Aquaculture 479 (2017) 798–807

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

The effect of commercial, natural and grape seed extract supplemented diets on gene expression signatures and survival of greenlip abalone (*Haliotis laevigata*) during heat stress

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ARTICLE INFO

Keywords:
Abalone
Differential gene expression
RNA-seq
Aquaculture
Heat stress
Diet
Genetic

ABSTRACT

Summer mortality is a phenomenon associated with high temperature water spikes that can result in mass mortalities of abalone and other molluscs. This is a particular concern for aquaculture industries due to the economic impacts of such events. Diets containing algal supplements have been suggested as pre-emptive solutions for preventing these mass mortalities. The same has also been suggested for diets containing grape seed extract. This is due to their potential as a source of antioxidative compounds, which reduce the accumulation of harmful reactive oxygen species. This study aimed to identify functional genes associated with high survival in abalone fed diets high in antioxidative compounds during heat stress. Tentacle transcriptomes of 40 greenlip abalone (*Haliotis laevigata*) were investigated to determine the combined effects of differing diets and temperature on the gene expression responses by abalone. Here we compare the functional gene expression changes at 22 °C and 25 °C in abalone fed common commercial, live macroalgal (*Ulva lactuca*) and grape seed extract supplemented commercial diets as a means to understand the resulting high survival of abalone fed grape seed extract during heat stress. Twenty-four genes were differentially expressed between high survival promoting diets (macroalgae or grape seed extract supplemented commercial) relative to the purely commercial diet. Many of these genes have been suggested to be involved in antioxidant and innate immunity responses. The identification of these genes and their functional roles has enhanced our understanding of processes that contribute to summer stress resilience in abalone. Our study supports the hypothesis that diet and gene expression signatures may be indicative of the survival capabilities of abalone when exposed to heat stress.

1. Introduction

The high demand for abalone as a luxury food source has caused a decline in wild stocks globally, but has also stimulated the growth of abalone aquaculture (Cook and Gordon, 2010). The transition of marine and freshwater species to aquaculture can prove challenging due to the potential increase of stressors, such as increased stock densities, increased handling of animals and the different climatic or chemical conditions experienced in the culture environment. Optimizing artificial feeds to replace natural diets is an additional challenge (Kurmaly et al., 1989) yet is essential due to the prohibitively high cost of natural diets. The diet composition of commercially important aquaculture species is vital for growth (Fernandez-Jover and Sanchez-Jerez, 2015), survival (DeGrandi-Hoffman et al., 2016; Rodney and Confred, 2016),

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http://dx.doi.org/10.1016/j.aquaculture.2017.07.025
Received 12 January 2017; Received in revised form 13 July 2017; Accepted 18 July 2017
Available online 18 July 2017
0044-8486/

reproductive success (Kennedy et al., 2016; Rojas et al., 2016) and product quality (Egea et al., 2016; Megersa et al., 2013).

Abalone are anatomically and biochemically adapted to digest macroalgae which forms an essential component of their natural diet in the wild (Garcıa-Carreno et al., 2003). The use of commercial aquaculture feeds typically result in high growth rates (Bautista-Teruel et al., 2003; Fleming et al., 1996), while abalone fed macroalgae diets have varied growth rates, depending on the algal species used (Naidoo et al., 2006). Macroalgae as an ingredient in abalone feed has numerous benefits for aquaculture including the promotion of sustained high feeding activity, optimal health and marketability (Bansemer et al., 2014). Feed containing macroalgae is also highly effective at preventing mass mortalities during heat stress events known as "summer mortalities" (Bansemer et al., 2016; Stone et al., 2014). However, due to the high costs of growing, harvesting and processing algal biomasses, compared to the costs of manufacturing commercial feed utilizing alternative ingredients, the use of algae as a direct feed source is generally confined to the early nursery life stages (Shields and Lupatsch, 2012).

High water temperature is the key driver of summer mortality, a phenomenon that occurs in both wild and culture environments during the summer months (Vandepeer, 2006). Summer mortality is known to be highly complex, partially driven by chemical water changes associated with increased temperatures (such as lower dissolved oxygen, and changes in nutritional factors and pH) which are thought to compromise the immune system and make abalone more vulnerable to infection by bacteria such as *Vibrio* (Cardinaud et al., 2015; Vandepeer, 2006). Summer mortality on abalone farms may be induced by sudden temperature spikes, and may also be exacerbated by a compromised metabolism, which can result from nutritionally unbalanced diets. This can cause cellular damage and ultimately death, potentially due to oxidative stress (Stone et al., 2014).

In general, abalone fed a macroalgae diet have higher antioxidant capabilities compared to those fed a commercial diet (Wan et al., 2004). Macroalgae have been reported to exhibit antimicrobial activities (Manivannan et al., 2011; Rattaya et al., 2015; Salvador Soler et al., 2007) as well as providing an array of beneficial nutrients and compounds when ingested (Ahn et al., 2002; Chandini et al., 2008; Cruz-Suárez et al., 2010). Given the high costs of macroalgae, interest has recently turned to trialing the use of food by-products and herbal extracts as a cost effective dietary supplement to commercial feeds in animal husbandry (Costa et al., 2013).

Grape seed extract (GSE) is rich in polyphenolic compounds and has well documented antioxidant, antimicrobial and anti-inflammatory properties when ingested (Perumalla and Hettiarachchy, 2011). In abalone, GSE has already been shown to act as an efficient dietary additive to improve productivity and reduce mortality during summer mortality events (Duong et al., 2016; Lange et al., 2014). However due to the complex nature of summer mortality and its association with numerous potential stressors, it is difficult to pinpoint the precise mechanism resulting in the increased survival of abalone.

