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RESEARCH ARTICLE

Changes in free amino acid concentrations and associated gene expression profiles in the abdominal muscle of kuruma shrimp (*Marsupenaeus japonicus*) acclimated at different salinities

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ABSTRACT

Shrimps inhabiting coastal waters can survive in a wide range of salinity. However, the molecular mechanisms involved in their acclimation to different environmental salinities have remained largely unknown. In the present study, we acclimated kuruma shrimp (*Marsupenaeus japonicus*) at 1.7%, 3.4% and 4.0% salinities. After acclimating for 6, 12, 24 and 72 h, we determined free amino acid concentrations in their abdominal muscle, and performed RNA sequencing analysis on this muscle. The concentrations of free amino acids were clearly altered depending on salinity after 24 h of acclimation. Glutamine and alanine concentrations were markedly increased following the increase of salinity. In association with such changes, many genes related to amino acid metabolism changed their expression levels. In particular, the increase of the expression level of the gene encoding glutamate-ammonia ligase, which functions in glutamine metabolism, appeared to be associated with the increased glutamine concentration at high salinity. Furthermore, the increased alanine concentration at high salinity was likely associated with the decrease in the expression levels of the the gene encoding alanine-glyoxylate transaminase. Thus, there is a possibility that changes in the concentration of free amino acids for osmoregulation in kuruma shrimp are regulated by changes in the expression levels of genes related to amino acid metabolism.

KEY WORDS: Alanine-glyoxylate transaminase, Glutamate-ammonia ligase, RNA-seq analysis, Osmolytes, Osmoregulation

INTRODUCTION

Shrimps belong to the subphylum Crustacea, which forms a large and diverse group in invertebrates, and some of them have exploited their niche using adaptation to different temperatures as an isolation factor (David, 2014; Jorde et al., 2015; Martin and Davis, 2001). Several shrimps inhabiting coastal areas can survive in a wide range of salinity, by changing intracellular free amino acid concentrations to maintain osmotic pressures (Camien et al., 1951; Freire et al., 2008; Henry et al., 1980; McNamara et al., 2004). It has been reported that the concentrations of total free amino acids were increased in the muscles of crayfish *Procambarus clarkii* and kuruma shrimp, *Marsupenaeus japonicus*, following the increase of environmental salinity, and changes were largely due to those of glycine and L-alanine (Abe et al., 2005; Okuma and Abe, 1994). Therefore, the two amino acids are considered to be important osmolytes for these invertebrates (Abe et al., 1999, 2005; Fujimori and Abe, 2002; Okuma and Abe, 1994). Another experiment indicated that the concentrations of total free amino acids were decreased in the muscle of Pacific white shrimp, *Litopenaeus vannamei*, acclimated at low salinity, whereas those of glycine and L-serine were increased in the hemolymph and were associated with a decrease in the osmotic pressure in the muscle, suggesting that tissue amino acids were released into the hemolymph to lower the osmolarity of the tissue (Shinji et al., 2012).

Despite such results, the acclimation to different salinities may also be regulated by the particular genes. Suppression subtractive hybridization and real-time PCR revealed the relationship between environmental salinity and gene expression levels. For instance, black tiger shrimp, *Penaeus monodon*, (Shekhar et al., 2013, 2014), Pacific white shrimp (Gao et al., 2012; Sun et al., 2011) and ridgetail white prawn, *Exopalaemon carinicauda* (Li et al., 2015), increased the expression levels of the gene encoding the Na^+/K^+-ATPase α-subunit in various tissues such as gills, gut, hepatopancreas and antennal glands exposed to both high and low salinities. Na^+/K^+-ATPase is known to be one of the ion transporters that exchanges ions between the cytoplasm and the hemolymph to maintain inorganic ion concentrations in shrimp (Boudour-Boucheker et al., 2014; Faleiros et al., 2010; Havird et al., 2014; Holliday, 1985). Therefore, Na^+/K^+-ATPase plays an important role in osmoregulatory systems at both high and low salinities. Black tiger shrimp exposed to high salinity also increased the expression levels of genes encoding intracellular fatty acid binding proteins in gut tissues (Shekhar et al., 2013), whereas Pacific white shrimp decreased those encoding hemocyanin, chitinase, ecdysteroid-regulated protein, trypsin and chymotrypsin 1 in the hepatopancreas (Gao et al., 2012; Sun et al., 2011).

RNA sequencing (RNA-seq) analysis has been demonstrated to be a powerful method to examine the effects of salinity or temperature on gene expression levels in several invertebrates (Huang et al., 2017; Lv et al., 2013; Meng et al., 2013; Santos et al., 2014; Sellars et al., 2015; Zhao et al., 2012). It has been reported that the swimming crab *Portunus trituberculatus*, acclimated for 10 days at different salinities, changed the expression levels of osmoregulation-related genes such as those encoding ion transporters and amino acid metabolism-related proteins in their gills (Lv et al., 2013).

As mentioned above, many genes including ion and amino acid transporters seem to participate in acclimation of crustaceans to the

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List of symbols and abbreviations

FPKM	fragments per kilobase of transcript per million fragments
gDNA	genomic DNA
MHC	myosin heavy chain
MYH	myosin heavy chain (gene family)
RNA-seq	RNA sequencing
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase

salinity change. However, the molecular mechanisms involved have remained unclear, as the regulatory mechanisms underlying changes of free amino acid concentrations are not well understood. Thus, it is necessary to identify the relationship of the concentrations of free amino acids with the expression levels of the genes related to amino acid metabolism using crustaceans exposed to different salinities. It is also important to examine the expression levels of genes in crustaceans acclimated at different salinities.

In the present study, we targeted kuruma shrimp as experimental animals, as they are widely cultured and a commercially available, important species. Because kuruma shrimp inhabit inland bays or brackish-water regions and can survive in a wide range of salinity, it is considered that they are suitable animals with which to examine the osmoregulation system. We acclimated the shrimp at different salinities and determined free amino acid concentrations in their abdominal muscle to identify the amino acid related to osmoregulation. In addition, we performed RNA-seq analysis on the same shrimp using a next-generation sequencer to identify the genes related to the salinity response.

MATERIALS AND METHODS

Animals

Approximately 40 specimens of kuruma shrimp were obtained from Matsumoto Suisan Co., Ltd, an aquaculture company in Miyazaki Prefecture, Japan. They were cultured in outdoor ponds under conditions of approximately 3.0% salinity. The ranges of water temperature and pH were from 10°C to 31°C and from 7.5 to 9.0, respectively, through the year. The dissolved oxygen concentration was greater than 4.0 mg l^{-1}. The shrimp were transported to Kitasato University in Kanagawa Prefecture, Japan, under cold and wet conditions with sawdust. First, we acclimated the shrimp at 3.4% salinity, because wild specimens inhabit approximately this salinity, although the salinities in the culture ponds were approximately 3.0%. The shrimp were acclimated in recirculating water tanks with filtration systems at 25°C for 3 days. Then, they were divided into three groups using 60 liter tanks at 1.7%, 3.4% and 4.0% salinities. Shrimp were fed commercially available pellets for shrimp *ad libitum* under a 14 h:10 h light:dark cycle. After acclimating at 25°C for 6, 12, 24 and 72 h, three specimens were collected each from the three tanks. The body lengths and masses of kuruma shrimp are shown in Table 1. Kuruma shrimp at different molting states were mixed, whereas male and female could not be identified owing to their premature stage in this experiment.

Determination of free amino acid concentrations

The second abdominal segments (Fig. 1) of three kuruma shrimp each from different salinity tanks were dissected after different periods of acclimation. The abdominal muscle was then collected and homogenized individually with eight volumes of 10% perchloric acid (w/w), and centrifuged at 12,000 ***g*** for 20 min at 4°C. The resulting supernatant was neutralized with an appropriate amount of 12 mol l^{-1} and 2 mol l^{-1} KOH, and centrifuged at 12,000 ***g*** for 20 min at 4°C to collect the supernatant containing free amino acids. Free amino acids were derivatized with *O*-phthalaldehyde and 3-mercaptopropionic acid, and their concentrations were determined using a high performance liquid chromatography LC-2000 series (Jasco, Tokyo, Japan) with a reverse-phase column TSK gel ODS-80Ts (length, 250 mm; inside diameter, 46 mm; Tosoh, Tokyo, Japan). Mobile phase A consisted of 50 mmol l^{-1} sodium acetate buffer (pH 5.63) and mobile phase B consisted of 20% 50 mmol l^{-1} sodium acetate buffer (pH 5.63) plus 80% absolute methanol (v/v). Amino acids were eluted at room temperature with a linear gradient from A:B=100:0 to A:B=10:90 in 75 min at a flow rate of 1.0 ml min^{-1}. Excitation and emission wavelengths to detect derivatized amino acids were 340 and 450 nm, respectively. The present method cannot distinguish between L- and D-amino acids.

Water content

The fourth and fifth abdominal segments (Fig. 1) of three specimens each from different salinity tanks were dissected after various periods of acclimation. Then, the abdominal muscle was collected and minced individually. The water content was measured with an MA35 moisture meter (Sartorius, Göttingen, Germany) according to the manufacturer's instructions.

Construction of cDNA libraries

Total RNA was extracted from the abdominal muscles in the third abdominal segments (Fig. 1) of each of three specimens acclimated at different salinities for 24 h, using ISOGEN II solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, where 30 µg of total RNA from each of three specimens acclimated at 1.7%, 3.4% or 4.0% salinity were mixed. Total RNA was treated with DNase I (Takara, Otsu, Japan) to digest contaminated genomic DNA (gDNA) according to the manufacturer's instructions, although we did not check any possible DNA remaining. Then, mRNA was purified using the Poly(A)$^+$ Isolation Kit from Total RNA (Nippon Gene). Subsequently, complementary DNA (cDNA) libraries were constructed from purified messenger RNA (mRNA) using the Ion Total RNA-Seq Kit v2 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The average size of each cDNA library was determined with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An

Table 1. Body length and mass of kuruma shrimp acclimated at different salinities

	6 h		12 h		24 h		72 h	
Salinity (%)	Body length (cm)	Body mass (g)	Body length (cm)	Body mass (g)	Body length (cm)	Body mass (g)	Body length (cm)	Body mass (g)
1.7	12.2±0.21	15.5±0.28	11.1±0.38	12.1±1.45	11.8±0.16	10.9±0.33	12.7±0.21	15.3±0.82
3.4	12.4±0.24	15.0±0.56	11.2±0.21	11.1±0.41	11.4±0.37	11.5±0.34	12.4±0.08	15.5±0.61
4.0	12.4±0.05	15.3±0.33	11.9±0.05	11.9±0.33	11.7±0.19	13.6±0.37	12.2±0.24	15.8±0.54

Values are given as means±s.d. (*n*=3).

