1 **Comparison of resting egg gene expression with different hatchability related to**

2 **salinity variations in the marine rotifer *Brachionus manjavacas***

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22

23 **Abstract** Salinity is a significant factor to affect resting egg hatching in the euryhaline rotifers. In
24 order to clarify the effects of salinity on resting egg hatchability, this study investigated gene
25 expressions of resting eggs subjected two different incubation salinities (at 17 and 33 ppt) in the rotifer
26 *Brachionus manjavacas*. The resting eggs formed at 17 ppt showed higher hatching rate at 17 ppt of
27 incubation salinity, compared to those at 33 ppt. Related to these circumstances, the resting eggs
28 incubated at 17 ppt expressed genes which have putative functions implying cellular differentiation and
29 embryonic development: late embryogenesis abundant protein (LEAs-1), α-amylase, and deaminase.
30 The resting eggs incubated at 33 ppt highly expressed the genes related to the environmental stresses:
31 AP2 transcription factors (AP2TF), and ATP decomposition: ABC transporter permease (ABC-TP),
32 NAD+ synthase, Copper-translocating P-type ATPase (CTP-ATPase). It is expected the resting eggs
33 incubated at 33 ppt may need a greater energy (ATP) to endure saline stress during incubation. The
34 obtained results indicated that the resting eggs regulate their hatching with the mechanisms of energy
35 allocation between embryo development and self-defense against environmental conditions like salinity
36 stresses.
37
38 **Keywords** Rotifera ・ Resting eggs ・ Salinity ・ Hatchability ・ Gene expression
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45 **Introduction**

46

47 Rotifer resting eggs are the final product of sexual reproduction, and can endure harsh environmental
48 conditions during diapause period. After a certain diapause period, the eggs hatch into amictic
49 females. It is generally known that exogenous (e.g., temperature, salinity, food) and endogenous factors
50 (e.g., hereditary, generation, aging) (Snell 1987; Hagiwara and Hino 1990; Gilbert 2003; Kim and
51 Hagiwara 2011a) regulate the quality and quantity of resting eggs (John 2016). Among the several
52 exogenous factors, salinity can modulate the mixis induction. Lubzens et al. (1980) found that mixis
53 did not occur in 100% seawater (salinity ca. 38‰ at 30 °C) in the rotifer *Brachionus plicatilis*.
54 However, when it was transferred to 50% or 25% seawater, mixis occurred with a high rate. Moreover,
55 salinity also affects the resting egg hatching. The hatchability of resting eggs can be modified with
56 saline conditions during formation and hatching. Resting eggs showed higher hatchability when the
57 eggs were incubated at the same salinity as during formation (Kim and Hagiwara 2011b), and the
58 resting eggs formed at lower salinity showed low hatchability with incubation at higher salinity
59 (Pourriot and Snell 1983).

60 Rotifers have sensitive molecular systems that trigger the expression of cellular defense and
61 homeostasis genes in response to environmental stressors (e.g., temperature, salinity, UV radiation)
62 (Kim et al. 2011, 2013; Kim et al. 2014a, b; Rhee et al. 2011; Wheelock et al. 1999). The unsuitable
63 salinity conditions affect the reproduction and distribution of hydrosphere organisms like rotifers
64 (Chinnery and Williams 2004), and stressful conditions increase energy demand required for
65 osmoregulation (Devreker et al. 2009). Hence, this process may decrease energy allocated to other
66 biological processes in a limited energy pool (Michalec et al. 2010). On the other hand, the molecular

