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A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a *Vibrio* infection

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Abstract:

Understanding of antimicrobial defence mechanisms of penaeid shrimp should help in the design of efficient strategies for the management and disease control in aquaculture. In this study, we have specifically analysed the expression in circulating hemocytes of antimicrobial peptides (AMPs) encoding genes, such as PEN2 and PEN3, ALF, crustin, lysozyme and a putative cysteine-rich peptide. We evidenced a relationship between the level of expression of some AMPs and the successful response of the shrimp, *Litopenaeus stylirostris*, to circumvent a pathogenic *Vibrio penaeicida* infection. Additionally, significant differences in some AMP transcript amounts are evidenced between control, non-selected shrimp line and the third generation breeding of shrimp selected for their survival to natural *V. penaeicida* infections. On the basis of these results, it will now be of great interest to determine if these AMPs are directly involved in the resistance of shrimp to infection or if they only reflect other acquired defence mechanisms which can confer a resistance.

Keywords: Crustacean; Decapoda; Penaeid; Vibrio penaeicida; Immune response; Penaeidins; Lysozyme; Anti-LPS factor; Crustin; Cysteine-rich peptide; Real-time PCR

Abbreviations: AMP, antimicrobial peptide; PEN, penaeidin; ALF, anti-lipopolysaccharide factor; EF-1α, elongation factor-1 alpha; PCR, polymerase chain reaction; rtPCR, real-time PCR; Ct, cycle threshold; PL, post-larvae; G3, third generation

1. Introduction

Understanding of antimicrobial defence mechanisms in penaeid shrimp (order: Decapoda, family: Penaeidae) is of a prime importance to develop approaches for limiting the impact of infectious diseases which affect the shrimp aquaculture. Advances have been made in the field of immunity with the molecular characterization of several antimicrobial peptide (AMP) families by both biochemical and genomic approaches. Penaeidins have been the first AMP family characterized from the shrimp *Litopenaeus vannamei* (Destoumieux *et al.*, 1997). Isolated from hemocytes, the blood cells, they have been intensively studied regarding their *in vitro* antimicrobial activities and their gene expression in response to microbial stimulation and *Vibrio* infections (for review Bachère *et al.*, 2004). With the development of large scale genomic methods, penaeidins have been identified in almost all the shrimp species studied. They have been reported following several EST (Expressed Sequence Tag) pilot projects from individual non-immune challenged *L. setiferus* and *L. vannamei* (Gross *et al.*, 2001) or from *Penaeus monodon* (Lehnert *et al.*, 1999; Supungul *et al.*, 2002; Tassanakajon *et al.*, 2006) and *Fenneropenaeus chinensis* (Shen *et al.*, 2004). This peptide family is now known to be ubiquitous for penaeid shrimp and original regarding structural characteristics (Yang *et al.*, 2003; Cuthbertson *et al.*, 2005, Cuthbertson *et al.*, 2008). The penaeidin family is constituted by three subgroups of peptides (PEN2, -3 and -4) which present characteristic features according to amino acid sequences and motifs (Gueguen *et al.*, 2006). The functional significance of such peptide diversity in animals is not yet well understood. Apart penaeidins, several antimicrobial peptides have been also identified by genomic approaches. These studies reveal a high level of conservation between immune effector cDNAs from the different shrimp species, *L. vannamei* and *L. setiferus* (Gross *et al.*, 2001), *L. stylirostris* (de Lorgeril *et al.*, 2005), *P. monodon* (Supungul *et al.*, 2004), *F. chinensis* (Shen *et al.*, 2004; Dong and Xiang, 2007) and *P. japonicus* (Rojtinnakorn *et al.*, 2002; He *et al.*, 2004). These effectors include anti-lipopolysaccharide (anti-LPS) factors initially characterized in horseshoe crab (Morita *et al.*, 1985), lysozyme (Hikima *et al.*, 2003; Sotelo-Mundo *et al.*, 2003) and crustins, polypeptidic molecules first evidenced in crab (Relf *et al.*, 1999; Bartlett *et al.*, 2002).

Until now, the involvement of AMPs in shrimp defence have begun to be approached with *in vitro* antimicrobial activity studies (Destoumieux *et al.*, 1999; Hikima *et al.*, 2003; Somboonwiwat *et al.*, 2005; de-la-Re-Vega *et al.*, 2006; Zhang *et al.*, 2007a, b; Supungul *et al.*, 2008) and characterization of their gene expression following microbial challenge or

infections (Munoz *et al.*, 2002; Munoz *et al.*, 2004; Liu *et al.*, 2005; Somboonwiwat *et al.*, 2006; Burge *et al.*, 2007; Kang *et al.*, 2007; Okumura *et al.*, 2007). However, the real role of these immune effectors in the elimination of pathogens or the resistance to diseases in shrimp remains largely unknown. In other animals, several studies have evidenced the importance of AMPs in fighting pathogens. In crayfish, *Pacifastacus leniusculus*, study using RNAi technology has showed that inhibition of anti-lipopolysaccharide factors (ALF) expression result in higher rates of viral propagation (Liu *et al.*, 2006). In *Drosophila*, immune potentiation and genetic studies demonstrated that the pathogen, *Pseudomonas aeruginosa*, in the initial stages of its host interaction, suppresses the defence response of the fly by limiting AMP gene expression (Apidianakis *et al.*, 2005). In vertebrates, transgenic mice that constitutively overexpressed porcine cathelicidin AMP (PR39) have been shown to display an increase resistance to *Streptococcus* skin infection (Lee *et al.*, 2005).