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Fig. 1. Kuruma shrimp. I–V correspond to the number of individual abdominal segments.

acclimation period of 24 h was selected because significant differences in free amino acid concentrations were observed at this period between kuruma shrimp acclimated to different salinities, as described in Results.

Sequencing

cDNA libraries prepared as above were treated with an Ion PGM Sequencing 200 Kit (Life Technologies) supplied with an Ion 318 chip (Life Technologies) according to the manufacturer's instructions. Sequencing was performed using an Ion PGM next-generation sequencer (Life Technologies). Sequencing data were subjected to the Maser analysis platform provided by the National Institute of Genetics in Japan.

Statistical analysis

Data were analyzed with one-way or two-way ANOVA, and differences shown in ANOVA were analyzed with the Tukey's method. To determine whether the assumptions of a normal distribution and homogeneity of variance were met, to qualify for parametric testing using ANOVA, we used the Kolmogorov–Smirnov test and Bartlett's test, respectively. Because data for alanine and glutamine concentration did not conform to a normal distribution, they were processed using natural logarithm and Box–Cox power transformations prior to ANOVA, respectively (Box and Cox, 1964; Clark et al., 2016; Cyr et al., 1998; Little et al., 2013).

The statistical analysis was also carried out using Student's *t*-tests.

RESULTS

Free amino acid concentration

The concentration of total free amino acids extracted from the abdominal muscle in the starting samples of kuruma shrimp (0 h) after acclimating at 3.4% salinity for 72 h was 260.7±30.6 µmol g^{-1} tissue (Fig. 2). The most abundant free amino acid was glycine, followed by arginine, glutamine and alanine in most cases. ANOVA analysis revealed that the concentration of total free amino acids at 3.4% salinity did not change significantly during a further 72 h of acclimation (P>0.05). The average concentration in the shrimp acclimated for 24 h at 1.7% salinity was significantly lower than that at 4.0% salinity as well as that at 3.4% salinity (Student's *t*-test, P<0.05). After 72 h, the concentration of total free amino acids at 4.0% salinity was significantly higher than that at 1.7% salinity (P<0.01) and 3.4% salinity (P<0.05). The concentration at 3.4% salinity was also significantly higher than that at 1.7% salinity (P<0.05). The concentration of total free amino acids was also higher at 3.4% than at 1.7% salinity after acclimating for 6 h (P<0.05).

The major free amino acids were glycine, arginine, glutamine and alanine, irrespective of different salinities and acclimation periods (Table S1). Changes in the major free amino acids during

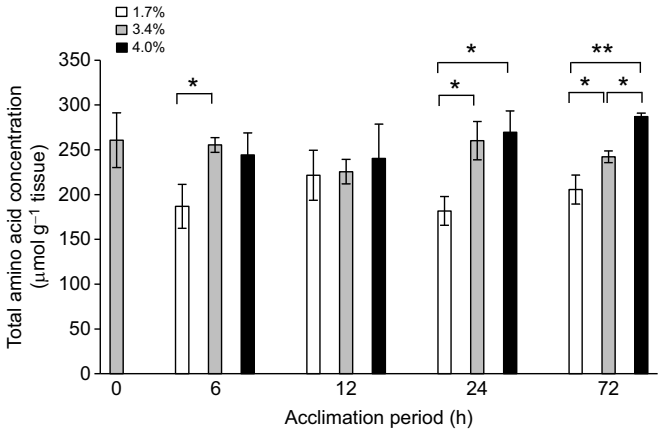

Fig. 2. Concentrations of free amino acids in the second abdominal segments of kuruma shrimp acclimated at different salinities for various periods up to 72 h. Open, grey and black bars indicate the concentrations in the shrimp acclimated at 1.7%, 3.4% and 4.0% salinity, respectively. Significance by Student's *t*-test at *P<0.05, **P<0.01.

acclimation were compared individually. No statistical differences were observed for glycine at any salinity (Fig. 3A), although the concentration of glycine was the highest among all free amino acids. The concentration of arginine, with the second largest concentration, showed a significant difference between the shrimp only at 1.7% and 4.0% salinity after 72 h (Fig. 3B).

Marked changes were observed in the concentration of glutamine, as shown in Fig. 3C. The statistical analysis did not show any significant differences for the shrimp at 3.4% salinity for any acclimation period, although the concentration at 0 h was apparently higher than that at 6–72 h. Student's *t*-test revealed that the concentration at 4.0% salinity was significantly higher than that at 1.7% salinity after acclimating for 24 and 72 h (P<0.01). The concentration at 3.4% salinity was also significantly higher than that at 1.7% salinity after 72 h. ANOVA analysis demonstrated that the concentration at 1.7% salinity after 6 h was significantly higher than that after 12 h, whereas the concentration at 4.0% salinity after 12 h was significantly lower than that after 6, 24 and 72 h at the same salinity.

The concentration of alanine showed changes similar to those of glutamine, as shown in Fig. 3D. Highly significant differences (P<0.01) were observed in the concentration between the shrimp at 1.7% and 4.0% salinity after 24 and 72 h as well as between shrimp at 3.4% and 4.0% salinity after 72 h. The difference between the shrimp at 1.7% and 3.4% salinity was also significant (P<0.05). ANOVA analysis demonstrated that alanine concentrations after 24 and 72 h were significantly higher than those after 6 and 12 h for the shrimp at 4.0% salinity. Taken together, these data show that the concentrations of glutamine and alanine changed clearly after acclimating kuruma shrimp at 1.7% and 4.0% salinity.

Water content

Fig. 4 shows changes in the water content of kuruma shrimp acclimated at different salinities. The water content was 74.9% initially. ANOVA analysis demonstrated that the water content was not significantly changed when the shrimp were acclimated for 72 h at 3.4% and 4.0% salinity. In contrast, the water content for the shrimp acclimated for 24 h at 1.7% salinity was significantly higher than that after 6 and 72 h. The differences in water content after acclimating for the same period at different salinities were significant between 1.7% and 4.0% salinity after

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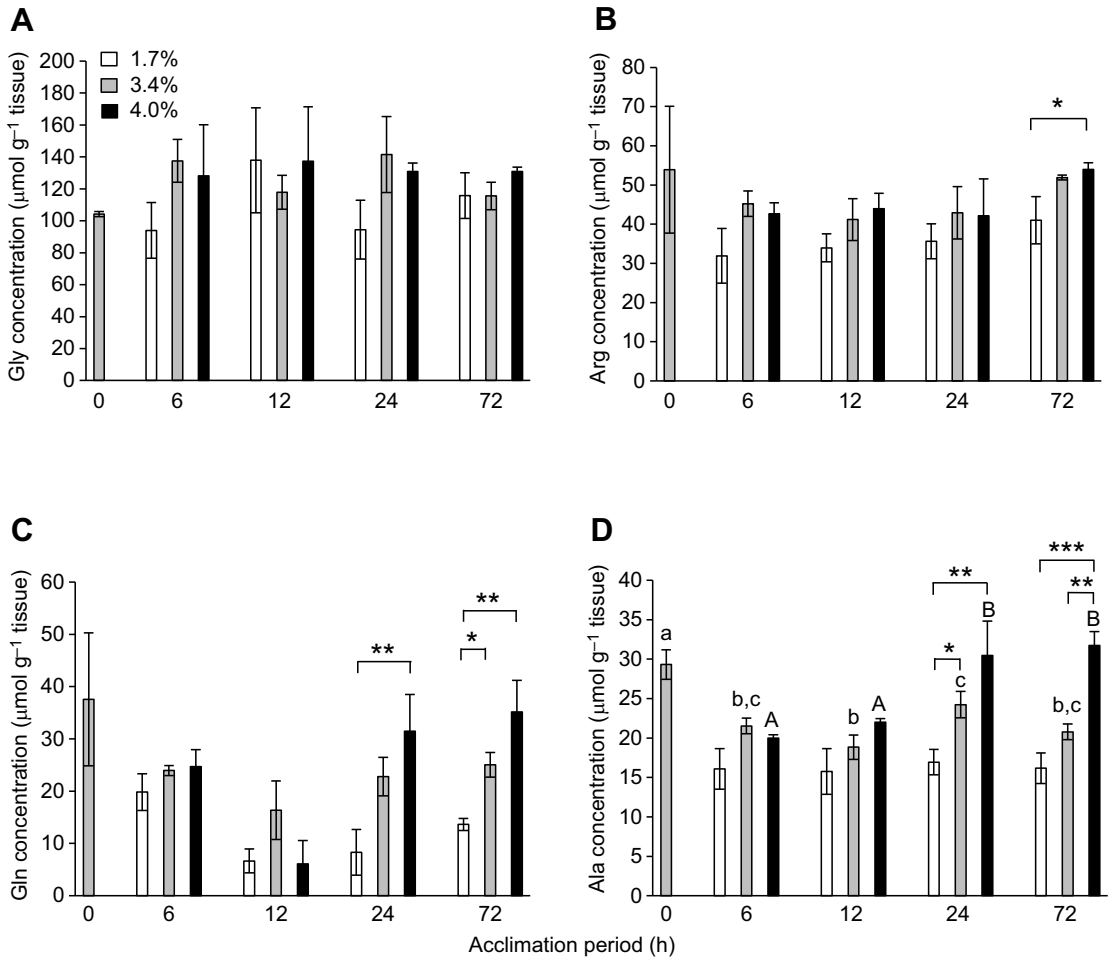

Fig. 3. Concentrations of amino acids in the second abdominal segments of kuruma shrimp acclimated at different salinities for various periods up to 72 h. (A) Glycine, (B) arginine, (C) glutamine and (D) alanine. Open, grey and black bars indicate the concentrations of glycine in the shrimp acclimated at 1.7%, 3.4% and 4.0% salinities, respectively. Significance by Student's *t*-test at **P*<0.05, ***P*<0.01, ****P*<0.001. Different letters indicate significant differences by ANOVA. Lowercase and uppercase letters indicate the differences among alanine concentrations at 3.4% and 4.0% salinity, respectively.