67 correlation between salinity-induced stress and defense/tolerance mechanism in rotifer resting eggs
68 have not been elucidated so far.
69 The resting eggs under different salinity conditions have possibility to show different gene
70 expression related to energy allocation between osmoregulation and embryo development of rotifer
71 resting eggs in the euryhaline rotifer *B. manjavacas*. This study firstly observed the resting egg
72 hatchability under different incubation salinity conditions (17 and 33 ppt). Secondly, the gene
73 expression was analyzed in relation to the observed phenomena.
74
75
76 **Materials and methods**
77
78 **Resting egg production and hatching**
79
80 This study employed the L-type rotifer *Brachionus manjavacas* (Australian strain) which maintained in
81 Aquaculture biology laboratory (Nagasaki University, Japan) over a decade at 22 ppt (part per thousand)
82 under room temperature. The experimental scheme is described in Figure 1. For the mass production of
83 resting eggs, the rotifers were cultured in 30 l of 17 ppt artificial seawater with gentle aeration at 25 °C
84 with daily feeding of the commercial *Chlorella vulgaris* (Super Fresh Chlorella V-12, Chlorella
85 Industry Co., LTD, Fukuoka, Japan) for 12 days. Resting eggs were harvested on the last day of culture
86 and then preserved at 4 °C in total darkness for two weeks. After diapause period, the hatching rate of
87 the preserved resting eggs was confirmed with the following methods. Twenty resting eggs were
88 randomly selected and transferred into a well of 6-well microplate, and incubated at 25 °C under two

89 different photoperiods i.e., 4 and 24 h using 3000-lux fluorescent light. The incubation salinity was
90 adjusted to 17 ppt for control and to 33 ppt for experimental groups. To compare their hatchability, a
91 mean of five replicates (*n* = 5) was calculated under each salinity and photoperiod.
92
93 **RNA extraction and cDNA synthesis**
94
95 The preserved resting eggs (about 20,000 eggs) were transferred into 500 ml glass beakers containing
96 200 ml of either 17 or 33 ppt of sterilized seawater. These eggs were incubated at 25 °C under two
97 different photoperiods (4 and 24 h). The total RNA was extracted with ISOGEN (Nippon gene, Tokyo,
98 Japan) according to the manufacturer's protocol. Subsequently, genomic DNA was removed using
99 TURBO DNA-free™ (Ambion®, Carlsbad, CA, USA) and then cDNA was synthesized using
100 Advantage® RT-for PCR kit (Clontech, Tokyo, Japan). The composed cDNA was preserved at -20 °C
101 until use.
102
103 **Differential display reverse transcription-PCR (DDRT-PCR)**
104
105 DDRT-PCR was performed using the Delta™ Differential Display Kit (Clontech, Tokyo, Japan)
106 according to the supplied protocol. The PCR cycle included one cycle at 94 °C for 5 min, 40 °C for 5
107 min, 68 °C for 5 min; two cycles at 94 °C for 30 s, 40 °C for 30 s, and 68 °C for 5 min; 30 cycles at
108 94 °C for 20 s, 60 °C for 30 s, 68 °C for 2 min; and one cycle at 68 °C for 7 min. The 30 cycles of
109 synthesis were carried out with an annealing temperature of 60 °C. With the PCR products,
110 electrophoresis was performed using the high resolution agarose gel (1.2%, Metaphor® Agarose,

111 Cambrex, Rockland, USA) followed by staining with SYBR Green (Molecular Probes Inc., Invitrogen,
112 Carlsbad, CA, USA) for 30 min. Using the photographic data, the gene expressions with salinity
113 treatments were compared, and differentially expressed gene fragments were excised from the gels and
114 preserved at -20 °C. The selected gene fragments were purified and re-amplified with the same
115 methods as the prior amplification. The statement of PCR products was confirmed with electrophoresis
116 using 1.8% of agarose gel (Agarose-LE, Classic type, nacalai tesque, Kyoto, Japan) and staining with
117 ethidium bromide (0.5 μg/ml). The target fragments were excised from the gels and purified again
118 using Wizard® SV Gel and the PCR Clean-Up System (Promega, Tokyo, Japan) according to the
119 technical manual. The extracts of purified cDNA fragments were kept at -20 °C.
120
121 **TA-cloning and sequencing**
122
123 Purified cDNA fragments were cloned using pGEM®-T and pGEM®-T Easy Vector Systems (Promega,
124 Medison, WI, USA). Blue/white selection were used for screening the recombinant. Eight white
125 colonies were selected per a cDNA fragment. Colony direct PCR was performed as follow conditions:
126 1 μl of template, 0.5 ul of M13 forward and reverse primer (10 pmol/μl), 0.5 ul of dNTP, 0.2 ul of
127 Ex-Taq (Takara Bio, Japan), 2 ul of Ex-Taq buffer (10×), in a total volume of 20 μl. PCR cycling:
128 95 °C for 5 min; 35 cycles of 95 °C for 10 s, 58 °C for 10 s, 72 °C for 30 s; and 72 °C for 7 min. The
129 sequencing of target fragments was handled by biology company (MAP BIOTHCH, Shanghai).
130
131 **Gene annotation**
132

