Host transcriptomic response to oyster oedema disease (OOD) in *Pinctada maxima*

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All other research described in this report is my own original work.

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Table of Contents

Abstract

Pinctada maxima is the most commercially important pearl oyster species in Australia. However production is currently suffering from oyster oedema disease (OOD), the symptoms of which include oedema of interstitial tissues resulting in swelling of tissues such as the kidney and mantle. There have been two well documented mass mortality events linked to OOD, one in the summer of 2006 and another in 2013, but a definitive cause is yet to be identified.

Here, the goal was to analyse the host transcriptomic response to disease, to better classify and understand OOD, and to test whether anti-viral responses were evident in OOD-affected oysters. RNA-seq next generation nucleotide sequencing analysed the host transcriptome of OOD-affected and OOD-unaffected *P. maxima* and a comparison of transcriptional responses identified a number of up- and down-regulated genes. These genes were further classified into functional biological pathways regulated during the disease state, providing an insight into the causative agent of OOD. Differentially expressed genes were able to be segregated into two main categories – general stress response related genes and immune response/wound healing related genes. Although the response was not characteristic to a viral infection, therefore ruling out a viral stressor, the response profile was not able to further implicate what remaining stressor could be the cause of OOD.

1. Introduction

Worldwide aquaculture production has increased dramatically over the last few decades. It is now comparable to the aquatic wild-harvest industry, at a production rate of 90.43 million tonnes per annum [1]. The rapid development of the global aquaculture industry has largely been a response to the growing food crisis and inability to maintain sustainable wild populations of aquatic species [2]. Shellfish, such as pearl oysters, have multiple uses as aquaculture species. They are reared not solely for food, but also for the harvest of non-edible products such as pearls [3]. This separates shellfish from other typical aquaculture species, as they have a place in multiple markets. Worldwide, approximately 27% of total aquaculture production is attributed to shellfish aquaculture [4]. However, after a period of rapid growth and expansion, the shellfish aquaculture industry is experiencing a major reduction in production, both due to economic pressure and a lack of major advancements in management practices, resulting in issues such as mass mortalities from diseases [5].

High production aquaculture industries are especially prone to mass mortalities, which lead to substantial economic loss. For example, shrimp white-spot disease plagued South East Asia in the early 1990s, with losses amounting to several billion dollars [6]. One major contributor to these mass mortality events are diseases driven by pathogenic agents. This is especially true for the shellfish aquaculture industry. Jones and Creeper [7] tallied many pathogens implicated in production losses among shellfish species, including *Vibrio spp.* in pearl oysters, haplosporidan parasites in rock oysters, microsporidan parasites in mussels, nematodes in scallops; and trematodes and *Perkinsus spp.* in abalones. In addition to pathogenic agents, the diseases suffered by aquaculture shellfish can also be caused by environmental factors [8], poor management practices [9], or a combination of factors [10]. For example, in 1987, mass mortality events of the pearl oysters (*Pinctada maxima*) in Western Australia were attributed to mass infection by *Vibrio spp.* resulting from sub-optimal management practices [11].

The diversity of disease-causing agents and affected species pose a problem with respect to disease identification. In many situations, extensive studies may be required to elucidate and validate the cause of a disease. In France, the first attempt to identify the cause of summer mortalities of juvenile *Crassostrea gigas* in the Bay of Morlaix failed to conclusively implicate any infectious agents, leading to an entirely different approach

required to eventually identify a *Vibrio* strain as the infectious agent [12]. In other cases of shellfish diseases, the causes remain completely unidentified [13]. Oyster oedema disease (OOD) [14] in pearl oysters is one such uncharacterised disease. OOD has caused mass mortalities in populations of farmed *P. maxima* in various areas of northern Australia. However, no conclusive cause of OOD has been identified through traditional approaches [15, 16]. Without a clear understanding of the aetiology of the disease, the formulation of improved management practices, cures or preventions is difficult, and alternative methods to analyse the cause of disease that can overcome the drawbacks of the traditional methods are required.

The analysis of host transcriptomic responses to disease is one such alternative method. Examination of the host transcriptome can help improve our understanding of disease states, identify common responses exhibited by different species, and help design more robust methods to identify and combat disease.

1.1 Management responses to disease

The prevalence of diseases result in constant shifts in management practices. Adapting practices to the presence of diseases to avoid mass mortality events involves three main strategies. The first is to improve animal husbandry techniques. For example, major improvements in survivability of pearl oysters came about after a mass mortality event in 1987 [11], when it was discovered that transportation practices back then placed undue stress on the oysters, resulting in greater susceptibility to infection. By altering transportation practices, the oysters were able to better cope with *Vibrio spp.* infections and therefore greatly reduced mortality.

The second management strategy involves the application of therapeutic chemicals as a prophylactic measure against infectious agents. This has been applied successfully to protect various aquaculture species, primarily fish, from serious biotic agents such as ectoparasites in *Piaractus mesopotamicus* [1] and *Streptococcus iniae* in hybrid striped bass [17]. However, therapeutic chemicals have yet to be widely explored in shellfish aquaculture [18]. Therapeutic chemicals in the aquatic environment can also negatively affect native organisms such as local microalga [19]. In addition, residual therapeutic chemicals can result in the development and retention of disease resistance in microbes, making disease outbreaks more difficult to treat [20-22].

The third strategy to control disease involves selective breeding for individuals with certain traits, such as disease resistance or accelerated growth. This is sometimes seen as a preferable alternative to therapeutic chemicals, because selection for disease-resistant individuals negates the necessity for repetitive usage of therapeutic chemicals [23, 24]. Selective breeding allows for disease resistance and other desirable traits to be established in the population and transferred from one generation to another, such as with the protection against the protistan parasite *Bonamia ostreae* by the oyster species *Ostrea edulis* in Rossmore, Ireland [25]. One disadvantage is the selection for particular traits often involves evolutionary trade-offs with other desirable characteristics. Long term inbreeding can, for example, cause reduction in yield and growth rate in the Pacific oyster *C. gigas* [26].

1.2. Disease identification and examination

In most cases, improvements in management practices need to be based on an understanding of the cause of a disease. The most common method used to identify the cause of a disease in shellfish aquaculture is histopathology. Here, the histopathological symptoms of the disease are used to identify potential causes, and each cause is investigated in turn. This can allow the direct identification of disease causing microbes. Histopathology has been varyingly successful in identifying the cause of disease in molluscs, dependent on the disease's aetiology. The major drawback of histopathology is that a lack of discovery may not indicate absence of pathogenic agent(s) [12].

Transcriptomic analysis of the host responses to disease now represents one of a number of alternatives to histopathology for disease identification. Many advancements have been made in genetic technologies over the last decade, making it possible to potentially identify the pathogenic agent/s responsible for the disease. Rather than identifying visible signs of a disease, whole transcriptomic analysis focuses instead on the host gene response to the disease, and how expression of specific genes changes under different stresses and disease states. Analysis of the host transcriptomic responses to diseases does not require focusing on investigating one cause at a time, and therefore has a higher potential for discovery of the cause of disease, regardless of whether the cause is a biological agent [27-29] or an abiotic factor [30]. This broadens the range of aetiological agents that can be identified using these methods. Whole transcriptome sequencing provides a broad understanding of the biology and aetiology of disease, based on host responses [31].

Finally, since shellfish are sessile or semi-sessile organisms, mass mortalities can occur very rapidly [32]. A diagnostic tool developed through understanding a host's transcriptional response to a disease allows for early detection of diseases, which is extremely beneficial to shellfish aquaculture industries.

1.3. Common technologies for host transcriptomic response analysis

Analysis of the host transcriptomic responses at various stages of different diseases has been done in numerous studies, and on a wide range of species, and therefore has broad applications. For instance in animals, the transcriptome analysis of the host response of non-human primates to an infection by the virus H5N1 has indicated that the severity of disease is dependent on differential gene expression during the early and late phases of disease [33]. In plants, the analysis of the *Rehmannia glutinosa* leaf transcriptome suffering from replanting disease found that there were excessive differential expression of genes involved in both the ethylene signalling and metabolism pathways [34].

Initially, transcriptome analyses were undertaken on a gene by gene basis. However, many multi-gene analytical methods have now been established. One well established tool for transcriptomic analysis is cDNA microarrays, which have the capability to quantify the expression levels of many genes of interest [35]. However, there are a number of disadvantages to utilizing microarrays. The design of microarray probes requires sequence information of genes known to be involved with the disease. Such tools requiring gene-specific probes are therefore only effective when specific sequence information is available. The amount of sequence data in the public domain for most shellfish aquaculture species is limited and therefore only a subset of genes can be analysed without additional sequencing projects. In addition, gene products are known to undergo substantial changes in terms of deletions, additions and realignments to form the final gene product. The purpose of these changes is to confer specific functions to the resultant strand that may not be available otherwise, such as for mediators of RNA-protein interactions [36]. Finally, the cost of transcriptome-wide screening by microarrays can be impractical, as the number of probes required can be very high. This also applies to similar technology such as in situ hybridization [37-39], which generally requires known expressed sequence tags [40] or cDNA sequences to act as a basis for hybridization. Additionally, there is a likelihood for cross-hybridization, which results in 'background noise' (visible cues for expression levels that are actually false readings) [41, 42].

Other methods that are commonly used for the transcriptomic analysis of disease are serial analysis of gene expression [43, 44], cap analysis of gene expression [45-47] and other technologies [24, 48, 49]. These approaches have the advantage of being high throughput and they allow for quantification of expression levels. However, they are based on Sanger sequencing technology, and so are generally costly and do not have the ability to distinguish between gene isoforms.

The most powerful and encompassing technology for host transcriptomic analysis is next-generation RNA-seq technology. This method is capable of transcriptome-wide analysis of gene responses without prior sequence information [50]. Next-generation RNA-seq technology is a novel high-throughput tool that produces large amounts of sequence data allowing transcriptome-wide analysis of RNA expression levels [50].

By sequencing the entire transcriptome, RNA-seq technology broadly identifies major differences in expression levels for many gene pathways between samples, providing substantially more comprehensive information on host transcriptomic responses than any other tool. In addition, RNA-seq allows for the discovery of novel genes and/or transcripts [51]. This insight into the biological machinery of a host substantially increases the amount of information available, including differences in expression levels of alternative splicing of transcripts [52] and isoforms [53, 54].

Its relative high cost is the singular limitation of utilizing RNA-seq technology. This forces researchers to be highly selective of the targets of their study, and generally they cannot select as many test subjects as desirable. This tends to limit the scope of a study, when experiments have to focus on a particular stage of a host's life cycle or a particular stage of a disease. Even when studies have been done on the same species at different stages of their life, the specifics may not be directly comparable [55, 56].

1.4. Response profiles

For RNA-seq technology to allow the aetiology of unknown diseases to be identified through the discovery of transcriptomic response of hosts, the transcriptomic response profile identified through RNA-seq analysis must be matched to response profiles to known stressors. A response profile to a specific stressor must contain a list of genes that are

differentially expressed in response to the stressor, so that a match to the response profile would then implicate the stressor as the cause of disease (and a limited or non-existent match would indicate otherwise). Response profiles must fulfil a number of requirements. Firstly, profiles must be distinct enough to allow for differentiation between stressors. The specificity will depend on the individual stressors, and whether or not genes exist that can be utilized across species to determine the source of stresses. Secondly, profiles must contain enough information to allow for comparison with results of existing transcriptomic studies, so that the tested response event can be determined as being consistent or inconsistent with previous response profiles.

A transcriptomic response study will yield its own list of up- or down-regulated genes/gene products, and when matched against these response profiles, can determine or at least narrow down the stressor involved in uncharacterised diseases. Figure 1 illustrates the overlap between genes that are regulated in response to varying stressors by *C. gigas*. In this study, 80 unique genes were found to be expressed only in response to extremes of temperature, and not to altered salinity, metal contamination or air exposure. Therefore, a host response profile matching those 80 unique genes in diseased animals would implicate temperature as a contributing factor. This is similar for all other stressors.

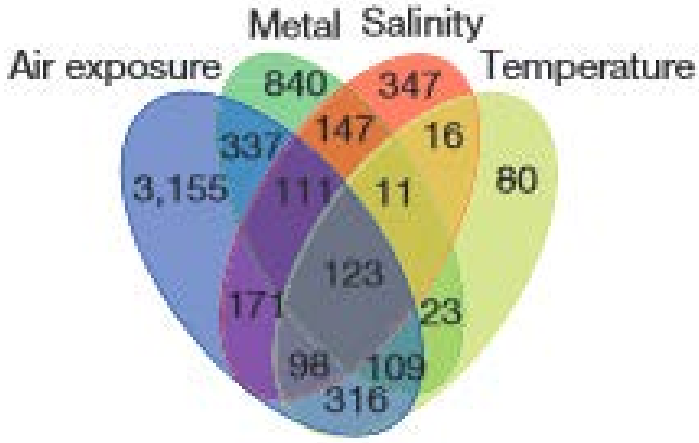


Figure 1: Adapted from Zhang et al. [57]. Venn diagram of common and unique genes expressed by *C. gigas* in response to temperature, salinity, air exposure and heavy-metal stress (zinc, cadmium, copper, lead and mercury), showing overlap of responses.

One current gap in our knowledge is that many specific response profiles remain unknown. The study by Zhang et al. on *C. gigas* only investigated four potential stressors [57], whereas there are many other stressors, including infectious agents that can affect productivity. In such cases, the number of unique genes found to be expressed in response to specific stressors will be limited.

