Expression profile of immune-related genes in *Artemia franciscana* challenged with luminescent and non-luminescent variants of *Vibrio harveyi* wild type and quorum sensing mutants

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Abstract. The invertebrates rely solely on their only defense system i.e. innate immunity, which is essential for host defense to eliminate invaders. The purpose of this study is to investigate changes in transcription levels of eight immune-related genes, including the *lgbp*, *dscam*, *proPo*, *ftn*, *tgase*, *pxn*, *mip* and *spn* in *Artemia franciscana* challenged with luminescent and non-luminescent variants of *Vibrio harveyi* wild-type strain and a triple quorum sensing mutant. Our results show that the virulent strains or strains producing more virulent factors induce *proPo* expression only at 6h after exposure while *spn/mip* are upregalated later on. Expression of *lgbp* gene is sustained in the challenge with a more virulent strain. For the non-virulent strains, JMH634L and JMH634NL, most of the tested genes (except the *spn*) showed no significant difference in gene expression between these treatment groups at either time point. Results of this study indicated that alteration of virulent factors such as luminescence and enzyme activities due to phenotypic variation might influence to the pathogen-induced processes and host-pathogen interaction, enabling bacteria to modulate the host defense reaction based on the pathogenic invader.

Key Words: bioluminescence, brine shrimp immunity, QS regualation, *Vibrio* pathogenesis.

Introduction. *Vibrio harveyi*, a marine bacterium that belongs to the Vibrio clade, is considered one of the most important bacterial pathogens in aquaculture, affecting a wide range of cultured marine organisms, including fish, crustaceans, and molluscs (Austin & Zhang 2006). Many studies on crustaceans have reported that the expression levels of immune-related genes in these organisms were markedly elevated in response to *V. harveyi* infection (Angthong et al 2010; Shanthi et al 2014; Liu et al 2016). The invertebrate crustaceans, unlike vertebrates, rely on their innate immunity as their defense system, which is essential for host defense to eliminate the invader. The innate immune system has two principal components: the cellular and humoral, both of which are activated upon immune challenge. These two components work synergistically to detect, eliminate, and combat foreign organisms. The cellular component involves those processes mediated by hemocytes, such as encapsulation, nodule formation, and phagocytosis. Whereas the humoral components involve the cell-free components of the hemolymph such as clotting cascade, anti-oxidant defense enzymes (like super-oxidase dismutase, peroxidase, catalase, and nitric oxide synthase), defensive enzymes (like lysozyme, acid phosphatase and alkaline phosphatase), reactive oxygen and nitrogen intermediates and antimicrobial peptides (Amparyup et al 2013).

The innate immune system recognizes self and non-self molecules by a limited number of germline-encoded receptors called pattern recognition receptors (PRRs). PRRs are proteins that are present on the cell membrane or in the cytoplasm as sensors for various pathogens. PRRs recognize pathogens by binding to molecular patterns/

structures rather than to a specific component of a given pathogen. These molecular patterns, called pathogen-associated molecular patterns (PAMPs) are typically polysaccharides and glycoproteins on the surface of microbes, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipotechoic acid (LTA) from Gram-positive bacteria, and glucans from fungal cells. The recognition process leads to the rapid induction of cellular and humoral responses by the host (Patnaik et al 2024). However, Vance et al (2009) reported that the host response to pathogens is not only limited to the recognition of microbial structures but also to the recognition of distinct pathogen-induced processes that contribute to the progression of the disease. The authors also proposed that recognition of pathogen-induced events would provide the host with strategies for distinguishing a virulent organism from one that has lower disease-causing potential. For example, it has been suggested that the host could sense translocated PAMPs as a signal for cytosolic access. In fact, as an example, translocated PAMPs sensed in the cytosol are flagellin. Since the delivery of flagellin to the host cell is strictly dependent on type III or IV secretion systems, the cytosolic presence of flagellin is a strong signal to the immune system that a pathogen (opposite to the commensal) is present (Vance et al 2009).

Several genes involved in the shrimp innate immune response have been identified and characterized, including those of pattern recognition receptors (Rajendran et al 2022; Patnaik et al 2024), proteins associated with cell adhesion activity (Liu et al 2004), and proteins involved in blood coagulation such as proteinase inhibitors, proPO activation or cytokine activation (Angthong et al 2010; Mansour et al 2022). Our previous study showed that the non-luminescent variants of the wild-type *V. harveyi* BB120 and its quorum-sensing mutant JMH634 respond differently in terms of virulence factor production and virulence towards *Artemia* compared to its parental luminescent strains (Hong et al 2016). In this study, the gnotobiotic *Artemia* model organism (GART) system is used to verify the hypothesis that phenotypic variation in *V. harveyi*, resulting in differences in *in vitro* virulence factors production and virulence to *Artemia* in luminescent and non-luminescent wild type *V. harveyi* and a quorum sensing mutant, affect the defense system of *Artemia*. For that, germ-free *Artemia* was challenged with the luminescent and non-luminescent variants of the *V. harveyi* wild-type strain and a triple quorum sensing mutant, and the transcript levels of key genes related to innate immunity in *Artemia* were determined. The following genes were studied: lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*), Down syndrome cell adhesion molecule (*dscam*), peroxinectin (*pxn*), prophenoloxidase (*proPO*), transglutaminase (*tgase*), ferritin (*ftn*), melanin inhibition protein (*mip*) and serpin (*spn*).

