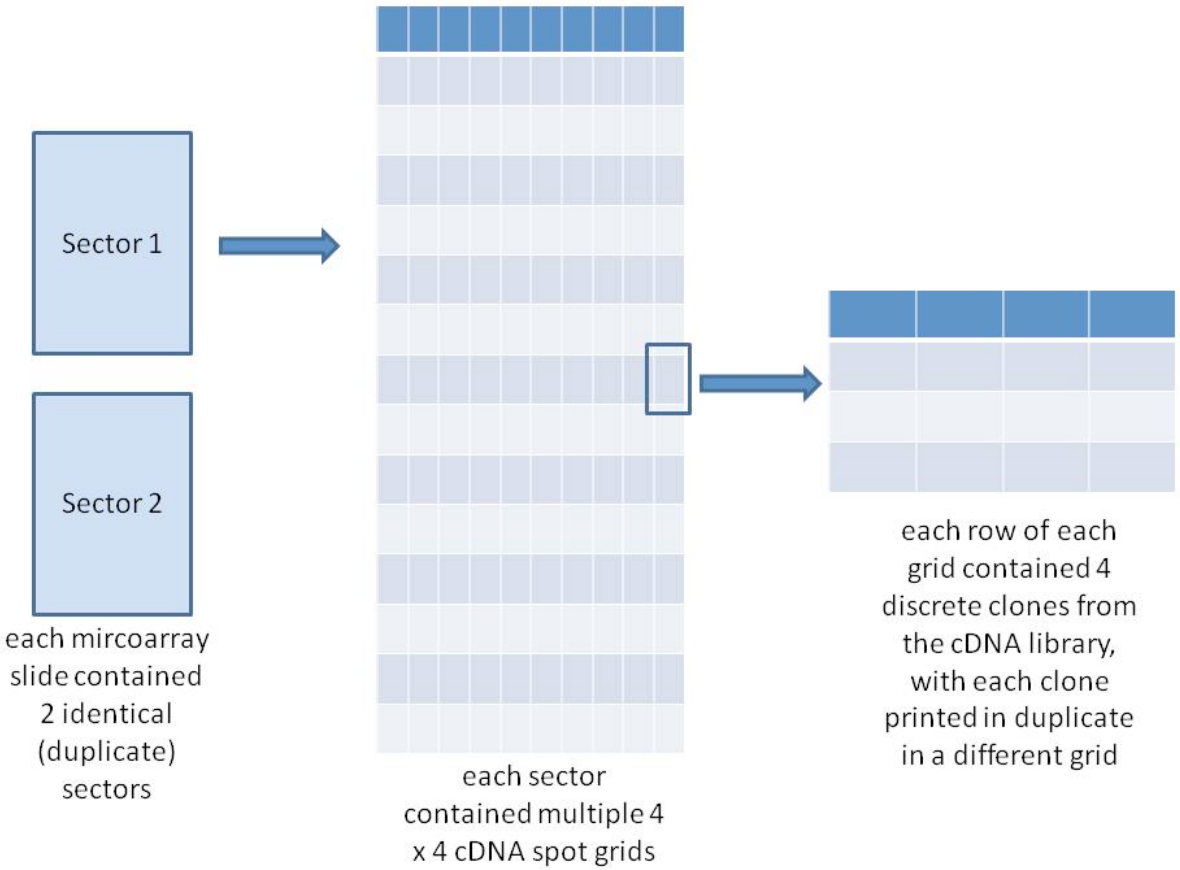

Figure 4. Layout of cDNA microarray slides. Each slide incorporated two identical sectors, with each sector containing more than 3,000 unique clones from the cDNA library.

Trial microarray comparison

Microarray comparisons were performed by Dr Nair, Dr Burger and Adam Wilkins at Macquarie University. For the initial microarray comparisons, a common reference (control) design was adopted. This is shown in Figure 5. RNA from each treated oyster was compared with RNA from control oysters via competitive hybridisations using commonly established protocols. Each comparison was undertaken in duplicate. The samples used to prepare probes for microarray analysis were same as those for the cDNA library from which the microarray was constructed. For the treatment (stressed oyster) samples, equal proportions of RNA from the various time points were combined and reverse transcribed into cDNA using the SuperScript Plus Indirect cDNA Labeling System (Invitrogen). The cDNAs were coupled to Alexa Fluor 555 or Alexa Fluor 647 fluorescent dye, and a dye swap strategy was adopted (see Figure 5). The fluorescently-labelled cDNAs were allowed to hybridise overnight to targets on the microarrays at 65^{o}C. After washing, the slides were washed, dried and stored in the dark until they were scanned using the Genepix4000B microarray scanner. The spot intensity files (GPR files) were then analysed using the Genepix 6 and BrBArray software packages to identify differentially-expressed spots.

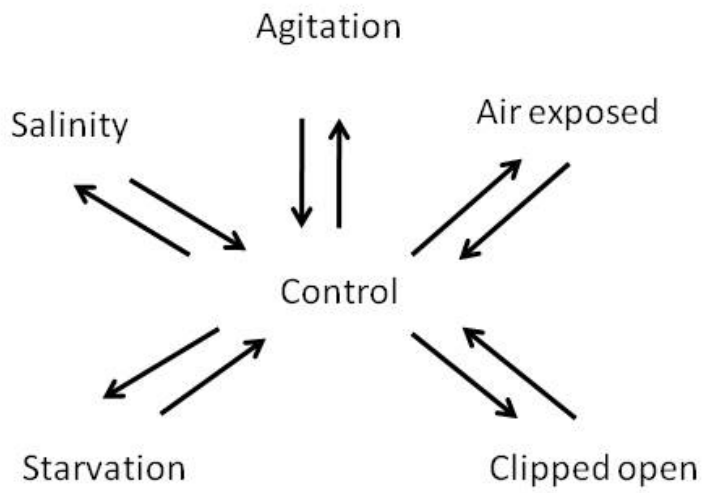

Figure 5. Microarray comparison strategy. RNA from control oysters was used as the common reference. For each comparison, a total of four slides were used. That is; Alexa Fluor 555-stress treatment vs Alexa Fluor 647-control (two slides) and Alexa Fluor 647- stress treatment vs Alexa Fluor 555-control (two slides). Thus, a total of 20 comparisons were made covering all 5 stress treatments.

An example of the data analysis with a single stress sample (air exposure) that was used to ensure the viability of the hybridisation protocol is shown in Figure 6. The analysis of this sample indicated that a total of 152 spots were differentially expressed between air-exposed and control oysters. This consisted of 12 spots that were significantly over-expressed in control oysters and 140 spots that were over-expressed in air-exposed oysters.

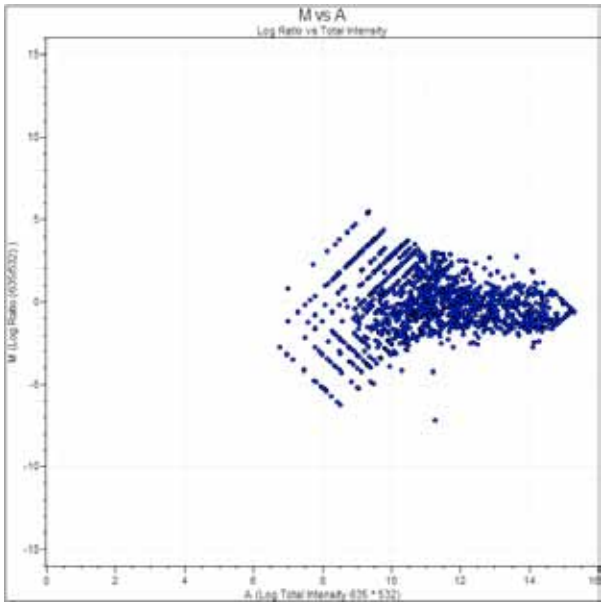

Figure 6. M-A plot of air-exposed oyster RNA vs control oyster RNA. The intensities of replicate spots and grids were averaged and normalised (whole slide normalisation). Statistical analysis of the data indicated that spots with a fold change (i.e. log2 (I657/I555), where I = normalised spot intensity) of ≥ 2.5 were differentially expressed.

Marker discovery: testing samples from initial stress experiments using the microarray to identify potential stress response genes

Once the viability of the hybridisation protocol had been confirmed using a single stress vs. control comparison, a complete microarray analysis incorporating all of the different stressors (salinity, mechanical agitation, "clipped open", air exposure and starvation) was undertaken by Dr Nair and Mr Wilkins using the protocol described in section 5.4 (above). cDNA probes for each treatment comparison incorporated mRNA from between 15 and 26 different oysters sampled at a range of time points during each of the "stress" treatments (between 5 and 9 oysters per time point). Each oyster and each time point contributed equally in terms of RNA content of the final pooled sample. In total, there were five two-way comparisons made between different "stress" conditions and controls, as shown in Table 1.

Table 1. Nomenclature for microarray comparisons of stressors vs. non-stressed controls

Comparison #	Stressor
1	air exposure
2	mechanical agitation
3	"clipped open"
4	low salinity
5	starvation

For each comparison, heat maps of hybridisation (fluorescence) intensity were generated, as were numeric data on relative fluorescence intensities for each spot on the array. An example of a heat map for a representative region of the array hybridised with samples from comparison #4 (low salinity stress) is shown in Figure 7. It shows a clear delineation in hybridisation intensity between different clone spots within the array.

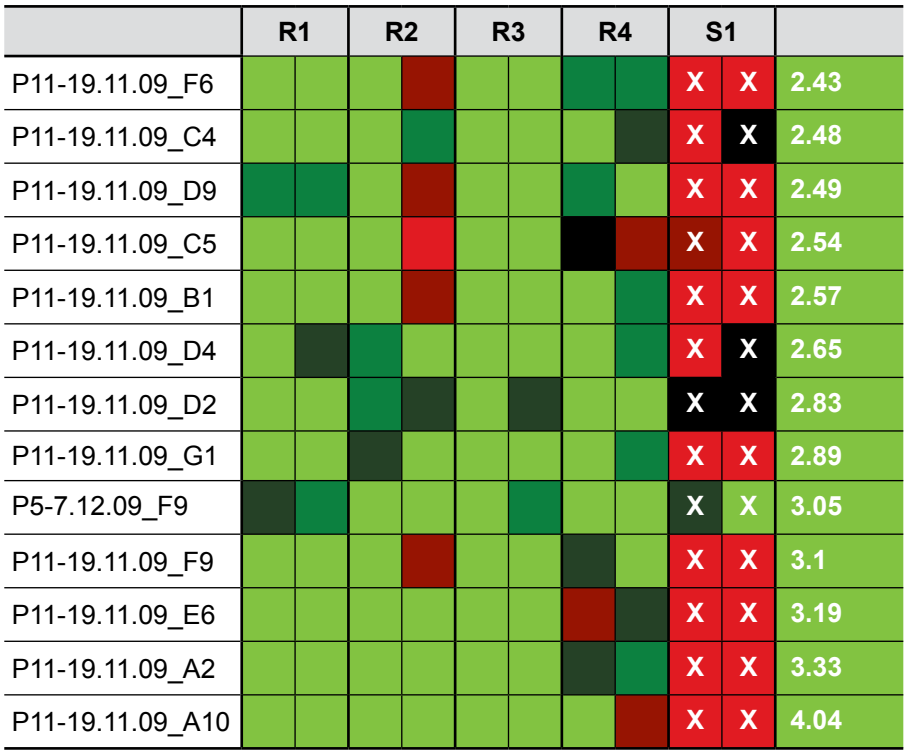

Figure 7. Heat map for a small region of a microarray hybridised with samples from Comparison 4 (low salinity stress vs. controls). Each box represents the data from a single microarray sector for each clone on the array. Each of the comparisons is represented by 4 boxes, each of which represents one of the 4 technical replicates used for each comparison (R1-4). The two boxes on the far right represent dye swaps (S1). Numbers on the left represent the clone # from the cDNA library. Numbers on the right are normalised fold change relative to controls. Colours show the relative level of expression in the treatment vs. controls. The brightest green represents the highest fold change, and the darkest red represents the lowest fold change in treatments relative to controls. This heat map shows only clones that differed between treatment and controls by folds of ≥2.

Heat maps for the complete analysis with all 5 comparisons are shown Appendix 1. When mean fold differences for fluorescence intensities between treatments and controls were set at ≥2.0, 448 clones were found to be significantly up or down regulated in stress treatments relative to controls (Figure 8). Among these 448 clones, 30 were significantly ($p<0.05$) down-regulated relative to controls, with the remainder up regulated. Mean fold differences in fluorescence intensity ranged from -4.05 (down-regulation) to +5.0 (up-regulation). Air exposure (Comparison 1) had the greatest effect on gene expression, with 126 differentially regulated clones relative to controls, whilst the low salinity treatment (comparison 4) had the least effect (57 clones). Many clones were differentially regulated by more than one of the stress treatments, meaning that there were 166 differentially regulated clones across all treatments. This represents 7% of all clones printed onto the array.