Additionally, many response studies do not meet the criteria for forming response profiles. Studies that have managed to identify a large range of differentially expressed genes, or broad biological pathways, may not be specific enough to generate response profiles for diagnostic purposes. For example, Fu et al. was able to identify five major biological pathways differentially expressed in response to thermal stress in the Zhikong scallop *Chlamys farreri* [58]. However, they did not investigate specific genes/gene products in detail limiting the diagnostic value of the study. Many studies also lack information on the direction (up or down regulation) of the differential expression [59, 60], which may be important in dictating the presence of a disease state.

1.5. Transcriptomic response of shellfish to stressors

In the shellfish aquaculture industry, the focus of transcriptomic response studies has been on high value species, including oysters, mussels, scallops and abalones [61]. Overall, the research has been focused on gene discovery and broader expression differences, as opposed to uncovering specific genes that can act as diagnostic markers. Table 1 lists examples of commonly studied stressors, and the response of the host to the stressors. Although there have been studies of responses to both abiotic and biotic factors, the focus has been on abiotic stressors, predominantly environmental contaminants [62-65] and changing water quality parameters [58, 66-68].

Table 1: Differentially expressed genes in host responses to various commonly studied stressors of shellfish. Expression is marked with either a downward or upward arrow, to respectively identify down/up-regulation of the gene, gene family or biological pathway involved (if it was stated in the study). Those that were unclear/unstated are marked as '-'.

Stressor	**Differentially expressed genes/gene products**	**Species**
Abiotic		
Thermal	Apoptosis regulation-related genes (↓) mRNA binding-related genes (↓) Mitochondrial envelope formation-related genes (↓) Oxidation reduction-related genes (↓) Cytoskeletal protein binding/chaperones (↑)	*Chlamys farreri* [58]
	Translation-related genes (↓) Protein folding related genes (↑) Genes involved in chitin metabolism (↑)	*Mytilus galloprovincialis (Lam.)* [67]
Copper	*NADH2* gene (↓ 1-4 days, ↑ day 6 and 8) Heat shock proteins (molecular chaperones) (↑) GPx (a peroxidase enzyme) (↑) Cavortin (↑) Pernin (↑) Ferritin (↑) *Phr1* gene (↑) *IGF1* gene (↑) Alpha tubulin (↑) *EF1A* gene (↑) Tributylin binding protein type 1 (↑) Cellulase (↓)	*Argopecten purpuratus* (post-larvae) [65]
	Toll-like receptors (-) NOD-like and RIG-like receptors (-) Apoptosis pathway (-) Lysosome and C-type lectin (-)	*Mizuhopecten yessoensis* (gills) [63]

Copper (continued)	NADH dehydrogenase subunit 4 (↑ <24h, ↓ 24-168h) Ferritin (↓) Laminin (↓) Senescent protein (↓) *EF1α* gene (↓) EF2 genes (↓) Calponin 2 (cytoskeleton structural protein) (↓) *vdg3* gene (↓) LPr1 (signalling/multifunctional scavenger molecule) (↓)	*Haliotis rufescens* [64]
Cadmium	ABC, HSP and CYP protein families (↑) ADH dehydrogenase (↑) ATPase (↑) cytochrome c oxidase-related genes (↑) cytochrome P450 families (↑)	*M. yessoensis* [62]
Ocean Acidification (CO2)	Chitinase (catalyze the degradation of chitin polymers) (↓) Calponin-like protein (↓) TYR1 (a tyrosinase) (↑) F-ATPase subunits (from F.sub.O] and the [F.sub.1] complex) (↓) EFalpha (support binding of aminoacyl tRNA to ribosomes) (↑)	*Mytilus edulis* [66]
Salinity	*LTrpC-8* gene (↓) Na/Pi-cotransporter (↓) C-type lectin (↑) thioester-containing protein (↑) C1q domain containing proteins (↑) Molecules related to antimicrobial activity (↓) Heat shock protein 70 (↑) Ca^{2+}-binding proteins (↓) Apoptosis (↓) Ankyrins (↑) Tubulin (↑) Actin (↑) MEGF10 (↓)	*Crassostrea gigas* [68]

Biotic		
Bacterial	Scavenger receptors cysteine-rich (SRs) (-) C1qDC proteins (-) C-type lectins (-) Serine protease inhibitor-2 (↑) Glutathione s-transferase (-) Cytochrome p450 (-) Heat shock proteins (molecular chaperones) (-) Inhibitor of apoptosis (IAP) (-) GTPase of the immunity-associated protein (GIMAP) (-) epididymal secretory protein E1 (-) Cadherin (-) Legumain (-) vdg3 gene (-) Dermatopontin 2 (-) Apextrin (-) Furin (↑) Interleukin 17 (IL-17) (-) Arginase (nitric oxide modulator) (-)	*Crassostrea virginica* [59]
Viral	Myeloid differentiation 88 (MyD88) (↑) Interferon induced protein 44 (IFI44) (↑) Glypican (Gly) (↓) Inhibitor of nuclear factor kappaB kinase beta (IkB2) (↑) Inhibitor of Apoptosis (IAP) (↑)	*C. gigas* [69]
Combined Stressors		
Heat and nickel	Proteolysis-related genes (↑) Genes encoding small molecular chaperones (↑) Genes involved in the chitin metabolic process (↑)	*M. galloprovincialis* [70]
Heat and copper	Translation-related genes (↑) Genes encoding heat shock proteins (molecular chaperones) (↑) "Microtubule-based movement" proteins (↑)	*M. galloprovincialis (Lam.)* [67]
Heat and salinity	Molecular chaperones (↑) NADH dehydrogenase (-) Arginine kinase (-) Actin-binding regulatory proteins (-) GTPases (-) Mitogen-activated protein kinases (-)	Mytilus spp. [60]

Oysters have been more intensively studied than other molluscs, with *C. gigas* being a model organism for many genome-wide studies. These studies have delved into the interplay between differentially expressed genes in response to various stressors. They have revealed that genes can be differentially expressed in response specifically to a particular stressor, or in response to multiple stressors (Figure 1). Pearl oysters in particular are sought after for their pearls, more so than many other molluscs. Therefore, in addition to stressors, there have also been transcriptomic studies of pearl oyster species to understand the mechanics behind biomineralization, and its relationship to both biotic and abiotic cues [71-73]

There are a number of genes, gene products and pathways that have been identified in multiple response studies. Molecular chaperones (heat shock proteins) are differentially expressed in response to thermal [58], salinity [60], copper [67], nickel [70] and bacterial [59] stressors, indicating that they play a wide, generalistic stress-response role. In almost all cases, there is an up-regulation of molecular chaperones in response to stress.

Apoptosis-related genes and gene products are also commonly differentially expressed, being involved in response to thermal [58], copper [63], salinity [60], and viral [69] stress., with viral apoptosis-related genes specifically belong to a singular family (IAP), characterized by amino-terminal baculovirus IAP repeats (BIRs) [74].

NADH dehydrogenases are among the most common differentially expressed genes in response to stress, even though their expression is temporally variable. In response to copper contamination, expression of NADH2 in post-larval *A. purpuratus* was found to decrease over the first four days of exposure, before increasing once again between day six and eight post-copper contamination [65]. In contrast, expression of NADH dehydrogenase subunit 4 in *Haliotis rufescens* under the same environmental conditions has been shown to increase within the first 24 hours, before decreasing expression between 24 and 168 hours [64]. This highlights the significance of addressing the various stages of both the host and disease cycle.

Anderson et al. [75] undertook a meta-analysis of the effects of environmental stress on edible to identify the most commonly affected genes by stressors. Overall, in response to stress, they found more up-regulation than down-regulation of genes. The most commonly differentiated genes were also identified, and the top ten are listed in Figure 2. Anderson et

al. also identified that in response to certain broad categories of stressors, biological pathways are affected differently. Genes involved in transcription and/or translation were more commonly differentially expressed in response to temperature than other stressors. In contrast, metabolism-related genes were more commonly differentially expressed in response to hypoxia and contamination than the other two stressors.

These authors were also able to identify a general response pathway, whereby response to stress induces energy production, which produces by-products (reactive oxygen species) that could lead to apoptotic cell death, if not controlled by molecular chaperones and the anti-oxidant system.

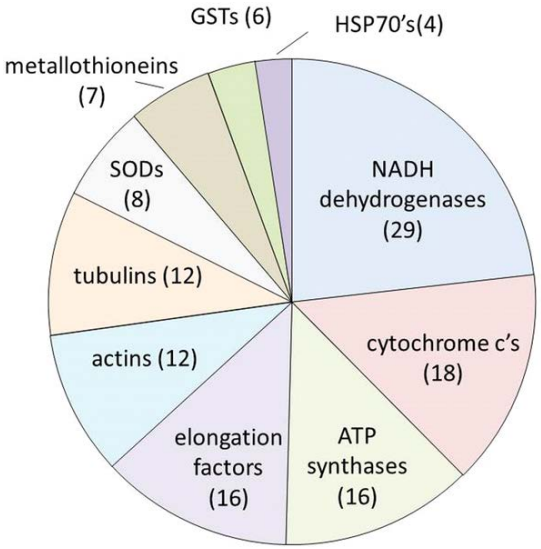


Figure 2: Adapted from Anderson et al. [75]. The top ten most commonly identified differentiated genes (not including those encoding ribosomal proteins) in response to ten different stressors. Numbers in brackets denote the number of times a gene was identified as differentially expressed in all treatments.

1.6. Shellfish response profiles to stressors

One clear finding of existing transcriptomic studies is that there are relatively unique genes/gene products that can be used to construct disease specific response profiles, particularly for viral diseases. Of the genes/gene products identified in Table 1, response to viral infection by *C. gigas* involves the up-regulation of two specific genes, myeloid differentiation factor 88 (MyD88) and interferon induced protein 44 (IFI44) [69]. These two genes are of significance, as they have been previously identified to be specific to viral infections [76-78]. Green and Montagnani [79] reported in addition toll-like receptor (TLR)

and protein kinase R (PKR) as also up-regulated in response to viral infection. A different study also found the inhibitor of Rel/NF-KB (IKB) and interferon-inhibiting cytokine (I), are also up-regulated during viral infections [80]. None of the genes/gene products involved in response to viral infection have been classified as up- or down-regulated in response to other stressors, including biotic stresses such as bacterial infections (Table 1). In non-shellfish models, interferon has also been implicated in responses to viral infections. For example, interferon is part of the response of *Marsupenaeus japonicus* to viruses such as white spot syndrome virus [81] and Taura syndrome virus [82]. However, the interferon involved in response to white spot syndrome virus by *M. japonicas* has been found to not be an alpha-interferon, but rather a mitochondrial F0-ATP synthase [83]. This could be the case in other organisms as well. Nonetheless, these data suggest that the current and main value of transcriptomic analyses of disease in molluscs is in the differentiation of viral diseases from those caused by other types of stress.

Table 2: A collated list of anti-viral responses in molluscs (*C. gigas* as a model organism), as reported by various authors.

Affected gene/gene products	**Response**	**Species**
Toll-like receptor (TLR)	Up-regulated	*Crassostrea gigas* [79]
MyD88	Up-regulated	*C. gigas* [69, 79]
Interferon regulation/induction (IRF/IFI44)	Up-regulated	*C. gigas* [69, 75, 79]
Protein kinase R (PKR)	Up-regulated	*C. gigas* [79]
Glypican (Gly)	Down-regulated	*C. gigas* [69, 75]
Inhibitor of nuclear factor kappaB kinase beta (IkB2)	Up-regulated	*C. gigas* [69, 80]
Inhibitor of Apoptosis (IAP)	Up-regulated	*C. gigas* [69]

On the other hand, bacterial infections and abiotic stressors induce very similar responses, as can be seen by response to bacterial infection by *Crassostrea virginica* in Table 1. Here, a C-type lectin, cytochrome p450, molecular chaperones, GIMAP, vdg3 gene and arginase are all shown to be differentially expressed mainly in response to thermal, metal and salinity stressors. Therefore, at the current state of knowledge, only transcriptional responses to viral infections are distinct enough for molluscs to generate a definitive response profile. The key genes involved in this antiviral response profile are shown in Table 2.

1.7. Transcriptomic response profile to OOD

In the current study, the Australian South Sea pearl oyster, *P. maxima*, has been chosen as the focus of differential gene expression analysis to develop a response profile for the currently uncharacterized disease, OOD. The goal is to determine whether OOD may be caused by viral infection.

Mass mortalities in populations of *P. maxima* have been reported in Western Australia since 2006 and no definitive causative agent has been identified to date. These mass mortalities have been attributed to oyster oedema disease (OOD), which Jones et al. [7] characterise by the reported loss of epithelial cells, mantle retraction and mild watery swelling (oedema) of the mantle tissues and palps, digestive gland, pericardial region and intestines of diseased *P. maxima* specimens. Affected oysters are slow to close their shells and high mortality (80 to 100%) can be evident, with thousands of freshly dead oyster shells (valves still attached at the hinge) present on cultured panels. No mortality was reported for other species settled on the affected panels (including other *Pinctada* species such as *P. margaritifera*).

Whilst OOD can be characterized by gross signs and histopathology, the cause of the disease remains unknown. No study has been able to show conclusively that the disease is caused by environmental or infectious factors (or a combination of the two) [15, 16]. One implication of a histopathological study that yields no conclusive results, is that the causative agent is not visible upon examination – such as is the case with many viruses. The enigmatic nature of OOD provides a valuable opportunity to functionally annotate and group transcriptomic data available for *P. maxima* suffering from this disease to identify potential gene families or pathways that can be informative in regards to identification of a causative agent. Specifically, comparison to known viral infection response profiles from other species provides the opportunity to test whether OOD is a viral disease.

