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ORIGINAL ARTICLE

Effects of 5-Aminolevulinic Acid on Gene Expression, Immunity, and ATP Levels in Pacific White Shrimp, *Litopenaeus vannamei*

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Abstract

With the emergence of several infectious diseases in shrimp aquaculture, there is a growing interest in the use of feed additives to enhance shrimp immunity. Recently, the use of 5-aminolevulinic acid (5-ALA), a non-protein amino acid that plays a rate-limiting role in heme biosynthesis, has received attention for its positive effect on immunity in livestock animals. To evaluate the effect of 5-ALA in the Pacific white shrimp, *Litopenaeus vannamei*, we conducted microarray analysis, a *Vibrio parahaemolyticus* immersion challenge test, an ATP level assay, and gene expression analysis of some hemoproteins and genes associated with heme synthesis and degradation. Out of 15,745 *L. vannamei* putative genes on the microarray, 101 genes were differentially expressed by more than fourfold ($p < 0.05$) between 5-ALA-supplemented and control shrimp hepatopancreas. 5-ALA upregulated 99 of the 101 genes, 41 of which were immune- and defense-related genes based on sequence homology. Compared to the control, the 5-ALA-supplemented group had a higher survival rate in the challenge test, higher transcript levels of porphobilinogen synthase, ferrochelatase, catalase, nuclear receptor E75, and heme oxygenase-1 and higher levels of ATP. These findings suggest that dietary 5-ALA enhanced the immune response of *L. vannamei* to *V. parahaemolyticus*, upregulated immune- and defense-related genes, and enhanced aerobic energy metabolism, respectively. Further studies are needed to elucidate the extent of 5-ALA use in shrimp culture.

Keywords 5-aminolevulinic acid · Microarray · ATP level · Heme synthesis · AHPND

Introduction

Shrimp aquaculture has rapidly expanded in recent years but still experiences periodic losses from disease outbreaks (Wilson et al. 2015; Flegel 2012). Approximately 22% of the total shrimp production is lost as a result of diseases (Flegel et al. 2008), and thus, there is a great need to understand the immune defenses of farmed shrimp to reduce this loss. Currently, Pacific white shrimp *Litopenaeus vannamei* make the biggest contribution to worldwide shrimp production. However, a bacterial disease called acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus*, has resulted in huge economic losses in *L. vannamei* farms since 2009 (Lai et al. 2015).

In animal husbandry, the incorporation of antibiotics in feeds has helped to prevent bacterial diseases (Lara-Flores et al. 2003). However, due to the heavy use and abuse of these prophylactic antibiotics in aquaculture, they have been strictly regulated or even prohibited in many countries (Cabello 2006). Aside from the emergence of antibiotic-resistant bacteria, antibiotic residues can also build up in animal tissues and the surrounding environment and are subsequently passed on to consumers (Baquero et al. 2008). Therefore, an alternative strategy for improving the health status of shrimp is necessary (Miandare et al. 2016).

The use of 5-aminolevulinic acid (5-ALA) as a feed additive for livestock animals has been receiving growing attention for its ability to enhance immunity and growth. 5-ALA, an endogenous non-protein amino acid, is generally known for its use in clinical photodynamic diagnosis and therapy

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(Ishizuka et al. 2011). After its production cost was reduced, its application in fields other than medicine has greatly expanded (Wang et al. 2009a). 5-ALA is synthesized by condensing succinyl-CoA and glycine with aminolevulinic acid synthase (ALAS) as the catalyst. 5-ALA is the sole precursor of heme (Hara et al. 2016), which is a prosthetic group of several hemoproteins. Hemoproteins have roles in oxygen transport (hemoglobin and myoglobin), respiration (cytochromes), antioxidant defenses (catalase and peroxidase) (Tsiftsoglou et al. 2006), nitric oxide biosynthesis (nitric oxide synthase) (Yao et al. 2010), ecdysone synthesis (nuclear receptor E75) (Reinking et al. 2005), and metabolism of drugs and xenobiotics (cytochrome P450) (Annalora et al. 2017). Heme also regulates several metabolic pathways and the synthesis of several proteins (Jover et al. 2000). Heme biosynthesis, which involves eight enzymes, is limited by ALAS. ALAS activity appears to decrease due to stress and aging (Atamna 2004). Thus, exogenous 5-ALA may enhance the synthesis of heme in cells, and thereby enhance the activity of the above-mentioned hemoproteins.

Among the beneficial results of incorporating 5-ALA in feeds of farmed animals are the following: increased serum iron concentration and egg quality (Chen et al. 2008a) and modulated growth performance and inflammatory response (Sato et al. 2012) in chickens; increased lymphocyte concentrations (Yan and Kim 2011), immunity (Chen et al. 2008b), apparent total tract digestibility of nutrients in weaned piglets (Hossain et al. 2016); and, increased blood iron status of sows and suckling pig and enhanced the efficiency of iron transfer from sows to piglets through elevated milk iron concentrations (Wang et al. 2009a). While several studies have investigated the effect of 5-ALA feed supplementation on land animals, few data are available for aquaculture species. In eels, supplementing 5-ALA in feeds has improved growth performance (Yasumoto et al. 2012). To our knowledge, there have been no studies on the effect of 5-ALA supplementation in shrimp or other invertebrates.

In view of the beneficial effects of 5-ALA in previous studies, we examined the effects of 5-ALA on gene expression, immunity, and ATP levels of Pacific white shrimp *L. vannamei*.

Materials and Methods

Shrimp, Diet Preparation, and Experimental Design

A single batch of 200 *L. vannamei* juveniles with an average body weight of 1.29 g ± 0.037 (mean ± SEM) was used in this study. Shrimp were divided into two groups in 100-l tanks with recirculating water system: control group ($n = 100$) and 5-ALA group ($n = 100$). Shrimp were acclimatized for 1 week prior to the feeding experiment.

For the experimental diet, 5-ALA powder, which was kindly donated by SBI Pharmaceuticals Co., Ltd. (Tokyo, Japan), was mixed with basal diet to a final concentration of 15 ppm and was given to the 5-ALA group. The control group was fed with basal diet. Feeding frequency was 4× daily (8:00, 13:00, 18:00, 23:00), and feeding rate was 5% of the shrimp's body weight. Feeding ration was computed and adjusted based on the weekly weight measurements. Salinity (30 ± 2 ppt) and temperature (25 °C) were maintained and monitored daily.

After 2 weeks of feeding, the following experiments were conducted: (1) For the determination of gene expression profile by microarray analysis, hepatopancreas from 4 shrimp per group were used. (2) The result of the microarray analysis was then validated by real-time quantitative PCR (qPCR) analysis. (3) For the measurement of mRNA transcript levels of some hemoproteins and genes involved in heme synthesis and degradation, hepatopancreas were collected from 4 to 5 shrimp (3 shrimp were also collected 3 days after the feeding experiment). (4) For the immersion challenge test, 40 shrimp from each group were used, divided equally for high ($n = 20$) and low ($n = 20$) doses. (5) For the ATP level assay, 3 shrimp from each group were sampled and ATP levels of the hepatopancreas were determined colorimetrically using a commercial kit.

