Lysozyme gene expression and hemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis*, after injection of various bacteria or temperature stresses

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Abstract:

The aim of the present study was to evaluate the expression of the *Mytilus galloprovincialis* lysozyme gene in different *in vivo* stress situations, including injection of bacteria *Vibrio splendidus* LGP32, *Vibrio anguillarum* or *Micrococcus lysodeikticus*, as well as heat shock at 30 °C and cold stress at 5 °C. Injection of *V. splendidus* LGP32 resulted in: (i) a general down-regulation of lysozyme gene expression, as quantified by Q-PCR; (ii) reduction in the number of circulating hemocytes; (iii) decrease in the percentage of circulating hemocytes expressing lysozyme mRNA which was now restricted to only small cells, as observed by ISH; and (iv) accumulation of hemocytes expressing lysozyme in the muscle sinus where injection took place. Injection of *V. anguillarum* or *M. lysodeikticus* induced significant up-regulation of lysozyme gene expression, but only 2–3 days post-injection, with no change in the total hemocyte counts but an increased percentage of hemocytes expressing lysozyme mRNA. Neither the control injection of PBS-NaCl nor temperature stress modified the lysozyme expression pattern. Consequently, the hemocyte population appears to be capable of discriminating between stress factors, and even between 2 *Vibrio* species.

Keywords: *Vibrio splendidus*; *Vibrio anguillarum*; *Micrococcus lysodeikticus*; Heat shock; Lysozyme; Gene expression; Q-PCR; ISH; *Mytilus*; Molluscs

1. Introduction

Lysozymes are small ubiquitous antibacterial enzymes that hydrolyze β-1, 4-linked glycoside bonds of peptidoglycan, a major cell wall component of Gram-positive bacteria. Several studies have shown that lysozymes are also able to kill Gram-negative bacteria, demonstrated in bivalves [1, 2] and in shrimp [3, 4]. Anti-protozoan and anti-fungal activities of lysozymes, mediated by cleaving N-acetylglucosamine in chitin, have been also documented [5, 6]. In addition, lysozyme can kill bacteria by non-enzymatic activity [7]. Thus, lysozymes appear to constitute an important component of immune defence against diverse microbial infections.

Several types of lysozymes have been purified, from the best-known chicken-type (c-type) and goose-type (g-type) [8], to the more recently identified invertebrate-type (i-type) [1, 9]. Such i-type lysozyme has been identified in several bivalve molluscs, including the Icelandic scallop, *Chlamys islandica* [1], the blue mussel, *Mytilus edulis* [10], several mytilids and vesicomyids [11], and the Mediterranean mussel, *Mytilus galloprovincialis*, the hydrothermal-vent mussel, *Bathymodiolus azoricus* and the cold-seep clam, *Calyptogena* sp. [12]. Although numerous reports concern the different types of lysozymes and their activities [10, 12-15], only the genes from the blue mussel, *M. edulis* [12], and from the Icelandic scallop, *Chlamys islandica* [16], have been sequenced. Curiously, the *M. edulis* lysozyme gene comprises five exons instead of the classical four exons of the c-type lysozyme gene, such as the *C. islandica* ones.

The Mediterranean mussel, *M. galloprovincialis*, is a filter-feeding bivalve and lysozymes are believed to be involved in digestive processes [17] as well as in host defence [18]. In *M. edulis*, lysozyme has been found localised within granular hemocytes [19] and higher levels of activity have been detected in hemocytes compared with plasma [20] in both *M. edulis* and the carpet shell clam, *Ruditapes decussatus* [21]. However, mRNA transcripts of g-type lysozyme were most abundantly expressed in gills, hepatopancreas and gonad, but only weak expression was evident in hemocytes and mantle from the Zhikong scallop, *Chlamys farreri* [22]. In the Pacific oyster, *Crassostrea gigas*, lysozyme mRNA was expressed in all tissues except the adductor muscle and ISH (*in situ* hybridization) analyses revealed strong expression in basophilic cells from the digestive tubules [23]. Similarly, ISH located lysozyme gene expression in the mantle and gill cells of the eastern oyster, *C. virginica*, with significantly higher mRNA content in labial palps and mantles than in gills, digestive glands and hemocytes [14]. For a long time, hemocytes have been considered as primary mediators of anti infectious defence, yet no work has been done on the kinetics of lysozyme gene expression in response to various challenges.

The aim of the present report was to study the kinetics of expression of the *M. galloprovincialis* lysozyme gene in response to various stress factors: high temperature, low temperature, and bacterial injection. Quantification of lysozyme transcripts was done using Q-PCR (quantitative polymerase chain reaction) with 28S ribosomal RNA as the house keeping gene. Visualisation of circulating hemocytes containing lysozyme mRNA was done by ISH and completed by histological observations of the posterior adductor muscles where injection took place.

2. Materials and methods

2.1. Bacterial growth and mussel challenges

Vibrio splendidus LGP32 is a Gram-negative marine bacterium isolated from juvenile oysters, *C. gigas*, during summer mortalities in 2001 [24], while *V. anguillarum* was from the Institut Pasteur-France (ATCC 19264). Both *Vibrio* species (50 µl of overnight-cultured inoculum) were grown at 20°C in 10 ml trypsin-casein-soya (TCS, AES Laboratoire, Bruz-France) for 4-6 h to ensure bacteria were in the exponential growth phase, then centrifuged for 10 min at 500xg, and adjusted to 10^8 CFU/ml with phosphate buffered solution isotonic to sea water (PBS-NaCl: 2 mM KH_2PO_4, 10 mM Na_2HPO_4, 3 mM KCl, 600 mM NaCl in distilled water, pH 7.4) according to 1 $OD_{600\ nm}$ = 5 x 10^8 CFU/ml. *Micrococcus lysodeikticus* from the Institut Pasteur-France (ATCC 4698) was grown at 37°C in Luria Broth (Sigma Chemical Co, St Louis MO-USA) until bacteria were in the exponential growth phase, then centrifuged 10 min at 500xg, and adjusted at 10^8 CFU/ml with PBS-NaCl according to 1 $OD_{600\ nm}$ = 0.36 x 10^8 CFU/ml.

