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1 # Osmoregulation of glutamine synthetase from Giant freshwater
2 # prawn (*Macrobrachium rosenbergii*) under osmotic stress

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21 **Abstract**

22 Glutamine synthetase is a key enzyme that catalyzes the biosynthesis of glutamine (Gln)
23 from glutamate and ammonia. Gln a vital amino acid acts as a precursor for protein
24 synthesis and also assist in ammonia repressor and a key osmoregulators in aquatics.
25 Here, we report the cloning and characterization of the GS gene from *Macrobrachium*
26 *rosenbergii* (*Mr*-GS). The complete nucleotide and deduced amino acid sequences were
27 determined that phylogenetically shared highest identity with other crustaceans. GS
28 mRNA was differentially expressed in 6 different tissues, with high to low order as
29 muscle > gills > heart > stomach > brain > haemolymph. *Mr*-GS expression and the
30 glutamine concentrations were analyzed in the gills and muscle tissues of prawn under
31 hyper/hypo-osmotic stress conditions. Under hyper-osmotic stress, the mRNA
32 expression of *Mr*-GS was significantly increased in both gills and muscle at 3, 6 and 12
33 h post-treatment with 2.54, 4.21 and 10.83 folds, and 11.66, 17.97 and 45.92 folds,
34 respectively. Protein analysis by western blot (WB) and Immunohistochemistry (IHC)
35 further confirmed the *Mr*-GS expression was increased at 12 h post treatment. On the
36 other hand, under hypo-osmotic stress, the mRNA expression of *Mr*-GS was also
37 significantly increased in both gills and muscle at 3, 6 and 12 h post treatment with
38 1.63, 3.30 and 3.52 folds, and 4.06, 42.99 and 26.69 folds, respectively. Furthermore,
39 under hyperosmotic stress, Gln concentration was increased in both gills and muscle at
40 6 and 12 h post treatment with 1.83, 2.02 folds, and 1.41, 1.29 folds, respectively. While,
41 under hypo-osmotic stress, Gln concentration was increased in both gills and muscle at
42 3, 6 and 12 h post treatment with 3.99, 3.40, 2.59 folds, and 1.72, 1.83, 1.80 folds,

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43 respectively. Taken together, these results suggest that *Mr*-GS might play a key role in

44 osmoregulation in *M. rosenbergii*.

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46 **Introduction**

47 Giant freshwater prawn (*Macrobrachium rosenbergii*) is one of the world's largest
48 freshwater cultured crustaceans and has a wide distribution in tropical and subtropical
49 areas of the world [1, 2]. There have been reported that the prawn could mature and
50 spawn in the freshwater area [3]. However, they must migrate to the brackish water
51 with salinity range between 9-19 ‰ for hatching and nursing of the larvae [2, 4]. As a
52 result of migration, this species exhibits an excellent tolerance to a wide range of
53 salinity, which is a characteristic of the prawn [5]. There are a number of reports about
54 the salinity tolerance of the prawn [2, 4-10]. However, the mechanism underlying the
55 osmoregulation of the prawn remains enigmatic.

56 Glutamine synthetase (GS, EC 6.3.1.2) is an enzyme catalyzes a reaction that
57 incorporates ammonium into glutamate and generates Glutamine (Gln), i.e., Glutamate
58 + ATP + $NH_3$ → Glutamine + ADP + phosphate [11]. The Gln plays crucial roles in an
59 array of biochemical functions, including protein synthesis, lipid synthesis, cell growth,
60 energy supply, as well as ammonia carrier [12]. The GS gene has been reported in many
61 species which included not only vertebrate species such as Chinese hamster [13],
62 chicken [14] and human [15], but also invertebrate species like *Procambarus clarkii*
63 [16], *Crassostrea gigas* [17], *Fenneropenaeus chinensis* [12] and *Litopenaeus*
64 *vannamei* [18]. However, up to date, there is no information about the GS gene of *M.*
65 *rosenbergii*. Previous studies had mainly focused on salinity-related changes in oxygen
66 consumption, ammonia excretion, and ion osmoregulation in *M. rosenbergii* [5]. There
67 have been reported that with the increase of water salinity, the levels of some free amino

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68 acid (FAA), including glycine, proline, arginine, glutamate, and alanine in the tissues
69 and haemolymph also raised in *M. rosenbergii* and *M. nipponense* species [19, 20], due
70 to the catabolism of proteins and amino acids (AA) [21] . Ammonia which is toxic for
71 aquatic animals and it must be catalyzed into Gln with the effort of GS enzyme as a
72 nontoxic transporter in the haemolymph circulation [22].