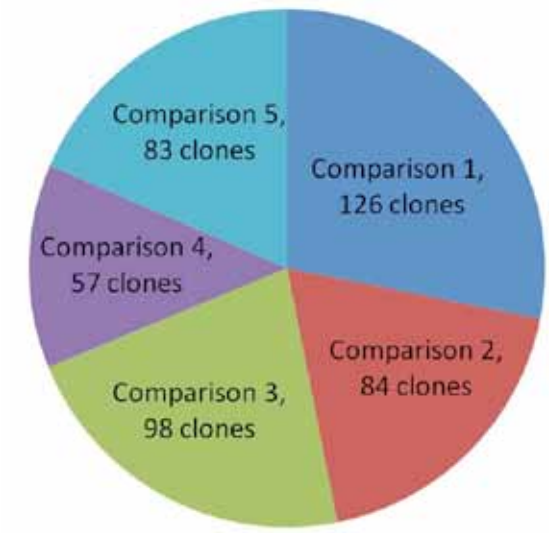

Figure 8. Numbers of clones that were differentially regulated (up or down regulated) by stress treatments (comparisons 1-5) relative to controls.

The data shown in Figure 8 suggest that there is a clear demarcation between the relative impacts of different environmental stressors. Even though a number of clones were differentially expressed in all of the stress treatments, others were specific to individual stressors. We took this into account when selecting clone sequences for more quantitative analyses to identify stress associated molecular biomarkers.

Multivariate analysis of microarray data

Multivariate clustering analysis of the microarray data, in which expression data for all differentially expressed clones was analysed simultaneously (Figure 9), shows that clones responding to the five different stress treatments formed five major primary clusters (A-E in Figure 9). It is possible that these clusters represent distinct suites of genes that are under the control of discrete gene regulatory networks. The largest and most significant cluster is D, which incorporates almost half of the differentially expressed clones. The majority of these clones were affected by exposure to air, and many of them were affected only by air exposure. This supports one of our major conclusions (see section 7, Conclusions and Recommendations), which is that exposure to air is the most significant stressor applied to oysters. Clusters A and B are also of interest, because they seem to reflect networks of genes that are affected by a broad range of stressors.

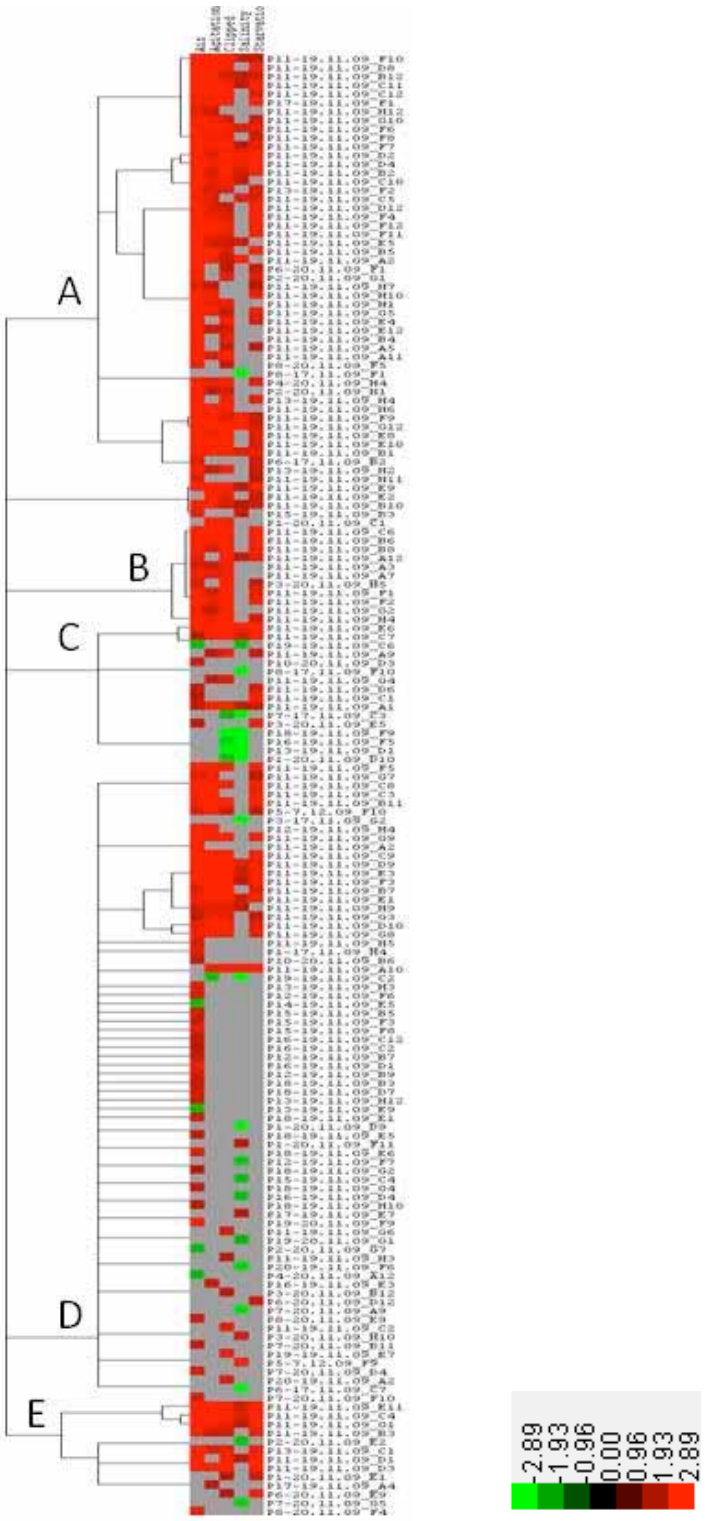

Figure 9. Hierarchical clustering of the differential gene expression data from microarray analyses. The data show the relative effects of the 5 different stressors on the expression of differentially expressed clones on the microarray. The coloured columns from left to right are data for the air exposure, agitation, salinity, “clipped” and starvation treatments. Shades of red indicate up-regulation, green represents down-regulation (see legend below for relative fold changes). The changes in intensity values (fold changes) were analysed using Cluster 3.0 (Eisen et a., 1998). The expression data was centrally weighted and a Spearman Rank Correlation Coefficient similarity matrix was used to perform the hierarchical clustering. The data was visualised using JavaTreeView (Saldanha 2004). The letters A-E represent major primary clusters.

6.2 CDNA sequencing to identify and characterise differentially expressed genes

cDNA sequencing and BLAST database searches

Of the 488 differentially expressed clones (at the ≥2 fold difference cut off), 192 were selected for sequencing. These included a mix of clones that were differentially expressed in more than one treatment, clones that were differentially expressed in just one treatment, and a number of clones that did not differ in expression between treatments and controls (for subsequent use as potential housekeeping genes in later real time PCR analyses). Preference was given to clones that yielded the highest fold differences in expression between stress treatments and controls. The full results of BLAST searches based on the nucleotide sequences of these clones are shown in Appendix 3.

After vector sequences were removed from the sequence list, 21% of the sequences did not return e-values in BLAST searches that warranted a robust identification in the sequence databases searched (NCBI non redundant sequence database; Figure 10). 15% of sequences matched known sequences from *Pinctada* (pearl oyster) species. The matches to *Pinctada* sequences were generally supported by extremely low e-values, suggesting high homology. The remaining 64% of sequences were related to species other than *Pinctada*.

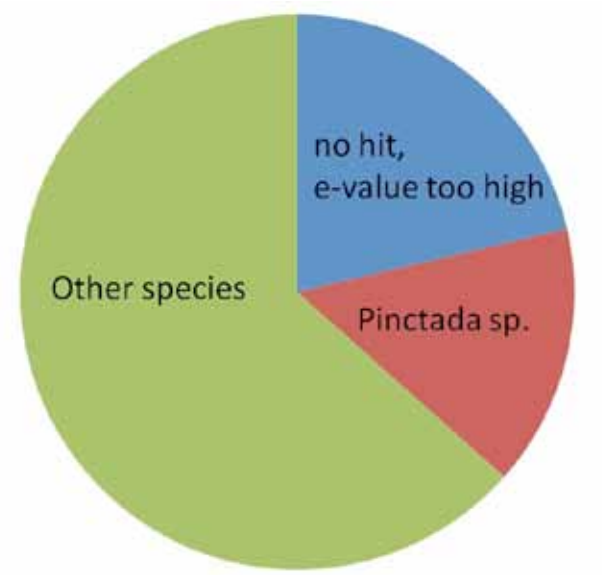

Figure 10. Phylogenetic distribution of BLAST matches for sequences from the cDNA library.

The sequence data also reveal sequence redundancy in the cDNA library (i.e. more than one clone encoded that same cDNA sequence). This was expected because cDNA was not normalised prior to microarray fabrication. As a result, 54% of sequences overlapped between more than one of the clones. Overall, 68 unique cDNA sequences were identified among the 192 clones sequenced. Clones homologous to know *Pinctada* nucleotide, EST or protein sequences comprised 18% of the unique sequences identified. There was also redundancy between unique cDNA clone sequences and the genes that they matched in BLAST searches. This meant that 37 different genes were identified among the original 192 sequences.

Gene ontologies

Gene ontology analyses based on functional annotations of the homologous genes (Figure 11), indicated that genes involved in translation (including both ribosomal proteins and ribosomal RNAs) were the most common (33%), whilst genes associated with microsatellite markers represented 18% of the identified sequences. 31% of the genes had no known function.

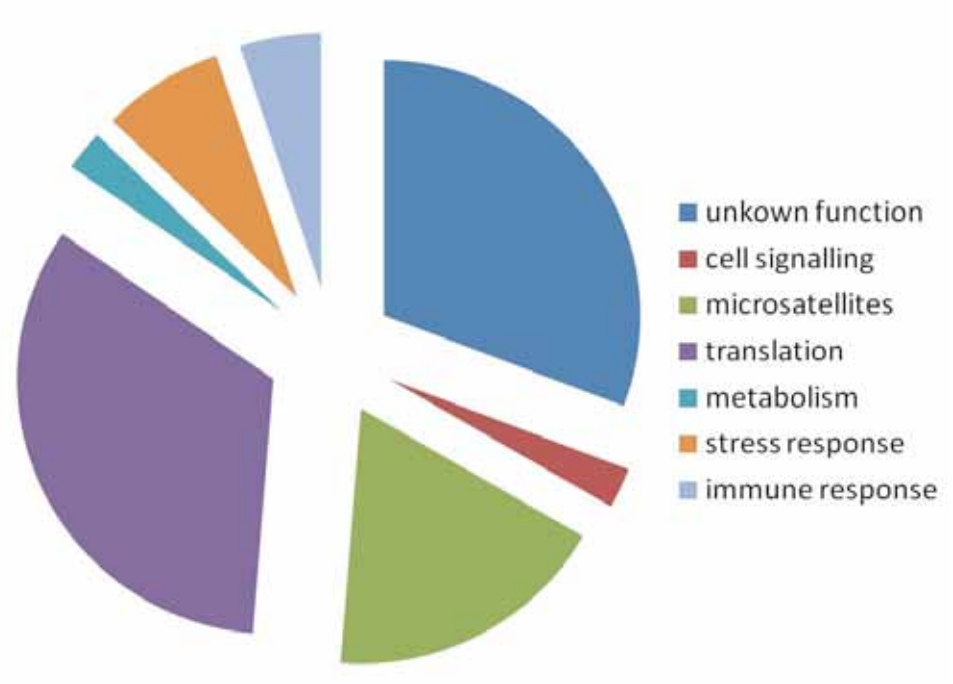

Figure 11. Functional ontologies for the genes identified by cDNA sequencing.

These gene annotations fit our current understanding of genomic and molecular processes in bivalve molluscs. Other studies of environmental stress in oyster species (including a substantial body of evidence from our laboratory) suggest that genes involved in translation (primarily genes encoding ribosomal proteins and RNAs) make up a substantial component of the functional systems that are differentially expressed in response to environmental stress (Thompson et al., 2011a,b).

The proportion of translation-associated genes that we identified in the current analysis is also likely to be biased because of the limited sequence resources for *Pinctada* species that are available in publically accessible sequence databases. As of April 2011, NCBI held approximately 1500 complete nucleotide sequences for *Pinctada*, the vast majority of which are for ribosomal genes and DNA microsatellites. There is also a very substantial EST database for *Pinctada* species, but these ESTs are largely unannotated with no information on their genetic homologies or function. Given these limitations, it is not surprising that many of the genes that we identified encode ribosomal proteins and ribosomal RNAs.

The limited availability of annotated *Pinctada* sequences also explains the disproportionately high number of matches to microsatellite sequences in the NCBI database. Microsatellites are regions of repetitive DNA that often flank expressible, encoded genes. Clearly, substantial effort has been placed in the past on population genetic analyses of pearl oysters, which has necessitated the identification and publication of microsatellite sequences for *Pinctada* species. This focus means that there is an overabundance of sequences in the available databases that are designated as “microsatellite” sequences, and so we returned a substantial number of hits to

these purported "microsatellite" gene sequences. However, most of these matches in the NCBI database incorporate substantial regions flanking microsatellite repeats that probably encode expressed genes that have yet to be characterised or annotated. For this reason, we could have included the "microsatellite" sequences that we identified in the "unknown function" category.