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Adult *M. galloprovincialis* were purchased from the marine farm Les Compagnons de Maguelone (Palavas-France) in May-June 2006 and April 2007. They were maintained in the laboratory in oxygenated sea water at 20°C for 1-3 days prior to experimentation. Four batches of 10 mussels each per sampling end-point were injected with 100 µl (10^7 bacteria) into the posterior adductor muscle, through a hole created by light filing on the shells. After injection, mussels were returned to 20°C sea water. Control injections consisted of 100 µl of PBS-NaCl. To test temperature stress, four batches of 10 mussels each per sampling end-point were subjected to 90 min immersion in sea water at either 30°C or 5°C, with their subsequent return to 20°C sea water. Four batches of 10 untreated mussels each (referred to as untreated) were sampled simultaneously with each corresponding stress to minimize seasonal variations. Thus, the experiments involved a grand total of 2,160 mussels.

2.2. Primers for lysozyme and 28S ribosomal RNA

Primers for lysozyme were designed from the M. galloprovincialis lysozyme mRNA (AF334665)[12]:
forward 5'-ATGTGGAATCTGAAGGACTTGT-3' (position 140-161) and
reverse 5'- CCAGTATCCAATGGTGTTAGGG-3' (position 486-507), giving an expected amplicon of 368 bp. Presence of a unique amplicon was checked by melting curve analysis (see 2.4) and gel electrophoresis on 2% agarose in Tris-borate-EDTA buffer stained with ethidium bromide. Specificity has been confirmed by several complete sequencings performed by Millegen (Labège-France).
28S rRNA was used as the house-keeping gene according to Cellura et al [25] using the previously reported primer sequences:
forward, 5'-AAGCGGAGGAAAAGAAACTAAC-3' and
reverse, 5'-TTTACCTCTAAGCGGTTTCAC-3', giving an amplicon of 378 bp with a melting temperature of 90.17 ± 0.04°C, also sequenced for confirmation of identity.

2.3. Hemolymph collection and cDNA synthesis

Hemolymph (0.8 ml per mussel) was collected from the posterior adductor muscle at 1, 3, 6, 9, 12, 24, 48 and 72 h post-injection, or immediately after the temperature shock (time 0) and after 3, 6, 9, 12, 15, 18 and 24 h recovery at 20°C. Hemolymph was extracted into a 1 ml disposable syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution (MAS)[26]. Hemolymph from 10 mussels was pooled and the hemocytes pelleted by 15 min centrifugation at 800xg at 4°C. Four pools of 10 mussels each, as replicates, were used for each sampling end-point. Total RNA was extracted with Trizol Reagent, according to the manufacturer's protocol (Invitrogen, Cergy Pontoise-France) and resuspended in 40 µl Tris-EDTA buffer. First strand cDNAs were synthesized from RNA adjusted to 5 µg using hexaprimers (Invitrogen, Cergy Pontoise-France) and murine leukemia virus reverse transcriptase (Promega, Charbonnières-France), and purified with Wizard SV gel and PCR clean-up system (Promega, Charbonnières-France), then kept in nuclease-free water at -20°C until use.

2.4. Quantification of lysozyme transcripts by Q-PCR

Q-PCR was performed on the 4 pools for each sampling end-point of similarly treated mussels using SYBR Green chemistry on a LightCycler 480 384 well-plate (Roche Diagnostics, Meylan-France). The Q-PCR mixture contained the following: 1 µl first strand cDNA (10 ng), 0.75 µl of each specific primer at a concentration of 25 µM, and 2.5 µl of reaction mix (Roche Diagnostics, Meylan-France) containing FastStart Taq DNA polymerase, reaction buffer 2x, dNTP mix, SYBR Green 1 dye and $MgCl_2$. The PCR amplification programme started with initial Taq polymerase activation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 65°C for 10 sec and 72°C for 15 sec. Melting temperatures were measured by returning to 65°C for 30 sec and gradual heating to 95°C. Negative control reactions contained sterile water in place of the cDNA template and were included in each run to ensure the absence of contamination. Calibration curves were obtained using 10-fold serial dilutions

of lysozyme amplicon in 10 µg/ml sonicated salmon sperm DNA (Sigma-Aldrich Chimie, St Quentin-France).

2.5. Data analysis

Crossing point values expressed in cycle numbers were measured according to a threshold position of 4.2 and converted into equivalent target amounts (ETA) by the LightCycler 480 software, using calibration curves. Lysozyme expression level was calculated from the ratio of ETA for lysozyme on ETA for 28S rRNA. Normalization of the ratios was calculated considering each ratio equal to 1 in untreated mussels and expressed as x-fold the ratio for untreated mussels. Data were presented as the arithmetical mean of the four replicates ± SEM. Normality of data distribution was assayed using the Shapiro-Wilk test available at http://cran.fr.r-project.org. To compare the data of individual end-points with expression in untreated mussels, Student's *t*-test using t-Ease 2.8 ISI software was employed. Differences were considered significant when $p < 0.05$.