Microarray Analysis

Total RNAs from the hepatopancreas of 5-ALA and control groups were isolated using RNAiso (Takara, Japan) following the manufacturer's instructions. RNA quality and quantity were then assessed using Qubit 2.0 fluorometer (Invitrogen by Life Technologies, USA). For microarray sample preparation and processing, the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Inc.) was followed. Briefly, the recommended volume of RNA spike-in controls (RNA Spike-in Kit, One Color) was added to 200 ng of total RNA and was reverse-transcribed. Then, cRNA was labeled with Cyanine-3 CTP using Low Input Quick Amp Labelling Kit, One Color (Agilent Technologies, Inc.). Amplified or labeled cRNA samples were then purified using RNeasy Mini Kit (Qiagen, Germany). Hybridization samples were then prepared and subjected to hybridization for 17 h at 65 °C (Agilent Technologies, Inc.) using Custom Gene Expression Microarray GE 8x15k (Agilent Technologies, Inc.) containing 15,745 probes for known genes and expressed sequence tags from *L. vannamei*. Hybridized slide was then washed and scanned immediately using a DNA Microarray Scanner with Surescan High-Resolution Technology (Agilent Technologies, Inc.). Data were extracted from the scanned slides using Agilent Feature Extraction Software 10.7.3.1 using default parameters. To determine differentially expressed genes (DEGs), data were log-transformed, normalized, and analyzed using Subio Platform

software (Subio Inc., Amami-shi, Japan). Only DEGs with fourfold difference between the two groups were used for *t* test analysis with $p < 0.05$. BLASTX searches were performed in Blast2GO (http://www.blast2go.org/) with an e-value cutoff of 1e-5. To create a heatmap of DEGs, Heatmap Illustrator v1.0 (Deng et al. 2014) was used. For the Gene Ontology (GO) annotations, Blast2GO (http://www.blast2go.org/), the current literature and public databases were used. To give an insight on the attributes of a gene product, GO annotation results were classified with respect to molecular function, biological process and cellular components using Blast2GO (http://www.blast2go.org/).

Validation of Microarray Analysis Results by qPCR

To validate the success of microarray hybridization, 5 DEGs were selected for qPCR analysis: peritrophin-A (Per-A), invertebrate-type lysozyme (i-Lys), c-type lectin (CTL), nitric oxide synthase (NOS), and heme-binding protein (HBP). In the microarray results, the first three genes were upregulated by more than tenfold, while NOS is a hemoprotein upregulated by more than fourfold in 5-ALA group compared with the control. HBP, a downregulated gene in 5-ALA group, was also chosen for validation. All the primers (Table 1) used in this study, except for catalase (Wang et al. 2009b; see below), were based on an EST library previously obtained by our laboratory and designed using Primer 3 (Untergasser et al. 2012). One microgram of total RNA previously extracted for microarray analysis was used to synthesize cDNA using a High-Capacity Reverse Transcription kit (Applied Biosystems, USA) following the manufacturer's instructions. Synthesized cDNA was diluted five times and was used as template for qPCR analysis using Thunderbird™ SYBR® qPCR Mix (Toyobo). The amplification reaction was performed using a MicroAmp Optical 96-well reaction plate (Applied Biosystems, USA), with each well containing a total volume of 20 μl (10 μl of the qPCR Mix, 0.6 μl of each of the primers. 0.4 μl of ROX reference dye, 2 μl of cDNA template). Cycling conditions were performed as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. At the end of each qPCR reaction, dissociation analysis was performed to confirm detection of only one product. Validation of microarray analysis by qPCR was repeated three times.

Gene Expression of Some Hemoproteins and Genes Associated with Heme Synthesis and Degradation

Hepatopancreases were collected after 3 days and 2 weeks of feeding. Total RNA was extracted using RNAiso (Takara, Japan), and cDNA was synthesized using a High-Capacity Reverse Transcription kit (Applied Biosystems, USA), following the manufacturer's instructions. The relative mRNA expression of some hemoproteins (catalase (CAT) and nuclear receptor ecdysone-induced protein 75 (E75)), genes involved in the heme synthesis pathway (ALAS, porphobilinogen

Table 1 List of gene targets with corresponding primer sequences, amplicon size, and mean PCR efficiency ± SEM used for qPCR analysis in this study. F: forward primer. R: reverse primer

Gene target	Primer sequence (5′ to 3′)	Amplicon size (bp)	Mean PCR efficiency ± SEM
Elongation factor-1 alpha	F: ATTGCCACACCGCTCACA R: TCGATCTTGGTCAGCAGTTCA	55	2.0 ± 0.008
Peritrophin A	F: GGTATCTTCGCCGACAACCA R: TCTTCCTGTCGAAGTGCAGG	103	1.9 ± 0.008
Invertebrate-type lysozyme	F: CCTACCCAGGGGCATACTTC R: CTGCTTGTCAGCATTGTCGC	101	2.0 ± 0.011
C-type lectin	F: CAAGATGGCTCCCACCAACA R: GTCGAACTCGGCGTTATCGG	103	1.9 ± 0.007
Nitric oxide synthase	F: GGAAGACCCACGTCTGGAAG R: TCGAGCGATCTCCTTGAAGC	92	2.0 ± 0.008
Heme-binding protein	F: CACATCCCACCTCTCCATCT R: TCACAGTTGGGTCCTTCTCC	297	1.9 ± 0.010
Aminolevulinic acid synthase	F: CACTCCATGACTGGTGCTGT R: GACCCACAGCATGTACCTCA	100	1.9 ± 0.017
Porphobilinogen synthase	F: CAATGGCAGACATCCAACAC R: CACTGGTTGCTCTGCTTCAG	90	2.0 ± 0.012
Catalase	F: TCAGCGTTTGGTGGAGAA R: GCCTGGCTCATCTTTATC	120	2.0 ± 0.007
Ferrochelatase	F: CATGGCTGACTCCAGCTACA R: CCATCTCATGCAGGGTTTCT	147	2.0 ± 0.010
Nuclear receptor E75	F: GCCTACAACAAGCCCCATAA R: GCCAGAGAGGAAGTCTGGTG	131	1.7 ± 0.003
Heme oxygenase-1	F: CTGAGGAGCTCGATGAGGAG R: CATGGCCACAACACTACCAG	116	1.9 + 0.006

synthase (PBGS) and ferrochelatase (FECH)), and a gene involved in heme degradation (heme oxygenase-1 (HO-1)) were measured by qPCR using Thunderbird™ SYBR® qPCR Mix (Toyobo). The reaction volume mixture as well as the cycling conditions are similar as described above.

Immersion Challenge Test

A pathogenic *V. parahaemolyticus* AHPND D6 strain was used with two doses: the final concentrations were 1.08×10^6 and 3.60×10^5 cfu/ml for the high and low doses, respectively. At the end of the 2-week feeding trial, a total of 40 shrimp per group were sampled and were divided equally for high ($n = 20$) and low ($n = 20$) doses. Mortality was recorded daily up to 2 weeks, and survival curves were determined from 20 animals per group, per dose. During the entire duration of the challenge experiment, shrimp were fed continuously with the experimental diet (5-ALA group) and basal diet (control group).

ATP Level Assay

ATP concentration in the hepatopancreas was determined using an ATP Colorimetric Assay Kit (cat# ab83355, Abcam®, Cambridge, MA, USA) following the manufacturer's instructions. This assay is based on the phosphorylation of glycerol in order to generate a product that can be easily determined colorimetrically. Briefly, ~ 10 mg of tissues was harvested and washed in cold PBS. Samples were then deproteinized using a PCA/KOH deproteinization step described by the manufacturer. After deproteinization, tissue lysates were neutralized and were used for the assay. ATP standard was prepared to generate a standard curve estimating the amount of the ATP between the range of 0–10 nmol. Fifty microliters of samples and sample background controls was then loaded in a 96-well plate with a clear flat bottom. ATP reaction and background control mix were then added with the corresponding samples and sample background controls. After gentle mixing, the plate was incubated for 30 min protected from light, and the optical densitometry at 570 nm was measured using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific Inc., MA, USA).