RNA sequencing (RNA-seq) reveals a snapshot of an organisms gene expression at a specific time (Chu and Corey, 2012) and has proven to be a useful tool for identifying important gene pathways affected by different diets in chickens (Li et al., 2014; Xu et al., 2015), fish (Reyes-Becerril et al., 2013; Yarahmadi et al., 2014), shrimp (Zhang et al., 2013), oysters (Joubert et al., 2014) and abalone (Wu et al., 2010; Zhang et al., 2010; Mateos et al., 2012b). For abalone, gene expression analysis has been shown to be an efficient tool used to study targeted biological pathways involving the effects of dietary oil (Mateos et al., 2011, 2012a, b), zinc (Wu et al., 2011), selenium (Y. Zhang et al., 2011), iron (Wu et al., 2010) and α-lipoic acid (Zhang et al., 2010).

The aim of this study was to utilize a RNA-seq approach to investigate the interactive effects of water temperature (heat stress) and diet composition on abalone gene expression. Differences in gene expression in response to heat stress between abalone fed live *Ulva lactuca* or a commercial diet with or without 5% GSE supplementation were examined. Mechanisms and diet related gene pathways that may be responsible for promoting abalone survival during a heat stress event are discussed.

2. Methods

Details of study animals, temperature challenge procedure and resulting survival for abalone fed commercial and GSE supplemented diets for this study are described in Duong et al. (2016). Treatment groups of abalone fed a 100% live *U. lactuca* diet were run simultaneously alongside the commercial diet treatment groups described in Duong et al. (2016) under the same conditions.

2.1. Experimental treatments and diets

Five treatments were used in this study: (i) commercial diet fed abalone maintained at 22 °C, (ii) commercial diet fed abalone maintained at 25 °C, (iii) commercial + 5%GSE diet fed abalone maintained at 25 °C, (iv) live *U. lactuca* fed abalone maintained at 22 °C and (v) live *U. lactuca* fed abalone maintained at 25 °C. The "commercial" diet was provided by Eyre Peninsula Aquafeeds and consisted of Abgrow diet 5 mm chips. The "commercial + 5%GSE" diet consisted of 5% Australian GSE (GSeedEX grape seed tannin, Tarac Technologies Pty Ltd., Nuriootpa, SA, Australia) formulated into Abgrow mash as described in Lange et al. (2014). The live *U. lactuca* was collected from the Outer Harbor area of St Vincent Gulf, South Australia. *U. lactuca* was cultured at South Australian Research and Development Institute, South Australian Aquatic Science Centre (SARDI SAASC) under natural light and an ambient photoperiod in 4000-L parabolic tanks containing sand-filtered seawater, as described by Stone et al. (2014). The live *U. lactuca* diet will hereafter be referred to as the "*Ulva*" diet. All abalone diet treatments were fed to excess at 16:00 with cleaning and collection of uneaten food performed daily at 8:30 as detailed in Duong et al. (2016).

2.2. Experimental system

Three-year-old greenlip abalone (*Haliotis laevigata*) were obtained from South Australian Mariculture (Boston Point, Port Lincoln, South Australia). One month prior to the experiment, abalone were transferred to 500 L flow through holding tanks at SARDI with aerated seawater (21 °C), photoperiod and fed 5 mm commercial diet chips. Ten abalone at a time were removed from the holding tanks and interspersed among four replicate tanks per treatment. The experiment ran for 38 days including a one week acclimatization period where temperatures were raised by ~1 °C per day from 21 °C to the treatment temperatures of 22 °C and 25 °C. 22 °C was selected as the control temperature as it is the highest temperature at which no mortalities have been recorded and is considered an optimal temperature for growth (Lange et al., 2014). Animals that died throughout the experiment were weighed and recorded and replaced with similar sized tagged abalone to maintain stocking densities. Abalone replaced throughout the experiment to maintain stocking densities were not selected for final sampling and RNA-seq. The experimental procedure is described in detail in Duong et al. (2016).

2.3. Sampling collection and RNA extraction

On the final day of the experiment, 2–3 epipodial tentacles of two abalone from each of the treatment tanks were sampled using dissection scissors (four tanks per treatment). This resulted in eight samples per experimental treatment. Sampling occurred within 2 min after abalone were removed from the tank. Tentacles were immediately stored in *RNAlater*, placed on ice and subsequently stored at − 80 °C.

Epipodium can be clipped without harming the animal, has been found to be responsible for up to 14% of oxygen uptake (Taylor and

Ragg, 2005) and contains the second largest volume of haemolymph, second to the kidney (Jorgensen et al., 1984), and therefore, sampling this tissue enables us to develop practical and non-lethal means for studying the response of the transcriptome to summer stressors, diets and other factors of importance to abalone aquaculture. The transcriptome of tissue and haemolymph from the epipodium has been successfully applied to study the response of abalone to temperature stress (Liang et al., 2014), the basal gene expression of summer mortality resilient and susceptible abalone (Shiel et al., 2017), and to find genes differentially expressed in association with fast growth in abalone (Choi et al., 2015).

RNA was extracted from epipodial tentacle samples using an RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol "Purification of Total RNA from Animal Tissue". Tissue samples were disrupted and homogenized using a desktop homogenizer (*Janke & Kunkel, Ultra-Turrax T25*). RNA quality and quantity was estimated using a *Thermo Scientific Nanodrop* (2000). Library preparation and 100 base pair (bp) single-end RNA sequencing (Illumina HiSeq2000) (two lanes) was outsourced to the Australian Genome Research Facility (AGRF). Gene expression sequence data used in this study is available in the NCBI Short Read Archive under Bioproject PRJNA286263.

2.4. Sequence mapping

In the absence of a reference genome for *H. laevigata*, a *de novo* transcriptome was previously assembled as a reference for read mapping and tentacle gene expression profiling (Shiel et al., 2015). Individual genes for each sample were mapped back to the transcriptome with the alignment program *Bowtie* (Version 1.0.0) (Langmead et al., 2009) as implemented in *Trinity* (Version 10.5.2012) (Grabherr et al., 2011). Transcript abundance of *de novo* assembled genes was calculated using *RSEM* (Li and Dewey, 2011).