133 The sequences were compared with other sequences in National Center for Biotechnology Information

134 (NCBI) Gene Bank database using BLASTX at NCBI server (http://blast.st-va.Ncbi. nlm.nih.gov /

135 Blast.cgi?PROGRAM=blastx&PAGE_TYPE = BlastSearch & LINK_ LOC = blastblas) (Table 1).

136 Using the highest score (>50) and E value (<1.0×e-18), it was inferred that the relevant gene function

137 associated with experiment conditions.

138

139 **Real-Time PCR**

140

141 The real-time PCR was used to validate the mRNA levels of certain genes from DD-PCR. Reaction

142 conditions as follows: 1 µl of cDNA template, 0.5 µl of forward and reverse primer (10 pmol/µl) for

143 each gene, 10 µl of SYBR Premix Ex Taq (2×) (Takara Bio, Japan), 0.4 µl of Rox Reference Dye II

144 (50×), in a total volume of 20 µl. Thermal cycling: 94 °C for 4 min; 39 cycles of 94 °C for 30 s, 55 °C

145 for 30 s, 72 °C for 30 s; and 72 °C for 10 min. With the CFX96 real-time PCR System (Bio-Rad, USA).

146 The 18S rRNA gene which is a stable housekeeping gene (Kim et al. 2015) was used for a reference

147 gene to normalize the transcript level. The $2^{-\Delta\Delta Ct}$ method was applied to calculate each transcriptional

148 level (Livak and Schmittgen 2001).

149

150 **Statistical analysis**

151

152 The effects of salinity and photoperiod on resting egg hatching rate were analyzed by two-way ANOVA

153 followed by Tukey HSD test. Significant differences in the gene expression levels with different

154 salinities and photoperiods were analyzed with one-way ANOVA followed by Tukey HSD. These

155 statistical analyses were conducted with SPSS version 17.0 (SPSS Inc, IL, USA).

156

157

158 **Results**

159

160 **Hatching rate of resting eggs under the different salinities**

161

162 The hatching rates of resting eggs were affected by salinity (two-way ANOVA, $P < 0.001$) and
163 photoperiod ($P < 0.001$). With the 4 h illumination, the resting eggs incubated at 17 ppt (control)
164 showed higher hatching rate (44.0±14.7%) than those at 33 ppt (7.0±5.7%). The same pattern was
165 observed with 24 h illumination: the higher hatching rate at 17 ppt (60.0±6.1%) compared to at 33 ppt
166 (34.0±5.5%) (Fig. 2).

167

168 **Transcriptional expression analysis of DDRT-PCR**

169

170 We detected 42 differentially expressed gene fragments associated with the incubation conditions,
171 although, the BLASTX results only include 15 putative functional genes (Table1). The resting eggs at
172 17 ppt under 4-h photoperiod expressed late embryogenesis abundant protein 1 (LEAs-1),
173 alpha-amylase (α-amylase) and deaminase. The transcripts for regulatory proteins (Histidine kinase,
174 HK/Histidine kinase regulator, HHKR) and cellular component proteins (TSP1 and CBM14 domain
175 containing protein, TCcP; SRY-related HMG box C protein, SRY-HCP) were observed in the resting
176 eggs incubated at 17 ppt with 24-h continuous light. These proteins have functions such as signal

