1 # Transcriptomes and molecular pathway analysis of the liver in the
2 red-eared slider turtle *Trachemys scripta elegans* under chronic
3 salinity stress

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16 **ABSTRACT**

17 The red-eared slider (*Trachemys scripta elegans*), identified as one of the 100 most invasive
18 species in the world, is a freshwater turtle originally from the eastern United States and
19 northeastern Mexico. Field investigations have shown that *T. s. elegans* can survive and lay
20 eggs in saline habitats. In order to understand the molecular mechanisms of salinity
21 adaptation, high-throughput RNA-Seq was utilized to identify the changes in gene expression
22 profiles and their associated metabolic pathways in the liver of *T. s. elegans* in response to
23 elevated salinity. We exposed individuals to 0, 5, or 15 psu (practical salinity units) for 30
24 days. A total of 157.21 million reads were obtained and assembled into 205,138 unigenes with
25 an average length of 620 bp and N50 of 964 bp. Of these, 1,019 genes were significantly
26 differentially expressed in 0 vs 5 psu, 1,194 genes in 0 vs 15 psu and 1,180 genes in 5 vs 15
27 psu. GO analysis identified 122, 106 and 13 significantly enriched GO terms in 0 vs 5, 0 vs 15
28 and 5 vs 15 psu groups, respectively. Major GO terms and important genes related to osmotic
29 adjustment among these significant enrichments were selected in order to understand their
30 implications for salinity adaptation. KEGG analysis identified immunity-related pathways
31 involved in salinity adaptation including cell adhesion molecules, antigen processing and
32 presentation, phagosomes, hematopoietic cell lineages, and natural killer cell mediated
33 cytotoxicity. The expression patterns of nine differentially expressed genes were validated by
34 quantitative real-time PCR (qRT-PCR) yielding mean correlation coefficients of 0.743, 0.862
35 and 0.897 in 0 vs 5, 0 vs 15 and 5 vs 15 psu groups, respectively. These findings improve our
36 understanding of the underlying molecular mechanisms involved in salinity adaptation and
37 provide general guidance to illuminate the invasion potential of *T. s. elegans* into saline

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41 **INTRODUCTION**

42 The red-eared slider turtle (*Trachemys scripta elegans*) has been introduced into diverse

43 aquatic habitats worldwide (including many countries in Africa, Asia, and Europe as well as

44 Australia) via the pet-release pathway and, as a result, is classified as a highly invasive

45 species by the International Union for Conservation of Nature (*Luiselli et al., 1997; Martins*

46 *et al., 2014*). It is native to freshwater habitats in 19 states of the eastern United States and

47 two states of northeastern Mexico (*Mittermeier et al., 2015*). Recently these turtles have been

48 found to lay eggs in the low salinity (0.1-26 ‰) estuary of the Nandujiang in Hainan

49 Province, China (*Liu et al., 2011; Yang & Shi 2014*). The extent of the saltwater adaptability

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50 of *T. s. elegans* is not fully understood. Studies of endocrine stress responses by *T. s. elegans*

51 in the Lake Pontchartrain Basin of Louisiana suggest that these turtles may serve as a sentinel

52 species for elevated salinity in environments where salinity is rising due to saltwater intrusion

53 (*Thompson et al., 2011*). These studies indicate that *T. s. elegans* can invade not only fresh

54 water, but also saline water environments. Therefore, the invasion potential and the

55 mechanism of response to salinity adaptation of *T. s. elegans* is of serious concern.

56 Changing levels of salinity are a crucial environmental stress factor for aquatic species

57 that can disrupt electrolyte balance, cell energetics, and various other physiological responses,

58 including activating stress hormones (*Lushchak 2011*). Species show altered composition the

59 osmolality of body fluids in response to changing salinity (*Charmantier et al., 2011*) and

60 adaptation to salinity change typically involves the physiological solution: tolerance of

