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Expression of immune-related genes in the oyster *Crassostrea gigas* during ontogenesis

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Abstract:

The work presented here reports the expression of immune-related genes during ontogenesis in the oyster *Crassostrea gigas*. Expression patterns of 18 selected genes showed that RNAs detected in oocytes and 2–4 cell embryos are of maternal origin and that gene transcription starts early after fertilization. The expression patterns of 4 genes (*Cg-timp*, *Cg-tal*, *Cg-EcSOD* and *Drac3*) suggested that hemocytes appear in the gastrula-trochophore stages. The localization of *Cg-tal* expression suggested that hematopoietic cells were derived from vessels and/or artery endothelia cells. Moreover, a bacterial challenge affected the level of expression of genes. Indeed, a change in expression levels was observed for *Cg-LBP/BPI*, *Cg-timp*, *Drac3* and *Cg-MyD88* genes in larval stages upon exposure to non-pathogenic bacteria. In early juveniles, a modulation was also observed for *Cg-LBP/BPI*, *Cg-timp*, *Cg-MyD88* and for *Cg-tal*, according to the concentration of bacteria. Altogether, the results showed that studying the appearance of immunocompetent cells through their ability to express immune-related genes is a tool to gain insight the ontogenesis of the oyster immune system.

Keywords: Bivalve mollusk; Hemocytes; Mollusk immunity; Hematopoiesis; Cell proliferation; Rel/NF-κB pathway

1. Introduction

In adult mollusks, the immune system is based on an innate system [1-3]. The internal defense mechanisms can be split into cell-mediated and humoral mechanisms: it has become increasingly apparent that both are interrelated and closely associated with hemocytes which are the main immunocompetent cells [4-7]. Hemocyte populations in bivalve mollusks have been the subject of extensive studies but the localization of hematopoietic site(s) remain(s) uncertain. The generally accepted belief is that hemocytes rise from differentiation of connective tissue cells [5].

To gain insight into the immune response in oysters, genomic approaches have been developed and therefore several immune related genes have been characterized in *Crassostrea gigas*. Escoubas [8] and Montagnani [9] isolated and characterized two proteins, oIKK (oyster IκB kinase like protein) and *Cg-rel*, which share structural and functional properties with elements of the Rel/NF-κB pathway in vertebrates and in *Drosophila* [10,11]. Moreover, three additional cDNAs of the Rel/NF-κB pathway were isolated including the adapter proteins *Cg*-MyD88, *Cg*-ECSIT, *Cg*-TRAF3 [9,12]. Based on homology with the Rel/NF-κB pathway in insects, the function of the Rel/NF-κB pathway in oysters may serve to regulate genes that are involved in innate defense and/or development [10,13]. A transcription factor, Tal1/SCL, belonging to the transcription factor class of bHLH (basic helix-loop-helix), has been characterized and has been shown to be specifically expressed in adult oyster hemocytes [14]. Moreover, several effectors have been studied for their potential involvement in the oyster immune response, e.g. *Cg-timp*, a tissue inhibitor of metalloproteinase [15], that is expressed specifically in hemocytes and is inducible after bacterial challenge and shell damage. *Cg-timp* may be an important factor implied in defense mechanism and in wound healing like in Vertebrates [16]. A second effector is *Cg-EcSOD*, an extracellular superoxide dismutase

65 which is involved in LPS-binding and which is also specifically expressed in hemocytes [17].
66 In addition, *Cg-def*, *Cg-defh1* and *Cg-defh2* are antimicrobial peptides (defensins) isolated
67 from mantle edge and hemocytes, respectively [18,19]. *Cg-def* showed an unaffected
68 expression following a bacterial challenge, whereas *Cg-defh2* expression was affected.
69 While, the immune response has been characterized for the oyster adult, so far, during
70 ontogenesis very little is known. At the cellular level, Elston [20] described the presence of
71 phagocytic cells (referred as coelomocytes) in the visceral cavity of veliger larvae in
72 *Crassostrea virginica*. Moreover, Elston and Leibovitz [21] observed motile phagocytes
73 containing bacterial fragments in the visceral cavity, and recorded the extrusion of redundant
74 phagocytes through the velum in oyster larvae (*C virginica* and *C. gigas*). At the molecular
75 level, only Herpin [22,23] has identified and characterized the transforming growth factor β
76 (TGF β) superfamily that includes bone morphogenetic proteins (BMPs), activins (*Cg*-ALR1)
77 and TGF- β *sensu stricto*. These effectors would play a key role in numerous biological
78 processes including early embryonic development and immune regulation.
79 In this paper, 18 genes potentially involved in immune system in adult oyster and in
80 other organisms were selected in order to study the immune system during the oyster
81 development. The expression pattern of each gene was analyzed in various development
82 stages of *Crassostrea gigas*. The expression level of selected genes was also studied
83 following an experimental bacterial challenge.
84

2. Materials and methods

2.1. Oyster developmental stages

Oysters at various developmental stages were produced at the IFREMER hatchery, La Tremblade (France), from mature oysters (5 females and 3 males) collected at the oyster parks in Marennes-Oléron bay. Several genitors were used to take account oyster genetic variability at population level and consequently to minimize individual variability [24,25]. One hour after fertilization, embryos were placed into 150 L tanks at a density of 100 embryos/L. All steps of fertilization and rearing were performed in 0.2 µm-filtered seawater at 23°C and aerated under air bubbling. Three independent spawns were performed.

The following stages were identified microscopically and collected: oocytes before fertilization, 2-4 cell stage embryos, morula, blastula, gastrula, trochophore larvae, D-hinged larvae, veliger larvae, larvae aged of 7 and 22 days post-fertilization , metamorphosing larvae and spat (Fig. 1). For total RNA extraction, one million individuals were pooled from oocytes to D-hinged larvae stages; whereas for the later developmental stages only 250,000 individuals were collected. For the spat, total RNA from three organisms was extracted in individuals.

2.2. Experimental bacterial challenge

Three non-pathogenic bacterial strains (*Vibrio tasmaniansis*, *V. anguillarum* and *Micrococcus luteus*) were grown separately overnight at 20-25°C in saline peptone water (peptone 15g/L ; NaCl 15g/L) for *Vibrio* strains, or at 30°C in Luria-Bertani medium for *M. luteus*. Experimental bacterial challenges were carried out by immersing the 200,000 embryos or larvae from 3 independent biparental fertilizations, in 2.5 L aquarium filled with filtered seawater at 20°C under air bubbling. The experimental exposure was performed with a

