Immune responses of white leg shrimp, Litopenaeus vannamei (Boone, 1931) to bacterially expressed dsRNA specific to VP28 gene of white spot syndrome virus

Journal:	*Journal of Fish Diseases*
Manuscript ID:	JFD-2014-38
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	03-Mar-2014

Complete List of Authors:	G, Taju; C. Abdul Hakeem College, Zoology N, Madan; C. Abdul Hakeem College, Zoology S, Abdul; C. Abdul Hakeem College, Zoology T, Rajkumar; C. Abdul Hakeem College, Melvisharam, Vellore District, Tamil Nadu 632 509, India., OIE Reference Laboratory for WTD S, Thamizvanan; C. Abdul Hakeem College, Zoology S, Otta; C. Abdul Hakeem College, Zoology Sahul Hameed, A.S.

SCHOLARONE™
Manuscripts

For Review Only

1 **Immune responses of white leg shrimp, *Litopenaeus vannamei* (Boone, 1931) to bacterially**
2 **expressed dsRNA specific to VP28 gene of white spot syndrome virus**

3

4 G. Taju[1], N. Madan[1], S. Abdul Majeed[1], T. Raj Kumar[1], S. Thamizvanan[1], S.K. Otta[2], A.S. Sahul
5 Hameed[1]*

6

7

8 [1]OIE Reference Laboratory for WTD, PG & Research Department of Zoology, C. Abdul
9 Hakeem College, Melvisharam – 632 509, Vellore District, Tamilnadu, India.

10

11 [2]Aquatic Animal Health and Environment Division, Central Institute of Brackishwater
12 Aquaculture, 75, Santhome High Road, RA Puram, Chennai – 600028, India.

Review Copy

13
14
15
16
17
18
19
20
21

22 *Corresponding author:
23 A.S.SahulHameed,
24 Tel/Fax: +91-416-269487.
25 E-mail address: cah_sahul@hotmail.com

26
27
28
29
30
31

32 **Abstract**

33 In the present study, dsRNA specific to VP28 gene of white spot syndrome virus
34 (WSSV) of shrimp was synthesized in *Escherichia coli* in large-scale and studied the immune
35 response of shrimp to dsRNA-VP28. The hematological parameters such as clotting time and
36 total haemocytes counts, and immunological parameters such as prophenoloxidase (proPO),
37 superoxide dismutase, superoxide anion and malondialdehyde content, as well as the mRNA
38 expression of ten immune-related genes were examined in order to estimate the effect of dsRNA-
39 VP28 on the innate immunity of *Litopenaeus vannamei*. The activities of proPO, SOA and SOD
40 significantly increased in haemocyte after dsRNA-VP28 treatment, whereas MDA content did
41 not change significantly. Among the ten immune related genes examined, only the mRNA
42 expression of proPO, cMnSOD, hemocyanin, crustin, BGBP, lipopolysaccharides (LPs), lectin
43 and lysozyme in haemocyte, gill and hepatopancreas of *L. vannamei*, was significantly up-
44 regulated at 12 h after dsRNA-VP28 treatment, while no significant expression changes were
45 observed in toll receptor and tumor receptor genes. The increase of proPO and SOD activities,
46 and SOA level and mRNA expression level of proPO, cMnSOD, hemocyanin, crustin, BGBP,
47 LPs, lectin and lysozyme after dsRNA-VP28 stimulation indicate that these immune related
48 genes were involved in dsRNA-VP28-induced innate immunity in shrimp.

49 **Keywords:** *Litopenaeus vannamei*, Double-stranded RNA, Immunological parameters,
50 Hematological parameters, Immune genes expression.

51

52 **1. Introduction**

53 White spot syndrome virus (WSSV) is one of the most devastating shrimp viral
54 pathogens and has a wide host range of several crustacean species, including shrimp, crab, and
55 crayfish. WSSV is responsible for 100% mortality within a few days after onset of the infection
56 and is a serious threat to the shrimp culture industry worldwide (Lightner, 1996). Transmission
57 of the virus is mainly through oral ingestion and waterborne routes in farms and vertical
58 transmission in the case of shrimp hatcheries (Rosenberry, 2002). Considering the global
59 economic and sociological importance of shrimp farming, development of new control measures
60 becomes inevitable against the spread of WSSV. Protective effects of various immunostimulants
61 such as oral administration of peptidoglycan (Itami et al., 1998), lipopolysaccharide (Takahashi
62 et al. 2000; Tsutsui et al. 2007), glucan (Song et al. 1997; Chang et al. 2003), and aqueous

Review Copy

63 extract of *Cynodondactylon* (Balasubramanian et al. 2007) have been reported against WSSV
64 infection. In contrast to the well studied effect of microbial immunostimulants on the immune
65 system of shrimp (Liu et al. 2005; Perez et al. 2005), Venegas et al. (2000) have also reported a
66 new concept of quasi-immune response against WSSV in *Penaeus japonicus*. Various vaccines
67 like inactivated WSSV vaccine (Namikoshi et al. 2004; Huang et al. 2005), antibacterial
68 components (George et al. 2006), and subunit recombinant vaccines (Kim et al. 2004; Li et al.
69 2005, 2006; Wei and Xu 2005; Jha et al. 2006) have also been tried so far against WSSV with
70 notable results. Because crustaceans have only a nonspecific innate immune response and no
71 long memory (Warren et al. 2006), developing a vaccine and using it as an effective control
72 strategy is difficult.

73 Double-stranded RNA (dsRNA) is an intermediate produced in the replication of many
74 viruses, which induces multifaceted immune responses in mammals, including RNA interference
75 (RNAi) and the production of interferon (IFN) (Kapadia et al., 2003). RNAi is a sequence
76 dependent antiviral mechanism, which can inhibit the replication of virus by suppressing the
77 expression of virus genes in host cells (Li et al., 2002; Silhavy et al., 2002; Li et al., 2004). In
78 addition to RNAi, another sequence-independent immune mechanism, IFN-related immune
79 responses are also induced by dsRNA in mammals (Robalino et al., 2004). In this process,
80 dsRNA is recognized by Toll like receptor 3 (TRL3) which then activates innate antiviral
81 immune responses via My88-dependent and independent pathways, and finally leads to the
82 production of interferons (IFNs) (Robalino et al., 2004). IFNs induce the expression of many
83 proteins including RNA-dependent protein kinase (PKR), the Mx (myxovirus) protein and
84 oligoadenylatesynthetase, which have been demonstrated to play important roles in antiviral
85 immunity. Moreover, dsRNA can also directly activate intracellular PKR and induce antiviral
86 responses. Activated PKR inhibits cellular and viral protein synthesis via phosphorylation of
87 eukaryotic translation initiation factor 2a (eIF2a) which further inhibits the replication of virus
88 (Robalino et al., 2004; Meurs et al., 1990).

89 The RNAi pathway is thought to be an ancient mechanism for protecting the host and its
90 genome against viruses and rogue genetic elements by the process of mRNA degradation that is
91 induced by double-stranded RNA (dsRNA) in a sequence-specific manner (Jones et al. 1999;
92 Zamore et al. 2000; Bernstein et al. 2001). Several recent papers have reported that siRNAs
93 (Westenberg et al. 2005; Xu et al. 2007) or dsRNA synthesized via *in vitro* methods (Robalino et

Review Copy

94 al. 2004; Robalino et al. 2005; Kim et al. 2007) serve as potential therapeutic agents for treating
95 white spot syndrome disease. In the marine shrimp *Litopenaeus vannamei*, the antiviral response
96 can be induced by sequence-independent or sequence-specific dsRNA, which may activate
97 RNAi-like mechanisms (Robalino et al. 2004, 2005). Westenberg et al. (2005) have used siRNAs
98 specific to the VP15 and VP28 genes of WSSV and observed that shrimp injected with VP15 or
99 VP28 siRNASs before WSSV challenge had a significantly lower mortality. Xu et al. (2007)
100 have used a specific 21-bp short interfering RNA targeting the VP28 gene of WSSV and the
101 results revealed that the transcription and expression of the VP28 gene were silenced. The
102 introduction of long dsRNAs corresponding to viral proteins is very effective in blocking WSSV
103 infection in *Penaeus chinensis* (Kim et al. 2007). Yodmuang et al. (2006) reported the protection
104 of *P. monodon* against yellow head virus (YHV) infection by silencing the genes of YHV using
105 long dsRNA encoding structural and non-structural proteins of YHV. Recently, Sarathi et al.
106 (2008, 2008a) reported the possibility of protecting *Penaeus monodon* against white spot
107 syndrome virus (WSSV) infection via RNAi technology by oral administration of bacterially
108 expressed VP28 (VP28dsRNA) gene of WSSV.

109 The detailed mechanism of this dsRNA-induced antiviral immunity is still not well
110 understood. In present study, bacterially synthesized dsRNA specific to VP28 gene of WSSV
111 promoted gene-specific interference with the WSSV infection in shrimp and the effect of
112 dsRNA-VP28 on the immune response of *L. vannamei* was explored for obtaining more insight
113 into the mechanism of dsRNA-VP28 induced immunity in shrimp. The temporal activity changes
114 of superoxide dismutase (SOD), prophenoloxidase (proPO), superoxide anion (SOA) and
115 malondialdehyde (MDA) variation; hematological parameters such as clotting time and total
116 haemocytes count (THC) changes, as well as the mRNA expression of some immune-related
117 genes in *L. vannamei* were examined after dsRNA-VP28 treatment to estimate the effect of
118 dsRNA-VP28 on the immune system to determine the dsRNA-VP28induced immune responses
119 in *L. vannamei*.

120

121 **2. Materials and Methods**

122 **2.1. Collection of experimental animals**

123 White leg shrimp, *L. vannamei* (10–15 g body weight), were collected from
124 Nagapatinam, Tamilnadu, Indiaand were maintained in 1000-l fiberglass tanks with air-lift

Review Copy

125 biological filters at room temperature (27–30 °C) with salinity between 20 and 25 parts per
126 thousand (ppt) for 1 week prior to experiment. During the experiment, the shrimp were fed with
127 commercial pellet feed (CP feed, Thailand), and water was totally exchanged daily.

128 **2.2. Isolation of dsRNA-VP28**

129 dsRNA-VP28 was synthesized according to the method of Sarathi et al. (2008) with
130 slight modification. For the isolation ofdsRNA-VP28, IPTG-induced bacterial cells were pelleted
131 for 15min at 3000 g (4 °C) and resuspended in 1.25 ml of TE buffer (10 mM Tris–HCl, 1 mM
132 EDTA, pH 7.5). A minimum volume of 0.75 ml of the cell suspension was replenished with an
133 equal volume of phenol–chloroform–isoamyl alcohol and heated at 65°C for 10 min. The
134 samples were then centrifuged for 10 min at 13,000 rpm and the aqueous phase transferred into a
135 fresh tube. Nucleic acid was then precipitated with isopropanol and kept at –20°C for 10 min and
136 then centrifuged. Supernatant was discarded and the pellet was washed with 70% ethanol. After
137 drying, the pellet was resuspended in 100 µl of Tris–NaCl–$MgCl_2$–dithiothreitol buffer. Eighteen
138 microliters of this solution was supplemented with 1 µl of DNaseI (3 mg/ml) and digested for 15
139 min at room temperature to remove DNA, followed by extraction with phenol/chloroform and
140 precipitation with ethanol. The pellet was resuspended in 50 µL RNase-free PBS buffer and
141 subjected to electrophoresis for RNA integrity confirmation. The dsRNA concentration was
142 quantified by measuring the absorbance using UV spectrophotometer at the wavelength of 260
143 and adjusted to a final concentration of 1 µg/ml. dsRNA was injected intramuscularly at the third
144 abdominal segment of shrimp using 1 mL syringe.

145 **2.3. Injection of dsRNA-VP28 in shrimp**

146 The shrimp were divided into two groups and maintained in aquarium tanks
147 (10shrimp/tank) as described above. In the Group I, the shrimp were injected intramuscularly
148 with 50 µL dsRNA at the concentration of 1 µg/µl specific to VP28 gene of WSSV at third
149 abdominal segment of shrimp. In Group II, the shrimp were injected intramuscularly with 50 µL
150 PBS (pH 7.0) and this group of shrimp was used as negative control. During the experimental
151 period, three shrimp were randomly collected from each group at 0 h, 3 h, 6 h, 12 h, 24 h and 48
152 h post-injection. The haemolymph was drawn from the heart using a 23 gauge needle attached to
153 a 1 mL syringe. After collection, the haemolymph was quickly mixed with ice cold anticoagulant
154 solution (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 4.6 at 28 °C).
155 Samples were immediately centrifuged at 800 x g at 4°C for 10 min to collect the haemocytes.

Review Copy

156 Cell pellet was gently washed with 500 µL of wash solution (415 mmol/L NaCl, 100 mmol/L
157 glucose, pH 7.0) and centrifuged again at 800 g for 5 min. The haemocytes were resuspended in
158 1 mL lysis buffer (415 mmol/L NaCl, 100 mmol/L glucose, 10 mmol/L cacodylic acid, 5
159 mmol/L $CaCl_2$, pH 7.0) and sonicated at 40% power for 2 min. Cell debris was removed by
160 centrifuging at 13,000 g for 10 min, and the resultant haemocyte lysate supernatant was used for
161 analyzing proPO, SOD and SOA activity and MDA content.

162 **2.4. Immunological parameters**

163 **2.4.1. Prophenoloxidase activity**

164 Prophenoloxidase activity of PBS or dsRNA-VP28 injected shrimp was analyzed
165 spectrophotometrically by the method of Hernandez-Lopez et al. (1996) by recording the
166 formation of dopachrome produced from L-dihydroxyl phenylalanine (L-DOPA). Briefly,
167 hemolymph collected from normal or experimental shrimp was diluted and centrifuged at 800 x
168 *g* at 4 °C for 20 min and the pellet was collected. Then the pellet was suspended gently in
169 cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate,
170 pH 7.0). The sample was again centrifuged as described above. After removing the supernatant,
171 the pellet was resuspended in 100 µL cacodylate buffer. The sample was incubated after adding
172 50 µL trypsin (Sigma, 1 mg ml^{-1}) at 25°C for 10 min, which served as an activator; 50 µL DOPA
173 was then added followed by 800 µL of cacodylate buffer 5 min later. The optical density was
174 measured at 490 nm wave length by UV–VIS spectrophotometer (Shimadzu, UV 2450).