Material and Method

Bacterial strains and growth conditions*.* *V. harveyi* strains used in this study are shown in Table 1. The strains were stored in 20% glycerol at -80°C. Rifampicin-resistant (RR) strains were produced as described by Hong et al (2016).

Table 1

Bacterial strains and their relevant feature (Hong et al 2016)

Strain	*Relevant feature*
BB120L	Rifampicin-resistant luminescent
BB120NL	Rifampicin-resistant non-luminescent
JMH634L	Rifampicin-resistant luminescent
JMH634NL	Rifampicin-resistant non-luminescent

Autoclaved *Aeromonas* sp. LVS3 bacteria were used as feed for *Artemia* (Marques et al 2004). LVS3 was grown in marine broth (MB) 2216 (Difco laboratories, MI, USA), and centrifuged at 2200 x g for 15 min. The supernatant was discarded and the pellet was re-suspended in filtered autoclaved seawater (FASW; 35 g L^{-1}, Aquarium Systems, Sarrebourg, France). Subsequently, the cell suspension was autoclaved at 121°C for 20 min. The bacterial densities were determined spectrophotometrically at an optical density (OD) of 600 nm assuming that an OD of 1.0 corresponds to 1.2 x 10^9 CFU mL^{-1} (McFarland standard; BioMerieux, France).

Axenic hatching of Artemia. Axenic hatching of *Artemia* cysts was performed following decapsulation and hatching procedures as described previously (Baruah et al 2010). Briefly, 4 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 mL of sterile distilled water for 1 h. Sterile cysts and nauplii were obtained after decapsulation by using 3.5 mL NaOH (32%) and 50 mL NaOCl (50%). The reaction was stopped after 2 min by adding 70 mL $Na_2S_2O_3$ (10 g L^{-1}). Thereafter the decapsulated cysts were washed with sterile FASW artificial seawater and then suspended in 1 L glass bottles containing FASW, and then incubated at 28°C for 28 h with constant illumination of approximately 27 μE m^{-2} sec^{-1} for hatching. After 28 h of incubation, the axenicity of the hatched *Artemia* nauplii was verified both by spread plating (100 mL) and by adding (500 μL) hatching water on Marine Agar and in Marine Broth (Difco, Detroit, USA), respectively followed by incubation at 28°C for 5 days. Bottles containing non-sterile nauplii were excluded from the experiment.

Artemia challenge test. After 28 h of incubation at 28°C (starting from hatched cysts), swimming nauplii at the instar II stage were collected, counted volumetrically, and then transferred into 1 L glass bottles containing sterile artificial seawater. The bottles were placed in a rectangular tank containing water maintained at 28°C using a thermostatic heater with constant illumination (approximately 27 μE m^{-2} sec^{-1}) and aeration. The nauplii were fed once with 107 CFU mL^{-1} of autoclaved LVS3. Simultaneously, they were challenged with luminescent (group 1) or non-luminescent (group 2) variants of *Vibrio* at 106 CFU mL^{-1}. *Artemia* nauplii that were non-challenged (group 3) were used as controls. Three replicates were maintained for each group. Samples containing 0.1 g of live nauplii were harvested from each glass bottle at 6 and 12 h post-challenge, rinsed in sterile distilled water, immediately frozen in liquid nitrogen, and preserved at -80°C for further analysis.

Quantitative real-time reverse transcription PCR. Total RNA was extracted from different *Artemia* samples using the SV Total RNA Isolation System (Promega, Leiden, The Netherlands) according to the manufacturer's instructions. The RNA quantity was measured spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to 500 ng mL^{-1} in all samples and the RNA samples were stored at -80°C for subsequent use.

Reverse transcription was done with the RevertAidTM H minus First strand cDNA synthesis kit (Fermentas, Fisher Scientific, Erembodegem, Belgium) according to the manufacturer's instructions. Briefly, 9 mL of reaction mixture containing 4 mL of 5x reaction buffer (0.25 mol^{-1} of Tris –HCl pH 8.3, 0.25 mol^{-1} of KCl, 0.02 mol^{-1} of $MgCl_2$, 0.05 mol^{-1} of DTT), 2 mL of 0.01 mol^{-1} of dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid H minus M-MuLV Reverse Transcriptase (Fisher Sciencetific, Erembodegem, Belgium), 1 mL of random hexamer primer. Then, 500 ng of RNA was added to the reaction mixture. Subsequently, the reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min and then cooled to 4°C. Complementary deoxyribonucleic acid (cDNA) samples were checked by polymerase chain reaction (PCR) and stored at -20°C for further use.