Here, for the first time, we have evidenced the relationship between the expression of antimicrobial peptides/proteins and the successful response of shrimp to circumvent a highly pathogenic bacteria infection. *Vibrio penaeicida* is a bacterium which predominantly affects juvenile shrimp, *L. stylirostris* (Goarant *et al.*, 1999) and *P. japonicus* (Ishimaru *et al.*, 1995) whereas earlier developmental larvae are not affected. This acquisition of susceptibility would be correlated with immunological or physiological changes occurring with the last post-larvae molt (Goarant *et al.*, 1998), and natural mortality associated was named “Syndrome 93”. *V. penaeicida* infection kinetics is well known in *L. stylirostris*, that allowed us to evidence, in a previous work, differences in the amount of immune effector transcripts between shrimp that survived acute infection and those which did not survived (de Lorgeril *et al.*, 2005). These results prompted us to specifically analyse the expression of AMP encoding genes, such as *Litsty* PEN2 and PEN3, ALF, crustin, and lysozyme. In addition, we have also considered the expression profile of a new anionic cysteine-rich peptide, with unknown function, that we previously identified in *L. stylirostris* as being regulated upon *Vibrio* infection (de Lorgeril *et al.*, 2005). The expression profiles of these AMP genes have been compared, on the one hand, according to survival capacities of shrimp, and on the other hand, between control, non-selected shrimp and shrimp issued from the third generation breeding of animals selected for their survival to natural *V. penaeicida* infections. Our results suggest that significant differences in basal levels of some AMP transcript amounts are evidenced between non-selected and selected shrimp lines and that according to the expression profile observed during infection, some AMP genes can reveal in shrimp their further capability to circumvent acute infection.

2. Material and methods

2.1. Shrimp

Juvenile *L. stylirostris* (20-30g) of two selected shrimp lines were obtained from the French New Caledonia Ifremer laboratory (Ifremer, BP2059, 98846 Noumea cedex, New Caledonia, France). For three generations, an experimental line has been developed by mating animals which had survived "Syndrome 93" episodes in commercial-like grow out ponds during the first 6 months of their life (initial densities : 20-25 PL/m^2). At each generation, the selected animals were then reared at lower densities (1-2 shrimp/m^2) to be reproduced at the age of 12 months. A non-selected control line was maintained in parallel by mating animals reared in unaffected "traditional" brood stock ponds in which the occurrence of the "Syndrome 93" is very low (initial density: 2 PL/m^2). In both lines, inbreeding was managed by the use of tags which avoided mating close relatives. The animals used in the experiments described in this paper were sampled among the third generation of the selected line and among the third generation of the control line.

2.2. Experimental infections

The infections were carried out by immersing individual shrimp for 2 hours in seawater tanks containing 1 x 10^3 colony forming unit (CFU) of *V. penaeicida* strain AM101 per ml, corresponding to LD20 conditions (lethal dose 20%) (Saulnier *et al.*, 2000). Animals were then rinsed with filtered seawater and transferred into 100 L tanks. Non-infected animals were kept in a separate 100 L tank. Hemolymph of animals at the intermoult stage was taken from the ventral sinus located at the base of the first abdominal segment, under an equal volume of anticoagulant Modified Alsever Solution (MAS) (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7; Rodriguez *et al.*, 1995).

For the first experimental infection, three groups of animals (15 shrimp per group) from each shrimp lines (control and selected) were put in separate tanks. For the first group, hemolymph was collected from each shrimp line 24 h prior to the experimental infection (-24) and these samples were considered as uninfected control. Samples for the two other groups were collected from each shrimp line at 12 h (+12) and 24 h (+24) post-infection.

The second experimental infection is described in figure 1. This experiment was conducted for real time PCR analyses at individual level and performed using the same experimental conditions. Hemolymph sampling was done at three different times for both

shrimp lines. In this experiment, shrimp were individually identified using coloured silicone injection under the third abdominal segment of the cuticle and hemolymph RNA samples were individually distinguished between animals that had survived the infection from those that died before 96 h following infection. In this experiment, hemolymph was collected without causing the death of the shrimp. Gene expression profiles were considered in the shrimp collected before infection as control (T-24). In shrimp collected 24 h post-infection, a distinction was done between animals which had survived the infection (T+24S) and those which had not survived (T+24NS) (N = 5-6). After hemolymph collection, hemocytes were isolated by centrifugation (800 g for 15 minutes at 4°C) and the pellets of hemocyte were resuspended in 1 ml of RNAlater (Ambion), incubated for 24 h at 4°C and them stored at -20°C for further RNA isolation.

2.3. Antimicrobial peptide cDNA sequences

To investigate AMP genes expression in shrimp circulating hemocytes, we have selected six cDNA sequences of AMPs (or putative AMPs) from *L. stylirostris* described in recent studies (Munoz *et al.*, 2004; de Lorgeril *et al.*, 2005). The GenBank accession numbers of the penaeidins, *Litsty* PEN2 and -PEN3, are AY351656 and AY351655, respectively. The GenBank accession numbers of lysozyme and the putative “cysteine rich peptide” isolated in a previous work (de Lorgeril *et al.*, 2005) are CV699332 and CV699287, respectively. The crustin cDNA sequence partially identified with the subtractive cDNA library (de Lorgeril *et al.*, 2005) was recently fully characterized. Finally, ALF cDNA sequence was obtained from post larvae *L. stylirostris* cDNA library (M. G. Janech, unpublished) (GenBank accession no. DQ010421).

2.4. In situ hybridization experiments

In situ hybridization analyses were carried out according to the method described by Munoz *et al.* (Munoz *et al.*, 2002). In brief, plasmids containing *Litsty* PEN3 and *L. stylirostris* ALF cDNA (Genbank accession number AY351655 and DQ010421, respectively) were used as template for the preparation of the probes. Digoxigenin (DIG)-UTPlabelled antisense and sense riboprobes were generated from linearized cDNA plasmids by *in vitro* transcription using RNA labelling kits, T3 RNA polymerase (Roche). DIG-labelled riboprobes were hybridized to hemocyte preparations as described previously (Munoz *et al.*, 2002).