175 **2.4.2. Superoxide anion detection**

176 Superoxide anion was quantified in PBS or dsRNA-VP28 injected shrimp by the method
177 of Song and Hsieh, (1994). Briefly, 100 µL of hemolymph was collected from PBS or dsRNA-
178 VP28 injected shrimp and centrifuged at 800 *g* for 5 min. After centrifugation, the supernatant
179 was removed and 100 µL of HBSS was added to haemocytes including normal sample tubes and
180 allowed to react for 30 min at 37°C. After incubation, haemocytes were washed three times with
181 HBSS and then stained with Nitro blue tetrazolium (NBT) solution (0.3%, 100 µL) for 30 min at
182 37°C. Removing the NBT solution and adding absolute methanol stopped the staining reaction.
183 The stained hemocytes were washed three times with 70% methanol and air dried. Added 120 µL
184 of 2 M KOH and 140 µL DMSO to the sample to dissolve the cytoplasmic formazan. The optical
185 density of the dissolved formazan was read on a UV–VIS spectrophotometer (Shimadzu, UV
186 2450) at the wave length of 630 nm.

Review Copy

187 **2.5. Antioxidant parameters**

188 **2.5.1. Assay of superoxide dismutase**

189 SOD activity was determined in PBS or dsRNA-VP28 injected shrimp by the method of
190 Beauchamp and Fridovich (1971) using NBT in the presence of riboflavin. Briefly, 100 µL of
191 hemolymph was obtained from PBS or dsRNA-VP28 injected shrimp and homogenized in a
192 mechanical homogenizer containing 0.5 mL of phosphate buffer (50 mM, pH 7.8). The
193 homogenate was centrifuged for 5 min at 5700 *g* at 4°C and the supernatant recovered was
194 heated for 5 min at 65°C to obtain a new supernatant after centrifugation, which was stored at
195 –20 °C until use. Samples were maintained on ice at all times to avoid protein denaturation. A
196 mixture of NBT, 20 µM of reaction mixture (0.1 Mm EDTA, 13 µM methionine, 0.75 mM NBT
197 and 20 µM riboflavin in phosphate buffer 50 mM, pH 7.8) and 100 µL of the crude extract was
198 placed under fluorescent light for 2 min or until A560 in normal sample tubes reached 0.20–0.25
199 OD. The results were expressed as relative enzyme activity.

200 **2.5.2. Malondialdehyde content**

201 In this method described by Beuge and Aust (1978), a mixture of 100 µL Tris buffer
202 (150mM, pH 7.1), 10 µL ferrous sulfate (100mM), 10 µL ascorbic acid (150 mM), 780 µL
203 distilled water and 100 µL of cell extract (haemocytes) was incubated at 37°C for 15min.
204 Thiobarbituric acid (0.375%, 2mL) was then added to the mixture and allowed to react at 100°C
205 (in water bath) for 15min. The reaction mixture was then centrifuged (800×*g* for 10min) and
206 supernatant was read at 532nm against blank. The MDA content was expressed as nmol per mg
207 of protein (nmol/mg protein).

208 **2.6. Hematological assays**

209 **2.6.1. Clotting time**

210 The clotting time of hemolymph was measured according to the method described by
211 Sachdev (1983). The hemolymph was collected from PBS or dsRNA-VP28injected shrimp
212 directly into a pre-cooled glass capillary tube of 1.2 mm diameter and 50 mm length. After the
213 hemolymph was inserted, the tube was kept at vertical position with the sample at the upper end.
214 Then the tube was maintained vertically until the gravity forced hemolymph to reach the lower
215 end of the tube, after which the tube was turned to 180°. This was repeated until the hemolymph
216 clotted. The clotting time is defined as the point when the flow of hemolymph ceases and
217 presumably total clotting occurs. The clotting time was recorded.

Review Copy

218 **2.6.2. Total haemocytes count**

219 Total haemocyte count (cells/ml) was performed using a Burker hemocytometer as
220 described by Sachdev (1983). One mL of hemolymph was drawn directly from the heart of
221 shrimp injected with dsRNA-VP28 or normal shrimp by inserting a 23 gauge needle attached to
222 a 2 ml syringe containing 1 ml of ice-cold anticoagulant Alsever solution (27 mM sodium citrate,
223 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 4.6 at 28 °C). A drop of hemolymph with
224 anticoagulant mixture was placed on a hemocytometer to measure the total haemocytes using a
225 phase contrast microscope and counted manually in all 25 squares (0.1 mm^3).

226 **2.6.3. Oxyheamocyanin levels**

227 For measurement of oxyheamocyanin level, 50 μL of hemolymph from normal or
228 experimental animal was immediately diluted with 900 μl of distilled water in a 10-mm quartz
229 cuvette and the absorbance was measured at 335 nm using a UV−VIS spectrophotometer
230 (Shimadzu, UV 2450). The concentration of oxyhemocyanin was determined based on the
231 method of Nickerson and Van Holde (1971) and Hagerman (1983).

232 **2.7. Quantification of immune-related gene expressionby real-time PCR**

233 The haemolymph, hepatopancreas and gill tissues were collected separately from each
234 shrimp for extracting total RNA using Trizolreagent). Two mg of DNase-treated (RQ1 DNase I,
235 Promega) total RNA and oligo (dT)-adaptor primer were used to synthesize first-strand cDNA
236 using M-MLV reverse transcriptase (NEB, USA). The cDNA was quantified using the
237 NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, USA) at 260 nm. The
238 mRNA level of immune-related genes was determined using SYBR green quantitative real-time
239 PCR in ABI PRISM 7300 Sequence Detection System (ABI, USA). The amplification was
240 performed in a 10 µL reaction volume containing 5 µL of 2 x SYBR Green Master Mix (ABI,
241 USA), 1µL of diluted cDNA, 0.5µL of each primer and 3.5 µL of DEPC-treated water. DEPC-
242 water for the replacement of cDNA template was used as the negative control. The thermal
243 profile for the SYBR green real-time PCR was 50 °C for 2 min and 95 °C for 10 min followed
244 by 40 cycles of 95 °C for 15 s and 72 °C for 1 min. The expression of immune-related genes was
245 normalized to the expression of β-actin gene for each sample. The details of primersets used in
246 this assay are shown in Table 1. Data analysis was executed using mathematical model for
247 relative quantification in real-time PCR by Pfaffl. (2001). ΔC_T represented the difference
248 between C_T for target gene and the internal control, and $\Delta\Delta C_T$ was obtained by subtracting the

Review Copy

249 ΔC_T for the control (PBS) from the ΔC_T for each sample. The obtained data were subjected to
250 statistical analysis.

251 **2.8. Data analysis**

252 Statistical analysis was executed using Excel software. All data obtained from the
253 experiments were analyzed using one-way ANOVA ($P<0.05$ as significant level). Statistical
254 calculations were performed using SPSS (version 16) software. Results are expressed as
255 mean±SD. A significance level of $p<0.05$ was used for statistical testing.

256

257 **3. Results**

258 The gene encoding VP28 protein of WSSV was amplified by PCR and amplified product
259 was cloned into LITMUS38i vector. The recombinant plasmid bearing the VP28 gene was
260 transformed to *E. coli* HT115DE3 and dsRNA specific to VP28 was expressed under induction
261 of 1 mM IPTG. After induction with IPTG at 37°C, dsRNA was isolated from induced
262 LITMUS38i-VP28 *E. coli* HT115DE3. The isolated dsRNA was analyzed via agarose gel
263 electrophoresis (Fig.1). A band of dsRNA-VP28 corresponding to the molecular weight of
264 615 bp was observed in 1.2% agarose gel electrophoresis in the sample of IPTG-induced
265 LITMUS38i–VP28–*E. coli* HT115DE3 (Fig.1, lane 3), whereas no dsRNA band was found at
266 the same position in both the non-induced LITMUS38i-VP28– *E. coli* HT115DE3 and induced
267 LITMUS38i–*E. coli* HT115DE3 (Fig.1, lanes 1 and 2). The result of agarose gel indicates the
268 successful expression of dsRNA-VP28in *E. coli*.

269 In the experiment, no mortality was recorded in shrimp injected with dsRNA-VP28 and
270 significant immune response was observed when compared to shrimp injected with PBS.
271 Significant change in the level of proPO, SOD and SOA was observed in shrimp injected with
272 dsRNA-VP28 when compared to shrimp injected with PBS (Figs.2, 3 and 4). The level of proPO
273 activity increased to 8.3 Unit/mg protein in shrimp injected with dsRNA-VP28 at 24 h.p.i.
274 whereas the level was about 4.9 Unit/mg protein in shrimp injected with PBS (Fig. 2). The
275 proPO activity gradually increased, reached highest level at 24 h p.i. in dsRNA-VP28 injected
276 shrimp. Significant change in the level of SOA and SOD was observed in shrimp injected with
277 dsRNA-VP28 and the level was found to be the highest at 24 h p.i. when compared to the shrimp
278 injected with PBS (Figs. 3 and 4). The SOA level remained at a low level during the initial 6
279 hours and increased 3 to 4 folds after 12 h post injection of dsRNA-VP28. No significant change

Review Copy

280 was observed in MDA content in shrimp injected with dsRNA-VP28 in comparison with PBS-
281 injected shrimp (Fig. 5).

282 The clotting time of hemolymph was estimated in shrimp injected with dsRNA-VP28 and
283 shrimp injected with PBS, and the results are shown in Fig. 6. There is no significant change in
284 the clotting time between shrimp injected with PBS and shrimp injected with dsRNA-VP28 at
285 different time interval after injection. Like clotting time, no significant change was observed in
286 total haemocyte count in shrimp injected with dsRNA-VP28 and normal shrimp (Fig.7). The
287 level of oxyhemocyanin (OHC) was determined in shrimp injected with dsRNA-VP28 and
288 shrimp injected with PBS at different time intervals. The results revealed that significant change
289 was found in shrimp injected with dsRNA-VP28 after 12 h p.i. The OD value increased to the
290 highest value of 0.61 in dsRNA-VP28 injected shrimp whereas the OD value for OHC in PBS
291 injected shrimp was found to be 0.342 at 24 h p.i. (Fig.8).

292 The mRNA expression level of ten immune-related genes was quantified in haemocytes,
293 hepatopancreas and gill tissue of shrimp injected with dsRNA-VP28 by quantitative real-time
294 PCR in order to investigate immune response todsRNA-VP28ds, and the results are presented in
295 Table 2. Lowered C_T value and enhanced mRNA expression level were observed in the genes of
296 proPO, cMnSOD, hemocyanin, crustin, BGBP, lipopolysaccharides (LPs), lectin, lysozyme, toll
297 receptor and tumor receptor in haemocytes, hepatopancreas and gill tissue of dsRNA-VP28
298 injected *L. vannamei* (Figs. 9A - J). The β-actin gene served as internal control in the
299 haemocytes, hepatopancreas and gill of *L. vannamei* during the experiment. Among these
300 immune-related genes, the expression level of proPO gene was enhanced about 12.17 fold, 8.9
301 fold and 5.8 fold in haemocytes, gill and hepatopancreas, respectively in dsRNA-VP28 injected
302 shrimp (Fig.9A). In the case of cMnSOD gene, the expression level was increased about 10.2
303 fold, 4.8 fold and 5.4 fold in haemocytes, gill and hepatopancreas, respectively in response to
304 dsRNA-VP28 injection in shrimp (Fig.9B). The expression level of hemocyanin gene was
305 enhanced about 6.01 fold, 5.1 fold and 3.9 fold in haemocytes, gill and hepatopancreas,
306 respectively in dsRNA-VP28 injected shrimp (Fig.9C). In the case of crustin gene, the
307 expression level was increased about 6.3 fold, 6.1 fold and 2.7 fold in haemocytes, gill and
308 hepatopancreas, respectively in response to dsRNA-VP28 injection in shrimp (Fig.9D). The
309 enhanced mRNA expression level was observed in all the tissues tested in shrimp in response to
310 dsRNA-VP28 in the case of BGBP (Fig.9E), LPs (Fig.9F), lectin (Fig.9G) and lysozyme

Review Copy

311 (Fig.9H). But no change in the mRNA expression level was observed in the case of toll receptor
312 gene (Fig.9 I) and tumor receptor gene (Fig.9J) in response to dsRNA-VP28 injection in shrimp.
313 At 12 h, there was a significant (*$P<0.05$, dsRNA-VP28 treatment) increase in the relative
314 abundance of proPO, cMnSOD, Hemocyanin, Crustin, BGBP, LPs, Lectin and Lysozyme genes
315 mRNA. As time progressed, the expression of immune genes dropped gradually, and decreased
316 to its original level at 48 h.post dsRNA-VP28 injection.
317

318 **4. Discussion**

319 Application of RNAi technology against WSSV is a possible solution to control the quick
320 spread of this deadly disease (Robalino et al., 2004, 2005; Westenberg et al., 2005; Xu et al.,
321 2007; Kim et al., 2007). It is not possible to apply *in vitro* synthesized dsRNA and siRNA
322 available for RNAi therapy in the shrimp culture ponds, because they cannot be produced in
323 large quantities. dsRNA can stimulate different immune responses in mammals based on their
324 length (Dong et al., 2009). dsRNA shorter than 30 bp, called small interference RNA (siRNA),
325 induces RNA interference (Elbashir et al., 2001, 2001a) while dsRNA longer than 30 bp induces
326 the production of IFNs and therefore triggers strong antiviral immunity (Fire et al., 1998; Tuschl
327 et al., 1999). In invertebrates, there is no dsRNA-induced IFN-related immunity because of the
328 lack of IFN-related genes (Robalino et al., 2004), and long dsRNA is often used to induce RNA
329 interference (Williams et al., 1979; Misquitta and Paterson, 1999; Caplen et al., 2000; Robalino
330 et al., 2005). Recently, antiviral immune responses were observed in some crustacea after
331 challenging with dsRNA (Robalino et al., 2004, 2005; Dong et al., 2009). But this antiviral
332 immunity was not as strong as it is in mammals and could be overcome by a high dose of
333 infectious virus (Robalino et al., 2004). The mechanism of this immunity is suspected to be
334 different from the mechanism in mammals because there is no report of the presence of IFN-
335 related genes in invertebrates. Moreover, the antiviral immunity induced by dsRNA in
336 crustaceans is sequence-independent which is different from RNAi.