The primers used for analyses of *proPO, tgase*, and *ftn* genes were previously described by Niu et al (2014). The gene $ef_{\alpha}1$ was used as a housekeeping gene in this study. For the genes *pxn*, *dsam*, *lgbp*, *spn*, and $ef_{\alpha}1$, specific primers were designed using the *Artemia* Genome database (Sony, not published). Amplification products were re-sequenced to confirm homology with the genes identified in *Pacifastacus* and/or other invertebrates. Evidence for their functionality awaits further research. Primers were designed using the online software Primer Express® Software v3.0.1 (Primer 3, IDT) and obtained from Eurogentec (Seraing, Belgium).

Quantitative reverse transcription real-time PCR was used to quantify the gene expression level in *Artemia*. The reaction was carried out with Maximal® SYBR Green/ROX qPCR master Mix (Fisher Scientific, Erembodegem, Belgium) and was carried out in a StepOne Real-Time PCR System thermal cycler (Applied Biosystems, Gent, Belgium) in a total volume of 25 mL, containing 12.5 mL of 2 x SYBR green master mix, primer (concentration see Table 4) and 2 mL of template cDNA. The thermal cycling consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing and elongation at 60°C for 1 min. Melting curve analysis was done to check for the amplification of untargeted fragments. Data acquisition was done with the STEPONE software.

Based on the result of the optimization experiment, the amplification efficiency of the reference and target genes were found to be equal allowing to analysis of real-time PCR data with the 2-ΔΔCT method (Livak & Schmittgen 2001). The expression was normalized to the endogenous control ($EF_{\alpha 1}$) by calculating ΔC_T:

$$\Delta C_t = C_{t\ target} - C_{t\ EF\alpha 1}$$

and expressed relative to the calibrator sample by calculating $\Delta\Delta C_t$:

$$\Delta\Delta C_t = \Delta C_t - \Delta C_{t\ calibrator}$$

The sample of unchallenged *Artemia* at 6 h was used as a calibrator. The relative expression was then calculated as:

$$\text{Relative expression} = 2^{-\Delta\Delta CT}$$

The primer sequences of study genes were described in Table 2.

Table 2

Real-time quantitative RT-PCR primers

Gene	*Efficiency*	*Primer concentration*	*Annealing temperature*	*Strand*	*Primer sequence (5′-3′)*
$ef_{\alpha}1$	2.0	0.20 μM	60°C	F	GGTCGGGGTTGAAACTGGTAT
				R	AGGGATTCGTGGGCATTT
ftn	2.2	0.20 μM	60°C	F	TCCAAGGATTATCCGATGAACA
				R	ATGACCAAGTGAGTGCTTCTTCT
tgase	2.2	0.20 μM	60°C	F	CCCCACAAGAACCATCTGAAG
				R	TCTCTCCGTGTCTCTCCAAAAG
proPO	2.2	0.20 μM	60°C	F	TCTGCAAGGAGGATTTAAGGA
				R	TGACTGAAAGGAGATGGGAC
lgbp	2.0	0.20 μM	50°C	F	GTGGACTGATGCTGAATG
				R	TGGTGGTCCAGATGATAC
pxn	2.0	0.20 μM	60°C	F	GAGCTACCGATGAAGATCCAG
				R	CGTTTCCTGAACAGCGAATAAA
dscam	1.9	0.25 μM	51°C	F	CATAGTCCAAGAGTGAATG
				R	GCCACATATTCAGTTAGAA
mip	2.0	0.20 μM	54°C	F	CGTGGTAATGCTAGTGA
				R	CACCATCCACCTTCATA
spn	2.0	0.20 μM	62°C	F	AAACCAGGCTTGCCGTT
				R	GCCGTGATTATTGTCCTTTGC

Note: F = forward; R = reverse.

Statistical analysis. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 using a significant level of 5%. The homogeneity of variances and normality of data were not fulfilled for an ANOVA analysis. The data was log-transformed prior subject to statistical analysis. All treatments were compared to the control and to each of the other treatments individually using independent samples t-test for each time point. Per time point, a matrix was obtained with an overview of all the results from the independent samples t-test.

Results. This study aimed to verify differences in *in vitro* virulence factors production or virulence to *Artemia* in luminescent and non-luminescent *V. harveyi* resulting from phenotypic variation that affects the defense system of *Artemia*. To verify the proposed hypothesis, germ-free *Artemia* and virulent/non-virulent strains of *V. harveyi* (for details, see Table 1) were used as host-pathogen model systems. As readouts of host immune responses, a battery of key immune-related genes, namely the *lgbp*, *dscam*, *proPo*, *ftn*, *tgase*, *pxn*, *mip*, and *spn* were analyzed. The germ-free *Artemia* culture system was employed to eliminate interference from environmental microbial communities, allowing for a focused study of the interaction effects between the host and the tested bacteria. Under this experimental condition, it was observed that there was a significant alteration in the transcription levels of the immune receptors and a few downstream genes known to be involved in the humoral and cellular innate immune response in *Artemia* in response to challenges with luminescent or non-luminescent variants of the wild-type *V. harveyi* and their corresponding mutants (Tables 3 and 4).