Statistical Analysis

mRNA transcripts were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and expression levels of target genes were normalized using the reference gene elongation factor-1 alpha, which had been validated as a stable reference gene (Dhar et al. 2009; Leelatanawit et al. 2012) and used in previous shrimp studies (Maeda et al. 2014; Maralit et al. 2015). Data were log transformed (log base 2) before being analyzed by *t* test. PCR amplification efficiencies were calculated using the LinRegPCR program (Ruijter et al. 2009). Mean PCR efficiency range of 1.7–2.0 is considered acceptable (Lilly et al. 2011). Survival data were analyzed with the Kaplan-Meier method. ATP levels were calculated according to the manufacturer's instructions, and data were analyzed by *t* test. Figures were obtained, and statistical tests of their data were done with GraphPad Prism v6 (GraphPad, San Diego, CA, USA), unless otherwise stated. In all cases, differences between groups were considered to be significant or highly significant when $*p < 0.05$ or $**p < 0.01$, respectively.

Results

Gene Expression Profile After 5-ALA Supplementation

Of the 15,745 *L. vannamei* putative hepatopancreas genes spotted on the microarray, 101 were DEGs, i.e., transcript levels differed by more than fourfold, between the 5-ALA-supplemented and control groups at $p < 0.05$ (Online Resource 1). Since a particular gene can be associated with more than one GO or can have multiple functions/be involved in different biological processes depending on the amount and completeness of available information (Ashburner et al. 2000), key function/s of the DEGs are summarized in Table 2. Among these DEGs, 41 were immune- and defense-related genes based on sequence homology (Table 2). Twelve genes showed the highest fold-change (> 8-fold) at $p < 0.05$ (Fig. 1). With a cutoff value of 1e−5 used in BLASTX, 8 genes have uncharacterized description (8%), with some having conserved domains (Table 2).

GO annotations were classified into three high-level categories: biological process, molecular function, and cellular component. For biological process, the majority of the DEGs are involved in cell surface receptor signaling pathways, oxidation-reduction processes, system development, cellular protein modification processes, and cellular component organization (Fig. 2a. For molecular function, the majority of the DEGs are involved in protein binding, ATP binding, oxidoreductase activity, nucleic acid binding, kinase activity, and ATPase activity (Fig. 2b. For cellular component, most of the DEGs are integral component of the membrane, located in/a subcomponent of the nucleus, plasma membrane, and mitochondrion (Fig. 2c). For the complete list of GO at all levels based on biological process, molecular function and cellular component, please see Online Resource 2.

Validation of Microarray Analysis Results by qPCR

The gene expressions of Per-A, i-Lys, CTL, and NOS were significantly upregulated in the 5-ALA group compared to the control (Fig. 3a–d). HBP was also significantly downregulated (Fig. 3e) in 5-ALA group. The hepatopancreas mRNA

Table 2 Sequence description of differentially expressed genes with more than fourfold difference (*t* test, $p < 0.05$) between 5-ALA-supplemented and the control groups with their putative function/s and fold change values ($n = 101$)

Probe name	Blast2GO sequence description	Accession # of top hit	Putative function/s	Fold change
Upregulated				
Defense- and immune-related				
gnl|UG|Lva_S62275146	Penlectin 5–3	ASA69503.1	Pattern recognition protein	61.130
gnl|UG|Lva_S46116066	C-type lectin 4	ACJ06432.1	Pattern recognition protein	43.501
gnl|UG|Lva_S46171149	Invertebrate-type lysozyme 2	AMR70307.1	Anti-microbial peptide	39.855
gnl|UG|Lva_S46123758	Chitin-binding peritrophin a	CRK90713.1	Defense	22.410
gnl|UG|Lva_S46168815	Gastrolith protein 30	AGZ62051.1	Defense	11.579
gnl|UG|Lva_S62305143	Procollagen-lysine, 2-Oxoglutarate 5-dioxygenase 1-like	XP_018016972.1	Response to stress, oxidoreductase	11.235
gnl|UG|Lva_S46109860	Strongly chitin-binding protein	BAM99303.1	Defense	9.426
gnl|UG|Lva_S62266293	Regulator of G protein signaling	XP_021917975.1	Signal transduction	6.947
gnl|UG|Lva_S62297109	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1-like	XP_018016972.1	Response to stress, oxidoreductase	6.422
gnl|UG|Lva_S62307334	Spatzle protein	AEK86524.1	Cytokine-like ligand	6.160
gnl|UG|Lva_S62255860	Tyrosine-protein phosphatase non-receptor type 23-like	XP_018011683.1	Tumor suppressor, protein tyrosine phosphatase activity	6.131
gnl|UG|Lva_S62274562	c-Maf-inducing protein-like	XP_014679731.1	T cell signaling	6.035
gnl|UG|Lva_S46258442	Heat shock protein HSP70	ABG90491.1	Response to stress	5.733
gnl|UG|Lva_S62256116	Katanin p80 WD40 repeat-containing subunit	XP_018008283.1	Signal transduction, ATPase activity, cell cycle	5.623
gnl|UG|Lva_S62261227	Putative cuticle protein	ABU41025.1	Defense	5.610
gnl|UG|Lva_S46150288	Alpha2 macroglobulin	ACU31809.1	Protease inhibitor	5.518
gnl|UG|Lva_S62297550	4-Coumarate--CoA ligase 1-like	XP_015589274.1	Defense and cell rescue- related	5.296
gnl|UG|Lva_S62266725	Dual specificity protein phosphatase 7-like	XP_023240484.1	Signal transduction	5.148
gnl|UG|Lva_S62249924	Baculoviral IAP repeat-containing protein	XP_012230156.1	Apoptosis, cell proliferation	4.894
gnl|UG|Lva_S62245446	Rho guanine nucleotide exchange factor 18	XP_021936117.1	Innate immune response, developmental protein	4.862
gnl|UG|Lva_S62283971	Endophilin b	EDS26080.1	Apoptosis, autophagy, vitellogenesis	4.699
gnl|UG|Lva_S62259172	MAP-kinase death activating protein domain	PNF35125.1	Apoptosis, cell proliferation	4.560
gnl|UG|Lva_S62279066	Chorion peroxidase-like	XP_011559330.1	Response to oxidative stress	4.527
gnl|UG|Lva_S62259184	Tyrosine-protein phosphatase non-receptor type 9-like	XP_021941822.1	Cellular response to cytokine stimulus, protein tyrosine phosphatase activity	4.506
gnl|UG|Lva_S55408693	Nitric oxide synthase	ADD63793.1	Anti-tumor, anti-microbial, cell redox homeostasis	4.492
gnl|UG|Lva_S62277020	Cell death specification protein	XP_015916806.1	Apoptosis, transcription regulation	4.363
gnl|UG|Lva_S62257348	NACHT and WD40 repeat domain-containing protein 2-like	XP_023215298.1	Signal transduction	4.283
gnl|UG|Lva_S62276046	Serine/threonine-protein phosphatase	XP_020605584.1	Signal transduction	4.237
gnl|UG|Lva_S62253807	Protein ecdysoneless homolog	XP_018028226.1	Defense, molting, cuticle biosynthesis	4.206
gnl|UG|Lva_S62296081	Serine protease 30-like	KDR12496.1	Anti-microbial peptide	4.162
gnl|UG|Lva_S62234151	Centaurin-alpha 1-like protein	ACN32215.1	GTPase activator activity, response to viral infection	4.139
gnl|UG|Lva_S62303631	Dopamine beta-hydroxylase	ANA78437.1	Neuroendocrine-immune regulation, oxidoreductase	4.099