2.6. Tissue sampling

Hemocytes were collected in MAS and immediately fixed by dilution with 5 vol of 10% neutral buffered formalin (NBF: 46 mM Na_2HPO_4, 30 mM NaH_2PO_4, 3.7 % formaldehyde in distilled water, pH 7). After centrifugation (800xg, 15 min, 4°C), hemocytes were resuspended in 10 ml 10% NBF and incubated overnight at 4°C. After washing in 70% ethanol by centrifugation (800xg, 15 min, 4°C), hemocyte concentration was adjusted to 10^7 cells/ml and one drop of 5 µl was deposited on poly-lysine coated glass slides, air dried and kept at 4°C. Total hemocyte numbers were determined in freshly collected hemolymph using Malassez' hemocytometer. Counts were done in duplicate for each of the 4 batches of untreated or experimentally treated mussels. Results are expressed as the arithmetic means ± SEM.

The adductor muscle from each sampled mussel was carefully detached from surrounding tissues and shell, and immediately immersed in Davidson fixative (33% of 95% ethanol, 22% of 37% formaldehyde, 11% glacial acetic acid and 34% distilled water). After 2 days of fixation, muscles were kept in 70% ethanol. They were embedded in paraffin after dehydration in gradual, increased ethanol concentrations, sectioned at 2 µm and laid on histological poly-lysine coated slides kept at room temperature.

2.7. Probe synthesis and ISH (in situ hybridization) assay

The lysozyme amplicon was synthesized by PCR using the same primers as for Q-PCR but using GoTaq DNA polymerase (Promega, Charbonnières-France). PCR conditions were: 2 min denaturation at 95°C, followed by 30 cycles of 95°C for 40 sec, 60°C for 30 sec and 72°C for 40 sec, ended by final extension at 72 °C for 10 min. After analysis on 2 % agarose gel, the PCR amplicon was cloned using TOPO TA Cloning kit (Invitrogen, Cergy Pontoise-France) with pCR II-TOPO plasmid and the inserted sequence controlled by sequencing (Millegen, Labège-France). Antisense and sense digoxigenin (DIG) labeled riboprobes were synthesized from the plasmid using the DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics, Meylan-France).

Cells on slides were permeabilised with 3 µg/ml proteinase-K (Sigma-Aldrich Chimie, St Quentin-France) for 10 min at 37°C then fixed with 0.4% cold formaldehyde and rinsed 5 min with 2X sodium chloride/sodium citrate buffer (2X SSC: 150 mM NaCl, 15 mM sodium citrate in distilled water, pH 7). Histological sections of muscles were heated at 65°C during 45 min, gradually hydrated in gradual, decreased ethanol concentrations, then permeabilised with 6 µg/ml proteinase-K for 10 min at 37°C.

Lysozyme riboprobes (50 ng/ml) were denatured for 15 min at 95°C, and hybridisation was performed overnight at 37°C in a humid chamber with 50% formamide, 1X Denhard's, 5% dextran sulphate and 0.5 mg/ml salmon sperm DNA in 4X SSC. Unbound riboprobes were removed by washing twice (30 min, room temperature) with 2X SSC, then twice (5 min, 37°C) with 1X SSC and twice (5 min, 37°C) with 0.5X SSC. Cells and muscle sections were equilibrated for 5 min in Buffer 1

(1M Tris-HCl, pH 7.5, 1.5 M NaCl) then incubated (15 min, 37°C) with 500 µl of 0.5 % blocking reagent (Roche Diagnostics, Meylan-France) in Buffer 1. Cells and muscle sections were then incubated with sheep anti-DIG Fab fragments (Roche Diagnostics, Meylan-France) conjugated to alkaline phosphatase (30 min, 37°C), then washed twice for 5 min in Buffer 1. The colorimetric reaction occurred during 3 h incubation in a freshly prepared solution of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Promega, Charbonnières-France) diluted in Buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1% PolyVinyle Alcohol). The slides were subsequently rinsed with Buffer 4 (100 mM Tris-HCl, pH 8, 10 mM EDTA).

To enhance the contrast, counter-staining was done with 0.5% Bismarck Brown Y solution (Sigma-Aldrich Chimie, St Quentin-France). After being dehydrated in 95 and 100% ethanol and treated three times with non-toxic LMR-SOL solvent (Labo-Moderne, Paris-France), hemocytes and muscle sections were mounted with a cover-slip using the permanent mounting medium Histalaque (Labo-Moderne, Paris-France). Observations were made with a photonic microscope Leica DMR (Leica Microsystems, Wetzlar-Germany) equipped with a Leica DC200 camera.

3. Results

3.1. Specificity of lysozyme amplicon

The sequences of both mRNAs from *M. galloprovincialis* (AF334665) and the one deduced from *M. edulis* gene (AF334662) present a high degree of identity with only 43 nucleotides different out of 531, representing 8%. In addition, such differences were homogeneously spread all along the cDNA sequences with only a single substitution at each difference. The exception was 3 consecutive bases located in position 417-419. The only cDNA amplicon obtained in Q-PCR was of 368 bp (Fig. 1) and corresponded to the expected size deduced from the reported cDNA sequence. Amplicons from several Q-PCR were analyzed for DNA melting temperature revealing only one symmetrical peak at 82.88 ± 0.08°C (Fig. 1). Definite confirmation of the primer specificity was obtained from several sequencings, showing total nucleotide sequence alignment with the expected *M. galloprovincialis* cDNA sequence reported in databank (AF334665) as belonging to i (invertebrate) type of lysozyme [12]. In addition, the nucleotide sequence of the *M. galloprovincialis* amplicon possessed only 45% and 51% identity with type-c lysozyme (*Marsupenaeus japonicus*, AB080238) and type-g lysozyme (*Chlamys farreri*, DF718947) including 9 to 15 gaps to enforce alignment, respectively.