Hence, the aims of the current study are to:

- Determine changes in gene expression in *P. maxima* associated with OOD.
- Identify specific pathways regulated during the disease state that may provide an indication of the causative agent of OOD, and specifically,
- Determine whether the transcriptomic response of *P. maxima* to OOD is characteristic of a viral infection or otherwise.

2. Materials and Methods

2.1. Sampling, RNA extraction and sequencing

A total of 20 *P. maxima* (adult production stock approximately 80-90 mm in size) were collected from two sites in north-western Australia in October 2013. The two sites were separated by approximately 600km of coastline. Additional site-specific information is confidential and cannot be disclosed by request of the pearling industry. Of these specimens, ten from one site fitted the formal case definition for OOD as defined by AusVet Animal Health Services and had obvious histopathological signs consistent with the criteria for this disease described in Jones et al. [16] (henceforth referred to as 'OOD-positive' specimens). The ten oysters from the other site did not fit the case definition, and did not have obvious histopathological signs of OOD (henceforth referred to as 'OOD-negative' specimens).

Tissue from five major organs (gills, gonads, mantle, foot and digestive tract) were collected from all 20 specimens. Immunocompetent cells in oysters are preliminarily hemocytes [84], which generally reside in high numbers in oyster gills [85, 86]. As such, the gills used for sample preparation would have contained large numbers of hemocyte suitable for analysis. Half of each tissue was stored in 4% formaldehyde in seawater for histopathological analysis to determine if oysters had clinical signs of OOD, and the other half was stored in *RNAlater*® (Ambion) at 4 °C overnight, then at -20 °C until RNA extraction. A modified Tri-Reagent (Sigma-Aldrich) protocol was used to extract RNA from the five tissues of each specimen [87].To ensure that the highest possible quality RNA was submitted for sequencing, a number of stringent quality control (QC) measures were undertaken including NanoDrop quantification and Illumina Bioanalyzer (Agilent) analysis.

Of the 20 specimens, four OOD-positive and four OOD-negative specimens were randomly selected and RNA from the five tissue types was pooled in equimolar amounts before being sent for library preparation and sequencing on an Illumina HiSeq 2000 platform at the Ramaciotti Centre for Genomics at the University of New South Wales. During library preparation using a TruSeq RNA Access kit, polyadenylation selection of mRNA molecules was undertaken and mRNA was fragmented to 200-300bp in length. mRNA fragments were then barcoded and pooled to be sequenced on one lane of Illumina's HiSeq 2000 platform (100bp paired-end reads).

2.2. Quality control

FastQC was used to access the quality of all eight libraries (4 OOD-positive specimens and 4 OOD-negative specimens), through an evaluation of various parameters. The ‘per base sequence quality’, ‘per sequence quality score’, ‘per base sequence content’ and ‘per base GC content’ were investigated to determine the quality of raw sequence data. For the eight libraries examined, a large portion of the reads were of high quality (bases with a quality score of 28 or above). A number of reads had quality scores that fell below the quality score of 28 and therefore trimming the reads based on their quality score was necessary.

QC of all sequences was conducted in Trimmomatic Version 0.32 [88] using the following parameters: a sliding window of 4:25, leading quality score trimming of 3, trailing quality score trimming of 3 and a minimum read length of 30. Trimming at this stringency ensured that the remaining reads were of high quality. The leading and trailing trimming scores were used to remove all bases of qualities 1 or 2, which are common unwanted artefacts from sequencing. This was verified by utilizing FastQC after quality trimming, to double-check the improved quality of the reads within each library.

Trimmomatic retained paired-end information of reads during sequence clean-up and removed entire sequences that post-enhancement consisted of lower than 30 base pairs. Some reads were retained post-enhancement, but were left without a complementary read. As sequence data is most valuable when existing as pairs, these sequences were ignored in further steps. Therefore, only paired, high quality sequences were kept for further analysis.

2.3. De novo assembly

As *P. maxima* does not have existing whole transcriptome or genome information available, the construction of the *P. maxima* transcriptome was performed *de novo* using the CLC Genomics workbench. First, the optimal kmer length was determined. The initial

assembly used an automated determination of the optimal kmer length. Once the kmer length provided by the automated software was obtained, kmer lengths both lower and higher in steps of 5 were tested manually (lengths 20, 25, 30, 35, 40, 45 and 50).

Two assembly procedures were also tested after defining the optimal kmer length of 25. The first procedure constructed a transcriptome using pooled data from all eight libraries at the optimal kmer length. The second procedure assembled libraries independently and removed redundancy in all assemblies resulting from the different kmer lengths using CD_Hit, a clustering software designed specifically for this purpose. A significance threshold of 0.95 was used, all other parameters were set to default. The output data showed that assembling all libraries using CD_Hit did not improve the quality of the final assembly. Therefore, the first procedure (simultaneous assembly of all libraries) was chosen to generate the assembly used in later steps.

2.4. RNA-seq analysis

RNA-seq analysis of the eight libraries (four OOD-positive versus four OOD-negative) was carried out in the CLC Genomics workbench. The analysis was performed for a number of candidate assemblies, chosen based on optimal values of contig length and quality. The RNA-seq analysis (legacy) tool from the CLC Genomics workbench was used to generate these initial analyses. The following parameters were chosen; a maximum number of mismatches of 2, minimum length fraction of 0.8, minimum similarity fraction of 0.8 and a maximum number of hits for a read of 20. All other parameters were set to default. This generated read count data for all eight libraries. The differential expression of contigs in the OOD-positive libraries versus the OOD-negative libraries was then undertaken in the CLC workbench. Empirical analysis of DGE (EDGE) was then performed to yield more statically precise results, allowing for better extraction of information on differential expression.

Contigs were deemed to be significantly differentially expressed between the OOD-positive and OOD-negative libraries when the FDR adjusted EDGE test p-value was <0.05. The differentiated contigs were then subjected to verification and manual annotation as described below.

RNA-seq results were subjected to principal component analysis (PCA) depicting either all contigs or the final subset of significantly differentially expressed contigs, relying on original expression values to group the eight libraries. Heat maps were also generated to graphically represent the expression levels of different contigs in all eight libraries, by sample clustering the libraries (again based on original expression values). Distance correlation (statistical dependence between two variables) is measured using Pearson correlation, with single linkage. Both the PCA and heat map were generated on the CLC Genomics workbench.

2.5. Annotation

Differentially expressed sets of transcripts were matched to known sequences (homologs) via Blast algorithms to determine the identity and putative functions of each differentially-regulated transcript. Blastx searches were performed against the NCBI Molluscan non-redundant protein sequence database.

Blast x annotations were then matched to Gene Ontology (GO) terms, through a mapping process that matched GO protein IDs to those sequences. Specific GO terms were then extracted from the pool for each mapped sequence, using default parameters (E-value of 1.0E-06, GO Weight of 5). This filtered the terms based on reliability and specificity. Domain/motif information was also extracted for each sequence within the blastx results, predicted through InterProScan 5.0 [89]. The scan processed sequences based on the presence of valid sequence strings.

Manual validation of the 147 most differentially expressed contigs was conducted to ensure their validity. The results were verified by cross-referencing with public databases, whereby the automated annotations was either validated and accepted, or modified if there was a discrepancy. For automated annotations without a clear result, manual annotation relied on Blasting against the NCBI non-redundant database first using blastn, before moving on to blastx if there are no conclusive results from the blastn similarity search.

2.6. Validation of expression levels

To accurately quantify the expression levels of genes of interest (identified through NGS), RT-qPCR was performed. This was to ensure that the semi-quantitative differential expression fold differences obtained from the RNA-Seq analysis were reliable.

Gene specific primers were designed using OligoCalc [90] for 15 genes that were found to be most differentially expressed in the RNA-seq analysis (see Table 3). Four reference genes were also chosen. A total of 24 primer sets were generated for the 15 contigs of interest and four potential reference genes, with two of the contigs of interest and three of the reference genes having a second primer set.

Table 3: Contigs of interest and RT-qPCR primer information. For the sequence information, 'F' denotes the forward primer, whilst 'R' denotes the reverse primer. A (Y) next to the primer name denotes the primer set chosen for the final validation of relative expression step.

Contig Number	Reference Gene?	Primer Name	Sequence (5'-3')	Amplicon Size
11368	No	P11368 (Y)	F: GATTCCTCACAAAGAAGCCTG R: GAATCGACCCATAATGATCCAC	83bp
12018	No	P12018	F: CTTCTTGGTGAACTGATAATCTG R: CTCTCTAATGTCCGCTCAAAG	90bp
1456	No	P1456-1	F: GACCTTTAACCTCACTTACCAG R: CTGTATGGCTACGTCAATCTTG	118bp
		P1456-2 (Y)	F: CTGTGATAGAACGACCAACAC R: CTGATCTGACTTCGCCCTC	83bp
14728	No	P14728	F: GATGCTGCCTTGTGAGAATG R: CATCCCAGCAGTGTCATAATG	110bp
30564	No	P30564 (Y)	F: CTGGTATAAGGTTCAACACGAG R: GTGTCCCATTCAACCAAACAG	110bp
3489	No	P3489	F: GACCCAACGACAGCGTTTG R: GAGTTCTGCGGATAATTCGTTC	94bp
3750	No	P3750 (Y)	F: GACGCGTGTCACGTACAG R: GATCCTATTGGTCATCGCAATC	112bp
3889	No	P3889 (Y)	F: GACCATATAGAAGGCAACCTC R: GTGCCACTCAAGGAAACTATC	87bp
48	No	P48	F: CACGACAACCGATACACCAG R: GACAGTTCGGTCCCTATCTG	104bp

6728	No	P6728	F: CTATGATGGCAAAGACGTGTC R: CACTTCCTCTACGATATGTATC	100bp
73	No	P73-1	F: GTATCGAATGGGAATTCATTGAC R: CATGCATTCCTCTTCAAGGATG	104bp
		P73-2 (Y)	F: CACTGCCAATAATCTGGAGAG R: GATTCTCTATTGACGCGATCTAC	111bp
73887	No	P73887 (Y)	F: GTTCCAATCAAATCTGCTTCATG R: GAACATCCACTGGGGAAATTAC	81bp
78929	No	P78929 (Y)	F: GAAATTGATGATGCAGAACCTTC R: CATCGTGCCATTTCCTTCATTC	90bp
78949	No	P78949 (Y)	F: CAAGGGACATTAGACTTTCCATC R: CTTTCCAGATATGATTACGTGTG	102bp
990	No	P990	F: CACCATCAATCGGTATCGAC R: GTGGTTCTGTTACCCTGGTC	103bp
1342	Yes	P1342-1	F: GAACATAATGAGAGCCAGTCTC R: GATCTATCCTCCTCTCCATC	89bp
		P1342-2 (Y)	F: GAAGATGGCAGCCACTGTC R: CAGTCCCAGAGCTTACTCAG	98bp
20	Yes	P20-1 (Y)	F: GTTGTCACCATGCCATCCAG R: GAGGCACGTTTCAGTGAAATTC	111bp
		P20-2	F: CTCTGACCTCAATTCAAACTGTG R: CAATAAGATGGACAGCACTGAC	115bp
21224	Yes	P21224	F: GAATTACCCGATGGTCAAGTC R: CACCTGGAGCGTTCAGTC	100bp
2161	Yes	P2161-1	F: CTGTGCAAGTTGTAGCTGTC R: CTTATACTGACCATGTGTAGAG	92bp
		P2161-2 (Y)	F: GTAGTGATTTCTGCTCCATCTC R: CACATTAAGATCGTTGGTGTAC	84bp

The amplification efficiency equation was **$E = [10(^{-1/M})] - 1$**, whereby an E (amplification efficiency value) of 100% indicated a doubling of amplicons for every RT-qPCR reaction, signifying that the primers were working correctly. A primer set did not meet the selection criteria if the E value was significantly higher than 100%, which signified that there was more than a doubling of RT-qPCR products per reaction; or a low value of E which signified that the primers were not able to properly amplify the target genes. A final nine out of the 17 contigs of interest primer sets and three of the seven reference gene primer sets (primer sets marked with a '(Y)' in Table 3) were selected from those that produced a single amplicon via the melt curve analysis for validation and had a coefficient (R^2) value

higher than 0.9. Here, three reference primer sets were tested to ensure a higher probability that at least one was able to act as a reference.

cDNA of four OOD-positive and four OOD-negative libraries was used for validation purposes. Whenever possible, the libraries chosen were the same as those that underwent RNA-seq analysis. The purpose of this validation step was simply to confirm that the RNA-seq analysis results were accurate in terms of fold difference. Hence validation focused on the eight libraries analysed by NGS, rather than all 20 libraries collected, to best avoid variabilities that may otherwise be captured. As C6A (an OOD-negative library) cDNA was not available, another OOD-negative library was chosen by random to make up the four x four analysis. RT-qPCR runs were performed for each primer set against each of the eight libraries.

To select the reference gene from the three potential reference primer sets chosen, standard deviation (sd) and coefficient of variation (cv) was calculated for each. The primer set (P1342-2) with the smallest sd and cv was chosen as the reference for all further analysis. The Livak method [91] was used to calculate relative eaxpression. All expression values are normalized to the reference gene (contig number 1342), and each OOD-positive expression value was then calculated to be relative to an OOD-negative expression value of 1.

Wilcoxon rank sum tests were carried out to compare the expression levels of each target amplicon to determine whether there were statistically significant differences in expression between cDNAs from four OOD-positive and four OOD-negative libraries.

The normalized relative expression values of the significantly differentially expressed contigs as determined by the Wilcoxon rank sum test were compared to the normalized relative expression values of the same contigs obtained from the RNA-seq analysis using the correlation coefficient similar to Liu et al. [92]. This determined if the relative expression values obtained from RT-qPCR validated the values from the RNA-seq analysis.