109 mixture containing the three strains at 3.10^7 live bacteria/L. Non exposed embryos and larvae
110 were used as the unchallenged controls. Samples were collected 10 and 24 h post-challenge
111 for RNA extraction. For spats of 3 months old, 50 individuals per experimental condition
112 were used. Experimental exposure was carried out by immersing early juvenile oysters in 20
113 L seawater tanks with the bacterial mixture described above (3.10^7 and 5.10^8 bacteria/L). In
114 parallel, some spats were not exposed and used as controls. Samples were collected 24 h after
115 bacterial challenge then processed for RNA extraction.
116
117 *2.3. RNA extraction and reverse transcription*
118 Total RNA was extracted using TRIzol™ Reagent (Invitrogen) according to the
119 manufacturer's protocol. After total RNA treatment with DNase I (2U, Invitrogen), a second
120 TRIzol extraction was achieved to inactivate DNase. The total RNA concentrations were
121 determined by OD_{260} measurements and the RNA integrity was checked by electrophoresis.
122 Two µg of total RNA extracted from each developmental stage were reverse transcribed using
123 200 units of M-MLV reverse transcriptase (Invitrogen) according to the supplier's
124 instructions.
125
126 *2.4. In situ hybridization (ISH)*
127 All samples were fixed in a solution containing 35% formaldehyde, 40% ethanol and 2%
128 ammonium hydroxide. After dehydration, animals were embedded in Paraplast and seriate 7
129 µm sections were cut, mounted on poly-L-lysine coated slides, and stored at 4°C until use.
130 Digoxigenin (DIG)-UTP-labelled and [^{35}S]UTP-labelled anti-sense and sense riboprobes were
131 generated from linearized DNA plasmids by *in vitro* transcription using RNA labelling kits,
132 T3 and T7 RNA polymerase (Roche) and [^{35}S]UTP (Amersham). DIG-labelled riboprobes

(40–100 ng/slide) and ^{35}S-labelled riboprobes (10^6 c.p.m. per slide) were hybridized on tissue sections according to the previously developed protocol [26].

2.5. Conventional polymerase chain reaction (*PCR)*

Primer sequences, designed with primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were selected to amplify products in the size range of 125 to 391 bp. Primer sequences are showed in Table 1. PCR were performed using buffer (2 mM $MgCl_2$), dNTPs (0.4 mM of each), primers (0.2 µM of each), 1 unit of Taq polymerase (Promega), first strand cDNA sample (0.5 µl), and water. All reactions were performed in 25µl volume in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) for 35 cycles. Each sample was amplified twice from each independent spawns. Ten µl of the amplification products were driven on agarose gel (1.5%). The elongation factor 1α (*Cg-EF-1α*) cDNA [27] was amplified for every sample as a positive control for PCR.

2.6. Quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) analyses were performed using the Lightcycler system version 3.5 (Roche Molecular Biochemicals) with the Lightcycler-FastStart DNA Master SYBR Green I (Roche Applied Science). Q-PCR reactions were achieved for 40 cycles in 10µl volume with final concentration 1X Lightcycler-FastStart DNA Master SYBR Green I (3 mM $MgCl_2$), primers (0.5 µM of each) and first strand cDNA (0.5 µl). The primer sequences, designed with primer 3 software, and conditions are listed in Table 2. Melting curve analysis was performed with continuous fluorescence acquisition (65 to 95°C at a temperature transition rate of 0.05°C/s) to determine the amplification specificity. Amplification efficiencies (E) were calculated according to the following equation: $E=10^{(-1/slope)}$ [28].

158 The relative expression level of each gene during the oyster ontogenesis was calculated
159 for 100 copies of the housekeeping gene (*Cg-EF-1α*) by using the following formula:
160 $N = 100 \times 2^{(Ct EF\text{-}1\alpha - Ct\ target\ gene)}$ [29].
161 For the challenged developmental stages, the relative level of target gene expression was
162 based on a comparative method [28,30]. The relative quantification value of sample,
163 normalized to the *Cg-EF-1α* gene (internal control) and relative to the unchallenged sample, is
164 expressed as $2^{-\Delta\Delta Ct}$, where ΔCt = (Ct (target gene) – Ct (*EF-1α*) and $\Delta\Delta Ct = \Delta Ct$ (challenged
165 sample) - ΔCt (unchallenged sample).
166
167 *2.7. Statistical analysis*
168 Data were analyzed using ANOVA and Student's t-test and differences were considered
169 statistically significant at $p<0.05$. All results are represented as mean ± standard error.
170
171 *2.8. Selection of immune related genes*
172 In order to acquire data on the transcription of immune-related genes during development
173 in *C. gigas*, we have analysed the expression of 18 genes chosen from a *C. gigas* EST library
174 [12], identified by DD-RT-PCR (differential display reverse transcription PCR) [14,15] or
175 cloned by PCR using degenerated primers [9]. Genes were selected according to their putative
176 function, such as their implication in antimicrobial response, in signaling pathways, in cellular
177 proliferation and in cell cycle (Table 3).
178
179 **3. Results**
180
181 *3. 1. Expression pattern of 18 immune-related genes during oyster ontogenesis*

First, a qualitative screening by PCR was performed in order to examine the expression patterns of each selected gene during development. Then, quantitative screening by Q-PCR was performed for 7 genes. The results for both PCR and Q-PCR analysis from the three independent spawns and three individual spats did not show significant differences meaning that the genetic variability of genitors did not affect the results.

Qualitative PCR was performed with total RNA isolated from 11 development stages (oocytes, 2-4 cell embryos, morula, blastula, gastrula, trochophore larvae, D-hinged larvae, veliger larvae (2 and 7 days), pediveliger larvae (22 days) and spat; Table 3). The reference gene, *Cg-EF-1α,* was amplified in all stages and in the three independent experiments. Thus *Cg-EF-1α* gene was used as a control.

The PCR results have led to divide the selected genes in 3 clusters (Table 3) according to transcript detection during development. In the first cluster, including the *Cg-def* (mantle), *Cg-defh2* (hemocyte) and *ficolin3* genes, no transcript detection was observed whatever the developmental stage. In the second cluster, including the *α-2 macroglobulin, MMP*, *Drac3*, *Cg-tal, Cg-timp and galectin 8* genes, the transcripts were always detected in early stages (oocytes and 2-4 cell embryos) and in older stages (trochophore to spat). Detection of transcripts was variable in morula, blastula and gastrula stages. In the third cluster, transcripts were systematically detected for every gene during all developmental stages. In this group, 4 components belong to signal transduction pathways, *i.e., Cg-MyD88*, *Cg-ECSIT*, *Cg-TRAF3* and *Cg-rel*. The 5 others comprised the *Cg-LBP/BPI*, *Cg-EcSOD*, *Ring3*, *Lyn* and *vav* genes.

Quantitative PCR expression patterns were performed for 7 genes (*Drac3*, *Cg-tal*, *Cg-timp*, *Cg-LBP/BPI*, *Cg-MyD88, Cg-EcSOD* and *galectin 8*) during various stages of development. These genes were chosen because they are representative of the clusters 2 and 3 previously described (Fig. 2). Moreover, these genes are involved in adult immune response as protease inhibitor (*Cg-timp)*, as recognition receptors by binding to lipopolysaccharide and

207 saccharide ligands on the microbial surface to trigger a host immune response (*Cg-LBP/BPI*,
208 *Cg-EcSOD* and *galectin 8*), as proteins involved in cell proliferation (*Drac3* and *Cg-tal*), and
209 as proteins of signaling pathways (*Cg-MyD88)* (Table 3).