177 transduction (Wolanin et al. 2002), cellular differentiation and metabolic processes including anabolism
178 and catabolism for the embryonic development. The resting eggs incubated at 33 ppt showed gene
179 expressions related to energy metabolism regardless of photoperiod; i.e., ABC transporter permease
180 (ABC-TP), NAD+ synthase, Copper-translocating P-type ATPase (CTP-ATPase), 3-oxoacid
181 CoA-transferase subunit B (3-OCoAT) and NADH dehydrogenase subunit 5 (NADHDnase),
182 Transcription factor with AP2 domains (AP2TF).
183
184 **Transcriptional validation by real-time PCR**
185
186 Using the real-time PCR, we tested expression dynamics of 15 target genes. These target genes showed
187 significant differences associated with the incubation salinities and photoperiods. The resting eggs at 17
188 ppt up-regulated genes related to embryo development and biological metabolism, such as LEAs-1,
189 α-amylase, deaminase, HK and SRY-HCP expressed (Fig. 3a). However, the higher salinity 33 ppt
190 induced up-regulation of ATPOE, ABC-TP, ECoAH, NAD+ synthase, CTP-ATPase, 3-OCoAT and
191 AP2TF which involved in the process of energy transportation and environmental stresses (Fig. 3b).
192
193
194 **Discussion**
195
196 Rotifers and its resting eggs have sensitive molecular systems of cellular defense and homeostasis
197 related to environmental stressors e.g., temperature, UV radiation, salinity, toxicants (Kim et al. 2013;
198 Kim et al. 2014a, b; Rhee et al. 2011; Wheelock et al. 1999; Clark et al. 2012). On the other hand, the

199 molecular defense to environmental stress has not been clarified so far. Recently, euryhaline rotifer
200 *Brachionus plicatilis* is justified as an osmoregulator and their stress level increased at high salinities
201 with the activity of Na^{+}/K^{+} ATPase (Lowe et al. 2005). In this study, we observed different hatching
202 rate of resting eggs related to incubation salinity (Fig. 2). Even under the same photoperiod, the
203 employed resting eggs showed the different hatching rate related to the incubation salinity. The higher
204 incubation salinity (33 ppt) induced the lower hatching rates. This circumstance proposed that the
205 resting eggs may have the same molecular system as the rotifer individuals own.

206 The resting eggs incubated at 17 ppt for 4 h lighting showed the active embryonic development
207 which can be explained by the following gene expressions (Table 1, Fig 3a). Highly-expressed genes at
208 17 ppt LEAs-1, α-amylase and deaminase are discovered in the late stages of plant seed embryonic
209 development (Galau et al. 1986), and have a function like desiccation tolerance, nutritional synthesis
210 and decomposition (Berjak et al. 2007). According to the former research, the LEAs are the only
211 desiccation tolerant proteins identified in *B. plicatilis* (Kim et al. 2011). LEAs are a member of highly
212 hydrophilic proteins which act as a "molecular shield" playing a role in anti-aggregation and protein
213 stabilization (Tunnacliffe et al. 2010). The α-amylase is a protein enzyme that hydrolyses
214 polysaccharides, such as starch and glycogen, yielding glucose and maltose (Stedman's 2000). These
215 sugars could be used as a primary energy source for the embryonic development, prior to lipid
216 utilization from the lipid bodies. Deaminase is an enzyme encoded by the CDA gene in humans (Kuhn
217 et al. 1993; Demontis et al. 1999) and involved in the regulation of 5-FU metabolism, which is related
218 to the DNA repair. In the initial period of incubation with light, resting eggs expressed environmental
219 tolerance genes to prepare sudden changes of environment. The previous study (Kim et al. 2015)
220 showed that during 4 h of illumination, a lot of genes associated with light stimulation and embryonic

221 development were expressed, and the similar expression pattern was observed at 17 ppt of incubation
222 salinity in this study. On the other hand, the resting eggs at 33 ppt showed significantly low expression
223 level of those genes (Fig. 3a). It is possible that the high salinity repressed expression of the indicated
224 genes, resulting in delayed development. On the other hand, the salinity may cause the delayed
225 development, and the changed gene expression patterns may be the result of the delay. These
226 circumstances exhibit possibility that higher salinity also plays as an inhibitor of resting egg hatching.