Table 3

Overview of the statistically significant results for the tested immune-related genes in *Artemia* larvae. The effects of a challenge with L or NL *V. harveyi* on gene transcription are shown

Gene	*120L*			*120NL*			*634L*			*634NL*		
	6 h	*12 h*	*24 h*	*6 h*	*12 h*	*24 h*	*6 h*	*12 h*	*24 h*	*6 h*	*12 h*	*24 h*
lgbp	↓	↑	↑	↓	↓	-	-	↓	-	-	↓	-
dscam	↓	-	-	↓	-	-	-	-	-	-	-	-
proPO	-	-	-	↓	-	-	↓	-	-	-	-	-
fnt	↓	↑	-	↓	-	-	-	-	-	-	↑	-
tgase	-	-	-	↓	-	-	-	-	-	-	-	-
pxn	-	-	-	↓	-	-	-	↑	-	↓	↑	-
mip	↓	↓	-	↓	↓	-	↑	↓	-	↑	↓	-
spn	-	↓	-	↓	↓	-	-	↓	↓	↑	↓	-

↑: significant up-regulation of the gene compared to the control at this time point; ↓: significant down-regulation; -: no significant difference between the treatment and the control.

Table 4

Summary of significant differences in immune-related genes expression

Gene	*120L vs. 120NL*			*120L vs. 634L*			*634L vs. 634NL*		
	6 h	*12 h*	*24 h*	*6 h*	*12 h*	*24 h*	*6 h*	*12 h*	*24 h*
lgbp	↑	↑	↑	↓	↑	↑	-	-	-
dscam	↑	-	-	-	-	-	-	-	-
proPO	↑	-	-	↑	-	-	-	-	-
ftn	↑	-	-	↓	-	-	-	-	-
tgase	↑	-	-	-	-	-	-	-	-
pxn	↑	-	-	-	↓	-	-	-	-
mip	↑	-	-	↓	-	-	-	-	-
spn	↑	↑	-	↓	↑	↑	↓	-	↓

↑: significant up-regulation; ↓: significant down-regulation; -: no significant difference.

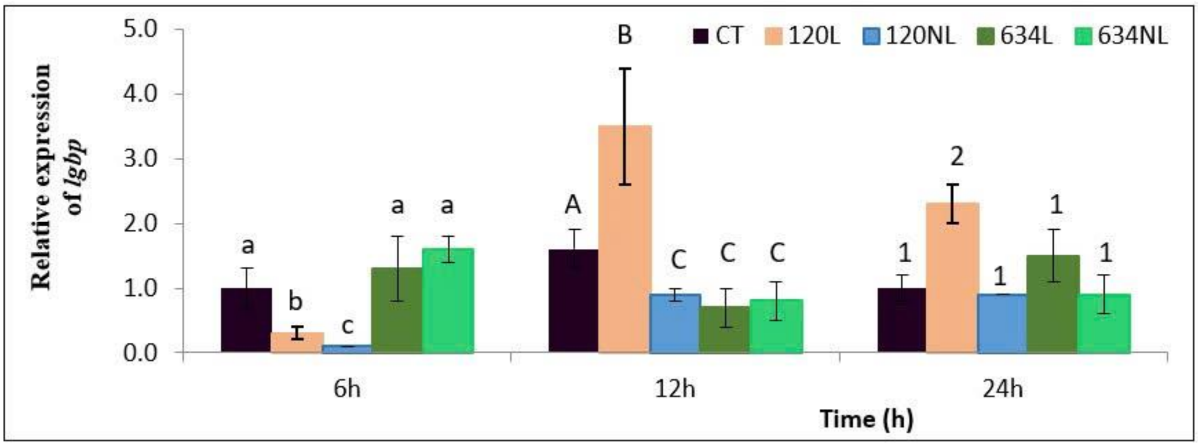
PRRs are involved in the first step of immune responses in invertebrates by binding to highly conserved pathogen structures, such as peptidoglycan and lipopolysaccharide (LPS) from bacteria or to danger-associated molecular patterns, such as Hsp70 and Hmgb1 (Harris et al 2012). In this study, we investigated the transcriptional level of two important PRRs in invertebrates i.e. lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*) and Down syndrome cell adhesion molecule (*dscam*).

The lipopolysaccharide and β-1,3-glucan binding protein (*lgbp*), also known as Gram-negative bacteria-binding protein (GNBP), is a pattern recognition receptor that can recognize and bind lipopolysaccharide (LPS) and b-1,3-glucan. In invertebrates, *lgbp* plays a vital role in the innate immune defense against Gram-negative bacteria and fungi. It induces cell and humoral-mediated immune responses like encapsulation, phagocytosis, nodule formation, clotting, synthesis of antimicrobial peptides, and activation of the prophenoloxidase (*proPO*) system. A recent study showed that the expression of *lgbp* in shrimp *Penaeus monodon* increased at 24 h post-injection with the pathogenic bacterium *V. harveyi* (Amparyup et al 2013). Another PRR is *dscam*, which is a hypervariable protein created by alternative splicing, involved in both general innate immunity and pathogen-specific immune response in invertebrates (Ng et al 2014).