62 elevated inorganic ion concentrations (mainly sodium and chloride) in plasma (*Gordon & Tucker 1965*), and accumulation of organic osmolytes (e.g. urea) to counteract cell-volume changes. The most dramatic changes in urea concentration are seen in plasma and tissues such as skeletal muscle, resulting from the up-regulation of hepatic urea (*Wright et al., 2004*). Accumulation of intracellular free amino acids via hepatic protein degradation or de novo amino acid synthesis can also contribute to osmotic balance (*McNamara et al., 2004; Yancey 1985*). When subjected to ambient salinity change, *T. s. elegans* increased serum glucose levels, and the activities of creatine kinase (CK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) in liver (*Shu et al., 2012*). Our previous studies have shown that *T. s. elegans* can increase blood osmotic pressure by balancing the entry of sodium and chloride ions with a decrease in the secretion of aldosterone, and by accumulating plasma urea for osmoregulation when ambient salinity was lower than 15‰ (*Hong et al., 2014*). However the molecular basis of these adaptive responses has not been studied in *T. s. elegans*.

Recently, high-throughput next-generation sequencing techniques have allowed researchers to broadly explore the extent and complexity of the transcriptomes of a wide range of eukaryotic species including to gain novel information about the gene responses of aquatic species to physiological and environmental stresses (*Wang et al., 2009*). RNA-seq is an efficient technique to probe the gene responses to physiological stress (*Li et al., 2013; Smith et al., 2013; Xia et al., 2013*), particularly when working with species that do not have a sequenced genome. The present study used RNA-seq to analyze the transcriptomic response of *T. s. elegans* to salinity stress and identify the genes (and their metabolic functions) that are

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involved in salinity adaptation of turtles challenged by a brackish water environment. These results provide insights into the molecular mechanisms underlying osmoregulation in *T. s. elegans* and address the potential for this species to further invade and spread through new aquatic and brackish territories.

MATERIALS AND METHODS

Animals

Healthy *T. s. elegans* were obtained from a local turtle farm in Hainan Province, China and were acclimated in three cement pools half-filled with freshwater for two weeks. After acclimatization, nine healthy *T. s. elegans* (BW: 424-478g, 2 years old) were divided into three groups in pools (190 cm × 65 cm × 32 cm) of differing salinity: one in freshwater serving as the control (0 practical salinity units, psu), and the other two challenged with 5 ‰ (5 psu) or 15 ‰ (15 psu) saltwater. Turtles were fed a commercial diet each Monday and Thursday and 24 h after feeding, unused feed was siphoned out followed by replacement of one-third of the water in each pool. Water salinity was measured every day and adjusted to the proper salinity as needed. Other water quality parameters were monitored 2 3 times a week with steady values of pH 7.5-7.9, total ammonia nitrogen of <0.02 mg L^{-1} and temperature 26-28℃. Photoperiod was 12h:12h L:D throughout.

After 30 days of exposure to the three experimental conditions, the three turtles from each group were subjected to a 24 h fast, then anesthetized by cryo-anesthesia moving turtles to -20℃ for 0.5-1 h. Experimental animal procedures had the prior approval of the Animal Research Ethics Committee of Hainan Provincial Education Centre for Ecology and

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110 Environment, Hainan Normal University (permit no. HNECEE-2014-004). Following

111 euthanasia, the liver of each individual was sampled and divided into two sections, flash

112 frozen in liquid nitrogen, and stored at -80°C until used for RNA extraction. One liver section

113 was used for quantitative real-time PCR (qRT- PCR), and the other was mixed from each

114 group of three turtles for RNA-seq analysis.

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115 **Total RNA extraction, library construction and sequencing**

116 Extraction of total RNA from liver samples used TRIzol® Reagent, following

117 manufacturer's instructions. Total RNA purity and concentration were determined using a

118 NanoDrop 2000. The sequencing library was then constructed from high-quality RNA

119 ($OD_{260/280}$=1.8-2.2, $OD_{260/230}$≥1.5, RIN≥8.0, 28S:18S≥1.0, >10 μg). RNA-seq analysis was

120 provided by Novel Bioinformatics Co., Ltd using the Sanger/Illumina method. Subsequently,

121 cDNA libraries were made using the Hiseq4000 Truseq SBS Kit v3-HS using 5 μg total RNA,

122 following manufacturer's instructions. Poly(A) mRNA was isolated with Dyabeads (Life

123 Technologies, USA), fragmented with RNaseIII and purified. The fragmented RNA was

124 added and ligated with ion adaptor. Then double-stranded cDNA was synthesized and purified

125 using magnetic beads. The molar concentration of the purified cDNA in each cDNA library

126 was then quantified with a TBS-380 fluorometer using Picogreen. The paired-end RNA-seq

127 library was sequenced with an Illumina HiSeq 4000. The RNA-Seq data were deposited in the

128 NCBI with accession number GSE117354

129 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE117354).