2.5. RNA isolation and real time PCR analysis

Total hemocyte RNAs from infected and uninfected shrimp were isolated using Trizol reagent (Gibco BRL) (1 ml/10^7 cells). Pooled hemocyte total RNA (15 individuals per tested condition) collected from uninfected shrimp (T-24) and infected shrimp (12 and 24 h post-infection) from both shrimp lines (control and selected), were subjected to preliminary quantitative real-time polymerase chain reaction (rtPCR) analysis to determine whether acute changes in RNA abundance of immune effectors could be detected earlier from 12-24 h post infection. Total RNAs were treated with DNase (TURBO DNase, Ambion) to remove contaminating genomic DNA and then, DNase was eliminated by phenol chloroform extraction. First-strand cDNA was synthesized from 1 µg of total RNA, using SuperScript II reverse transcription kit, according to the manufacturer's instruction (Invitrogen), in 20µl of volume reaction. 0.5µl of each reverse transcription reaction served as template in 10µl of rtPCR reaction containing 1X SYBR Green master mix (Qiagen) and 0.5 µM of each primer. A list of oligonucleotide primers used to amplify gene products are shown in Figure 2A. Each rtPCR reaction was done in triplicate with an initial denaturation step of 900s at 95°C followed by an amplification of the target cDNA (35 cycles of denaturation at 95°C for 15 seconds, annealing between 54°C and 64°C for 15 seconds and extension time at 72°C for 15 seconds) and performed with the LightCycler (Roche Molecular Biomedicals). In addition, to determine the rtPCR efficiencies of each primer pair used, standard curves were generated using five serial dilutions of plasmid containing the insert of interest (10^3 to 10^7 copies/µl). Results are presented here as changes in relative expression normalized to the reference gene, elongation factor-1α (EF-1α, GenBank accession AY117542) using the method described by Pfaffl (Pfaffl, 2001). rtPCR efficiencies of tested genes varied between 1.87 and 1.98 (Figure 2A), however, as these efficiencies were not exactly 2.00 (representing 100% amplification efficiency at each cycle), we calculated the relative abundance using the corrected equation for differences in efficiency, as described by Pfaffl (Pfaffl, 2001).

A second rtPCR experiment was conducted only on randomly selected samples taken from 5 or 6 individual shrimp from each shrimp line. Shrimp are labelled as follows: surviving shrimp at 96 h post infection (+24S); not surviving shrimp at 96 h post infection (+24NS) and uninfected control shrimp sampled at 24 h before infection (T-24). This analysis was conducted to verify differences observed in the first experiment and to incorporate statistical data. The rtPCR conditions and analytical procedures were identical to the one from the first experiment and statistical significance was determined using ANOVA-Test LSD

between experimental conditions tested. Significant differences were considered when $P<0.05$.

3. Results

3.1 AMP gene expression profiles during Vibrio infection differ in selected shrimp line and control line.

Expression profiles of genes coding for the AMPs, *Litsty* PEN2 and -PEN3, ALF, crustin, lysozyme and cysteine-rich peptide, were analyzed by rtPCR to examine the general pattern of expression during the course of *V. penaeicida* infection. This preliminary analysis of pooled hemocytes from 15 animals was performed for a shrimp line selected from natural surviving capacity to *Vibrio* infection (selected line) and a control line which has not been subject to a selection (control line) (Figure 2).

Given the criteria that approximately two-fold changes in transcript abundance represent differential expression (Larkin *et al.*, 2003; Wang *et al.*, 2001), the abundance of RNAs for ALF, lysozyme, crustin and cysteine-rich peptide appeared to be modulated during the course of infection compared to uninfected animals in both tested shrimp lines. Among tested AMPs, only ALF displayed different expression pattern during the infection between the two shrimp lines. Indeed, ALF transcript abundance increases at 12 and 24 h post-infection for the control line (relative increase of 3 and 2.1-fold *versus* uninfected shrimp, respectively), but this increase is delayed at 24 h post infection for the selected line (by a factor of 2.7-fold). On the other hand, a fall of transcript abundance was showed for the other AMPs in the first 12 h post-infection. This is particularly evident for lysozyme, crustin and the cysteine-rich peptide in the control shrimp line (relative decrease of 3.2, 3.3 and 4.5-fold, respectively *versus* uninfected shrimp) and in the selected shrimp line (relative decrease of 25, 3.5 and 9.1-fold, *versus* uninfected shrimp, respectively). This fall in transcript abundance of crustin and cysteine-rich peptide is similar for both lines, and followed by a tendency to return to levels observed in uninfected shrimp at 24 h. This increase in crustin and cysteine-rich peptide transcripts between 12 and 24 h post infection appears significantly more important in selected shrimp line (relative increase of 5.8 and 9.8-fold, respectively) than in control line (relative increase of 2.5 and 2.6-fold, respectively). Same expression profile was found for lysozyme until 12 h post infection, but these lysozyme transcripts remained in lower abundance at 24 h for selected line (relative decrease of 3.57-fold *versus* uninfected shrimp), than in the control shrimp line. In this first analyse, penaeidin expression patterns were

observed moderately modulated, particularly for *Litsty* PEN3 in the control shrimp line. However, for the selected shrimp line, *Litsty* PEN2 and -PEN3 displayed an increase of transcript abundance at 24 h post-infection (relative increase of 3.93 and 2.73-fold, respectively *versus* shrimp collected 12 h post infection).

3.2. Abundance of ALF and Litsty PEN3 transcripts and composition of circulating hemocyte populations.

In previous work, modulation of penaeidin transcripts from hemocytes has been shown to be related to changes in the composition of circulating hemocyte populations (Munoz *et al.*, 2002). To better understand the modulations of expression profiles of ALF and penaeidins in our experiments, we have determined the percentage of circulating hemocytes which expressed, respectively, *Litsty* PEN3 and ALF transcripts, during the infection and in the two shrimp lines (Figure 3). Pooled hemocytes (N=15 shrimp) were analysed both by *in situ* hybridization and by rtPCR from uninfected, and infected shrimp at 12 and 24 h post infection. Two-fold changes in percentages of positive cells have been considered as representative. Regarding *Litsty* PEN3, the relative number of hemocytes, that express *Litsty* PEN3, decrease slightly from the circulating populations within the first 12 h and stay under the level of uninfected shrimp at 24 h after infection in the control line. However, in the selected animals the number of *Litsty* PEN3-expressing hemocytes together with the relative abundance of transcripts increase between 12 and at 24 h (relative increase by a factor of 2-fold of positive hemocytes) at a higher level than observed before infection. At the contrary, in the first 12 h post infection of control shrimp line, the relative number of hemocytes that express ALF increases in the blood circulation (relative increase by a factor of 2-fold), related to an increase in transcript abundance compared to non infected shrimp. At 24 h, the number of positive cells continues to increase (factor of 2.8-fold) with a relative abundance of ALF transcripts which stay in higher level than uninfected shrimp. For the selected shrimp, a continue increase of both relative number of ALF-positive hemocytes (factor of 2-fold) and relative abundance of transcript is observed from non infected shrimp to 24 h post-infection.