3. Results and Discussion

The RNA-seq analysis showed substantial differences in the transcriptional profiles of OOD-positive versus OOD-negative libraries, providing evidence that OOD is a distinct disease state in pearl oysters. The data indicated the presence of a typical wound healing/immune response that is characteristic of the general stress response pathway characterized by Anderson et al. [75]. There was a lack of genes that are known in other species to be differentially expressed in response to viral infection. The general stress response pathway identified, and the lack of differentially expressed viral response genes, suggests that the causative agent of OOD is not viral.

3.1. Quality control

The number of reads obtained per library ranged from 19 to 32 million, with a total of over 192 million reads over the eight libraries. Quality checking via FastQC yielded average quality scores in the medium to high range (a quality score of 20 to 28; and 28 and above respectively) for all eight libraries.

The trimming process removed 6% of all reads, comprising those that did not meet the 30bp cut-off point post-trimming. A further 19% of reads did not have complimentary reads (either forward or reverse) after the trimming process, and hence were not included in the downstream analysis. Therefore, 75% of reads (144 million) were retained after the QC process. All these reads were of high quality (quality scores of 28 or above).

3.2. Assembly results

3.2.1. Optimal assembly kmer length

Parameters for each assembly with kmers from 20 to 50 at steps of 5 were used to determine the optimal assembly settings (Table 4). As expected, the N50 values and average lengths decreased as kmer length increased, although the variations in N50 values were not large. The relative consistency of N50 results did not significantly promote one assembly over the other. However, the highest number of contigs of 1000 bp in length, the largest contig and the highest number of resultant bp peaked for the assembly using a kmer length of 25. A higher number of larger contigs were desirable for this study, as the

focus was for *de novo* transcriptome assembly. This process prefers a larger number of better, more fully assembled contigs, as *de novo* annotation relies on the ability to generate similarity matches to non-*P. maxima* organisms. Hence, the kmer length of 25 was deemed optimal. To confirm this, a further examination of the largest contigs for each assembly was conducted. The results indicated that there was little redundancy or contigs that could have potentially been merged together.

Table 4: Results for the different assemblies for each kmer length tested (at steps of kmer length 5). All other parameters were the same in each assembly.

Parameter	**Kmer 20**	**Kmer 25**	**Kmer 30**	**Kmer 35**	**Kmer 40**	**Kmer 45**	**Kmer 50**
Number of contigs	69,579	81,236	82,900	82,521	79,696	75,711	70,257
Total number of bp	34,113,692	39,246,796	38,919,791	37,492,046	35,187,020	32,255,854	28,869,006
Number of contigs with length of 1000 bp	5,351	5,547	5,212	4,537	4,036	3,389	2,771
N75	328	329	322	314	307	298	290
N50	509	498	480	459	442	422	401
N25	1,122	990	929	858	811	755	796
Average contig length	490	483	469	454	442	426	411
Largest contig (bp)	23,447	25,381	17,607	15,473	12,535	12,989	10,490

3.2.2. Optimal assembly method

To test all assembly options that could potentially yield viable, desirable results, a number of further assembly methods were examined. These included generating assemblies for individual libraries, before assembling all eight assemblies (designated ‘separate’ in Table 5) and generating an assembly of assemblies of all seven kmer lengths tested (designated ‘multiple kmer’ in Table 5). The output of these alternative methods were compared to the original method of a ‘single’ assembly of all eight libraries, using a kmer length of 25. From the results, it was determined that a single assembly of all eight libraries was preferable to either the ‘separate’ or ‘multiple kmer’ assemblies.

Although both the N50 and average contig length were higher for the assembly of individual library assemblies, the number of large contigs (determined by higher values for number of contigs of 1000 bp in length, the largest contig and the highest number of resultant bp) still indicated that the single assembly of kmer length 25 was the preferable of the two. Examination of the resultant contigs from the assembly of individual libraries identified more redundancy due to the collapsing of sequences for each library separately. This prevented many contigs from being further matched together during the second assembly. Conversely, individual assemblies meant that there were a larger number of shorter contigs produced that could not be assembled either, and hence were removed from the assembly. This explained the lower number of contigs obtained by this method (almost a six-fold decrease), despite a much higher proportion of larger contigs.

Table 5: A comparison of different assembly methods.*

	(A) Single Assembly	(B) Separate Assemblies	(C) Multiple kmer assembly
Number of Contigs	81,236	13,573	161,283
Total number of bp	39,246,796	11,240,225	77,092,263
Number of contigs with length of 1000 bp	5,547	2,803	10,873
N50	498	962	342
Average Length	483	828	478
Largest Contig (bp)	25,381	20,201	25,381

*Method A involves a single assembly of contigs from all libraries, using a kmer length of 25. Method B involves the assembly of contigs from each assembly separately, before assembling those assemblies together, both using a kmer length of 25. Method C involves the assembly of all libraries using different kmer lengths first (20 to 50, step of 5) before assembling all assemblies together.

In contrast, the multiple kmer assembly consisted of approximately double the number of contigs, total number of base pairs, and number of contigs with length of 1000 bp compared to the single assembly. A comparison of the distribution of contigs in terms of proportions indicated that it was very similar for both the multiple kmer and single assemblies (Figure 3). This suggests that the assembly of assemblies of different kmer lengths only doubled the contigs already present, producing more redundancy.

In summary, both of the two 'assembly of assemblies' yielded contigs that were unsuitable for further analysis. Regardless of the method, the assembly of assemblies yielded unnecessary duplicates, which would pose problems downstream in the RNA-seq analysis. It was therefore concluded that a single assembly was the optimal output for RNA-seq.

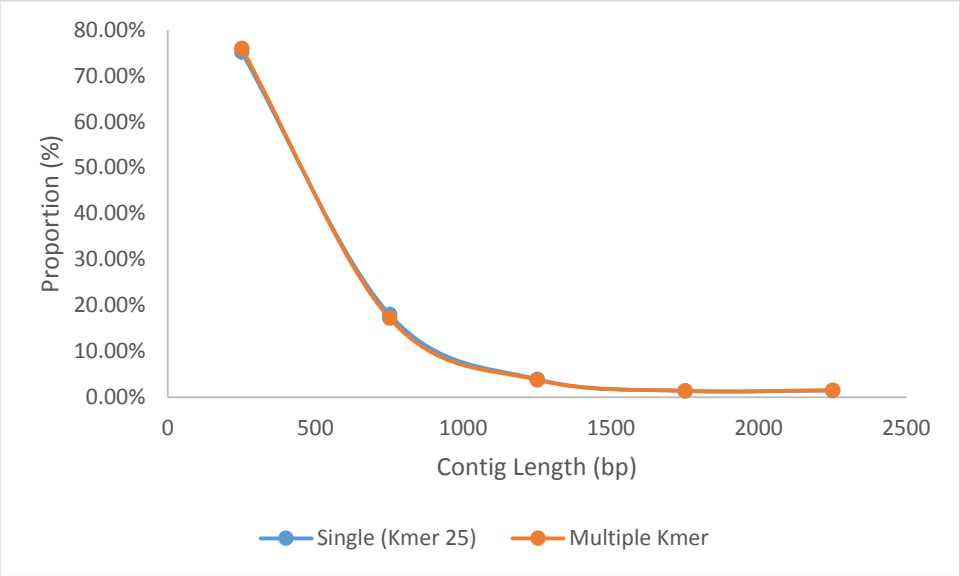


Figure 3: The contig length distribution for both the individual assembly of kmer 25, and the combined assembly of assemblies of all tested kmer lengths. The two distributions overlap over the majority of the contig length range.

3.3. Differential gene expression

RNA-seq analysis and subsequent DGE analysis identified 147 genes that were significantly differentially expressed between OOD-positive and OOD-negative libraries (FDR adjusted p-value < 0.05) in either direction (up-regulated and down-regulated in OOD-positive libraries versus OOD-negative libraries). The selection criteria used resembled 'double-filtering' [93], whereby large fold changes without significant p-values (as those fold changes can be caused by one or two large outliers) were ignored, as were those with low fold changes (yet with significant p-values), as these could be false signals simply due to the low variance. Through this method, both potentially undesirable types of outliers were ignored. Figure 4 depicts this selection procedure graphically, with red dots representing contigs of interest whilst blue dots represents non-differential contigs.

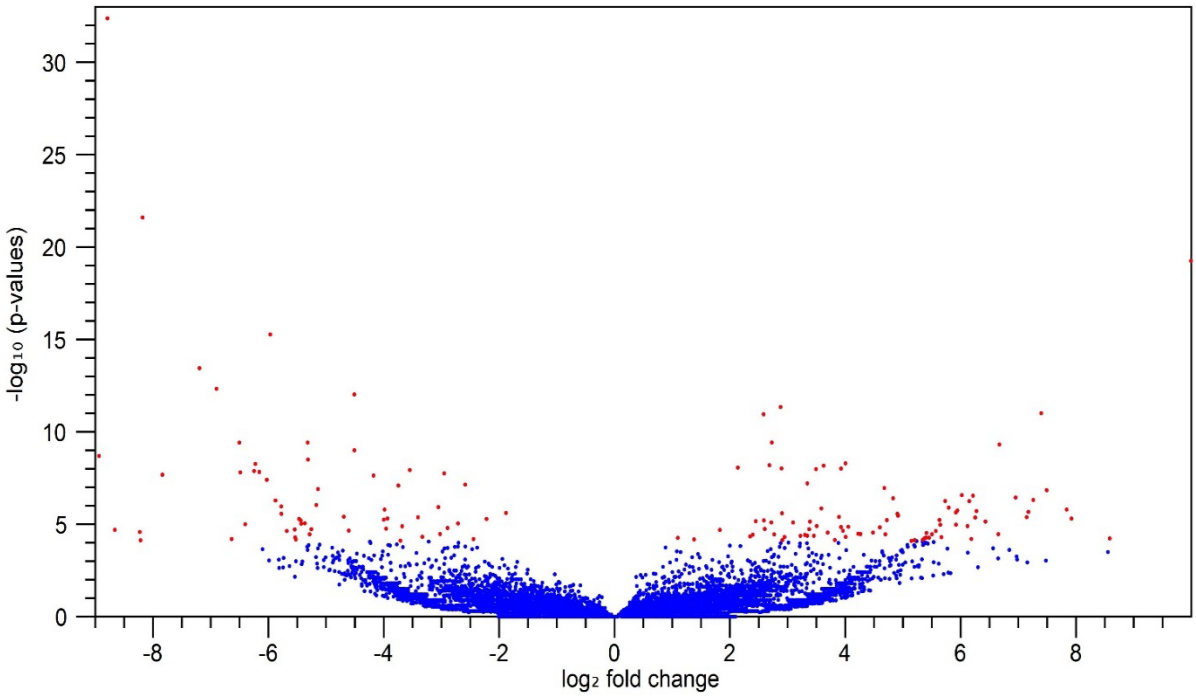
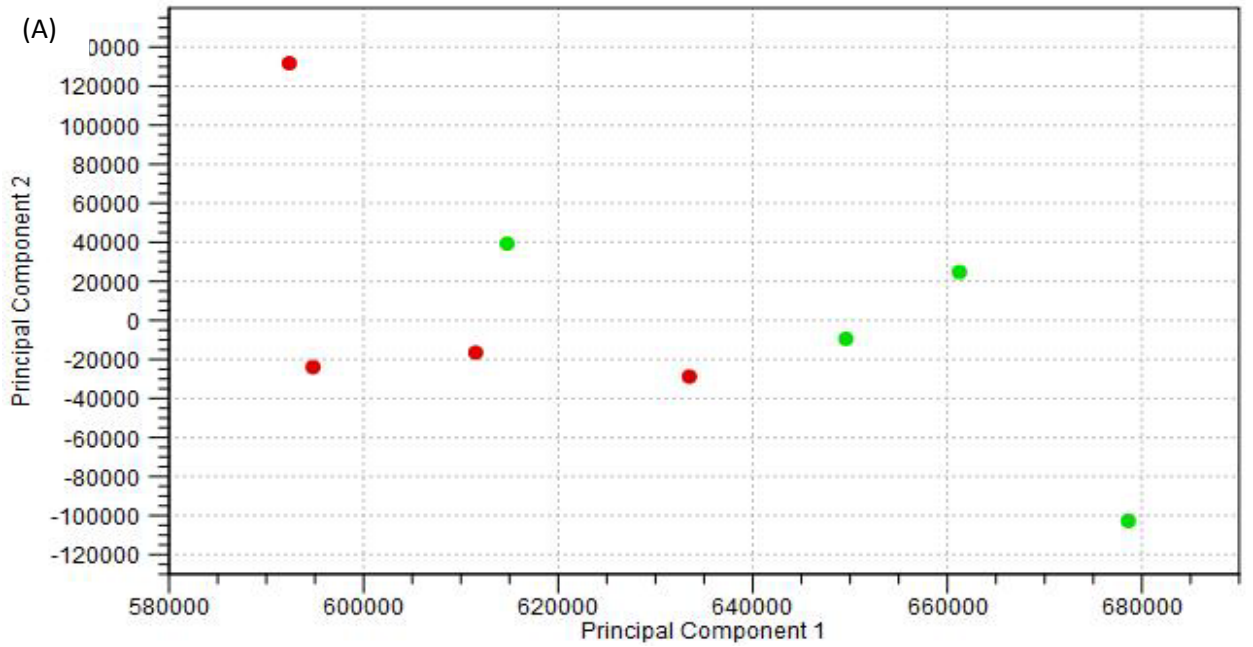


Figure 4: Volcano plot of the distribution of contigs in terms of fold change and p-value (non-FDR-adjusted) in RNA-seq comparisons of OOD-positive versus OOD-negative libraries. Red dots denote contigs that were deemed to be significantly differentially expressed. Blue dots denote no significant difference.