130 **De novo assembly and annotation**

133 Raw reads were trimmed and quality controlled using SeqPrep (https://
134 github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) using default
135 parameters. High-quality trimmed sequences were used for sequence assembly with Trinity
136 (https://github.com/trinityrnaseq/trinityrnaseq/wiki) (*Grabherr et al., 2011*). All assembled
137 transcripts were identified by using BLASTX against the NCBI protein nonredundant (NR),
138 String, Swissprot, and KEGG databases. A typical cut-off E-value was set at $<1.0\times10^{-5}$. The
139 BLAST2GO (http://www.blast2go.com/b2ghome) program (*Conesa et al., 2005*) was used to
140 obtain gene ontology (GO) annotations of unique assembled transcripts for describing
141 biological processes, cellular components, and molecular functions. KEGG pathway analysis
142 used KEGG databases (http://www.genome.jp/kegg/)(*Kanehisa & Goto 2002*).

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143 **Analysis of differential expression and functional enrichment**

144 Expression levels of transcripts were calculated as fragments per kilobase of exon per
145 million mapped reads (FPKM). RNA-Seq by Expectation-Maximization (RSEM;
146 http://deweylab.biostat.wisc.edu/rsem/) was used to quantify gene transcripts, and edgeR
147 (http://www.bioconductor.org/packages/2.12/ bioc/html/edgeR.html) (*Robinson et al., 2009*)
148 was used to conduct differential expression analysis. For functional-enrichment analyses we
149 used GO (using GOatools https://github.com/tanghaibao/goatools) and KEGG (using KOBAS
150 http://kobas.cbi.pku.edu.cn/home.do) (*Xie et al., 2011*). Statistical analyses of enrichment
151 were carried out through Fisher's exact tests. Corrected p-values less than 0.05 for GO or
152 KEGG analyses were considered significant enrichment.

153 **Experimental validation by qRT-PCR**

154 Nine genes identified as significantly expressed from the KEGG pathways were randomly

156 selected and used for validation by qRT-PCR. Table 1 shows the specific primers used.
157 TRIzol® Reagent (Invitrogen, Carlsbad, USA) was used to extract total RNA from liver,
158 followed by reverse-transcription using First-strand cDNA Synthesis Kit (Invitrogen,
159 Carlsbad, US). The qRT-PCR for gene expression was analyzed by an Applied Biosystems
160 7500 Fast Real-Time PCR System in 96-well plates with a 20 µl reaction volume containing
161 1×SYBR Green qPCR Master Mix, gene-specific forward and reverse primers (0.4 µM) and
162 cDNA (8 ng). The cycling conditions were 95°C for 2 min followed by 40 cycles of 95°C for
163 5 s and 60°C for 30 s. *β-actin* was chosen as the reference gene, as it was expressed in the
164 three groups and unaffected under salinity stress, and relative fold changes were determined
165 using Relative Expression Software Tool v.2009 based on the cycle threshold (Ct) values
166 generated by qRT-PCR.

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167 **RESULTS**

168 **Sequencing and de novo assembly**

169 A total of 157.21 million reads and 23.58 billion bases were obtained from the liver
170 transcriptome of *T. s. elegans*, including 50.68 million in 0 psu, 57.91 million in 5 psu
171 and 48.62 million in 15 psu groups (Table 2). After filtering, trimming, and adapter
172 clipping, 152.52 million reads (97.02% of total reads) were retained including 48.92
173 million in 0 psu, 56.50 million in 5 psu and 47.10 million in 15 psu (Table 2).
174 Subsequently, 205,138 unigenes with an average length of 620 bp and N50 of 964 bp
175 were obtained by de novo assembly. The largest and smallest unigenes were 22,866 bp
176 and 201 bp, respectively (Table 3).

Annotation and differential expression of genes

BLASTX searches against five databases recovered a subset of unambiguous alignments relative to references: Pfam (15,565 read, 7.59%), KEGG (16,603 reads, 8.09%), String (12,825 reads, 6.25%), Swissprot (23,298 reads, 11.36%), and NR (35,572 reads, 17.34%) (Table 4).