Despite these observations on the paucity of existing sequence data for *Pinctada* species, in terms of the current project (which aims to identify effective biomarkers of environmental stress), the nature and function of the sequences that we have identified is largely irrelevant. The most pressing criterion to meet our specific goal is that the sequences were significantly up or down regulated in stress treatments relative to controls. The precise function of those gene sequences is a matter of biological interest, and will help to explain the cellular processes that respond to environmental stress. This is an important consideration if we are to understand the biology of pearl oyster aquaculture. But in the context of identifying relevant markers of "stress", the precise function of biological markers is not consequential. Hence, in the sections below, we used a range of sequences, even those with unknown homology or function, as potential biomarkers of environmental stress.

6.3 Real time PCR validation of Microarray Data

Real time PCR strategy

The microarray analysis described above is only semi-quantitative because it does not include biological replicates of each treatment and control. To confirm that the microarray analyses represent a robust quantification of differences in gene expression between treatments and controls, a fully replicated analysis of gene expression was undertaken using quantitative real time reverse transcription PCR (qRT-PCR) on a subset of the differentially expressed clones identified in the microarray analysis.

The current consensus is that, to confirm the veracity of expression data from microarrays, differential expression needs to be confirmed by qRT-PCR for 5% to 10% of the differentially expressed genes. Hence, 18 sequences (from among the 68 unique sequences, and 37 genes, described in Section 6, above) were selected for PCR primer design to validate the quantification of results in the microarray analysis by qRT-PCR. Five sequences were selected because they represented clones that did not differ significantly in expression between stress treatments and controls. These sequences were selected as potential housekeepers (reference genes) for subsequent qRT-PCR analyses. Primers for an additional housekeeping gene (cytochrome c oxidase subunit I, CO1) were designed from known sequences for *Pinctada* species. The remaining 13 sequences comprised a range of clones that exhibited significant differential expression in comparisons between stress treatments and controls. These sequences represent potentially robust biomarkers of environmental stress. Primer sequences for these "stress response" clones are shown in Table 2. They were selected by balancing a number of criteria, which included:

- a preference for clones with obvious homology to known *Pinctada* genes
- high fold differences in expression between stress treatments and controls
- whether or not they were differentially expressed in all, a number, or just one of the stress treatments (to give a spread of generic and stress-specific markers), and,
- whether primers could be designed to provide a single annealing temperature for qRT-PCR

Table 2. Clones selected for primer construction, showing the clone designation, the stress treatments (1-5) in which the clones were differentially expressed relative to controls (x indicates significant differential expression), the matching sequences found in BLAST searches, forward and reverse primer sequences, and the expected length of PCR products.

Unique Clones	1	2	3	4	5	Best match	Forward primer (5'→3')	Reverse primer (5'→3')	Estimated product size
P10-20.11.09_B6	x					*Pinctada maxima* microsatellite M412 sequence	TCAGTGCACTTACCCAATCG	CATTTACCGCCTGCCATAGT	155
P11-19.11.09_B12	x	x	x	x	x	*Pinctada maxima* mitochondrial gene for 16S rRNA	GGGTAACAGGACGCTTTTGA	ACTGGCTTACGCCGTTCTAA	158
P11-19.11.09_E5	x	x	x	x		*Pinctada maxima* PmaxCL270Contig1	CAGAGTGGCCATTTGGTGTA	ACCAGCAGAGCACAGACCTT	156
P11-19.11.09_F7	x	x	x	x	x	No decent hits (e-vals too high)	GGTAGAAGGACCCCGAACAT	TCCAAATCTGATGCAGGAAA	192
P11-19.11.09_H1	x	x	x			No decent hits (e-vals too high)	GCCGGATATTTACCTCACGA	ACCAGCATCACTTCCAGGTC	156
Unique Clones	**1**	**2**	**3**	**4**	**5**	**Best match**	**Forward primer**	**Reverse primer**	**Product size**
P11-19.11.09_H12	x	x				No decent hits (e-vals too high)	AACAACTTCCCAGCCGTAAA	TCCAAATAATGGCAGGGATT	152
P11-19.11.09_H5	x					*Pinctada maxima* mitochondrial gene for 12S rRNA	TCCAGACAGGGGAACTTGAC	TTTCCCCCAAACCATACAAA	150
P1-20.11.09_D9				x		*Pinctada martensi* clone 03-46 tissue-type heart microsatellite sequence	GAGGGGTATCAACCCCAAGT	GAAAATGACCACACCCTTTTTC	159
P12-19.11.09_H4	x	x				No decent hits (e-vals too high)	ACGCCAAGCCCTTTTATTTT	TAGCCACCCTCTGCACTTTT	289
P19-19.11.09_E7			x			No decent hits (e-vals too high)	TTGGACTTCATCCGATCTCC	AGAACTCTCGCCCAAGATGA	292
P6-20.11.09_E9					x	*Dracaena cambodiana* clone DC522 microsatellite sequence	CTGGCTGGTGGTTTTGTTTT	GCCAGCCTTATCGCTGTTAG	232
Unique Clones	**1**	**2**	**3**	**4**	**5**	**Best match**	**Forward primer**	**Reverse primer**	**Product size**
P7-17.11.09_C3			x	x		*Bombyx mori* BNGR-A32 mRNA for neuropeptide receptor A32, complete cds	TGCTTTTGTTGACGTTTTGG	CTCAAGCTATGCATCCAACG	209

Reverse phase purified primers were constructed for all of these clones and tested by semi-quantitative (colony) PCR to confirm that they could effectively amplify the corresponding target gene from the cDNA library. Representative results of that colony PCR are shown in Figure 12. Most primer sets effectively amplified products of the appropriate sizes in colony PCRs of clones from the cDNA library against which the primers were designed. None of these PCR products incorporated more than one band, suggesting that the primer pairs were highly specific. Four of the primer pairs shown in Table 2 did not yield products in colony PCR. However, two of these primer pairs did yield robust amplification in qRT-PCR (see below). This is probably due to the different DNA polymerases used in the two forms of PCR.

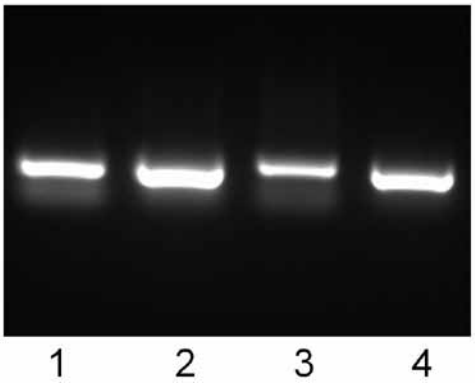

Figure 12. Representative examples of PCR products generated by colony PCR of clones from the cDNA library amplified with four different sets of primers from the list shown in Table 2.

qRT-PCR was performed using an Eppendorf Mastercycler Realplex thermal cycler with KAPA qPCR SYBR green PCR kits (Geneworks) according to the manufacturers' instructions. Reaction mixtures consisted of 10µl 2x KAPA SYBR green mastermix, 0.3µmol of each forward and reverse primer, 2µl of template (1x, 5x, 25x and 125x dilutions) and water adjusted to 20µl. Primer pairs included seven of the differentially expressed genes identified in the microarray analysis, and CO1 as a housekeeping (reference) gene. Templates were reverse transcribed RNAs from each of the stress treatments (3 replicates of pooled RNA from a range of time points for each treatment and non-stressed controls). Reaction mixtures were loaded into 96well PCR plates that were subjected to the following PCR cycling conditions: 15 min at 95˚C, 35 cycles of 15s at 94˚C, 30s at 60˚C and 30s at 72˚C. The design of a typical 96-well plate is shown in Figure 4. Four dilutions of each template were included in order to calculate the amplification efficiency for that sample and primer pair, and triplicates of each sample were analysed for each primer pair. Relative expression of the seven target genes was then calculated by the ΔΔCt method (Table 3).

Table 3. Relative expression of transcripts that were amplified by the primers indicated (A2-H5). The values in the table indicate fold change (ΔΔCt), relative to the housekeeper, COI. NA indicates that data is not available.

	A2	B8	E8	F9	H2	G11	H5
Agitation	0.10	0.42	0.55	0.00	0.53	0.03	0.00
Air	NA	1.63	0.06	NA	0.67	0.00	3.72
Clipped	NA	0.86	0.16	3.81	NA	NA	NA
Salinity	NA	2.46	0.74	10.47	NA	NA	NA
Starvation	NA	1.10	NA	NA	NA	NA	NA

6.4 Testing field samples using validated markers

Collection of RNA samples from pearl oysters after they had been subjected to routine de-fouling

Field trials were undertaken by Dr. Crockford (Fisheries WA) in May 2011 at the Kimberley Marine Research Station, Cygnet Bay, WA, with the assistance of Cygnet Bay Pearls. Groups of 36 oysters were subjected to two conditions. The oysters were approximately 5 cm to 10 cm shell diameter, and sizes were similar in the two treatment groups. All oysters were unseeded.

One group of 36 oysters were removed from the water and subjected to routine de-fouling, which is a normal and relatively frequent part of the farming practice. The defouling process involves oysters being removed from the water, pressure sprayed with seawater to remove soft fouling, with calcareous fouling organisms removed manually with a chisel. These oysters are referred to as "treatment" or "stressed". The remaining 36 oysters were used as controls ("healthy"). They were not subjected to the de-fouling treatment.

The experiment followed a fully replicated design, such that each group (treatment or control) was divided into 3 replicates of 12 oysters, each from 3 different zones in Cygnet Bay (Table 4).

Table 4. Replication of the control and treatment groups in different zones in Cygnet Bay.

	Healthy (controls, no recent antifouling)	Stressed (treatment, recent antifouling)
Zone 1	12	12
Zone 2	12	12
Zone 3	12	12
Total	**36**	**36**

Controls (sampled 9.5.11 from 11.30 am) labelled as:

- A1-12 (zone 1)
- C1-12 (zone 2)
- E1-12 (zone 3)

Treated (cleaned midday 9.5.11, sampled from 6 pm) labelled as:

- B1-12 (zone 1)
- D1-12 (zone 2)
- F1-12 (zone 3)

Zone 1 = JVO2007 2007 Hatchery Hospital Section

Zone 2 = JVO2010 2010 Hatchery Shellbank

Zone 3 = JVO2008 2008 Spat B 30

Preparation of oyster RNA for analysis of field samples

Gill tissues from each oyster were harvested into RNAlater (in duplicate) and stored at 4ºC overnight. Samples were then frozen at -20ºC, before being transported on dry ice to Macquarie University for analysis. Protocols for mRNA purification and cDNA construction were identical to those described in Section 7.

Primer sets used for quantitative PCR analysis of field samples

Thirteen "stress response" genes identified by microarray analysis of samples from laboratory-based "stress" treatments were selected for the analysis of field trials. These included genes that showed the highest fold differences between laboratory based stress treatments and unstressed controls in the microarray analysis and subsequent real-time qRT-PCR validation. A range of different housekeeping (reference) genes were also selected from the validated microarray data.

Real time PCR of gene expression in field samples

qRT-PCR was performed using an Eppendorf Mastercycler Realplex thermal cycler with KAPA qPCR SYBR green PCR kits (Geneworks) according to the manufacturers' instructions. The conditions used for qRT-PCR were identical to those employed to validate microarray data in section 7. Briefly, reaction mixtures consisted of KAPA SYBR green mastermix, forward and reverse primers, and template (1x, 5x, 25x and 125x dilutions) (Table 5). Templates were reverse transcribed RNAs from each of the stress treatments (3 replicates of pooled RNA from a range of time points for each treatment and non-stressed controls). Reaction mixtures were loaded into 96-well PCR plates that were subjected to the following PCR cycling conditions: 15 min at 95˚c, 35 cycles of 15s at 94˚c, 30s at 60˚C and 30s at 72˚C.

Table 5. Reaction mixtures used for qPCR of field samples

Reaction Mix			
Component	**Starting Conc.**	**Amount**	**Final**
Kappa Master mix	2x	10µl	1x
Forward Primer	20µM	0.2µl ea.	0.2µM
Reverse Primer	20µM	0.2µl ea.	0.2µM
Template	0.8-100ng/µl	1µl	0.8-100ng
H_2O		8.6µl	
		Total 20µl	

Relative expression of the 13 putative stress response genes was calculated by both the 2-ΔΔCt and modified Pfaffl (Pfaffl, 2001) methods. The relative expression (up- or down- regulation) of all 13 target genes in the de-fouling treatment, relative to controls, is shown in Figure 13. Fold differences in expression relative to controls ranged from up regulation of 28-fold (gene SP1-C9) to 1.4 fold down-regulation (gene SP2-E9) (note that fold differences shown in Figure 9 are log2 values). Across all genes, there were 11 instances of up-regulation and 2 cases of down-regulation in de-fouled (treatment) samples relative to untreated controls. Six out of the 13 target genes were up-regulated by more than 4-fold in de-fouled oysters relative to controls. Variability between zones within the treatment or control groups was limited, except for gene SP2-E5.