6 h ($P<0.01$), 12 h ($P<0.05$), 24 h ($P<0.01$) and 72 h ($P<0.01$). Significant differences were also observed between the shrimp at 1.7% and 3.4% salinity after 12, 24 and 72 h ($P<0.05$). However, within an acclimation period, no significant differences in water content were observed between the shrimp at 3.4% and 4.0% salinity.

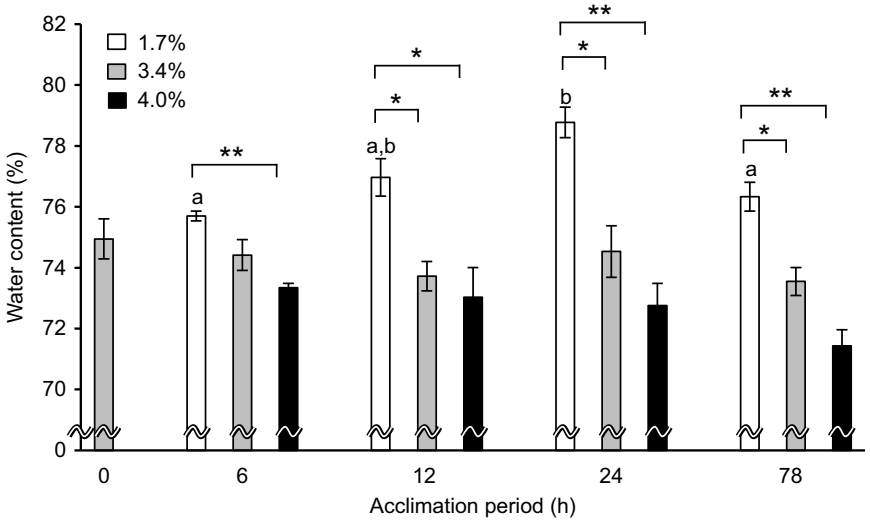

Fig. 4. Water content in the fourth and fifth abdominal segments of kuruma shrimp acclimated at different salinities for various periods up to 72 h. Open, grey and black bars indicate the water content in shrimp acclimated at 1.7%, 3.4% and 4.0% salinity, respectively. Significance by Student's *t*-test at **P*<0.05, ***P*<0.01. Different letters indicate significant differences by ANOVA.

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The concentrations of total free amino acids were inversely proportional to the water content, with a significant relative coefficient value of $r=-0.54076$ ($P<0.01$).

RNA-seq analysis

To minimize any possible individual variations, we mixed mRNA prepared each from three specimens with the same amount (30 µg) for all sampling points, as described in the Materials and methods. Table S2 shows the average sizes of the constructed cDNA libraries for the shrimp acclimated for 24 h at 1.7, 3.4 and 4.0% salinity, together with corresponding Ion PGM sequencing data. RNA-seq data for the shrimp acclimated at 1.7% and 4.0% salinity were subjected to MA plot analysis (Wang et al., 2010), together with those for the shrimp acclimated at 3.4% salinity as a reference. At 1.7% salinity, the numbers of genes with expression levels that were increased more than twofold and decreased less than 50% than those at 3.4% salinity were 8696 and 5367, respectively. In contrast, the corresponding numbers at 4.0% salinity compared with those at 3.4% salinity were 3407 and 3683, respectively.

Tables S3–S6 show the genes with expression levels that were increased by more than 10-fold or decreased less than 10% during acclimation for 24 h at 1.7% or 4.0% salinity compared with those at 3.4% salinity as a reference, together with gene names, fragments per kilobase of transcript per million fragments (FPKM) values and fold change. A total of 2065 genes showed increased expression levels at 1.7% salinity, among which 124 genes were identified (Table S3). In contrast, 2618 genes had decreased expression levels at 1.7% salinity, among which 113 genes were identified (Table S4). Meanwhile, the numbers of genes with expression levels that were increased and decreased at 4.0% salinity were 444 and 302, respectively, among which 16 and 31 genes were identified, respectively (Tables S5 and S6).

Gene expression profiles in glutamine- and alanine-related metabolic pathways

Glutamine-related metabolic pathways are shown in Fig. 5, where changes in the expression levels of the genes encoding glutamate synthase, glutamate–ammonia ligase, glutaminase, glutamine–fructose-6-phosphate transaminase and amidophosphoribosyltransferase determined by RNA-seq analysis for the shrimp acclimated for 24 h at different salinities are depicted, together with changes in the concentrations of glutamine and glutamate after the same acclimation period (Fig. 3C). The expression levels of these genes, except that encoding glutamate–ammonia ligase, were decreased in association with the increase of salinity. In contrast, the expression levels of the gene encoding glutamate–ammonia ligase were increased following the increase of salinity.

Alanine-related metabolic pathways are shown in Fig. 6, where changes in the expression levels of the genes encoding alanine transaminase and alanine–glyoxylate transaminase determined by RNA-seq analysis for the shrimp after 24 h at different salinities are depicted, together with changes in the concentration of alanine after the 24 h acclimation period at different salinities (Fig. 3D). The expression levels of the gene encoding alanine

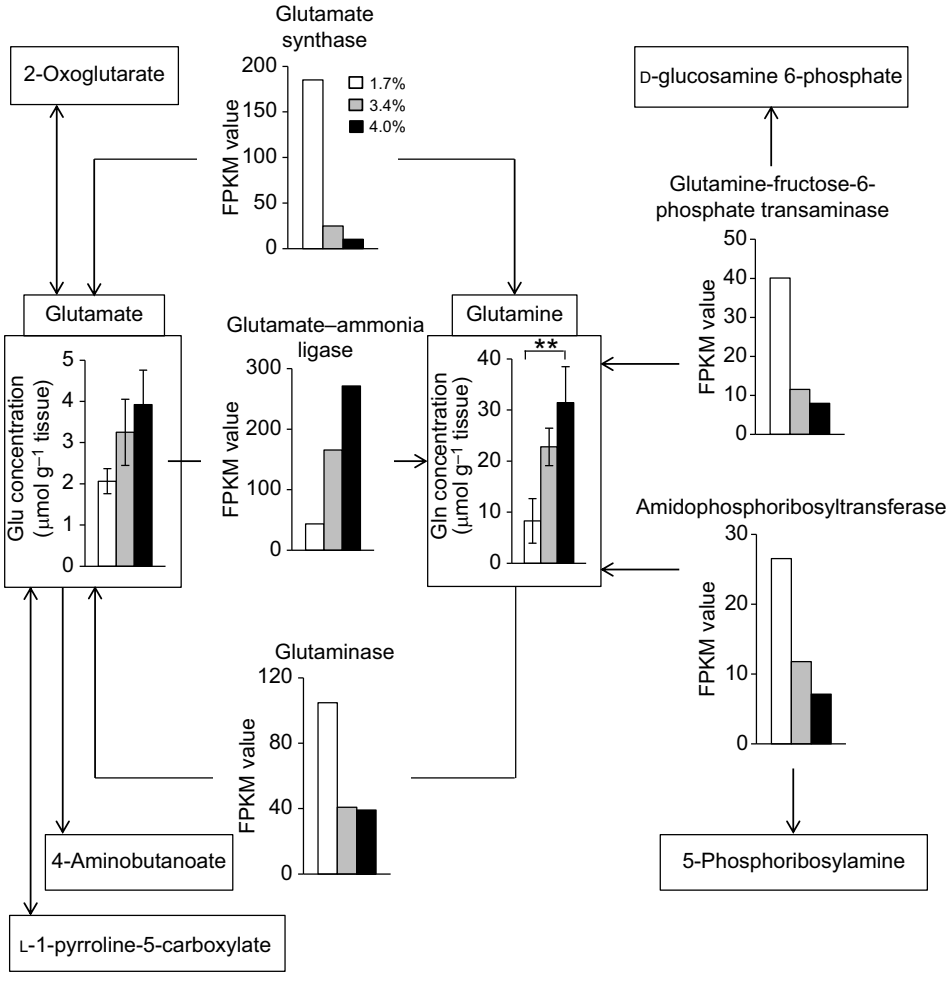

Fig. 5. Genes and metabolites related to glutamine metabolism. Open, grey and black bars indicate the concentrations of glutamine and its related metabolites or the FPKM values of the genes related to glutamine metabolism in kuruma shrimp acclimated for 24 h at 1.7%, 3.4% and 4.0% salinity, respectively.

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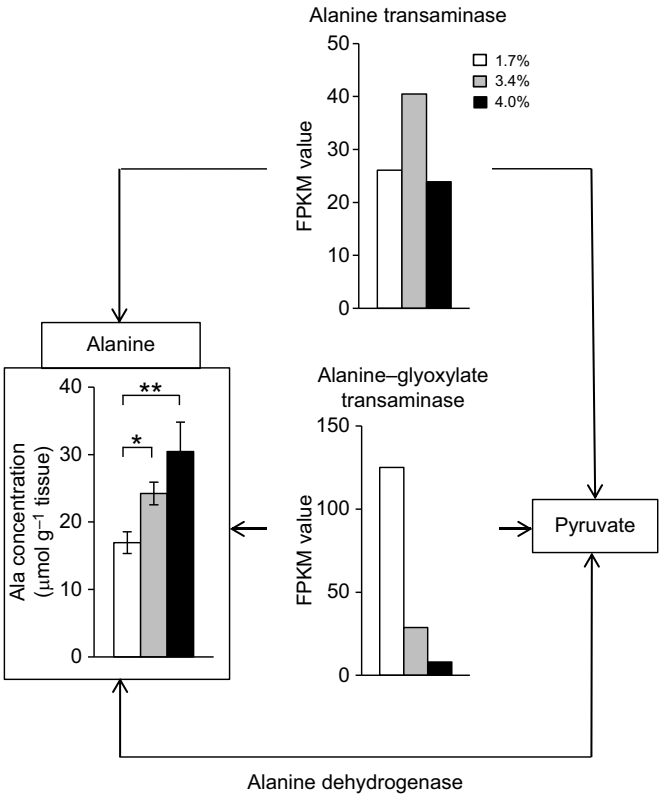

Fig. 6. Genes and metabolites related to alanine metabolism. Open, grey and black bars indicate the concentration of alanine or the FPKM values of the genes related to alanine metabolism in kuruma shrimp acclimated for 24 h at 1.7%, 3.4% and 4.0% salinities, respectively.

dehydrogenase were decreased following the increase of salinity, whereas those of the gene encoding alanine–glyoxylate transaminase were higher at 3.4% salinity than at 1.7% and 4.0% salinity. Unfortunately, the gene encoding alanine dehydrogenase could not be detected in the present RNA-seq analysis.