Table 2 (continued)

Probe name	Blast2GO sequence description	Accession # of top hit	Putative function/s	Fold change
gnl|UG|Lva_S46149472	Zinc finger CCCH domain-containing protein	XP_021928951.1	Antiviral	4.054
gnl|UG|Lva_S62277107	Ankyrin repeat protein in complex with MAPK Erk2	3ZU7_B	Signal transduction	4.006
ATPase activity				
gnl|UG|Lva_S62234436	ATPase family AAA domain-containing protein 1-like	XP_018026005.1	ATPase activity	6.041
gnl|UG|Lva_S62298865	ATP-dependent RNA helicase	XP_013113540.1	ATPase and ATP-dependent multisubstrate helicase, mitochondrial RNA metabolism and maintenance	6.108
gnl|UG|Lva_S62263335	Phospholipid-transporting ATPase	KZS13761.1	ATPase activity, phospholipid transport	5.472
Cytoskeletal and extracellular matrix				
gnl|UG|Lva_S62306892	Fast-type skeletal muscle actin 18	AUB30297.1	Microfilament protein	7.194
gnl|UG|Lva_S62263162	Myosin-IIIa-like	XP_023490416.1	Microfilament protein	4.549
gnl|UG|Lva_S62241613	Tubulin gamma-2 chain	ANJ04738.1	Microtubule protein	4.533
gnl|UG|Lva_S62266557	Spectrin alpha chain-like	XP_022198458.1	Cytoskeletal scaffold protein, MAPK cascade[a]	4.325
gnl|UG|Lva_S46256645	Troponin C	AET36896.1	Microfilament protein	4.284
gnl|UG|Lva_S62298266	Myosin-I heavy chain-like	XP_018020635.1	Microfilament protein	4.210
gnl|UG|Lva_S62306849	Fast-type skeletal muscle actin 15	AUB30161.1	Microfilament protein	4.033
gnl|UG|Lva_S62273241	Fibrillin-3-like	XP_018028143.1	Extracellular matrix structural constituent	4.007
Biosynthesis and metabolism				
gnl|UG|Lva_S46189263	Putative Beta-hexosaminidase fdl	ANV82809.1	Carbohydrate metabolism	9.941
gnl|UG|Lva_S46131571	Chitooligosaccharidolytic beta-N-acetylglucosaminidase-like	XP_023339719.1	Carbohydrate/chitin metabolism	8.324
gnl|UG|Lva_S62297638	Type III iodothyronine deiodinase-like	XP_013381375.1	Hormone biosynthesis	6.584
gnl|UG|Lva_S62276901	Abhydrolase domain-containing protein 2	XP_018008977.1	Acylglycerol catabolism	6.228
gnl|UG|Lva_S62296086	Cyclase family protein	WP_026452667.1	Tryptophan catabolism	5.674
gnl|UG|Lva_S62263753	Peroxisomal acyl-coenzyme A oxidase 1-like	XP_019628198.1	Fatty acid beta-oxidation, oxidoreductase response to fungus[a]	5.170
gnl|UG|Lva_S62261229	GPI ethanolamine phosphate transferase 2	KZS03515.1	Glycosylphosphatidylinositol (GPI) anchor biosynthesis	5.012
gnl|UG|Lva_S62277057	Carbohydrate sulfotransferase 5-like	XP_018900167.1	Carbohydrate synthesis	4.825
gnl|UG|Lva_S46139796	Apolipoprotein D	ODM95517.1	Lipid metabolism, regulates resistance to stress[a]	4.762
gnl|UG|Lva_S62248295	Putative phosphatidylinositol 4-kinase alpha	OWR54925.1	Phosphatidylinositol biosynthesis	4.568
gnl|UG|Lva_S62264639	Dephospho-CoA kinase	XP_014283345.1	CoA biosynthesis	4.238
gnl|UG|Lva_S62235881	Phosphoribosylformylglycinamidine synthase, putative	EEB09999.1	De novo' inosine monophosphate biosynthesis	4.215
gnl|UG|Lva_S46106207	Putative RNA-directed DNA polymerase from transposon BS-like protein	KXZ75766.1	RNA-dependent DNA biosynthesis	4.151
gnl|UG|Lva_S46208394	Homocysteine S-methyltransferase family protein	WP_069134484.1	Methionine biosynthesis, response to DNA damage[a]	4.146
gnl|UG|Lva_S62264342	ADP-dependent glucokinase-like	XP_018118418.1	Carbohydrate metabolism	4.102
Cell differentiation and development				
gnl|UG|Lva_S62269495	Netrin receptor unc-5-like with Ig-like domain	XP_018024919.1	Multicellular organism development, axon guidance, apoptosis[a]	6.766
gnl|UG|Lva_S62269135	Low-density lipoprotein receptor-related protein 4	XP_023024784.1	Animal organ development	5.591

Table 2 (continued)

Probe name	Blast2GO sequence description	Accession # of top hit	Putative function/s	Fold change
gnl|UG|Lva_S62297772	Contactin-6-like, partial, with Ig domain	XP_018019989.1	Neuron differentiation	4.714
gnl|UG|Lva_S62243681	Protein SpAN-like isoform X2	XP_018024457.1	Multicellular organism development	4.462
gnl|UG|Lva_S62303410	CDK5 and ABL1 enzyme substrate	XP_021942726.1	Cell cycle regulation, nervous system development	4.263
gnl|UG|Lva_S62241181	Rabconnectin, putative	EEB16015.1	Lumen formation, regulation of Notch signaling pathway	4.237
gnl|UG|Lva_S62274130	Discoidin domain-containing receptor 2	XP_021937558.1	Regulates cell differentiation, fibrillar collagen receptor	4.083
gnl|UG|Lva_S62241396	Dentin sialophosphoprotein-like	XP_018027454.1	Ossification, extracellular matrix organization	4.055
gnl|UG|Lva_S62261352	Zinc finger protein castor	XP_018008763.1	Cell differentiation, tumor-suppressor[a]	4.000
Transport				
gnl|UG|Lva_S62242385	Multivesicular body subunit 12B-like	XP_018008997.1	Endosomal transport	6.132
gnl|UG|Lva_S62265716	Dynactin subunit 1-like	XP_013781001.1	Transport along microtubule	4.582
gnl|UG|Lva_S62271984	Sortilin-related receptor-like	XP_018326357.1	Low density lipoprotein binding and transport, multifunctional endocytic receptor	4.500
gnl|UG|Lva_S62259734	Monocarboxylate transporter 13	XP_008468265.1	Transmembrane transport	4.366
gnl|UG|Lva_S62301315	Kinesin-like protein unc-104	XP_021916356.1	Transport along microtubule, molting, ATPase activity	4.062
gnl|UG|Lva_S62243781	Epithelial chloride channel protein	XP_003220166.1	Chloride transport	4.059
gnl|UG|Lva_S62256243	Kinesin heavy chain	XP_018023092.1	Transport along microtubule	4.023
Transcription, processing and protein modification				
gnl|UG|Lva_S46109293	Histone-arginine methyltransferase CARMER-like	XP_018006811.1	Protein methylation, chromatin organization	10.238
gnl|UG|Lva_S62257277	NEDD8-activating enzyme E1 catalytic subunit-like	XP_018016262.1	Protein neddylation	5.840
gnl|UG|Lva_S62304626	Enhancer of split mbeta protein-like	XP_018024920.1	Transcription regulation	5.438
gnl|UG|Lva_S62304532	Carboxypeptidase B-like	XP_018017271.1	Metallocarboxypeptidase activity, proteolysis	5.120
gnl|UG|Lva_S46255192	ER membrane protein complex subunit 1-like	XP_018022828.1	Protein folding in ER	4.985
gnl|UG|Lva_S62242316	Protein Smaug homolog 1-like	XP_023227770.1	Regulation of transcription, DNA-templated	4.973
gnl|UG|Lva_S62272200	Mind bomb, putative	EEB19732.1	Protein ubiquitination	4.968
gnl|UG|Lva_S62241062	F-box only protein 9	KDR08236.1	Protein ubiquitination, innate immune response[a]	4.675
gnl|UG|Lva_S62257835	Cytoplasmic tRNA 2-thiolation protein 1-like	XP_013785076.1	tRNA wobble uridine modification	4.173
Others				
gnl|UG|Lva_S46176872	Acylphosphatase-2-like	XP_018022187.1	Acylphosphatase activity	5.838
gnl|UG|Lva_S62241534	Phytanoyl-CoA dioxygenase domain-containing protein 1	KZS15483.1	Dioxygenase activity, iron-binding	5.194
gnl|UG|Lva_S62279592	Hemicentin-2-like with Ig-like domain	XP_022903610.1	Sensory transduction	4.886
gnl|UG|Lva_S62243353	PiggyBac transposable element-derived protein 3-like	XP_020454066.1	Transposase, DNA-binding	4.481
gnl|UG|Lva_S62298684	Synapsin	XP_014258317.1	Neuron development, neurotransmitter secretion	4.618
gnl|UG|Lva_S62256608	Cytochrome c-type heme lyase	KZS09082.1	Cytochrome c-heme linkage	4.387
Uncharacterized proteins				
gnl|UG|Lva_S62285626	UP LOC106153133	XP_013382401.1	Conserved domain (CD): LicD superfamily	8.587
gnl|UG|Lva_S62290099	UP LOC108679145	XP_018023216.1	CD: none	6.245