210 A transcript level decrease of *Drac3*, *Cg-tal*, *Cg-timp* and *galectin 8* (cluster 2), *Cg-*
211 *LBP/BPI*, *Cg-MyD88* and *Cg-EcSOD* (cluster 3) were observed in morula, blastula and
212 gastrula stages (Fig. 2) compared to previous stages (oocyte, 2-4 cell embryo). *Drac3* and *Cg-*
213 *tal* transcript abundance significantly increased ($p<0.05$) in the trochophore larvae compared
214 to gastrula stage expression levels. From the D-hinged larvae to the spat, while the *Drac3*
215 transcript level decreased significantly ($p<0.05$) but not uniformly, the *Cg-tal* transcript level
216 increased progressively and significantly ($p<0.05$) (Fig. 2). *Cg-timp* transcript level (Fig. 2)
217 increased 25-fold in the pediveliger larvae ($p<0.05$) comparatively to D-hinged larvae. This
218 increase was followed by a significant decrease of the level in spat ($p<0.05$). Concerning *Cg-*
219 *LBP/BPI*, the number of transcripts increased significantly from trochophore larvae to spat
220 ($p<0.05$). For *Cg-MyD88,* transcript level increased significantly ($p<0.05$) from D-hinged
221 larvae to pediveliger larvae. The highest level of expression was observed in the
222 metamorphosing larvae. Finally, both for *Cg-EcSOD* and *galectin 8* effectors, transcript
223 numbers strongly increased in metamorphosing larvae and spat. In this older stage increases
224 of 177-fold and 50-fold were seen for *Cg-EcSOD* and *galectin 8*, respectively.

225

226 *3. 2. Localization of Cg-timp, Cg-EcSOD, Cg-tal, and Drac3 expression*

227 In order to identify the potential site of hematopoiesis and to better understand the
228 immune response, transcripts of 4 genes (*Cg-timp, Cg-EcSOD, Cg-tal,* and *Drac3)* were
229 detecting using *in situ* hybridization (ISH) in 4 larval stages (D-hinged larvae, veliger larvae
230 (2 and 7 days), pediveliger larvae) and in spat.

No labelling was obtained whatever the larval stages and the labelling methods (DIG-UTP or [^{35}S] UTP) for the 4 tested genes, while unlabelled hemocytes were always observed. In spat, a strong hybridization signal was observed for *Cg-EcSOD* and *Cg-tal* only (Fig. 3). *Cg-EcSOD* hybridization signal was localized in hemocytes attached to blood vessel endothelium, in circulating hemocytes and in infiltrating hemocytes in gills, mantle and digestive gland. *Cg-tal* hybridization labelling was observed only in hemocytes attached to blood vessel endothelium.

3. 3. Relative quantification of immune-related gene expression after a bacterial challenge during development

The impact of bacteria challenge was studied by evaluating the differential expression of the 7 genes studied above, i.e. *Cg-tal, Cg-LBP/BPI, Cg-timp, Drac3, Cg-MyD88, Cg-EcSOD* and *galectin 8*. The transcript levels of these genes were quantified by Q-PCR in 4 development stages (gastrula, D-hinged larvae, veliger larvae, pediveliger larvae). No expression difference was observed for *Cg-EF-1α* between unchallenged and challenged animals (data not shown) for all developmental stages. Hence, *Cg-EF-1α* was used to normalize the transcript levels.

The low levels of expression exhibited by *Cg-EcSOD* and *galectin 8* genes during development (Fig. 2) was unaffected by the bacterial challenge (data not shown). It appeared that the transcript number of *Cg-tal* increase in D-hinged and veliger larvae, however these differences were not statistically significant (Fig. 4).

Cg-LBP/BPI transcript abundance increase significantly (3 fold, $p<0.05$) in veliger larvae 24 h post stimulation comparatively to unchallenged animals. These results reveal that *Cg-LBP/BPI* expression is modulated by the bacterial challenge according to developmental stages (Fig. 4).

256 *Cg-timp* relative expression in the D-hinged larvae 24 h post stimulation (Fig. 4) was
257 more than 8 fold higher than in the control ($p<0.05$). In veliger larvae, a significant increase
258 of the *Cg-timp* transcript level (4 fold, $p<0.05$) was observed 10 h post stimulation. *Cg-timp*
259 expression was affected by the bacterial challenge.

260 A significant increase of the relative expression of *Drac3* ($p<0.05$, Fig. 4) was observed
261 both in D-hinged larvae and in veliger larvae (3 and 3.4 fold, respectively) 10 h post
262 stimulation. In pediveliger larvae, a significant increase of the relative expression (2.4 fold,
263 $p<0.05$) was obtained 24 h post stimulation. *Drac3* was the only gene to be significantly
264 inducible in pediveliger larvae among all the genes that were tested. These results showed a
265 *Drac3* transcript level increase 10 h post stimulation in early larval stages, whereas in
266 pediveliger larvae, just before the metamorphosis, the induction took more time since the
267 transcript level increased after 24h.

268 *Cg-MyD88* relative expression in D-hinged larvae was more than 6 and 33 fold higher 10
269 h and 24 h post stimulation, respectively compared to unchallenged animals ($p<0.05$, Fig. 4).
270 In veliger larvae, the transcript level was significantly higher ($p<0.05$) in challenged animals
271 compared to unchallenged ones (15 fold). The *Cg-MyD88* transcript abundance was strongly
272 increased in D-hinged and veliger larvae following bacterial stimulation.

273

274 *3. 4. Relative quantification of gene expression in spat after a bacterial challenge*

275 To investigate the possible inductibility and involvement in immune response of *Drac3*,
276 *Cg-EcSOD, galectin 8*, *Cg-timp*, *Cg-tal*, *Cg-LBP/BPI* and *Cg-MyD88* in spat, relative
277 expression of these genes were monitored using Q-PCR in whole challenged and
278 unchallenged individuals. The transcript abundance was measured 24 h after exposure (Fig 5).

279 *Drac3*, *Cg-EcSOD* and *galectin 8* gene expressions were not affected by bacterial
280 stimulations. *Cg-timp*, *Cg-tal* and *Cg-LBP/BPI* transcript levels were significantly higher (2.1,

281 2, 2.1 fold respectively, $p<0.05$) in challenged spats *vs* unchallenged spats following exposure
282 to 5.10^8 bacteria/L. *Cg-MyD88* relative expression, in challenged animals, was more than 1.7
283 and 4 fold higher with 3.10^7 and 5.10^8 bacteria/L respectively, than in unchallenged animals
284 ($p<0.05$). However, relative expression was significantly lower when animals were exposed to
285 3.10^7 bacteria/L than relative expression measured in animals exposed to 5.10^8 bacteria/L (2.5
286 fold). These results reveal that in spat, expression of some genes was affected by the bacterial
287 challenge and that the magnitude was dependent on the concentration of the bacteria.
288

4. Discussion

In oysters, differences in the susceptibility to pathogens are observed between larvae, juveniles and adults. While some studies of immunity in oyster adults have been performed [1,31], so far, little is known about the immune system ontogenesis [32,33]. In this study we investigated the expression of 18 genes, potentially involved in immunity during development of the pacific oyster and studied the impact of a bacterial challenge.