3.3. AMP transcript abundance can be related to shrimp survival capacity and to differences between the two shrimp lines.

A second round of real-time PCR analyse was made to compare RNA abundance of AMPs between uninfected shrimp (-24) and shrimp collected 24 h after infection which survived to the infection (+24S) and which did not survived (+24NS), from both the selected

and control shrimp lines (Figure 4). Transcript abundances were determined using individual samples (N = 5-6) to infer differences by statistical methods between all samples for all six AMPs.

ALF showed an increase of RNA abundance 24 h after infection in both shrimp lines. In control line, significant differences were found between surviving shrimp and uninfected shrimp (1.01±0.09 versus 1.64±0.35, respectively, $p<0.05$), but not for non surviving animals. At the contrary, in the selected shrimp line, a significant increase of ALF transcript abundance was detected in non surviving shrimp compared to uninfected shrimp (1.65±0.15 *versus* 1.08±0.21, respectively, $p<0.05$). However, no significant difference was observed between surviving and non surviving shrimp in both lines ($p>0.05$).

For penaeidin family, *Litsty* PEN2 and -PEN3 transcripts decrease 24 h after infection. *Litsty* PEN3 showed significant difference in transcript abundance only for control line, with a decrease of transcripts in the non surviving shrimp compared to uninfected shrimp (0.35±0.10 versus 1.06±0.15, respectively, $p<0.05$) but also compared to surviving animals (0.35±0.10 versus 0.83±0.20, respectively, $p<0.05$). On the other hand, significant differences were observed for *Litsty* PEN2 in both lines (control and selected). In control shrimp line, a significant decrease of *Litsty* PEN2 transcripts were observed both for surviving and non surviving shrimp compared to uninfected shrimp (0.62±0.12 *versus* 1.07±0.17 and 0.32±0.25 *versus* 1.07±0.17, respectively, $p<0.05$). In the selected shrimp line, however, significant differences were seen between surviving and non surviving shrimp (0.93±0.12 *versus* 0.69±0.10, respectively, p<005), and the decrease in *Litsty* PEN2 transcripts was also significant between non surviving and uninfected shrimp (0.69±0.10 *versus* 1.04±0.14, respectively, p<005). However, no difference was observed between uninfected and surviving shrimp from this selected line.

Crustin and cysteine-rich peptide transcripts appear modulated in both shrimp lines with similar patterns of expression. Non surviving shrimp from both lines showed a very weak abundance of crustin and cysteine-rich peptide transcripts in comparison with uninfected animals (0.22±0.06 *versus* 1.08±0.12 and 0.16±0.06 *versus* 1.12±0.23, respectively for control line) (0.60±0.16 *versus* 1.07±0.16 and 0.25±0.08 *versus* 1.06±0.16, respectively for selected line). Both shrimp lines displayed significant higher abundance in crustin and cysteine-rich transcripts in surviving animals comparatively to non-surviving ones (1.10±0.30 *versus* 0.22±0.06 for crustin and 0.79±0.35 *versus* 0.16±0.06 for cysteine-rich peptide in control line, and respectively, 1.03±0.14 *versus* 0.60±0.16 and 0.91±0.23 *versus* 0.25±0.08, in selected line).

Lysozyme showed a distinct pattern of expression from other AMPs, which differ also between the two shrimp lines. In control line, we observed a dramatic decrease in transcript abundance in the non surviving shrimp compared to uninfected shrimp (0.07±0.05 *versus* 1.03±0.16, respectively, $p<0.05$) and compared to surviving shrimp (0.07±0.05 *versus* 1.71±0.64, respectively, $p<0.05$). In the selected line, both surviving and non surviving shrimp showed a significant decrease in abundance of transcripts compared to uninfected shrimp (0.13±0.02 *versus* 1.23±0.39 and 0.16±0.05 *versus* 1.23±0.39, respectively, $p<0.05$).

3.5. Basal levels of gene expression before infection differ between the two shrimp lines, control and selected

An ultimate round of real time PCR analyses was performed to compare the basal level of gene expression for lysozyme and *Litsty* PEN3 observed 24 h before the infection in the two shrimp lines (selected and control). Individual analyses revealed significant differences between the shrimp selected for survival and the control shrimp (Figure 5). In control lines, hemocytes showed a lower abundance of lysozyme transcripts compared to those from the shrimp selected line (1.21±0.29 *versus* 4.44±1.45, respectively, $p<0.05$) (Figure 5A). On the other hand, *Litsty* PEN3 transcripts were detected in higher abundance in hemocytes from control shrimp than in hemocytes from selected shrimp line (1±0.03 *versus* 0.71±0.08, respectively, $p<0.05$) (Figure 5B).

4. Discussion

This study describes for the first time differences in AMP gene expression or abundance of AMP transcripts according to the survival capacity of the shrimp, *L. stylirostris*, to survive a *Vibrio* infection. In this work, we also evidenced differences related to the genetic selection of shrimp that have survived in their environment to natural *Vibrio* infection named “Syndrome 93”. Recent advances have been made in the characterization of AMPs in penaeid species through biochemical and genomic approaches. All AMP families described to date appear to be ubiquitous in penaeid shrimp. In *L. stylirostris*, penaeidin *Litsty* PEN2 and -PEN3 have been identified by Suppression Subtractive Hybridization (SSH) from hemocytes of shrimp that have survived a *V. penaeicida* infection (de Lorgeril *et al.*, 2005). In the SSH library, we also have identified lysozyme transcripts as being modulated in response to *Vibrio* infection as well as a putative cysteine-rich antimicrobial peptide, presenting some similarities with cysteine-rich peptide from *Mus musculus*, named cryptidin (Ouellette and Lualdi, 1990).