Expression profiles of genes other than those related to amino acid metabolism

RNA-seq analysis revealed that many genes other than those related to amino acid metabolism exhibited altered expression levels when the shrimp were acclimated for 24 h at different salinities (Tables S3–S6). The expression levels of the genes encoding myosin heavy chain (MYH) type 1 (MYH1) and MYH2 were increased at 1.7% salinity (Table S3). The expression levels of MYH3 were decreased at 1.7% salinity (Table S4) and increased at 4.0% salinity (Table S5).

The expression levels of the genes encoding sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and ATP synthase subunit 9 mitochondrial precursor were decreased at 1.7% salinity. In contrast, the expression levels of the gene encoding Na^{+}/K^{+}-ATPase α-subunit were increased at 1.7% salinity (Table S3).

DISCUSSION

In order to understand the mechanisms involved in the acclimation of kuruma shrimp to different environmental salinities, we determined the concentrations of free amino acids in the abdominal muscle of shrimp acclimated at different salinities for various time periods (up to 72 h). As shown in Fig. 2, the concentrations of total free amino acids were changed following the alteration of acclimating salinity. It has been reported that the concentrations of total free amino acids in kuruma shrimp were increased following the increase of salinity over 4 days (Abe et al., 2005). We obtained similar results in the present study. In addition, the present study demonstrated that it took 24 h to change the concentrations of free amino acids following the alteration of salinity.

Fig. 3 shows changes in the concentrations of major amino acids – glycine, arginine, glutamine and alanine. The concentration of glycine did not change significantly (Fig. 3A), although previous investigations observed an increase in the glycine concentration in the muscles of kuruma shrimp and crayfish (Okuma and Abe, 1994; Abe et al., 2005). It has also been reported that the concentration of alanine increased in adductor muscle of hard clam *Meretrix lusoria* at high salinity, whereas that of glycine did not increase (Okuma et al., 1998). It was not clear why the concentration of glycine did not change depending on salinity in the present study. The concentration of arginine also did not change significantly following the increase of salinity (Fig. 3B), whereas those of glutamine and alanine were increased at high salinity and decreased at low salinity after 24 h of acclimation (Fig. 3C,D). Therefore, in the present study, these two amino acids were found to possibly act as osmolytes, taking at least 24 h to adjust the cellular osmotic pressure to the environmental salinity. We previously reported changes in the accumulation of metabolites in the brackish water clam *Corbicula japonica* exposed to different salinities, where the concentration of L-alanine was also increased in association with the increase of environmental salinity, although that of L-glutamine did not change significantly (Koyama et al., 2015; Okamoto et al., 2012). Therefore, it is considered that alanine is an osmolyte common to invertebrates with open vascular systems.

The increase in water content was almost proportional to the decrease in the concentration of total free amino acids (r=−0.54076). Although the concentrations of free amino acids were increased following the decrease in water content, the difference in water content between the shrimp acclimated for 72 h at 1.7% and 4.0% salinity (5%) was much less than the difference in the concentration of total free amino acids between the same shrimp (30%). Thus, the increase in the concentration of total free amino acids following the increase of salinity is not likely the simple effect of the decrease in water content, but seems attributable to the acclimation of kuruma shrimp to high salinity by increasing the concentration of free amino acids, as reported previously (Okuma and Abe, 1994; Abe et al., 2005).

In the present study, we could partly explain the accumulation of glutamine and alanine at high salinity by the functions of the genes participating in metabolism of the respective amino acids (Figs 5 and 6).

Fig. 5 shows the metabolic pathways of glutamine and its related compounds. The concentrations of glutamine and glutamate and the FPKM value of five genes are also shown. Kuruma shrimp acclimated at high salinity increased the expression level of the gene encoding glutamate–ammonia ligase (Fig. 5). Therefore, it is considered that glutamine was synthesized from glutamate by this enzyme in response to high salinity, increasing the concentration of glutamine. In contrast, the expression levels of four other enzymes – glutamate synthase, glutaminase, glutamine–fructose-6-phosphate transaminase and amidophosphoribosyltransferase – were increased at low salinity (Fig. 5). These results suggest that glutamine was catabolized to glutamate, D-glucosamine 6-phosphate or 5-phosphoribosylamine by the four abovementioned enzymes, thus decreasing the concentration of glutamine at this low salinity.

Fig. 6 shows the metabolic pathways of alanine and its related compounds. As in the case of glutamine, the concentration of alanine was increased following the increase of environmental salinity after 24 h (Fig. 3D). The FPKM value of the alanine–glyoxylate transaminase gene was increased at low salinity, whereas that of the alanine transaminase gene was not changed markedly at different salinities. Thus it seems that alanine was catabolized to pyruvate by the alanine–glyoxylate transaminase gene at low salinity, decreasing the concentration of alanine at this low salinity. However, we could not detect the transcripts encoded by the alanine dehydrogenase gene. Thus, the mechanisms involved in changes of the alanine concentration at different salinities remain unclear. In this regard, it has been reported that pyruvate was not detected in the gill and foot muscle of brackish water clam, although the concentration of alanine was increased at high salinity (Koyama et al., 2015). Pyruvate, once accumulated at low salinity, might have been quickly catabolized into another substance.

The expression levels of the genes encoding MYH1 and MYH2 were increased at 1.7% salinity (Table S3). MYH is the major muscle protein and two genes encoding MYH, MHC1 and MHC2, which correspond to the genes encoding MYH1 and MYH2, respectively, have been reported to be expressed in the abdominal muscle of kuruma, black tiger and Pacific white shrimp (Koyama et al., 2012a,b). These are expressed only in flexor muscle (*MHC1*) and in both flexor and extensor muscles (*MHC2*) in anaerobic metabolism. In the present study, the expression levels of MYH1 at 3.4% salinity were approximately 1.5-fold more than that of MYH2, and the expression levels of MYH2 at 1.7% salinity were approximately 3-fold more than that of MYH1. The expression levels of MYH3 were decreased at 1.7% salinity (Table S4) and increased at 4.0% salinity (Table S5). The gene encoding MYH3 has been reported as *MHC3* to be expressed in the pleopod muscle having aerobic metabolism of kuruma shrimp and black tiger shrimp (Koyama et al., 2012a, 2013). However, the relationship between MYH gene expression level and osmoregulation remains unclear.

SERCA plays an important role in regulating the calcium concentration in the cytoplasm (Clapham, 1995). It has been reported that the expression levels of SERCA in the muscle of Pacific white shrimp acclimated at high salinity (40 psu) were 9.7-fold higher than those acclimated at low salinity (20 psu) (Wang et al., 2013). Although the expression levels of the gene encoding SERCA were decreased at 1.7% salinity, we did not observe an increase at 4.0% salinity in this study (Table S4). It has been reported that European lobster *Homarus gammarus* acclimated at low salinity (2.21%) from seawater (3.50%) increased their Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchange activities in epipodites (2.6- and 1.2-fold, respectively) and branchiostegites (1.6- and 1.2-fold, respectively), and decreased these activities in gills (0.30- and 0.52-fold, respectively) (Flik and Haond, 2000). Thus, the activity of SERCA might be changed depending on salinity and may modulate the calcium concentration in kuruma shrimp. The expression levels of the ATP synthase subunit 9 mitochondrial precursor gene were also decreased at 1.7% salinity (Table S4). Such decreased expression levels of this gene have been reported in the gills of Pacific white shrimp acclimated at low salinity (Gonçalves-Soares et al., 2012).

The expression levels of the Na^{+}/K^{+}-ATPase α-subunit gene were increased at 1.7% salinity (Table S3). Such enhanced expression of the Na^{+}/K^{+}-ATPase α-subunit gene has been reported in the gills of black tiger shrimp acclimated at low salinity (Shekhar et al., 2013). In addition, when cinnamon shrimp, *Macrobrachium amazonicum*, which live in freshwater, were acclimated at 2.5% salinity, the expression level of the Na^{+}/K^{+}-ATPase α-subunit gene and Na^{+}/K^{+}-ATPase activity in their gills, along with their hemolymph osmolality and chloride ion concentration, were increased, and their hemolymph osmolality and chloride ion concentration were also increased within 24 h (Faleiros et al., 2010). It has also been reported that freshwater prawn *Macrobrachium rosenbergii* acclimated at high salinity (two-thirds seawater and full seawater) increased the concentrations of total free amino acids, sodium ion and chloride ion in their hemolymph (Huong et al., 2001). In the case of blue crab, *Callinectes sapidus*, acclimated at low salinity, the expression level of the Na^{+}/K^{+}-ATPase α-subunit gene and Na^{+}/K^{+}-ATPase activity in their gills were also increased (Lucu and Towle, 2003). It is thus suggested that ion transporters such as Na^{+}/K^{+}-ATPase play an important role in adjusting the cellular osmotic pressure to the environmental salinity together with changes in free amino acid concentrations.

Although we did not carry out real-time PCR to confirm the data obtained from global gene expression analysis by RNA-seq in the present study, we previously demonstrated that the RNA-seq data were satisfactorily verified by real-time PCR experiments with our work on global gene expression analyses of muscle tissues from medaka acclimated to low and high environmental temperatures (Ikeda et al., 2017) and of gill tissues from normal and thermally selected strains of rainbow trout (Tan et al., 2012).

In conclusion, we examined the concentrations of free amino acids and gene expression profiles of kuruma shrimp acclimated at different salinities. A number of genes changed their expression levels in response to changes in environmental salinity. In addition, the concentrations of free amino acids were considered to be regulated by various genes related to amino acid metabolism. For instance, the concentration of glutamine was increased at high salinity in association with the increase of the expression level of glutamate–ammonia ligase. The concentration of alanine was increased at high salinity in association with the decrease of the expression level of alanine–glyoxylate transaminase. In future studies, further investigation is required regarding the participation of D-amino acids and their related enzymes in the adaptation of shrimp to environmental salinity change.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.W.; Methodology: K.Y., M.J., D.I., S.W.; Software: S.A.; Validation: D.I., S.P.; Formal analysis: H.K., E.T., T.Y.; Investigation: H.K., N.M., M.H., E.T., T.Y.; Data curation: H.K., N.M., S.W.; Writing - original draft: H.K.; Writing - review & editing: K.Y., M.J., T.Y., S.P., S.W.; Supervision: S.A., S.W.; Project administration: S.W.; Funding acquisition: S.W.