2.5. Differential expression

RSEM count data analyses were performed using R (version 3.3.0). We utilized the *Limma* RNA-seq differential gene expression method (Smyth, 2005), which uses the *Voom* module to transform the data based on observational-level weights derived from the mean-variance relationship (Ritchie et al., 2015). This method calculates the non-parametric estimates of mean-variance relationships to estimate weights for a linear model analysis of log-transformed counts (normalized for sequence depth) with the empirical Bayes Shrinkage of variance parameters. Differential expression analyses were performed to examine the differences in gene expression between feed and temperature treatments of interest by first fitting a linear model to estimate the variability in the data with *lmFit* (Smyth, 2005) including all five treatments with commercial diet representing the baseline. Grouping of abalone within tanks was accounted for by including tank as random effect with the *duplicateCorrelation* function within *Limma* (Smyth, 2005) that estimates a common value for the intra-duplicate correlation.

Tests for differential expression were performed by constructing the following pairwise contrasts based on this model (i) *Ulva* diet at 22 °C vs. a commercial diet at 22 °C, (ii) *Ulva* diet at 25 °C vs. a commercial diet at 25 °C, (iii) commercial + 5%GSE diet at 25 °C vs. a commercial diet at 25 °C, (iv) *Ulva* diet at 22 °C vs. an *Ulva* diet at 25 °C, (v) commercial diet at 22 °C at vs. a commercial diet at 25 °C. In order to identify genes showing differential expression, an omnibus test (F-test) was used to analyze the effects of diet (contrasts i, ii & iii) and the effects of temperature (contrasts iv & v). Genes differentially expressed were selected by *F*-test with a 5% false discovery rate (FDR) (Benjamini and Hochberg, 2000). Multidimensional scaling was used to visualize relatedness of the experimental groups (control commercial diets versus the GSE supplemented and *Ulva* diets). Given a set of expression values for genes under the five experimental conditions, a matrix of up/down gene signature patterns was constructed with a comparison of the experimental group means to the global mean (Fig. 2). A heatmap was generated in R using the *heatmap.2* function (omitting row and dendrograms) of the *gplots* package (Warnes, 2016).

2.6. Functional annotation and key transcript validation

Differentially expressed genes were annotated using the *Trinotate* pipeline (version 1.1; http://trinotate.github.io/). *Trinotate* provides functional annotations for transcriptome sequences by combining protein prediction (via *Transdecoder*) (http://transdecoder.github.io/) and BLAST (Altschul et al., 1990) homology with the UniProt database, identification of Pfam (Finn et al., 2013) domains using HMMER (Finn et al., 2011), prediction of signal peptides using *SignalP* (Petersen et al., 2011), transmembrane regions using tmHMM (Krogh et al., 2001) and rRNA using RNAMMER (Lagesen et al., 2007). *Trinotate* assigned SwissProt identifiers (Farriol-Mathis et al., 2004) to differentially expressed genes. For the genes of interest presented in this study, if no SwissProt identifier was found, BLAST searches against non-redundant protein and nucleotide databases were attempted (1e-5 threshold). Gene Ontology (GO) terms were retrieved from *QuickGO* (Binns et al., 2009). These GO terms were used to determine the higher-level immune response GO classifications using *CateGOrizer* (www.animalgenome.org/tools/catego/) through the *Immune System Gene Classes* classification method with consolidated single occurrences.

3. Results

3.1. Survival

After the 38 day trial, the survival of greenlip abalone fed a commercial diet at 22 °C was 85% and was significantly higher than the 40% survival recorded for abalone fed the same diet at 25 °C (n = 40; $P < 0.001$; Kaplan-Meier; Log-Rank test; Fig. 1). Dissimilarly, the survival of abalone fed commercial + 5%GSE diet at 25 °C (77.5%) was not significantly reduced in comparison to the same 22 °C commercial diet control abalone (n = 40; $P = 0.402$; Kaplan-Meier; Log-Rank test; Fig. 1). The survival of abalone fed an *Ulva* diet at 22 °C was 72.5% and was not significantly less in abalone fed the same diet at 25 °C (52.5%) (n = 40; $P = 0.078$; Kaplan-Meier; Log-Rank test; Fig. 1). Pairwise comparison Log-Rank tests for all treatments available in Fig. S1.

3.2. Differential expressed genes related to diet

Twenty-four genes were found to be differentially expressed (FDR < 0.05) using an F-test across the three diet and temperature

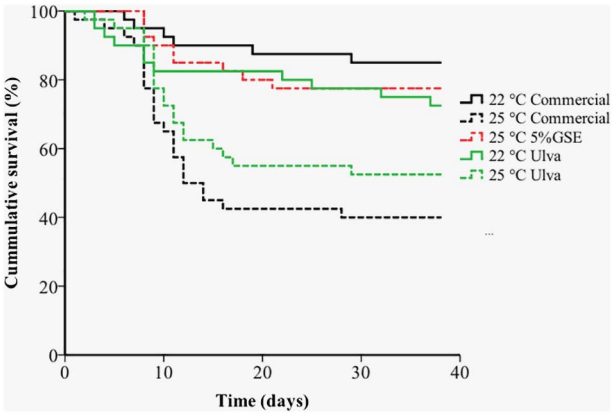

Fig. 1. Kaplan-Meier survival curves of greenlip abalone (*Haliotis laevigata*) fed the commercial and *Ulva* diets at 22 °C, and the commercial diet, *Ulva* diet and commercial + 5%GSE diet at 25 °C.

Table 1
Differentially expressed genes due to diet. Log_2 fold-change from commercial diet controls (C22 & C25) are displayed for *Ulva* 22 °C (U22), *Ulva* 25 °C (U25) and the commercial + 5%GSE diet at 25 °C (G25). Adjusted *P* value corresponds to an F-test across all three of the contrasts for which Log_2 fold-changes are shown. Log_2 average CPM (counts per million) of each gene is displayed.