337 Changes in the level of prophenoloxidase activity observed in the present study indicate
338 the involvement of dsRNA-VP28-induced immunity in shrimp as observed in crab by Dong et al.
339 (2009). A significant elevation of proPO activity observed in the haemolymph of *L. vannamei*
340 injected with dsRNA-VP28 might be due to the dsRNA treatment which triggered the
341 prophenoloxidase activating system and transformed the prophenoloxidase into phenoloxidase.

Review Copy

342 In crustacea, melanization occurs when the cellular defense reactions are initiated (Ratcliffe et
343 al., 1985; Soderhall et al., 1986). Phenoloxidase, the key enzyme in the synthesis of melanin,
344 occurs in hemolymph as an inactive proenzyme prophenoloxidase (proPO) (Chang et al., 2003;
345 Balasubramanian et al., 2008). ProPO is activated to form phenoloxidase when it reacts with
346 zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPs), urea, calcium
347 ions, trypsin, or heat (Soderhall et al., 1984, 1986). Results from several experiments have
348 confirmed that apart from their role in melanization, components of the putative proPO
349 activating system stimulate several cellular defense reactions, including phagocytosis, nodule
350 formation, encapsulation, and haemocytes locomotion (Soderhall et al., 1986; Johanson et al.,
351 2000). The increase of phenoloxidase activity might defend the animal against invading
352 pathogens by promoting the melanization reaction, and generating highly cytotoxic quinones that
353 could inactivate viral pathogens or by enhancing other cellular activities such as phagocytosis
354 and encapsulation (Dong et al., 2009). This mechanism is especially important to protect the
355 shrimp against viral infection, because viral infection can inhibit the activity of phenoloxidase
356 activity in crustaceans (Mathew et al., 2007), while any reductions in the activity of
357 phenoloxidase activity might lead to the failure of phagocytosis (Mathew et al., 2007) and make
358 the animals susceptible to infection. The dsRNA-induced mechanism help to improve the
359 condition of enhancement phenoloxidase activity and similar observation was reported in
360 Chinese mitten crab *Eriocheirsinensis*by injection of two double-stranded RNA (GFP-dsRNA
361 and NoPSD-dsRNA) (Dong et al., 2009). In addition, there are other reports about the
362 enhancement of shrimp resistance to viral infection after stimulation with LPs (Takahashi et al.,
363 2000), or 1, 3-glucans (Chang et al., 2003). The exact mechanism of dsRNA activation on the
364 prophenoloxidase system still is not known and requires further investigation.

365 The superoxide anion activity increased significantly in dsRNA-VP28 administered
366 shrimp when compared to normal control shrimp. The production of O_2^- has been reported as an
367 accurate method to measure the effectiveness of potential immunostimulants (Munoz et al.,
368 2000). The higher level of O_2^- in dsRNA-VP28 administered shrimp indicates that dsRNA-VP28
369 may be the potential immunostimulant. Downs et al. (2001) reported that increase in the
370 superoxide anion against pathogens is considered to be beneficial after exposing shrimp to
371 immunostimulants. These facts suggested that increase in the superoxide anion is considered to
372 be beneficial with respect to increased immunity.

Review Copy

373 Antioxidation systems play an important role in clearing excess ROS and protecting
374 organisms from injury by ROS (Dong et al., 2009). In the present study, a significant increase of
375 SOD activity was observed in *L. vannamei* after dsRNA-VP28 injection, which indicated that
376 this antioxidation system was involved in the dsRNA-VP28-induced immune response. The high
377 level SOD could convert superoxide anion into hydrogen peroxide, maintain the balance of
378 pro/antioxidant and protect cells against injury from lipid peroxidation. It is well known that
379 pathogen infection always results in inhibition of the SOD activity and a dramatic increase in the
380 level of ROS (Schwarz, 1996; Dong et al., 2009). High level ROS can lead to lipid peroxidation
381 of cell membranes or reduction in the PO activity (Mathew et al., 2007), and can result in high
382 mortality. The foreign stimuli enhancing the SOD activity will augment the host resistance to a
383 pathogen. The enhanced SOD activity in response to dsRNA-VP28injection indicates that
384 dsRNA-VP28 could strengthen the innate immunity in *L. vannamei* via this antioxidation system.
385 Malondialdehyde (MDA) content is an indicator of lipid peroxidation and is often used to
386 evaluate the damage of membranes resulting from oxidative stress. In the present study, MDA
387 content was also measured to examine the lipid peroxidation resulting from dsRNA-VP28
388 stimulus. There was no significant variation of MDA content in haemolymph of *L. vannamei*
389 treated with dsRNA-VP28 compared to control animals as reported by Dong et el. (2009) in crab
390 injected with dsRNA.This suggests that dsRNA-VP28, although a viral-related molecular
391 patternhas no role in excessive ROS production as in the case of viral infection. This is
392 consistent with the previous report (Robalino et al., 2004) in which different doses of dsRNA did
393 not result injury to shrimp. It is also supported by an earlier report that structural proteins of the
394 virus rather than dsRNA were responsible for ROS production in mouse splenocytes (Schwarz,
395 1996).
396 Balasubramaiyam et al. (2008) reported that there were no significant changes in clotting
397 time (CT) and total haemocytes count (THC) between normal and plant (*Cynodondactylon*)
398 treated shrimp at different day of post injection. Similar resultswere also observed in clotting
399 time (CT), total haemocytes count (THC) and oxyhemocyanin of hemolymph in the present
400 study. No significant changes were observed in clotting time, total haemocytes count and
401 oxyhemocyanin level between control shrimp and dsRNA-VP28 treated shrimp.
402 Hepatopancreas and haemocytes which have a vital role in the immune response, are the
403 major sites for the synthesis of immune defence molecules and are involved in eliminating

Review Copy

404 pathogens or other particulate matter (Johnson, 1987; Vogt, 1996; Gross et al., 2001). Gill is an
405 important organ of respiration as well as of osmoregulation (Mantel and Farmer, 1983; Pequeux,
406 1995). These organs were selected for quantification of expression of immune genes in response
407 to dsRNA-VP28 injection. One of the interesting hypotheses derived from our observation of
408 activation of RNAi and innate immunity by dsRNA-VP28 is that these two pathways interact
409 functionally to confer immunity to a viral pathogen. Such a possibility was previously
410 unrecognized among invertebrates, as *L. vannamei* is the only invertebrate in which dsRNA has
411 been shown to induce both innate immune reactions and RNAi-like antiviral immunity (Robalino
412 et al., 2005).

413 Among the ten immune-related genes examined, proPO, cMnSOD, hemocyanin, crustin,
414 BGBP, LPs, lectin and lysozyme genes responded to dsRNA-VP28 challenge at different time
415 intervals in hemocyte, gill and hepatopancrease of shrimp by qPCR analysis. The expression of
416 these eight genes (proPO, cMnSOD, hemocyanin, crustin, BGBP, LPs, lectin and lysozyme) was
417 up-regulated by dsRNA-VP28 challenge, which indicated that these eight immune genes were
418 involved in the immune response of the *L. vannamei* in response to dsRNA-VP28. Another two
419 genes namely toll receptor and tumor receptor genes were not significantly up-regulated in
420 dsRNA-VP28 treated shrimps. Recently, a TLR (Toll Receptor) was characterized in *L.*
421 *vannamei*, but was shown to play no role in dsRNA-induced antiviral immunity (Labreuche et
422 al., 2009). Wang et al. (2011) reported investigate Tumor necrosis factor receptor (TNFR)-
423 associated factor 6 (TRAF6) function in invertebrate innate immune responses, *Litopenaeus*
424 *vannamei* TRAF6 (LvTRAF6) was identified and characterized.

425 Superoxide dismutases (SODs), which are important antioxidant enzymes, are present in
426 almost all oxygen respiring animals. In the present study, cMnSOD was found to have basal
427 expression level in shrimp injected with PBS while in shrimp injected with VP28dsRNA, the
428 mRNA expression was found to be increased significantly in haemocytes, gill and
429 hepatopancreas. This indicates that dsRNA-VP28 stimulates immune genes reaching maximum
430 level at 12 hp.i., thereafter decreasing gradually after 24 hp.i. and finally reaching normal level at
431 48 hp.i. The prophenoloxidase system forms an important part of an immune recognition process
432 of the defense mechanism and enzyme production during proPO system activation. Takahashi et
433 al. (2000) observed increased proPO activity in *M. japonicas* fed with diet containing LPS for 7
434 days at a dose of 20 mg kg^{-1} shrimp body weight. In *Penaeus monodon* that had been fed a diet

Review Copy

435 containing glucan for 20 days, proPO activity was significantly increased when compared to non
436 glucan group (Chang et al., 2003). In the present study, enhanced mRNA expression was found
437 in haemocytes, gill and hepatopancreas in response to injection of dsRNA-VP28 in *L. vannamei*.
438 This result agrees with previous works carried out by various workers with different
439 immunostimulants. Crustin, one of the important AMPs in crustacean has gained the attention of
440 many researchers. Crustin has been demonstrated to be markedly up regulated in the kuruma
441 prawn, *Marsupenaeus japonicus* from day 1 to 7 with oral administration of peptidoglycon
442 (Rattanachai et al., 2005). Similarly, increased expression of crustin was found in shrimp
443 injected with dsRNA-VP28at 12 hp.i. and gradually decreased to normal level at 48 hp.i.
444 Immune gene expression profile showed that a yeast incorporated diet up-regulated all the six
445 AMP (antimicrobial peptide genes) genes (ALF, Crustin-1, Crustin-2, Crustin-3, Penaeidin-3 and
446 Penaeidin-5) and immune gene (proPO) to a significant level and that the genes were
447 differentially expressed in the haemocytes of *P. monodon* during pre-and post-challenge period
448 (Divya et al., 2013). Although the detailed mechanism of proPO, cMnSOD, hemocyanin, crustin,
449 BGBP, LPs, lectin and lysozyme remain unknown, their up-regulation induced by dsRNA-VP28
450 indicates the potential effects of dsRNA-VP28 on the defense responses against bacterial and
451 viral diseases.
452

Review Copy

453 **Acknowledgements**

454 The authors are grateful to the Management of C. Abdul Hakeem College, Melvisharam,
455 India, for providing the facilities to carry out this work. This work was funded by the Department
456 of Biotechnology, Government of India, New Delhi, India.
457

458 **Reference**

459 Balasubramanian G., Sarathi M., Rajesh Kumar S. & SahulHameed A.S. (2007). Screening the
460 antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp.
461 *Aquaculture* 263, 15–19.
462 Balasubramanian G., Sarathi M., Venkatesan C., John Thomas. & SahulHameed A.S. (2008).
463 Studies on the immunomodulatory effect of extract of *Cyanodondactylon* in shrimp,
464 *Penaeusmonodon* and its efficacy to protect the shrimp from white spot syndrome virus
465 (WSSV). *Fish & shellfish immunology* 25, 820–828.

466 Beauchamp C., Fridovich I. (1971). Superoxide dismutase: Improved assay and an assay
467 applicable to acrylamide gels. *Analytical biochemistry* 44, 276–87.
468 Bernstein E., Caudy A.A., Hammond S.M. & Hannon G.J. (2001). Role for a
469 bidentateribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366.
470 Beuge J.A. & Aust A.D. (1978). Microsomal lipid peroxidation. *Methods enzymology* 52, 302–
471 310.
472 Caplen N.J., Fleenor J., Fire A. & Morgan R.A. (2000). dsRNA mediated gene silencing in
473 cultured Drosophila cells: a tissue culture model for the analysis of RNA interference.
474 *Gene* 252, 95–105.
475 Chang C.F., Su M.S., Chen H.Y. & Liao I.C. (2003). Dietary-1,3-glucan effectively improves
476 immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus.
477 *Fish & shellfish immunology* 15, 297–310.
478 Cheng, W., Liu, C.H., Tsai, C.H. & Chen, J.C. (2005). Molecular cloning and characterisation of
479 a pattern recognition molecule, lipopolysaccharide- and b-1,3-glucan binding protein
480 (LGBP) from the white shrimp *Litopenaeus vannamei*. *Fish & Shellfish Immunology* 18,
481 297-310.
482 Divya T.B., Swapna P., Antony, Simi P.J., Ann Rose B. & Rosamma P. (2013). Marine yeast
483 Candida aquaetextoris S527 as a potential immunostimulant in black tiger shrimp
484 *Penaeus monodon*. *Journal of invertebrate pathology* 112, 243–252.
485 Dong C., Zhao J., Song L., Wang L., Qiu L., Zheng P., Li L., Gai Y. & Yang G. (2009). The
486 immune responses in Chinese mitten crab *Eriocheirsinensis* challenged with double-
487 stranded RNA. *Fish & shellfish immunology* 26, 438–442.
488 Downs C., Fauth J.E. & Woodley C.M. (2001). Assessing the health of grass shrimp
489 (*Palaemonetespugio*) exposed to natural and anthropogenic stressors: a molecular
490 biomarker system. *Marine biotechnology* 3, 380–97.
491 Elbashir S.M., Lendeckel W. & Tuschl T. (2001). RNA interference is mediated by 21- and 22-
492 nucleotide RNAs. *Genes & development* 15,188–200.
493 Elbashir S.M., Harborth J., Lendeckel W., Yalcin A., Weber K. & Tuschl T. (2001a). Duplexes,
494 of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*
495 411, 494–8.