Expression pattern analysis led to classify the 18 genes into three clusters (Table 3). Most transcripts were detected in early stages (oocytes and 2-4 cell embryos) but not in later stages as morula, blastula and gastrula stages. Assuming that embryonic transcription in Mollusks only starts at the compacted morula stage [22], these results suggest that the transcripts detected during the first two developmental stages (oocytes and 2-4 cell embryos) are not produced by the embryo itself but are of maternal origin. RNAs of maternal origin are probably degraded in the older stages explaining the decreases observed in morula, blastula and gastrula stages. The early development of animals is programmed by maternally synthesized RNAs and proteins that are loaded into the developing oocyte by the mother [34]. Because the subsequent phases of embryogenesis require products encoded by zygotically synthesized transcript, these maternal RNAs are rapidly degraded but their stability is regulated in space as well as in time. The complete degradation of these transcripts is achieved differently according to the species [35,36]. Since, most likely the mechanisms that regulate transcript stability are evolutionarily conserved in all metazoa [36], it is assumed that maternal RNA might be regulated in a similar way in oysters.

Hemocytes, which are the immunocompetent cells of the oyster *Crassostrea gigas*, participate in a variety of functions [5]. To investigate the putative site of hemocyte generation during development, we quantified the expression and localized the expression

313 sites of *Cg-timp*, *Cg-EcSOD*, *Cg-tal* and *Drac3*. These genes were selected because in the
314 adult oyster they are specifically expressed in hemocytes and are, therefore, considered as
315 hemocyte markers [9,14,15,17]. During oyster development, *Cg-timp, Drac3 and Cg-tal*
316 transcription levels increase from the trochophore stage. These results suggest that hemocytes
317 are generated at the gastrula-trochophore stages, relatively early after fertilization (10-12 h).
318 At the cellular level, it has been shown that hemocytes are phagocytic cells attached to the
319 surface of visceral cavity in veliger larvae [20]. Increases of *Drac3* and *Cg-tal* expression
320 levels were observed in trochophore stage (Fig. 2). These results suggest that hematopoiesis
321 has been activated and could lead to an increase of hemocyte precursor cell number because
322 *Drac3* and *Cg-tal* are specifically markers of the hemocyte precursor cells. Sasamura, *et al*.,
323 [37] showed in *Drosophila* that *Drac3* is essential for hemocyte differentiation, and Barreau-
324 Roumiguière *et al*., [14] demonstrated that *Cg-tal* belongs to the Tal1/SCl family, which is
325 crucial for the generation of hematopoietic cells in early embryos [38-40]. Moreover, Tal1 is
326 one of the earliest markers of mammalian hematopoietic development *i.e*. its expression
327 precedes the formation of the hematopoietic sites in mouse [41].

328 During development, only *Cg-EcSOD* and *Cg-tal* expression sites were clearly observed
329 in serial sections from spat, suggesting that the ISH method is not sufficiently sensitive.
330 Detection with ISH is however dependent on the transcript number per cell. This could
331 explain the lack of detection of transcripts for some genes in early developmental stages. A
332 strong *Cg-EcSOD* positive ISH signal was observed in circulating and infiltrating hemocytes
333 throughout the animal. A *Cg-tal* signal was localized only in hemocytes attached to blood
334 vessel endothelium. No labelled cell was detected in connective tissue. By analogy with the
335 Tal/SCL role in hematopoiesis in other organisms, these results suggest that hematopoietic
336 cells could not arise from differentiation of connective tissue cells as suggested previously [5]
337 but could rather derive from the vessel or/and artery endothelia cells. Recently, it was

demonstrated in mouse, human and chicken embryos that aortic endothelia cells can also give rise to hematopoietic cells [42-44].

The quantification of *Cg-tal, Drac3*, *Cg-LBP/BPI*, *Cg-timp* and *Cg-MyD88* transcripts following a bacterial challenge showed that transcription of these genes was activated according to (i) the developmental stage, (ii) the time period between stimulation beginning and quantification, and (iii) the amount of bacteria used for challenge. This suggests that these genes are involved in protection against bacteria during development. In this study, the increase in *Cg-tal* transcription observed after bacterial stimulation in D-hinged, veliger larvae and spat could mean that haematopoiesis was activated and contributed to increase the number of hemocytes. This increase in *Cg-tal* expression was concomitant to that of *Drac3*. Since *Drac3* is essential for hemocyte differentiation in *Drosophila* [37], these results suggest that bacterial stimulation could activate hemocyte differentiation following proliferation. Moreover, since hemocytes are oyster immunocompetent cells capable of non-self recognition and where antimicrobial effectors are produced [1,5], their proliferation could contribute to reinforce the immune response against bacteria in oyster. The hemocyte proliferation should be involved in immune response in Drosophila [45,46] and crustacean [47,48].

Cg-LBP/BPI are thought to play a significant role in transducing cellular signal from LPS and thus involved in the immune response [49,50]. During oyster development, *Cg-LBP/BPI* transcripts were detected in all stages and a progressive increase in *Cg-LBP/BPI* transcription was observed from D-hinged larvae to spat. In the larvae, the visceral mass has two prominent components: the velum and the fluid-filled coelomic cavity containing the visceral organs [51]. Various epithelia, especially in the velum and the mantle constitute the first barrier against bacterial invasion. The progressive increase in *Cg-LPB/BPI* transcription seems to correspond to the apparition of larvae epithelia. These results suggest that *Cg-LBP/BPI* gene could participate actively in the first line of defense during development and also to an acute

363 immune response following a microbial infection since *Cg-LBP/BPI* transcription was
364 activated in larvae stages after a bacterial challenge. *Cg-LBP/BPI* transcription began 10 h
365 post-stimulation and increased at 24 h. This may result from a hemocyte proliferation, as also
366 sustained by the *Cg-tal* and *Drac3* expression increases.

367 *Cg*-Timp is a member of the tissue inhibitor metalloprotease family, which the most
368 widely recognized action is inhibition of matrix metalloproteases (MMPs). These genes
369 regulate the proteinaceous extracellular matrix homeostasis and a wide range of physiological
370 processes that include embryonic development, connective tissue remodeling, wound healing,
371 glandular morphogenesis and angiogenesis [52]. In this study, *MMP and Cg-timp* transcripts
372 were detected from the gastrula to the 22 days larvae suggesting that a local balance between
373 MMPs and TIMPs could be set up during the oyster development. The strong induction of
374 *Cg-timp* was observed in veliger and pediveliger larvae just before metamorphosis. During
375 metamorphosis, drastic morphological changes affect the specific larval organs [53]. These
376 results reinforced and completed the initial study of Montagnani *et al*., [32] and suggest that
377 *Cg-timp* plays an important role in tissue remodeling during the oyster metamorphosis. Like
378 in adults [15], *Cg-timp* transcription in D-hinged larvae, veliger larvae and in spat was
379 activated meaning that *Cg-timp* transcription is inducible in larvae and would participate in
380 host defense mechanism. In adult, optimal expression was obtained 9 to 12 h post-challenge
381 whereas in D-hinged larvae, the optimal expression was observed 24 h post-challenge. This
382 could result from the trigger of hemocyte proliferation and suggests that D-hinged larvae
383 immune system may not be completely mature.