Transcript	SwissProt ID	Protein description	Immune class gene ontology	Log_2 fold change			Adjusted *P* value (FDR)	Average CPM (Log_2)
				U22 vs. C22	U25 vs. C25	G25. vs C25		
comp103470_c1	ACT_PLAMG	Actin, adductor muscle	–	− 0.62	− 0.55	− 0.15	0.0474	11.43
comp96445_c0	EIF3J_XENTR	Eukaryotic translation initiation factor 3 subunit J	GO:0008152 - metabolism GO:0019538 - protein metabolism	− 0.56	− 0.16	0.24	0.0383	8.27
comp102190_c0	CATA_DROME	Catalase	GO:0005739 - mitochondrion GO:0006950 - stress response GO:0008152 - metabolism GO:0009056 - catabolism	− 0.37	− 0.44	0.22	0.0474	7.95
comp102157_c0	6PGD_HUMAN	6-phosphogluconate dehydrogenase, decarboxylating	GO:0005975 - carbohydrate metabolism GO:0008152 - metabolism	− 0.55	− 0.45	− 0.13	0.0474	7.50
comp90124_c0	–	–	–	− 0.66	− 0.15	0.26	0.0474	7.45
comp97610_c0	S6A13_MOUSE	Sodium- and chloride-dependent GABA transporter 2	–	− 0.65	− 0.32	− 0.03	0.0460	6.23
comp104372_c1	MARH4_DANRE	E3 ubiquitin-protein ligase MARCH4	GO:0008152 - metabolism GO:0019538 - protein metabolism	0.70	0.45	− 0.02	0.0460	6.21
comp91191_c0	PSA5_MOUSE	Proteasome subunit alpha type-5	GO:0008152 - metabolism GO:0009056 - catabolism GO:0019538 - protein metabolism GO:0042590 - antigen presentation, exogenous antigen via MHC class I	− 0.48	− 0.27	− 0.14	0.0474	5.96
comp87791_c0	ERP29_BOVIN	Endoplasmic reticulum resident protein 29	GO:0000165 - MAPKKK cascade GO:0006915 - apoptosis GO:0006950 - stress response GO:0008152 - metabolism GO:0019538 - protein metabolism GO:0042981 - regulation of apoptosis GO:0043408 - regulation of MAPKKK cascade	− 0.75	0.24	0.21	0.0474	5.96
comp107321_c1	–	–	–	− 0.32	− 0.30	− 0.13	0.0474	5.87
comp98011_c0	–	–	–	− 0.32	− 0.48	− 0.38	0.0474	5.61
comp97842_c0	PCY2_HUMAN	Ethanolamine-phosphate cytidylyltransferase	GO:0006629 - lipid metabolism GO:0008152 – metabolism	− 0.36	− 0.22	− 0.16	0.0460	5.60
comp90914_c0	G6PI_PIG	Glucose-6-phosphate isomerase	GO:0005125 - cytokine activity GO:0005975 - carbohydrate metabolism GO:0008152 - metabolism GO:0009056 – catabolism	− 0.49	− 0.53	− 0.07	0.0474	5.48
comp91012_c1	NFAT5_HUMAN	Nuclear factor of activated T-cells 5	GO:0001816 - cytokine production GO:0006950 - stress response GO:0008152 - metabolism GO:0009628 - response to abiotic stimulus	0.45	0.57	0.34	0.0474	5.13
comp77500_c0	BL1S2_XENTR	Biogenesis of lysosome-related organelles complex 1 subunit 2	–	− 0.45	− 0.34	− 0.20	0.0258	4.82
comp91841_c0	–	–	–	− 1.00	− 0.18	− 0.32	0.0474	4.57
comp104589_c0	MANF_HUMAN	Mesencephalic astrocyte-derived neurotrophic factor	GO:0006950 - stress response	− 1.15	0.00	− 0.11	0.0474	4.40
comp35390_c0	–	–	–	0.38	0.52	0.26	0.0383	4.18
comp103377_c0	FACE1_MOUSE	CAAX prenyl protease 1 homolog	GO:0008152 - metabolism GO:0009056 - catabolism GO:0019538 - protein metabolism	− 0.50	− 0.17	0.10	0.0258	4.17
comp103327_c0	128UP_DROME	GTP-binding protein 128up	–	− 0.61	− 0.20	− 0.09	0.0474	4.01
comp97852_c0	–	–	–	− 0.56	− 0.36	− 0.42	0.0310	3.94
comp101706_c0	CE051_DANRE	UPF0600 protein C5orf51 homolog	–	− 0.61	− 0.55	− 0.27	0.0474	3.46
comp101200_c1	ABCA1_MOUSE	ATP-binding cassette sub-family A member 1	GO:0006629 - lipid metabolism GO:0006897 - endocytosis GO:0006909 - phagocytosis GO:0006950 - stress response GO:0008152 - metabolism GO:0009605 - response to external stimulus GO:0019538 - protein metabolism	− 0.58	− 0.08	0.48	0.0474	3.35
comp102246_c1	PATS1_DICDI	Probable serine/threonine-protein kinase pats1	GO:0000910 - cytokinesis GO:0008152 - metabolism GO:0019538 - protein metabolism	− 0.85	− 0.64	− 0.37	0.0474	2.86

contrasts described in Section 2.5 (Table 1). Average expression of these genes ranged from 2.86 to 11.43 counts per million (log_2) with relatively low fold changes (Table 1).

Eighteen of the 24 differentially expressed genes discovered were assigned a homologous Swissprot gene ID (Table 1). The remaining six genes were unable to be annotated with a further NCBI non-redundant BLASTX database search (E value cutoff: 1×10^{-5}). Seventeen of the 18 annotated genes were assigned Gene Ontology (GO) terms (Table S1). In total, 260 GO terms were assigned to the differentially expressed genes. Eighty-eight of these GO terms were assigned to 19 higher immune related classification GO terms (Table S2). A large majority of the genes have known involvement in metabolism (n = 12) and more specifically protein metabolism (n = 7). A smaller proportion of genes were identified to have known involvement in stress responses (n = 5; Table 1).