In total, 3,393 genes were significantly up- or down-regulated ($FDR < 0.05$ & $\log_2|FC| \geq 1$), including 1,019 in 0 vs 5 psu, 1,194 genes in 0 vs 15 psu and 1,180 genes in 5 vs 15 psu. In the 0 vs 5 psu group 445 genes were significantly up-regulated and 574 genes were significantly down-regulated (Fig. 1A). A total of 526 genes were significantly up-regulated and 668 genes were significantly down-regulated in the 0 vs 15 psu group (Fig. 1B). Similarly, 548 genes were significantly up-regulated and 632 genes were significantly down-regulated in the 5 vs 15 psu group (Fig. 1C). Further analysis revealed a common subset of 374 genes increased and 423 decreased in both salinity treatments.

Gene Ontology

Gene Ontology (GO) annotation by BLAST2GO was used to identify differentially expressed genes. In total, 1,251, 1,064, and 388 GO terms were obtained in 0 vs 5, 0 vs 15, and 5 vs 15 psu groups, respectively. And there were 122, 106, and 13 significantly enriched GO terms in 0 vs 5, 0 vs 15, and 5 vs 15 groups, respectively. As for osmotic adjustment, there were 15 GO terms that showed significant enrichment related to osmotic function in turtle liver in the 0 vs 5 psu comparison (Table 5) and 12 in the 0 vs 15 psu comparison (Table 6). Commonly enriched GO terms in both salinity treatments were ion binding (GO:

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207 0043167), ion transmembrance transport (GO: 0034220), hydrogen ion transmembrane
208 transporter activity (GO: 0015078), hydrogen ion transmembrane transport (GO: 1902600),
209 hormone activity (GO: 0005179), monovalent inorganic cation transport (GO: 0015672),
210 anion binding (GO: 0043168), and ion transport (GO: 0006811). There were no GO terms that
211 showed significant enrichment related to osmotic function in the comparison of 5 vs 15. As
212 for the salinity stress of 5 psu, the unique GO terms were inorganic ion transmembrane
213 transport (GO:0098660), cellular amino acid metabolic process (GO:0006520), ion
214 transmembrane transporter activity (GO:0015075), nitrogen compound metabolic process
215 (GO:0006807), inorganic cation transmembrane transport (GO:0098662), inorganic cation
216 transmembrane transporter activity (GO:0022890), and cation transmembrane transport
217 (GO:0098655). However, the unique GO terms in the 15 psu group were iron ion binding
218 (GO:0005506), calcium ion binding (GO:0005509), proton transport (GO:0015992),
219 hydrogen transport (GO:0006818), transmembrane transport (GO:0055085), metal ion
220 binding (GO:0046872), cation binding (GO:0043169), and transaminase activity
221 (GO:0008483).

222 Based on the enrichment analysis, a list of candidate genes involved in salinity acclimation
223 in *T. s. elegans* were identified. Many of these were identified as encoding proteins involved
224 in ion transport, hormone regulation and energy metabolism: *TAT* (tyrosine aminotransferase),
225 *STK* (serine/threonine-protein kinase), *SIK* (salt inducible kinase), *APOE* (apolipoprotein E),
226 *glnA/GLUL* (glutamine synthetase), *NPPA* (natriuretic peptide A), *ACDC* (adiponectin),
227 *SCN1B* (voltage-gated sodium channel type I beta), *KCNH5* (potassium voltage-
228 gated channel Eag-related subfamily H member 5), *INSRR* (insulin receptor-related receptor),

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231 *GCK* (glucokinase), *CYP17A* (steroid 17 alpha-monooxygenase /17 alpha-

232 hydroxyprogesterone deacetylase), *ASS1* (argininosuccinate synthase), *GST* (glutathione S-

233 transferase), and *OAZ3* (ornithine decarboxylase antizyme 3). The fold-change of these

234 important genes in relation to osmotic adjustment in these three groups are shown in Table 7.

235 Compared to the control, the genes that increased in both salinity groups included: *STK*, *SIK*,

236 *APOE*, *GLUL*, *ACDC*, *GCK*, and *OAZ3*. However, *TAT*, *NPPA*, *SCN1B*, *CYP17A*, *GST*, and

237 *ASS1* decreased in both salinity groups.