Principle component analysis (PCA) of the differential expression analysis of all 81,236 contigs showed a segregation of contigs from the OOD-positive libraries compared to those belonging to OOD-negative libraries (Figure 5A). This segregation is far more distinct for the subset of 147 contigs deemed to be significantly differentially expressed, as seen in the PCA of this subset (Figure 5B). It is also interesting to note that the cluster of OOD-negative libraries in the PCA of the 147 differentially expressed contigs appear to be more variable in transcriptomic responses. This suggests that OOD causes a restricted transcriptomic response in oysters, hence reducing the variability. However, it cannot be discounted that some of the OOD-negative oysters had sub-clinical infections that could lead to distinct transcriptomic responses, leading to greater variability in this group.



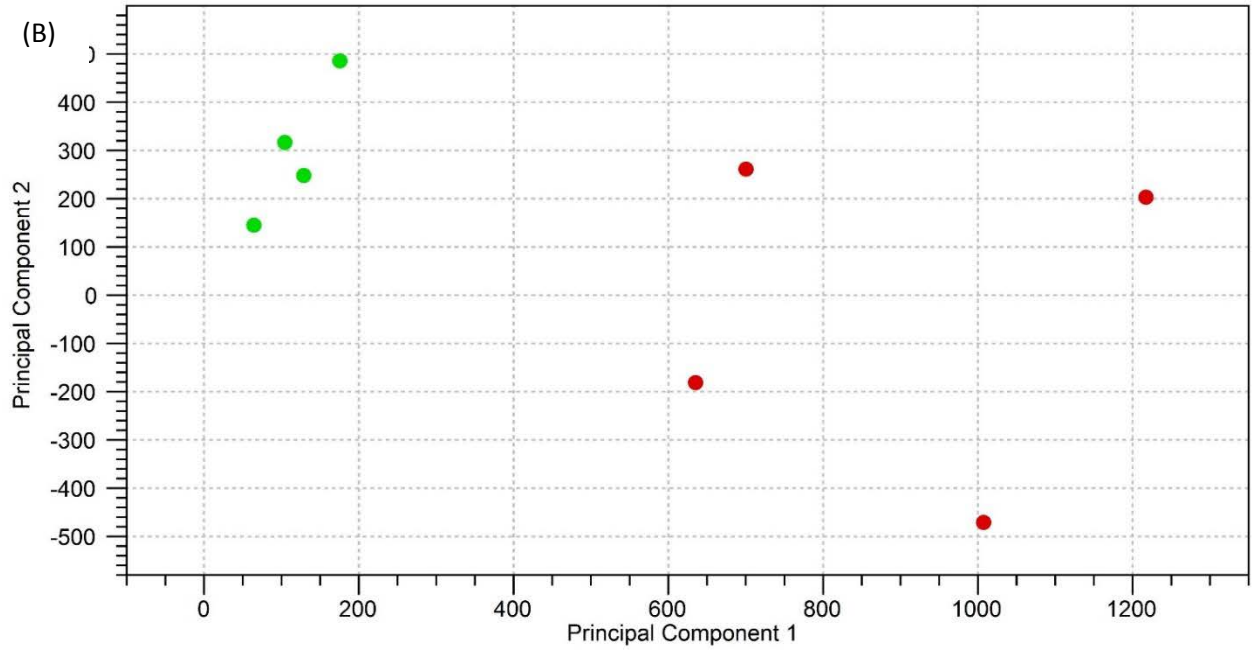


Figure 5: Principle component analysis of relative expression data for (A) the entire array of contigs obtained from the *de novo* assembly process and (B) the subset of 147 contigs deemed to be significantly differentially expressed between OOD-positive versus OOD-negative libraries. For both PCA plots, red dots signify OOD-negative libraries, whilst green dots signify OOD-positive libraries.

The validity of the 147 differentially expressed contigs was confirmed through a heatmap representation of their expression levels (Figure 6). Due to the large range of fold-differences (in either direction), not all differential contigs are visible under the resolution shown in Figure 6. However, the heat maps clearly show a marked division between OOD-positive and OOD-negative libraries.

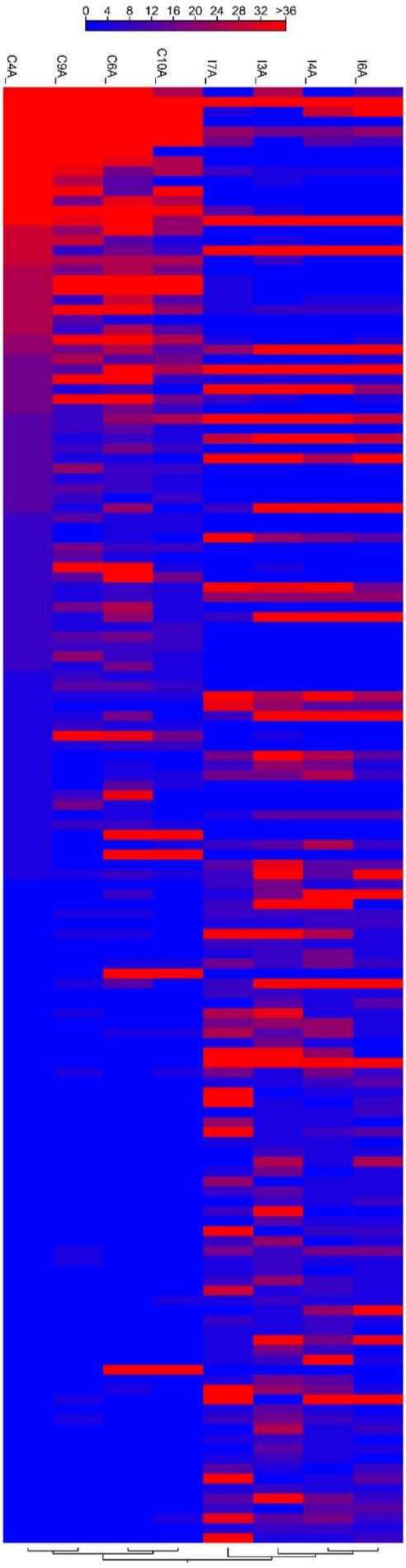


Figure 6: Heatmap showing the expression levels of the 147 contigs of interest calculated by RNA-seq analysis. Here, a scale from blue to red signifies the level of expression, with blue signifying no or undetectable expression, whilst increasing red colours signify increasingly levels of expression. Due to the level of resolution required for the generation of the heat map, not all differences between gene expression levels are represented. The four OOD-negative libraries (C4A, C9A, C6A and C10A) are on the left (numbered 1-4 in order), the four OOD-positive libraries (I7A, I3A, I4A, I6A) are on the right (numbered 5-8 in order).

In terms of fold-differences for the subset of 147 contigs, the majority of differentially expressed contigs (126) had fold-differences of less than 100 for OOD-positive versus OOD-negative libraries. Of the remaining contigs, 20 had fold-differences of between 100 and 500 (Figure 7). One contig returned a fold difference of over 1000 fold.

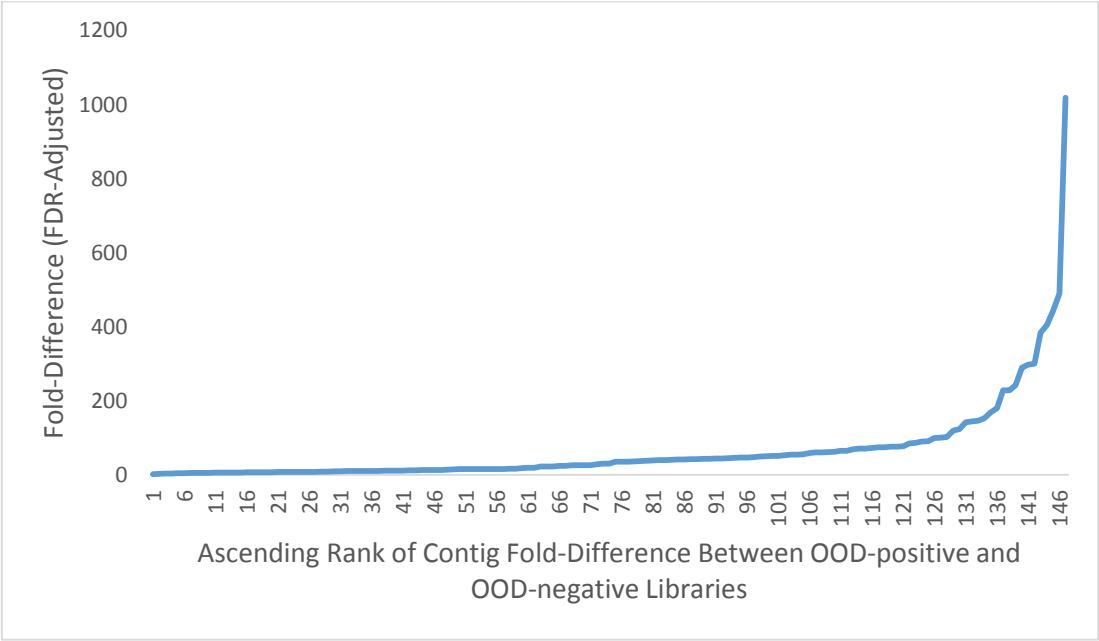


Figure 7: Fold-change (OOD-positive versus OOD-negative libraries) distribution for all 147 contigs, based on RNA-seq data, ordered from the smallest to largest fold difference.

3.4. Complete Transcriptome Annotation

Of the 81,236 contigs making up the whole transcriptome of the individuals tested in this study, 28,732 contigs returned significant Blastx hits (35% hit rate) in searches of the NCBI database. Of these hits, 17,796 (22%) could be assigned GO terms (Figure 8A). The subset of 147 contigs that were found to be differentially expressed between OOD-negative and OOD-positive libraries had a higher percentage of Blastx hits at 46% (80/147 contigs), although the percentage of contigs assigned GO terms was similar at 21% (31 contigs) in the subset (Figure 8B).

The three top species with which contigs shared significant homology were *C. gigas* (24,039 hits), *Lottia gigantea* (1,857 hits) and *Aplysia californica* (1,030 hits). All of these species have complete genome sequences [57, 94, 95]. Together, these three species of molluscs comprised 94% of the top hits. Of these species, *C. gigas* has the closest phylogenetic relationship to *P. maxima*, with both belonging to the family Bivalvia [96].

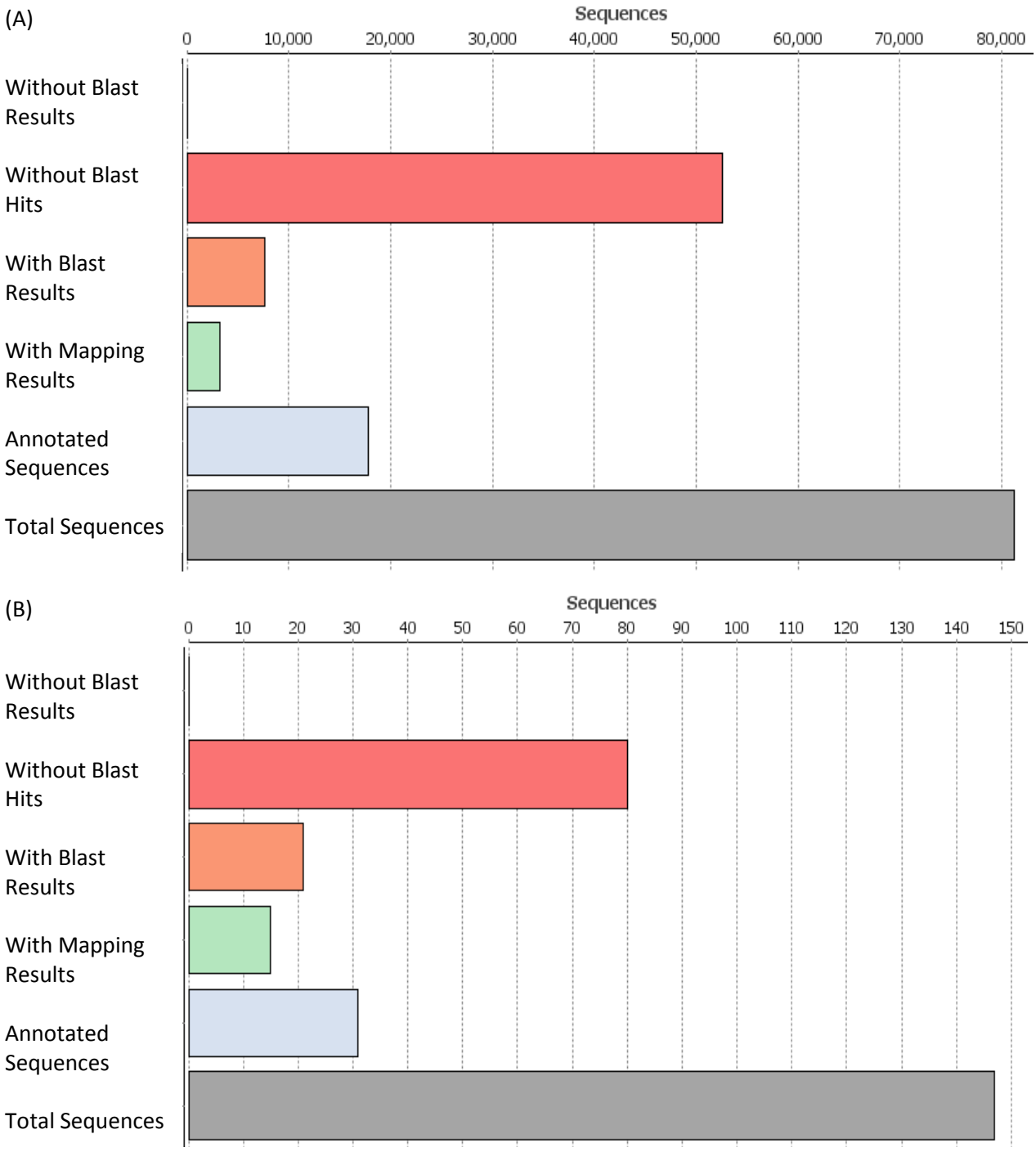


Figure 8: Blast and annotation results for (A) all 81,236 contigs that make up the transcriptome of the *P. maxima* libraries sequenced, and (B) the subset of 147 significantly differentially expressed contigs.