3.3. Gene signature profiling of abalone fed commercial and Ulva diets

The greatest difference in the expression levels of treatments was detected between abalone fed the *Ulva* and commercial diet treatments at 22 °C. Of the genes that were differentially expressed, 21 were expressed at significantly lower levels in the *Ulva* treatment, while only three genes were expressed at higher levels in abalone fed *Ulva* relative to abalone fed the commercial diet (Fig. 2). These genes possess a diverse array of immune class GO terms including involvement in metabolism, stress response and cell and apoptosis signaling (Table 1).

Many of the gene expression signatures due to differences in diet recorded at 22 °C were maintained in abalone after exposure to heat stress at 25 °C (Fig. 2; central panel). Here, 18 genes showed distinct expression signatures between the *Ulva* and the commercial diet (Fig. 2). Four genes demonstrated higher expression levels in *Ulva* fed abalone in comparison to those fed a commercial diet. Three of these genes were assigned functional immune class GO terms including; involvement in metabolism (NFAT5_HUMAN, MARH4_DANRE, ERP29_BOVIN), stress response (ERP29_BOVIN) and apoptosis (ERP29_BOVIN; Table 1). ERP29_BOVIN demonstrated opposite expression signatures between the commercial and *Ulva* diets at 22 °C (high expression in *Ulva*, low expression in commercial), expressed in the opposite manner at 25 °C (low expression in *Ulva*, high expression in commercial; Fig. 2). Notable genes found to be expressed at low levels in abalone fed the *Ulva* diet relative to abalone fed the commercial diet at 25 °C included; CATA_DROME, G6PI_PIG, 6PGD_HUMAN, PATS1_DICDI, PCY2_HUMAN, BL1S2_XENTR, FACE1_MOUSE, PSA5_MOUSE, ACT_PLAMG, CE051_DANRE. These genes possess numerous immune class GO term functions, primarily including involvement in metabolism, stress response and cell signaling (Table 1).

Despite their distinct expression differences at 22 °C, small to no gene expression differences due to diet was seen for several genes at 25 °C between abalone fed *Ulva* and commercial diets (including; 128UP_DROME, MANF_HUMAN, ABCA1_MOUSE and EIF3J_XENTR; Fig. 2). Functional immune class GO terms describe involvement in stress response (MANF_HUMAN), metabolism (ABCA1_MOUSE, EIF3J_XENTR), response to stress, external stimuli, and phagocytosis (ABCA1_MOUSE; Table 1).

3.4. Gene signature profiling of abalone fed a GSE supplemented diet

Abalone fed a commercial + 5%GSE diet expressed 14 genes at higher levels, nine at lower levels and one (comp107321) at average levels relative to the expression levels of abalone from the *Ulva* or commercial diet treatments (Fig. 2). The expression profile of abalone fed a GSE supplemented diet was similar to those abalone treated with both *Ulva* and commercial diets at 25 °C. Genes expressed at low levels in abalone fed the GSE supplemented diet, such as 6PGD_HUMAN, PATS1_DICDI, PCY2_HUMAN and BL1S2_HUMAN demonstrated similar expression levels in abalone fed *Ulva* diet at 25 °C (Fig. 2). Functional immune class GO terms assigned to these genes included involvement in metabolism (PATS1_DICDI, PCY2_HUMAN, 6PGD_HUMAN) and cytokinesis (PATS1_DICDI; Table 1). The high expression level of NFAT5_HUMAN and ERP29_BOVIN detected in abalone fed the GSE supplemented diet also displayed a similar expression pattern demonstrated by abalone fed the *Ulva* diet at 25 °C (Fig. 2).

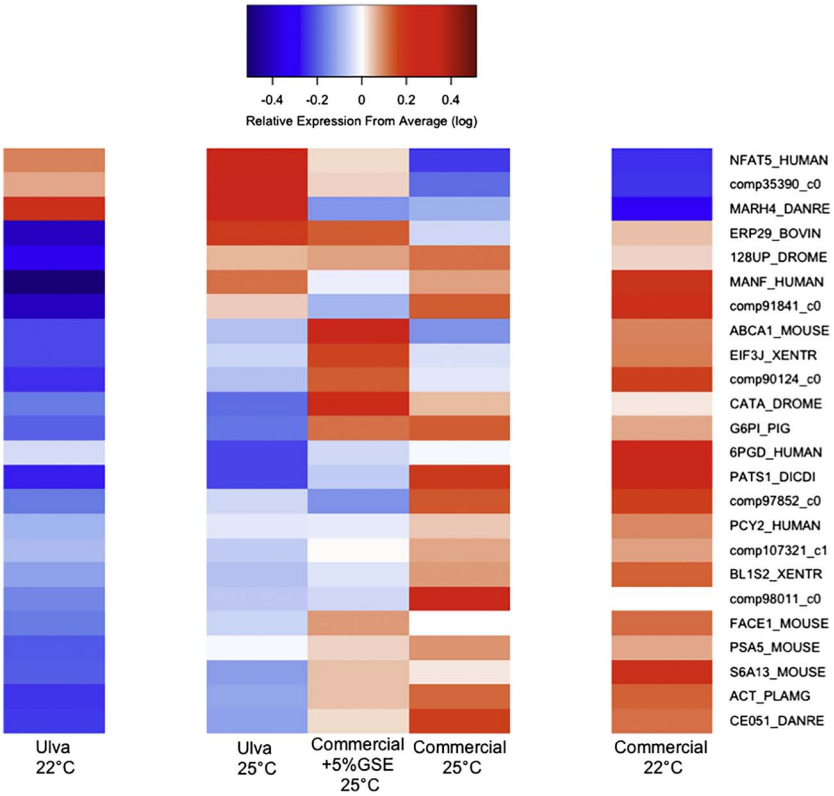

Fig. 2. Heatmap of gene expression differences between feed and temperature stressed treatments. The colour comparison represents (log_2) gene expression differences in treatments relative to the total average expression of all treatments. Blue heatmap bars represent lower expression levels and red bars represent higher expression levels relative to the average expression of all treatments for each of the 24 genes. Differentially expressed genes (listed to the right) were ordered by hierarchal clustering of the 22 °C *Ulva* and commercial diets. Treatments groups are indicated along the bottom track of the graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Several highly expressed genes in abalone fed the GSE supplemented diet (FACE1_MOUSE, PSA5_MOUSE, S6A13_MOUSE, ACT_PLAMG and CE051_DANRE, CATA_DROME, G6PI_PIG) also demonstrated similar expression levels in abalone fed the commercial diet at 25 °C (Fig. 2). Immune class GO terms assigned to these genes include involvement in metabolism (FACE1_MOUSE, PSA5_MOUSE, CATA_DROME, G6PI_PIG), antigen presentation (PSA5_MOUSE), cytokine activity (G6PI_PIG) and stress response (CATA_DROME; Table 1). The low expression level of MARH4_DANRE was also common between abalone fed the GSE supplemented diet and abalone fed a commercial diet at 25 °C (Fig. 2).