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238 **KEGG pathway**

239 Differentially expressed genes (including up &down regulated genes) of these three

240 groups identified via the KEGG database fell into five branches of cellular systems: (a)

241 metabolism, (b) genetic information processing, (c) environmental information

242 processing, (d) cellular processes, and (e) organismal systems (Fig. 2). Within the

243 organismal systems branch, a subset of genes were involved in categories of metabolism

244 of other amino acids (111 genes), lipid metabolism (577 genes), glycan biosynthesis and

245 metabolism (349 genes), energy metabolism (532 genes), carbohydrate metabolism (468

246 genes), and amino acid metabolism (596 genes). In addition, a subset of genes, were

247 involved in signal transduction (2,171 genes), transport and catabolism (1,012 genes),

248 endocrine system (1,089 genes), and immune system (1,159 genes).

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249 A total of 130, 156, and 128 pathways were identified from KEGG in the comparisons of 0

250 vs 5, 0 vs 15, and 5 vs 15 psu, respectively. However, only four, two, and six pathways related

251 to immunity were significantly enriched in the comparisons of 0 vs 5, 0 vs 15, and 5 vs 15

252 psu, respectively (corrected $P < 0.05$, Table 8). The immunity-related pathways found were

261 cell adhesion molecules (CAMs), phagosome, and antigen processing and presentation in the
262 0 vs 5 psu group, and hematopoietic cell lineage in the 0 vs 15 psu group, as well as natural
263 killer cell mediated cytotoxicity with the expression of many genes decreased in both groups
264 (Table S1 and S2). The enrichment in the other pathways was not significant although the
265 expression of some genes were significantly expressed.

266 **qRT-PCR to validate RNA-seq**

267 Nine genes were randomly selected for qRT-PCR analysis in order to validate the
268 differentially expressed genes that were identified by RNA-Seq and gain detailed quantitative
269 information on their differing expression patterns. All genes tested revealed a single product
270 as assessed by melting-curve analysis and, in all cases, fold changes from qRT-PCR
271 corresponded to the RNA-seq expression results (Fig. 3). The qRT-PCR results were
272 significantly correlated with the RNA-seq results with correlation coefficients of 0.743 in 0 vs
273 5 psu, 0.862 in 0 vs 15 psu groups, and 0.897 in 5 vs 15 psu groups ($p < 0.05$). This provides
274 strong evidence that RNA-Seq data can be a reliable indicator of the expression patterns of
275 the hundreds of genes identified as differentially expressed in the current study.

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276 **DISCUSSION**

277 Osmoregulation in some aquatic animals can be a complex process because individuals
278 must deal with fluctuating salinity levels in their natural habitats, often on a daily or seasonal
279 basis. As a normally freshwater species, the red-eared slider *T. s. elegans*, does not possess
280 salt glands, and requires osmoregulation to survive when entering environments of higher
281 salinity (e.g. brackish or estuarine water). The physiological research has shown that *T. s.*
282 *elegans* can increase blood osmotic pressure by balancing the entry of NaCl with the

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288 decreased secretion of aldosterone, and accumulating urea and free amino acids in blood

289 (*Hong et al. 2014*). Results of GO enrichment analysis in the study showed that ion binding,

290 ion transmembrance transport, hydrogen ion transmembrane transporter activity, hydrogen ion

291 transmembrane transport, hormone activity, monovalent inorganic cation transport, anion

292 binding, and ion transport were significantly differentiated, which provide a strong evidence

293 for the physiological mechanism.