Table 2 (continued)

Probe name	Blast2GO sequence description	Accession # of top hit	Putative function/s	Fold change
gnl|UG|Lva_S62303248	UP LOC108673930	XP_018017307.1	CD: none	4.281
gnl|UG|Lva_S62301535	Uncharacterized protein (UP) LOC105285227	XP_011347620.1	CD: GRIP (golgin-97, RanBP2alpha, Imh1p and p230/golgin-245) domain	4.245
gnl|UG|Lva_S62255538	UP LOC108677090	XP_018020728.1	CD: fibronectin 3	4.167
gnl|UG|Lva_S62240556	UP LOC108672397	XP_018015531.1	CD: zinc peptidase	4.157
gnl|UG|Lva_S62273258	UP LOC108669733, partial	XP_018012627.1	CD: none	4.093
gnl|UG|Lva_S62240969	UP LOC108677311	XP_018021013.1	CD: none	4.089
Downregulated				
gnl|UG|Lva_S46211088	NADH dehydrogenase 1 alpha subcomplex subunit 5	AAH91561.1	Mitochondrial electron transport	0.211
gnl|UG|Lva_S62297940	Heme-binding protein 2-like	XP_013406655.1	Negative regulation of mitochondrial membrane potential, positive regulation of necrotic cell death	0.099

[a] Putative immune-related function of some multifunctional genes not listed under "defense- and immune- related"

expressions of the selected genes by qPCR are consistent with those determined by the microarray analysis, validating our results. For all the genes evaluated, the mean PCR efficiencies were within the acceptable range of 1.7–2.0 (Lilly et al. 2011).

Expressions of Some Hemoproteins and Genes Associated with Heme Synthesis and Degradation

The transcript level of the ALAS gene was higher in the 5-ALA group compared to the control after 3 days of feeding but was almost equal between the two groups after 2 weeks of feeding (Fig. 4a). For two other genes involved in the heme synthesis pathway (PBGS and FECH), the transcript levels were higher in the 5-ALA group after both 3 days and 2 weeks of feeding (Fig. 4b–c). For CAT, which is a hemoprotein, transcript levels were also higher in the 5-ALA group at both sampling times (Fig. 4d). Another hemoprotein, E75, was also significantly higher in 5-ALA group after 2 weeks of feeding (Fig. 4e). HO-1, which is responsible for heme degradation, was also higher in the 5-ALA group after 3 days, while it was only slightly higher after 2 weeks (Fig. 4f).

Immersion Challenge Test

In the challenge tests (for each concentration of bacteria), the survival rates were higher for the 5-ALA group. For the lower dose of bacteria (3.60×10^5 cfu/ml), 5-ALA group showed 95% survival while all the shrimp in the control group died after 13 days post-immersion (Fig. 5a). For higher dose of bacteria (1.08×10^6 cfu/ml), survival in 5-ALA group was twofold higher compared to the control (Fig. 5b).

ATP Level Assay

Hepatopancreas ATP levels were twofold higher in the 5-ALA group than in the control group (Fig. 6).

Discussion

By the early 2000s, 5-ALA had been used in several fields besides medicine (Sasaki et al. 2002). However, so far, only few studies have used it in aquaculture and no studies have used it for shrimp or other invertebrates. The microarray analysis revealed that 5-ALA upregulated 41 defense- and immune-related genes, including some genes having immune functions ([a] in Table 2) different from their main biochemical functions, a case of gene multifunctionality (Pritykin et al. 2015). Upregulation of some of these genes was validated by qPCR (Fig. 2). One gene, identified as c-type lectin 4, which functions as a pathogen recognition protein (PRP) (Alenton et al. 2017) was reported to facilitate in the clearance of *Vibrio anguillarum* in vivo (Wang et al. 2009c). Another PRP characterized in *Penaeus monodon* named as

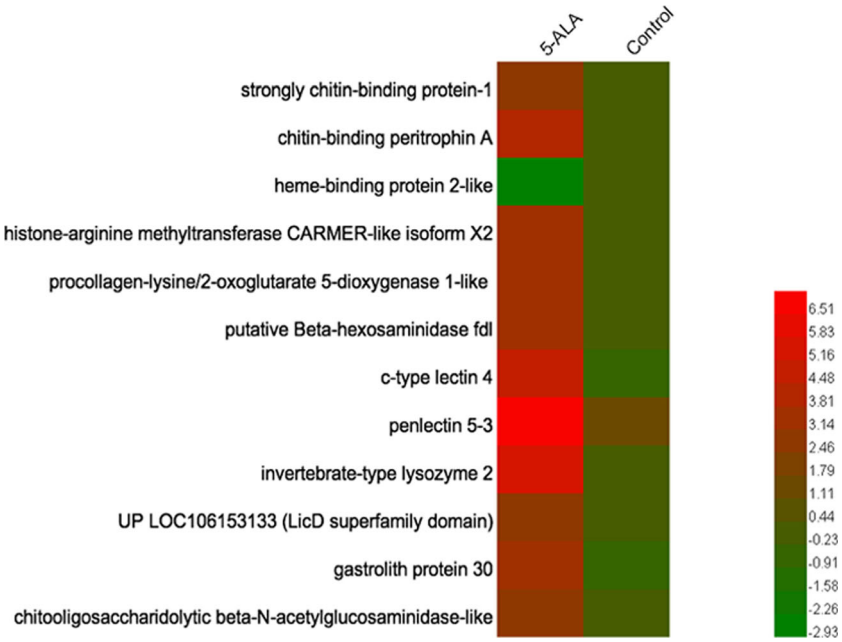

Fig. 1 Heatmap of differentially expressed genes from the microarray results with more than eightfold difference, $p < 0.05$ by t test analysis