ALF and crustin transcripts have also been characterized from *L. stylirostris* larvae using a cDNA library (M. Janech, personal communication).

The availability of these various AMPs identified in *L. stylirostris* led us to analyse and to compare their expression patterns during *Vibrio* infection in circulating hemocytes. First analyses of expression profiles were considered from pooled hemocyte RNA samples obtained from shrimp at the main phases of the immune response previously characterized (Bachère *et al.*, 2004) and according to the *Vibrio* infection course. The time 12 h post-infection corresponds to the first phase immune reaction with a migration of circulating granular hemocyte population towards the site of infection (Muňoz *et al.*, 2002). The point 24 h corresponds to the peak of mortalities induced by *V. penaeicida* but also to the second phase of the immune response characterized by a stimulation of hematopoiesis and increase in penaeidin-3 gene expression (Muňoz *et al.*, 2004). At that time, most of the mechanisms involved in a successful elimination of the *Vibrio* may occur. Among tested AMP genes, namely *Litsty* PEN2, *Litsty* PEN3, cysteine-rich peptide, crustin, ALF and lysozyme, different expression patterns can be observed for both shrimp lines (Figure 2B). Apart ALF gene, all the tested genes showed a fall of transcript abundances during the first hours post-infection followed by an increase in the late infection phase (*Litsty* PEN2 and -PEN3, lysozyme, crustin and cysteine-rich). These expression profiles are in agreement with previous studies on penaeidins in *L. stylirostris* (Muňoz *et al.*, 2004) as well as on crustin in *L. vannamei* (Burge *et al.*, 2007) and lysozyme in *P. monodon* (Supungul *et al.*, 2004). The fall in AMP transcripts during the first phase (12 h) post-infection may correspond to a migration of AMP expressing hemocytes from the blood circulation and the lyse of granular hemocytes within the tissues at the site of infection (Bachère *et al.*, 2004). At 24 h, transcripts for *Litsty* PEN2 and -PEN3, lysozyme, crustin and cysteine-rich peptide increase again in circulating hemocytes that corresponds to the second phase of the immune response. The expression pattern of ALF differs with, at the contrary, an increase of their transcripts within the first phase, which is in agreement with previous study in *P. monodon* during *V. harveyi* challenge (Supungul *et al.*, 2004). ALF may be expressed in different hemocyte populations than the other studied AMPs. *In situ* hybridization assays on circulating hemocytes seem to confirm this hypothesis (Figure 3), but it is not excluded that the expression of this AMP is transcriptionally regulated during the second phase of the immune response. Concerning shrimp from the selected line, the patterns of expression appeared to be more modulated than for the control line, particularly in the later phase of the immune response. Indeed, a higher increase of *Litsty* PEN2 and -PEN3, crustin and cysteine-rich transcripts are seen at 24 h after infection in the shrimp selected for

survival, than in the control line. These differences in expression patterns between both shrimp lines could be the result of a differential capacity to produce through hematopoiesis new hemocytes which present a higher transcriptional activity of these effectors as shown for penaeidins (Muňoz *et al.*, 2002; Muňoz *et al.*, 2004). Concerning ALF expression profile, the increase in abundance of transcripts appears to be delayed in the selected line compared to the control one and no rapid increase is seen at 12 h during the first phase of the immune response. Lysozyme expression profile during infection differs also between the selected and non selected lines. The abundance of lysozyme transcripts remains lower in the selected shrimp. Based on the similarity between the gene expression patterns, these results suggest that first ALF and lysozyme would be expressed in different hemocyte population than penaeidins, crustins and the cysteine-rich. However, differences could also be related to differences in the regulation of their expression. This emphasizes the need of further fundamental studies on hemocyte lineage and on the co-localisation of the different peptides in the hemocyte populations but also at the level of shrimp tissues during the infection.

Based on these observations, we have focused our attention on the second phase of the immune response (24 h post infection) to compare at individual level the relative amount of AMP transcripts between non infected stages, and surviving or not surviving shrimp to a *Vibrio* infection, for the two shrimp lines (Figures 1 and 4). Among the different AMPs, only ALF does not show differences in transcript abundance between surviving and not surviving shrimp whatever the shrimp line. However, due to the increase in transcripts during the infection, it could be used as a prophylactic marker, revealing an immune response in shrimp. At the contrary, all other AMPs display expression profiles discriminating, at 24 h, the shrimp which will survive or will not survive the infection. These results reveal a potential link between abundance of AMP gene transcripts and the surviving capacity of shrimp. In addition, penaeidin and lysozyme expression profiles show significant differences between the two shrimp lines. For penaeidins, expression pattern of *Litsty* PEN2 can discriminate both shrimp lines with higher expression levels in the selected shrimp line, while *Litsty* PEN3 can discriminate shrimp according to surviving capacity only from control shrimp line. These observations support the idea that selected shrimp showed better capacity to produce this antimicrobial peptide family in the late phase of the immune response. However, at the contrary, lysozyme transcripts are dramatically poorly represented in the selected shrimp compared to the control animals as the result of a survival to infection. Considering our present knowledge, this phenomenon cannot be clearly explained but merit further attention. We can hypothesise that lysozyme-expressing hemocytes could be mobilized in selected

shrimp towards the site of infection where they may produce and release the molecule. Indeed, it has been shown that recombinant lysozyme is effective *in vitro* against *Vibrio* strains (Hikima *et al.*, 2003; de-la-Re-Vega *et al.*, 2006), reinforcing the potential role of this molecule in the bacterial defence capability of the shrimp.

To go further in these expression analyses as potential markers for infection resistance, we have then focused our attention on penaeidin *Litsty* PEN3 and lysozyme but considering the basal levels of transcripts before an infection in the two shrimp lines. Surprisingly, not only these genes discriminate the shrimp lines, the survival and non survival capacity during infection, but their transcript abundances significantly differ in animals before their infection, between the selected and control line (Figure 5). In a perspective of application in genetic selection of resistant shrimp, these two genes present striking predictive interest concerning the potential capacity of shrimp to survive an infection. On the basis of these results, it will be now of great interest to better understand the real function of these effectors in the immune capacity of the shrimp and to determine if there are directly involved in the resistance of shrimp to infection or if they only reflect other acquired defence mechanisms which can confer a resistance.