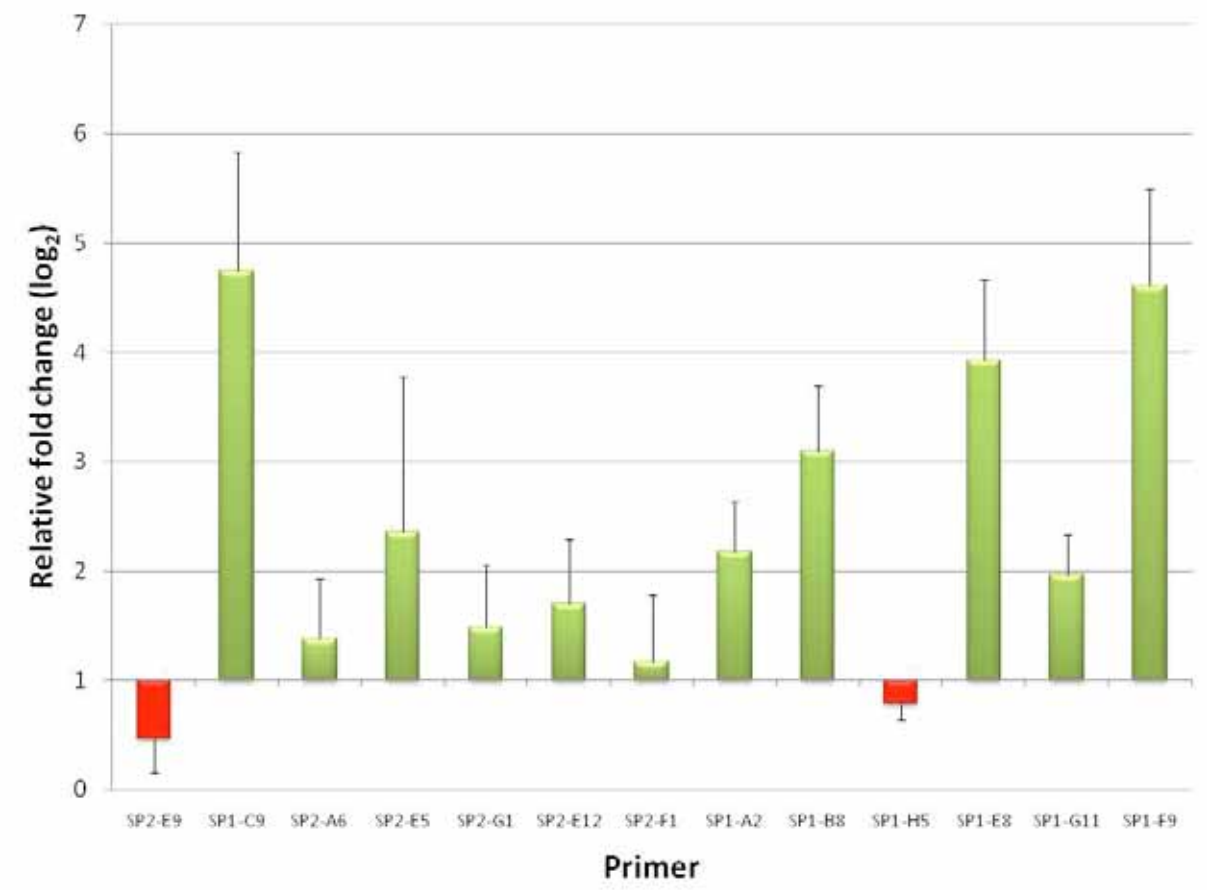

Figure 13. Changes in the expression of stress response biomarkers in P. maxima. The data show relative expression (de-fouling treatment vs. controls) of 13 putative stress response genes tested by qRT-PCR. Note that relative expression is shown as $\log_2$ values. Values > 0 represent up-regulation of the gene in the de-fouling treatment relative to untreated controls, whilst values < 0 represent down regulation. Relative fold change was calculated from $2^{-\Delta\Delta Ct}$ values for each samples, using invariant gene Sp1-E12 as a reference (housekeeper). Error bars = SEM, n = 3.

7.0 Discussion

This project used cDNA microarray technology to analyse environmental stress responses in *P. maxima*. Its goal was to identify stress response genes that can be used as biomarkers in analyses of the oyster farming practice to identify stressful processes that might affect oyster health. There is growing body of evidence that stress in aquaculture alters the gene expression profiles of farmed species, altering key physiological systems. Our previous studies have shown that stressors associated with oyster farming, such as exposure to air and handling, have detrimental effects on the oyster immune system, presumably leaving oysters more susceptible to infectious diseases (Butt et al., 2006, 2007; Butt et al., 2006, 2007, 2008; Kuchel, et al. 2010, 2011a,b). And we already know that there are hormonally based stress responses in oysters that have the potential to affect a range of physiological systems, that, in their most extreme cases, induce the programmed death of critical cells involved in immune responses.

This FRDC project took our work in a practical direction by identifying suites of genes that can be used as tools to monitor stress during the farming practice, with the goal of identifying, modifying or ameliorating particularly stressful processed. The stressors that we tested included altered salinity, mechanical agitation, air exposure, "clipped open" and starvation. Samples from control (non-stressed) oyster were also included in the analysis so that genes down regulated by stress could be identified. cDNA library development was initially affected by a range of technical problems, which troubleshooting indicated were primarily due to the low amounts of RNA that could be obtained from oyster blood cells (haemocytes). To resolve this problem, we changed our target tissue from haemocytes to gills, which greatly increased RNA yields and allowed the cDNA library to be effectively constructed. It is unlikely that this change in target tissue had a substantial impact on our ability to identify genes associated with stress responses, which are primarily expressed by haemocytes, because there are substantial numbers of haemocytes in gills as well as hemolymph (blood).

Recommendation #1 *– that future studies of gene expression and stress in oysters focus on gill tissue because it is a superior source of mRNA for analysis.*

Recommendation #2 *– that further studies be conducted to examine the spatial distribution of haemocytes in* Pinctada maxima *and to determine whether the relative lack of haemocytes in the blood was a feature of pearl oysters sampled prior to 2006.*

Once the cDNA library and resulting microarray had been constructed, the microarrays were analysed to identify genes affected by stress. This gene discovery phase of the project used samples from the initial, laboratory-based stress experiments. The data indicated that expression of 448 clones on the array differed significantly in expression ($p < 0.05$ and ≥ 2.5 fold difference in expression levels) between stress treatments (altered salinity, mechanical agitation, air exposure, "clipped open" and starvation) and non-stressed controls. The impacts of the different stressors, in terms of the number of clones on the array affected, indicated that exposure to air was the most stressful treatment, whilst altered salinity had the least effect on gene expression. Multifactorial analysis of the microarray data also suggested that these is a discrete set of genes affected by exposure to air. This fits with other work that we have done with pearl oysters, which has shown that exposure to air has by far the biggest impact on the expression oxidative stress response genes in *P. imbricata*, and on phenoloxidase activity in *P. maxima* (Kuchel et al., unpublished data).

Recommendation #3 – *that further studies be conducted to confirm the significant impact on oysters of exposure to air relative to other potential stressors, with the intention of advising industry to limit exposure to air during the farming process.*

cDNA sequencing of differentially expressed clones identified 37 unique gene sequences. Of these, 51% corresponded to genes involved in translation (both ribosomal RNAs and proteins), cell signalling, metabolism, stress responses and immune responses. This range of biological functions fits with a growing body of evidence from studies of other oyster and invertebrate species in our laboratory and elsewhere, which suggests that environmental stress affects a range of physiological systems, not just genes normally associated with "stress" responses (Thompson et al., 2011a,b). The main group of differentially expressed genes were putatively involved in translation, indicating that the ability of cells to synthesise proteins is substantially affected by stress. Again, this fits with emerging evidence from a range of species that environmental stress substantially impacts cellular protein synthesis, which may have diverse effects on a range of biochemical processes in cells. The identification of genes involved in immune responses also indicates that the stressors applied may substantially modulate immunocompetence, affecting disease susceptibility and resistance.

Perhaps the most surprising result from the initial microarray analysis was the large number of differentially expressed clones (>50%) that either did not match any known sequences in the available databases, or matched genes with no known function. This represents a very low identification hit rate for this type of analysis. The most likely reasons for this is the very low number of fully characterised sequences for *Pinctata* species in the publically available databases, and the relatively large evolutionary distance between *Pinctada* and species with a fully sequenced genome. The availability of complete genome sequences in closely related species substantially increases the likelihood of successful gene and protein identification. However, there are currently no bivalve mollusc species with complete genome sequences, even though the costs and time required for genome sequencing have decreased substantially in recent years.

Recommendation # 4 *– that industry considers supporting the sequencing of the* Pinctada maxima *genome to provide the genetic resources necessary for detailed scientific analysis of gene expression, disease resistance, pearl production and other key needs.*

Despite this low hit rate, in the context of this project (which had the focused aim of identifying potential stress biomarkers), the limited number of differential genes that could be fully characterised was relatively inconsequential. The function of biomarker genes does not affect their ability to detect stressful events. To confirm that results of microarray analyses, we performed qRT-PCR on samples from laboratory based stress experiments using primers for a subset of the differential genes identified on the microarray. This type of validation is essential because microarrays in themselves are not fully quantitative, so that levels of differential expression need to be confirmed by qRT-PCR. Happily, the analysis did confirmed that the majority of genes chosen for primer design were differentially regulated.

Having identified and validated a range of potential biomarker genes, the final stage of the project was to confirm the utility of those biomarkers in assessing the impacts of stress in the field. The field trials, in which oysters were subjected to a routine farming practice involving both exposure to air and mechanical de-fouling, strongly supported our identification of "stress response" genes in *P. maxima* that can be used as effective biomarkers to differentiate healthy oysters from those subjected to stresses associated with farming practices. The most striking observation from the field trials was the level of change in the expression of some genes in

response to the stress of de-fouling. Fold differences in expression among these genes were far higher than we observed using samples from laboratory-based stress experiments. This suggests that combining air exposure and mechanical agitation (both of which are factors in de-fouling) may have synergistic effects on stress response genes, when compared to the response to each of these stressors tested in isolation under controlled laboratory conditions.

8.0 Benefits and adoption

The beneficiaries of the project are the pearling industry and the state government agencies in the Northern Territory and Western Australia. The project identified a number of genes that could be adopted immediately by industry as biomarkers of stress in pearl oysters. Industry now have access to a powerful new technology showing molecular activity in oysters subjected to stress. This can be used at the industry level to refine industry management practice. The state animal health laboratories have access to a suite of PCR's that can be added to existing tools used to indicate the health status of oysters.

It is difficult to quantify the research in terms of value to industry. However, it should be noted that the cost of a PCR for "stress" based on a pool of 5 animals would be about $70, while the cost of histology on 5 oysters, as a "health check" would be between $115 and $350.

9.0 Further development

Even though this project was successful, it highlighted the lack of publically available DNA sequence data for pearl oysters. This limited our ability to understand the biological processes being affected by stress, although it did not prevent us from identifying potential biomarker genes. One next step for industry should be the sequencing of the pearl oyster genome. This would allow putative biological functions to be assigned to genes that are affected by stress, allowing us to develop a broad understanding of the broad biological processes that are affected by stress. Further developments should also include additional work to understand the biological activities of individual biomarker genes that could be exploited to develop simple "litmus" tests of stress that can be applied on-farm during the farming practice. These tests may be particularly important is assessing the effectiveness of future selective breeding programs designed to enhance oyster health at the genetic level.

A number of recommendations came from this project, background to which is provided in the discussion. These recommendations were:

Recommendation #1 *– that future studies of gene expression and stress in oysters focus on gill tissue because it is a superior source of mRNA for analysis.*

Recommendation #2 *– that further studies be conducted to examine the spatial distribution of haemocytes in* Pinctada maxima *and to determine whether the relative lack of haemocytes in the blood was a feature of pearl oysters sampled prior to 2006.*

Recommendation #3 *– that further studies be conducted to confirm the significant impact on oysters of exposure to air relative to other potential stressors, with the intention of advising industry to limit exposure to air during the farming process.*

Recommendation # 4 *– that industry considers supporting the sequencing of the* Pinctada maxima *genome to provide the genetic resources necessary for detailed scientific analysis of gene expression, disease resistance, pearl production and other key needs.*

10.0 Planned outcomes

In terms of practical outcomes for industry, this project has delivered:

- Additional *Pincatada maxima* genetic sequence data;
- A cDNA microarray that can be used by industry in future to test the effects of a range of environmental variables in the field.
- The identification of a suite of gene biomarkers that can be used to assess the relative impacts of different processes within the farming practice, with the intention of ameliorating or mitigating particularly harmful processes. These have potential as "markers" for stress in pearl oysters. Discussions have been had with industry managers on potential uses for this technology to improve industry practices. Early evidence is that some environmental stressors, particularly exposure to air, may be relatively more harmful than other factors and further work (for example the contribution to time and temperature) will allow managers to make informed decisions about farm procedures.
- Assays to identify "stress" in oysters. There are three ways in which these genes might be used in a routine assay of stress. The first is the quantitative real time PCR method that we used in the project. A second, less expensive option is to use normal semi-quantitative PCR. The level of difference in expression for some of the genes that identified is probably great enough that semi-quantitative PCR could identify simple on/off differences in expression of those genes. Both of these PCR methods require samples to be rapidly frozen in the field and then transported to a laboratory for analysis. The most realistic and cheapest option would require more work to develop antibodies against proteins encoded by the differentially expressed genes. That way the concentration of proteins from the corresponding genes could be measured in relatively simple colorimetric assays, potentially on site. The advantage of this is that proteins are more stable than mRNA and so sample collection and storage would not be so problematic.