A breakdown of the GO term assignment reveals that of the GO terms obtained for all contigs in the whole transcriptome, 49% were biological processes, 33% were cellular components and 18% had molecular functions (Figure 9A). These are similar proportions to the subset of 147 contigs, of which 47% were biological processes, 31% were cellular components, and 22% had molecular functions (Figure 9B).

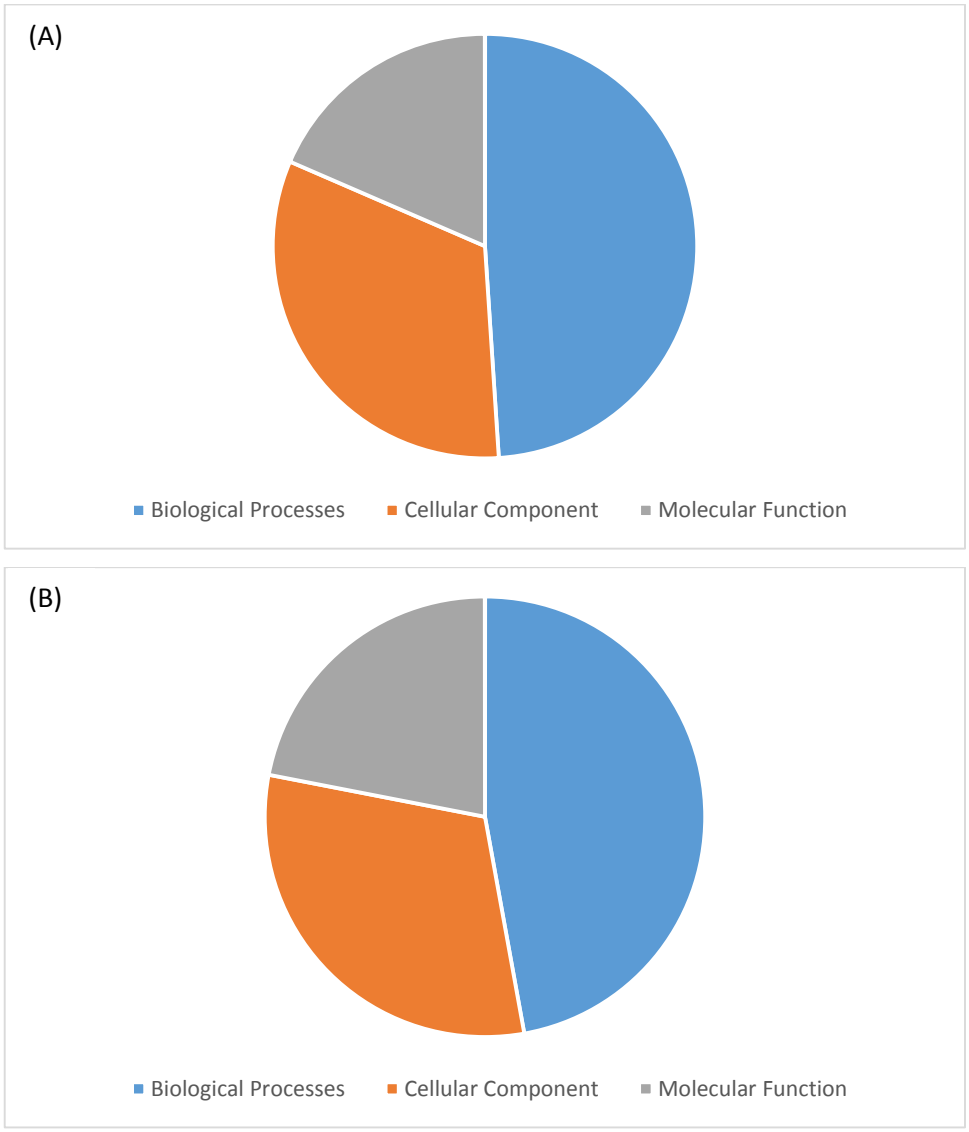
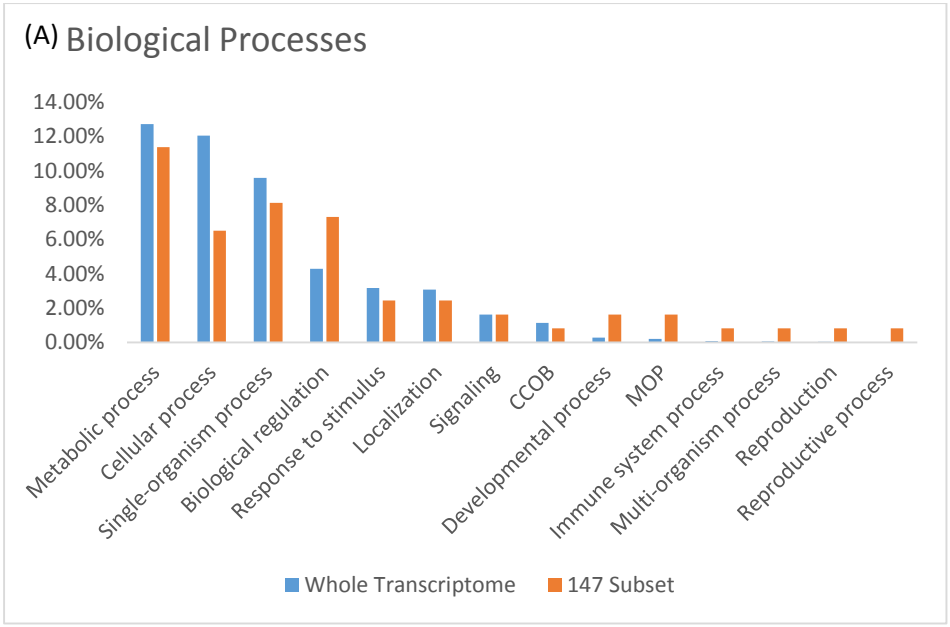


Figure 9: GO terms divided by broad ontologies for (A) the whole transcriptome, and (B) the 147 differentially expressed contigs identified through RNA-seq analysis.

The three broad categories could be further broken down into more specific GO terms, with a total of 18 for biological functions, 14 for cellular components and ten for molecular functions. Of these, GO terms assigned to the subset of 147 significantly differentially expressed contigs only consisted of 14 biological functions (Figure 10A), seven cellular components (Figure 10B) and six molecular functions (Figure 10C).

Figure 10: Deep hierarchical division of GO terms shared by the whole transcriptome and the 147 significantly differentially expressed contigs, with a layered breakdown of the ontologies into specific pathways. The terms are divided into (A) biological processes, (B) cellular components, and (C) molecular functions. Abbreviations are as follows: CCOB = cellular component organization or biogenesis, MOP = multicellular organismal process, MC = macromolecular complex, MEL = membrane-enclosed lumen.

(Figure overleaf)



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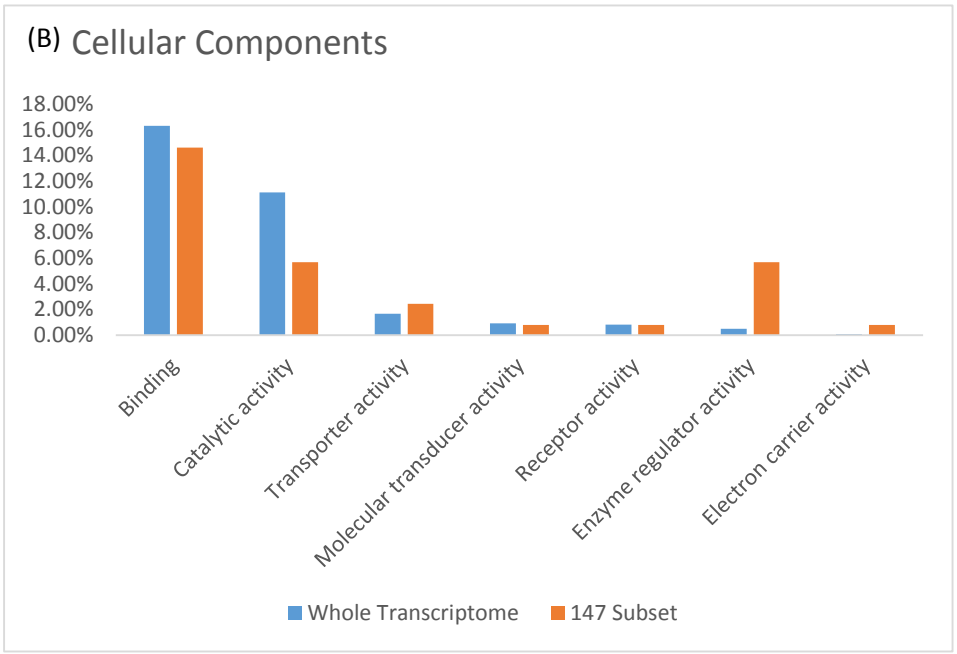

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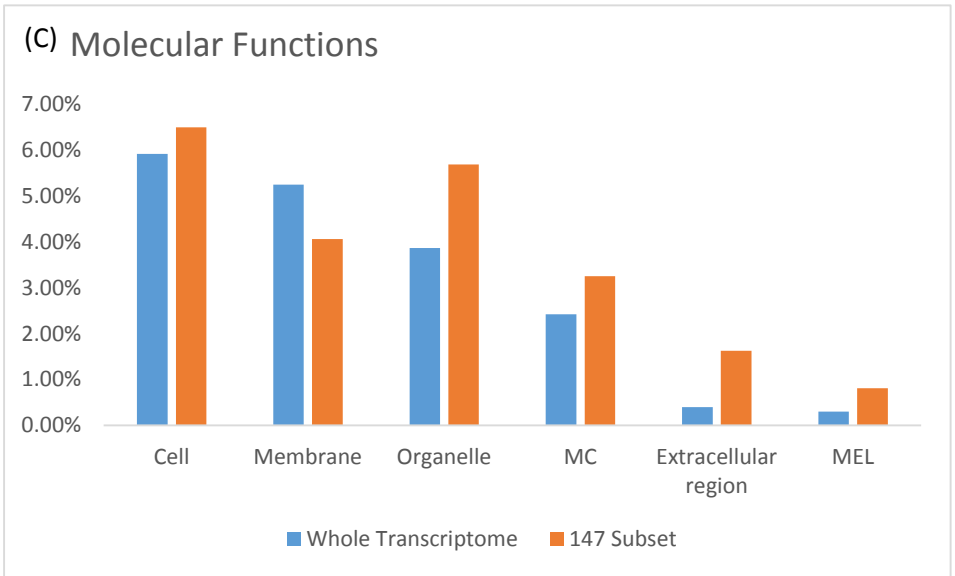

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To determine whether for each GO term the proportion of assigned contigs were significantly different between the whole transcriptome and the subset of 147 contigs, a z-test statistical analysis was performed for each GO term. Table 6 displays all GO terms that were significantly different proportionally between the whole transcriptome and the subset of 147 contigs. The proportion of contigs that were assigned cellular process and/or catalytic activity GO terms in the subset of 147 significantly differentiated contigs was significantly less than that for the whole transcriptome, suggesting that response to OOD by *P. maxima* involved less cellular processes and catalytic activities than average. All other GO terms had a higher proportion of assigned contigs in the subset of 147 differentially expressed contigs. This suggests that there is a higher degree of involvement by genes with these GO terms in response to OOD than would be expected on average. A large proportion of these GO terms (8) were biological processes (Figure 4A), indicating that broadly, biological processes are most significantly affected during OOD.

Similar *de novo* transcriptome sequencing of both molluscs (*Crassostrea hongkongensis*, *Crassostrea angulata*, *Bathymodiolus azoricus*, and *Mizuhopecten yessoensis*) [97-100] and non-molluscs (*Apostichopus japonicus* and hybrid catfish) [101, 102] yielded between 20,056 and 144,746 (mean of 95,331) contigs, with 24-43% (mean of 33%) of the contigs having Blast hits, and 12-30% (mean of 18%) assigned GO terms. The RNA-seq analysis results of this study lies within these ranges from other similar studies, and is very similar to the mean values provided. This indicates that outputs from our transcriptome analysis are within a typical range.

Table 6: All GO terms that had a significantly different proportion of assigned contigs between the whole transcriptome and the subset of 147 contigs. There were a total of 66283 annotations for the whole transcriptome, and 123 annotations for the subset of significantly differentiated contigs. The z-test scores and corresponding p-values are shown, with all being <0.05. Comparisons that were not significant are not included in the table.