Significant differences in the expression levels of *lgbp* among the different groups were observed at different time points post-challenge (Figure 1A). At 6 h post-challenge, the expression level in the group exposed to luminescent or non-luminescent wild-type *V. harveyi* BB120L was significantly down-regulated compared to the control. There was a significant difference in *lgbp* expression level between the *Artemia* challenged with luminescent BB120L and its non-luminescent variant (BB120NL) or luminescent JMH634L. However, at 12 h, the expression level of *lgbp* was significantly up-regulated in the BB120L-challenged group compared to the BB120NL and JMH634L-challenged groups, and control group, whereas in all the other (treated) groups, there was a significant down-regulation in the expression level. At 24 h, the BB120L-challenged group exhibited a significant increase in the expression level of *lgbp* compared to all other groups.

The expression pattern of *dscam* showed a significant decrease at 6 h post-challenge in the BB120L and BB120NL compared to the JMH634L-exposed group and control group. However, the expression level of *dscam* in the group exposed to either luminescent or non-luminescent variants of the mutant JMH634L was not significantly different from that of the control. No significant differences among the groups were observed either at 12 and 24 h time points (Figure 1B).

(A)



(B)

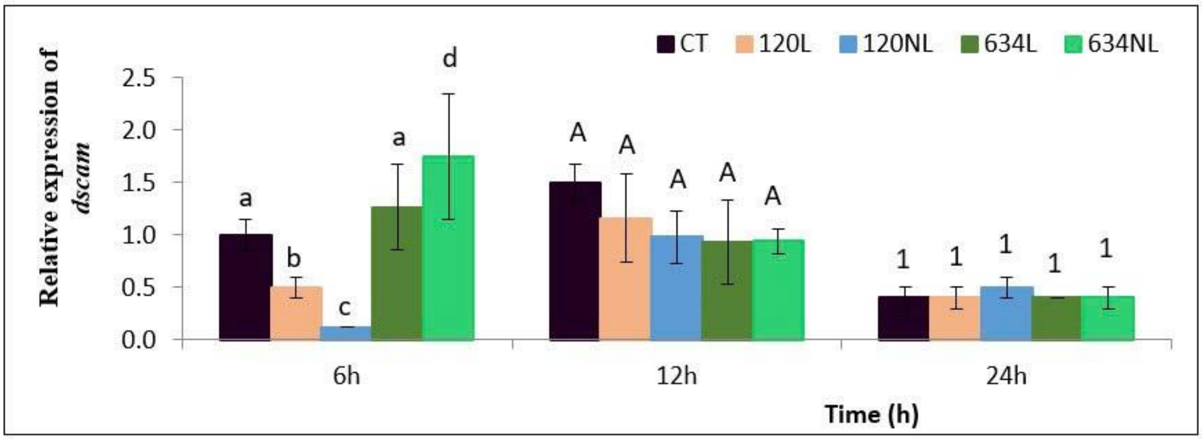


Figure 1. Relative expression of *lgbp* (A) and *dscam* (B) genes in *Artemia* larvae.

The larvae were challenged with luminescent (L) or non-luminescent (NL) of the wild type BB120 and its quorum sensing mutant JMH634 or not. The unchallenged *Artemia* larvae served as control. Sample was collected for *lgbp* and *dscam* gene expression at 6, 12 and 24 h post challenge. The expression was calculated relative to the *ef*$_{\alpha}$*1* gene; expression in unchallenged *Artemia* at 6 h was set at 1 and the other data points were normalized accordingly. Error bars in Figure 1 indicated the standard deviation. Different letters/numbers (small and capital for 6 and 12 h, respectively and number for 24 h) were indicated for significant differences among groups ($p < 0.05$).

Next, we analyzed the expression of a set of four genes related to the humoral and cellular immune system: *proPO*, *ftn*, *tgase*, and *pxn* as these immune components were previously reported to be involved in inducing resistance in invertebrates against bacterial infection (Park et al 2014). As shown in Figure 2A, at 6 h post-challenge, the group challenged with BB120NL exhibited a significant decrease in the *proPO* expression level compared to the unchallenged control group and to the group challenged with either luminescent BB120 strain. The expression level of *proPO* was significantly down-regulated in the JMH634-exposed groups relative to the BB120L, and there is no significant difference in gene expression in these treatment groups (JMH634L and JMH634NL). No significant differences were observed among the groups at both 12 and 24 h time points (Figure 2A).