11.0 Conclusion

This project has shown that practices that are commonly applied to pearl oysters during routine farming have wide-ranging effects on the expression of oyster genes. It is likely that the broad changes in gene expression evident after these stressful events will markedly alter the physiology of oysters, with ramifications for the overall health of oysters and their ability combat additional environmental changes, such as exposure to infectious diseases. In practical terms our work has identified a number of genes that could be used as robust markers to assess "stress" in oysters. These markers could be used assess overall oyster health, and to study farming practices in detail so that unduly stressful processes can be eliminated or modified. Our work already suggests that exposing oysters to air may be one of the most stressful components of current farming practices.

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13.0 Appendices

13.1 Intellectual property

The research described in this report is for the public domain. The report and any resulting manuscripts are intended for wide dissemination and promotion. All data and statistics presented conform to confidentiality arrangements.

13.2 Staff

As with any project of this size, a number of staff contributed to the project. These were:

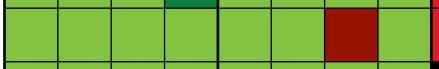
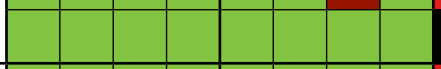
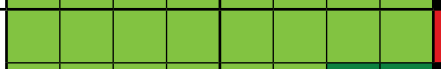
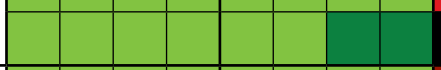
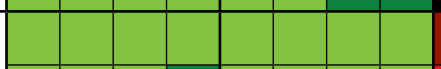
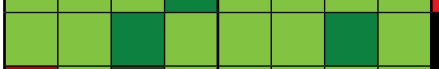
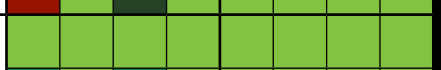
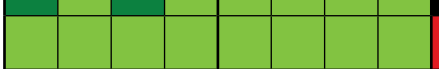
Name	Affiliation	Funding
Dr David Raftos	Macquarie University	FRDC and in-kind
Dr Sham Nair	Macquarie University	FRDC and in-kind
Dr Brian Jones	Fisheries WA	FRDC and in-kind
Dr Melanie Crockford	Fisheries WA	FRDC and in-kind
Mr. Adam Wilkins	Macquarie University	FRDC and in-kind
Ms. Camille LeCroix	Macquarie University	FRDC and in-kind
Ms. Druime Nolan	Fisheries WA.	FRDC
Dr. Meike Berger	Fisheries WA	FRDC

13.3 Heat maps showing complete replicated analysis of the microarray

Heat maps showing the complete replicated analysis of the microarray. Each slide represents one of the 3 biological replicates used from each stress treatment. Each box represents a single cDNA spot on the microarray slide. Colours represent the gradient of divergence from the fluorescence intensity of the corresponding spot in control slides. Green = up regulation relative to controls; reds = downregulation relative to controls. Clone numbers are shown on the left and the mean difference in fluorescence intensity for each clone relative to controls (n=3). This Figure shows only clones that differed in mean intensity from controls by fold differences of great than 2.

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P10-20.11.09_B6			X X X X	2.19	1
P10-20.11.09_D3			X X X X	2.31	1
P11-19.11.09_A1			X X X X	2.17	1
			X X X X	2.8	2
			X X X X	3.56	3
			X X	2.17	4
			X X X X	2.19	5
P11-19.11.09_A10			X X X X	4.51	2
			X X X X	3.56	3
			X X	4.04	4
			X X X X	3.54	5
P11-19.11.09_A11			X X X X	3.2	1
			X X X X	2.52	2
			X X X X	2.9	5
P11-19.11.09_A12			X X X X	2.59	1
			X X X X	2.9	3
			X X	2.14	4
			X X X X	2.56	5
P11-19.11.09_A2			X X X X	3.73	1
			X X X X	3	2
			X X X X	2.11	3
			X X	3.33	4
P11-19.11.09_A3			X X X X	2.41	1
			X X X X	2.32	2
			X X X X	2.71	3

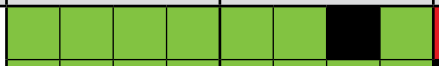
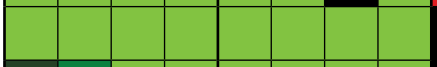
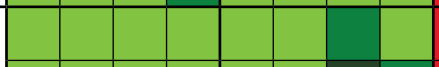
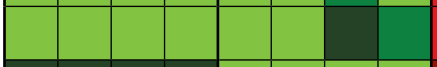
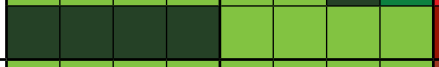
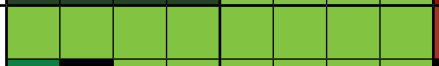
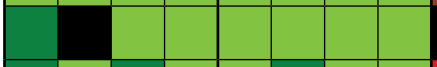
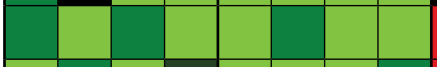
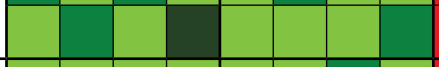
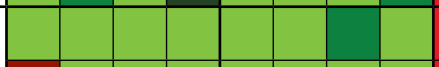
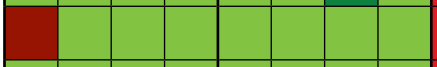
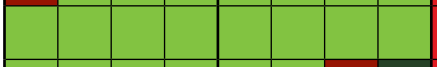
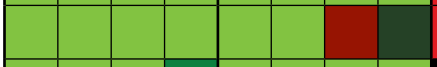
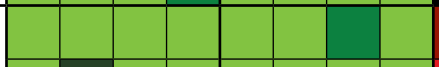
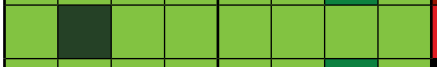
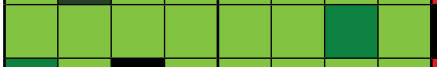
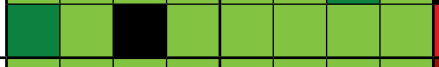
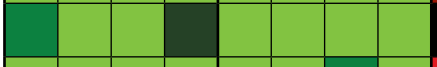
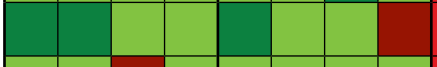
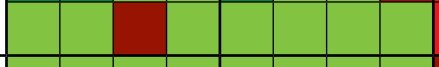
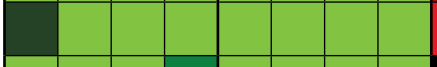
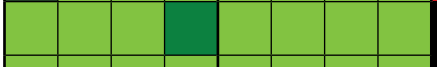
Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_A5			X X X X	2.78	1
			X X X X	2.22	3
			X X X X	2.17	5
P11-19.11.09_A7			X X X X	2.77	1
			X X X X	2.52	2
			X X X X	2.78	3
P11-19.11.09_A9			X X X X	2.1	2
			X X X X	2.7	3
			X X X X	2.21	5
P11-19.11.09_B1			X X X X	4.08	1
			X X X X	3.56	2
			X X X X	4.01	3
			X X	2.57	4
			X X X X	2.56	5
P11-19.11.09_B10			X X X X	3.11	1
			X X X X	3.11	2
			X X X X	2.33	3
			X X	2.01	4
			X X X X	2.31	5
P11-19.11.09_B11			X X X X	3.29	1
			X X X X	3.77	2
			X X X X	2.91	3
			X X X X	2.88	5
P11-19.11.09_B12			X X X X	3.7	1
			X X X X	3.25	2
			X X X X	2.28	3
			X X	2.16	4
			X X X X	2.27	5
P11-19.11.09_B2			X X X X	4.28	1
			X X X X	2.32	2
			X X X X	4.01	3
			X X	2.27	4
			X X X X	3.47	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_B3			X X X X	2.95	1
			X X X X	2.28	2
			X X X X	2.36	3
			X X X X	2.72	5
P11-19.11.09_B4			X X X X	3.06	1
			X X X X	2.66	3
P11-19.11.09_B5			X X X X	3.76	1
			X X X X	3.6	2
			X X X X	2.5	3
			X X X X	3.04	5
P11-19.11.09_B6			X X X X	3.24	1
			X X X X	2.85	2
			X X X X	3.39	3
			X X X X	3.11	5
P11-19.11.09_B7			X X X X	2.43	1
			X X X X	3.76	2
			X X X X	3.4	3
			X X X X	2.46	5
P11-19.11.09_B8			X X X X	3.84	1
			X X X X	2.44	2
			X X X X	4.78	3
			X X X X	2.78	5
P11-19.11.09_C1			X X	2.03	1
			X X X X	2.88	5
P11-19.11.09_C10			X X X X	3.35	1
			X X X X	2.63	2
			X X X X	2.82	3
			X X X X	2.12	5
P11-19.11.09_C11			X X X X	4.19	1
			X X X X	3.84	2
			X X X X	3.24	3
			X X	2.08	4
			X X X X	3.04	5

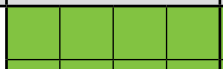
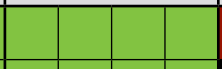
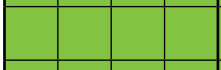
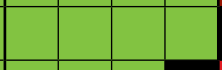
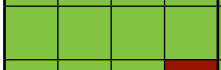
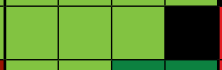
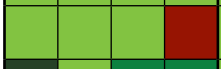
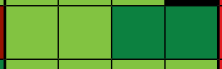
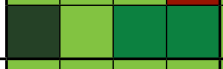
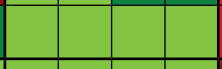
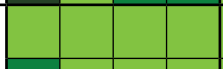
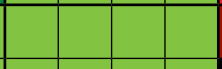
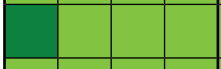
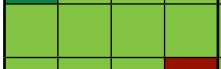
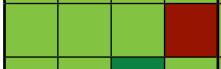
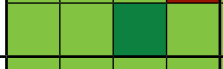
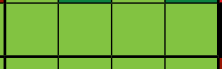
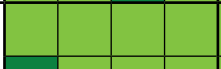
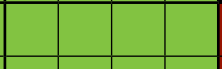
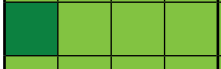
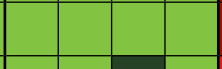
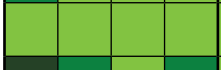
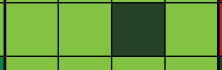
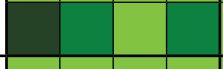
Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_C12			X X X X	3.46	1
			X X X X	3.22	2
			X X X X	3.06	3
			X X X X	2.54	5
P11-19.11.09_C2			X X X X	2.58	3
P11-19.11.09_C3			X X X X	3.26	1
			X X X X	5	2
			X X	2.68	5
P11-19.11.09_C4			X X X X	3.41	1
			X X X X	3.35	2
			X X X X	3.1	3
			X X	2.48	4
			X X X X	3.3	5
P11-19.11.09_C5			X X X X	3.59	1
			X X	2.44	2
			X X	2.54	4
			X X X X	2.5	5
P11-19.11.09_C6			X X X X	3.77	1
			X X X X	3.23	2
			X X X X	4.05	3
			X X X X	3.23	5
P11-19.11.09_C7			X X X X	2.12	1
			X X X X	2.91	2
			X X X X	3.39	3
			X X	2.3	4
			X X X X	3.12	5
P11-19.11.09_C8			X X X X	3.69	1
			X X X X	4.35	2
			X X X X	3.19	3
			X X X X	2.8	5