3.2. Relative concentrations of 28S rRNA transcript

The expression of 28S rRNA gene was measured by Q-PCR in all the cDNA samples collected from each time point. Samples collected from untreated mussels on the day of the corresponding challenge did not possess identical 28S rRNA content. ETA ranged from $3.69 \times 10^4 \pm 0.39 \times 10^4$ to $7.15 \times 10^4 \pm 0.56 \times 10^4$ according to the various batches of mussels, with no statistically significant differences (not shown). ETA was not stable throughout the duration of the various challenges but corresponded closely to the concentration in untreated animals (fold change from 0.77 to 1.19). None of the stress factors had a significant effect on 28S rRNA expression, confirming that the use of the 28S rRNA gene to normalize levels of gene expression in mussels is appropriate.

3.3. Effect of bacterial and PBS-NaCl injection on hemocyte lysozyme mRNA expression

Injection of *V. splendidus* LGP32 resulted in a general decrease in lysozyme mRNA levels as shown in Fig. 2. The maximum decrease of -3.47 ± 0.11 fold the *ratio* in untreated mussels (p=0.00001) was observed 6 h after injection. Injections of both *V. anguillarum* and *M. lysodeikticus* resulted in an increase in lysozyme mRNA with a maximum observed after 48 h for *V. anguillarum* (4.15 ± 0.71; p=0.004) and 72 h for *M. lysodeikticus* (5.78 ± 0.87; p= 0.005). Only a transient,

statistically significant decrease was observed after 3 h for *V. anguillarum* (-2.10 ± 0.17; p=0.002), whereas small, but statistically significant increases, were recorded with PBS-NaCl injection at 9 h (1.67 ± 0.14; p=0.033) and 48 h (1.61 ± 0.07; p=0.19).

3.4. Effect of heat shock and cold stress in hemocyte lysozyme mRNA expression

Limited increases in lysozyme mRNA levels were obtained after heat shock, with a maximum (but not significant value) of 2.60 fold the *ratio* in untreated mussels after 12 h of recovery (Fig. 3). The only statistically significant value was recorded after 3 h of recovery (2.16 ± 0.08; p=0.002). In contrast, cold stress resulted in a general decrease in the quantity of lysozyme mRNA, being minimal after 15 h of recovery (-3.84 ± 0.08; p=0.022), the only significant difference from untreated mussels.

3.5. Lysozyme expression in circulating hemocytes

ISH with antisense lysozyme riboprobe has been performed on hemocytes collected from all the sampling end-points of all the injected mussels, plus the untreated. In untreated mussels, labelling of lysozyme mRNA was observed only in some granulocytes (Fig. 4). The small hemocytes referred to as hyalinocytes appeared unstained. Six hours after injection of *V. splendidus* LGP32, the total number of circulating hemocytes was significantly lower than in untreated (1.42 x 10^6 ± 0.14 x 10^6 versus 2.37 x 10^6 ± 0.22 x 10^6, p=0.005; not shown) and very few hemocytes were labelled (Fig. 4). In addition, the labelling was restricted to small cells, the large cells, mainly granulocytes were no longer stained. Forty eight hours after injection of *V. anguillarum* or *M. lysodeikticus*, numerous, but not all, small and large hemocytes were stained by the lysozyme riboprobe. At that time, total numbers of circulating hemocytes did not vary significantly from untreated, ranging from 2.50 x 10^6 ± 0.15 x 10^6 in the corresponding untreated to 3.00 x 10^6 ± 0.23 x 10^6 (p=0.094) after injection of *V. anguillarum* and from 2.27 x 10^6 ± 0.21 x 10^6 in the corresponding untreated to 2.90 x 10^6 ± 0.19 x 10^6 (p=0.0502) after injection of *M. lysodeikticus.* The specificity of hybridization was controlled by incubation with sense riboprobe, resulting in the absence of blue coloration.

3.6. Accumulation of hemocytes within muscle

Histological sections of adductor muscle from untreated mussels revealed the presence of narrow sinuses between the muscular fibres. The lumen of the sinuses contained few hemocytes including some labelled granulocytes, and liquid hemolymph was observed as open spaces after histological treatments (Fig. 5). Six hours after injection of *V. splendidus* LGP32, the sinuses were enlarged and the lumen was almost completely filled with numerous aggregated hemocytes, the cytoplasm of which being labelled. In muscles injected 48 h before with *V. anguillarum*, the size of the sinuses were similar to those of untreated mussels, but the lumen was completely filled with unstained aggregated hemocytes. In contrast, injection of *M. lysodeikticus* resulted in sinuses resembling the ones of untreated mussels, with open spaces, few hemocytes and rare labelling.

4. Discussion

For a long time, mollusc hemocytes have been reported to be responsible for bactericidal activity mediated by numerous toxic compounds, such as lysozyme [17], superoxide [27], lysosomal enzymes [21], nitric oxide [28], phenoloxidase [29] and antimicrobial peptides [30, 31]. In addition, signal transduction pathways in *M. galloprovincialis* [32] and *Crassostrea gigas* [33, 34] have been identified. Few studies, however, concentrated on the *in vivo* regulation of the immune genes, with those to date reporting that expression of immune-related genes have been modulated by physical stress, bacterial challenges or exposure to poly aromatic hydrocarbons [35-39]. In the present study, the kinetics of expression of the *M. galloprovincialis* lysozyme gene in response to different *in vivo* challenges was established. According to single melting curve of PCR amplicon, to total nucleotide

sequence identity with type-i lysozyme, and to low nucleotide sequence identities with type-c and type-g lysozymes, we considered both mRNA quantification and ISH referred to the unique type-i lysozyme of *M. galloprovincialis*.