384 *Cg-MyD88* is expressed in all development stages (Table 3), but expression level remains
385 relatively low and the increase observed in metamorphosing larvae is moderate. *Cg-MyD88*
386 would be involved in regulation of genes involved in development. Indeed, in *Xenopus,*
387 XMyD88 protein function is required for axis formation [54] and in *Drosophila*, MyD88 is

388 implicated in the dorsal-ventral pattern of embryo [55]. Interestingly, transcription level
389 increased strongly after bacterial challenge in all larval stages and in spat. The prominent role
390 of MyD88 in host innate immunity and inflammation has been studied in various vertebrates
391 and invertebrates species [56-58]. Our results indicate that *Cg-MyD88* may be a key
392 component of the immune response because of its role in the Rel/NF-κB signal transduction
393 pathway [9]. Components of this pathway plus other adapter proteins such as ECSIT, TRAF,
394 oIKK, IκB [8,9], appear to be similar to Toll or TLR/IL-1 signalling effectors in *Drosophila*
395 and mammals respectively [11]. In Drosophila and mammals, Toll and TLR/IL1 pathways
396 regulate the expression of many genes involved in various mechanisms of the immune
397 response [59].

398 *Cg-EcSOD* is weakly expressed during development except in metamorphosing larvae and
399 in spat, where it is strongly expressed (Fig. 2). This gene which encodes an extracellular
400 superoxide dismutase, was only detected in hemocytes both in adults [17,60] and during early
401 stages. Moreover, the transcript level was unaffected by bacterial challenge. These results
402 suggest that *Cg-EcSOD* is constitutively expressed and is part of defences against reactive
403 oxygen species (ROS) and especially superoxide anion [61,62].

404 Altogether, the results showed that studying the appearance of immunocompetent cells
405 through their ability to express immune-related genes is a tool to gain insight the ontogenesis
406 of the oyster immune system. First, we showed that while transcription of immune genes in
407 embryos is initiated during the compacted morula stage, it remained low in gastrula and was
408 unaffected following a bacterial challenge. This may be due to the fact that immune system is
409 rudimentary and immature in early embryonic stages. Second, the major maturation events
410 leading to immunocompetence occurred between D-hinged larvae and veliger larvae. At these
411 stages, hemocyte generation/proliferation and induction of immune related genes are
412 concomitant. However, in pediveliger larvae, only *Drac3* transcription level was increased.

413 This stage, which is the last stage of oyster planktonic life, corresponds to anatomic and
414 physiologic intense changes which could be responsible for the reduced capacity of larvae to
415 respond to bacterial infection. Taken together, these results could explain the variability of
416 susceptibility to infections during development.
417

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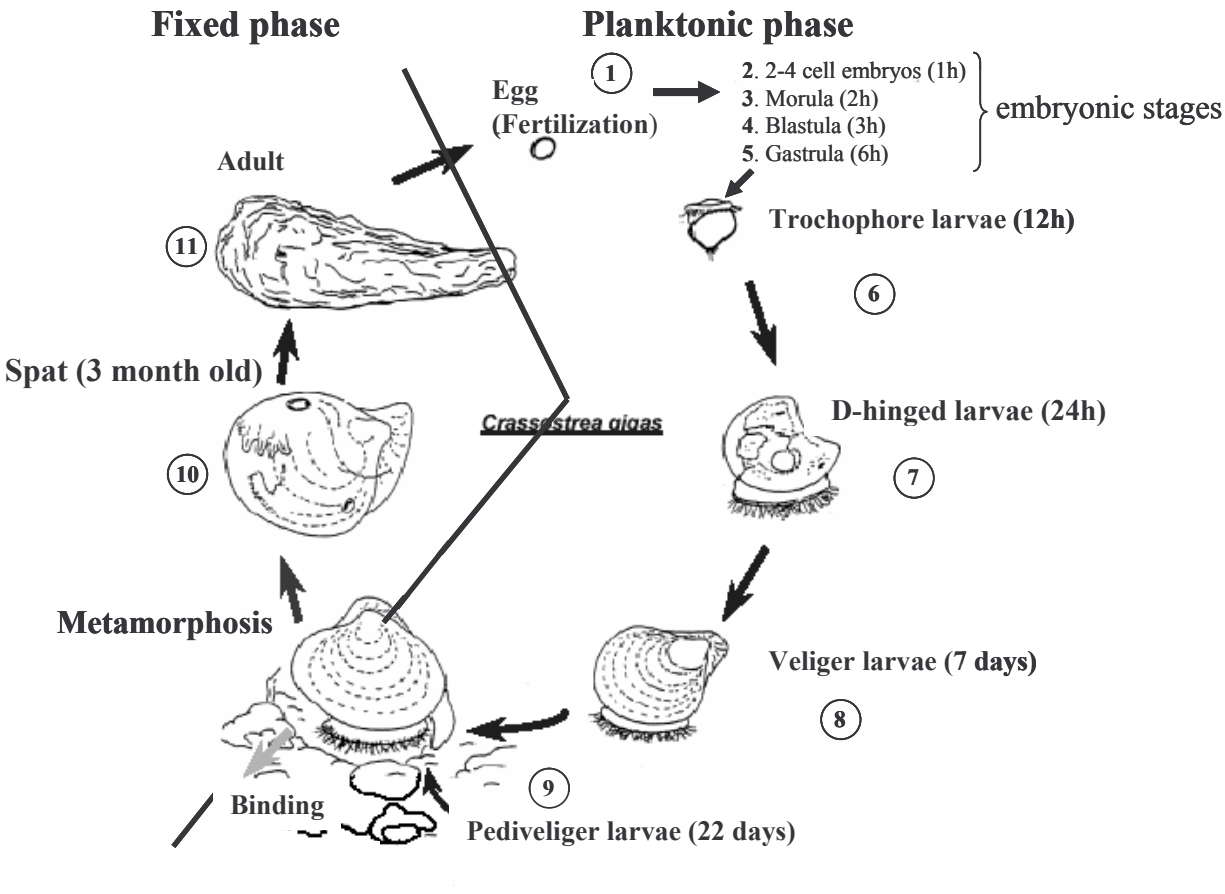
597