Review Copy

496 Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E. & Mello C.C. (19980. Potent and
497 specific genetic interference by double stranded RNA in *Caenorhabditiselegans*. *Nature*
498 391, 806–11.
499 George M.R., Maharajan A., John K.R. & Prince Jeyaseelan M.J. (2006). Shrimps survive white
500 spot syndrome virus challenge following treatment with Vibrio bacterin. *Indian journal*
501 *of experimental biology* 44, 63–67.
502 Gross P.S., Bartlett T.C., Browdy C.L., Chapman R.W. & Warr G.W. (20010. Immune gene
503 discovery by expressed sequence tag analysis of haemocytess and hepatopancreas in the
504 Pacific white shrimp, *Litopenaeusvannamei*, and the Atlantic white shrimp, *L. setiferus*.
505 *Developmental & comparative immunology* 25, 565–77.
506 Hagerman L. (1983). Haemocyanin concentration of juvenile lobster (*Homarusgammarus*) in
507 relation in molting cycle and feeding conditions. *Marine biology* 77, 11–17.
508 Hernandez-Lopez J., Gollas-Galvan T. & Vargas,-Albores F. (1996). Activation of the
509 prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes).
510 *Comparative biochemistry and physiology*. 113C, 61–66.
511 Huang R., Xie Y., Zhang J. & Shi Z. (2005). A novel envelope protein involved in White spot
512 syndrome virus infection. *Journal of general virology* 86, 1357–1361.
513 Itami T., Asano M., Tokishige K., Kubono K., Nakagawa A., Takeno N., Nishimura H., Maeda
514 M., Kondo M. & Takahashi Y. (1998). Enhancement of disease resistance of kuruma
515 shrimp, *Penaeusjaponicus*, after oral administration of peptidoglycan derived from
516 *Bifidobacteriumthermophilum*. *Aquaculture 1*6, 277–288.
517 Jha R.K., Xu Z.R., Shen J., Bai S.J., Sun J.Y. & Wei F.L. (2006). The efficacy of recombinant
518 vaccines against white spot syndrome virus in *Procambarusclarkii*. *Immunology Letters*
519 105, 68–76.
520 Johanson M.W., Keyser P., Sritunyalucksana K. & Soderhall, K. (2000). Crustacean
521 haemocytess and haematopoiesis. *Aquaculture* 91, 45–52.
522 Johnson P.T. (1987). A review of fixed phayocytic and pinocytotic cell of decapods crustaceans,
523 with remarks on haemocytess. *Developmental comparative immunology* 11, 679–704.
524 Jones L., Hamilton A.J., Voinnet O., Thomas C.L., Maule A.J. & Baulcombe D.C. (1999).
525 RNA–DNA interactions and DNA methylation in post transcriptional gene silencing.
526 *Plant Cell* 11, 2291–2301.

Review Copy

527 Kapadia S.B., Brideau-Andersen A. & Chisari F.V. (20030. Interference of hepatitis C virus
528 RNA replication by short interfering RNAs. *Proceedings of the national academy of*
529 *sciences* USA 100, 2014–8.
530 Kim D.K., Jang I.K., Seo H.C., Shin S.C., Yang S.Y. & Kim J.W. (2004). Shrimp protected from
531 WSSV disease treatment with egg yolk antibodies (IgY) against a truncated fusion
532 protein derived from white spot syndrome virus. *Aquaculture* 237, 21–30.
533 Kim C.S., Kosuke Z., Nam Y.K., Kim S.K. & Kim K.H. (2007). Protection of shrimp
534 (*Penaeuschinensis*) against white spot syndrome virus (WSSV) challenge by double-
535 stranded RNA. *Fish & shellfish immunology* 23, 242–246.
536 Labreuche, Y., O'Leary, N.A., de la Vega, E., Veloso, A., Gross, P.S., Chapman, R.W., Browdy,
537 C.L., and Warr, G.W., 2009. Lack of evidence for *Litopenaeus vannamei* Toll receptor
538 (lToll) involvement in activation of sequence-independent antiviral immunity in shrimp.
539 *Developmental & comparative immunology* 33(7), 806-810.
540 Li H., Li W.X. & Ding S.W. (2002). Induction and suppression of RNA silencing by an animal
541 virus. *Science* 296, 1319–21.
542 Li W.X., Li H., Lu R., Li F., Dus M. & Atkinson P. (2004). Interferon antagonist proteins of
543 influenza and vaccinia viruses are suppressors of RNA silencing. *Proceedings of the*
544 *national academy of sciences* USA 101, 1350–5.
545 Li H.X., Meng X.L., Xu J.P., Lu W. & Wang J. (2005). Protection of crayfish, *Cambarusclarkii*,
546 from white spot syndrome virus by polyclonal antibodies against a viral envelope fusion
547 protein. *Journal of fish diseases* 28,285–291
548 Li L.J., Yuan J.F., Cai C.A., Gu W.G. & Shi Z.L. (2006). Multiple envelope proteins are
549 involved in white spot syndrome virus (WSSV) infection in crayfish. *Archives of virology*
550 114, 1–9.
551 Lightner D.V. (Ed.). (1996). A Handbook of Shrimp Pathology and Diagnostic Procedures for
552 Diseases of Cultured Penaeid Shrimp (Baton Rouge, LA:World Aquaculture Society),
553 305 pp.
554 Liu F., Liu Y., Li F., Dong B. & Xiang J. (2005). Molecular cloning and expression profile of
555 putative antilipopolysaccharide factor in Chinese shrimp (*Fenneropenaeus chinensis*).
556 *Marine Biotechnology* 7, 600–608.

Review Copy

557 Mantel L.H. & Farmer L.L. (1983). Osmotic and ionic regulation. In: Farmer, L.L. (Ed.), Internal
558 Anatomy and Physiological Regulation. Academic Press, New York, 53–161.
559 Mathew S., Kumar K.A., Anandan R., Viswanathan, Nair P.G. & Devadasan K. (2007). Changes
560 in tissue defence system in white spot syndrome virus (WSSV) infected *Penaeus*
561 *monodon*. *Comparative biochemistry and physiology Part C* 145, 315–20.
562 Meurs E., Chong K., Galabru J., Thomas N.S., Kerr I.M. & Williams B.R. (1990). Molecular
563 cloning and characterization of the human double-stranded RNA-activated protein kinase
564 induced by interferon. *Cell* 62, 379–90.
565 Misquitta L. & Paterson B.M. (1999). Targeted disruption of gene function in Drosophila by
566 RNA interference (RNAi): a role for nautilus in embryonic somatic muscle formation.
567 *sciences* USA. 96, 1451–6.
568 Munoz M., Cedeno R., Rodriquez J., Van der knap W., Mialhe E. & Bacher E. (2000).
569 Measurement of reactive oxygen intermediate production in haemocytes of Penaeid
570 shrimp, *PenaeusVannamei*. *Aquaculture* 91, 89–107.
571 Namikoshi A., Wu J.L., Yamashita T., Nishizawa T., Nishioka T., Arimoto M. & Muroga K.
572 (2004). Vaccination trials with *Penaeus japonicas* to induce resistance to white spot
573 syndrome virus. *Aquaculture* 229, 25–35.
574 Nickerson K.W. & Van Holde K.E. (1971). A comparison of molluscan and arthropod
575 hemocyanin: I. Circular dichroism and absorption spectra. *Comparative biochemistry and*
576 *physiology Part B* 39, 855–872.
577 Pequeux A. (1995). Osmotic regulation in crustaceans. *Journal of crustacean biology* 15, 1–60.
578 Perez F., Ortiz J., Zhinaula M., Gonzabay C., Calderon J. & Volckaert F.A. (2005). Development
579 of EST-SSR markers by data mining in three species of shrimp: Litopenaeusvannamei,
580 *Litopenaeusstylirostris*, and Trachypenaeus birdy. *Marine Biotechnology* 7, 554–569.
581 Pfaffl M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR.
582 *Nucleic acids research* 29(9):e45.
583 Ratcliffe N.A., Rowley A.F., Fitzgerald S.W. & Rhodes C.P. (1985). Invertebrate immunity:
584 Basic concepts and recent advances. *International review of cytology* 97, 183–350.
585 Rattanachai A., Hirono I., Ohira T., Takahashi Y. & Aoki T. (2005). Peptidoglycan inducible
586 expression of a serine proteinase homologue from kuruma shrimp (*Marsupenaeus*
587 *japonicus*). *Fish & shellfish immunology* 18, 39–48.

Review Copy

588 Robalino J., Browdy C.L., Prior S., Metz A., Parnell P., Gross P. &Warr G. (2004). Induction of
589 antiviral immunity by double-stranded RNA in a marine invertebrate. *Journal of virology*
590 78, 10442–10448.
591 Robalino J., Bartlett T., Shepard E., Prior S., Jaramillo G., Scura E., Chapman R.W., Gross P.S.,
592 Browdy C.L. & Warr G.W. (2005). Double-stranded RNA induces sequence-specific
593 antiviral silencing in addition to nonspecific immunity in a marine shrimp: convergence
594 of RNA interference and innate immunity in the invertebrate antiviral response?. *Journal*
595 *of virology* 79, 13561–13571.
596 Rosenberry B. (2002). World shrimp farming (San Diego: Shrimp News International).
597 Sachdev K.N. (1983). Clinical Pathology and Bacteriology. Jaypee Brother New Delhi.133 pp.
598 Sarathi M., Simon M.C., Ishaq Ahmed V.P., Rajesh Kumar S. & SahulHameed A.S. (2008).
599 Silencing VP28 gene of white spot syndrome virus of shrimp by bacterially expressed
600 dsRNA. *Marine Biotechnology* (NY). 10, 198–206.
601 Sarathi M., Simon M.C., Venkatesan C. & Hameed A.S. (2008a). Oral administration of
602 bacterially expressed VP28dsRNA to protect *Penaeus monodon* from white spot
603 syndrome virus. *Marine Biotechnology* (NY). 10, 242-249.
604 Schwarz K.B. (1996). Oxidative stress during viral infection: a review. *Free Radical Biology &*
605 *Medicine* 21, 641–9.
606 Silhavy D., Molnar A., Lucioli A., Szittya G., Hornyik C. & Tavazza M. (2002). A viral protein
607 suppresses RNA silencing and binds silencing-generated, 21 to 25 nucleotide double-
608 stranded RNAs. *Embo journal* 21, 3070–80.
609 Soderhall K. & Hall L. (1984). Lipopolysaccharide-induced activation of Prophenoloxidase
610 activating system in crayfish haemocyte lysate. *Biochimica et biophysica acta* 797, 99–
611 104.
612 Soderhall K., Smith V.J. & Johansson M. (1986). Exocystosis and uptake of bacteria by isolated
613 haemocyte populations of two crustaceans: evidence for cellular co-operation in the
614 defense reactions of arthropods. *Cell & Tissue Research* 243, 43–9.
615 Song Y.L. & Hsieh Y.T. (1994). Immunostimulation of tiger shrimp *Penaeus monodon*
616 haemocytes for generation of microbicidal substances: analysis of reactive oxygen
617 species. *Developmental & comparative immunology* 18, 201–09.

Review Copy

618 Song Y.L., Liu J.J., Chan L.C. & Sung H.H. (1997). Glucan-induced disease resistance in tiger
619 shrimp (*Penaeus monodon*). *Fish vaccines* 90, 413–421.
620 Takahashi Y., Kondo M., Itami T., Honda T., Inagawa H. & Niahizawa T. (2000). Enhancement
621 of disease resistance against penaeid acute viraemia and induction of virus-inactivating
622 activity in haemolymph of kuruma shrimp, *Penaeusjaponicus* by oral administration of
623 Pantoeaagglomerans lipopolysaccharide (LPS). *Fish & shellfish immunology* 10, 555–
624 558.
625 Tsutsui N., Ohira T., Kawazoe I., Takahashi A. & Wilder M.N. (2007). Purification of sinus
626 gland peptides having vitellogenesisinhibiting activity from the white leg shrimp
627 *litopenaeus vannamei*. *Marine Biotechnology* 9, 360–369.
628 Tuschl T., Zamore P.D., Lehmann R., Bartel D.P. & Sharp P.A. (1999). Targeted mRNA
629 degradation by double-stranded RNA *in vitro*. *Genes & development* 13, 3191–7.
630 Venegas C.A., Nonaka L., Mushiake K., Nishizawa T. & Muroga K. (2000). Quasi-immune
631 response of *Penaeus japonicus* to penaeid rod shaped DNA virus (PRDV). *Diseases of*
632 *aquatic organisms* 42, 83–89.
633 Vogt G. (1996). Cytopathology of Bay of Piran shrimp virus (BPSV), a new crustacean virus
634 from the Mediterranean Sea. *Journal of invertebrate pathology* 68, 239–45.
635 Warren A.A., Meola D.M., Wang Y., Guo X., Zhou L., Xiang J., Moss S., Arce S., Warren W.,
636 Xu Z. & Bell K. (2006). Isolation and mapping of telomericpentanucleotide (TAACC)n
637 repeats of the Pacific white leg shrimp, *Penaeus vannamei,* using fluorescence in situ
638 hybridization. *MarineBiotechnology* 8, 467–480.
639 Wei K.Q. & Xu Z.R. (2005). Effect of white spot syndrome virus envelope protein VP28
640 expressed in silkworm (*Bombyxmori*) pupae on disease resistance in *Procambarusclarkii*.
641 Shi Yan Sheng WuXueBao 38, 190–198.
642 Westenberg M., Heinhuis B., Zuidema D. & Vlak J.M. (2005). siRNA injection induces
643 sequence-independent protection in *Penaeus monodon* against white spot syndrome virus.
644 *Virus Research* 114, 133–139.
645 Williams B.R., Gilbert C.S. & Kerr I.M. (1979). The respective roles of the protein kinase and
646 pppA20 p50 A20 p5 A-activated endonuclease in the inhibition of protein synthesis by
647 double-stranded RNA in rabbit reticulocyte lysates. *Nucleic acids research* 6, 1335–50.

Review Copy

648 Xu J., Han F. & Zhang X. (2007). Silencing shrimp white spot syndrome virus (WSSV) genes by
649 siRNA. *Antiviral Research* 73, 126–131.
650 Yodmuang S., Tirasophon W., Roshorm Y., Chinnirunvong W. & Panyim S. (2006). YHV-
651 protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality.
652 *Biochemical and biophysical research communications* 341, 351–356.
653 Zamore P.D., Tuschl T., Sharp P.A. & Bartel D.P. (2000). RNAi: double stranded RNA directs
654 the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25–33.

Review Copy

Figure legend

Fig.1 Bacterially expressed dsRNA specific to VP28 gene of white spot syndrome virus. Lane M-100 bp Marker; Lane 1 - Control *E.coli*HT115(DE3) containing LITMUS38i vector withoutinsert; Lane 2 -Un-induced *E. coli*HT115(DE3) containing LITMUS38i with V28 gene; Lane 3 - IPTG induced *E.coli*HT115(DE3) containing LITMUS38i with V28 gene.

Fig.2 Prophenoloxidase activity in haemolymph of *L. vannamei* injected with dsRNA-VP28. Bar represents mean value for ten shrimp with standard error. Asterisk indicates significant difference (*$P < 0.05$) between control and treated shrimp.

Fig.3 Superoxide dismutase activity in haemolymph of *L. vannamei* injected with dsRNA-VP28. Bar represents mean value for ten shrimp with standard error. Asterisk indicates significant difference (*$P < 0.05$) between control and treated shrimp.