73 Once the crustaceans were stressed by various environmental factors, GS
74 expression and the concentration of Gln were found to be increased, illustrating that GS
75 plays an essential role in environmental stress resistance and adaptation [12, 23].
76 Furthermore, Gln as a transporter of ammonia in the haemolymph provides an abundant
77 FAA as osmolytes that further utilized by other cells or protein synthesis [18, 20]. Since
78 the gills is a vital tissue for osmoregulation and the muscle is the largest storehouse of
79 protein and amino acid for providing energy, therefore, we focused our studies on gills
80 and muscle in this report. We have cloned a GS gene from *M. rosenbergii* (*Mr*-GS),
81 and the osmoregulation of the GS was characterized under osmotic stress.

82 **Materials and methods**

83 **Experimental design and samples collection**

84 Adult *M. rosenbergii* (approximately 12-15 g) were obtained from Jin Yang
85 Aquaculture Co. Ltd., Guangzhou, China. First of all, the prawns were acclimatized to
86 freshwater in the tank at 25 °C for at least one week before experiments. The prawns
87 were cultured in freshwater and used as negative control. For hyper-osmotic stress
88 treatment, some of the prawns were shifted directly into brackish water with 13 ‰
89 salinity. For hypo-osmotic stress treatment, the prawns which have been adapted to the

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90 water with 13 ‰ were shifted directly to freshwater. The tissues from six individuals
91 were sampled for the RNA extraction at 0, 3, 6, and 12 h post the stress treatment. The
92 samples of the gills and muscle were frozen immediately in liquid nitrogen for western-
93 blot analysis, while part of them was instantly fixed and processed for
94 immunohistochemistry (IHC).

95 **RNA isolation, cDNA synthesis and gene cloning of Mr-GS**

96 The total RNA from the various samples were extracted using RNAiso plus (TaKaRa,
97 Dalian, China) and the first-strand cDNA was synthesized using HIScript® Q Select RT
98 SuperMix for qPCR (Vazyme, Nanjing, China) according to the manufacturer's
99 instructions. Specific primers was designed based on the sequences of the GS gene
100 identified from the *M. rosenbergii* transcriptomic data in our laboratory (unpublished
101 data), so as to amplify the complete open reading frame ORF of *Mr*-GS. All used
102 primers were shown in Table 1. PCR amplification was performed under the following
103 conditions: pre-denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 10
104 s, 55 °C for 30 s and 72 °C for 1 min, post extension at 72 °C for 10-min and finally
105 kept at 4 °C. The amplified specific PCR products were electrophoresed on 1% agarose
106 gels, and the target products were purified with a TaKaRa Agarose Gel DNA
107 Purification KitVer.2.0 (TaKaRa, Japan). The purified DNA fragments were ligated
108 into the pET-32a (+) plasmid (TaKaRa, Japan) and transformed into competent
109 *Escherichia coli* DH5α cells. Positive clones containing inserts of the expected size
110 were sequenced using M13 primers and sequenced at Invitrogen, Shanghai.
111

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112 **Table 1.** Primers used in the present study

113

| Primers name | Sequences (5' - 3') | Purpose |
|---|---|---|
| **GS-F1** | CTTCGCATCCCAGGAGGAATT | RT-PCR |
| **GS-R1** | GGACAGCTGGAACATCTCCTT | RT-PCR |
| **GS-F2** | CGCGGATCCCTTCGCATCCCAGGAGGAATT | Recombinant plasmid |
| **GS-R2** | CCCAAGCTTGGACAGCTGGAACATCTCCTT | Recombinant plasmid |
| **GS-F3** | AGCCTGCCTCTACACTGGTA | qRT-PCR |
| **GS-R3** | TGACGCCGAAATCTTCAGCT | qRT-PCR |
| **β-actin-F** | GTCGTGACTTGACCGATTACCT | qRT-PCR |
| **β-actin-R** | ATCTCCTGCTCGAAGTCCAATG | qRT-PCR |