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Data availability
RNA-seq data are available in the DNA Data Bank of Japan database under the accession number DRA 006082.

Supplementary information

Supplementary information available online at
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Supplementary Table S1. The concentrations of free amino acids in the second abdominal segments of kuruma shrimp acclimated at 1.7 %, 3.4 % and 4.0% salinity for various acclimating periods up to 72 h.

	Gly	Arg	Gln	Ala	Tau	Asn	Glu	Ser	Val	Leu	Tyr	Asp	His	Ile	Phe	Met	Lys	Total
3.4% 0h	104.23 ± 1.63	53.90 ± 16.16	37.55 ± 12.72	29.32 ± 1.87	6.33 ± 0.58	5.35 ± 0.18	1.61 ± 0.64	3.18 ± 1.24	4.54 ± 1.07	3.59 ± 0.83	2.44 ± 0.01	1.45 ± 0.95	1.75 ± 0.81	2.61 ± 0.68	1.41 ± 0.34	1.17 ± 0.11	0.24 ± 0.34	260.67 ± 30.58
1.7% 6h	93.93 ± 17.43	31.92 ± 6.97	19.81 ± 3.52	16.08 ± 2.57	6.35 ± 0.96	4.02 ± 1.11	1.88 ± 0.18	2.26 ± 0.91	1.19 ± 0.85	1.36 ± 0.68	1.14 ± 0.04	4.19 ± 3.10	0.59 ± 0.28	1.14 ± 0.55	0.63 ± 0.33	0.39 ± 0.10	0.00	186.89 ± 24.51
3.4% 6h	37.50 + 13.44	45.24 + 3.21	23.95 + 0.96	21.52 + 0.99	7.25 + 0.98	2.50 + 0.50	2.29 + 0.30	3.09 + 1.66	2.69 + 0.89	2.01 + 0.75	1.45 + 0.17	2.32 + 0.51	0.60 + 0.43	1.30 + 0.58	0.97 + 0.35	0.73 + 0.30	0.00	255.40 + 8.12
4.0% 6h	28.20 + 31.92	42.63 + 2.83	24.69 + 3.23	20.00 + 0.42	7.76 + 1.02	3.05 + 0.37	2.39 + 0.55	2.61 + 1.16	2.91 + 0.86	3.05 + 2.02	1.28 + 0.08	1.58 + 0.36	0.59 + 0.31	1.92 + 0.95	0.94 + 0.26	0.65 + 0.29	0.00	244.24 + 24.46
1.7% 12h	37.88 ± 32.84	33.97 ± 3.59	6.65 ± 2.30	15.74 ± 2.89	6.98 ± 1.47	2.51 ± 1.62	2.97 ± 0.55	6.28 ± 1.45	0.76 ± 0.14	0.59 ± 0.02	0.75 ± 0.30	3.29 ± 1.16	0.35 ± 0.02	2.18 ± 2.58	0.36 ± 0.07	0.31 ± 0.05	0.00	221.57 ± 27.90
3.4% 12h	17.84 ± 10.56	41.18 ± 5.31	16.33 ± 5.60	18.83 ± 1.55	9.14 ± 2.51	3.22 ± 1.90	2.10 ± 0.82	3.62 ± 1.45	1.91 ± 0.80	1.49 ± 0.78	1.54 ± 0.26	5.24 ± 3.05	0.88 ± 0.52	0.86 ± 0.41	0.93 ± 0.50	0.54 ± 0.28	0.00	225.64 ± 13.77
4.0% 12h	37.31 ± 34.13	43.95 ± 3.92	6.08 ± 4.49	21.99 ± 0.47	7.68 ± 2.51	3.52 ± 3.66	1.79 ± 0.61	3.51 ± 1.05	2.29 ± 0.62	3.97 ± 2.62	1.72 ± 0.14	1.97 ± 0.44	0.63 ± 0.32	1.69 ± 0.45	0.81 ± 0.08	1.53 ± 1.02	0.00	240.40 ± 38.41
1.7% 24h	94.48 ± 18.45	35.63 ± 4.47	8.30 ± 4.36	16.94 ± 1.62	7.10 ± 2.05	1.82 ± 0.37	2.06 ± 0.30	8.62 ± 2.19	0.77 ± 0.16	0.67 ± 0.14	0.69 ± 0.13	3.26 ± 0.41	0.43 ± 0.08	0.38 ± 0.06	0.34 ± 0.07	0.26 ± 0.05	0.00	181.74 ± 16.00
3.4% 24h	41.46 ± 23.75	42.94 ± 6.69	22.79 ± 3.67	24.22 ± 1.68	7.99 ± 1.34	3.02 ± 0.27	3.25 ± 0.80	1.78 ± 0.63	3.02 ± 0.19	2.26 ± 0.26	1.51 ± 0.27	2.30 ± 1.33	0.72 ± 0.29	1.51 ± 0.17	0.74 ± 0.05	0.73 ± 0.18	0.00	260.23 ± 21.46
4.0% 24h	130.83 + 5.36	42.16 + 9.39	31.45 + 7.05	30.47 + 4.35	8.02 + 0.88	3.90 + 0.96	3.92 + 0.83	1.70 + 0.72	3.48 + 0.54	2.59 + 0.52	2.21 + 0.43	3.62 + 1.64	0.71 + 0.18	1.74 + 0.31	0.78 + 0.06	0.67 + 0.30	1.24 + 0.94	269.49 + 23.98
1.7% 72h	15.78 + 14.35	40.99 + 6.02	13.63 + 1.16	16.16 + 1.94	8.21 + 0.70	1.74 + 0.13	1.78 + 0.34	1.64 + 0.45	1.28 + 0.11	0.87 + 0.07	0.56 + 0.20	1.51 + 0.35	0.27 + 0.39	0.61 + 0.09	0.34 + 0.20	0.29 + 0.09	0.00	205.68 + 16.26
3.4% 72h	115.57 ± 8.57	51.90 ± 0.62	25.06 ± 2.35	20.78 ± 1.00	7.94 ± 1.21	2.12 ± 1.41	1.82 ± 0.36	2.26 ± 0.91	3.46 ± 0.50	2.72 ± 0.24	1.23 ± 0.06	3.40 ± 3.49	0.82 ± 0.46	1.37 ± 0.50	1.05 ± 0.51	0.74 ± 0.19	0.00	242.25 ± 6.47
4.0% 72h	130.91 ± 2.69	53.98 ± 1.70	35.16 ± 6.04	31.72 ± 1.77	7.70 ± 0.59	3.58 ± 2.39	3.04 ± 1.32	2.64 ± 1.09	4.65 ± 0.89	4.79 ± 0.58	2.28 ± 0.34	1.24 ± 0.42	1.56 ± 0.78	1.73 ± 0.86	1.07 ± 0.59	1.08 ± 0.52	0.00	287.14 ± 3.83

Values are given as mean ± standard deviation (n = 3).

Supplementary Table S2. The average size of the constructed cDNA library and corresponding Ion PGM sequencing data for kuruma shrimp reared for 24 h at different salinity

Salinity (%)	cDNA library average length (bp)	Base number (Mb)	Read number	Average read length (bp)
1.7	272	478	3,723,179	127
3.4	291	517	4,743,277	102
4.0	247	316	3,181,239	196

Supplementary Table S3. The genes with expression levels increased more than 10 times in kuruma shirimp acclimated at 1.7 % salinity. Listed are the fragments per kilobase of transcript per million fragments (FPKM) values of genes from the shrimp acclimated at 1.7 % and 3.4 % salinity, and their fold changes (FC). Listed are the genes whose expression levels were increased more than 10 folds during acclimation for 24 h at 1.7 % salinity compared with those at 3.4 % salinity as a reference.

Gene	1.7 % FPKM	3.4 % FPKM	FC
Homarus americanus beta-I tubulin mRNA, complete cds	162.46	0.01	15708.4
Eriocheir sinensis truncated cathepsin A gene, complete cds	30.24	0.06	502.9

Fenneropenaeus chinensis glucose-regulated protein 78 mRNA, complete cds	83.55	0.17	479.7
Penaeus monodon lymphoid organ expressed yellow head virus receptor protein mRNA, partial cds	539.41	1.42	379.5
PREDICTED: Ailuropoda melanoleuca zinc finger CCHC domain-containing protein 24-like (LOC100484114), partial mRNA	15.13	0.05	305.8
Strongylocentrotus purpuratus kinesin heavy chain (LOC373178), mRNA	41.24	0.16	254.3
Scylla paramamosain isolate 2 I-connectin mRNA, partial cds	119.32	0.76	157.6
Marsupenaeus japonicus mjTSPc mRNA for thrombospondin, complete cds	1695.81	12.00	141.3
Marsupenaeus japonicus F1F0-ATP synthase beta subunit mRNA, complete cds	3509.10	27.45	127.8
Uncinocarpus reesii 1704 ATP-binding cassette sub-family E member 1, mRNA	105.91	0.96	110.3
PREDICTED: Monodelphis domestica protein flightless-1 homolog (LOC100619473), mRNA	22.91	0.24	93.5
Litopenaeus vannamei phosphofructokinase mRNA, partial cds	228.94	2.45	93.4
PREDICTED: Bombus terrestris hypothetical protein LOC100642686 (LOC100642686), mRNA	129.31	1.79	72.2
Procambarus clarkii mRNA for Kettin, complete cds	1.04	0.02	66.3
Penaeus monodon beta tubulin mRNA, partial cds	3555.86	55.04	64.6
PREDICTED: Nasonia vitripennis hypothetical protein LOC100117915 (LOC100117915), mRNA	108.15	1.71	63.3
PREDICTED: Monodelphis domestica cleft lip and palate associated transmembrane protein 1 (CLPTM1), mRNA	29.20	0.47	62.5
Marsupenaeus japonicus MYH2 mRNA for myosin heavy chain type 2, complete cds	60293.54	986.41	61.1
PREDICTED: Macaca mulatta actinin, alpha 4, transcript variant 5 (ACTN4), mRNA	168.32	2.91	57.8
Drosophila pseudoobscura pseudoobscura GA25794 (Dpse¥GA25794), mRNA	269.08	4.74	56.7
Nebalia hessleri RNA polymerase II largest subunit mRNA, partial cds	32.88	0.59	55.5
Cherax quadricarinatus clone cherax_263 mRNA sequence	70.26	1.27	55.5
Scylla paramamosain chaperonin zeta subunit mRNA, complete cds	166.40	3.11	53.4
Rattus norvegicus adaptor-related protein complex 2, beta 1 subunit, mRNA (cDNA clone MGC:124574 IMAGE:7929959), complete cds	21.63	0.41	52.8
Penaeus monodon receptor for activated protein kinase c1 (RACK1) mRNA, complete cds	764.01	14.84	51.5
Litopenaeus vannamei G protein beta 1 subunit mRNA, complete cds	77.80	1.53	51.0
Litopenaeus vannamei SID-1-like protein mRNA, complete cds	20.42	0.41	49.2
Drosophila persimilis GL25810 (Dper¥GL25810), mRNA	61.67	1.29	47.9
Anopheles gambiae str. PEST AGAP001519-PA (AgaP_AGAP001519) mRNA, complete cds	39.46	0.84	46.8
Penaeus monodon saposin isoform 1 mRNA, complete cds	940.33	20.63	45.6
PREDICTED: Bos taurus topoisomerase (DNA) II alpha 170kDa (TOP2A), mRNA	17.07	0.38	45.2
Culex quinquefasciatus myosin-Id, mRNA	2556.84	57.07	44.8