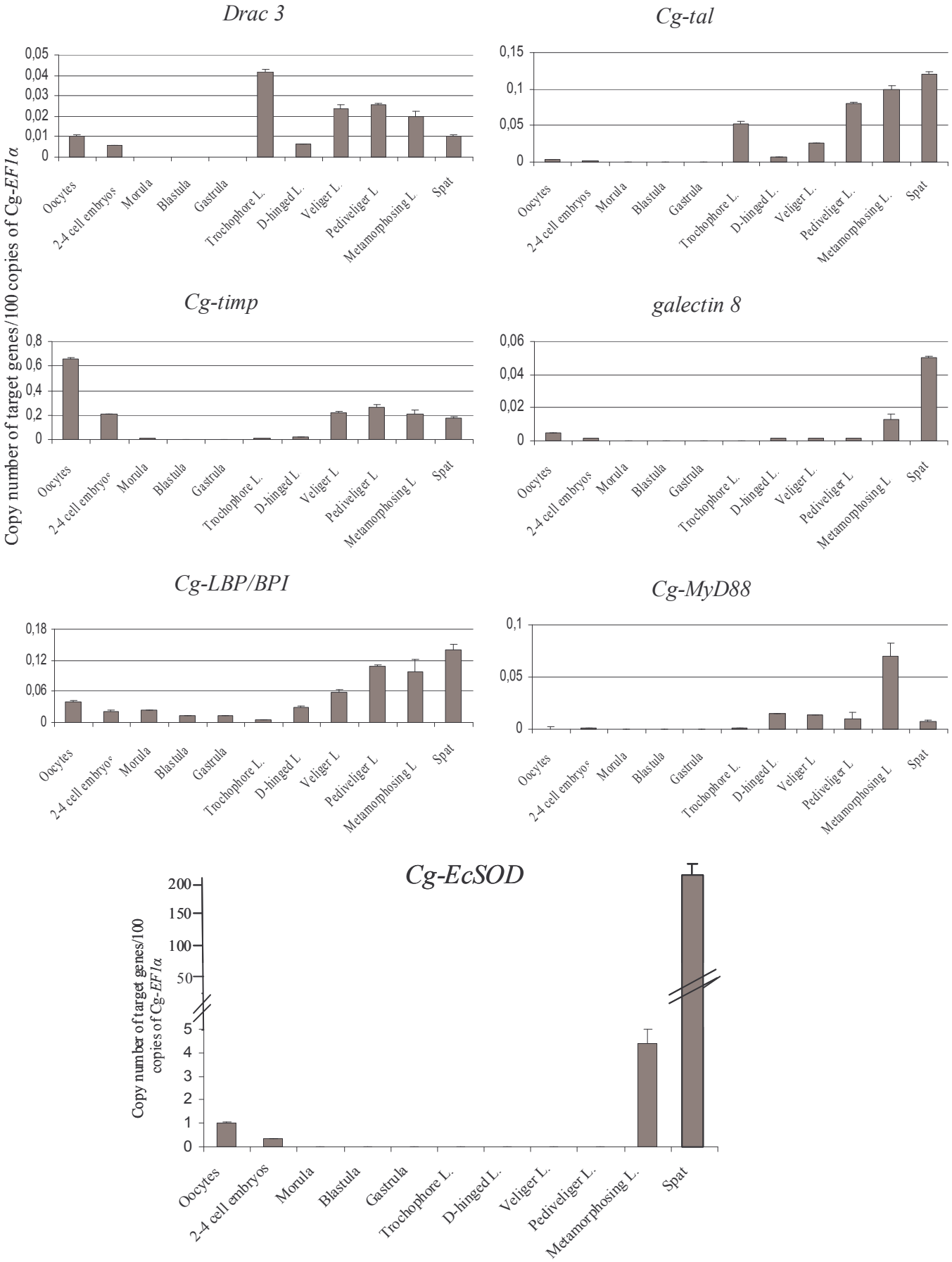
Figure 1


Fixed phase
Planktonic phase
Egg
(Fertilization)
1
2. 2-4 cell embryos (1h)
3. Morula (2h)
4. Blastula (3h)
5. Gastrula (6h)
embryonic stages
Adult
11
Trochophore larvae (12h)
6
Spat (3 month old)
Crassostrea gigas
D-hinged larvae (24h)
10
7
Metamorphosis
Veliger larvae (7 days)
8
9
Binding
Pediveliger larvae (22 days)