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Figure legends

Figure 1. Experimental protocol used for the AMP transcript expression analyses in both shrimp lines of *L. stylirostris* after a *Vibrio penaeicida* infection. Details on the time table sampling of shrimp hemocytes for individual expression analyses. Two groups from each shrimp lines were set up and each shrimp of these groups was individually identified by silicone tagging. Hemocytes of the first group from each lines were collected 24 h before the infection represent the samples of uninfected shrimp (-24). Then, second groups from each line were infected by *Vibrio penaeicida* in LD20 conditions (lethal dose 20%). For infected groups, hemocytes were collected 24 h post-infection (period of mortality), without causing the death of the shrimp. For the four experimental groups, hemolymph was individually sampled. Mortalities were monitored until 96 h (end of period of acute mortalities) and hemocytes samples were sorted out according to the surviving *versus* non surviving capacity of shrimp.

Figure 2. Expression patterns of six AMP gene transcripts during *V. penaeicida* infection in circulating hemocytes from two shrimp lines. A- PCR primers (forward and reverse) used to amplified ALF, *Litsty* PEN3, *Litsty* PEN2, lysozyme, crustin and cysteine-rich peptide in real time PCR procedure. Specific annealing temperature of each primer pair is noted as well as the PCR efficiency; calculated by the equation: E=10[-1/slope] (Pfaffl, 2001). **B-** Graphics show relative expressions of ALF, *Litsty* PEN3, *Litsty* PEN2, lysozyme, crustin and cysteine-rich peptide transcript in hemocytes collected from uninfected shrimp (-24), 12 h post infection (+12) and 24 h post-infection (+24) from a selected shrimp line in grey (selected line) and from a control shrimp line not selected in black (control line). Fifteen shrimp were used for each point analysed by real time PCR. Relative expressions were normalized with elongation factor-1α (EF-1α) and each value was calculated in reference to uninfected shrimp (relative expression = 1) according the $2^{-\Delta\Delta Ct}$ method corrected for efficiencies (Pfaffl, 2001).

Figure 3. *In situ* hybridization analyses of PEN3 and ALF on circulating hemocytes during *V. penaeicida* infection from two shrimp lines. A: results obtained for *Litsty* PEN3; B: results obtained for ALF. Pictures show ALF or *Litsty* PEN3 labelling on hemocytes from uninfected shrimp (-24) and from shrimp collected 12 h and 24 h post infection (+12 and +24)

for both shrimp lines (control and selected). Corresponding percentage of positive cells was noticed below and represents the means of positive cells from three cytospins, evaluated with image analysis software (ImageJ-1.32J). Relative numbers of positive cell during infection were calculated in reference to the number of positive cells from uninfected shrimp (relative number of positive cell = 1) and were reported on graphic histograms, where relative expressions of corresponding transcript already present in Figure 2 were superposed in curves.

Figure 4. Real time PCR analyses of AMP gene transcripts in hemocytes of surviving and non surviving shrimp to *V. penaeicida* infection from two shrimp lines. Relative expression of ALF, *Litsty* PEN3 (PEN3), *Litsty* PEN2 (PEN2), lysozyme, crustin and cysteine-rich peptide transcripts were obtained from two selected shrimp lines. Results are expressed as mean values from five or six shrimp collected before infection (T-24) and shrimp collected 24 h post-infection, which have survived infection (T+24S in black) or did not survive infection (T+24NS in grey). Results are expressed as mean values ± SDV from five or six shrimp per experimental conditions. Relative expression levels were normalized with elongation factor-1α and values during the infection were calculated in reference to the uninfected group (relative expression = 1) according to the $2^{-\Delta\Delta Ct}$ method corrected for efficiency (Pfaffl, 2001). Experimental conditions bracketed together present statistical difference, $p < 0.05$ (ANOVA-test LSD).

Figure 5. Real time PCR analyses of lysozyme and *Litsty* PEN3 basal transcript abundance from two shrimp lines. Graphics show relative expressions of lysozyme (**A**) and *Litsty* PEN3 **(B)** transcripts in hemocytes from a control shrimp line in black and from a selected shrimp line in white. Relative expression levels were normalised with elongation factor-1α and results are expressed as mean values ± SDV from five or six shrimp per shrimp line. Each value was calculated in reference to the control line group (relative expression = 1) according to the $2^{-\Delta\Delta Ct}$ method corrected for efficiency (Pfaffl, 2001). * denotes statistical difference between the two shrimp lines, $p < 0.05$ (Student's *t* test).

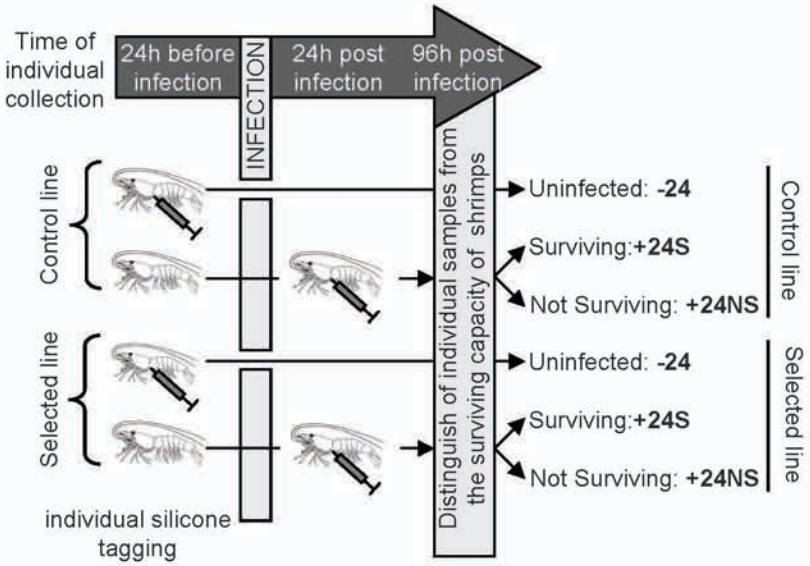

Time of individual collection
24h before infection
INFECTION
24h post infection
96h post infection
Control line
Selected line
individual silicone tagging
Distinguish of individual samples from the surviving capacity of shrimps
Uninfected: -24
Surviving:+24S
Not Surviving: +24NS
Uninfected: -24
Surviving:+24S
Not Surviving: +24NS
Control line
Selected line