According to previous reports [25, 39], we decided to maintain the quantification of 28S rRNA mRNA as representative of house keeping gene expression to be used in Q-PCR. However, the expression of 28S rRNA was not constant in untreated mussels collected during different weeks, at the time of the corresponding challenge. This observation confirmed the effect of the season on mussel physiology [29, 40] and underlines the absolute necessity to perform the challenges during the same season when comparisons are required. In addition, no one gene expression is strictly unaffected by experimental treatments. In our challenges, the quantities of 28S rRNA transcripts varied. No significant down regulation was observed, and only the injection of *V. splendidus* LGP32 was able to significantly up-regulate this gene. Although mathematically significantly different (p<0.05), the up-regulations were lower than 1.21 fold the expression in untreated mussels. Such slight variations may represent normal modulations of expression in a living organism. Consequently, and in agreement with the 4 to 10 fold up-regulation previously observed for antimicrobial peptides (AMPs) and HSP70 [25, 39], we decided that variations lower than 4 fold the one observed in untreated mussels will not be considered to reflect a biological response to the challenge.

Regarding lysozyme gene expression with this limitation, only up-regulations were observed, and only following *V. anguillarum* or *M. lysodeikticus* injection. Both were at the later end-points of 2 to 3 days post-injection, revealing the delay in the triggering of the lysozyme gene expression. Curiously, *M. lysodeikticus* was the only bacterium not capable of up-regulating the gene expression of the 3 AMPs and of HSP70 [39]. Only *V. splendidus* LGP32 has been suspected to induce mortality in bivalves [24], but neither lysozyme (this report) nor mytilin, myticin and HSP70 [39] gene expressions were up-regulated after injection of *V. splendidus* LGP32. One can hypothesize relationships between the nature/structure of the injected bacteria and the immune-related gene response. Neither heat shock nor cold stress resulted in significant modulation of lysozyme gene expression. A much earlier study reported both individual and seasonal variability of hemolymph lysozyme activity in oysters [41]. More recently, heat shock was demonstrated to cause rapid induction of HSP70 and AMP myticin gene expression in *M. galloprovincialis* [39]. Subsequently, in the present study, lysozyme gene expression in hemocytes appeared variable, but not influenced by temperature shock.

The different cell types found in the hemolymph of mussels were reported in the 1990s. The general assertion was that 2 cell categories existed in *M. edulis*: (i) hyalinocytes and (ii) granulocytes which might be subdivided according to granule size [42]. Staining capacities resulted also in 2 cell types: (i) basophils (about 40% of the total hemocytes) with a large majority of hyalinocytes and (ii) eosinophils (about 60%) with small and large granules [43, 44]. Also 2 cell types were described in *M. galloprovincialis*, (i) hyalinocytes with characteristics of undifferentiated cells, and (ii) granulocytes being acidophils, basophils or both [45]. Both phenoloxidase/peroxidase activities and lysozyme were found associated with the granules of eosinophils [19, 29]. Despite the fact that monoclonal antibodies revealed 3 cell types [46], we decided to consider the two easily distinguishable categories, hyalinocytes and granulocytes. In the present report, lysozyme mRNA has been detected by ISH within the granulocytes in untreated mussels, however, injection of bacteria dramatically modified that profile. A few hours after injection of *V. splendidus* LGP32, the number of circulating hemocytes was dramatically reduced. The decrease in lysozyme mRNA transcript number revealed by Q-PCR was not due to the collection of less hemocytes, as cDNA concentrations had been adjusted to 10 ng in all the samples. ISH confirmed that few of the circulating hemocytes contained lysozyme mRNA, whereas histological observation revealed numerous hemocytes aggregated within the enlarged muscle sinuses and expressing the lysozyme gene. The accumulation of hemocytes containing mytilin has been also observed after bacterial challenge [47]. Presumably, this neutralizes localized infection, yet accumulation of hemocytes at the injection site resulted at least temporarily in lowering the number of circulating immune cells, consequently reducing the capacity to counter infection in another location.

The response to *V. anguillarum* was totally different, with no difference in the number of circulating hemocytes but some accumulation in the muscle sinuses. The numerous hemocytes containing lysozyme observed in circulation, from both large and small cells, may explain the up-regulation detected by Q-PCR. Similarly, *M. lysodeikticus* challenge resulted both in up-regulation of the lysozyme gene expression, and observations of numerous hemocytes containing lysozyme in circulation. No obvious accumulation, however, was observed in the muscle sinuses and the number of circulating hemocytes was not significantly different to those from untreated animals, revealing different responses of the hemocytes according to the nature of the injected bacteria.

Other studies using clams have shown that defence-related factors were modulated in hemolymph in response to *V. tapetis* injection. For instance, lysozyme activity of cell-free hemolymph significantly increased 24-72 h post-challenge in *Ruditapes philippinarum* [48], as we observed in *M. galloprovincialis* challenged with both *V. anguillarum* and *M. lysodeikticus*. In contrast, no correlation was found between lysozyme activity from cell-free hemolymph and infection by the protozoan, *Bonamia ostreae* in the flat oyster, *Ostrea edulis* [49]. On the contrary to observations in mussels, lysozyme of the shrimp, *Litopenaeus vannamei*, is expressed in nearly all the circulating hemocytes [50]. Meanwhile, the shrimp response to an injection of *V. campbellii* resembled the one of *M. galloprovincialis* after injection of *V. splendidus* LGP32: i.e. decrease in lysozyme mRNA within the first hours following injection, with simultaneous recruitment and accumulation of hemocytes at the injection site.

In summary, based on quantification of lysozyme gene expression, total hemocyte counts, observation of hemocytes containing lysozyme mRNA and hemocyte accumulation at the site of injection, it appeared that hemocyte populations reacted differently according to the nature of the stress. Hemocytes were able to differentiate amongst bacterial species, even between two *Vibrio* species, whereas physical stress or PBS-NaCl injection did not strongly modulate the expression of the lysozyme gene. Further studies must address the behaviour of the other known immune molecules in order to obtain a complete overview of an innate immune response.