294 Under salinity stress, loss of water from the cells can cause cells shrink and potentially die.

295 In this sense, ion regulation is important for a cell to balance osmotic change. *KCNH5* (www.

296 ncbi.nlm.nih.gov/gene/27133) and *SCN1B* (*Qin et al., 2003*) are involved in

297 potassium/sodium voltage-gated ion channels and fluid balance that controls arterial blood

298 pressure by altering blood electrolyte composition and/or volume. Natriuretic peptide A

299 (*NPPA*) is well known to regulate body fluid levels and electrolytic homeostasis and has

300 natriuretic, diuretic, and vasodilatory actions (*Espiner et al., 2014*). NPPA is highly expressed

301 and associated with H_2O/Na^+ absorption and protein Ser/Thr phosphatases (*Espiner et al.*

302 *2014*). The salt inducible kinase (*SIK*) acts to modulate adrenocortical function particularly in

303 response to high plasma Na^+, K^+, ACTH, or stress (*Wang et al., 1999*). *SIK1* also has a role in

304 steroidogenesis whereas *SIK2* is implicated in gluconeogenesis regulation in liver; both

305 belong to the AMPK (AMP-activated kinase) subfamily of serine/threonine kinases

306 (*Berggreen et al., 2012*). The AMPK is a crucial regulator of cellular energy levels (*Hardie &*

307 *Ashford 2014*) and under stress conditions that deplete ATP, AMPK action promotes ATP-

308 producing catabolic pathways while inhibiting ATP-consuming anabolic functions (*Rider et*

309 *al., 2009; Rider et al., 2006*). Transcripts of adiponectin (*ACDC*) were also enriched in liver

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312 under both salinity stresses and this hormone participates in the pathway of fatty acid
313 oxidation by regulating AMPK (*Chong et al., 2013*).
314 Genes associated with transporting molecules related to metabolic processes were also
315 modulated in association with an up regulation of genes involved in ATP energy production.
316 As salinity level increases, acclimation of *T. s. elegans* to elevated salinity conditions requires
317 investment of energy to maintain their homeostatic balance (*Hong et al. 2014*). It is also
318 documented that changes in components related to the glycolysis, fatty acid metabolism, and
319 ATP production are often associated with raised salinity conditions in freshwater fish (*Lavado*
320 *et al., 2014; Tine et al., 2008*). In our study, the differentially expressed genes including
321 *ATP6*, *MTATP6*, and *ATPeFOA* were enriched, which are associated with the ATP needed for
322 the synthesis and operation of transport-related proteins that drive ion- and osmoregulatory
323 processes (*Lee et al., 2003*). The patterns of differently expressed genes suggest a requirement
324 for more energy by *T. s. elegans* during raised salinity conditions.
325 The ureogenesis is a strategy for diamondback terrapins (*Malaclemys terrapin*) that are
326 known to inhabit brackish water, and for the desert tortoise, *Gopherus agassizii* under
327 dehydration stress (*Dantzler & Schmidt-Nielsen 1966*). The red-eared slider turtle, *T. s.*
328 *elegans*, can also increase urea content in plasma and tissues by synthesizing organic
329 osmolytes (e.g. free amino acids and urea) in order to provide a colligative defense against
330 water loss (*Hong et al. 2014*). In this study, the genes involved in categories of metabolism of
331 other amino acids (111 genes) and amino acid metabolism (596 genes) were differentially
332 expressed, which seem to be logical categories with respect to osmolytes. Some of the
333 differentially expressed genes were associated with cellular amino acid metabolism including

334 *GLUL* (glutamine synthetase), *OAZ3* (ornithine decarboxylase antizyme 3), *STK33*

335 (serine/threonine kinase), and *GST* (glutathione S-transferase). Glutamine is the most

336 prevalent amino acid in body fluids and muscle, is mainly transported by a Na^+-dependent

337 neutral amino acid system, and its turnover rate exceeds those of other amino acids (*Zander et*

338 *al., 2015*). Glutamine is synthesized from the ATP-dependent conjugation of ammonia to

339 glutamate, and is a well-known defense against ammonia (*Cooper & Plum 1987; Essexfraser*

340 *et al., 2005*). When salinity returns to normal, glutamine pools can be utilized as precursors

341 for a variety of important cell molecules (e.g. purines, pyrimidines, mucopolysaccharides) or,

342 in the presence of glutaminase, glutamine can be deaminated for direct excretion of ammonia

343 in the kidney or used for urea synthesis in liver before excretion. The accumulation of urea in

344 response to high salinity can be due to both urea retention and elevated rates of urea synthesis

345 via the ornithine-urea cycle, uricolysis of uric acid, or hydrolysis of arginine (*Dépêche &*

346 *Schoffeniels 1975; Gordon & Tucker 1965*). *OAZ3*, involved in the ornithine-urea cycle, was

347 identified to be differentially expressed under salinity exposure in this study, which suggested

348 that ureogenesis may also be activated in response to hyperosmotic conditions in this species.