Danio rerio dynein, cytoplasmic 1, heavy chain 1 (dync1h1), mRNA	129.27	2.89	44.8
Penaeus monodon calreticulin mRNA, complete cds	137.61	3.09	44.5
Ciona intestinalis cDNA, clone:cilv008l08, full insert sequence	18.26	0.42	43.2
Danio rerio suppressor of Ty 16 homolog, mRNA (cDNA clone MGC:194708 IMAGE:9038340), complete cds	20.79	0.49	42.3
Gryllus bimaculatus mRNA, GBcontig31854	556.12	14.10	39.4
PREDICTED: Cricetulus griseus alkyldihydroxyacetonephosphate synthase, peroxisomal-like (LOC100756809), mRNA	86.97	2.24	38.8
Penaeus monodon innexin 2 mRNA, complete cds	49.93	1.29	38.7
Marsupenaeus japonicus mRNA for putative ovarian lipoprotein receptor, complete cds	81.40	2.20	37.0
PREDICTED: Anolis carolinensis 26S protease regulatory subunit 4-like (LOC100551981), mRNA	93.96	2.58	36.5
PREDICTED: Bombus impatiens polypyrimidine tract-binding protein 2-like (LOC100745429), mRNA	172.91	4.93	35.1
Marsupenaeus japonicus cytosolic MnSOD mRNA, partial cds	59.85	1.72	34.8
Penaeus monodon leucine-rich repeat protein mRNA, complete cds	98.35	2.85	34.5
Arabidopsis lyrata subsp. lyrata 1, 4-alpha-glucan branching enzyme protein soform SBE2.2 precursor, mRNA	30.23	0.94	32.1
Penaeus monodon microsatellite 2GG sequence	58.60	1.83	32.0
Culex quinquefasciatus plexin-A2, mRNA	63.68	2.08	30.6
Fenneropenaeus chinensis alpha2 macroglobulin isoform 2 mRNA, complete cds	36.39	1.29	28.2
Fenneropenaeus chinensis ATP-binding cassette transmembrane transporter mRNA, complete cds	80.84	2.87	28.2
Drosophila ananassae GF17067 (Dana¥GF17067), mRNA	289.09	10.41	27.8
Penaeus monodon Na+/K+-ATPase alpha subunit gene, complete cds	148.39	5.38	27.6
Drosophila mojavensis GI19300 (Dmoj¥GI19300), mRNA	76.67	2.80	27.4
Caenorhabditis briggsae C. briggsae CBR-SPC-1 protein (Cbr-spc-1) mRNA, complete cds	56.79	2.23	25.5
Penaeus monodon elongation factor 2 mRNA, complete cds	246.75	9.70	25.4
Acytostelium subglobosum clone ADB0000991 fumarate hydratase gene, complete cds	43.05	1.71	25.2
Litopenaeus vannamei pyruvate kinase 3 mRNA, complete cds	3344.01	137.19	24.4
Litopenaeus vannamei carbonic anhydrase I mRNA, complete cds	45.65	1.90	24.1
Marsupenaeus japonicus ATP/ADP translocase mRNA, complete cds	2168.67	91.94	23.6
Mus musculus NOL1/NOP2/Sun domain family member 2 (Nsun2), mRNA	59.11	2.54	23.3
PREDICTED: Bombus terrestris glycogen debranching enzyme-like (LOC100651379), mRNA	540.10	24.86	21.7
Halichondria sp. KJP-2009 phosphofructokinase (PFK) mRNA, partial cds	58.19	2.70	21.5

PREDICTED: Gallus gallus similar to protein phosphatase 2A B56-alpha (LOC421372), mRNA	60.53	2.86	21.2
Homarus americanus AMP-activated protein kinase gamma subunit mRNA, partial cds	71.10	3.36	21.1
PREDICTED: Hydra magnipapillata similar to topoisomerase (DNA) II alpha (LOC100214280), partial mRNA	105.46	5.13	20.6
PREDICTED: Tribolium castaneum similar to SLMAP protein (LOC656454), mRNA	182.03	9.20	19.8
Pediculus humanus corporis glycine dehydrogenase, putative, mRNA	90.47	4.59	19.7
Gasterosteus aculeatus clone CFW186-C01 mRNA sequence	131.84	6.70	19.7
Penaeus monodon Tudor staphylococcal nuclease (tsn) mRNA, complete cds	17.90	0.92	19.4
Anopheles gambiae str. PEST AGAP012169-PA (AgaP_AGAP012169) mRNA, complete cds	173.58	9.15	19.0
Litopenaeus vannamei argonaute 2 mRNA, complete cds	98.16	5.19	18.9
Fenneropenaeus chinensis actin 1 mRNA, complete cds	7773.69	416.02	18.7
Marsupenaeus japonicus MjGo mRNA for GTP binding protein alpha subunit Go, complete cds	46.08	2.49	18.5
Tetraodon nigroviridis full-length cDNA	30.63	1.66	18.4
Drosophila virilis GJ17652 (Dvir¥GJ17652), mRNA	144.47	7.90	18.3
Scylla paramamosain T-complex Chaperonin 5-like protein mRNA, complete cds	564.09	31.02	18.2
Bombyx mori Bm_191 SRP RNA, complete sequence	8417.22	470.08	17.9
Marsupenaeus japonicus putative RNA helicase (Pl10) mRNA, complete cds	84.89	4.74	17.9
Branchiostoma floridae hypothetical protein, mRNA	42.38	2.43	17.4
Penaeus monodon carbonic anhydrase I mRNA, complete cds	200.28	11.64	17.2
Marsupenaeus japonicus MjGi mRNA for GTP binding protein alpha subunit Gi, complete cds	70.91	4.14	17.1
PREDICTED: Sus scrofa phosphofructokinase, platelet (PFKP), mRNA	156.75	9.51	16.5
Marsupenaeus japonicus hsp90 mRNA for heat shock protein 90, complete cds	742.43	46.98	15.8
Gammarus duebeni type A1 myosin heavy chain mRNA, partial cds	113.64	7.41	15.3
Xenopus (Silurana) tropicalis isoleucyl-tRNA synthetase (iars), mRNA	31.55	2.15	14.6
Fenneropenaeus chinensis triosephosphate isomerase mRNA, complete cds	222.28	15.32	14.5
Fenneropenaeus chinensis peroxinectin mRNA, complete cds	49.36	3.43	14.4
PREDICTED: Nomascus leucogenys ubiquitin-like modifier activating enzyme 1 (UBA1), mRNA	130.01	10.91	11.9
Marsupenaeus japonicus MYH1 mRNA for myosin heavy chain type 1, complete cds	18532.09	1590.56	11.7
Litopenaeus vannamei single VWC domain protein 1 mRNA, complete cds	3071.32	290.45	10.6
Penaeus monodon cyclin A mRNA, complete cds	673.44	0.00	
Ixodes scapularis glucose dehydrogenase, putative, mRNA	196.76	0.00	
Salmo salar clone ssal-rgf-541-322 Spectrin alpha chain, brain putative mRNA, partial cds	159.64	0.00	
Trichoplax adhaerens hypothetical protein, mRNA	96.76	0.00	

PREDICTED: Oreochromis niloticus PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae) (PRPF8), mRNA	87.97	0.00
PREDICTED: Oreochromis niloticus probable ATP-dependent RNA helicase DDX17-like (LOC100692646), mRNA	75.96	0.00
PREDICTED: Nasonia vitripennis AP-2 complex subunit mu-1-like, transcript variant 2 (LOC100114041), mRNA	61.41	0.00
Litopenaeus vannamei partner of drosha-like protein (Pasha) mRNA, complete cds	60.19	0.00
Penaeus monodon mago-nashi mRNA, complete cds	59.25	0.00
Marsupenaeus japonicus mRNA for toll receptor 2, complete cds	55.79	0.00
Litopenaeus vannamei mitochondrial ATP synthase subunit alpha precursor, mRNA, complete cds; nuclear gene for mitochondrial product	49.82	0.00
Marsupenaeus japonicus MAPK mRNA for mitogen-activated protein kinase, complete cds	42.30	0.00
Bembix americana voucher Bxam811 RNA polymerase II gene, partial cds	39.64	0.00
Monosiga brevicollis MX1 predicted protein MONBRDRAFT_17492 mRNA, complete cds	39.14	0.00
Penaeus vannamei mRNA for phosphoenolpyruvate carboxykinase (pepck gene)	35.88	0.00
Penaeus monodon eukaryotic translation initiation factor 3 subunit G mRNA, complete cds	35.72	0.00
Libinia emarginata voucher LemMALA syntaxin mRNA, partial cds	35.70	0.00
TPA_inf: Amblyomma variegatum Golgi reassembly stacking protein GRASP65 mRNA, partial cds	35.41	0.00
PREDICTED: Cricetulus griseus ring finger protein 121 (Rnf121), mRNA	32.27	0.00
Danio rerio strawberry notch homolog 1 (Drosophila) (sbno1), mRNA	31.87	0.00
Thielavia terrestris NRRL 8126 chromosome 5, complete sequence	27.58	0.00
PREDICTED: Danio rerio adaptor-related protein complex 2, alpha 1 subunit, transcript variant 2 (ap2a1), mRNA	24.36	0.00
Penaeus monodon 14-3-3-like protein mRNA, complete cds	23.28	0.00
Penaeus monodon peroxiredoxin mRNA, complete cds	19.22	0.00
PREDICTED: Strongylocentrotus purpuratus similar to GA15522-PA (LOC581772), mRNA	17.64	0.00
Gryllus bimaculatus mRNA, GBcontig27912	15.57	0.00
Drosophila mojavensis GI18285 (Dmoj¥GI18285), mRNA	14.40	0.00
Litopenaeus vannamei V-H-ATPase subunit A mRNA, complete cds	13.38	0.00
Danio rerio zgc:76988 (zgc:76988), mRNA	12.78	0.00
Aplysia californica Src tyrosine kinase 2 (Src2), mRNA	9.20	0.00
PREDICTED: Ornithorhynchus anatinus SRSF protein kinase 2 (SRPK2), mRNA	8.51	0.00
PREDICTED: Ciona intestinalis similar to nicotinamide nucleotide transhydrogenase (LOC100177202), mRNA	4.70	0.00