Fig.4 Superoxide anion level in haemolymph of *L. vannamei* injected with dsRNA-VP28. Bar represents mean value for ten shrimp with standard error. Asterisk indicates significant difference (*$P < 0.05$) between control and treated shrimp.

Fig.5 Malondialdehyde content in haemolymph of *L. vannamei* injected with dsRNA-VP28. Bar represents mean value for ten shrimp with standard error. Asterisk indicates significant difference (*$P < 0.05$) between control and treated shrimp.

Fig.6 Clotting time (in seconds) of haemolymph from of *L. vannamei* injected with dsRNA-VP28.

Fig.7 Total hemocyte count in *L. vannamei* injected with dsRNA-VP28 and normal shrimp.

Fig.8 Oxyhemocyanin level in haemolymph of *L. vannamei* injected with dsRNA-VP28 and normal shrimp.

Review Copy

Fig.9 The relative expression level of proPO (A), SOD (B), hemocyanin (C), crustin (D), BGBP (E), lipopolysaccharides (F), lectin (G), lysozyme (H), Toll receptor (I) and Tumor receptor (J) genes in haemocytes, gill and hepatopancreas of *L. vannamei* injected with dsRNA-VP28. Bar represents mean value for ten shrimp with standard error. Asterisk indicates significant difference (*P < 0.05) between control and treated shrimp.