227 The delayed embryonic development related to environmental stresses were observed with the
228 resting eggs incubated at 33 ppt under 24 h of illumination (Table 1, Fig. 3b). Enoyl-CoA hydratase
229 (ECoAH) and 3-oxoacid CoA-transferase subunit B (3-OCoAT) are important for fatty acid
230 metabolism (Bahnson et al. 2002; Gautam and Liu 2003). Acyl-CoA dehydrogenase (ACAD) can
231 destroy the long fatty acid chains into acetyl CoA through catalyzes the fatty acid β-oxidation (Thorpe
232 and Kim 1995). Fatty acid oxidation was suggested to be involved in the resting egg hatching progress
233 (Hagiwara et al. 1995). The previous study (Kim et al. 2015) elucidated that the genes related to
234 hatching procedure are highly expressed with 30 min photoperiod. Therefore, these phenomena also
235 exhibit embryonic development delayed with the incubation at higher salinity (33 ppt). ABC
236 transporter permease (ABC-TP), NAD+ synthase, and Copper-translocating P-type ATPase
237 (CTP-ATPase) are expressed in the process of ATP decomposition (Inesi et al. 2014). The mitochondria
238 can produce cellular energy (ATP) for cellular metabolism, signaling transduction, and growth (Henze
239 and Martin 2003; McBride et al. 2006). Moreover, AP2 transcription factors (AP2TF) which showed
240 significantly higher expression level at 33 ppt in the 24 h incubation are activated in response to
241 environmental stresses such as high salinity (Abogadallah et al. 2011), heat (Sakuma et al. 2006),
242 freezing (Yang et al. 2005), osmotic stress (Zhu et al. 2010). This suggests that resting eggs under 33

243 ppt need a greater amount of energy to endure salinity stress because of the function of intracellular
244 lipid chaperone, AP2 involved in the energy metabolism (Cao et al. 2013). For the osmotic homeostasis,
245 there are a lesser amount of available energy to be used for cellular and embryonic development which
246 is the reason for lower hatchability of these resting eggs.

247 These expressed genes related to incubation salinity and photoperiod elucidate that the homeostatic
248 mechanism under saline stress. At the higher salinity, resting eggs allocate more energy for homeostasis
249 than for embryonic development. While resting eggs are incubated at the optimal salinity for hatching,
250 the eggs concentrate on the embryonic development. The obtained results figured out that rotifer
251 resting eggs have an inducible defense system responding to external abiotic stress.

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Table

Table 1 Putative functions of differentially expressed genes related to incubation salinities (17 and 33 ppt) and photoperiods (4 and 24 h) in the resting eggs of euryhaline rotifer *Brachionus manjavacas*. The score and E-value are the results of BLASTX based on the NCBI database.

Photoperiod	Salinity	Accession number	Clone size (bp)	Gene product name or probable function	Score	E-value
4 h	17 ppt	ADE05593.1	691	Late embryogenesis abundant-like protein 1 (LEAs-1)	79.3	2e-13
		WP_055756038.1	228	Alpha-amylase (α-amylase)	119.0	1e-29
		OGA61391.1	408	Deaminase	108.0	5e-27
24 h	17 ppt	WP_084601170.1	337	Hybrid sensor histidine kinase/response regulator (HHKR)	178.0	1e-49
		SDQ82225.1	604	Histidine kinase (HK)	239.0	3e-75
		CDW55082.1	543	TSP 1 and CBM 14 domain containing protein (TCcP)	56.6	4e-06
		CAY12635.1	563	SRY-related HMG box C protein (SRY-HCP)	133.0	2e-35
	33 ppt	WP_044750164.1	276	ATP-dependent OLD family endonuclease (ATPOE)	150.0	5e-41
		WP_034331136.1	176	MULTISPECIES: ABC transporter permease (ABC-TP)	74.7	5e-15
		WP_058951874.1	265	MULTISPECIES: enoyl-CoA hydratase (ECoAH)	162.0	7e-47
		WP_055399314.1	199	NAD+ synthase	77.8	3e-15
		WP_057267502.1	772	Copper-translocating P-type ATPase (CTP-ATPase)	414.0	3e-137
		SDQ37588.1	416	3-oxoacid CoA-transferase subunit B (3-OCoAT)	240.0	8e-79
		AQM37716.1	230	NADH dehydrogenase subunit 5 (NADHDnase)	52.8	4e-06
		XP_966125.2	638	Transcription factor with AP2 domain(s) (AP2TF)	94.4	1e-18

Figure legends

Fig. 1 Experimental scheme for the comparison of different gene expression related to the incubation salinity (ppt) and photoperiod (h).

Fig. 2 Hatching rates of resting eggs incubated at different salinities 17 (for control) and 33 ppt for two photoperiods 4 and 24 h. Each column and error bar indicates mean and standard deviation, respectively. The alphabets denote significant differences among treatments (a > b > c, Tukey HSD, $P < 0.05$, $n = 5$).