Rana catesbeiana clone rcat-evr-536-309 Ubiquitin putative mRNA, complete cds	4.57	0.00
Danio rerio spectrin alpha 2 (spna2), mRNA	1.63	0.00
Ixodes scapularis spectrin alpha chain, putative, mRNA	1.05	0.00

Supplementary Table S4. The genes with expression levels decreased less than 10 % in kuruma shrimp acclimated at 1.7 % salinity. Listed are the fragments per kilobase of transcript per million fragments (FPKM) values of each gene from the shrimp acclimated at 1.7 % and 3.4 % salinity, and their fold changes (FC). Listed are the genes whose expression levels were decreased less than 10 % during acclimation for 24 h at 1.7 % salinity compared with those at 3.4 % salinity as a reference.

Gene	1.7 % FPKM	3.4 % FPKM	1/FC
Marsupenaeus japonicus clottable protein mRNA, complete cds	0.01	3877.73	317586.8
Penaeus monodon sarcoplasmic calcium binding protein mRNA, complete cds	0.05	2358.55	44816.3
Marsupenaeus japonicus Rad23 mRNA for nucleotide excision repair protein, complete cds	0.01	83.72	7932.7
Penaeus monodon ribosomal protein L3 mRNA, complete cds	0.19	694.45	3582.3
Litopenaeus vannamei arginine kinase mRNA, complete cds	4.94	16595.90	3362.4
Litopenaeus vannamei ATP synthase subunit 9 mitochondrial precursor, mRNA, complete cds; nuclear gene for mitochondrial product	0.09	261.63	2939.4
Cherax quadricarinatus clone y9_B7 mRNA sequence	0.01	21.80	2771.5
Litopenaeus vannamei ryanodine receptor gene, partial cds	0.06	145.83	2624.7
Marsupenaeus japonicus DD5 mRNA, complete cds	0.02	44.62	2464.1
Marsupenaeus japonicus mRNA for astakine, complete cds	0.01	24.26	1948.6
Fenneropenaeus chinensis actin 1 mRNA, complete cds	0.15	156.05	1028.3
Gasterosteus aculeatus clone CFW45-F03 mRNA sequence	0.12	125.79	1016.3
Penaeus monodon calreticulin mRNA, complete cds	0.12	108.48	925.4
Penaeus monodon nuclear autoantigenic sperm protein (NASP) mRNA, complete cds	0.03	25.57	901.6
Litopenaeus vannamei actin T2 mRNA, complete cds	0.48	382.99	792.8
Penaeus monodon ribophorin I mRNA, complete cds	0.02	11.31	609.1
Drosophila grimshawi GH16493 (Dgri¥GH16493), mRNA	0.03	15.64	596.3
Ixodes scapularis nhl repeat-containing protein, putative, mRNA	0.01	8.37	575.7
Marsupenaeus japonicus DD9B mRNA, partial cds	5.73	2342.40	408.5
Fenneropenaeus chinensis nuclear receptor E75 protein mRNA, complete cds	0.22	82.54	371.7
Penaeus monodon Tudor staphylococcal nuclease (tsn) mRNA, complete cds	0.11	33.75	307.7
Marsupenaeus japonicus mRNA for Pjchi-2, complete cds	1.23	290.09	235.7
PREDICTED: Gallus gallus threonyl-tRNA synthetase-like 2 (TARSL2), mRNA	0.08	19.04	227.2

Litopenaeus vannamei adenine nucleotide translocase 2 (LVANT2) mRNA, complete cds	12.74	2437.86	191.3
Penaeus monodon actin 2 (act2) mRNA, complete cds	23.82	4008.58	168.3
Marsupenaeus japonicus lys-pj mRNA for c-type lysozyme, complete cds	0.11	17.61	160.3
Drosophila melanogaster peritrophin A mRNA, complete cds	0.15	22.06	142.4
Marsupenaeus japonicus DD9A mRNA, partial cds	11.01	1349.40	122.5
PREDICTED: Tribolium castaneum similar to Myosin heavy chain CG17927-PF, transcript variant 3 (LOC659358), mRNA	0.21	22.34	104.7
PREDICTED: Rattus norvegicus similar to procollagen, type IV, alpha 6 (LOC363458), miscRNA	0.31	32.03	104.0
Nasonia vitripennis cuticular protein analogous to peritrophins 3-D2 (Cpap3-d2), mRNA	0.23	22.48	96.1
Tetraodon nigroviridis full-length cDNA	3.55	324.58	91.4
Scylla paramamosain putative myosin regulatory light chain 2 smooth muscle mRNA, complete cds	0.42	34.88	82.6
PREDICTED: Cavia porcellus pre-mRNA-processing-splicing factor 8-like (LOC100712742), mRNA	0.05	3.36	66.4
Gecarcinus lateralis alpha-2-tubulin mRNA, complete cds	4.79	250.85	52.4
Litopenaeus vannamei Lit v 3 allergen myosin light chain mRNA, complete cds	23.61	1180.18	50.0
Procambarus clarkii mRNA for projectin, complete cds	5.88	275.93	46.9
Penaeus monodon inhibitor of apoptosis protein (IAP) mRNA, complete cds	0.08	3.54	46.2
Litopenaeus vannamei beta-N-acetylglucosaminidase mRNA, complete cds	2.41	105.05	43.5
Micromonas pusilla CCMP1545 predicted protein, mRNA	0.73	26.60	36.6
Procambarus clarkii sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) mRNA, complete cds	103.08	3684.76	35.7
Drosophila yakuba Tbp-1 (Dyak¥Tbp-1), mRNA	0.53	18.60	34.9
PREDICTED: Anolis carolinensis adenosine monophosphate deaminase 2 (ampd2), mRNA	0.31	10.56	34.2
Marsupenaeus japonicus myosin light chain mRNA, complete cds	389.29	12596.93	32.4
Litopenaeus vannamei Spz3 mRNA, complete cds	0.75	18.25	24.4
Penaeus monodon eukaryotic translation initiation factor 5A mRNA, complete cds	14.88	346.10	23.3
Marsupenaeus japonicus clone MjACP15-T 3' UTR	4.00	88.52	22.2
Heliconius cydno cordula C10 copy 2 hairy cell leukemia (HCL) gene, partial cds	2.91	60.34	20.7
Mythimna separata alpha-tubulin mRNA, complete cds	22.09	418.56	18.9
Marsupenaeus japonicus MYH3 mRNA for myosin heavy chain type 3	118.00	1785.97	15.1
Procambarus clarkii casp-2 mRNA for calcification associated soluble matrix protein 2, complete cds	0.00	2049.88	
Penaeus monodon ribosomal protein L7 mRNA, complete cds	0.00	498.06	
Portunus pelagicus cuticle protein CB6 mRNA, complete cds	0.00	406.33	
PREDICTED: Taeniopygia guttata tubulin, alpha 8 (LOC100227941), mRNA	0.00	243.55	

Pseudoalteromonas sp. SM9913 chromosome I, complete sequence	0.00	127.31
Fenneropenaeus chinensis glucose-regulated protein 78 mRNA, complete cds	0.00	115.20
Rimicaris exoculata alpha-tubulin mRNA, partial cds	0.00	97.85
Marsupenaeus japonicus mRNA for alpha2-macroglobulin homolog, complete cds	0.00	96.78
Marsupenaeus japonicus ADP-ribosylation factor 1 (Arf1) mRNA, complete cds	0.00	93.17
PREDICTED: Ailuropoda melanoleuca tubulin, alpha 1c (TUBA1C), mRNA	0.00	92.94
Branchiostoma floridae hypothetical protein, mRNA	0.00	90.42
Penaeus monodon clone NF228 alpha-tubulin mRNA, partial cds	0.00	87.10
Procambarus clarkii mRNA for Kettin, complete cds	0.00	81.03
Litopenaeus vannamei carbonic anhydrase I mRNA, complete cds	0.00	79.82
Penaeus monodon i-type lysozyme-like protein 2 mRNA, complete cds	0.00	66.92
T.pyriformis TU20 gene for ubiquitin	0.00	64.59
PREDICTED: Apis mellifera ribose-phosphate diphosphokinase (LOC552273), mRNA	0.00	63.07
Nematostella vectensis predicted protein (NEMVEDRAFT_v1g110807) partial mRNA	0.00	62.36
Pythium chamaihyphon strain PPRI8625 beta-tubulin gene, partial cds	0.00	60.67
PREDICTED: Oryctolagus cuniculus tubulin, beta 4 (LOC100348290), mRNA	0.00	60.07
Drosophila pseudoobscura pseudoobscura GA18162 (Dpse¥GA18162), mRNA	0.00	57.80
Marsupenaeus japonicus mRNA for farnesoic acid O-methyltransferase, complete cds	0.00	46.07
Cherax quadricarinatus clone cherax_250 mRNA sequence	0.00	42.80
Rivulus marmoratus parvalbumin 2 mRNA, complete cds	0.00	38.86
Hemimysis anomala isolate 1139hemi glutamyl-prolyl tRNA synthetase (EPRS) gene, partial cds	0.00	38.65
Metapenaeus ensis fushi tarazu-factor 1 mRNA, complete cds	0.00	36.76
Mus musculus histone cluster 1, H2bm, mRNA (cDNA clone MGC:171009 IMAGE:8862404), complete cds	0.00	34.43
PREDICTED: Cavia porcellus WD repeat-containing protein 5-like (LOC100719056), mRNA	0.00	33.56
P.vannamei mRNA for transforming growth factor beta-like protein	0.00	33.15
Xenopus laevis uncharacterized LOC100036902 (LOC100036902), mRNA	0.00	32.14
PREDICTED: Taeniopygia guttata similar to solute carrier family 6, member 17 (LOC100231778), mRNA	0.00	32.00
Drosophila virilis GJ15814 (Dvir¥GJ15814), mRNA	0.00	29.91
Penaeus monodon ALG-2 interacting protein x (Alix) mRNA, complete cds	0.00	29.85
Penaeus monodon small ubiquitin-like modifier 1 mRNA, complete cds	0.00	29.45
Apis mellifera filamin-like (LOC409697), mRNA	0.00	29.08
PREDICTED: Oreochromis niloticus creatine kinase M-type-like (LOC100702938), mRNA	0.00	28.67
Drosophila pseudoobscura pseudoobscura GA18477 (Dpse¥GA18477), mRNA	0.00	27.72