Review Copy

Fig.1

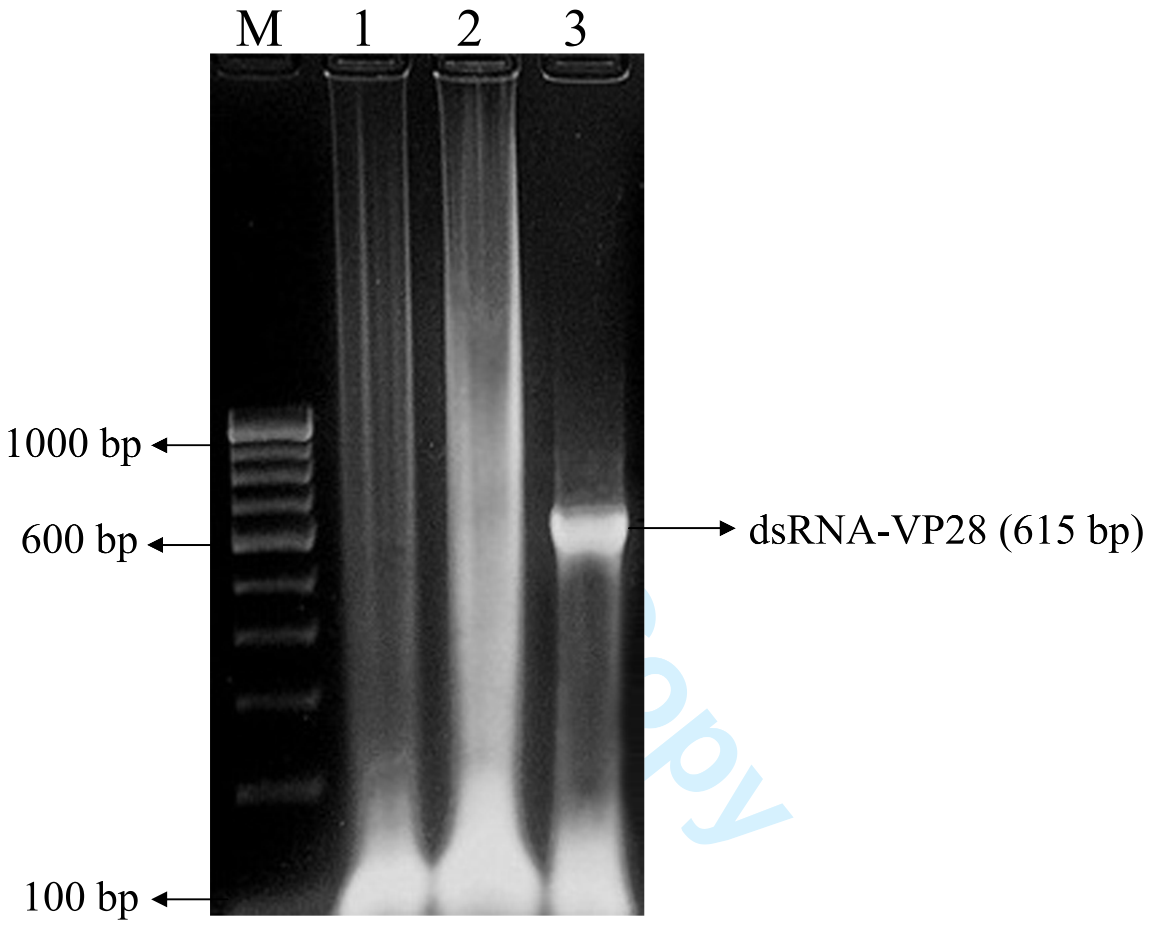


Fig.2

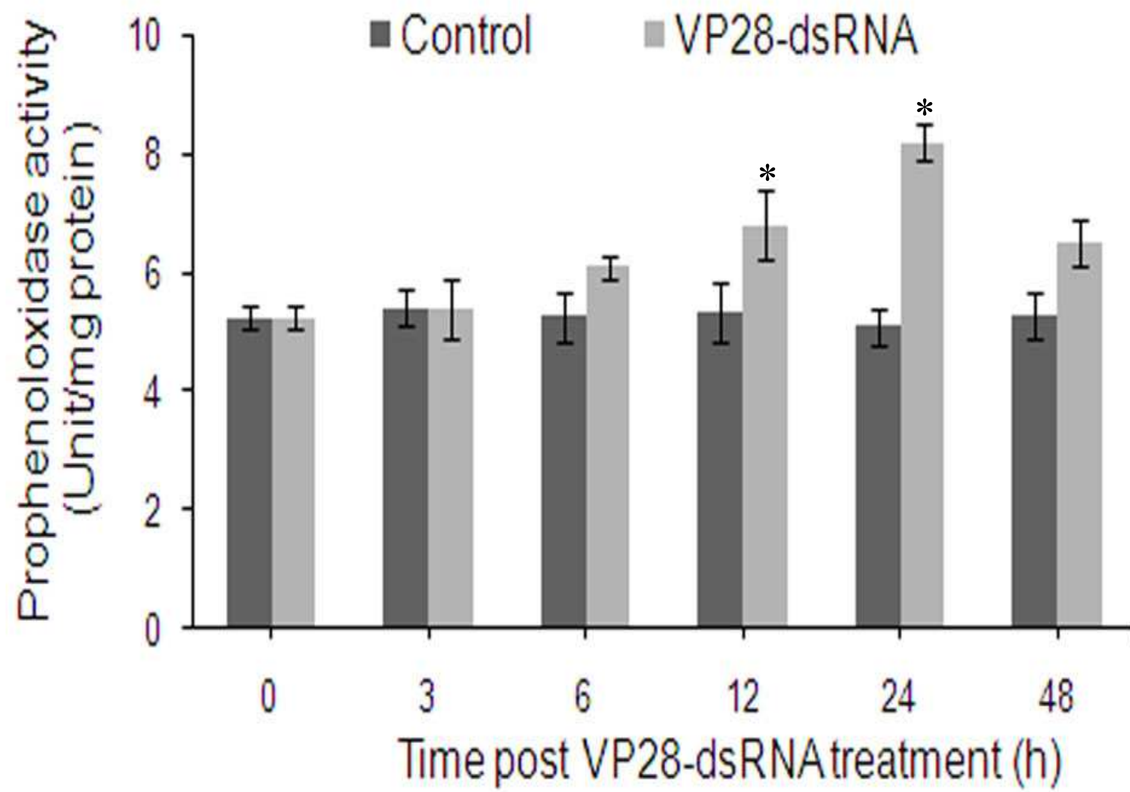


Fig.3

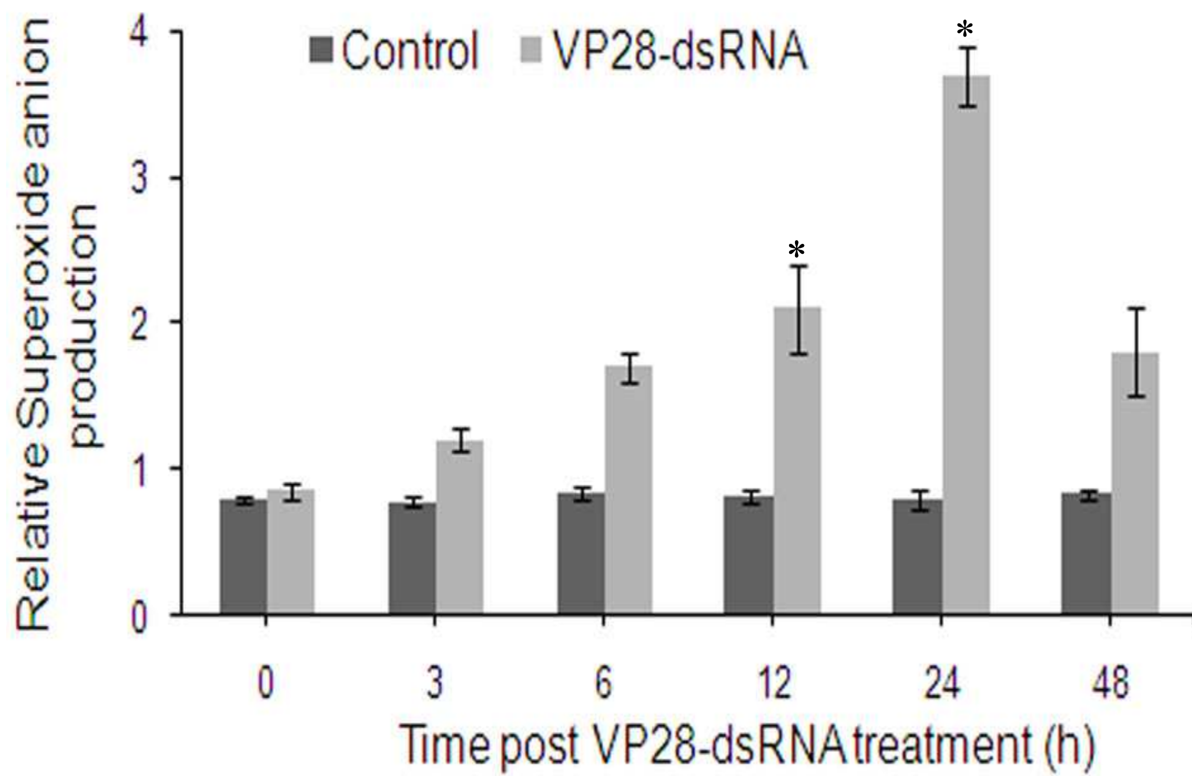


Fig.4

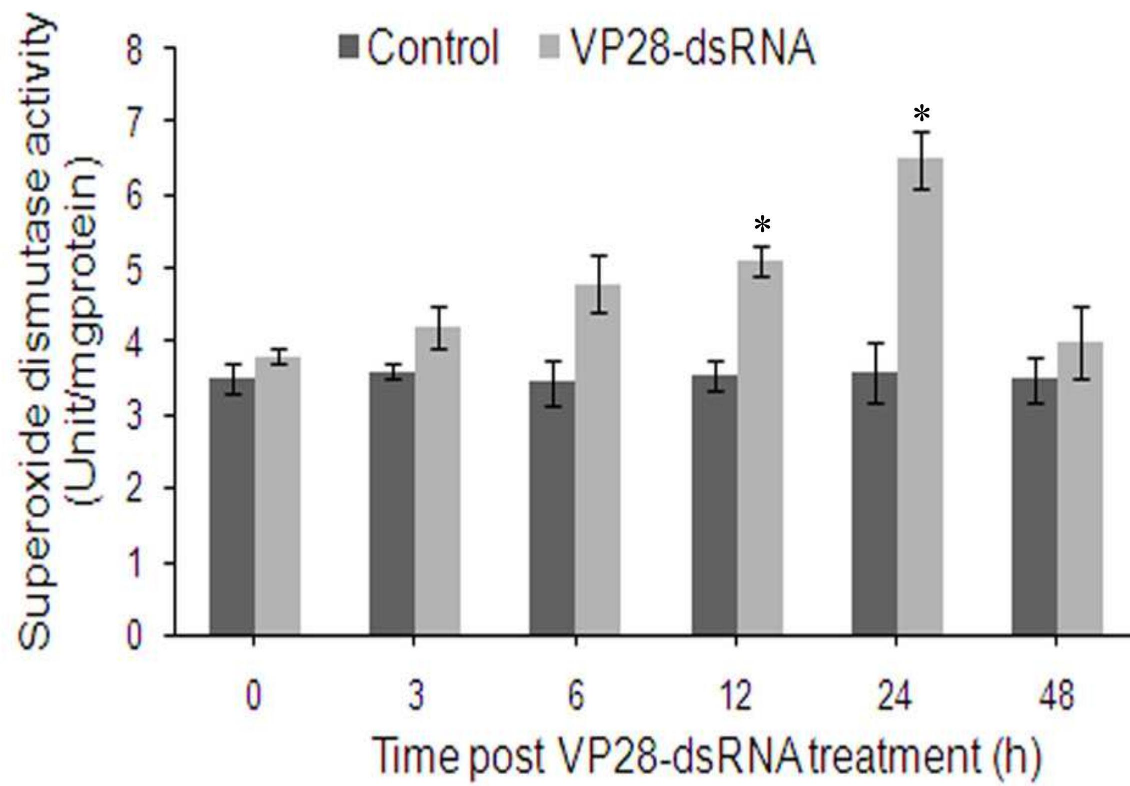


Fig.5

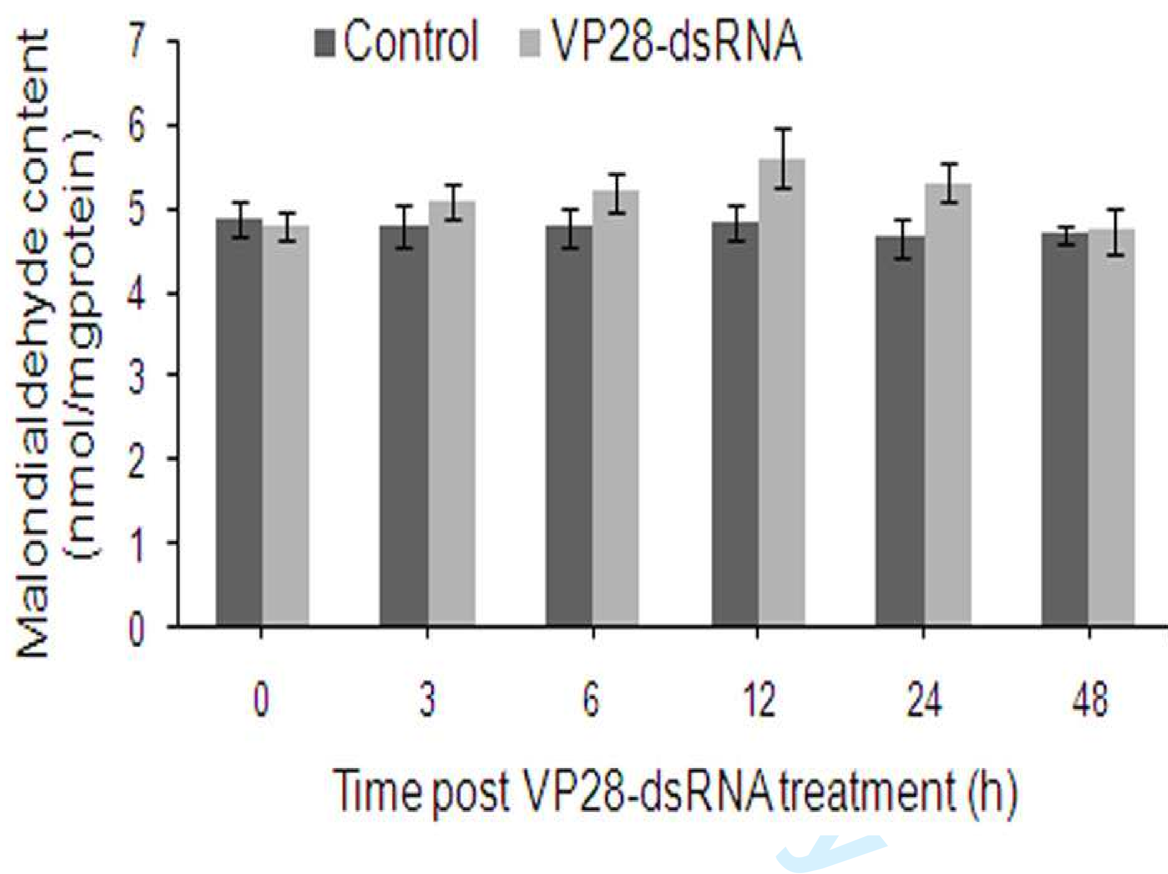


Fig.6