349 Our result correlated well with studies in crab-eating frog (*Fejervarya cancrivora*), where its

350 resistance to hyperosmotic environmental conditions is generally linked to the accumulation

351 of urea in plasma and tissues, urea resulting from an up-regulation of the hepatic urea

352 synthesis machinery (*Gordon & Tucker 1965*). Also, the striped catfish (*Pangasianodon*

353 *hypophthalmus*) can counteract osmotic imbalance by triggering a regulatory volume

354 increase, an internal process that initiates a net gain in osmolytes and/or water, increasing cell

355 volume that re-establishes normal values and prevents further cell shrinkage (*Nguyen et al.,*

356 *2016*).

357 The current study was designed not only to generate a catalogue of differentially
358 expressed genes involved with salinity exposure, but also to allow the data to be integrated to
359 identify the relationships on the adaptive response. Based on gene ontology information and
360 from data in published studies, functional categories of differentially expressed genes were
361 identified. Therefore, the potential interactions of the differentially expressed genes that
362 responded to high salinity in *T. s. elegans* are shown in Figure. 4. The pathways and processes
363 that are targeted provide us with numerous candidate genes for future investigations about the
364 molecular mechanisms that underlie high salinity tolerance.

365 Interestingly, only the pathways related immunity were significantly enriched when the
366 data was assessed by KEGG analysis, while the other pathways were not significantly
367 enriched although the expression of some genes were significantly expressed. Compared with
368 other species under stress, many KEGG pathways were enriched significantly. When the
369 zebrafish (*Danio rerio*) was infected with spring viremia of carp virus, 26 and 52 pathways in
370 KEGG were significantly changed in brain and spleen (*Gao et al., 2017*). The result of a very
371 few significantly enriched pathways in the study of transcriptome responses may be evidence
372 of salinity adaptation in *T. s. elegans* under 15 psu salinity for one month.

373 When *T. s. elegans* was subjected to salinity stress, the major histocompatibility complex
374 class (MHC) genes *MHC-I* and *MHC-II* were both differentially expressed. The cell surface
375 glycoprotein MHC-I is expressed in most nucleated cells and has a key role in the regulation
376 of the cytotoxic effector functions of T cells and natural killer (NK) cells (*Höglund & Brodin*
377 *2010*). NK cells can distinguish target cells that differ only in their expression of MHC I

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380 molecules (*Ulianich et al., 2011*), and can also have regulatory roles; for example, they are

381 significant early producers of interferon-γ (IFNγ), which can influence T-cell responses

382 (*Martín-Fontecha et al., 2011*). The MHC II pathway activates CD 4+ helper T cells through

383 the recognition of antigens on the surface of the professional antigen-presenting cells (*Xiang*

384 *et al., 2005*), which stimulate the proliferation and differentiation of T cells and B

385 lymphocytic cells, and activate macrophages (*Robinson & Delvig 2002*). The pathway of

386 natural killer cell mediated cytotoxicity was differentially expressed with more genes

387 decreased in both 0 vs 5 and 0 vs 15 psu, which indicated that they are more susceptible to

388 bacterial challenges in higher salinity conditions. The finding of immunity weakened might

389 restrict the ability of *T. s. elegans* to fully adapt as an invasive species in saline environments.

390 More field investigations of survival in saline environments in *T. s. elegans* need to be

391 conducted.

392 **CONCLUSIONS**

393 In this study, we report the first transcriptome analysis of *T. s. elegans* under salinity stress.

394 Significant enrichment of gene expression in response to elevated salinity included transcripts

395 of proteins involved in ion binding, hydrogen ion transmembrane transports, hormone

396 activity, monovalent inorganic cation transport, anion binding, and ion transport. In addition,

397 altered expression of gene transcripts related to osmotic adjustment, metabolism, signaling,

398 cell activities and immunity also occurred. Immunity-related genes identified in *T. s. elegans*

399 under salinity stress included cell adhesion molecules (CAMs), as well as genes involved in

400 functions including hematopoietic cell lineage, antigen processing and presentation,

401 phagosome, and natural killer cell mediated cytotoxicity. Finally, we combined the data on

functional salinity tolerance genes into a hypothetical schematic model that describe potential relationships and interactions among target genes to explain the molecular pathways related to salinity responses in *T. s. elegans*. Our results also indicate that the invasion potential of this widely-introduced species exhibit salinity adaptation by means of characteristic gene expression patterns especially the ureogenesis related to amino acid metabolism, and a very few significantly enriched pathways KEGG pathway.