Marsupenaeus japonicus ubiquitin-conjugating enyzme E2r mRNA, complete cds	0.00	26.50
Drosophila persimilis GL23091 (Dper¥GL23091), mRNA	0.00	22.99
Litopenaeus vannamei Spz1 mRNA, complete cds	0.00	22.46
Farfantepenaeus subtilis crustin mRNA, complete cds	0.00	21.85
TPA_exp: Amblyomma variegatum malate dehydrogenase mRNA, complete cds	0.00	21.31
Marsupenaeus japonicus cyclin B mRNA, complete cds	0.00	19.45
Homarus americanus ryanodine receptor (RyR) mRNA, partial cds	0.00	18.55
Ixodes scapularis conserved hypothetical protein, mRNA	0.00	18.36
Drosophila willistoni GK17504 (Dwil¥GK17504), mRNA	0.00	18.32
PREDICTED: Apis mellifera RING finger protein nhl-1-like (LOC408420), mRNA	0.00	16.40
Marsupenaeus japonicus clone MjACP4-0 3' UTR	0.00	15.80
Marsupenaeus japonicus MjALF2 mRNA for anti-lipopolysaccharide factor 2, complete cds	0.00	15.04
PREDICTED: Oreochromis niloticus ras-related protein Rab-1A-like, transcript variant 1 (LOC100707666), mRNA	0.00	14.76
Tetrahymena pyriformis beta-tubulin gene 1 (beta-TT1)	0.00	12.92
Penaeus monodon serine proteinase-like protein mRNA, complete cds	0.00	12.35
Yarrowia lipolytica YALI0E24013p (YALI0E24013g) mRNA, complete cds	0.00	11.36
Fenneropenaeus merguiensis ribosomal protein L10a mRNA, complete cds	0.00	10.00
Vorticella microstoma alpha-tubulin gene, partial cds	0.00	9.74
Calocarides chani phosphoenolpyruvate carboxykinase (PEPCK) gene, partial cds	0.00	8.29
Danio rerio proteasome (prosome, macropain) 26S subunit, ATPase 2 (psmc2), mRNA	0.00	6.97
Fenneropenaeus chinensis peroxinectin mRNA, complete cds	0.00	6.61
PREDICTED: Strongylocentrotus purpuratus hypothetical LOC578337 (LOC578337), mRNA	0.00	6.53
Litopenaeus vannamei arsenite-resistance protein 2-like protein (Ars2) mRNA, complete cds	0.00	6.44
Tetrahymena thermophila Phosphatidylinositol 3- and 4-kinase family protein, mRNA	0.00	6.09
Osmerus mordax clone omor-rgc-505-373 COP9 signalosome complex subunit 3 putative mRNA, complete cds	0.00	4.32
Libinia emarginata voucher LemMALA proteasome subunit mRNA, partial cds	0.00	4.06

Supplementary Table S5. The genes with expression levels increased more than 10 times in kuruma shrimp acclimated at 4.0 % salinity. Listed are the fragments per kilobase of transcript per million fragments (FPKM) values of each gene from the shrimp acclimated at 4.0 % and 3.4 % salinity, and their fold change (FC). Listed are the genes whose expression levels were increased more than 10 folds during acclimation for 24 h at 4.0 % salinity compared with those at 3.4 % salinity as a reference.

Gene	4.0 % FPKM	3.4 % FPKM	FC
Penaeus monodon actin 2 (act2) mRNA, complete cds	4668.53	24.79	188.3
Litopenaeus vannamei actin T2 mRNA, complete cds	3523.19	27.73	127.0
Marsupenaeus japonicus SPH mRNA for serine proteinase homologue, partial cds	30.11	0.29	105.6
Bombyx mori Bm_191 SRP RNA, complete sequence	28455.62	470.08	60.5
Penaeus monodon lymphoid organ expressed yellow head virus receptor protein mRNA, partial cds	80.84	1.42	56.9
Procambarus clarkii mRNA for projectin, complete cds	64.81	2.19	29.6
Marsupenaeus japonicus MYH3 mRNA for myosin heavy chain type 3	22.80	1.35	16.9
Marsupenaeus japonicus DD5 mRNA, complete cds	57.50	4.69	12.3
Penaeus monodon ribosomal protein L7 mRNA, complete cds	403.85	34.48	11.7
Drosophila virilis GJ15814 (Dvir¥GJ15814), mRNA	343.84	29.91	11.5
Litopenaeus vannamei ribosomal protein L8 mRNA, complete cds	596.88	52.86	11.3
Fenneropenaeus chinensis triosephosphate isomerase mRNA, complete cds	23.05	2.21	10.4
Marsupenaeus japonicus MjALF2 mRNA for anti-lipopolysaccharide factor 2, complete cds	24.13	0.00	
Penaeus monodon Ras-like nuclear protein (Ran) mRNA, complete cds	5.22	0.00	
Branchiostoma floridae hypothetical protein, mRNA	4.25	0.00	
Scylla paramamosain hypothetical protein mRNA, partial cds	2.08	0.00	

Supplementary Table S6. The genes with expression levels decreased lessthan 10 % in kuruma shrimp acclimated at 4.0 % salinity. Listed are the fragments per kilobase of transcript per million fragments (FPKM) values of each gene from the shrimp acclimated at 4.0 % and 3.4 % salinity, and their fold changes (FC). Listed are the genes whose expression levels were decreased less than 10 % during acclimation for 24 h at 4.0 % salinity compared with those at 3.4 % salinity as a reference.

Gene	4.0 % FPKM	3.4 % FPKM	1/FC
Mythimna separata alpha-tubulin mRNA, complete cds	0.22	600.55	2746.7
Litopenaeus vannamei carbonic anhydrase I mRNA, complete cds	0.03	79.82	2488.4
Marsupenaeus japonicus mjTSPc mRNA for thrombospondin, complete cds	3.54	5022.97	1418.7
Marsupenaeus japonicus CRP-2 mRNA for cortical rod protein-2, complete cds	2.71	3237.87	1194.7
Marsupenaeus japonicus mjTSPa mRNA for thrombospondin, complete cds	0.49	490.29	1001.5
Procambarus clarkii casp-2 mRNA for calcification associated soluble matrix protein 2, complete cds	4.37	2049.88	469.5
Penaeus monodon cyclin A mRNA, complete cds	0.53	81.00	153.6
Fenneropenaeus chinensis heat shock protein 90 mRNA, complete cds	0.60	88.20	147.0

Salmo salar phosphoglycerate mutase 2-1 (muscle) (LOC100194644), mRNA	0.61	69.37	113.5
Litopenaeus vannamei arginine kinase mRNA, complete cds	249.35	16595.90	66.6
PREDICTED: Apis mellifera ribose-phosphate diphosphokinase (LOC552273), mRNA	0.98	63.07	64.1
Marsupenaeus japonicus DD9B mRNA, partial cds	42.99	2342.40	54.5
Penaeus monodon actin 2 (act2) mRNA, complete cds	54.73	2313.15	42.3
Cherax quadricarinatus beta actin mRNA, complete cds	1.72	62.38	36.3
Penaeus monodon sarcoplasmic calcium binding protein mRNA, complete cds	81.32	2358.55	29.0
Penaeus monodon progestin membrane receptor component 1 (PGMRC1) mRNA, complete cds	0.00	485.75	
Portunus pelagicus cuticle protein CB6 mRNA, complete cds	0.00	406.33	
PREDICTED: Taeniopygia guttata tubulin, alpha 8 (LOC100227941), mRNA	0.00	243.55	
PREDICTED: Ornithorhynchus anatinus tubulin alpha-1C chain-like (LOC100088515), partial mRNA	0.00	218.34	
Equus caballus actinin alpha 3 (ACTN3) mRNA, complete cds	0.00	148.56	
Anoplopoma fimbria clone afim-evh-509-131 Proteasome subunit alpha type-6 putative mRNA, complete cds	0.00	146.94	
Pseudoalteromonas sp. SM9913 chromosome I, complete sequence	0.00	127.31	
Penaeus monodon elongation factor 2 mRNA, complete cds	0.00	122.13	
Fenneropenaeus chinensis glucose-regulated protein 78 mRNA, complete cds	0.00	115.20	
Tetraodon nigroviridis full-length cDNA	0.00	103.19	
PREDICTED: Tribolium castaneum similar to GTP binding protein (LOC658378), mRNA	0.00	90.52	
Penaeus monodon nuclear autoantigenic sperm protein (NASP) mRNA, complete cds	0.00	79.76	
Marsupenaeus japonicus heat shock protein 70 (hsp70) mRNA, partial cds	0.00	74.64	
Penaeus monodon thrombospondin protein mRNA, complete cds	0.00	72.40	
Procambarus clarkii mRNA for projectin, complete cds	0.00	46.56	
Fenneropenaeus chinensis serine protease-like protein mRNA, complete cds	0.00	17.95	