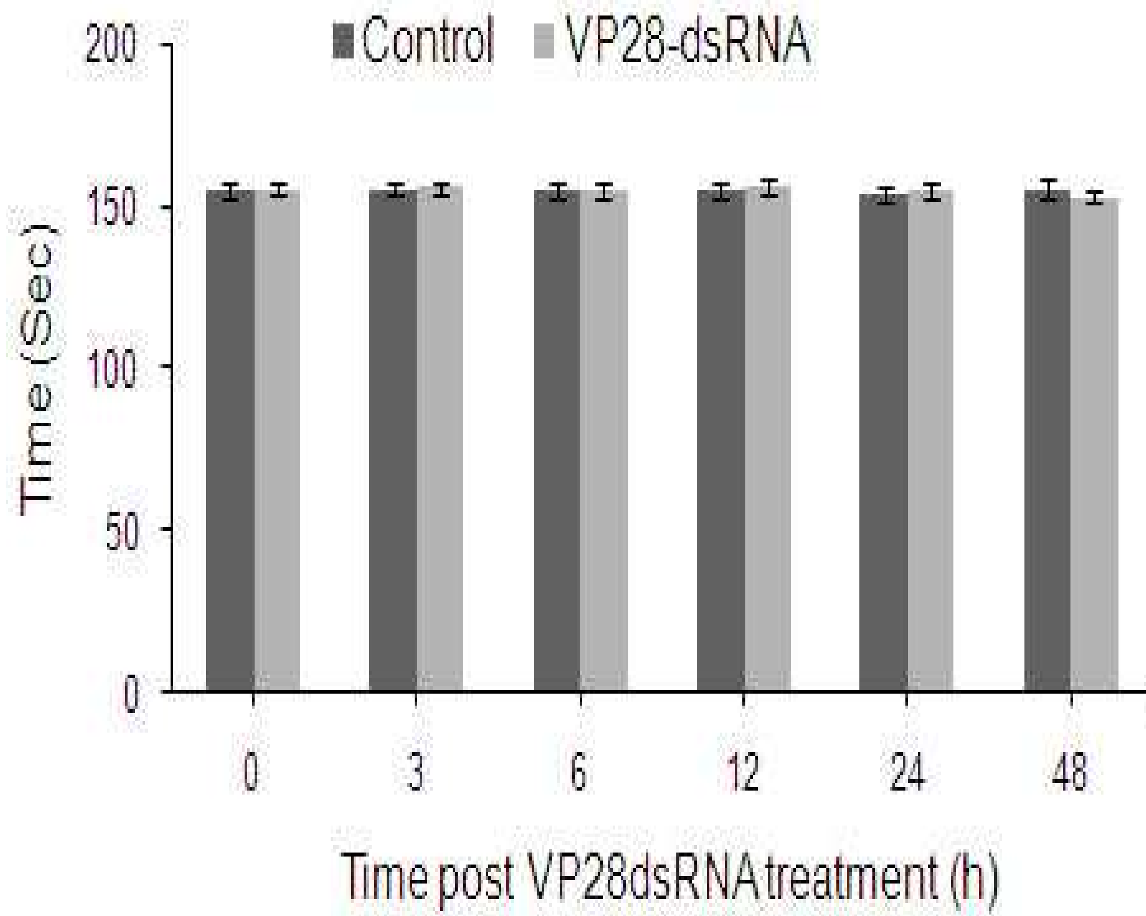


Fig.7

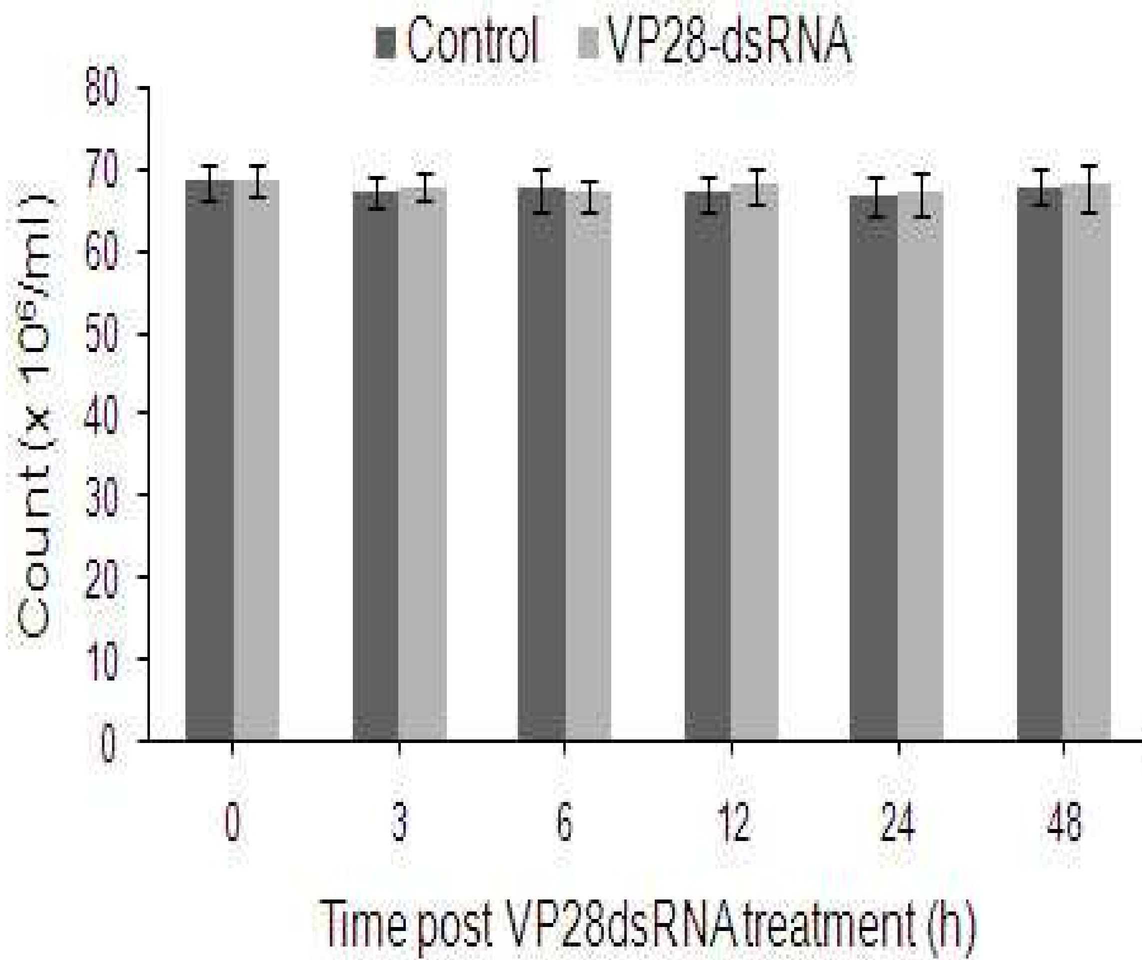


Fig.8

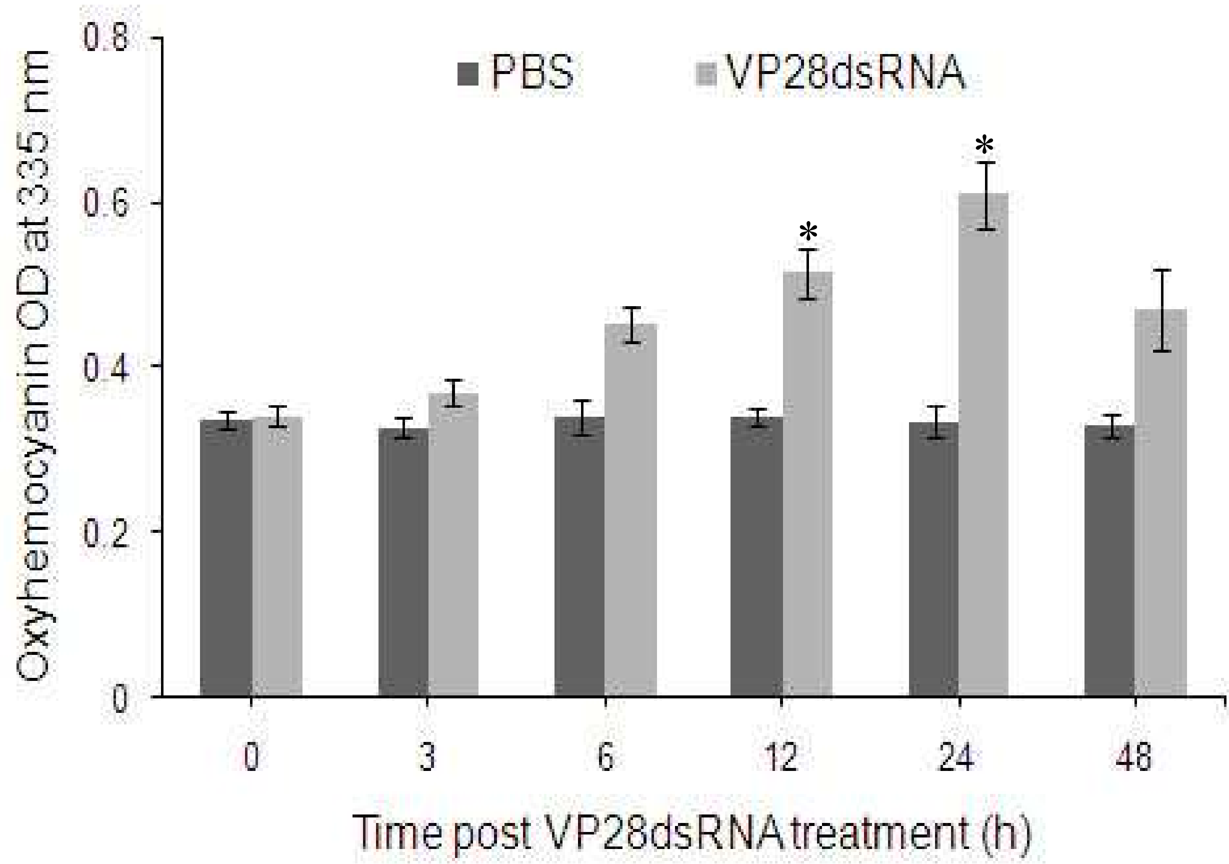
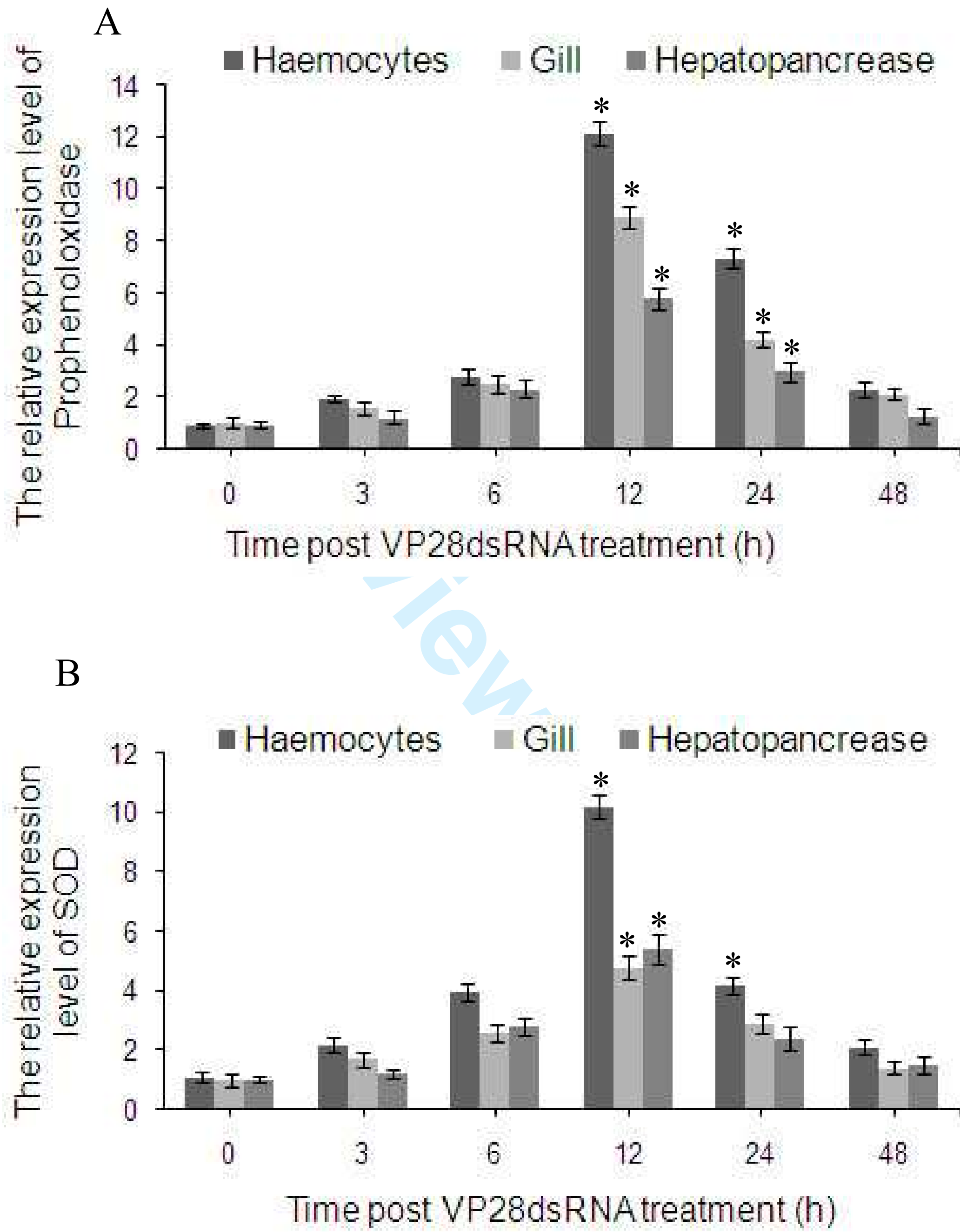
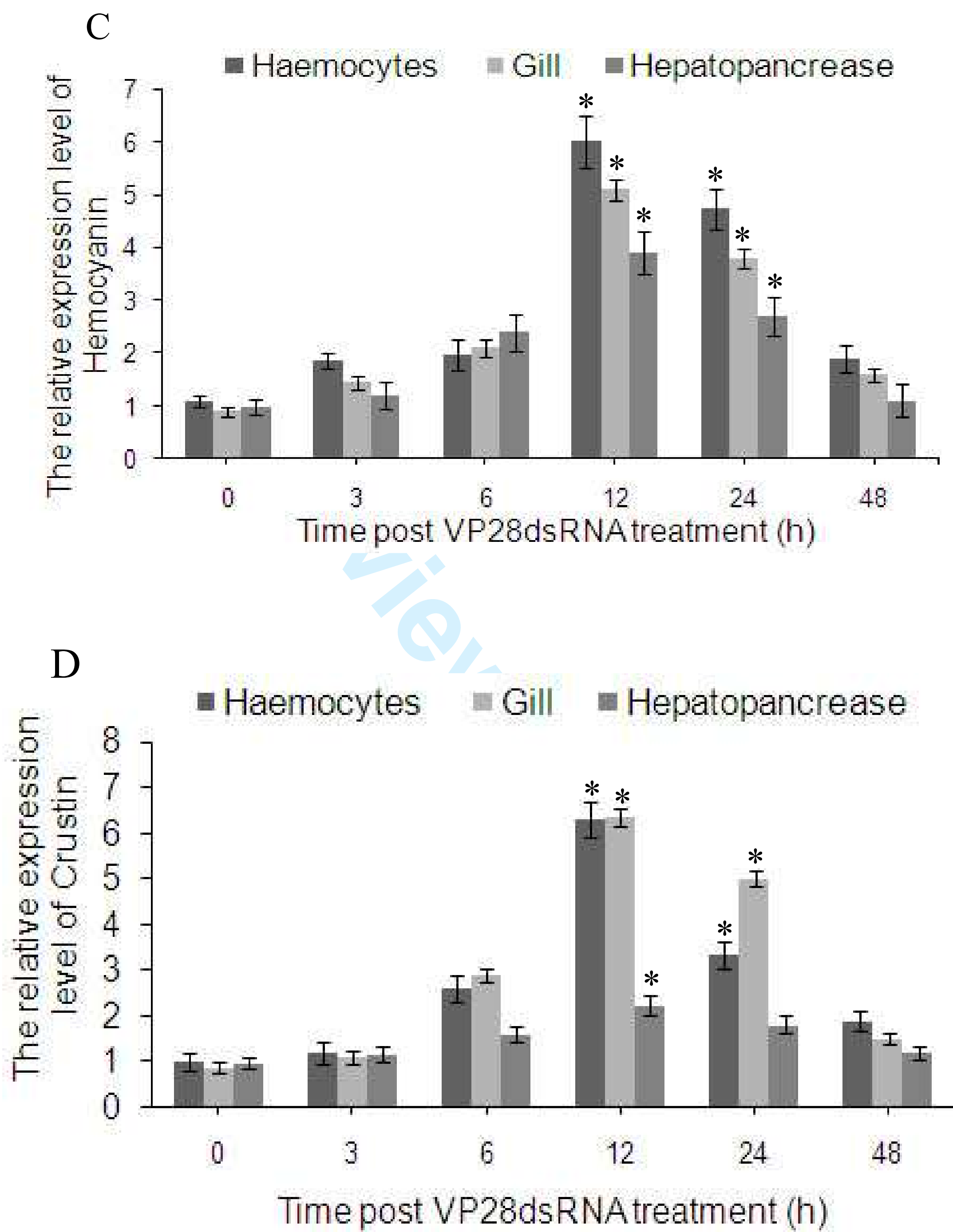
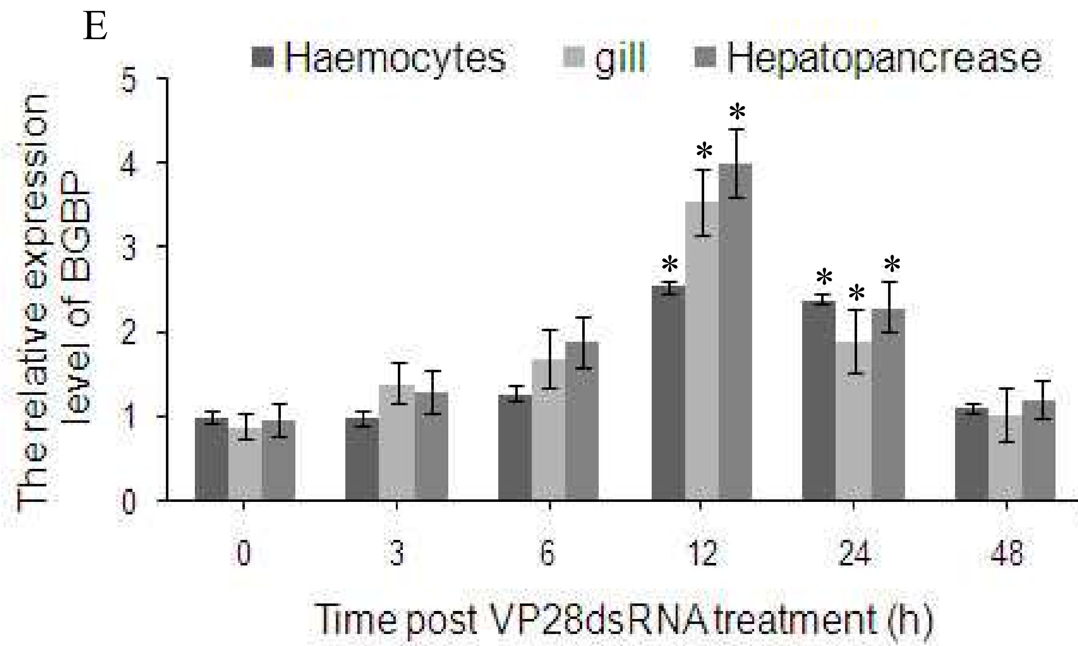