penlectin, exhibited binding, agglutination and antimicrobial activity against both Gram-positive and Gram-negative bacteria (Angthong et al. 2017). Moreover, two antimicrobial peptides, the invertebrate-type lysozyme (Chen et al. 2016) and serine protease 30-like (Tassanakajon et al. 2013), were upregulated in 5-ALA group. 5-ALA-induced upregulation of these PRPs together with the AMPs that directly interact with pathogenic bacteria may have contributed to the resistance of *L. vannamei* to *V. parahaemolyticus* (Fig. 5). Viral (WSSV) and fungal (*Fusarium* sp.) challenge tests were also conducted in this study (Online Resource 3), but there was no significant difference in the survival between 5-ALA and control groups for either pathogen. This suggests that 5-ALA is more effective against bacterial infection or that a higher concentration of 5-ALA may be necessary to protect against viral and fungal pathogens. Dietary administration of a lower concentration of 5-ALA (10 ppm) did not significantly protect against *V. parahaemolyticus* infection (data not shown). Interestingly, two genes having antiviral activity (a zinc finger CCCH domain-containing protein, 1A-like isoform X2 (Tassanakajon et al. 2013) and a centaurin-alpha 1-like (Wang et al. 2009d) were also upregulated by 5-ALA, which raises the possibility that 5-ALA also protects against viral infection.

Another immune-related DEG, peritrophin A, which binds to chitin and has potential antimicrobial activity (Tassanakajon et al. 2013), may protect *L. vannamei* against pathogens (Huang et al. 2015). GO annotations suggest that ALA-5 upregulated six other hepatopancreas genes that are involved in chitin binding and metabolism. These genes include gastrolith protein 30, penlectin 5-3, strongly chitin-binding protein-1, putative cuticle protein, chitooligosaccharidolytic beta-*N*-acetylglucosaminidase-like, and protein ecdysoneless homolog. Five of these genes were upregulated more than eightfold by 5-ALA (Fig. 1), which suggests that 5-ALA has an important effect on chitin metabolism. Chitin is one of the main components of the peritrophic membrane (PM) and cuticle of insects (Han et al. 2015; Zhao et al. 2010) and is highly relevant for the growth of shrimps, since it needs to be synthesized and cleaved during the molting process (Rocha et al. 2012). The PM is a non-cellular semipermeable membrane that covers the midgut of arthropods and is thought to function as a protective barrier against invading pathogens (Huang et al. 2015) as well as to help stimulate food digestion (Tellam et al. 1999). On the other hand, the cuticle, which is composed of chitinous matrix and chitin-associated proteins and lipids, is important for locomotion and protection against dehydration as well as pathogen invasion (Moussian 2010). 5-ALA also upregulated kinesin-like protein unc-104, which positively regulates the expression of amyloid precursor-like protein that is required for molting (Wiese et al. 2010). Interestingly, in an ALAS-mutant larvae of *Drosophila melanogaster*, the cuticle was detached from the epidermis and the basal region was frayed, which suggests that ALAS has a role in organizing the contact between the cuticle and epidermis (Shaik et al. 2012).

RNA-seq data (Nakaoka et al. 2017) also revealed that ALAS is highly expressed in the prothoracic gland (a major organ for ecdysone synthesis) of silkworm *Bombyx mori*, while the abundance of 20-hydroxyecdysone (the active form of ecdysone) was low in *D. melanogaster* RNAi larvae, suggesting that ALAS is indispensable for ecdysteroidogenesis. These studies together with the present microarray results suggest that the synthesis of 5-ALA has a role in the organization of chitin and cuticle, and possibly molting, in invertebrates.

Another DEG, NOS, which is a hemoprotein, was significantly upregulated in the 5-ALA group. In *L. vannamei*, LPS and poly I:C caused rapid changes in NOS transcript levels,

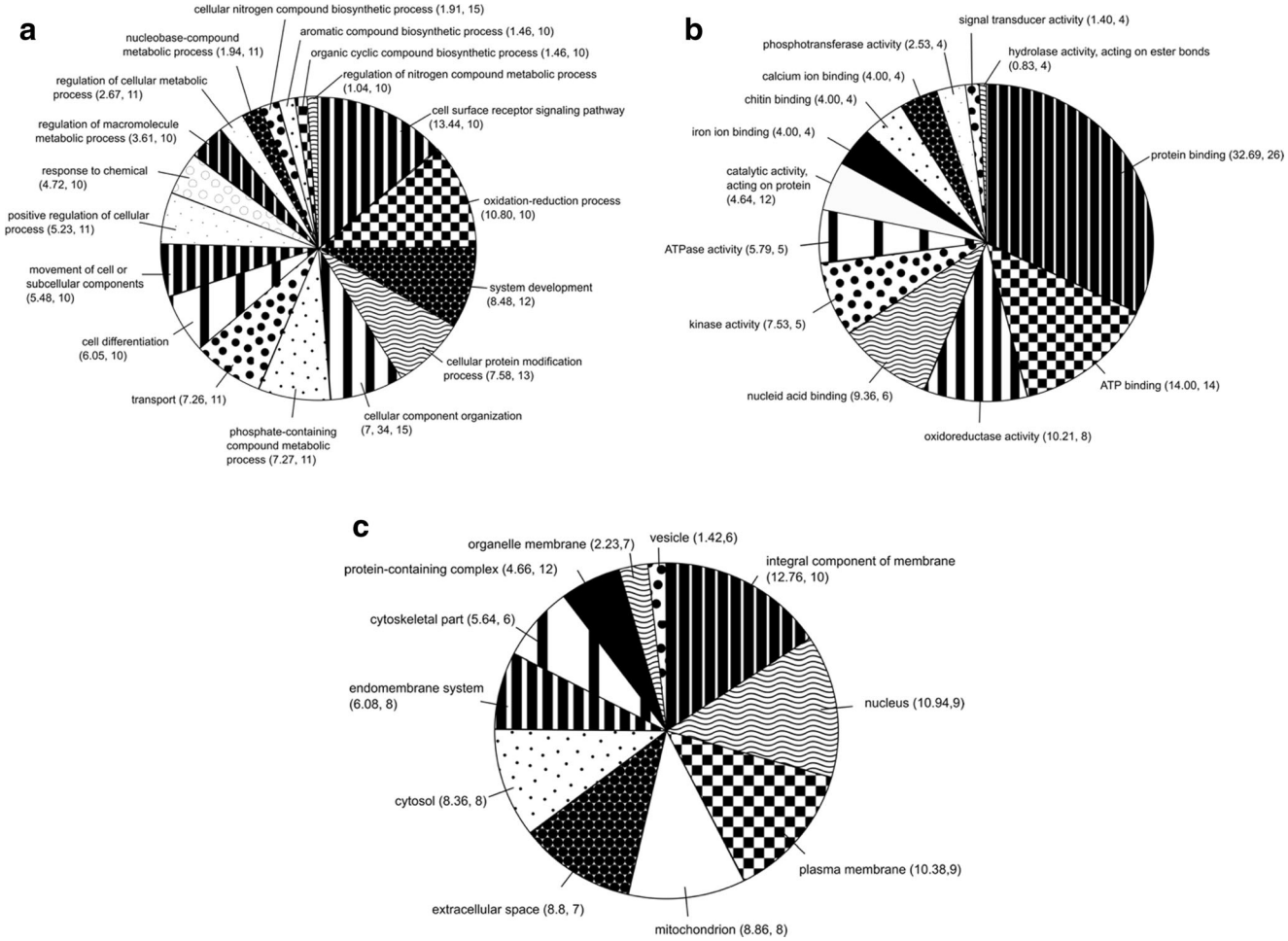

Fig. 2 Distribution of GO classification based on three high-level categories. **a** Multi-level pie chart of combined graph of GO biological process annotations (sequence filter = 10). **b** Multi-level pie chart of combined graph of GO molecular function annotation (sequence filter = 4). **c** Multi-level pie chart of combined graph of GO cellular component annotation (sequence filter = 6). Each part of the pie graph designates one GO term with corresponding node score and number of sequences in parenthesis. If the number of GO annotations were fewer than the sequence filter, then these GO's are not displayed in this graph