Acknowledgments

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Figures
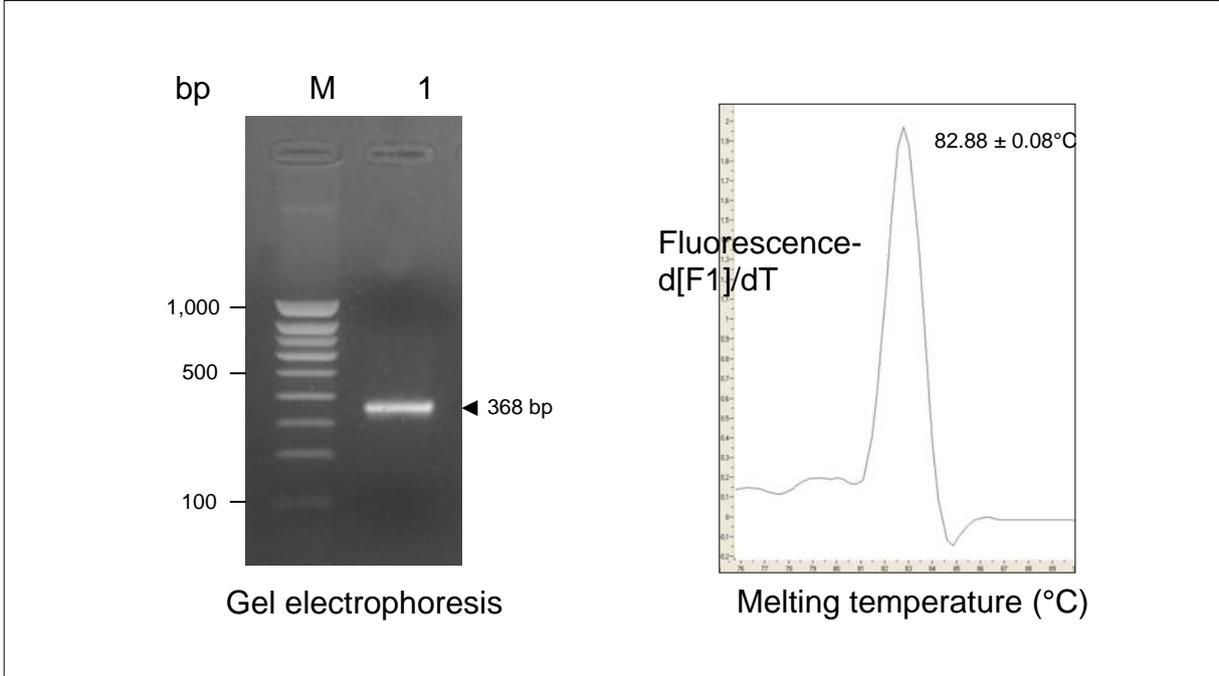
Figure 1

Figure 1 - Gel electrophoresis migration and melting curve of Q-PCR lysozyme amplicon revealing the presence of a unique product of amplification at 368 bp and 82.88 ± 0.08°C. M: DNA molecular weight marker. 1: Q-PCR amplicon.
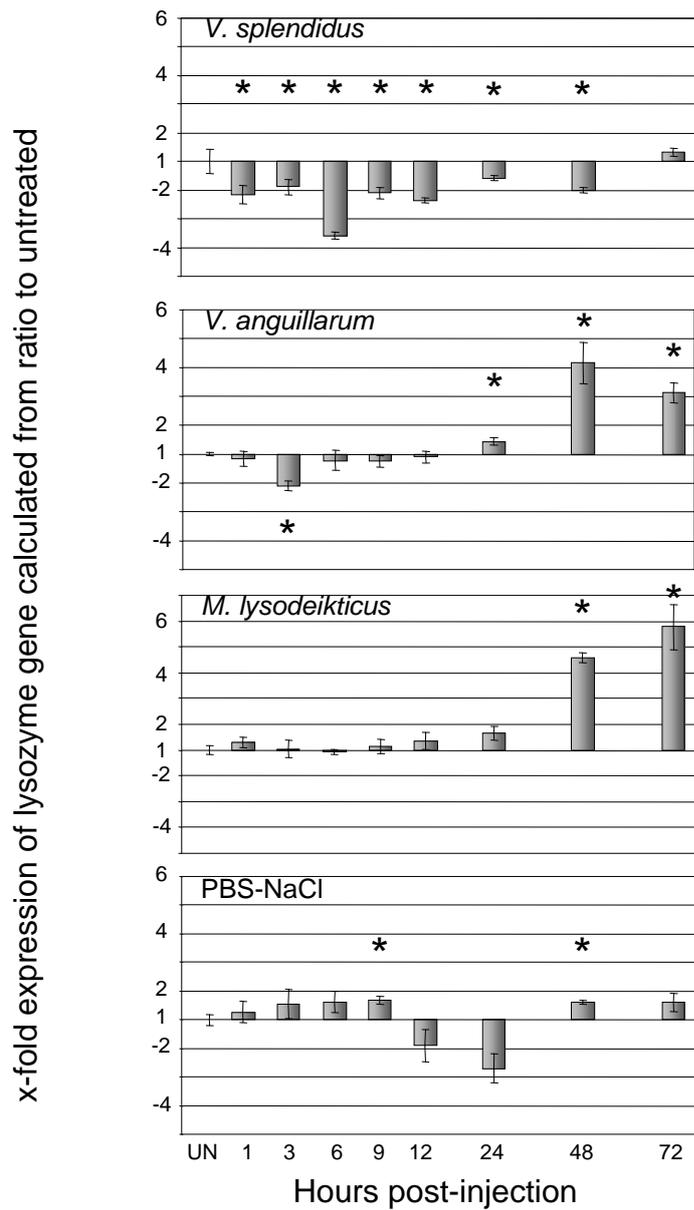
Figure 2