The *ftn* gene showed different expression patterns at different time points post-challenge (Figure 2B). At 6 h post-challenge, the expression level of *ftn* was significantly decreased in the *Artemia* exposed to either BB120L or BB120NL relative to the control and JMHL634-exposed groups. There was a significant difference in *ftn* gene expression between the BB120L and BB120NL-exposed group while no significant difference between the JMH634L and JMH634NL was observed. However, at 12 h post-challenge, there was no significant difference in *ftn* gene expression among *Vibrio*-exposed groups. At 24 h post-challenge, no significant differences among the groups were observed (Figure 2B). This result is in accordance with the finding of Ong et al (2005), who reported that mRNA expression of the horseshoe crab *ftn* was up-regulated 3 h after being challenged with *Pseudomonas aeruginosa*, and the plasma ferritin disappeared between 6-48 h post-challenge. This result suggested that during infection, *ftn* might be concealed intracellularly as it withholds iron from the invading pathogen.

Similar to what was observed for *ftn* at 6 h post-challenge, the expression level of *tgase* in *Artemia* challenged with BB120NL was significantly low compared to the control and the BB120L-exposed group (Figure 2C). No significant differences were observed between BB120L and JMH634 treatment groups. No significant differences were observed among the groups at both 12 and 24 h time points (Figure 2C).

Peroxinectin, encoded by the *pxn* gene, is a molecule first isolated and described from the freshwater crayfish *Pacifastacus leniusculus* and this protein combines a cell adhesion ligand and a peroxidase. In addition, crayfish *pxn* is also an opsonin, a degranulation, and an encapsulation-promoting factor (Johansson et al 1995). *Pxn* is a *proPO* system-associated protein and plays a crucial role in cellular defense for encapsulation enhancement in crustaceans. For example, when a foreign particle enters the hemolymph, hemocytes recognize the foreign intruder as non-self and change from non-adhesive to adhesive cells, strongly adhering to the foreign target, and subsequently form a multilayer sheath of cells during encapsulation (Sritunyalucksana et al 2001). Previous studies observed that the *pxn* gene was up-regulated significantly in white shrimp *Litopenaeus vannamei* and *Artemia franciscana* when the shrimp was challenge with Vibrio (Shanthi et al 2014; Roy et al 2022).

As shown in Figure 2D, 6 h post-infection, the expression level of *pxn* was significantly lower in the *Artemia* challenged with BB120NL compared to its luminescent variant (BB120L) and the control. The *pxn* expression level was not significantly different among BB120L, JMH634L, and JMH634NL-exposed groups (Figure 2D). After 12 h post-challenge, expression of *pxn* by the BB120L and BB120NL remained at the same level compared to the control. However, in the mutant JMH634-challenged group, expression levels of *pxn* were significantly increased compared to the BB120L-challenge group and the control group but no significant difference was observed among these treatments (Figure 2D).