3.5. Differentially expressed genes affected by temperature

Five hundred and twenty-one genes were found to be differentially expressed (FDR < 0.05) using an F-test across the two temperature contrasts between *Ulva* and commercial diet fed abalone at 22 °C and 25 °C (Table S3). Average expression of these genes ranged from $\log_2(-4.62)$ up to $\log_2(11.11)$ counts per million with fold changes ranged between −7.6 and 5.69 (Table S3). Several of these genes such as HSP10 (comp91833_c0), HSPA5 (comp99703_c0), HSP60 (comp73030_c0), and AHSA1 (comp95581_c0) are known to be involved in the heat shock response of molluscs (Artigaud et al., 2015; Clark et al., 2008; Falfushynska et al., 2016; Wang et al., 2014), and confirm the degree of heat stress exposure in this experiment. The expression of these four heat stress response genes were significantly up-regulated in heat stressed treatments with fold changes ranging from $\log_2(0.44)$ up to $\log_2(1.04)$ (Table S3). HSPA5 was an exception to this general pattern with a small decrease recorded in expression for heat stressed abalone fed a commercial diet at 25 °C ($\log_2(-0.2)$) (Table S3).

Out of the 521 genes differentially expressed between temperature treatments, two of these genes (comp102157_c0 and comp87791_c0) were also found to be significantly differently expressed between diet treatments. Comp102157_c0 (6PGD_HUMAN) was down regulated in abalone at 25 °C in both *Ulva* and commercial diet treatments relative to their 22 °C controls with $\log_2$ fold-changes of −0.35 and −0.4, respectively (Table S3). Comp87791_c0 (ERP29_BOVIN) was down regulated in abalone fed a commercial diet at 25 °C relative to abalone at 22 °C with $\log_2$ fold-changes of −0.21. Contrastingly, comp87791 (ERP29_BOVIN) expression was significantly up-regulated in abalone fed a *Ulva* diet at 25 °C relative to abalone at 22 °C with $\log_2$ fold-changes of 0.78 (Table S3).

4. Discussion

This is the first study to utilize Next-Generation sequencing techniques to undertake a transcriptome wide (tentacle transcriptome) analysis to investigate the combined effects of different diets and temperatures on abalone gene expression as a means of identifying genes associated with increased survival. Results of our RNA-seq and bioinformatic analyses identified that heat stress had a considerable transcriptomic response with 521 differentially expressed genes associated with differences in temperature. Of greatest interest in this study were the 24 differentially expressed genes that were influenced by diet. Many of these gene expression responses were diet specific. Our results demonstrate that diet could potentially alter the response of the innate immune system before and during heat stress.

A lower level of expression of differentially expressed genes was detected in abalone treated with an *Ulva* diet (generally promoting high survival) compared to abalone fed commercial feed (resulting in low survival). The comparably low survival of abalone fed the commercial diet relative to abalone sustained on an *Ulva* or GSE supplemented diet during heat stress, may be the result of a compromised immune system even before being subjected to heat stress. Yarahmadi et al. (2014) suggested that the low expression of stress response genes and coding proteins in stressful situations is possibly due to an increased resistance to common unwanted stressors during culture.

4.1. Heat shock protein response to temperature

Several commonly identified members of heat stress response gene families were identified to be differentially expressed between abalone maintained at 22 °C and 25 °C. HSP60, HSP10 and AHSAI genes were consistently up-regulated in heat stressed abalone fed either a commercial or *Ulva* diet relative to abalone held at the control temperature with expression patterns consistent with past heat stress response research of molluscs (Artigaud et al., 2015; Falfushynska et al., 2016). However the expression of HSPA5 was higher in abalone fed an *Ulva* diet in response to heat stress, while lower levels of expression were found in abalone fed a commercial diet, relative to the 22 °C. HSPA5 (GRP78_CHICK) is a member of the HSP70 family and considered to be involved in the heat stress response in molluscs (Clark et al., 2008; Wang et al., 2014).

4.2. Oxidative response pathways and diet related survival of abalone

Biogenesis of lysosomal organelles complex-1, subunit 2 (BL1S2) was down-regulated in greenlip abalone fed the *Ulva* or GSE supplemented diet. BL1S2 is required for the formation of lysosome-related organelles and plays a role in intracellular vesicle trafficking. Lysosome membrane proteins are an active contributor to apoptosis signaling induced by classic stimuli such as oxidative stress (Köbis et al., 2013). Oxidative stress and reactive oxygen species (ROS) destabilize the lysosomal membrane through lipid peroxidation (Persson et al., 2003), however, these effects can be neutralized by antioxidants (Roberg and Ollinger, 1998). As a result, lipid peroxidation is commonly used as a measure of oxidative stress (Vlahogianni et al., 2007). With both *Ulva* and GSE known to provide antioxidants when ingested, the lower expression of BL1S2 in abalone fed *Ulva* and GSE supplemented diets suggests an active antioxidant effect. Our results support those found in similar studies. For example, BL1S2 has been found to be expressed at significantly lower levels in fit and robust strains of rainbow trout (*Oncorhynchus mykiss)* naturally selected for survival while challenged with pathogen and temperature stress (Köbis et al., 2013). Similarly to our study, Köbis et al. (2013) acknowledged that nearly all differentially expressed genes associated with oxidative response pathways showed reduced expression in fitter rainbow trout. This suggests that fitter animals, such as abalone fed antioxidative compounds, may not need to express these genes at high levels.