114

115 **Sequence and phylogenetic analysis of Mr-GS**

116 The full-length AA sequence of *Mr*-GS ORF and protein domains were predicted by
117 Emboss (http://emboss.Bioinformatics/) and SMART (http://smart.embl-heidelberg.de)
118 tools. The sequences similarity was analyzed by the BLAST program
119 (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignments were performed by
120 Clustal X 2.0 program, and the breakpoints analyses were further determined by
121 DNAMAN software package (Lynnon Biosoft, Canada). The phylogenetic tree was
122 constructed based on the ORF AA sequences of *Mr*-GS proteins by MEGA 6.0 software
123 with the neighbor-joining (NJ) method with 1000 bootstraps replications.

124 **Quantitative real-time PCR (qRT-PCR) assay and data analysis**

125 The expression pattern of *Mr*-GS in various tissues at different time points was studied
126 using qRT-PCR in Roche LightCycler 480 (Roche, USA). All used primers were
127 presented in Table 1, where β-actin was used as an internal reference gene. The qRT-
128 PCR was conducted using AceQ® qPCR SYBR® Green Master Mix (Vazyme, Nanjing,
129 China). The reaction was performed in a final volume of 20 μl, containing 1 μl cDNA,
130 10 μl AceQ® qPCR SYBR® Green Master Mix, 1 μl each specific primer, and 7 μl

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131 dd$H_2O$ under following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for
132 15 s, 60 °C for 30 s and 72 °C for 20 s, finally at 4 °C for 5 min on. The relative expression
133 ratio of the target genes versus β-actin gene was calculated using $2^{-\Delta\Delta CT}$ method [24].
134 Each sample was measured at least triplicate, and all data were presented as mean ±
135 standard deviation (SD). Significant differences between samples were analyzed by
136 one-way analysis of variance (ANOVA) in GraphPad Prism 7. The difference was
137 considered significant, $P < 0.05$ (*), $P < 0.01$ (**) or not significant, $P > 0.05$ (NS).

138 **Western-blot assay**

139 Total protein from the lysates of frozen gills and muscle tissues were prepared by
140 homogenization as described [25]. Briefly, the tissue samples were weighed and
141 homogenized three times in 5 volumes (w/v) of ice-cold extraction buffer containing
142 50 mM imidazole (pH 7.0), 1 mM EDTA, 25 mM NaF, and 1 mM PMSF. Subsequently,
143 sonicated for 30 s and centrifuged at 10,000 x *g* at 4 °C for 10 min. The protein
144 concentrations were determined according to the method of Bradford Protein Assay Kit
145 (Beyotime, Shanghai, China). The total protein (about 50 μg) was separated in an SDS-
146 PAGE (10 %) and transferred onto nitrocellulose membrane (Bio-Rad, America). The
147 membranes were blocked with TBST (137 mM NaCl, 20 mM Tris, 1 % Tween-20, pH
148 7.6) containing 5 % skim fat milk at room temperature (RT) for 1 h. Then the
149 membranes were incubated at 4 °C overnight with the rabbit *anti*-*Mr*-GS (1/1000
150 dilution) primary antibody which was prepared in our laboratory. Subsequently, the
151 membranes were washed three times for 5 min with TBST and incubated with HRP-
152 conjugated secondary antibody of goat *anti*-rabbit IgG (1/10,000 dilution). The

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153 membranes were incubated for 1 h and then washed thrice for 5 min with TBST. The
154 immunoreactive bands were revealed by chemiluminescence (ECL Western Blotting
155 Substrate, Solarbio, Beijing, China) and measured by using ChemiScope 6000 (CliNX,
156 Shanghai, China).

157 **Immunohistochemistry assay**

158 The IHC assay was performed as previously described [26]. In brief, both the gill and
159 muscle tissues were fixed with 4 % paraformaldehyde for 24 h at 4 °C, and then paraffin-
160 embedded samples were cut into 4-μm sections and baked at 60 °C for 2 h. Sections
161 adhered to slides were deparaffinized with xylene and rehydrated, submerged into
162 EDTA antigenic retrieval buffer and microwaved for antigenic retrieval for 15 min.
163 Later, the sections were treated with 3 % hydrogen peroxide ($H_2O_2$) in methanol,
164 followed by incubation with 1 % bovine serum albumin (BSA) to block nonspecific
165 binding at RT for 1 h. Then the tissue sections were incubated with the primary antibody
166 rabbit *anti*-*Mr*-GS (1/200 dilution) overnight at 4 °C. After washing thrice with TBST,
167 slides were incubated with HRP-conjugated goat *anti*-rabbit IgG (1/1000 dilution) at
168 RT for 1 h and developed with DAB substrate solution (Guge Biotech, China). Finally,
169 the sections were counterstained with hematoxylin, mounted and photographed
170 (ECLIPSE E100, Nikon).