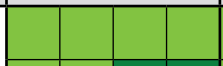
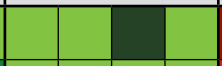
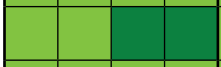
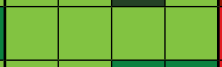
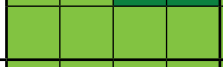
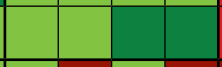
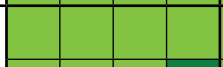
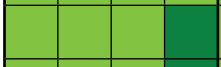
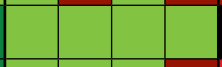
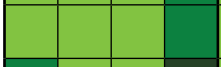
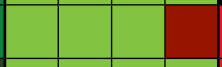
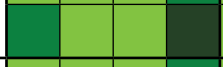
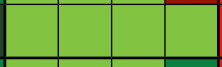
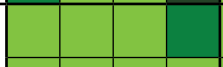
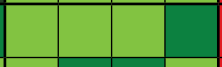
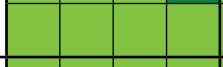
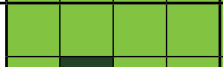
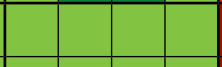
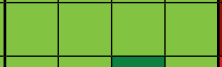
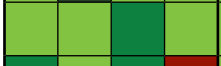
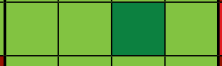
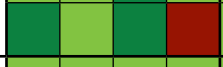
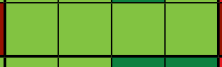
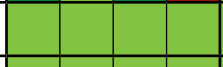
Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_C9			X X X X	3.73	1
			X X X X	4.12	2
			X X X X	3.99	3
			X X X X	3.35	5
P11-19.11.09_D1			X X X X	3.48	1
			X X X X	2.82	3
			X X	2.09	4
			X X X X	3.94	5
P11-19.11.09_D10			X X X X	2.63	1
			X X X X	3.07	2
			X X X X	3.42	3
			X X X X	2.64	5
P11-19.11.09_D12			X X X X	3.94	1
			X X X X	2.48	2
			X X X X	2.18	3
			X X X X	3.05	5
P11-19.11.09_D2			X X X X	3.82	1
			X X X X	2.41	2
			X X X X	3.04	3
			X X	2.83	4
			X X X X	3.14	5
P11-19.11.09_D3			X X X X	3.7	1
			X X X X	2.81	2
			X X X X	3.49	3
			X X X X	3.91	5
P11-19.11.09_D4			X X X X	4.3	1
			X X X X	2.85	2
			X X X X	3.19	3
			X X	2.65	4
			X X X X	4.03	5
P11-19.11.09_D6			X X X X	2.11	1
			X X X X	2.4	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_D8			X X X X	4.69	1
			X X X X	3.47	2
			X X X X	3.15	3
			X X X X	2.79	5
P11-19.11.09_D9			X X X X	3.34	1
			X X X X	4.29	2
			X X X X	3.59	3
			X X	2.49	4
			X X X X	2.64	5
P11-19.11.09_E1			X X X X	3.2	1
			X X X X	3.97	2
			X X X X	3.79	3
			X X	2.32	4
			X X X X	3.44	5
P11-19.11.09_E10			X X X X	3.16	1
			X X X X	2.87	2
			X X X X	3.1	3
			X X X X	2.41	5
P11-19.11.09_E11			X X X X	4.28	1
			X X X X	3.59	2
			X X X X	3.56	3
			X X	2.28	4
			X X X X	3.61	5
P11-19.11.09_E12			X X X X	3.01	1
			X X X X	2.16	2
			X X X X	2.33	3
			X X X X	3.33	1
P11-19.11.09_E2			X X X X	3.01	2
			X X X X	2.61	3
			X X X X	2.13	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_E3						3.32	1
						4.47	2
						3.61	3
						2.28	4
						2.78	5
P11-19.11.09_E4				4.42	1
				2.77	3
				2.45	5
P11-19.11.09_E5					3.62	1
					2.25	2
					2.13	3
					2.29	4
P11-19.11.09_E6						3.12	1
						3.19	2
						3.47	3
						3.19	4
						3.87	5
P11-19.11.09_E8					4	1
					2.66	2
					3.69	3
					2.58	5
P11-19.11.09_E9						3.95	1
						4.12	2
						3.33	3
						2	4
						2.93	5
P11-19.11.09_F1					3.13	1
					2.38	2
					3.21	3
					2.61	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_F10			X X X X	4.44	1
			X X X X	3.73	2
			X X X X	3.06	3
			X X	2.21	4
			X X X X	2.07	5
P11-19.11.09_F11			X X X X	3.55	1
			X X X X	3.22	2
			X X X X	2.68	3
			X X X X	3.14	5
P11-19.11.09_F12			X X X X	4.11	1
			X X X X	3.22	2
			X X X X	2.77	3
			X X X X	2.98	5
P11-19.11.09_F2			X X X X	3.13	1
			X X X X	2.67	2
			X X X X	3.74	3
			X X X X	3.04	5
P11-19.11.09_F3			X X X X	3.06	1
			X X X X	3.23	2
			X X X X	3.2	3
			X X	2.04	4
			X X X X	2.99	5
P11-19.11.09_F4			X X X X	3.52	1
			X X X X	2.67	2
			X X X X	2.42	3
			X X X X	3.08	5
P11-19.11.09_F5			X X X X	3.27	1
			X X X X	3.32	2
			X X X X	3.19	3
			X X X X	2.89	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_F6				4.09	1
				3.09	2
				2.78	3
				2.43	4
				2.58	5
P11-19.11.09_F7				3.92	1
				3.64	2
				3.24	3
				2.03	4
				2.63	5
P11-19.11.09_F8				4.51	1
				2.69	2
				2.65	3
				2.36	5
P11-19.11.09_F9				3.79	1
				3.54	2
				2.42	3
				3.1	4
				2.24	5
P11-19.11.09_G1				3.72	1
				4.8	2
				2.64	3
				2.05	4
				3.38	5
P11-19.11.09_G10				2.89	1
				2.69	2
				2.35	3
				2.33	5
P11-19.11.09_G12				3.58	1
				2.93	2
				2.89	3
				2.89	4
				2.32	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_G2				2.61	1
				2.15	2
				2.96	3
P11-19.11.09_G3				2.06	1
				2.77	2
				2.63	3
				2.12	5
P11-19.11.09_G4				2.18	2
				2.46	3
P11-19.11.09_G5				3.62	1
				3.22	2
				2.19	3
				2.66	5
P11-19.11.09_G6				2.32	3
P11-19.11.09_G7				2.5	1
				2.59	2
				2.02	5
P11-19.11.09_G8				2.74	1
				3.12	2
				3.62	3
				2.67	5
P11-19.11.09_G9				3.59	1
				3.67	2
				3.52	3
				2.68	5
P11-19.11.09_H1				3.55	1
				2.68	2
				2.66	3
P11-19.11.09_H10				3.56	1
				2.73	2
				2.62	5
P11-19.11.09_H11				2.54	1
				2.06	5

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P11-19.11.09_H12			X X X X	2.5	1
			X X X X	2.22	2
P11-19.11.09_H3			X X X X	2.07	3
P11-19.11.09_H4			X X X X	3.15	1
			X X X X	2.74	2
			X X X X	3.22	3
			X X X X	2.38	5
P11-19.11.09_H5			X X X X	2.45	1
P11-19.11.09_H6			X X X X	3.59	1
			X X X X	3.25	2
			X X X X	3.47	3
P11-19.11.09_H7			X X X X	2.51	1
			X X X X	2.19	2
			X X X X	2.02	5
P11-19.11.09_H9			X X X X	2.34	1
			X X X X	2.48	2
			X X X X	2.42	3
			X X	2.06	4
P1-17.11.09_H4			X X X X	2.47	1
P1-20.11.09_C1			X X	3.09	2
			X X X X	2.77	3
P1-20.11.09_D10				-2.19	3
			X X	-3.1	4
P1-20.11.09_D9			X X	-3.07	4
P1-20.11.09_E1			X X X X	2.07	3
			X X X X	2.31	5
P1-20.11.09_F11			X X	2.07	4
P12-19.11.09_B7			X X	2.17	1
P12-19.11.09_B9			X X X X	2.43	1
P12-19.11.09_F6			X X X X	2.45	1
P12-19.11.09_F7			X X	-2.29	4
P12-19.11.09_H4			X X X X	2.88	1
			X X X X	2.94	2

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P13-19.11.09_C1			X X X X	4.34	1
			X X X X	2.52	2
			X X X X	2.59	3
			X X X X	2.73	5
P13-19.11.09_D1			X X X X	-2.91	3
			X X	-4.05	4
P13-19.11.09_E9			X X X X	-2.07	1
P13-19.11.09_F2			X X X X	3.1	1
			X X X X	2.38	2
			X X X X	2.45	3
			X X X X	2.42	5
P13-19.11.09_H12			X X X X	2.24	1
P13-19.11.09_H2			X X X X	3.32	1
			X X X X	2.3	2
			X X X X	2.79	3
			X X X X	2.28	5
P13-19.11.09_H3			X X X X	2.52	1
P13-19.11.09_H4			X X X X	4.04	1
			X X X X	2.73	3
			X X X X	2.71	5
P14-19.11.09_E5			X X X X	-2.13	1
P15-19.11.09_B3			X X X X	2.64	1
			X X	2.25	4
P15-19.11.09_B5			X X X X	2.34	1
P15-19.11.09_C4			X X	-2.15	4
P15-19.11.09_F3			X X X X	2.24	1
P15-19.11.09_F8			X X X X	2.56	1
P16-19.11.09_C12			X X X X	2.09	1
P16-19.11.09_C2			X X X X	2.03	1
P16-19.11.09_D1			X X X X	2.63	1
P16-19.11.09_D4			X X	-2.17	4
P16-19.11.09_E3			X X X X	2.11	2

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P16-19.11.09_F5			X X	-2.47	3
			X X	-3.43	4
P17-19.11.09_A4			X X X X	2.04	2
P17-19.11.09_E7			X X	2.02	4
P17-19.11.09_F1			X X X X	3.98	1
			X X X X	3.32	2
			X X X X	3.06	3
			X X X X	3.03	5
P18-19.11.09_B3			X X X X	2.11	1
P18-19.11.09_D7			X X X X	2.34	1
P18-19.11.09_E1			X X X X	2.29	1
P18-19.11.09_E5			X X X X	2.14	1
P18-19.11.09_E6			X X X X	2.54	1
P18-19.11.09_F9			X X X X	-2.96	3
			X X	-3.9	4
P18-19.11.09_G2			X X X X	2.04	1
P18-19.11.09_G4			X X X X	2.17	1
P18-19.11.09_H10			X X X X	2.01	1
P19-19.11.09_C2			X X X X	-2.22	2
			X X	-3.86	4
P19-19.11.09_C6			X X X X	-2.15	1
			X X	-2.14	4
P19-19.11.09_E7			X X X X	2.54	3
P19-20.11.09_F9			X X X X	2.72	1
P19-20.11.09_G1			X X	-2.01	4
P20-19.11.09_A2			X X X X	2.49	3
P20-19.11.09_F6				-2.46	4
P2-20.11.09_E2			X X	-2.23	4
P2-20.11.09_G1			X X X X	3.44	1
			X X X X	2.54	3
			X X X X	2.55	5
P2-20.11.09_G7			X X	-2.09	4

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P2-20.11.09_H1			X X X X	3.1	1
			X X X X	2.13	2
			X X X X	2.31	3
P3-17.11.09_G2			X X	-2.84	4
P3-20.11.09_B12			X X X X	2.07	3
P3-20.11.09_E5			X X X X	2.72	5
			X X X X	2.08	1
P3-20.11.09_H10			X X	2.3	4
P3-20.11.09_H5			X X X X	2.51	1
			X X X X	2.97	3
			X X X X	2.23	5
P4-20.11.09_A12			X X	-2.05	4
P4-20.11.09_H4			X X X X	3.06	1
			X X X X	2.61	2
			X X X X	2.87	3
			X X X X	2.61	5
P5-7.12.09_F10			X X X X	2.5	1
			X X X X	3.22	2
			X X X X	2.28	3
			X X	2.18	5
P5-7.12.09_F9			X X	3.05	4
P6-17.11.09_B2			X X X X	2.44	1
			X X X X	2.33	5
P6-17.11.09_C7			X X	-3.43	4
P6-20.11.09_D12			X X X X	2.22	5
P6-20.11.09_E9			X X X X	2.02	3
			X X X X	2.76	5
P6-20.11.09_F1			X X X X	3.24	1
			X X X X	2.11	3
			X X X X	2.27	5
P7-17.11.09_C3				-2.07	3
			X X	-3.18	4
P7-20.11.09_A9			X X	-2.55	4

Clone ID	Slide 1	Slide 2	Slide 3	Mean	Comparison #
P7-20.11.09_B11			X X X X	2.18	1
P7-20.11.09_D4			X X X X	2.38	1
P7-20.11.09_F10			X X X X	2.48	1
P7-20.11.09_G5				-2.42	4
P8-17.11.09_F1				-2.97	4
P8-17.11.09_F10			X X	-3.06	4
P8-20.11.09_E9			X X X X	2.25	1
P8-20.11.09_F4			X X X X	2.66	1
P8-20.11.09_F5			X X X X	2.72	1
			X X X X	2.21	3

13.4 List of clones from the cDNA library that revealed fold differences of >2 in fluorescence intensity when stress treatments were compared to controls

List of clones from the cDNA library that revealed fold differences of >2 in fluorescence intensity when stress treatments were compared to controls.