Abalone fed the commercial diet had higher expression of ethanolamine-phosphate cytidylyltransferase (PCY2) relative to the *Ulva* or GSE supplemented diet. PCY2 is the main regulatory enzyme in the production of phosphatidylethanolamine, which plays a key role in regulation of cell growth and metabolic homeostasis (Pavlovic and Bakovic, 2013). PCY2 activity has also been linked oxidative stress levels (Basu et al., 2015) and has previously been found to be up-regulated in livestock and domestic animals experiencing what is commonly referred to as the “summer slump” (Bhusari et al., 2007). The summer slump that can occur in livestock and domestic animals is caused by ingesting long grasses infected with the fungus, *Neotyphodium coenophialum*, which has been found to be the cause for symptoms such as hyperthermia and decreases in feed intake, growth and reproductive fitness (Schmidt and Osborn, 1993). PCY2 has also been found to be up-regulated in European eels (*Anguilla anguilla*) from polluted sites (Baillon et al., 2015). High expression of this gene is generally associated with animals experiencing a form of stress and so may represent the lower stress threshold of abalone fed the commercial diet.

The enzyme 6-Phosphogluconate dehydrogenase (6PGD) contributes to antioxidant protection (Kozar et al., 2000; Puskas et al., 2000). The reaction catalysed by the 6PGD enzyme produces NADPH, which is an active component in gene pathways for protecting the cell

against oxidant agents (Kukielka and Cederbaum, 1990; Valderrama et al., 2006). The down-regulation of this gene in abalone maintained at 25 °C relative to those at 22 °C in both *Ulva* and commercial diet treatments suggests that the activation of this gene is affected by temperature. The basal expression of this gene however appears to be affected by diet. The lower expression of 6PGD in abalone fed the *Ulva* and GSE supplemented diet in comparison to the commercial diet suggests that abalone fed the commercial diet possess a higher physiological demand for oxidative defense or that the antioxidants in the *Ulva* and GSE supplemented feeds suppress the expression of some genes otherwise involved in promoting oxidative defense. 6PGD enzyme activity has been tied to heavy metal exposure in the grass carp (*Ctenopharyngodon idella*) (Hu et al., 2013), and high pesticide use (Ceyhun et al., 2010) and overstocking (Aksakal et al., 2011) in the rainbow trout (O.*mykiss)*.

Endoplasmic reticulum resident protein 29 (ERP29_BOVIN) is a member of the thioredoxin superfamily proteins, which have vital roles in oxidative protein folding (Lu and Holmgren, 2014). ERP29 expression can be induced by stress and may provide protection by facilitating the re-folding of denatured or aggregated proteins (Mkrtchiana et al., 1998). ERP29 has also been found to be induced with exposure to cadmium stress and is suggested to be part of the immune stress response in the mussel, *Mytilus galloprovincialis* (Wu et al., 2016). Interestingly, the expression of ERP29 was found to be expressed at lower levels in abalone fed a commercial diet when exposed to heat stress, yet expressed at higher levels in *Ulva* fed abalone relative to the unstressed controls on the same diets. The expression of ERP29 was also comparatively high in abalone fed the GSE supplemented commercial diet and exposed to heat stress. These patterns suggest that diets high in antioxidative compounds such as provided by an *Ulva* or GSE supplemented diet may provide the potential for abalone to respond to heat stress with increased expression of potentially vital oxidative defense genes. This may increase their chance of survival when exposed to stressors such as summer mortality.

Catalase is a key antioxidant enzyme that exists in all aerobic organisms (Klotz and Loewen, 2003). Catalase (CATA_DROME) expression was down-regulated in *Ulva* diet treatments with and without heat stress exposure. The expression of this gene was significantly higher in abalone fed the purely commercial diet at both the control and heat stress exposed treatments. Antioxidant enzymes are known to work as the first line of defense against free radicals (Roch, 1999). In the antioxidant enzymatic system, catalase promotes a high degree of resistance to hydrogen peroxide (W. Zhang et al., 2011) and is responsible for breaking down damaging compounds such as hydroxyl radicals, hypochlororous acid and singlet oxygen into oxygen and water (Anderson, 2001). Catalase activity has become a common measure of oxidative stress with pathogenic infection (W. Zhang et al., 2011) and exposure to pollution (Damiens et al., 2004; Oliveira et al., 2007; Vlahogianni et al., 2007) in molluscs. Catalase activity was also recorded to be higher in Pacific oysters (*Crassostrea gigas*) exposed to high temperatures relative to those that were not (Damiens et al., 2004). Considering the known function of catalase in the antioxidant defense system, the lower expression of the Catalase gene in abalone fed the *Ulva* diet in this study supports the idea that a *Ulva* diet provides some form of antioxidant defense when ingested (Wan et al., 2004). Interestingly, the expression of Catalase was relatively higher in abalone fed the GSE supplemented diet compared to those fed the *Ulva* and commercial diet treatments. The GSE supplemented diet resulted in significantly higher survival rates of abalone compared to the other heat stressed treatments which may suggest that abalone fed this diet may be better equipped to combat oxidative stress. Considering that the GSE supplemented diet contains both the regular commercial diet (high in protein and carbohydrate and fat) and a source of beneficial compounds (such as antioxidants) from the GSE, abalone on this diet may possess a relatively more complete diet when compared to either the purely commercial or *Ulva* diet tested in this study.