597

598

Figure 2



598
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Figure 3

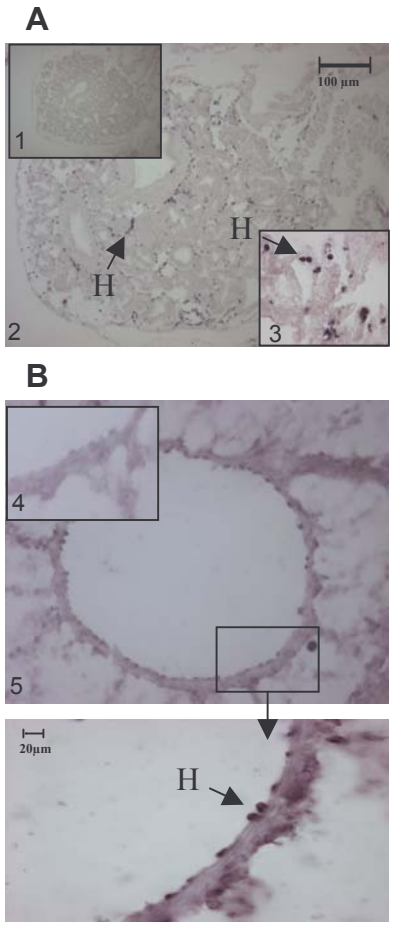

Figure 4

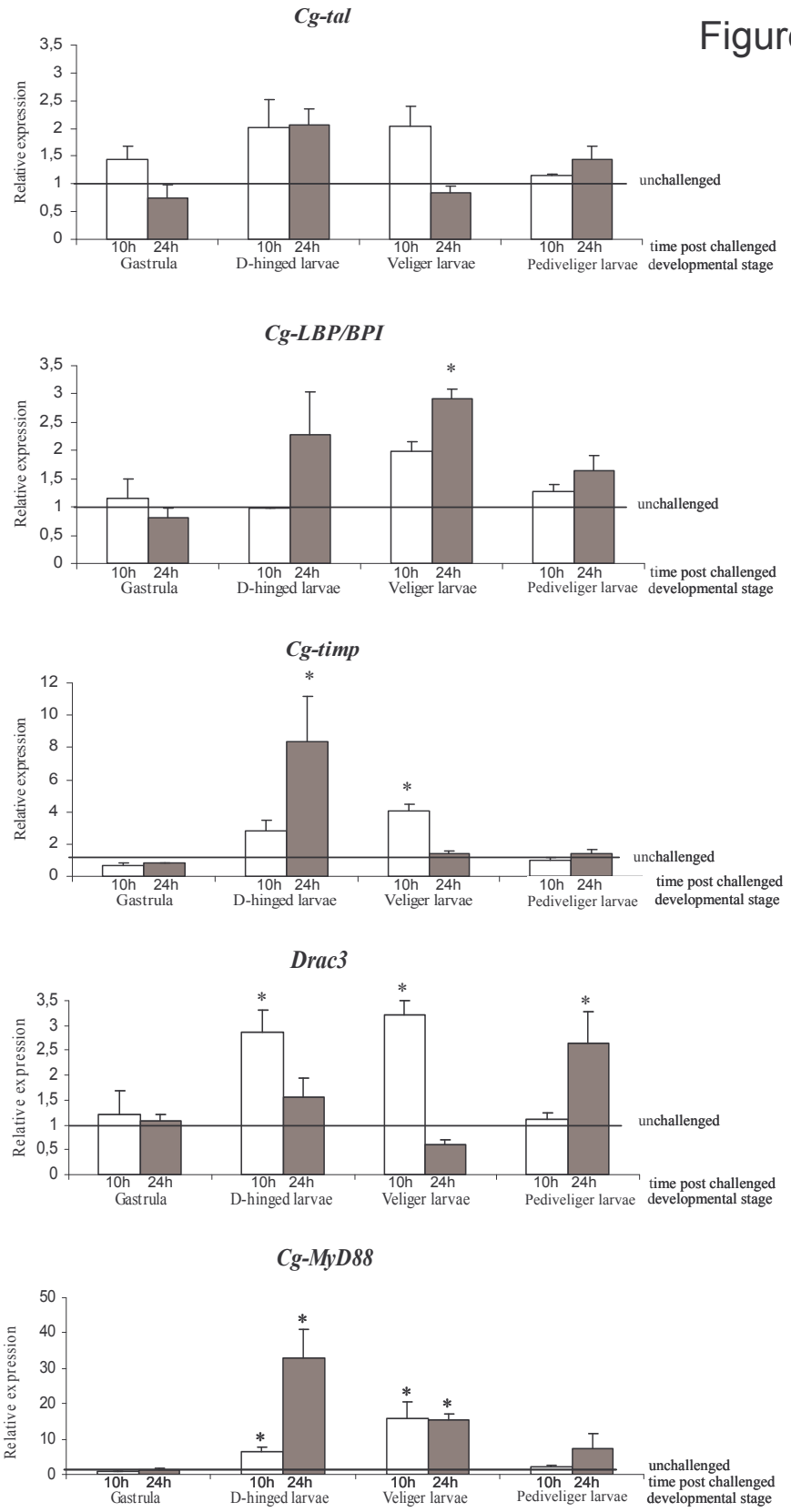
Cg-tal
Relative expression
unchallenged
time post challenged
developmental stage
10h 24h
Gastrula
D-hinged larvae
Veliger larvae
Pediveliger larvae
Cg-LBP/BPI
Cg-timp
Drac3
Cg-MyD88

Figure 5

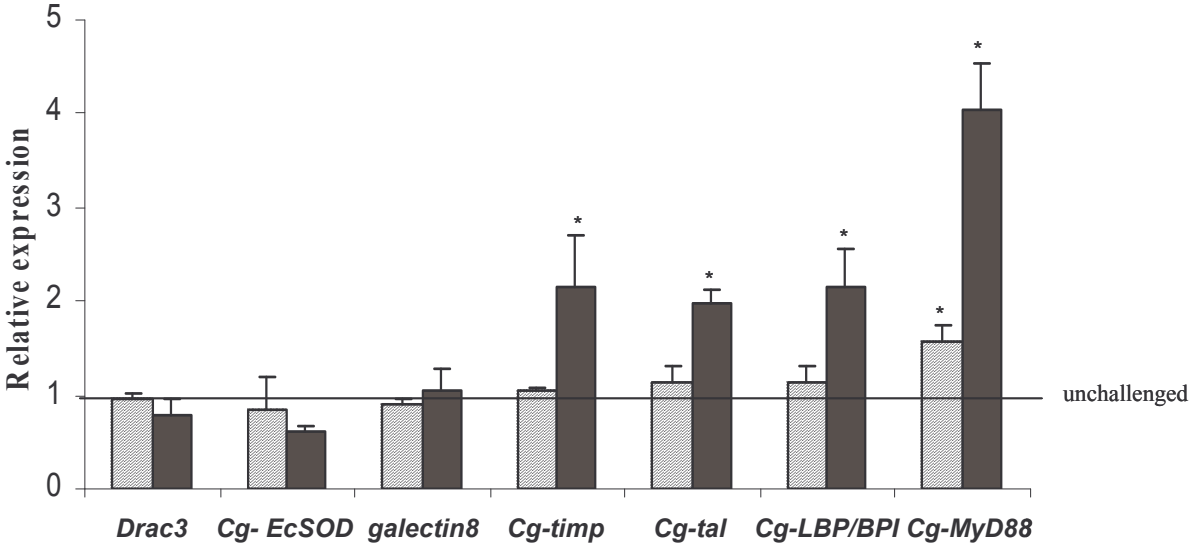
Relative expression
5
4
3
2
1
0
unchallenged
Drac3
Cg- EcSOD
galectin8
Cg-timp
Cg-tal
Cg-LBP/BPI
Cg-MyD88

Figure legends

Fig. 1. The life cycle of the oyster *Crassostrea gigas*. 1: Fertilized oocytes, 2. 2-4 cell embryos, 3. Morula, 4. Blastula, 5. Gastrula, 6: Trochophore larvae, 7: D-hinged larvae, 8: Veliger larvae, 9: Pediveliger larvae, 10: Spat and 11: Adult. Stage duration can be highly variable, depending on temperature, food supply, and other factors.

Fig. 2. Expression level of immune-related genes during ontogenesis in *Crassostrea gigas* by quantitative real time PCR. Each value is the mean ± standard error of embryo or larvae pools from three spawns and three individual spats. L: larvae.

Fig. 3. Detection of *Cg-EcSOD* and *Cg-tal* mRNAs in spat of *Crassostrea gigas* by *in situ* hybridization. A: *Cg-EcSOD in situ* hybridization using anti-sense probe showed positive signals in infiltrating hemocytes (arrows) in digestive gland (2), gill (3). B: *Cg-tal*, *in situ* hybridization labelled cells were attached to blood vessel endothelium (5), the shape and the localization of the positive cells evoke hemocytes (arrows). Control consisted of sections hybridized with sense probe, no labelling was observed (1 and 4).

Fig. 4. Relative expression of *Cg-tal*, *Cg-LBP/BPI*, *Cg-timp*, *Drac3* and *Cg-MyD88* in gastrula (6 h post-fertilization), D-hinged larvae (24 h), veliger larvae (2 days) and pediveliger larvae (22 days) of *Crassostrea gigas* by quantitative real time PCR according to Livack and Schmittgen method (2001), after a bacterial challenge (3.10^7 bacteria/L). Each value is the mean ± standard error of embryo or larvae pools from 3 independent biparental fertilizations. □10 h and ■ 24 h post stimulation; * Significant difference with not challenged animals ($p<0.05$). ─ : Relative expression of the unchallenged animals.