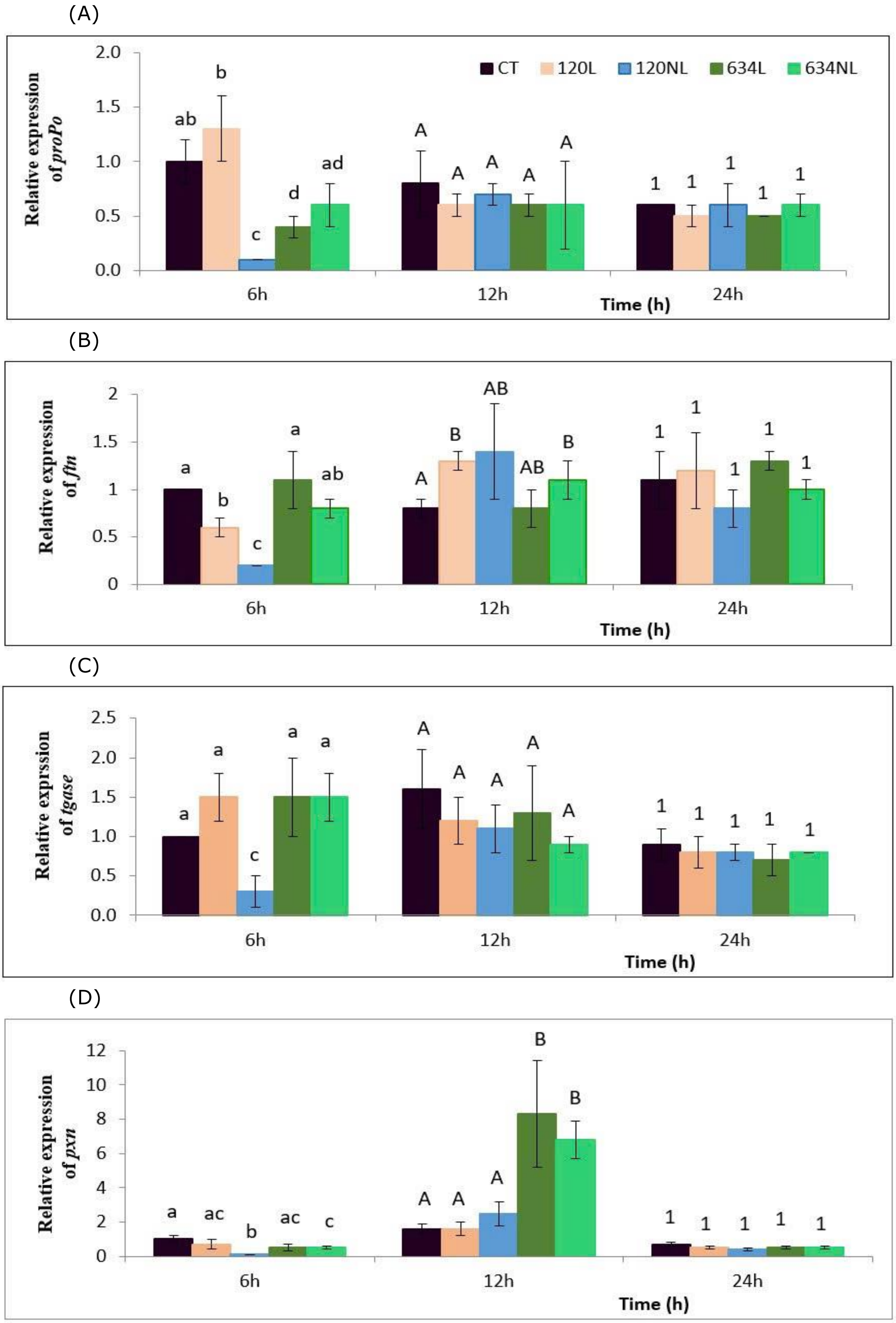


Figure 2. Relative expression of *proPo* (A), *ftn* (B), *tgase* (C) and *pxn* (D) genes in *Artemia* larvae.

Melanization plays a crucial role in defense reactions, such as wound healing encapsulation, sequestration of microbes, and the production of toxin intermediates, that are speculated to kill invading microorganisms. Melanin synthesis are essential for defense and development but must be tightly controlled because systemic hyperactivation of the *proPO* system, the excessive formation of quinones, and inappropriate excessive melanin synthesis is also deleterious to the hosts (Zhao et al 2005). The melanization inhibition protein (*mip*) is involved in the regulation of excessive production of quinones (Angthong et al 2010).

A study carried out by Angthong et al (2010) showed that *mip* was expressed in all the examined tissues in *Penaeus monodon* except hemocytes. The expression of this gene was very low during the larval stages and hardly present in eggs and at the nauplii stage upon the *V. harveyi* challenge.

In the present study, we found that a significant increase in the expression level of *mip* was detected in the JMH634L and JMH634NL-exposed groups relative to the BB120L and to the control group but no significant difference among the latter treatments was observed (at 6 h) (Figure 3A). Subsequently, the expression level of this gene was down-regulated significantly in all groups compared to the control at 12 h. Our result supports the findings of Angthong et al (2010), who suggested that upon *V. harveyi* infection the *mip* protein in *P. monodon* (PmMIP) is first released from the tissues into the hemolymph whereafter the *mip* protein is degraded during the bacterial infection to allow melanization.