171 **Determination of Glutamine concentration**

172 Frozen gills and muscle tissues were processed and measured using shrimp Glutamine
173 ELISA Kit (Kawanshu, Shanghai, China) following manufacturer instruction. Briefly,
174 to the microwells previously coated with *anti*-Gln antibodies, samples, standards, and

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175 HRP-labeled detection antibodies were sequentially added with appropriate incubations
176 and washing. Tetramethylbenzidine (TMB) was added to each microplate wells to form
177 a final yellow color from blue by the catalysis of peroxidase. The absorbance was
178 measured at a wavelength of 450 nm using a microplate reader (Molecular Devices,
179 USA), the intensity of color was measured.

## 180 Results

### 181 Sequence and phylogenetic analysis of *Mr*-GS

182 The full-length cDNA transcript of *Mr*-GS was 1965 bp with a 76 bp at 5′-untranslated
183 region (UTR), an 803 bp 3′-UTR containing a 13 bp poly (A) tail. Nucleotide sequence
184 analysis showed that the ORF of 1086 bp which encoded a putative protein of 361 AA
185 with estimated molecular weight (MW) of 40.75 kDa (Fig 1A). SMART analysis
186 displayed that *Mr*-GS protein contained two catalytic domains of Gln-synt_N located
187 at N-terminal region at about 21-101 bp and Gln-synt_C located at C-terminal region
188 at 107-356 bp (Fig 1B).

189 **Fig 1. The sequence analysis of *Mr*-GS.** (A) The full-length cDNA sequence and
190 deduced amino acid sequence of *Mr*-GS. The ORF of the nucleotide sequence was
191 shown in lowercase, while the 5' and 3'-UTR sequences were shown in upper-case
192 letters. The two potential *Mr*-GS binding domains were underlined in black. (B)
193 Architecture and location representation of two characteristic domains of *Mr*-GS.

194 Multiple sequence alignment indicated that the AA ORF sequences of *Mr*-GS
195 showed 87 % identity to the *Marsupenaeus japonicus* GS (*Mj*-GS), 83 % to *Hyalella*
196 *azteca* GS (*Ha*-GS), 81 % to *Pacifastacus leniusculus* GS (*Pl*-GS), 79 % to *Daphnia*

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197 *magna* GS (*Dm*-GS), 71 % to *Danio rerio* GS (*Dr*-GS), and 70 % identity to the *Homo*
198 *sapiens* GS (*Hs*-GS) (Fig 2). The results suggested that GS proteins were highly
199 conserved from invertebrates to vertebrates, and multiple alignments discovered five
200 conserved regions within *Mr*-GS. Besides, the phylogenetic tree demonstrated that GS
201 genes were separated into two groups consisting of invertebrate and vertebrate. The
202 invertebrates included crustacean, insecta, arachnida, and merostomata, and the
203 vertebrates contained actinopterygii and mammalians, correlating well with the
204 evolutionary origins. As shown in Fig 3, the GS of *M. rosenbergii* were clustered with
205 *Litopenaeus vannamei*, *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, *Penaeus*
206 *monodon*, *Hyalella azteca*, *Procambarus clarkii*, *Pacifastacus leniusculus* and *Daphnia*
207 *magna* together into the crustacean group.