suggesting that NOS has a role in defense against pathogens (Yao et al. 2010). In the tropical land crab, *Gecarcinus lateralis*, NOS was found to regulate ecdysteroid synthesis in the Y organ (Kim et al. 2004). NOS is responsible for the biosynthesis of nitric oxide, which coordinates metabolism, growth, and development via the nuclear receptor E75 (Cáceres et al. 2011). E75, on the other hand, is required for ecdysone synthesis and contains heme as a prosthetic group (Reinking et al. 2005). In this study, E75 transcript levels were higher in the 5-ALA group. Ecdysone and their homologs, generally termed ecdysteroids, are reported to regulate growth by molting (Hosamani et al. 2017). Thus, the upregulation of NOS, in addition to enhancing immunity, may also induce molting by increasing E75 transcript levels.

On the other hand, 5-ALA downregulated HBP, which has a SOUL domain. This was also validated by qPCR (Fig. 3e). SOUL was reported to be specifically expressed in the pineal gland and retina but is ~ 40% homologous to p22HBP, which is ubiquitously expressed in tissues (Sato et al. 2004). HBP/SOUL is also involved in cell death by inducing mitochondrial permeability transition under stress conditions (Szigeti et al. 2006). SOUL overexpression alone already induced a non-significant decrease in cell viability, while induction of SOUL with stress stimuli significantly increased the numbers of necrotic and apoptotic cells (Szigeti et al. 2006). In the present study, the downregulation of HBP/SOUL in the absence of stress stimuli may be favorable because overexpression of SOUL in untreated cells decreased cell viability.

While some hemoproteins and genes associated with heme synthesis and degradation were statistically higher in 5-ALA group compared to the control by qPCR analysis (including CAT gene), these were not shown as DEG's in the microarray results since the expression of these genes are lower. As previously reported, fold-change measurements appear to be somewhat similar for microarray and qPCR at higher expression levels but fold-change similarity between the two

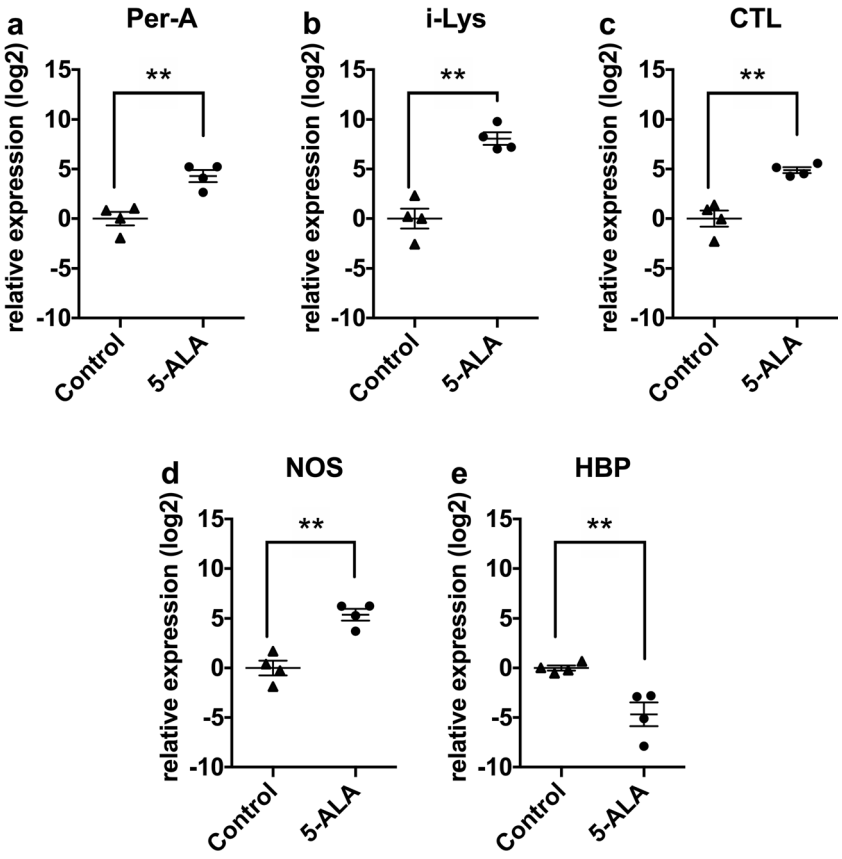

Fig. 3 Validation of microarrray results by quantitative RT-PCR analysis of the following genes: **a** peritrophin A (Per-A), **b** i-type lysozyme (i-Lys), **c** c-type lectin (CTL), **d** nitric oxide synthase (NOS), and **e** heme-binding protein (HBP). Data are plotted with means ± SEM. Asterisks indicate levels that are statistically significant by *t* test analysis; $^{**}p < 0.01$

disagree at lower expression levels and is likely due to the higher sensitivity of qPCR (Camarillo et al. 2011).

The expression of ALAS, the rate-limiting enzyme in the biosynthesis of heme, is regulated by heme levels via a negative feedback mechanism (Jover et al. 2000). It has been suggested that exogenous supplementation of 5-ALA bypasses this feedback regulation, which results in the production of more heme (Mateo et al. 2006). In this study, the transcript levels of ALAS