Figure 2 - Kinetics of expression level of lysozyme gene following injection of *Vibrio splendidus* LGP32, *V. anguillarum*, *M. lysodeikticus* or PBS-NaCl. Values were inferred from 4 experiments performed in duplicate and plotted as mean ± SEM (bar). *: values statistically significantly different from untreated (UN) with p<0.05.
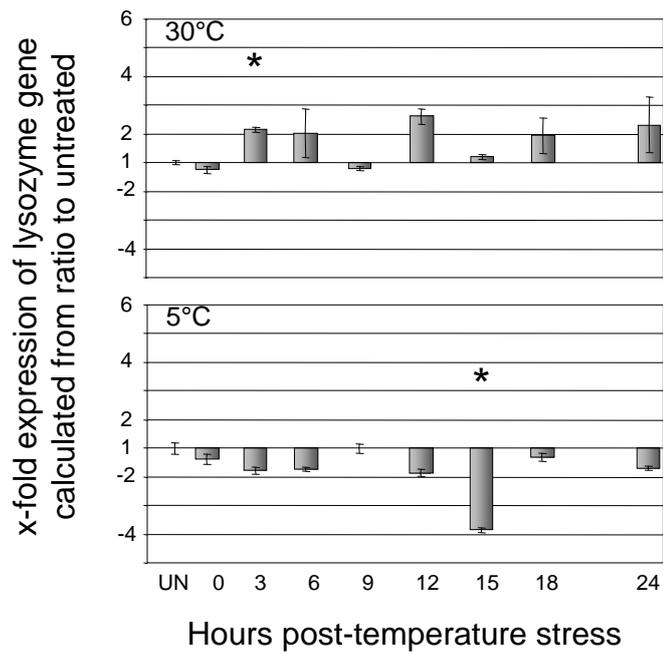
Figure 3