CLONE #	CLONE #	CLONE #
P10-20.11.09_B6	P10-20.11.09_D3	P11-19.11.09_A1
P11-19.11.09_A10	P11-19.11.09_A11	P11-19.11.09_A12
P11-19.11.09_A2	P11-19.11.09_A3	P11-19.11.09_A5
P11-19.11.09_A7	P11-19.11.09_A9	P11-19.11.09_B1
P11-19.11.09_B10	P11-19.11.09_B11	P11-19.11.09_B12
P11-19.11.09_B2	P11-19.11.09_B3	P11-19.11.09_B4
P11-19.11.09_B5	P11-19.11.09_B6	P11-19.11.09_B7
P11-19.11.09_B8	P11-19.11.09_C1	P11-19.11.09_C10
P11-19.11.09_C11	P11-19.11.09_C12	P11-19.11.09_C2
P11-19.11.09_C3	P11-19.11.09_C4	P11-19.11.09_C5
P11-19.11.09_C6	P11-19.11.09_C7	P11-19.11.09_C8
P11-19.11.09_C9	P11-19.11.09_D1	P11-19.11.09_D10
P11-19.11.09_D12	P11-19.11.09_D2	P11-19.11.09_D3
P11-19.11.09_D4	P11-19.11.09_D6	P11-19.11.09_D8
P11-19.11.09_D9	P11-19.11.09_E1	P11-19.11.09_E10
P11-19.11.09_E11	P11-19.11.09_E12	P11-19.11.09_E2
P11-19.11.09_E3	P11-19.11.09_E4	P11-19.11.09_E5
P11-19.11.09_E6	P11-19.11.09_E8	P11-19.11.09_E9
P11-19.11.09_F1	P11-19.11.09_F10	P11-19.11.09_F11
P11-19.11.09_F12	P11-19.11.09_F2	P11-19.11.09_F3
P11-19.11.09_F4	P11-19.11.09_F5	P11-19.11.09_F6
P11-19.11.09_F7	P11-19.11.09_F8	P11-19.11.09_F9
P11-19.11.09_G1	P11-19.11.09_G10	P11-19.11.09_G12
P11-19.11.09_G2	P11-19.11.09_G3	P11-19.11.09_G4
P11-19.11.09_G5	P11-19.11.09_G6	P11-19.11.09_G7
P11-19.11.09_G8	P11-19.11.09_G9	P11-19.11.09_H1
P11-19.11.09_H10	P11-19.11.09_H11	P11-19.11.09_H12
P11-19.11.09_H3	P11-19.11.09_H4	P11-19.11.09_H5
P11-19.11.09_H6	P11-19.11.09_H7	P11-19.11.09_H9
P1-17.11.09_H4	P1-20.11.09_C1	P1-20.11.09_D10
P1-20.11.09_D9	P1-20.11.09_E1	P1-20.11.09_F11
P12-19.11.09_B7	P12-19.11.09_B9	P12-19.11.09_F6
P12-19.11.09_F7	P12-19.11.09_H4	P13-19.11.09_C1
P13-19.11.09_D1	P13-19.11.09_E9	P13-19.11.09_F2
P13-19.11.09_H12	P13-19.11.09_H2	P13-19.11.09_H3
P13-19.11.09_H4	P14-19.11.09_E5	P15-19.11.09_B3

CLONE #	CLONE #	CLONE #
P15-19.11.09_B5	P15-19.11.09_C4	P15-19.11.09_F3
P15-19.11.09_F8	P16-19.11.09_C12	P16-19.11.09_C2
P16-19.11.09_D1	P16-19.11.09_D4	P16-19.11.09_E3
P16-19.11.09_F5	P17-19.11.09_A4	P17-19.11.09_E7
P17-19.11.09_F1	P18-19.11.09_B3	P18-19.11.09_D7
P18-19.11.09_E1	P18-19.11.09_E5	P18-19.11.09_E6
P18-19.11.09_F9	P18-19.11.09_G2	P18-19.11.09_G4
P18-19.11.09_H10	P19-19.11.09_C2	P19-19.11.09_C6
P19-19.11.09_E7	P19-20.11.09_F9	P19-20.11.09_G1
P20-19.11.09_A2	P20-19.11.09_F6	P2-20.11.09_E2
P2-20.11.09_G1	P2-20.11.09_G7	P2-20.11.09_H1
P3-17.11.09_G2	P3-20.11.09_B12	P3-20.11.09_E5
P3-20.11.09_H10	P3-20.11.09_H5	P4-20.11.09_A12
P4-20.11.09_H4	P5-7.12.09_F10	P5-7.12.09_F9
P6-17.11.09_B2	P6-17.11.09_C7	P6-20.11.09_D12
P6-20.11.09_E9	P6-20.11.09_F1	P7-17.11.09_C3
P7-20.11.09_A9	P7-20.11.09_B11	P7-20.11.09_D4
P7-20.11.09_F10	P7-20.11.09_G5	P8-17.11.09_F1
P8-17.11.09_F10	P8-20.11.09_E9	P8-20.11.09_F4
P8-20.11.09_F5		

13.5 Blast searches (against NCBI non-redundant database) of sequences for 192 clones from the cDNA library

BLAST searches (against NCBI non-redundant database) of sequences for 192 clones from the cDNA library that included clones that were differentially expressed in more than one treatment, clones that were differentially expressed in just on treatment, and a number of clones that did not differ in expression between treatments and controls. The table shows the clones library address, whether the clone was selected from control or treatment samples in the library, the length of overlap in the matching BLAST hit, the annotation of the best BLAST hit for each clone, and the e-value for the hit.

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P10-20.11.09_A3	Control	0-950	*Plasmodium falciparum* 3D7 chromosome 8	1.00E-04
P10-20.11.09_D8	Control	84-570	No decent hits (e-vals too high)	
P10-20.11.09_F10	Control	85-525	No decent hits (e-vals too high)	
P10-20.11.09_F12	Control	147-173	*Schistosoma mansoni* genome sequence supercontig Smp_scaff006942	2.00E-15
P10-20.11.09_G1	Control	44-147	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P1-20.11.09_D7	Control		Vector	
P12-19.11.09_A3	Control	0-140	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	3.00E-22
P13-19.11.09_A7	Control	0-145	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	8.00E-18
P15-19.11.09_B2	Control	0-171	TSA: *Arachis hypogaea* CL1Contig9404.Arhy mRNA sequence	3.00E-11
P15-19.11.09_D2	Control	0-284	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	6.00E-22
P15-19.11.09_H4	Control	46-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	8.00E-22
P17-19.11.09_D2	Control	51-379	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	3.00E-65
P19-20.11.09_G7	Control	109	TSA: *Arachis hypogaea* CL1Contig10955. Arhy mRNA sequence	3.05E-17
P20-19.11.09_H11	Control	44-823	No decent hits (e-vals too high)	

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P2-20.11.09_H11	Control	47-149	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	4.00E-19
P3-17.11.09_F12	Control	102-139	*Heterocypris* sp. 18S ribosomal RNA gene, complete sequence	9.00E-12
P4-20.11.09_H2	Control	44-146	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P5-20.11.09_G7	Control	121-148	*Aphanomyces euteiches* cDNA	2.60E-01
P6-17.11.09_A7	Control	0-476	No decent hits (e-vals too high)	
P6-17.11.09_F11	Control	43-144	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	7.00E-22
P7-20.11.09_A1	Control	44-146	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	4.00E-19
P7-20.11.09_B1	Control	47-146	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	7.00E-22
P8-17.11.09_A1	Control	45-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-20
P8-17.11.09_B7	Control	0-161	TSA: *Arachis hypogaea* CL1Contig9735.Arhy mRNA sequence	3.00E-11
P8-20.11.09_B10	Control	124-150	*Aphanomyces euteiches* cDNA	2.00E-05
P8-20.11.09_H8	Control	46-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-20
P10-20.11.09_B6	Experimental	43-261	*Pinctada maxima* microsatellite M412 sequence	3.00E-19
P10-20.11.09_D3	Experimental		Vector	
P11-19.11.09_A1	Experimental	100-138	No decent hits (e-vals too high)	
P11-19.11.09_A10	Experimental	67-444	No decent hits (e-vals too high)	
P11-19.11.09_A11	Experimental	99-144	*Salmo salar* clone ssal-evd-536-300 Gamma-interferon-inducible lysosomal thiol reductase precursor putative mRNA, complete cds	2.00E-10
P11-19.11.09_A12	Experimental	120-146	No decent hits (e-vals too high)	

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P11-19.11.09_A2	Experimental	43-145	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	4.00E-19
P11-19.11.09_A3	Experimental	114-140	*Aphanomyces euteiches* cDNA	2.00E-05
P11-19.11.09_A5	Experimental	124-150	*Aphanomyces euteiches* cDNA	2.00E-05
P11-19.11.09_A7	Experimental	97-124	Populus EST from mild drought-stressed leaves	4.00E-06
P11-19.11.09_A9	Experimental	97-145	TSA: *Arachis hypogaea* CL1Contig14946. Arhy mRNA sequence	4.00E-12
P11-19.11.09_B1	Experimental	44-334	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	4.00E-64
P11-19.11.09_B10	Experimental	45-150	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	2.00E-24
P11-19.11.09_B11	Experimental	66-169	No decent hits (e-vals too high)	
P11-19.11.09_B12	Experimental	42-370	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	4.00E-64
P11-19.11.09_B2	Experimental	45-147	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	8.00E-22
P11-19.11.09_B3	Experimental	43-122	*Homo sapiens* mRNA; cDNA DKFZp779E152 (from clone DKFZp779E152)	3.00E-12
P11-19.11.09_B4	Experimental		Vector	
P11-19.11.09_B5	Experimental	118-144	*Aphanomyces euteiches* cDNA	2.00E-05
P11-19.11.09_B6	Experimental		Vector	
P11-19.11.09_B7	Experimental	44-152	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	2.00E-22
P11-19.11.09_B8	Experimental	0-123	No decent hits (e-vals too high)	
P11-19.11.09_C1	Experimental	43-147	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	1.00E-19
P11-19.11.09_C10	Experimental		Vector	
P11-19.11.09_C11	Experimental	43-143	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240)	1.00E-19

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P11-19.11.09_C12	Experimental	45-377	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	3.00E-65
P11-19.11.09_C2	Experimental		Vector	
P11-19.11.09_C3	Experimental	0-237	*Ostertagia ostertagi* mRNA for heat shock protein 20 (hsp20 gene)	6.00E-22
P11-19.11.09_C4	Experimental	45-394	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	1.00E-83
P11-19.11.09_C5	Experimental	51-131	No decent hits (e-vals too high)	
P11-19.11.09_C6	Experimental	45-185	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	1.00E-21
P11-19.11.09_C7	Experimental		Vector	
P11-19.11.09_C8	Experimental	43-368	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	2.00E-69
P11-19.11.09_C9	Experimental	45-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P11-19.11.09_D1	Experimental	42-141	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P11-19.11.09_D10	Experimental	0-203	TSA: *Arachis hypogaea* CL1Contig13911.Arhy mRNA sequence	2.00E-07
P11-19.11.09_D12	Experimental		Vector	
P11-19.11.09_D2	Experimental	43-143	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240	6.00E-17
P11-19.11.09_D3	Experimental		Vector	
P11-19.11.09_D4	Experimental	0-950	*Dicentrarchus labrax* chromosome sequence corresponding to linkage group 1, top part, complete sequence	8.00E-12
P11-19.11.09_D6	Experimental	84-204	No decent hits (e-vals too high)	
P11-19.11.09_D8	Experimental		Vector	
P11-19.11.09_D9	Experimental	43-135	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	1.00E-17
P11-19.11.09_E1	Experimental	0-147	No decent hits (e-vals too high)	

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P11-19.11.09_E10	Experimental	101-149	No decent hits (e-vals too high)	
P11-19.11.09_E11	Experimental	43-146	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P11-19.11.09_E12	Experimental	86-335	No decent hits (e-vals too high)	
P11-19.11.09_E2	Experimental	46-145	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	4.00E-25
P11-19.11.09_E3	Experimental	44-201	*Pinctada fucata* 28S ribosomal RNA gene, partial sequence	1.00E-21
P11-19.11.09_E4	Experimental		Vector	
P11-19.11.09_E5	Experimental	43-283	TSA: *Pinctada maxima* PmaxCL270Contig1, mRNA sequence	3.00E-63
P11-19.11.09_E6	Experimental	45-147	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-20
P11-19.11.09_E8	Experimental	44-154	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240)	3.00E-27
P11-19.11.09_E9	Experimental		Vector	
P11-19.11.09_F1	Experimental	0-144	*Oreochromis mossambicus* ribosomal protein L9-like mRNA, partial sequence	2.00E-11
P11-19.11.09_F10	Experimental	45-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	6.00E-23
P11-19.11.09_F11	Experimental	43-142	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P11-19.11.09_F12	Experimental	0-117	No decent hits (e-vals too high)	
P11-19.11.09_F2	Experimental		Vector	
P11-19.11.09_F3	Experimental	39-505	Mouse DNA sequence from clone RP23-259J23 on chromosome 11 Contains a ribosomal protein S17 (Rps17) pseudogene, complete sequence	9.00E-05
P11-19.11.09_F4	Experimental	74-500	No decent hits (e-vals too high)	
P11-19.11.09_F5	Experimental		Vector	