208 **Fig 2. Analysis of protein sequences across species.** Five conserved regions were
209 marked with Ⅰ: the latch (F/Y-D-G-S-S), Ⅱ: (G-X(8)-E/K-V-X(3)-Q-W-E), Ⅲ: ATP-
210 binding site (K-P-X(4,5)-N-G-A-G-X-H-T-H-T-N-X-S), Ⅳ: Glutamate binding site
211 (N/S-R-X(3)-I-R-I-P-R), and Ⅴ: (F/L-E-D-R-X-P-S-X-N-X-D-P-Y), respectively.
212 Multiple-sequence alignment of *M. rosenbergii* (*Mr*-GS) with Dr-GS, *Danio rerio* GS
213 (NP 878286.3); Dm-GS, *Daphnia magna* GS (KZS15608.1); Ha-GS, *Hyalella azteca*
214 GS (XP 018023652.1); Pl-GS, *Pacifastacus leniusculus* GS (AFV39702.1); Hs-GS,
215 *Homo sapiens* GS (NP 001028216.1); Mj-GS, *Marsupenaeus japonicus* GS
216 (AWW43688.1).

217 **Fig 3. Phylogenetic analysis of full-length amino acid sequences of *Mr*-GS.** The
218 diagram was generated by the neighbor-joining method using the MEGA 6.0 program.

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219 Numbers next to the branches represent the percentage of replicate trees in the bootstrap
220 replication (1,000). Bar scale at the bottom indicates 5 % amino acid divergence. The
221 analysis involved 20 amino acid sequences including *Macrobrachium rosenbergii*
222 (marked with a triangle); *Litopenaeus vannamei* (AEO80035.1); *Marsupenaeus*
223 *japonicus* (AWW43688.1); *Fenneropenaeus chinensis* (AFN66649.1); *Penaeus*
224 *monodon* (AGA83299.1); *Hyalella azteca* (XP 018023652.1); *Procambarus clarkii*
225 (AKN79748.1); *Pacifastacus leniusculus* (AFV39702.1); *Daphnia magna*
226 (KZS15608.1); *Halyomorpha halys* (XP 014294596.1); *Leptinotarsa decemlineata* (XP
227 023022227.1); *Anoplophora glabripennis* (XP 018567831.1); *Anopheles darlingi*
228 (ETN61772.1); *Centruroides sculpturatus* (XP 023214668.1); *Limulus polyphemus*
229 (XP 013778191.1); *Parasteatoda tepidariorum* (XP 015929268.1); *Danio rerio* (NP
230 878286.3); *Homo sapiens* (NP 001028216.1); *Bos taurus* (AAI03100.1); *Mus musculus*
231 (AAA37746.1); *Rattus norvegicus* (NP 058769.4).

232 **Tissue distribution and mRNA expression profiles of Mr-GS**

233 GS gene expression in the control group (non-stress treated prawn) was analyzed by
234 qRT-PCR. The level of the *Mr*-GS mRNA could be detected in all tested tissues,
235 organized from high to low expression levels as muscle> gills> heart> stomach> brain >
236 haemolymph (Fig 4).

237 **Fig 4. Tissue distribution analysis of *Mr*-GS in non-salinity stress of *M. rosenbergii***
238 **by qRT-PCR.** Relative expression levels of *Mr*-GS in the gill, muscle, stomach, heart,
239 brain, and hemolymph, β-actin as an internal control gene.

240 In response to hyperosmotic stress (0→13 ‰), the expression of the mRNA levels

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241 were found to be increasingly up-regulated both in the gills and muscles in a timely
242 manner. Compared to that of 0 h, the mRNA expression was significantly increased in
243 the gills with 2.54, 4.21, and 10.83 folds at 3, 6 and 12 h post of the treatment (Pt).
244 While it was also considerably increased in the muscles with 11.66, 17.97, and 45.92
245 folds at 3, 6 and 12 h Pt (Fig 5A). On the other hand, under the hypo-osmotic stress
246 (13→0 ‰), compared to that of 0 h, the mRNA expression was significantly increased
247 in the gills with 1.63, 3.30, and 3.52 folds at 3, 6 and 12 h Pt. While it was also increased
248 dramatically in the muscles with 4.06, 42.99, and 26.69 folds at 3, 6 and 12 h Pt (Fig 6
249 A).

250 **Western blotting and Immunohistochemistry analyses**

251 To further investigate the function of GS protein in osmoregulation in *M. rosenbergii*,
252 we evaluated the GS protein expression levels in gill and muscle tissues. Compared
253 with that at 0 h, the results showed that the expression of *Mr*-GS protein was
254 considerably up-regulated in both the gills and muscle at 12 h Pt (Fig 5B, 6B).
255 Furthermore, the same tissue samples were subjected to the IHC assay. The results
256 showed a similar tendency of protein expression levels with that of the western blotting
257 in both the muscle and gill tissues (Fig 5B, 6B).