Figure 3 - Kinetics of expression level of lysozyme gene after temperature stress at 30°C and 5°C. Values were inferred from 4 experiments performed in duplicate and plotted as mean ± SEM (bar). *: values statistically significantly different from untreated (UN) with p<0.05.
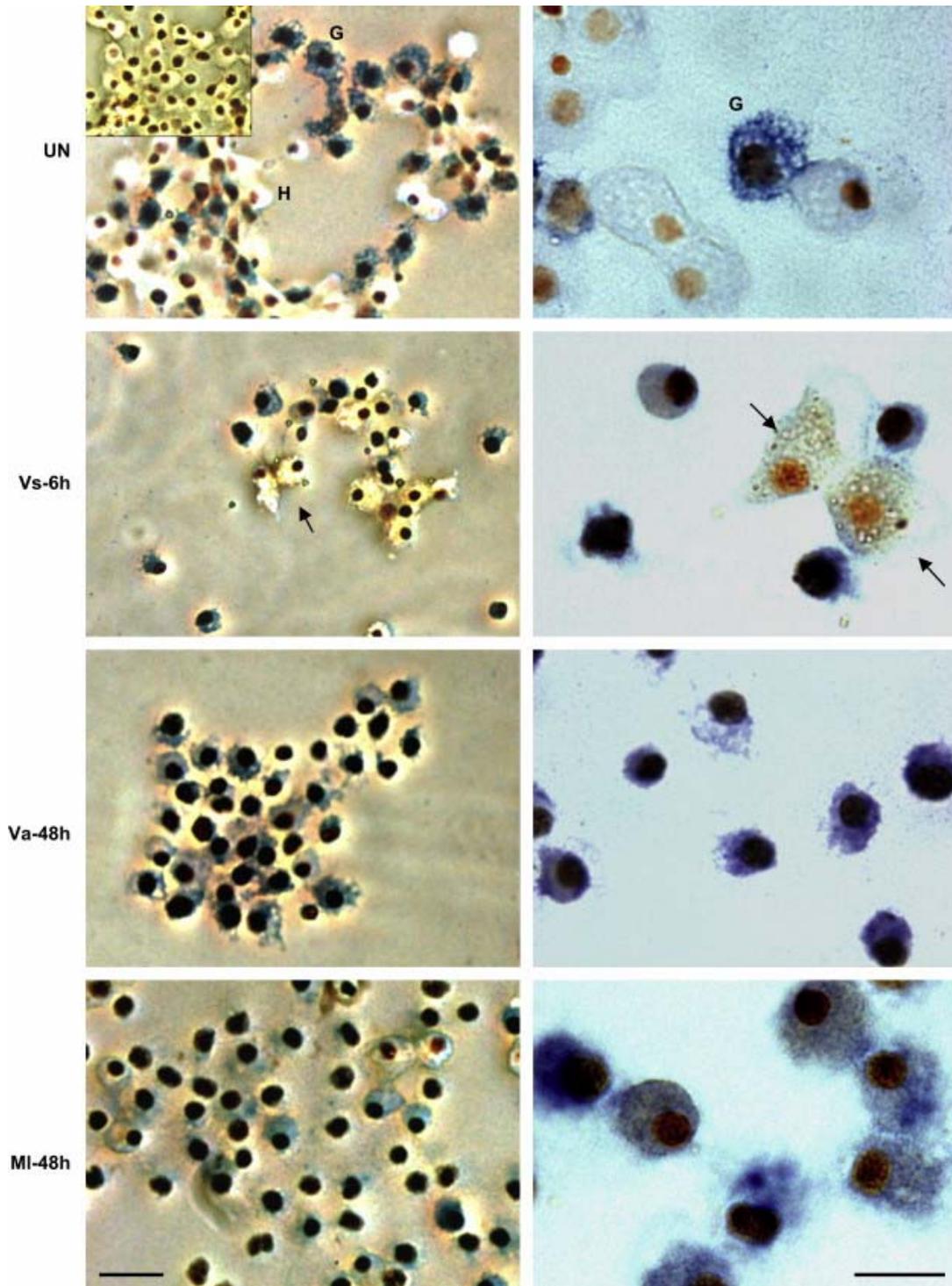
Figure 4 - Optical microscopy observations of ISH on circulating hemocytes from untreated (UN) and bacteria-injected mussels revealing lysozyme gene expression in blue. Counter staining of nuclei was with Bismarck brown. Vs-6h: *Vibrio splendidus* LGP32 6 h post-injection; Va-48h: *V. anguillarum* 48 h post-injection; Ml-48: *Micrococcus lysodeikticus* 48 h post-injection. G: granulocytes expressing lysozyme gene; H: hyalinocytes not expressing lysozyme gene. Upper left window in UN corresponded to hybridization with sense lysozyme probe. Note the absence of blue coloration in granulocytes from Vs-6h (arrows), the disappearance of granulocytes and the blue coloration of all the small hemocytes from Va-48h, and the mixed population of blue granulocytes and small hemocytes from Ml-48h. Magnification bars: 50 µm (left column) and 20 µm (right column).
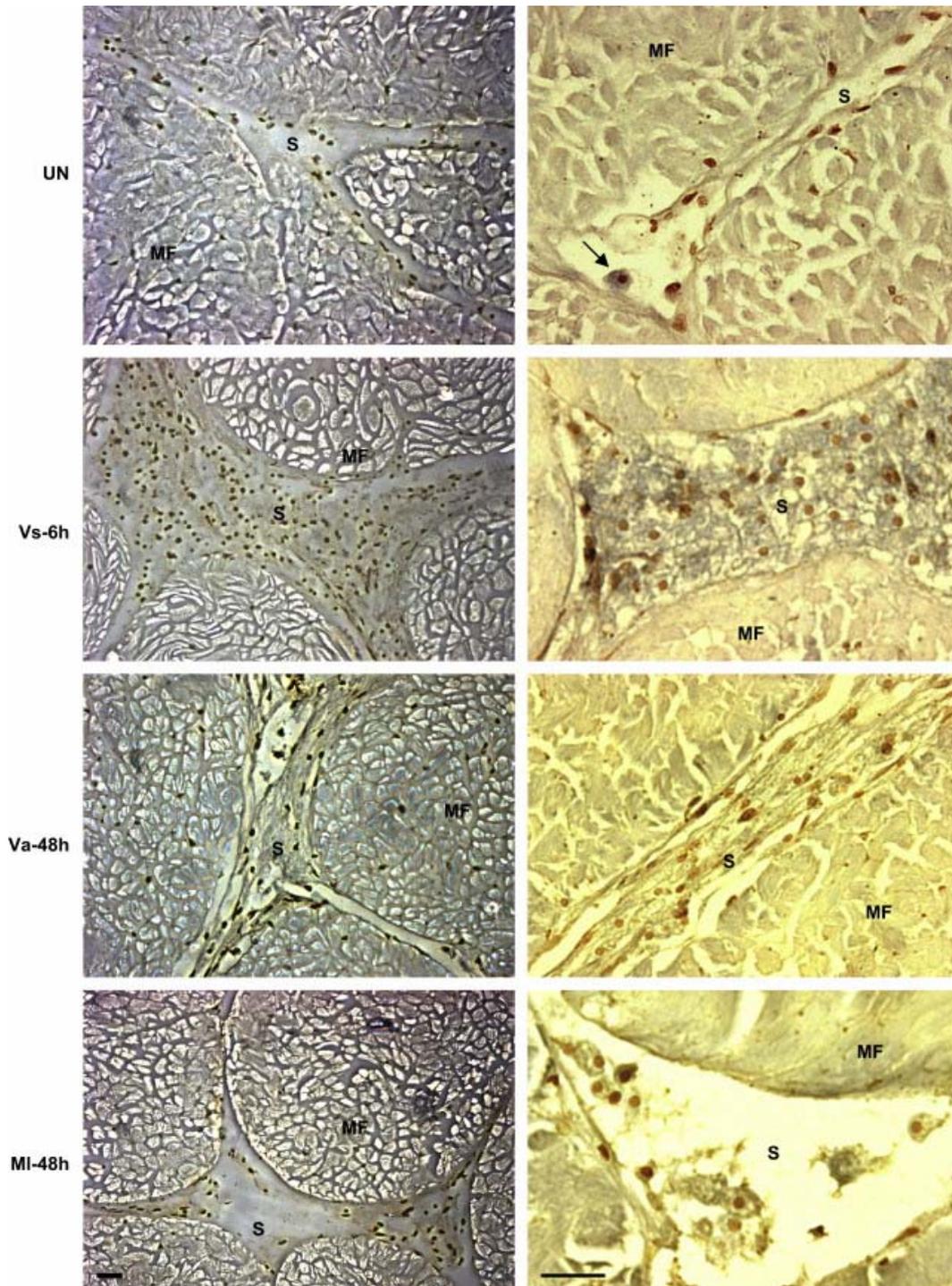
Figure 5 - Optical microscopy observations of ISH on posterior adductor muscle from untreated (UN) and bacteria-injected mussels revealing lysozyme gene expression. Counter staining of nuclei was with Bismarck brown. Vs-6h: *Vibrio splendidus* LGP32 6 h post-injection; Va-48h: *V. anguillarum* 48 h post-injection; Ml-48: *Micrococcus lysodeikticus* 48 h post-injection. MF: muscle fibers; S: sinus. Note the open clear sinus in UN containing one blue stained granulocyte (arrow), the enlarged sinus containing aggregated blue stained hemocytes from Vs-6h, the accumulation of unstained aggregated hemocytes within narrow sinus from Va-48h, and the open sinus containing few free hemocytes from Ml-48h. Magnification bars: 50 μm.