4.3. Innate immune response pathways and diet related survival of abalone

Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF_HUMAN) has been found to be up-regulated by various forms of endoplasmic reticulum stress, with silencing of MANF rendering cells more susceptible to endoplasmic reticulum stress-induced death and over expression improving cell viability (Apostolou et al., 2008). The high expression of MANF in both heat stressed *Ulva* and commercial diet treatments, as well as in the commercial diet treatment that was not exposed to heat stress, may suggest a stress reaction. Abalone fed the GSE supplemented diet did not appear to be affected in the same manner. The comparably lower expression of MANF in the GSE supplemented abalone may reflect a shift in gene pathways as a result of the supplementation. For example, different diets in the European sea bass (*Dicentrarchus labrax*) have been suggested to change in, or promote, alternate gene pathways involved in innate immune responses (Geay et al., 2011). It is also possible that abalone on the GSE supplemented diet may not have experienced the same degree of stress at the molecular level as the heat stressed *Ulva* and commercial diet fed abalone, given the high survival rate promoted by GSE.

This same distinction between the three heat stressed groups appears in the expression signature of ATP-binding cassette sub-family A member 1 (ABCA1_MOUSE) gene. ABCA1 is implicated in convergent functions including lipid metabolism, inflammation and apoptosis (Schmitz et al., 2000) such as preserving the viability of macrophages following exposure to oxidized phospholipids and apoptotic cells (Yvan-Charvet et al., 2010). However ABCA1 has been found to be negatively affected by external stressors resulting in down-regulation during heat (Tang et al., 2015) and inflammatory stress, which can result in lipid-mediated injuries in peripheral tissues (Ma et al., 2008). The gene expression levels of the abalone fed the commercial diet at 22 °C compared to the heat stressed abalone fed the commercial diet also suggests ABCA1 is down regulated with heat stress. However, the response of this gene with *Ulva* and GSE supplemented feed treatments do not. The highest level of ABCA1 gene expression was demonstrated in the GSE supplemented abalone, which suggests they have a greater ability to maintain this high expression during heat stress and potentially have an increased ability to preserve macrophages (Yvan-Charvet et al., 2010). Expression of ABCA1 was low in abalone fed *Ulva* even though the *Ulva* diet is known to promote survival during heat stress (Bansemer et al., 2016; Stone et al., 2014).

The Nuclear factor of activated T-cells 5 (NFAT5_HUMAN) gene is generally considered to be involved in the immune response during times of osmotic stress (Cheung and Ko, 2013; Neuhofer, 2010). The high expression of the NFAT5 in the *Ulva* and the GSE supplemented treatments along with their high survival rate after heat stress, may suggest a high immune response capacity. Gene expression analysis of the pearl oyster (*Pinctada fucata*) revealed that the NFAT5 genes may also be involved in the innate immune response to lipopolysaccharide and polyinosinic-polycytidylic acid, and in the nucleus inserting operation (Huang et al., 2015). NFAT5s increased expression has also been suggested to play a role in moderating osmotic stress in Atlantic salmon (*Salmo salar*) in response to changes between freshwater and seawater environments (Lorgen et al., 2017). NFAT5 activity has been demonstrated to be related to some cytokine activity such as that of Interleukin-1beta, which has been linked with the inhibition of apoptosis during hyperosmolar stress in human cell stress trials (Lee et al., 2008).

Ulvans are water-soluble sulfated polysaccharides derived from *Ulva* species of green seaweed that promote antioxidative effects (Govindan et al., 2012). Ulvans have also been found to increase the mRNA expression of cytokines such as Interleukin-1beta (Berri et al., 2016). Interleukin-1beta stimulates the immune responses by activating lymphocytes or promoting the release of other cytokines that then activate lymphocytes, macrophages or NK cells. In a study to describe the immunological benefits of nucleotide-supplemented turbot (*Scophthalmus*

maximus) feed, Interleukin-1beta was found to increase its expression significantly in comparison to the control diet and its high expression was indicative of tissue with high macrophage presence (Low et al., 2003). High Interleukin-1beta levels were also found to be associated with higher survival to bacterial challenge after guava leaf supplementation of the diet of rohu carp, *Labeo rohita*, (Giri et al., 2015). The high expression of the NFAT5 gene in the abalone fed *Ulva* without heat stress, and its continued high expression after heat stress exposure, suggests NFAT5 could be acting as a frontloading (Barshis et al., 2013) or preparative defense gene (Dong et al., 2008). Preparative defense genes usually possess a higher baseline expression prior to stress and help maintain physiological health by providing a faster protein level response when stress occurs. While the expression of NFAT5 found in this study is potentially linked to diets high in antioxidant compounds, there is also a general decrease in abalone appetite during heat stress (Bonga, 1997; Stone et al., 2014), which may suggest that the composition of the abalone diet immediately prior to heat stress may have a more important influence on survival than what the abalone consumes during the heat stress.

5. Conclusion

The nutrigenomic approach used here has revealed many genes with related processes affected by the different diets that may explain variation in survival of abalone during heat stress. In particular, genes involved in oxidative defense and innate immunity were influenced by diet. The general down regulation of many of these genes in the *Ulva* fed treatments relative to abalone fed the commercial diet may be explained by a high resistance to unwanted stressors. This suggests that commercial diets may lack nutritionally beneficial compounds that boost the immune system. The 5% GSE replacement diet may stimulate several alternate gene pathways involved in immune response. These pathways may be responsible for *Ulva* and GSE supplemented feeds resulting in high survival of greenlip abalone during heat stress. This information may assist future research to develop cost effective diets for improving the health, survival and productivity of abalone in aquaculture.

Acknowledgements

This genetic investigation was funded by the Australian Research Council (ARC) Discovery Project DP110100592 awarded to Jan Strugnell. Thanks to the Victorian Life Science Computation Centre for summer internship (BPS). Thanks to the La Trobe University School of Life Science Postgraduate Publication Award. This study was also partially funded by the Thriving Abalone Project (6251) as part of the Functional Food Focus Program being conducted by SARDI as part of the South Australian Government Primary Industries and Regions South Australia Agribusiness Accelerator Program. The authors also wish to thank Dr. Tom Coote and Kym Heidenreich (Eyre Peninsula Aquafeeds) for their technical and financial contributions to the supply and manufacture of the diets. The authors also wish to acknowledge Krishna-Lee Currie and Duong N. Duong of Flinders University for technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.aquaculture.2017.07.025.

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