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Table captions

Table 1 Sequences of primers for qRT-PCR validation

Table 2 Summary of Illumina expression short reads production and filtering

Table 3 Summary of *de novo* assembly results of illumina sequence data

Table 4 The functional annotation of unigenes

Table 5 Significant enrichment of GO terms related to osmotic regulation in 0 vs 5 psu groups

Table 6 Significant enrichment of GO terms related to osmotic regulation in 0 vs 15 psu groups

Table 7 The change fold of some important genes in relation to osmotic adjustment in these significantly enriched GO terms

Table 8 Differentiation of pathway analysis in the liver of *T. s. elegans* in 0 vs 5 psu, 0 vs 15 psu and 5 vs 15 psu groups

425

426 **Figure caption**

427 **Figure 1 Visualization of differentially expressed genes in liver from turtles exposed to 0**

428 **vs 5 psu (A) , 0 vs 15 psu (B) and 5 vs 15 psu (C).** The X axis shows differential fold

429 changes of genes (log_2 FC) and the Y axis shows -log P value for the differentially expressed

430 genes. Red points show the genes with markedly significant up-regulation, orange points

431 indicate significant up-regulation, dark blue points indicate markedly significant down-

432 regulation, light blue points indicate significant down-regulation, and black points show no

433 significant difference.

434

435 **Figure 2 KEGG annotation.** KEGG metabolic pathways of differentially expressed genes

436 from three groups can be divided into five branches: (A, orange) Metabolism, (B, yellow)

437 Genetic Information Processing, (C, green) Environmental Information Processing, (D, blue)

438 Cellular Processes, (E, purple) Organismal Systems. The vertical axis lists the KEGG

439 metabolic pathways and the horizontal axis shows the percentage of total genes in each

440 category. The number of genes in each category is listed to the right of each bar.

441

442 **Figure 3 Comparison of relative fold changes between RNA-seq and qRT-PCR results in**

443 ***T. s. elegans.* (A) 0 vs 5 psu; (B) 0 vs 15 psu; (C) 5 vs 15 psu.** Fold changes are expressed as

444 the ratio of gene expression after salinity challenge to the control group as normalized with *β-*

445 *actin* gene.

446

447 **Figure 4 Potential interactions of positively selected genes and differentially expressed**

448 **genes involved in the response of *T. s. elegans* liver to high salinity.** (1) *NPPA* plays roles in

449 the regulation of body fluid levels and electrolytic homeostasis pathways, whereas *ADPN* and

450 *SIK* are in the pathway of lipid (glucose) metabolism under regulation by AMPK; (2) *GLUL*

451 (glnA), *ASS1*, *STK33* and *OAZ3* play roles in the accumulation of free amino acids and urea,

452 while *KCNH5* and *SCN1B* are involved mainly in the process of H_2O/Na^+ absorption; (3)

453 *ATP6*, *MTATP6* and *ATPeFOA* are v-type-H+ ATPases and participate in Na+ uptake as

454 energy production.

455 Note: *NPPA* (natriuretic peptide A), ADPN (adiponectin), *SIK* (salt inducible kinase), AMPK

456 (AMP-activated kinase), *GLUL* (glutamine synthetase), *ASS1* (argininosuccinate synthase),

457 *STK* (serine/threonine-protein kinase), *OAZ3* (ornithine decarboxylase antizyme 3), *KCNH5*

458 (potassium voltage-gated channel eag-related subfamily H member 5), *SCN1B* (voltage-gated

459 sodium channel type I beta).

460

461 **Supplemental Information**

462 **Table S1** Key genes of differentially expressed of KEGG pathway analysis following low-

463 salinity change (0 vs 5 psu)

464 **Table S2** Key genes of differentially expressed of KEGG pathway analysis following high-

465 salinity change (0 vs 15 psu)

466

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