780

781 Fig. 5. Relative expression in *Crassostrea gigas* spat of *Drac3, Cg-EcSOD, galectin 8*, *Cg-*
782 *timp, Cg-tal, Cg-LBP/BPI* and *Cg-MyD88,* following a bacterial challenge by quantitative real
783 time PCR according to Livack and Schmittgen (2001) method. Each value is the mean ±
784 standard error of three individual spats at 24h post-stimulation. ▨: 3.10^7 bacteria/L, ■: 5.10^8
785 bacteria/L, * Significant difference with unchallenged samples (p<0.05). ── : Relative
786 expression of the unchallenged samples. These results were confirmed with two more
787 independent experiments.

Table 1. Specific primers and annealing conditions of studied immune-related genes used to do conventional PCR.

Gene name	GenBank	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)	Annealing (°C)
EF1α	AB122066	Published [27]		200	50
Cg-def	AJ565499	Published [18]		297	60
Cg-defh2	DQ400102	Published [19]		125	55
ficolin3	BQ426875	GGTATGACAATCTGTGCTGA	TGTAATCCGTCCAGTTACG	193	51
α 2-macroglobulin	BQ426790	TGTGGGACAAAGGAAGAGTTG	ACAAACAGGGCAGGACTTGA	159	58
MMP	BQ426653	CTACAAAGACAGGGAGGAAG	GTCAGGGCAAACACACTC	166	55
Drac3	BQ427023	GATAATAGTGCGACGGAGTG	CATCAGCATACAGGTCTTCC	184	55
Cg-tal	AY039650	Published [14]		234	60
Cg-timp	AF321279	Published [15,27]		523	58
galectin8	BQ427054	TGGAAGTTGAATCTGGTCTGG	TGCTGTTAAGAACCATCTCACG	171	58
Cg-LBP/BPI	AY165040	TGTCCTTGGCGACGGTCAGTTGTG	GTCCTCAAATTACCTATATCAGTAAC	199	55
Cg-MyD88	DQ530619	AGGTACCGGCTGTGATACGA	TTCAAACGCCACCAAGACTG	219	53
Cg-ECSIT	BQ427193	CCTCATCGGGAATCACACCATTA	CGTGCGAGGGCATATAGAGTTTG	227	65
Cg-TRAF3	BQ426746	CAGCCAGCCATTTTACACCAGTC	CCGTTTAAAACTGCTGCTTGTGG	247	65
Cg-rel	AY039648	Published [9]		409	65
Cg-EcSOD	DQ010420	Published [17]		160	58
Ring3	BQ426600	AGCAGACACAACCACTCCTG	CTTGGGCTTGATGATTTTCC	170	58
Lyn	BQ427191	GGAGCCAAGACCATTGAGG	TGTCCATTCTGTCCTTCTGC	172	55
vav	BQ427355	AAGAACACAGAGCTACACAGC	ACTGACTGGCATGACTGAAG	199	58

Table 2. Specific primers and annealing conditions used to measure the expression of immune-related genes by real time PCR.

Gene name	GenBank	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)	Annealing (°C)	PCR efficiency
EF1α	AB122066	Published [27]		200	62	1.94
Drac3	BQ427023	GATAATAGTGCGACGGAGTG	CATCAGCATACAGGTCTTCC	184	62	1.90
Cg-tal	AY039650	Published [14]		234	62	1.99
Cg-timp	AF321279	Published [15,27]		200	62	1.90
Cg-LBP/BPI	AY165040	TGTCCTTGGCGACGGTCAGTTGTG	GTCCTCAAATTACCTATATCAGTAAC	199	62	2.00
Cg-MyD88	DQ530619	AGGTACCGGCTGTGATACGA	TTCAAACGCCACCAAGACTG	219	62	2.00
Cg-EcSOD	DQ010420	Published [17]		160	60	1.99
galectin8	BQ427054	TGGAAGTTGAATCTGGTCTGG	TGCTGTTAAGAACCATCTCACG	171	62	1.94

Table 3. Expression patterns of selected-immune-related genes obtained from embryos and larvae pools by conventional PCR analysis.

	Genes	Expression site in adult oysters	Functional classification	Oocytes	2-4 cell embryos	Morula	Blastula	Gastrula	Trochophore	D-hinged	Veliger 2 days	Veliger 7 days	Pediveliger 22 days	Spat
hg	*EF1α*	ubiquitous	Translation elongation factor [27]	+	+	+	+	+	+	+	+	+	+	+
	Cg-def	mantle	Immunity: Antimicrobial peptide [18]	-	-	-	-	-	-	-	-	-	-	-
1	*Cg-defh2*	hemocytes	Immunity: Antimicrobial peptide [19]	-	-	-	-	-	-	-	-	-	-	-
	ficolin3[a]	nd	Immunity: Lectin	-	-	-	-	-	-	-	-	-	-	-
	α-2 macroglobulin[a]	nd	Immunity: Protease Inhibitor	+	+	-	+	+	+	+	+	+	+	+
	MMP[a]	nd	Immunity: Protease	+	+	+/-	+/-	+/-	+	+	+	+	+	+
2	*Drac 3*[a]	hemocytes	Cell cycle Barreau-Roumiguière, pers. comm.	+	+	+	-	+/-	+	+	+	+	+	+
	Cg-tal	hemocytes	Immunity: Cellular proliferation [14]	+	+	+/-	+/-	+	+	+	+	+	+	+
	Cg-timp	hemocytes	Immunity: Protease Inhibitor [15,32]	+	+	+/-	+/-	+	+	+	+	+	+	+
	galectin 8[a]	nd	Immunity: Lectin	+	+	-	-	-	+	+	+	+	+	+
	Cg-LBP/BPI	hemocytes/epithelia	Immunity	+	+	+	+	+	+	+	+	+	+	+
	Cg-MyD88	nd	Signaling Pathways [9]	+	+	+	+	+	+	+	+	+	+	+
	Cg-ECSIT	nd	Signaling Pathways [9]	+	+	+	+	+	+	+	+	+	+	+
	Cg-TRAF3	nd	Signaling Pathways [9]	+	+	+	+	+	+	+	+	+	+	+
3	*Cg-rel*	ubiquitous	Signaling Pathways [9]	+	+	+	+	+	+	+	+	+	+	+
	Cg-EcSOD	hemocytes	Immunity [17]	+	+	+	+	+	+	+	+	+	+	+
	Ring3[a]	nd	Cell cycle	+	+	+	+	+	+	+	+	+	+	+
	Lyn[a]	nd	Cell cycle	+	+	+	+	+	+	+	+	+	+	+
	vav[a]	nd	Immunity	+	+	+	+	+	+	+	+	+	+	+

[a]Genes from the EST library [12]. hg: housekeeping gene, nd: not determined , +: Detected by PCR, +/-: Low detected, -: Undetected