Fig. 3 mRNA levels of functional genes involved in (a) embryonic development, and (b) energy metabolism, which were detected from the resting eggs incubated under different photoperiods (h) and salinities (ppt). Each column and error bar indicates mean and standard variation of three replicates. The 18S rRNA gene was used as a reference gene to normalize the transcript level. The alphabets denote significant differences among the treatments (a > b > c, Tukey HSD, $P < 0.05$, $n = 3$).

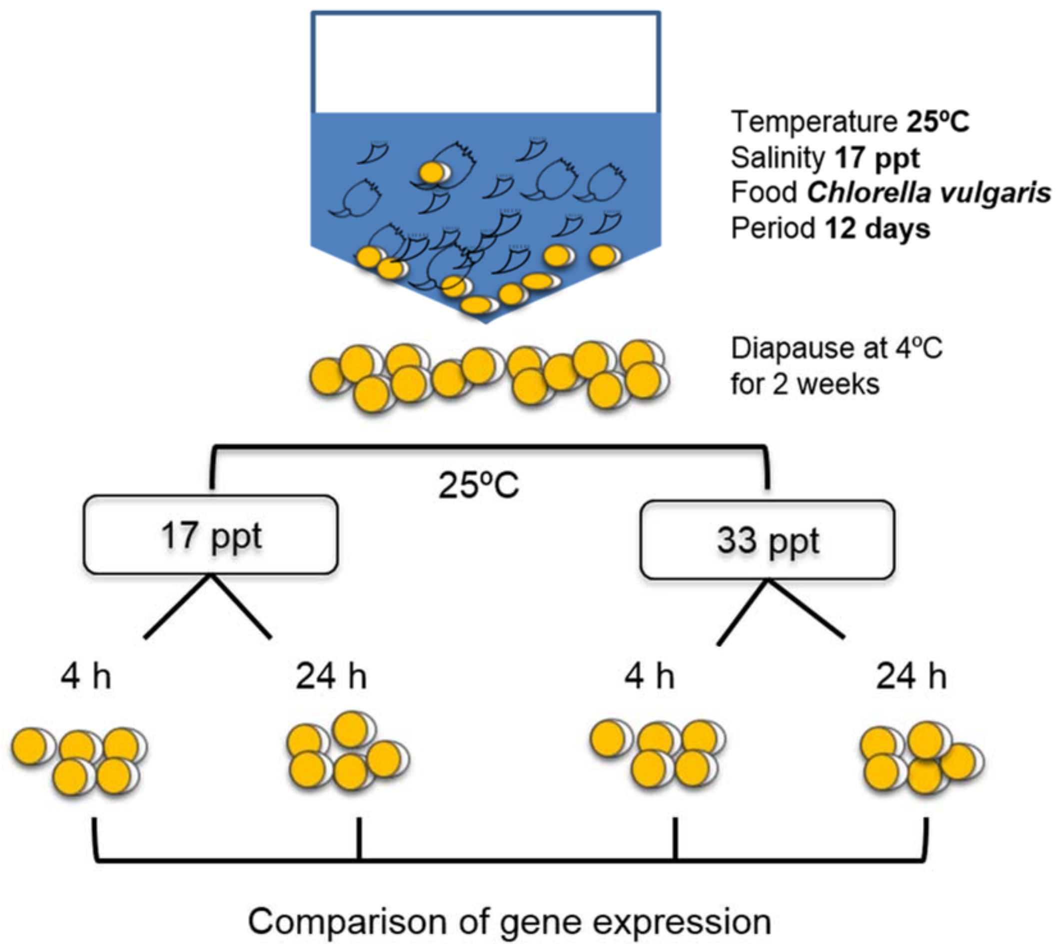


Fig 1

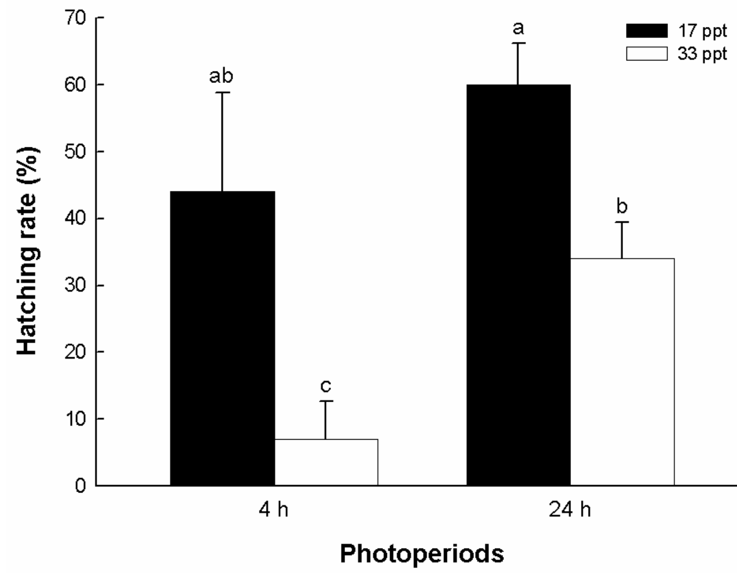
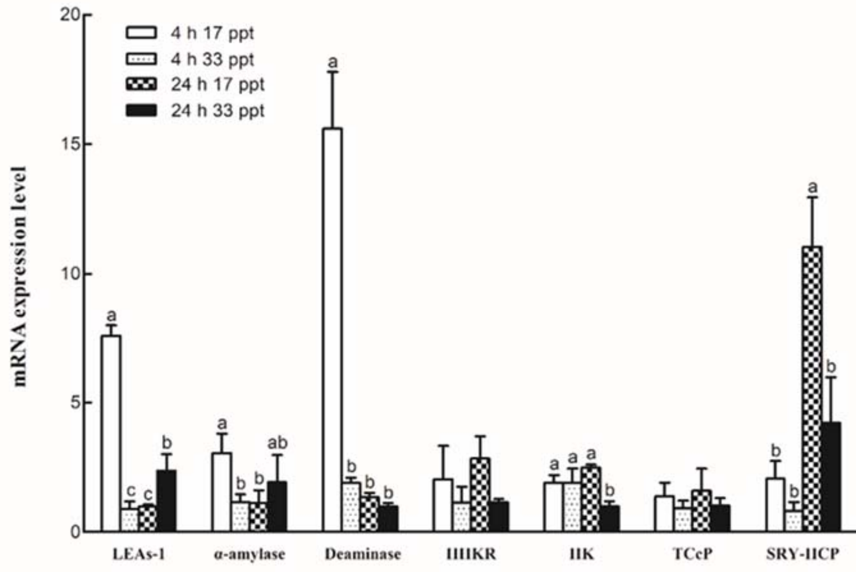
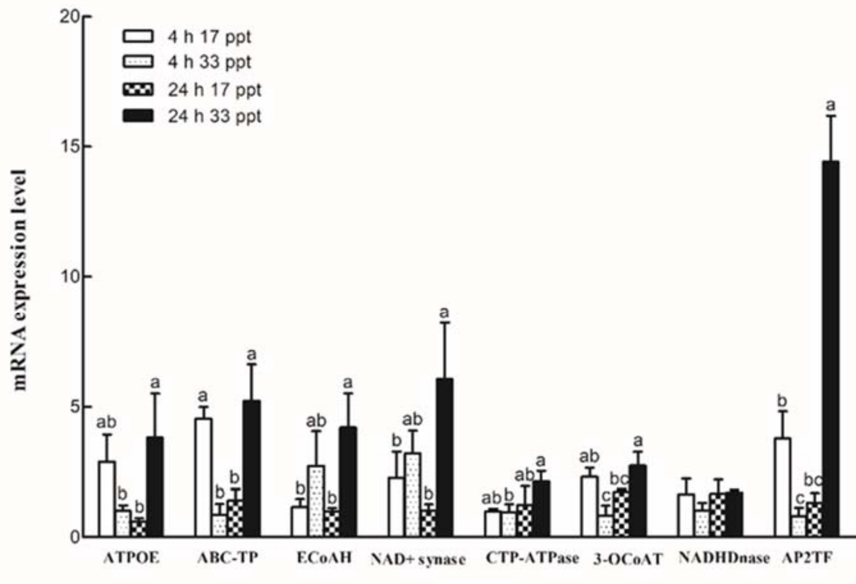


Fig 2



(a)



(b)

Fig 3