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P11-19.11.09_F6	Experimental	99-228	No decent hits (e-vals too high)	
P11-19.11.09_F7	Experimental	52-341	No decent hits (e-vals too high)	
P11-19.11.09_F8	Experimental	43-369	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	3.00E-65
P11-19.11.09_F9	Experimental	48-149	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	2.00E-23
P11-19.11.09_G1	Experimental	1-102	*Homo sapiens* 12 BAC RP11-686G8 (Roswell Park Cancer Institute Human BAC Library) complete sequence	1.00E-05
P11-19.11.09_G10	Experimental	0-950	No decent hits (e-vals too high)	
P11-19.11.09_G12	Experimental	43-142	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	1.00E-19
P11-19.11.09_G2	Experimental	47-189	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	6.00E-19
P11-19.11.09_G3	Experimental	47-151	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240)	1.00E-20
P11-19.11.09_G4	Experimental	124-150	*Aphanomyces euteiches* cDNA	2.00E-05
P11-19.11.09_G5	Experimental	42-136	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	1.00E-19
P11-19.11.09_G6	Experimental	46-196	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	7.00E-19
P11-19.11.09_G7	Experimental		Vector	
P11-19.11.09_G8	Experimental		Vector	
P11-19.11.09_G9	Experimental	43-369	*Pinctada maxima* mitochondrial gene for 16S rRNA, 3' partial sequence, country:Philippines	3.00E-65
P11-19.11.09_H1	Experimental	48-338	No decent hits (e-vals too high)	
P11-19.11.09_H10	Experimental	0-186	Populus EST from leave	1.00E-16
P11-19.11.09_H11	Experimental	46-153	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	7.00E-23

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P11-19.11.09_H12	Experimental	0-256	No decent hits (e-vals too high)	
P11-19.11.09_H3	Experimental	45-153	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P11-19.11.09_H4	Experimental	42-225	*Pinctada margaritifera* gene for 18S rRNA, complete sequence, country:Japan:Okinawa	8.00E-57
P11-19.11.09_H5	Experimental	43-519	*Pinctada maxima* mitochondrial gene for 12S rRNA, partial sequence, country:Japan: Okinawa, Aka Island	0.00E+00
P11-19.11.09_H6	Experimental	44-151	TSA: *Arachis duranensis* DurSNP_c10895. Ardu mRNA sequence	8.00E-25
P11-19.11.09_H7	Experimental		Vector	
P11-19.11.09_H9	Experimental	113-139	*Aphanomyces euteiches* cDNA	5.00E-05
P1-17.11.09_H4	Experimental	46-147	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	4.00E-19
P1-20.11.09_C1	Experimental	43-149	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	8.00E-22
P1-20.11.09_D10	Experimental	98-439	*Pinctada margaritifera* clone ac11 microsatellite sequence	4.00E-08
P1-20.11.09_D9	Experimental	0-269	*Pinctada martensi* clone 03-46 tissue-type heart microsatellite sequence	6.00E-23
P1-20.11.09_E1	Experimental		Vector	
P1-20.11.09_F11	Experimental	48-180	*Ompok pabo* voucher OB-02-CG-NBFGR-LKO 28S ribosomal RNA gene, partial sequence	1.00E-14
P12-19.11.09_B7	Experimental		Vector	
P12-19.11.09_B9	Experimental	66-411	No decent hits (e-vals too high)	
P12-19.11.09_F6	Experimental		Vector	
P12-19.11.09_F7	Experimental		Vector	
P12-19.11.09_H4	Experimental	43-950	No decent hits (e-vals too high)	
P13-19.11.09_C1	Experimental	89-341	TSA: *Pinctada maxima* PmaxCL440Contig1, mRNA sequence	0.007

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P13-19.11.09_D1	Experimental	65-362	*Pinctada margaritifera* clone ac11 microsatellite sequence	9.00E-09
P13-19.11.09_E9	Experimental	43-144	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240)	1.00E-24
P13-19.11.09_F2	Experimental		Vector	
P13-19.11.09_H12	Experimental	44-291	*Pinctada maxima* clone JCUPm 3_a12 microsatellite sequence	9.00E-08
P13-19.11.09_H2	Experimental	121-171	*Homo sapiens* mRNA; cDNA DKFZp781I1086 (from clone DKFZp781I1086)	4.00E-06
P13-19.11.09_H3	Experimental	43-94	TSA: *Arachis hypogaea* CL1Contig12162. Arhy mRNA sequence	1.00E-12
P13-19.11.09_H4	Experimental	42-123	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	2.00E-24
P14-19.11.09_E5	Experimental	0-950	Vector	
P15-19.11.09_B3	Experimental	43-282	Secondary symbiont of Sitobion miscanthi clone Jiangyou 16S ribosomal RNA gene, partial sequence	3.00E-42
P15-19.11.09_B5	Experimental	89-476	*Pinctada martensi* clone pearlsac03-32 microsatellite sequence	1.00E-08
P15-19.11.09_C4	Experimental	0-160	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929) [LysM, putative peptidoglycan-binding, domain containing 4]	3.00E-11
P15-19.11.09_F3	Experimental	44-141	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	7.00E-22
P15-19.11.09_F8	Experimental	88-366	No decent hits (e-vals too high)	
P16-19.11.09_C12	Experimental		Vector	
P16-19.11.09_C2	Experimental	44-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	5.00E-24
P16-19.11.09_D1	Experimental	99-146	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	8.00E-22
P16-19.11.09_D4	Experimental	47-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	7.00E-22
P16-19.11.09_E3	Experimental		Vector	
P16-19.11.09_F5	Experimental	83-518	No decent hits (e-vals too high)	

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P17-19.11.09_A4	Experimental	43-167	*Nodipecten nodosus* 28S ribosomal RNA gene, partial sequence	6.00E-12
P17-19.11.09_E7	Experimental	0-123	*Prodontorhabditis wirthi* strain DF5074 28S large subunit ribosomal RNA gene, partial sequence	3.00E-09
P17-19.11.09_F1	Experimental	49-138	TSA: *Arachis hypogaea* CL1Contig14645. Arhy mRNA sequence	1.00E-11
P18-19.11.09_B3	Experimental	45-148	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	6.00E-23
P18-19.11.09_D7	Experimental	43-121	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	5.00E-22
P18-19.11.09_E1	Experimental	42-143	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	6.00E-23
P18-19.11.09_E5	Experimental	100-126	*Aphanomyces euteiches* cDNA	2.00E-05
P18-19.11.09_E6	Experimental	98-144	*Rana catesbeiana* clone rcat-evr-518-076 Nucleoplasmin putative mRNA, complete cds	8.00E-07
P18-19.11.09_F9	Experimental	82-392	*Pinctada margaritifera* clone ac11 microsatellite sequence	6.00E-11
P18-19.11.09_G2	Experimental	44-145	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	7.00E-22
P18-19.11.09_G4	Experimental	43-234	TSA: *Arachis hypogaea* CL1Contig12314. Arhy mRNA sequence	4.00E-11
P18-19.11.09_H10	Experimental	99-246	*Bombyx mori* BNGR-A32 mRNA for neuropeptide receptor A32, complete cds	1.00E-12
P19-19.11.09_C2	Experimental	47-147	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	7.00E-22
P19-19.11.09_C6	Experimental	90-404	No decent hits (e-vals too high)	
P19-19.11.09_E7	Experimental	0-950	No decent hits (e-vals too high)	
P19-20.11.09_F9	Experimental	122-148	*Aphanomyces euteiches* cDNA	2.00E-05
P19-20.11.09_G1	Experimental	0-912	TSA: *Pseudochattonella farcimen* UiO 109 assembled mRNA CL28Contig1	2.00E-15
P20-19.11.09_A2	Experimental	44-414	No decent hits (e-vals too high)	
P20-19.11.09_F6	Experimental	44-685	*Pinctada martensi* clone 11-32 microsatellite sequence	1.00E-36

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P2-20.11.09_E2	Experimental	0-950	No decent hits (e-vals too high)	
P2-20.11.09_G1	Experimental	0-448	No decent hits (e-vals too high)	
P2-20.11.09_G7	Experimental	44-70	*Aphanomyces euteiches* cDNA	2.00E-05
P2-20.11.09_H1	Experimental	43-134	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	8.00E-21
P3-17.11.09_G2	Experimental	44-718	*Pinctada martensi* clone foot08-46 microsatellite sequence	8.00E-08
P3-20.11.09_B12	Experimental	125-151	*Aphanomyces euteiches* cDNA	2.00E-05
P3-20.11.09_E5	Experimental		Vector	
P3-20.11.09_H10	Experimental	101-182	TSA: *Arachis duranensis* DurSNP_c71468. Ardu mRNA sequence	3.00E-17
P3-20.11.09_H5	Experimental	123-149	*Aphanomyces euteiches* cDNA	2.00E-05
P4-20.11.09_A12	Experimental	191-216	Populus EST from leave	5.00E-05
P4-20.11.09_H4	Experimental	43-146	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	3.00E-21
P5-7.12.09_F10	Experimental	42-222	No decent hits (e-vals too high)	
P5-7.12.09_F9	Experimental	43-140	*Pongo abelii* mRNA; cDNA DKFZp469O0929 (from clone DKFZp469O0929)	4.00E-19
P6-17.11.09_B2	Experimental	45-212	No decent hits (e-vals too high)	
P6-17.11.09_C7	Experimental	46-569	TSA: *Pinctada maxima* PmaxCL486Contig1, mRNA sequence	1.30E-53
P6-20.11.09_D12	Experimental	46-149	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	6.00E-23
P6-20.11.09_E9	Experimental	0-950	*Dracaena cambodiana* clone DC522 microsatellite sequence	1.00E-04
P6-20.11.09_F1	Experimental	44-156	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240) [pancreatic lipase-related protein 1]	1.00E-26
P7-17.11.09_C3	Experimental	45-341	*Bombyx mori* BNGR-A32 mRNA for neuropeptide receptor A32, complete cds	9.00E-53

Library Clone ID	Origin of clone for sequencing	Position of overlap in hit sequence	Top hit (blastn, somewhat similar seqs, nr db)	e-value
P7-20.11.09_A9	Experimental	42-666	*Pinctada martensi* clone 11-32 microsatellite sequence	2.00E-20
P7-20.11.09_B11	Experimental	90-343	No decent hits (e-vals too high)	
P7-20.11.09_D4	Experimental		Vector	
P7-20.11.09_F10	Experimental	44-663	No decent hits (e-vals too high)	
P7-20.11.09_G5	Experimental	45-686	*Pinctada martensi* clone 11-32 microsatellite sequence	1.00E-36
P8-17.11.09_F1	Experimental	90-227	No decent hits (e-vals too high)	
P8-17.11.09_F10	Experimental	46-390	*Pinctada martensi* clone M5-42 microsatellite sequence	4.00E-26
P8-20.11.09_E9	Experimental		Vector	
P8-20.11.09_F4	Experimental		Vector	
P8-20.11.09_F5	Experimental	44-149	*Homo sapiens* mRNA; cDNA DKFZp779I1240 (from clone DKFZp779I1240)	1.00E-20

13.6 Media release

A study by researchers at WA Department of Fisheries and Macquarie University in Sydney has uncovered important genetic information about the Western Australia's most valuable aquaculture species – silver lipped pearl oysters, which produce South Sea pearls. WA is the world's top producer of silver lipped pearl oysters, with the industry worth an average of about $120 million annually.

The research project, funded by the pearling industry and the Fisheries Research and Development Corporation, used DNA microarray technology to develop a molecular test to assess the health status of pearl oysters.

Principal investigator, Department of Fisheries Senior Fish Pathologist Dr Brian Jones, said DNA microarray technology was used to identify stress markers in pearl oysters *(Pinctada maxima)*. This helped researchers to identify processes in oyster farming that stress oysters at a molecular level.

Dr Jones said the project had generated a large amount of new information about pearl oysters, which the project team has entered onto a worldwide database.

"Putting the DNA sequence material into an international database will mean that the information we uncover cannot be patented, and therefore will be freely available to scientists and pearling industries worldwide," Dr Jones said.

"The silver lipped pearl oyster is an iconic WA species and supports our most valuable aquaculture industry. We are delighted that the FRDC and the pearling industry have supported this project."