Figure 1

A

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing (°C)	PCR efficiency
ALF	CAAGAGGATACAGCTGCACTACAAGG	CTTGGCGTCTTGTTCGGAGATGAG	55	1,87
Litsty PEN3	CCATGCGCCTCGTGGTCTG	GAACGCGCTTGTAAGGTGGTAA	64	1,98
Litsty PEN2	CCATGCGCCTCGTGGTCTG	AGCAATTGCGGCATCTGGGAA	64	1,91
lysozyme	GGCTTGGCACCAGGGTTACC	CGTCTGCACGTCAGCTGTG	59	1,93
crustin	CCAAGTCCGTCCCACATGC	CTGTCGAAGCAGCACTTGTC	55	1,91
cysteine-rich	GCCCTAAGTGCCCATATGA	GCCGCATTCACATCCTATA	54	1,89
EF-1α	GGTGCTGGACAAGCTGAAGGC	CGTTCCGGTGATCATGTTCTTGATG	60	1,96

B

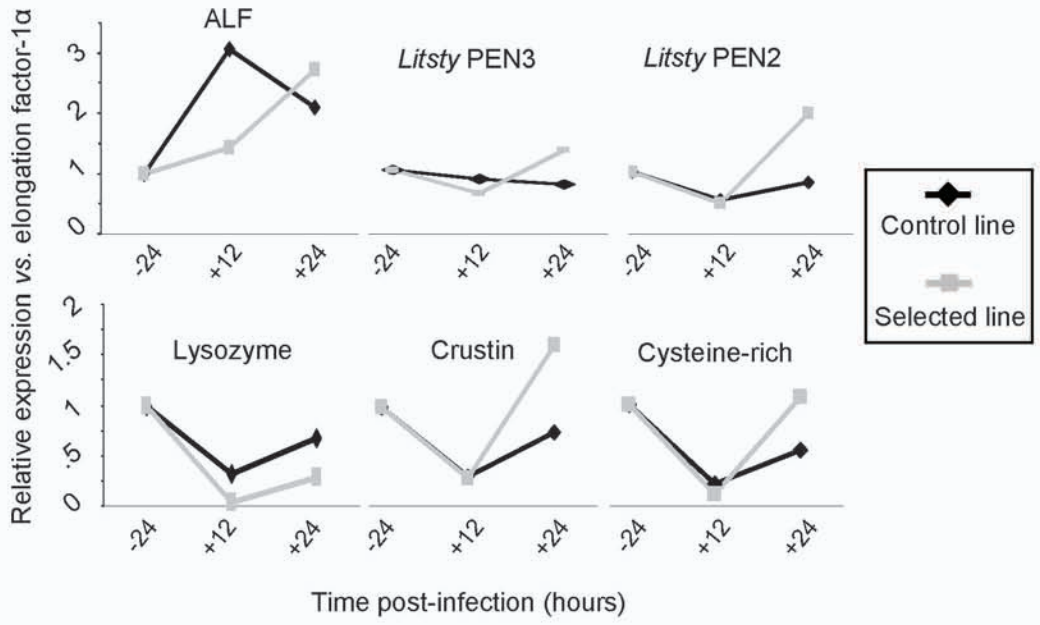
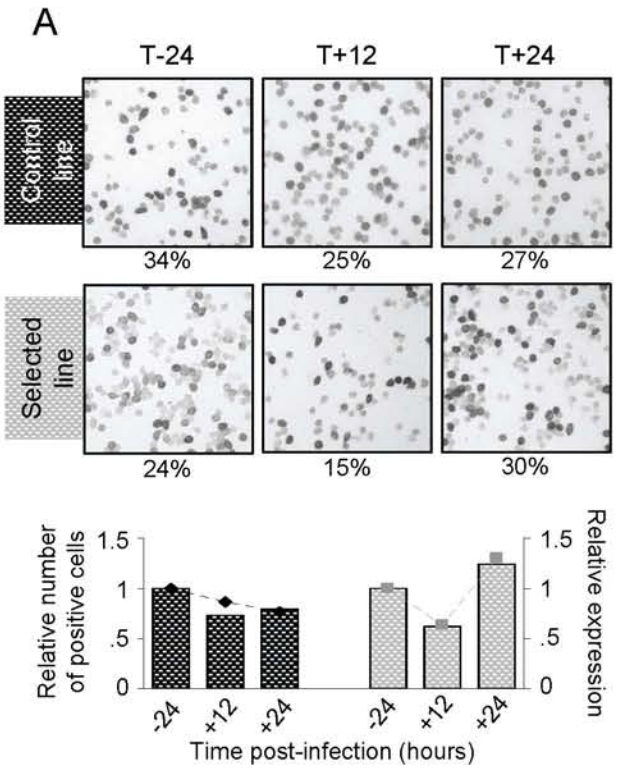


Figure 2


A
T-24
T+12
T+24
Control line
34%
25%
27%
Selected line
24%
15%
30%
Relative number of positive cells
Relative expression
1.5
1
.5
0
-24
+12
+24
Time post-infection (hours)