	Number of Contigs			
GO Annotation	**Whole Transcriptome**	**147 Subset**	**Z-test score**	**Corresponding p-value**
Cellular process	7985	8	-1.88744	0.02955
Biological regulation	2838	9	-1.66033	0.048424
Developmental process	191	2	-2.7537	0.002946
Multicellular organismal process	138	2	-3.42506	0.000307
Immune system process	44	1	-3.17905	0.000739
Multi-organism process	38	1	-3.4561	0.000274
Reproduction	23	1	-4.5371	2.85E-06
Reproductive process	15	1	-5.64262	8.37E-09
Catalytic activity	7378	7	-1.91725	0.027603
Enzyme regulator activity	325	7	-8.17029	1.54E-16
Electron carrier activity	52	1	-2.88214	0.001975
Extracellular region	264	2	-2.1537	0.015632

3.5. Expression validation

Six of the nine contigs of interest that underwent RT-qPCR analysis were found to be significantly differentially expressed based on the Wilcoxon rank sums test (p-value <0.05). The difference in expression levels for the reference contig (contig 1342) was not significant (p-value >0.05) under the same test, confirming that it was suitable as a reference.

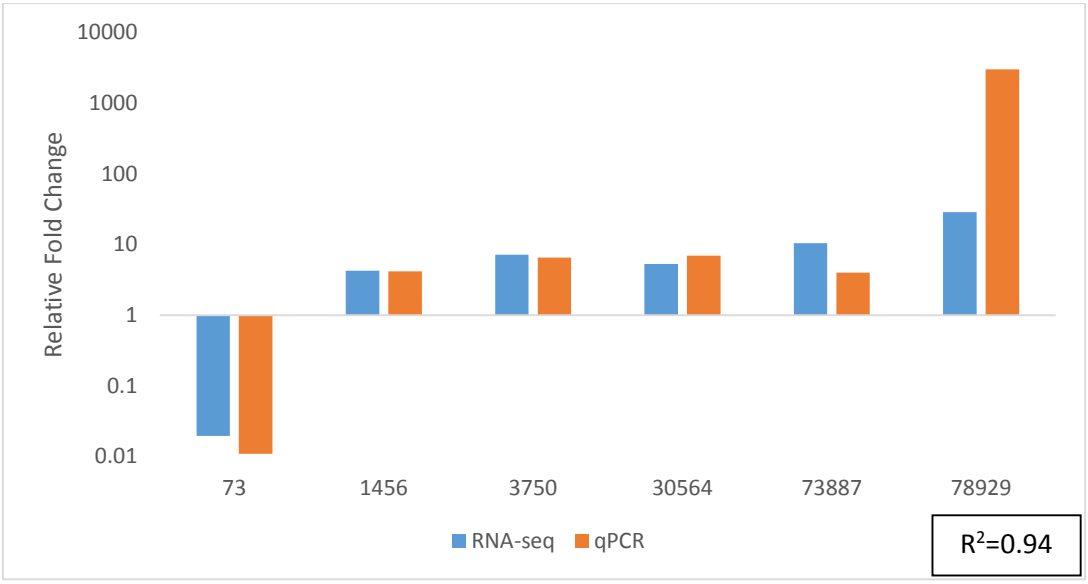

Figure 11: Relative fold differences (normalized) obtained from the RNA-seq analysis (blue) and RT-qPCR (orange) plotted against each other. The horizontal axis depicts the numerical identifiers of each of the six contigs found to be significantly differentially expressed through the RT-qPCR analysis.

The normalized relative expression values for the six contigs obtained through both the RNA-seq analysis and the RT-qPCR is shown in Figure 11, revealing a close correlation between the two analyses. The correlation coefficient (R^2 = 0.94) supports this, indicating that the RNA-seq analysis results are reliable as validated through RT-qPCR analysis.

3.6. Mollusc-specific genes that were differentially expressed

A total of 23 genes (discounting hypothetical proteins, uncharacterized, predicted or putative matches) matching entries in the molluscan database were found to be differentially expressed (Table 7). Of the 30 contigs that match these genes, 25 were up-regulated and five were down-regulated in OOD-positive relative to OOD-negative specimens. This is consistent with the finding by Anderson et al. [75] that in response to a stressor, there are generally more genes up-regulated than down-regulated.

Table 7: Automated annotated contigs matching known mollusc genes that were differentially expressed in the RNA-seq comparison of OOD-positive and OOD-negative specimens. The number of contigs with matches to known genes is shown, along with the contig ID for each match and top blast hit result information. Each gene is also assigned into a specific functional category.

Gene	Number of Contigs Matched	Contig ID	Blast Result				Functional Category
			Specific Match [Organism] / Accession Number	E-value	Identity	Fold Change (OOD-negative versus OOD-positive)	
Hydrolase	1	1790	hydrolase [*Helicobacter pullorum*] / WP_005023606.1	3.30E-35	79.45%	19.2	Unknown (bacteria)
chk1 checkpoint	1	5866	CHK1 checkpoint protein [*Echinococcus multilocularis*] / CDS35417.1	2.90E-20	76.56%	13.0	Cell cycle/apoptosis
carp-1	1	5848	clam ADP-ribosylating protein CARP-1 [*Meretrix lamarckii*] / BAF03560.1	1.60E-13	52.00%	-46.0	Immunity
Collagen alpha-2 chain	1	78949	Collagen alpha-2(VIII) chain [*Crassostrea gigas*] / EKC36664.1	4.40E-28	60.75%	61.6	Immunity/wound healing
Dispatched	1	73730	dispatched-like protein 1 [*Crassostrea gigas*] / EKC41449.1	5.80E-29	80.00%	10.4	Communication

DNA mismatch repair protein	1	13133	DNA mismatch repair protein Msh6 [*Crassostrea gigas*] / EKC25057.1	1.40E-24	76.50%	-46.6	DNA repair
Heat shock 70 kDa protein 8	1	990	Hsp70, partial [*Gallus gallus*], AAO44919.1	3.50E-48	94.30%	-7.7	Stress
Inter-alpha-trypsin inhibitor heavy chain h3	5	10635	Inter-alpha-trypsin inhibitor heavy chain H3 [*Crassostrea gigas*] / EKC29055.1	2.90E-61	63.75%	6.4	Protein regulation
		1456	Inter-alpha-trypsin inhibitor heavy chain H3 [*Crassostrea gigas*] / EKC23874.1	0.00E+00	63.60%	6.0	
		1458	Inter-alpha-trypsin inhibitor heavy chain H3 [*Crassostrea gigas*] / EKC23874.1	1.50E-29	78.00%	168.5	
		6990	Inter-alpha-trypsin inhibitor heavy chain H3 [*Crassostrea gigas*] / EKC27555.1	2.00E-61	48.20%	6.6	
		938	Inter-alpha-trypsin inhibitor heavy chain H3 [*Crassostrea gigas*] / EKC23874.1	1.10E-18	79.15%	7.4	

Inter-alpha-trypsin inhibitor heavy chain H4	2	10634	Inter-alpha-trypsin inhibitor heavy chain H4 [*Crassostrea gigas*] / EKC36390.1	3.00E-27	64%	5.3	Protein regulation
		12018	Inter-alpha-trypsin inhibitor heavy chain H4 [*Crassostrea gigas*] / EKC36390.1	3.00E-79	59.85%	5.5	
Macrophage mannose receptor	2	3750	Macrophage mannose receptor 1 [*Crassostrea gigas*] / EKC31065.1	1.90E-94	46.55%	10.1	Immunity
		7045	Macrophage mannose receptor 1, partial [*Chelonia mydas*] / EMP36986.1	5.50E-41	45.75%	7.5	
Metallothionein	1	16434	metallothionein [*Pinctada maxima*] / ACJ22893.1	2.40E-18	94.00%	50.8	Stress
Myosin heavy chain	1	73	Myosin heavy chain, striated muscle [*Crassostrea gigas*] / EKC37566.1	0.00E+00	82.15%	-36.0	Cytoskeleton

NADH dehydrogenase	1	5311	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial [*Crassostrea gigas*] / EKC27484.1	1.40E-14	46.00%	-35.3	Metabolism
NADH dehydrogenase subunit 2	2	988	NADH dehydrogenase subunit 2 (mitochondrion) [*Pinctada maxima*] / YP_006883012.1	4.90E-53	85.00%	-442.6	Metabolism
		989	NADH dehydrogenase subunit 2 (mitochondrion) [*Pinctada maxima*] / YP_006883012.1	2.70E-54	85.00%	1018.6	
Permease	1	195	permease [*Lactobacillus crispatus*] / WP_013085634.1	2.10E-58	71.50%	29.9	Unknown (bacterial)
Prism shell protein 18	1	78932	Prism uncharacterized shell protein 18 like [*Pinctada fucata*] / BAM76253.1	7.50E-13	71.00%	383.7	Biomineralization
ALO17	1	79139	Protein ALO17 [*Crassostrea gigas*] / EKC32504.1	1.60E-20	57.67%	35.6	Protein regulation

Tar1p	1	1962	Tar1p [*Saccharomyces cerevisiae S288c*] / NP_690845.1	2.10E-34	64.35%	8.5	Unknown (fungal)
Sacsin	2	11369	Sacsin [*Crassostrea gigas*] / EKC26842.1	9.40E-26	47.25%	18.7	Stress
		77244	Sacsin [*Crassostrea gigas*] / EKC26842.1	6.30E-23	44.70%	9.3	
Short-chain collagen c4	1	14728	Short-chain collagen C4 [*Crassostrea gigas*] / EKC34668.1	1.20E-44	56.70%	5.1	Immunity/wound healing
Transforming growth factor-beta-induced protein ig-h3	1	6728	Transforming growth factor-beta-induced protein ig-h3 [*Crassostrea gigas*] / EKC23900.1	0.00E+00	51.95%	4.4	Immunity
Nacre shell protein 20	1	7760	nacre uncharacterized shell protein 20 [*Pinctada margaritifera*] / CCE46184.1	1.20E-17	95.00%	10.5	Biomineralization
von willebrand factor d and egf domain-containing protein	1	73887	von Willebrand factor D and EGF domain-containing protein [*Crassostrea gigas*] / EKC28789.1	1.70E-55	49.33%	14.8	Immunity/wound healing

937

938

Manual annotation against the entire NCBI non-redundant database identified an additional 41 differentially expressed contigs that matched genes/gene products that were not hypothetical, putative nor predicted. A large proportion of these were bacterial (approximately 66%), with seven contigs matching to spirochaetes or similar organisms. Three matched a specific spirochaete species, *Spirochaeta sp. L21-RPul-D2*. All spirochaete genes, however, were down-regulated in OOD-positive individuals, suggesting no link in the causative effect of the presence of the bacterium.

A further six contigs were found to match five molluscan genes after the manual annotation process (Table 8). These represented matches not identified by the automated process. The failure of automated searches to identify these matches was likely due to new additions to the non-redundant NCBI database that did not exist when the molluscan database used for the Blast processes was constructed. Addition of the manually annotated genes brought the total number of differentially expressed contigs with a match to molluscan genes/gene products to 48, with 33 distinct genes/gene products implicated.

Table 8: Manually annotated contigs identified as host genes that were differentially expressed in the RNA-seq comparison of OOD-positive and OOD-negative specimens. The number of contigs with matches to known genes is shown, along with the contig ID for each match and top blast hit result information. Each gene is also assigned into a specific functional category.

Gene	**Number of Contigs Matched**	**Contig ID**	**Blast Result**				**Functional Category**
			Specific Match [Organism] / Accession Number	**E-value**	**Identity**	**Fold Change (OOD-negative versus OOD-positive)**	
ATP synthase beta subunit	1	3889	ATP synthase beta subunit [*Pinctada fucata*]/ ABC86835.1	2.00E-72	98%	-7.4	Metabolism

Metalloproteinase inhibitor 3	1	4712	Metalloproteinase inhibitor 3 [*Crassostrea gigas*]/ EKC31955.1	1.00E-13	30%	11.3	Protein regulation
Sacsin	2	11368	Sacsin [*Crassostrea gigas*]/ EKC26842.1	7.00E-22	29%	-8.3	Stress
		36151	Sacsin [*Crassostrea gigas*]/ EKC26842.1	4.00E-15	35%	47.5	
Sarcoplasmic calcium-binding protein	1	4537	Sarcoplasmic calcium-binding protein [*Crassostrea gigas*]/ EKC29122.1	1.00E-27	53%	-25.9	Cytoskeleton
Uromodulin	1	30564	Uromodulin [*Crassostrea gigas*]/ EKC21727.1	8.00E-41	35%	7.5	Unknown

The characterized differentially expressed genes were placed into ten broad categories as listed in Tables 6 and 7. These were cell cycle (including apoptosis), immunity, wound healing, communication, DNA repair, stress response, protein regulation, cytoskeleton, metabolism, and biomineralization. Seven of these corresponds to intracellular processes defined by Anderson et al. [75]. Here, metabolism, cell cycle and communication related genes were less commonly identified than in Anderson et al. [75], whilst protein regulatory genes and specifically immunity related genes are found at higher rates (Figure 12). However, the methodology used to assign genes of interest to intracellular processes may be different, and could be the source of some of the discrepancy. With the three remaining broad categories fitted into the intracellular processes as described by Anderson et al. [75], the proportions could differ again.