258 **Fig 5. Expression levels of *Mr*-GS exposed to hyperosmotic stress.** (A) Relative
259 mRNA expression levels of *Mr*-GS exposed to hyperosmotic stress at 0, 3, 6, and 12 h
260 in the gill and muscle, with β-actin as an internal gene. These results are means ± SD.
261 *$p$<0.05, **$p$<0.01 versus control. (B) Relative expression levels of *Mr*-GS protein
262 levels exposed to hyperosmotic stress in gill and muscle, detected by western blot

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263 analysis, β-actin as control, and immunohistochemistry of gill and muscle tissues at 0
264 and 12 h. Bars: 5 μm.

265 **Fig 6. Expression levels of *Mr*-GS exposed to hypoosmotic stress.** (A) Relative
266 mRNA expression levels of *Mr*-GS exposed to hypoosmotic stress at 0 h, 3 h, 6 h and
267 12 h in the gill and muscle, with β-actin as an internal gene. These results are means ±
268 SD. *$p$<0.05, **$p$<0.01 versus control, ns = non-significant. (B) Relative expression
269 levels of *Mr*-GS protein levels exposed to hyperosmotic stress in gill and muscle,
270 detected by western blot analysis, β-actin as control, and immunohistochemistry of gill
271 and muscle tissues at 0 and 12 h. Bars: 5 μm.

272 **Changes of Gln concentration**

273 There have been shown that Gln played a crucial role in osmoregulation; therefore, we
274 analyzed the Gln concentration in the gill and muscle of the prawns. Compared to that
275 of 0 h, in response to hyperosmotic stress, Gln concentration was almost increased in
276 both gills and muscle at 3, 6 and 12 h post treatment with 0.49, 1.83, 2.02 folds, and
277 1.16, 1.41, 1.29 folds, respectively. While, under hypo-osmotic stress, Gln
278 concentration was increased in both gills and muscle at 3, 6 and 12 h post treatment
279 with 3.99, 3.40, 2.59 folds, and 1.72, 1.83, 1.80 folds respectively (Fig 7).

280 **Fig 7. Change folds of Glutamine concentrations in the *M. rosenbergii* under**
281 **hyperosmotic stress**. (A) and hypoosmotic stress (B) at 3, 6 and 12 h in the gills and
282 muscles. These results are means ± SD.

283 **Discussion**

284 In the present study, we firstly cloned the glutamine synthetase (*Mr*-GS) of *M.*

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285 *rosenbergii*, then evaluated its roles in osmoregulation under osmotic stress. There are
286 two catalytic domains, named Gln-synt_N and Gln-synt_C in *Mr*-GS, which are
287 essential for the activity of GS. Multiple sequence alignment indicated that *Mr*-GS
288 proteins were highly conserved with five conserved regions that are present from
289 invertebrates to vertebrates [18]. Up to date, there are three types of the cytosolic GS
290 gene. GS I and III located mostly in prokaryotes, and GS II was identified from
291 eukaryotes [12, 27]. In this report, *Mr*-GS belonged to the GS Ⅱ. A phylogenetic tree
292 showed that it has a close association with other crustacean groups GS gene. Besides,
293 in the present study, the GS proteins were separated into six clades including the
294 crustaceans, insecta, arachnida, merostomata, actinopterygii, and mammalian groups,
295 which was consistently correlated with the evolutionary origin of GS.

296 The mRNA transcripts of *Mr*-GS could be detected in all examined tissues and
297 indicated that GS is a widely distributed enzyme, this was also observed in other species
298 such as *Litopenaeus vannamei* [4] and *Fenneropenaeus chinensis* [12]. There has been
299 reported that more energy was required to maintain the body metabolic balance and
300 osmoregulation when the shrimps were exposed to salinity stress [28, 29]. It is also
301 stated that crustaceans increase their oxygen consumption, respiratory quotient and
302 enhance the protein catabolism rate to maintain energy consumption to resist
303 environmental stresses [18, 28, 29]. Muscle tissue is the largest storehouse of protein
304 and AA in prawns [14]. During the stress, due to the more active catabolism of protein
305 and AA, the ammonia concentration usually raised in the tissues, resulting in the
306 toxicity to the host. Therefore, the extra ammonia must be secreted to the water or