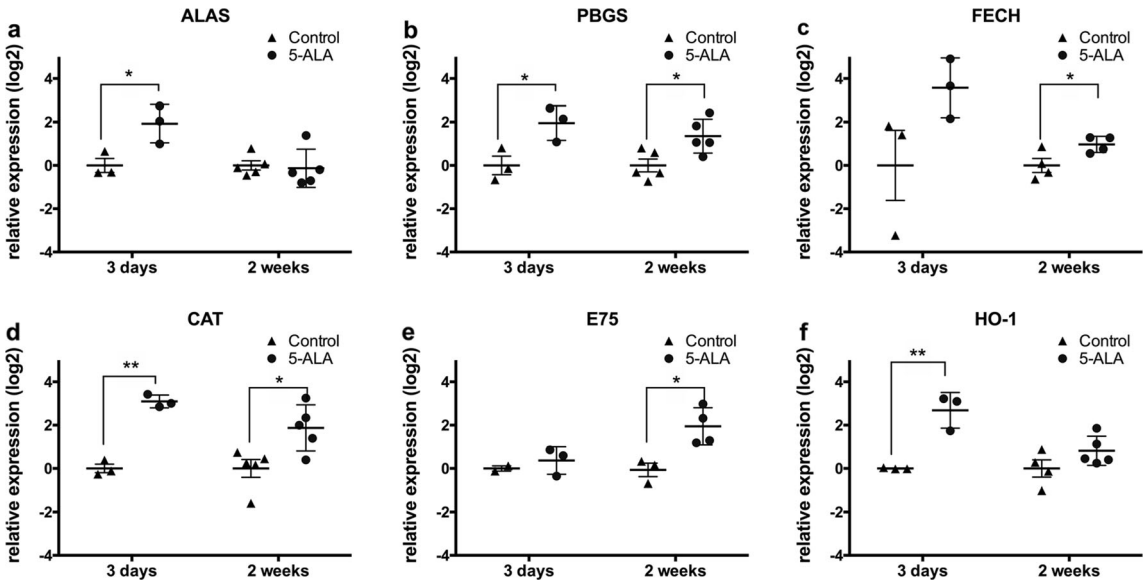

Fig. 4 Quantitative RT-PCR analysis of some hemoproteins and genes involved in heme synthesis and degradation: **a** aminolevulinic acid synthase (ALAS), **b** porphobilinogen synthase (PBGS), **c** ferrochelatase (FECH), **d** catalase (CAT), **e** nuclear receptor E75 (E75), and **f** heme oxygenase-1 (HO-1). Data are plotted with means ± SEM. Asterisks indicate levels that are statistically significant by *t* test analysis; $^{*}p < 0.05$, $^{**}p < 0.01$

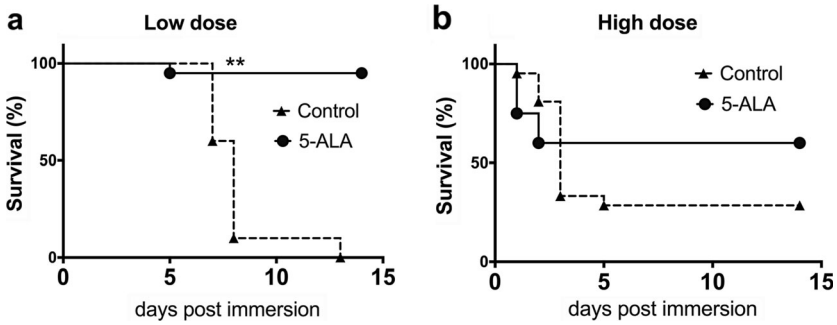

Fig. 5 Kaplan-Meier survival curves after *V. parahaemolyticus* immersion challenge using two doses of bacteria: **a** low dose = 3.60×10^5 cfu/ml and **b** high dose $=1.08 \times 10^6$ cfu/ml. Asterisks indicate levels that are statistically significant, relative to the control, $^{**}p < 0.01$ by survival analysis

were not statistically different between the two groups after 2 weeks of feeding (Fig. 4a), suggesting that exogenous 5-ALA did not affect endogenous ALAS expression at the transcript level, which agrees with a previous report (Liu et al. 2016).

In this study, the transcript levels of two of the enzymes (PBGS and FECH) involved in the heme synthesis pathway were higher in 5-ALA group compared to the control suggesting that the pathway was allowed to continue after the incorporation of 5-ALA in the cells. PBGS catalyzes the second step of heme synthesis while ferrochelatase is the last enzyme in the pathway that inserts iron in protoporphyrin to form heme (Layer et al. 2010).

On the other hand, catalase, a hemoprotein that functions as an antioxidant enzyme (Trasviña-Arenas et al. 2013), was statistically higher in the 5-ALA group. 5-ALA has also upregulated another hemoprotein and antioxidant, chorion peroxidase, and other DEGs having oxidoreductase activity (Table 2). This result suggests that 5-ALA has stimulated the expression of antioxidants, which provide functional role in innate immunity and thus introducing higher protection to infectious bacteria. Also, the fact that catalase and chorion peroxidase (antioxidants), NOS (which is also involved in cellular redox homeostasis), and E75 (vital for ecdysteroid synthesis) are hemoproteins supports the hypothesis that 5-ALA increases the expression of hemoproteins.

Heme oxygenase-1 was higher in the 5-ALA group, which suggests that 5-ALA enhanced the production of heme, which is one of the main inducers of HO-1 expression (Nishio et al. 2014). 5-ALA upregulated HO-1 mRNA levels in vertebrates (Nishio et al. 2014; Frank et al. 2007; Hagiya et al. 2008; Hou et al. 2013). This is the first report of the effect of 5-ALA on HO-1 expression in invertebrates, which suggests similar heme degradation pathway among animal groups. HO-1 degrades heme, producing iron, biliverdin, and carbon monoxide (Nishio et al. 2014). HO-1 expression is important because HO-1 metabolites have cytoprotective, anti-inflammatory, and antioxidant effects that protect shrimp against pathogens and environmental stress (Li et al. 2013; Otterbein et al. 2000; Barañano et al. 2002).

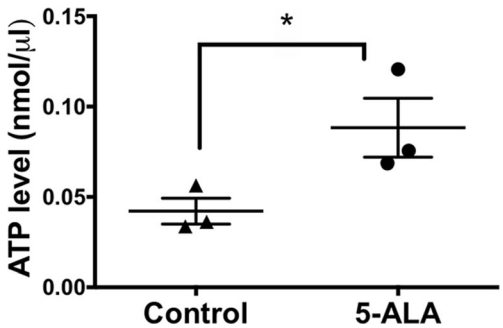

Fig. 6 ATP levels (nmol/µl) of control and 5-ALA groups. Asterisks indicate levels that are statistically significant, relative to the control, $^{*}p < 0.05$, by *t* test analysis

5-ALA has also been reported to increase the activity of the hemoprotein cytochrome c oxidase (complex IV), the last enzyme in the electron transport chain, which has a fundamental role in aerobic energy metabolism (Ogura et al. 2011). Along with the increase of complex IV activity was an increase in liver ATP levels in 5-ALA administered mice (Ogura et al. 2011). Similarly, 5-ALA increased ATP levels in the present study (Fig. 5). Cytochrome c-heme lyase, which covalently attaches heme to cytochrome c (Dumont et al. 1991) in the electron transport chain, was also upregulated by 5-ALA. Five of the DEGs that were upregulated by 5-ALA are involved in ATPase activity (Table 2). Increased ATP levels and expression of genes involved in the electron transport chain (one gene) and ATPase activity (five genes) suggest that 5-ALA enhances aerobic energy metabolism in *L. vannamei*. This is beneficial to shrimp since ATP is the primary energy currency of living systems, which is necessary to carry out shrimp's normal activities and even more during stress and microbial infections (Tiwari et al. 2002; Liang et al. 2015). Cellular ATP was also reported to play a role in disease resistance involving hypersensitive cell death, which is a response to bacterial infection (Hatsugai et al. 2012).

Taken together, our results support the idea that dietary 5-ALA affects heme synthesis and thus the activity of hemoproteins. 5-ALA administration also affects other pathways (Yamada et al. 2017) because heme is not only restricted to the synthesis of hemoproteins (Padmanaban et al. 1989). Heme synthesized in the mitochondria is transported across the mitochondrial membrane, regulating a number of metabolic processes and systems utilizing oxygen, e.g., transcription, translation, transport, processing, and cell differentiation (Padmanaban et al.

1989; Jover et al. 2000). These processes are also observed in the DEGs reported in this study (Table 2, Fig. 2a–c).

In conclusion, we demonstrated that dietary administration of 5-ALA upregulates various immune- and defense-related genes (including some hemoproteins), increases ATP level, and improves resistance to AHPND-causing *V. parahaemolyticus*. Further studies are needed to determine its effect on molting and growth as well as its effects when used for a longer period and in different concentrations.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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