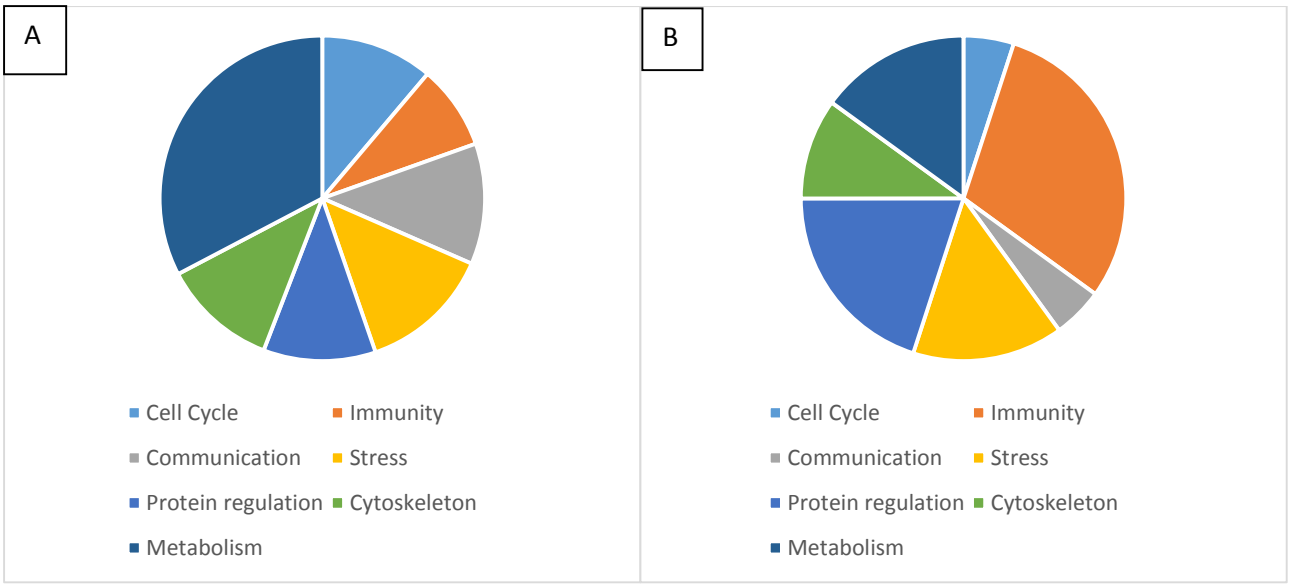

Figure 12: The proportion of genes belonging to each of the seven overlapping intracellular processes. (A) dictates the most commonly identified genes in proportion to each other by Anderson et al. [75], whilst (B) dictates the proportion of genes identified in the current study.

There were also a number of genes with unknown functions, two of which are bacterial in origin. Contig 1790 matches to a hydrolase, which is a large class of enzymes catalysing the hydrolysis of a chemical bond, such as those of mammalian hepoxilin and soluble epoxide [103]. As the match was to a bacterial (*Helicobacter pullorum*) hydrolase, the specific function of this gene in *P. maxima* cannot be identified. The second unknown bacterial match (contig 195) is a permease, which are membrane transport proteins of molecules such as L-tryptophan [104] oxyanions like molybdite [105]. Similarly to the hydrolase, the function in *P. maxima* is highly uncertain, as the match is to a bacteria (*Lactobacillus crispatus*). Tar1p (contig 1962) is another non-mollusc gene, which in *Saccharomyces cerevisiae* plays a role in respiratory regulation [106, 107], and has been shown to also play a similar role in at least one other yeast species (Kluyveromyces lactis) [108]. However, the role of Tar1p has yet to be identified in non-yeast species, and so it is unclear what role Tar1p plays in *P. maxima*. Uromodulin (contig 30564) was the final gene with an uncharacterized molluscan function.

The remaining genes could be grouped into two broad categories of responses – a general stress response, involving changes in communication, stress response, protein regulation, cytoskeleton, and metabolism; and a concurrent wound healing response, involving changes in the cell cycle, immunity, wound healing, DNA repair and biomineralization.

3.6.1. General stress response

Genes/gene products involved with the cytoskeleton are those that controls the structure of cells, and maintenance of such structure. Both sarcoplasmic calcium-binding protein and myosin heavy chain are cytoskeletal proteins. In molluscs, sarcoplasmic calcium-binding proteins are regulatory proteins in the muscle [109], whilst the myosin heavy chain is involved in muscle contraction [110]. In this case, both are down-regulated, which is consistent with the symptoms of OOD: loss of epithelial cells and mantle retraction. Myosin heavy chains are also known to fuse with anaplastic lymphoma kinase (ALK), which can also be fused to ALO17 [111, 112]. Contig 79139 was matched to ALO17, and was up-regulated in response to OOD. Whether there is a relationship between ALO17 and myosin heavy chains in this case must be further investigated. Von willebrand factor D and EGF domain-containing protein could also play a role in relation to the symptomology of OOD. The EGF (epidermal growth factor) links it to the outer layers of tissues and cells, whilst the Von willebrand factor D plays a role in blood clotting in humans [113]. However, its role in molluscs is unknown.

There were a number of gene products involved in general stress responses which are activated ubiquitously in response to varied stressors [75]. These generic stress response genes include molecular chaperones (heat shock proteins). Molecular chaperones are commonly accompanied by co-chaperones that form complexes together, such as that of the heat shock protein 70 (hsp70) DnaK and its co-chaperone, Dna-J [114]. In the current study, both a member of the hsp70 family (heat shock 70 kDa protein 8, HSPA8) and an hsp70 co-chaperone (sacsin) were found to be differentially expressed. HSPA8 antagonizes apoptosis [115-117], and therefore its reduction among OOD-positive individuals could indicate that apoptosis is uninhibited during OOD. Four contigs are found to match the hsp70 co-chaperone, sacsin, with three showing up-regulation in OOD-positive libraries and one being down-regulated. All four matches have the same accession number (EKC26842.1), suggesting that all four contigs could potentially be one single read. However, the fact that they are expressed at different levels from one another suggests that these could be different sacsin isoforms, and the one down-regulated sacsin isoform (contig 11368) could be the co-chaperone to the hsp70 found in this study.

Metallothionein also plays a role in general stress responses, specifically as a potential defence against metal toxicity [118] by trafficking metals (mainly zinc) and sequestering them [119]. Increased concentrations of vanadium [120] or cadmium [121] for example, has been reported to result in increased expression of metallothionein by mussels (*Mytilus sp.*). Metallothionein has therefore been investigated as a potential biomarker of metal toxicity. However it is known that there are other factors that can trigger expression of metallothioneins as well, including generalized stress [122].

In humans and other mammals, inter-alpha-trypsin inhibitor heavy chains inhibits proteases and binds to chemicals such as hyaluronan [123], and is also up-regulated in response to infections [124, 125]. Two forms (chains h3 and h4) were identified in the current study, with all matched contigs up-regulated in response to OOD. The number of matches found in this study and the ubiquitous role inter-alpha-trypsin inhibitor heavy chains supports the notion of a general response to infection in OOD-impacted oysters.

Genes involved in metabolism control the production of energy, and the catabolism/anabolism of chemicals within an organism. A major proportion of energy production takes place in the mitochondria. NADH dehydrogenase is located within the inner mitochondrial membrane, and is responsible for catalysing the dehydrogenation of NADH into NAD^+. The behaviour of NADH dehydrogenases are enigmatic in terms of their potential role in OOD. Whilst one NADH dehydrogenase subunit 2 (contig 988) had a 442.63 negative fold-change (down-regulation) in OOD-positive individuals, the other (contig 999) saw a 1018.64 positive fold-change (up-regulation). This was the biggest fold-change amongst the significantly differentially expressed contigs. Whilst it is known that NADH dehydrogenase subunits can show different patterns of expression based on different time points during responses to stress [64, 65], there is high sequence similarity between the two contigs, and actual expression values for the two contigs suggested that they were allelic and unique to the collection sites. Whilst the average expression value of contig 988 for OOD-positive oysters was zero, there was a much higher expression value for OOD-negative oysters. This was the reverse for contig 999, suggesting that contig 988 was exclusive to OOD-negative oysters, whilst contig 989 was exclusive to OOD-positive oysters. Also in terms of metabolism, ATP synthase beta subunit (contig 3889) was found to be down-regulated [126, 127].

Finally, biomineralization, especially in pearl oysters, may be in response to contamination or other abiotic and biotic factors [72, 73]. Nacre and prisms are two types of calcium carbonate deposits, which are controlled by two very different pathways [71], although they can form a highly desirable composite. Both nacre and prism related proteins were up-regulated in response to OOD, suggesting a more orchestrated stress response.

3.6.2. Immune response and wound healing

In general, genes involved in immune responses and wound healing act to protect the organism from harm and when not possible, repair damage done to the organism. A total of ten genes were identified in these categories during the current study.

There were a number of genes found to play a role in responding to infections. Metalloproteinase inhibitor 3 was found to be up-regulated in response to OOD. Metalloproteinase inhibitors are inhibitors of matrix metalloproteinase, with roles in the degradation and remodelling of tissues [128]. In molluscs, the gene is found to play a role in wound healing and defense mechanisms, specifically in response to shell damage and bacterial infections [129, 130]. Although uncovered by Zhang et al. for *C. gigas* [57], no specific function was described for uromodulin. In mammals such as humans, uromodulin is secreted by kidneys and seems to play a role in immune responses against urinary infections [131, 132]. It is unclear if uromodulin plays a similar role in *P. maxima*. Macrophage mannose receptor-1 is involved in inflammation and wound healing, specifically in times of infection [133]. It acts as a receptor for pathogen associated molecular patterns. Its differential expression in OOD-positive oysters may suggest that there is an infectious agent involved in OOD.

Chk1 is a regulator of the cell cycle and associated DNA damage in mammals such as humans [134, 135] and mice [136, 137], amongst other animals such as frogs [138], and even fungi [139, 140]. This suggests that the role of Chk1 is ubiquitous, including in *P. maxima*. Similarly, carp-1 is suggested to play a role in the cell cycle, specifically up-regulation of the gene induced apoptosis [141, 142]. It (contig 5848) was down-regulated in OOD-positive oysters, and therefore suggests a suppression of apoptosis. DNA mismatch repair proteins are a large class of proteins, including proteins such as the heterodimeric MutS homolog (MSH) [143]. Here, Msh6 (contig 13133) was found to be

down-regulated, which has been shown elsewhere to be the direct effect of the presence of a stressor [144, 145]. It has been found that Msh6 induces apoptosis [146, 147], and therefore the suppression of Msh6 in OOD-positive oysters may inhibit apoptosis.

Transforming growth factor-beta-induced protein ig-h3 (TGFBI), represented by contig 6728, plays a role in inducing collagen expression [148], of which two were identified to be differentially expressed: collagen alpha-2 chain (contig 78949) and short-chain collagen c4 (contig 14728). Collagen is known to play a role in immune responses by binding to pathogens, and antagonizing apoptosis by binding to apoptosis-inducing cells [149]. TGFBI is also known to play other roles, such as in inhibiting cell attachment [150]. The up-regulation of all three of these genes in OOD-positive oysters strongly suggests an immune-response, and once again exhibits a clear indication that apoptosis is antagonized in response to OOD.

Dispatched (contig 73730) is a protein that plays a role in membranal molecular trafficking, which along with a hedgehog gene is involved in cellular growth and development [151-153]. However, without the differential expression of the hedgehog gene in this case, it is unsure that dispatched plays this same role in response to OOD.

3.7. OOD response profile and comparison to the viral infection response profile

As determined in section *1.6.*, the viral infection response profile is very specific. Table 1 lists five proteins/protein families that are differentially expressed in response to a viral infection, which are distinct from those involved in response to other stressors. These proteins have been definitively implicated in response to viral infections in both oysters [79, 80, 154] and non-oysters [81, 82].

A total of 27 genes/gene products make up the OOD response profile. However, no gene/gene product determined through the current study matches with any of the protein/protein families that makes up the typical viral infection response profile. This excludes the viral stressor as a potential cause of OOD.

On the other hand, a large proportion of the OOD response profile matches to other genes/gene products identified in Table 1 that are associated with stress, wound healing and immune responses. NADH dehydrogenase was commonly found to be differentially

expressed, along with molecular chaperones (heat shock proteins and their co-chaperones). Many proteins that play a role in apoptosis inhibition were also identified. All of these changes are characteristic of responses to non-viral infections. Comparison of the broad intracellular processes identified in this study found that there was no significant difference (p-value=0.282, >0.05) between our data and the meta-analysis of Anderson et al. [75]. This indicated that there was a concerted, but generalized response to stress in OOD-affected oysters. This is in agreement with the observation that a large portion of the OOD response profile (21 genes out of 27) plays a role in stress responses (13 genes) or immune responses/wound healing (8 genes).

4. Conclusion

The transcriptomic analysis of the host response of the pearl oyster *P. maxima* to OOD has validated the method as a viable investigative tool for the future. Through RNA-seq analysis, a comparison of expression levels of OOD-positive and OOD-negative specimens was accomplished, and a set of differentially expressed genes obtained and collated into a response profile of 28 genes. A comparison of this response profile to the typical viral infection response profile of molluscs yielded no match. In contrast, there were clear indications that an alternative stressor, yielding a more 'general' response to stress, is involved in OOD. In particular, this generalised stress response involved the differential expression of genes involved in wound healing and immune responses.

This study has therefore been able to narrow the focus of further research, which will be able to concentrate on non-viral stressors. With more stressor response profiles built in the future, studies such as this will become more precise and powerful. Even now, this study has already established the feasibility of generating the data necessary for comparison.

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6. Appendix

6.1. Journal format instructions

The chosen journal is **Fish and Shellfish Immunology**, with instructions printed at http://www.elsevier.com/journals/fish-and-shellfish-immunology/1050-4648/guide-for-authors.

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Introduction

Types of Papers

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Short Communications: Where the subject matter does not warrant a full paper, a Short Communication is more likely to be acceptable for publication. Communications should be restricted in length to no more than the equivalent of two printed pages of Fish and Shellfish Immunology, that total length to include references and normally no more than one illustration and one short table. No abstract will be required, nor should a communication necessarily have subheadings or be subdivided as are full papers, but an introductory sentence or sentences must make its purport clear. In other respects submitted manuscripts should comply with the instructions given above. A Short Communication may be concerned with any subject within the scope of Fish and Shellfish Immunology but should be confined to a single point or issue of progress, and to such as an unusual occurrence, an interesting observation or a topical and timely finding.

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