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307 converted to glutamine [18, 21]. Our results indicated that during the stress exposure,
308 *Mr*-GS expression levels had significantly increased in the gills and muscles at various
309 time points. This might relate to the conversion of ammonia which is catalyzed by GS.
310 Previous reports proposed that the ammonia formed during catalysis of AA in aquatic
311 crustacean is mainly transported to gills and excreted as free ammonia by diffusion [30,
312 31]. Increased expression of GS in the gills and digestive tissues of *C. gigas,* and the
313 protein levels of GS were significantly regulated in the muscles of *Monopterus albus*
314 when exposed to stress conditions [17, 25]. In this report, the ammonia concentration
315 in the prawn was not measured, which needs to be investigated in the future. Similar
316 studies have reported that FAA function as an essential osmoregulators in crustaceans
317 such as *Panopeus herbstii* [32], *P. monodon* [33] and *M. nipponense* [20]. In *M.*
318 *rosenbergii,* the total FAA including glycine, proline, arginine, glutamate, and alanine
319 concentrations were maintained nearly 1 mM in freshwater; however, it dramatically
320 increased up to 2.1 mM in higher salinities [19]. Gln has been regarded as one of the
321 essential osmolytes in *P. motoro* [34], *L. vannamei* [18] and *M. albus* [25]. In summary,
322 Gln not only performing as a nontoxic transporter of ammonia but also a kind of
323 abundant FAA and as a major osmolyte for crustaceans.

324 **Conclusion**

325 In conclusion, our results revealed significant evidence that *Mr*-GS could be involved
326 in coordinate osmoregulation in *M. rosenbergii* exposed to osmotic stress. Our results
327 will shed new light on the osmoregulation of crustacean.

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328 **Acknowledgments**

329 This work was jointly supported by "Innovation and Strong Universities" special fund
330 from the Department of Education of Guangdong Province (KA170500G, TK222001G,
331 KA18058B3, KA1819604). Fund from the Department of Science and Technology of
332 Guangdong Province (KA1810312), V Sarath Babu was supported by Chinese
333 Postdoctoral Science Foundation (189103).

334 **Author Contributions:**

335 **Conceived and designed the experiments:** Li Lin, Gan Pan. **Performed the**
336 **experiments:** Zhijie Lu. **Analyzed the data:** Zhendong Qin, Chengkai Ye, Jiabo Li,
337 Sarath Babu V, Guang Yang, Haiyang Shen, Guomao Su. **Writing:** Zhijie Lu, Sarath
338 Babu V, Li Lin.

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**Fig 1.**

(A)