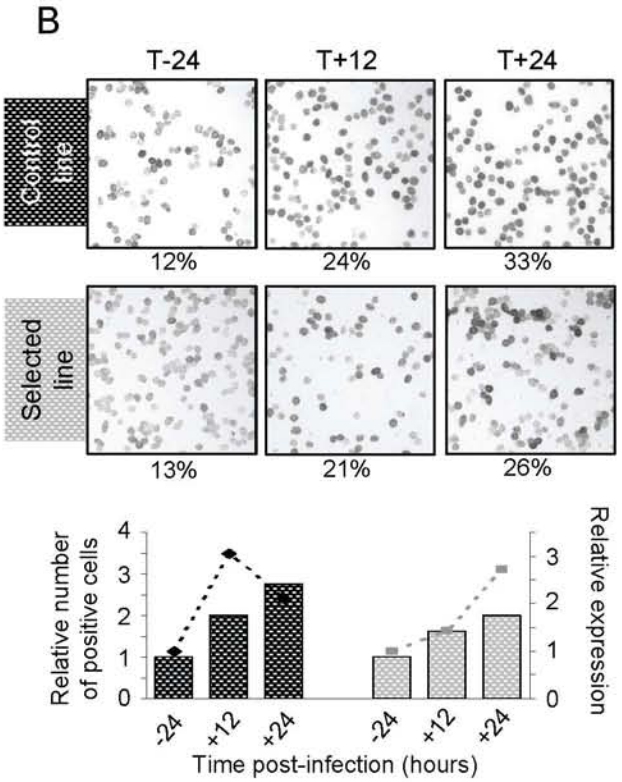

B
T-24
T+12
T+24
Control line
12%
24%
33%
Selected line
13%
21%
26%
Relative number of positive cells
Relative expression
4
3
2
1
0
-24
+12
+24
Time post-infection (hours)

Figure 3

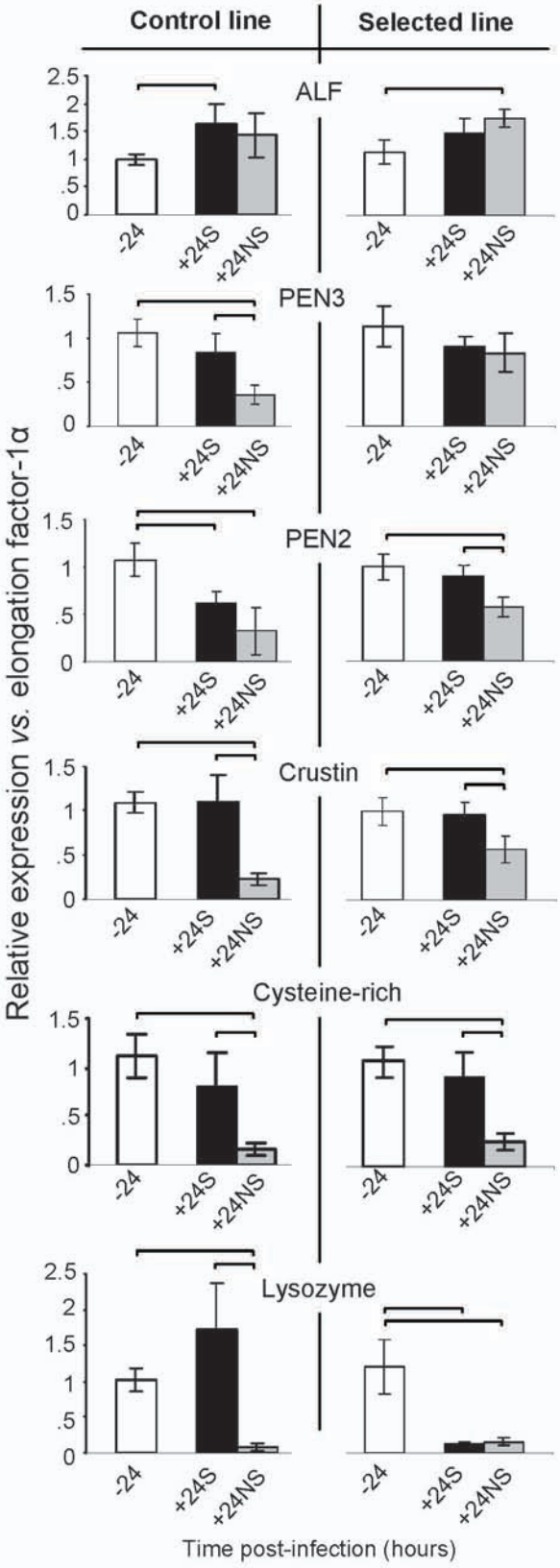

Control line
Selected line
ALF
PEN3
PEN2
Crustin
Cysteine-rich
Lysozyme
Relative expression vs. elongation factor-1α
-24
+24S
+24NS
Time post-infection (hours)

Figure 4

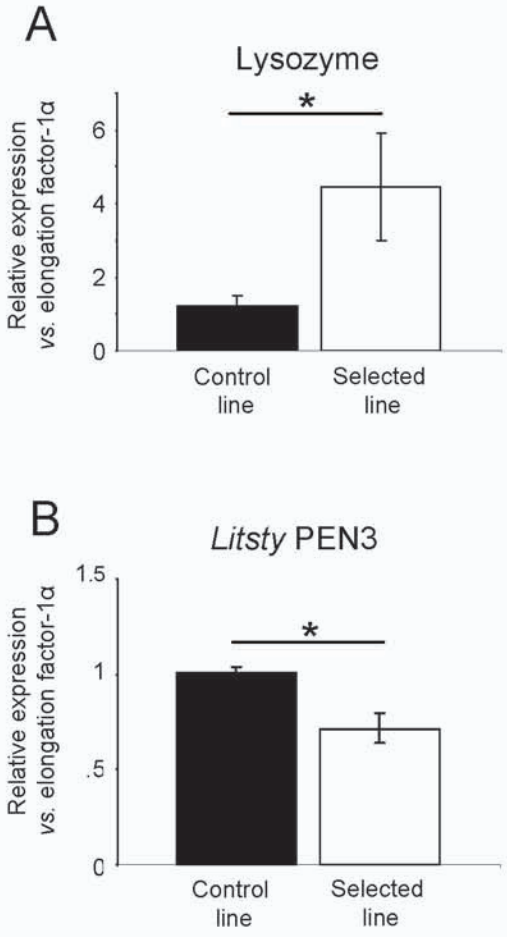

A
Lysozyme
*
Relative expression
vs. elongation factor-1α
6
4
2
0
Control
line
Selected
line
B
Litsty PEN3
*
Relative expression
vs. elongation factor-1α
1.5
1
.5
0
Control
line
Selected
line

Figure 5