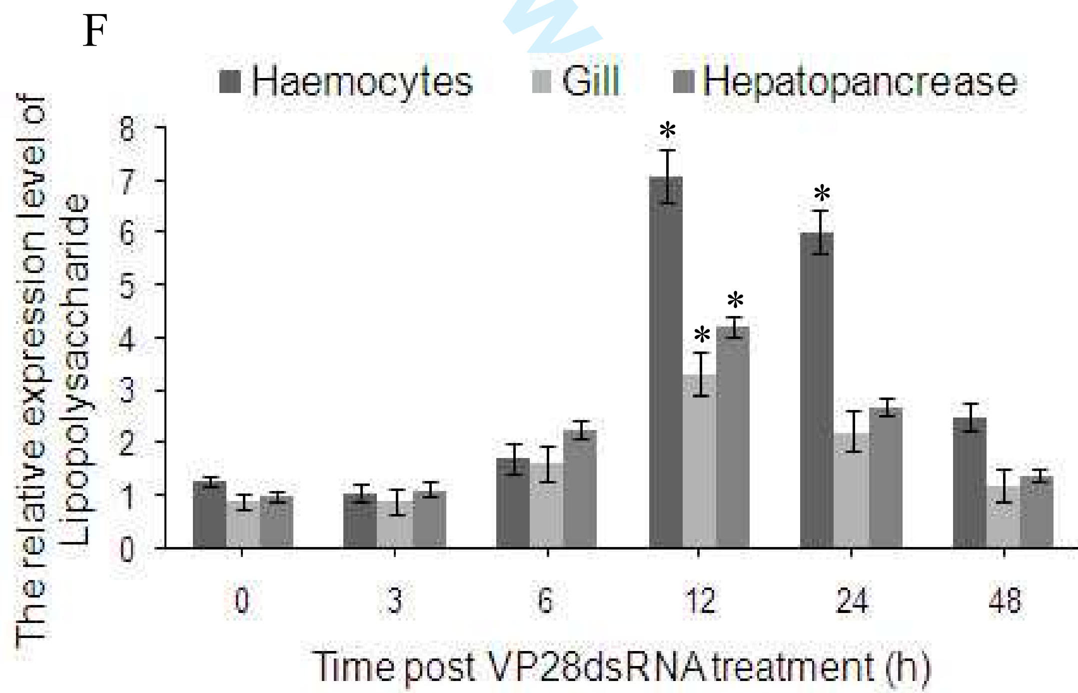
Fig.9

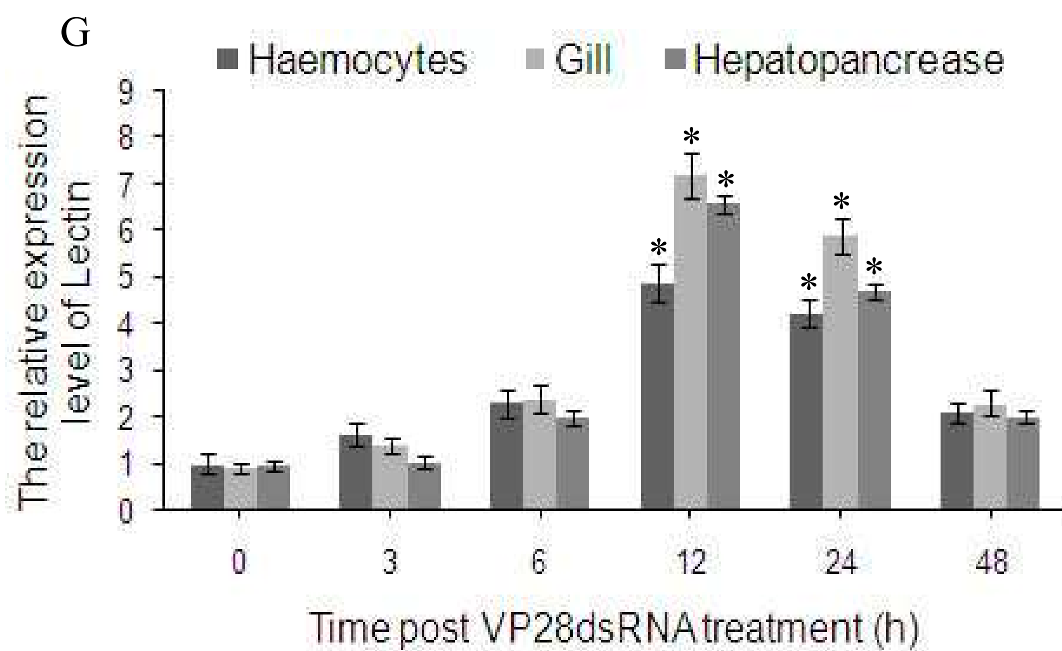


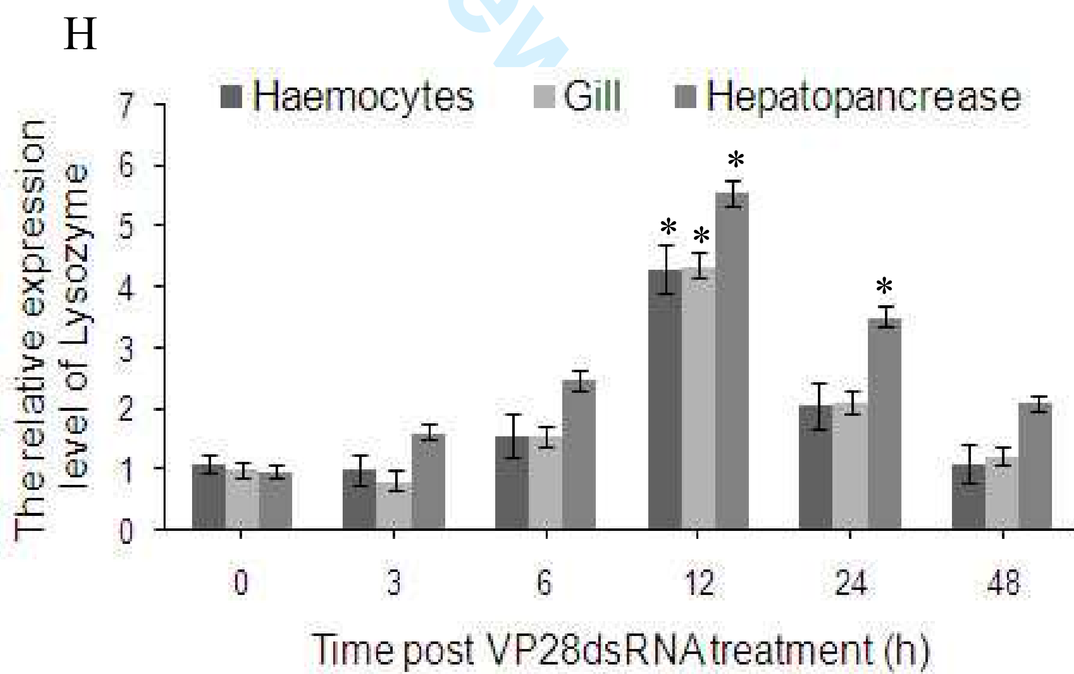


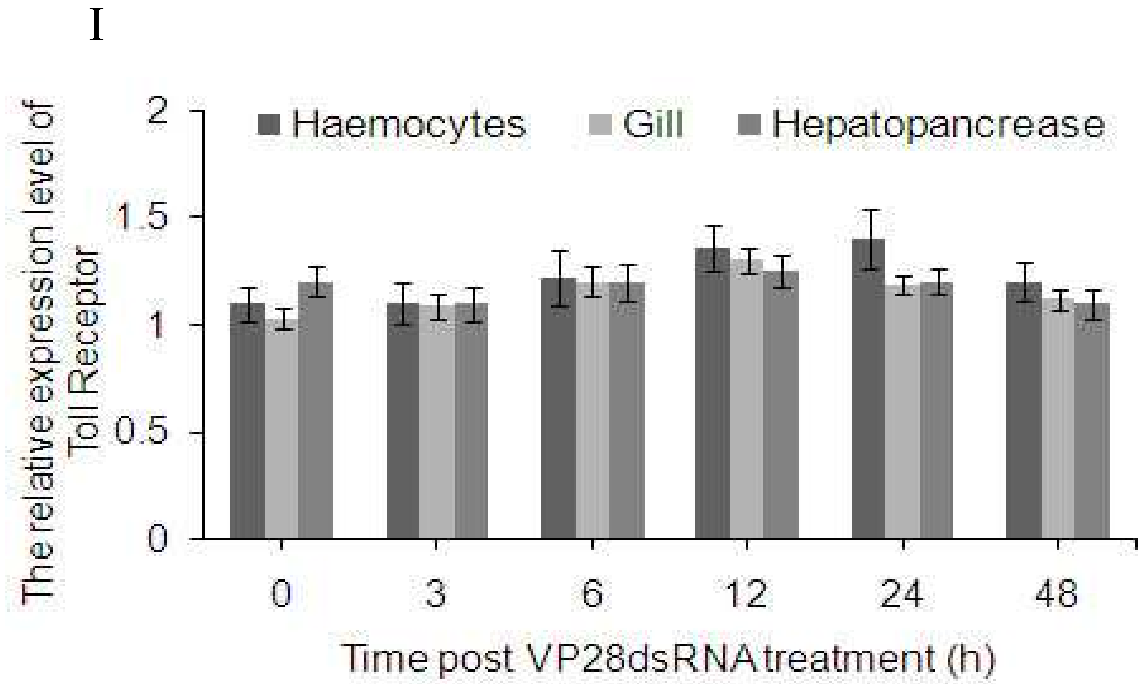
view


E
Haemocytes
gill
Hepatopancrease
The relative expression level of BGBP
5
4
3
2
1
0
*
0
3
6
12
24
48
Time post VP28dsRNA treatment (h)


F
Haemocytes
Gill
Hepatopancrease
The relative expression level of Lipopolysaccharide
8
7
6
5
4
3
2
1
0
*
0
3
6
12
24
48
Time post VP28dsRNA treatment (h)


G
Haemocytes
Gill
Hepatopancrease
The relative expression level of Lectin
Time post VP28dsRNA treatment (h)
0
3
6
12
24
48
0
1
2
3
4
5
6
7
8
9


H
Haemocytes
Gill
Hepatopancrease
The relative expression level of Lysozyme
Time post VP28dsRNA treatment (h)
0
3
6
12
24
48
0
1
2
3
4
5
6
7


I
The relative expression level of Toll Receptor
Haemocytes
Gill
Hepatopancrease
2
1.5
1
0.5
0
0
3
6
12
24
48
Time post VP28dsRNA treatment (h)

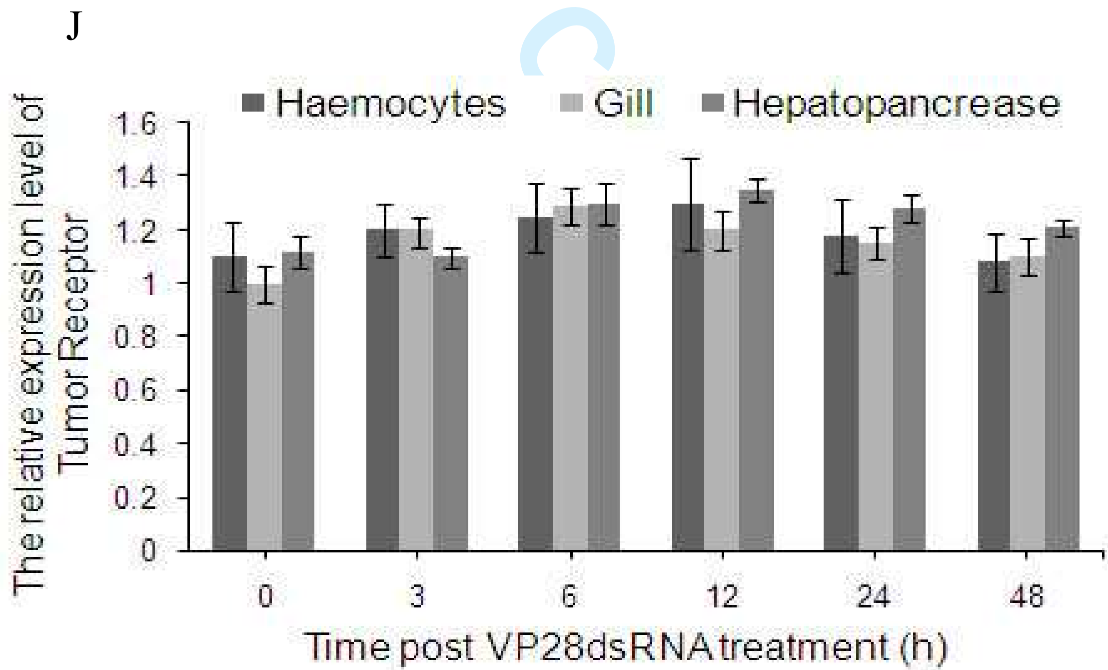

J
The relative expression level of Tumor Receptor
Haemocytes
Gill
Hepatopancrease
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
3
6
12
24
48
Time post VP28dsRNA treatment (h)

Table 1 Primers and their nucleotide sequences used immune genes in *Litopenaeus vannamei* in the present study.

Primers	Sequence (5'-3')	GenBank Accession No.
β-Actin F β-Actin R	AGTAGCCGCCCTGGTTGTAGAC TTCTCCATGTCGTCCCAGT	AF300705.2
Prophenoloxidase F Prophenoloxidase R	CTCCATTCCGTCCGTCTG CGGCTTCGCTCTGGTTAG	AY723296.1
cMnSOD F cMnSOD R	CGCGGATCCGATGGCTGAGGCAAAGGAA CCGGAATTCTGGGCAAACATCTGTGCTATCT	DQ005531.1
Crustin F Crustin R	GGCGGAGTAGGTGTTGGT TGTGGGCAGTCGAGTATCTT	AY486426.1
Lipopolysacharides F Lipopolysacharides R	CATGTCCAACTTCGCTTTCAGA ATCACCGCGTGGCATCTT	Chen et al., 2005
Lysozyme F Lysozyme R	CGCGGATCCGACTGATGCGGAAGCGACTA CCGGAATTCAGCCACCCAGGCAGAATA	AY170126.2
Lectin F Lectin R	TGTCGCCAGTGTTCGTTC CTACTTCATCTGTTGCTCCT	EF583939.1
BGBP F BGBP R	TACGGCTGCTCCCGAACT TACGAGGCAACATCGAAATA	AY249858.1
Toll Receptor F Toll Receptor R	CTATTGTGGTGCTTTCGT TCTCGTCCAACTCGCTCT	DQ923424.1
Tumor Receptor F Tumor Receptor R	CGCGGATCCGATGTTCACCGACACCTAT CCGGAATTCTTCCAACTTTGCCTTTAC	HM581680.1
Hemocyanin F Hemocyanin R	TCCTCGTCTCAACCACAA GCACATCAGGCACAGTAA	HQ709161.1

Review Copy

Table 2 Quantification of mRNA expression of immune-related genes in haemocytes, hepatopancreas and gill of shrimp injected with dsRNA-VP28 by real time PCR. Mean (±SE) values of cycle threshold (C_T)

Immune genes	Cycle threshold (C_T) values (Mean±SD) of triplicate assays for gene expression from normal and VP28dsRNA injected shrimp (*L. vannamei*)					
	Normal (0 h)	3 h	6 h	12 h	24 h	48 h
Crustin						
Haemocytes	31.77±0.78	30.95±0.27	29.38±0.14	27.38±1.20*	28.21±0.10*	30.44±0.90
Gill	32.45±0.33	32.05±0.61	30.29±0.28	28.90±1.62*	29.15±0.72*	31.87±1.13
Hepatopancreas	30.85±0.30	30.40±0.71	30.10±1.40	29.93±0.52*	30.12±1.03*	33.01±1.22
Lipopolysaccharide						
Haemocytes	22.97±0.06	22.50±0.20	21.73±1.04	18.27±0.22*	18.81±0.97*	21.90±0.36
Gill	26.79±0.19	26.88±0.41	25.99±0.72	24.65±0.92*	25.16±0.54*	26.01±1.35
Hepatopancreas	28.96±0.48	28.50±0.34	27.44±0.19	26.01±0.33*	27.20±1.02	27.98±0.28
Lysozyme						
Haemocytes	21.80±0.02	21.71±0.52	21.09±0.73	20.53±1.41*	20.70±0.20	21.01±1.26
Gill	27.17±0.35	27.54±0.56	26.57±0.33	25.87±0.90*	26.01±1.01*	26.99±0.81
Hepatopancreas	28.49±0.19	27.05±0.12	26.38±0.17	25.01±0.10*	25.42±0.29*	27.30±1.40
Tumor Receptor						
Haemocytes	32.98±0.39	31.92±0.51	30.84±0.54	31.33±0.25	31.10±0.39	31.90±0.82
Gill	31.92±0.41	31.41±0.19	31.02±0.41	30.47±0.34	31.12±0.64	31.57±0.70
Hepatopancreas	31.73±0.22	31.69±0.63	31.32±0.29	31.50±0.92	31.02±0.56	31.20±0.80
Lectin						
Haemocytes	32.55±1.24	31.10±0.12	30.89±0.72	29.34±0.22*	29.70±0.23*	31.90±1.04
Gill	31.76±0.45	30.78±0.74	30.01±0.29	27.56±0.17*	27.69±0.10*	30.97±0.58
Hepatopancreas	29.15±0.46	28.01±1.01	27.87±0.48	25.19±0.32*	25.31±0.10*	28.89±0.61
BGBP						
Haemocytes	29.23±0.18	30.66±0.31	29.01±0.11	27.62±1.57*	27.90±0.20*	28.75±0.29
Gill	31.10±0.64	31.06±0.76	30.76±0.65	28.77±0.70*	29.36±0.71*	30.95±0.93
Hepatopancreas	28.70±0.18	28.31±0.74	28.04±0.18	27.44±0.29*	27.79±0.48*	28.20±0.80

Table 2 Continued

Toll Receptor						
Haemocytes	25.75±0.46	25.13±0.30	24.42±0.38	24.65±0.81	24.13±0.17	24.90±1.20
Gill	30.88±0.13	30.59±0.46	30.10±0.27	29.55±0.63	29.13±0.99	29.96±0.30
Hepatopancreas	32.80±0.14	32.10±0.61	31.95±0.10	31.30±0.60	31.18±0.12	31.83±0.38
Prophenoloxidase						
Haemocytes	30.84±0.27	29.89±0.25	29.22±0.80	25.18±0.22*	26.65±0.84*	29.79±1.03
Gill	32.51±0.17	31.47±0.50	30.12±0.91	27.10±0.47*	29.96±0.22*	31.93±1.25
Hepatopancreas	31.53±0.37	30.75±0.61	30.95±1.08	28.44±0.40*	29.10±0.79	30.66±0.19
Hemocyanin						
Haemocytes	30.99±0.51	30.11±0.30	29.35±0.14	27.76±0.65*	28.01±0.26*	30.20±0.80
Gill	29.77±0.24	29.40±0.14	28.90±0.61	26.34±0.44*	28.80±0.75*	29.10±0.24
Hepatopancreas	28.98±0.05	28.66±0.19	28.14±0.37	27.10±0.14*	27.50±0.16*	28.42±0.28
SOD						
Haemocytes	33.84±0.20	32.68±0.23	31.98±0.63	29.12±0.10*	31.68±0.53	33.41±0.82
Gill	31.98±0.43	31.55±0.09	31.12±0.12	30.02±0.97*	31.50±0.18	31.88±0.64
Hepatopancreas	32.05±0.36	31.80±0.15	30.96±0.83	30.15±0.42*	31.11±0.35	31.90±0.40
β-Actin						
Haemocytes	27.33±1.20	27.56±0.79	27.95±1.20	27.82±0.19	28.15±0.71	28.01±0.95
Gill	28.01±0.43	28.12±0.48	28.10±0.70	28.08±0.71	28.90±0.50	29.01±0.20
Hepatopancreas	28.89±0.52	29.01±1.02	29.15±0.91	28.98±0.18	28.88±0.27	28.40±0.70