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   1 ACGTGTGCTCTTCCGATCTGGAAGCTTTTGTGTACTTCGCATCCCAGGAGGAATTTATGCTCAAAAGGCTATCAAGatggcatatggaactaataaaaca 100
   1                                                                            M  A  Y  G  T  N  K  T    8
 101 gtcttggacagatacctgagacttgaaatccctgatcagaaatgtcaggctatgtacgtctgggtagatggtacaggagagaacctgaggtctaagacaa 200
   9V  L  D  R  Y  L  R  L  E  I  P  D  Q  K  C  Q  A  M  Y  V  W  V  D  G  T  G  E  N  L  R  S  K  T  R 42
 201 gaactctcaacttcactcccaagagccctagtgaacttcccatctggaactttgacggctcttcaactggacaagctgaaggcagtaacagtgacgtgta 300
  43  T  L  N  F  T  P  K  S  P  S  E  L  P  I  W  N  F  D  G  S  S  T  G  Q  A  E  G  S  N  S  D  V  Y  75
                                  GS-synt_N
 301 tctgcatccagttgctatctacagagatcctttcagacttggtaacaacaaactggttctctgtgaaacctacaaatacaacaagaaacccactgacacc 400
  76  L  H  P  V  A  I  Y  R  D  P  F  R  L  G  N  N  K  L  V  L  C  E  T  Y  K  Y  N  K  K  P  T  D  T  108
 401 aacaaacgatggagctgtctggaagtcatgaaggaggcggaagaacagcatccttggtttggcatggaacaagaatatacccttttggatattgacaagc 500
 109N  K  R  W  S  C  L  E  V  M  K  E  A  E  E  Q  H  P  W  F  G  M  E  Q  E  Y  T  L  L  D  I  D  K  H 142
 501 accctcttggctggcccaagaacggttacccaggtccccagggccttactactgcggtgtaggtgctagcaaagtatatggccgtgacattgttgaagc 600
 143  P  L  G  W  P  K  N  G  Y  P  G  P  Q  G  P  Y  Y  C  G  V  G  A  S  K  V  Y  G  R  D  I  V  E  A  175
 601 tcactacagagcctgcctctacactggtattaacatttctggagaaaatgctgaggtcatgcctgcccagtgggaattccaggtcggcccttgcgaaggc 700
 176  H  Y  R  A  C  L  Y  T  G  I  N  I  S  G  E  N  A  E  V  M  P  A  Q  W  E  F  Q  V  G  P  C  E  G   208
 701 atcacgatgggagatgacctctggatggccagatatctcctccatcgcgtagctgaagatttcggcgtcattgtgtcactggaccctaagcctattcctg 800
 209I  T  M  G  D  D  L  W  M  A  R  Y  L  L  H  R  V  A  E  D  F  G  V  I  V  S  L  D  P  K  P  I  P  G 242
 801 [illegible]tcgaaatcgaatctgctattgacaagatgtc 900
 243[illegible] S  T  K  A  M  R  D  P  D  G  I  V  E  I  E  S  A  I  D  K  M  S  275
                                  GS-synt_C
 901 caaggtccacgacaagcacatcagggcctatgacccccatggaggcaaggacaacgagaggcgtttgactgggctccatgaaacctcctcaattcacgac 1000
 276  K  V  H  D  K  H  I  R  A  Y  D  P  H  G  G  K  D  N  E  R  R  L  T  G  L  H  E  T  S  S  I  H  D   308
1001 ttctcagcaggtgtagccaacagaggagcgtctataagaatccctcgtggcgttgccgaagaaaggacaggctacctagaggaccgtagaccatcttcca 1100
 309F  S  A  G  V  A  N  R  G  A  S  I  R  I  P  R  G  V  A  E  E  R  T  G  Y  L  E  D  R  R  P  S  S  N 342
1101 acgccgacccctacgttgtgtctgagagactggtacgcactatctgcctgaacgagcagtagAATCGCTGCTGCATATCATCGCATATCATATCAAGGAG 1200
 343  A  D  P  Y  V  V  S  E  R  L  V  R  T  I  C  L  N  E  Q  *                                          361
1201 ATGTTCCAGCTGTCCAGAGTACAATTATTGTGTTGTTAGCTAGATATACCTGTTCATATGTTATGTGTTCTCACCGGCTATGTCTTGTCTTGGCTGTAAC 1300
1301 GTGTCCCTGATCACGACGAATCGATAAAAACAGGAAACTGCTCTGGACACACTTAAAAAAAAATAGGACTACCTTCGTGGTGCACTGATGAAGAATTAGA 1400
1401 AAATCCTGAGCCTTGCTTTTAACAGTTCGTTTTATCGTTGGCATATACTGTATGCACTGTTATCTGATCGTCACAGTTGCTTTACAGTGCAAAATTATAT 1500
1501 ATATATATATATATCCCAAAGCAAGCTCTCTTAGGTTTTGTTTTACGCAATGCACGTACATCATAAACAATGTTATTTCTGATTACGCAAATGTAAAACA 1600
1601 GTTTTCAAAAGTCAATTTCAGGATTATCTATCTTCAAGAAACACATGTTATCTTAATCTCACCCAAATGTAACATATGATTTCATCACCAACTGCTGATT 1700
1701 CTCTAGTATCGGGGTATTTATACCATTCTATGTATTGTGTTTTGTCCGCGTGAAAGACTCAAGAGACATGAAACCGTTTACTTTACAAACATATCTTAAT 1800
1801 TAAAACACTTGGCTTTCATCGCCGACATTCTTTTACACACACACACACTCCTCACCTTCTTAGCACAGTGTTTGTGGATCTTTTTTTTTATAAAAAAAAT 1900
1901 TTCTCTGTATATAAATATTTTATGATAAATATATTTCTATAATGGTAATATGAAAAAAAAAAAAA 1965
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(B)

Figures