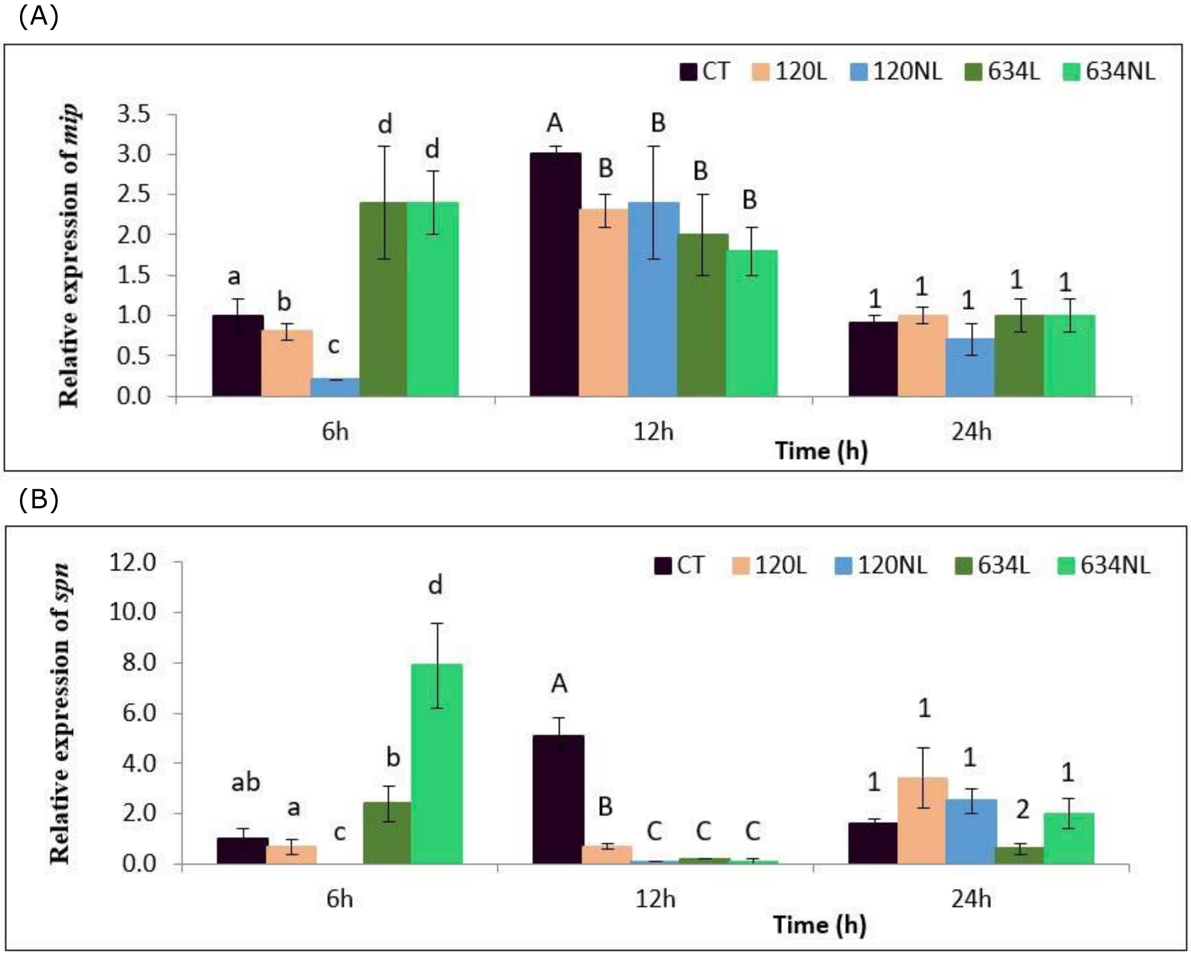


Figure 3. Relative expression of *mip* (A) and *spn* (B) genes in *Artemia* larvae.

The *spn* gene encoding the protein *spn* is involved in several biological processes, such as inflammation, angiogenesis, fibrinolysis, blood coagulation, complement activation, tumor suppression, and hormone transport (Potempa et al 1994). The *spn* protein in hemolymph of arthropods is likely to function in protecting their hosts from infection by pathogens or parasites through inhibiting fungal or bacterial proteinases or regulating endogenous proteinases involved in coagulation, *proPO* activation, or cytokine activation (Kanost 1999). In this study, the pattern of *spn* gene expression was different in different challenge groups (at 6 h) (Figure 3B). The decrease of the *spn* expression gene at 6 h post-challenge with either BB120L or BB120NL might indicate that low synthesis of *spn* and high expression of relevant serine proteinase occurred during this period. This phenomenon might be caused by the involvement of the serine proteinases in wound healing, *proPO* activity, phagocytosis, and other defense responses after bacterial challenge (Liu et al 2007). Then the expression of the *spn* gene remained significantly low at 12 h in these treatment groups compared to the control after that it slightly increased towards the end of the experiment. It corresponds to the re-increased synthesis of *spn* to inhibit over-expressed serine proteinase, as observed in *Fenneropenaeus chinensis* (Liu et al 2009). In contrast, for the *Artemia* challenged with JMH634 groups, the trend of the *spn* gene expression was inverse.

Discussion. Virulence factors are integrated multifactor systems or products expressed by a pathogen that is essential for allowing the pathogen to replicate in the host and cause clinical symptoms (Wilson et al 2019). Virulence factors are not constitutively expressed and their production tightly depends on the environmental conditions faced by the bacterium. Depending on the stage of infection, in the intestinal lumen, inside epithelial cells or professional phagocytes, or in the bloodstream, the set of virulence factors expressed in these different conditions has to vary accordingly. Different patterns of gene expression were observed in different challenge groups at different time points. BB120L and BB120NL are virulent to *Artemia*, and switching from L to NL status reduced the number of virulence factors in the wild-type BB120 strain in *in vitro* (except swimming mobility) and in *in vivo* conditions. In the present study, exposure to the BB120NL variant significantly decreased the mRNA level of *Artemia* in all tested genes at 6 h in comparison to its parental luminescent strain (BB120L). Based on the previous results and this study, it is suggested that the higher production of virulence factors such as luminescence, caseinase, and hemolytic activity as observed in the BB120L, might be involved in the induction at the transcriptional level of these genes in *Artemia*. However, the increased expression of all these genes does not need to be sustained except for the *lgbp* gene. It is unclear why sustained expression of the *lgbp* gene is needed in the BB120L challenge in the absence of higher expression of genes downstream in the defense.

The strains BB120L and JMH634L appeared to be virulent and non-virulent towards *Artemia* in the challenge test, respectively (Hong et al 2016). In addition, the production of some virulence factors was higher in vitro in the BB120L relative to JMH634L (Hong et al 2016). It has been shown that bacterial pathogens have co-evolved with their host, displaying remarkably refined molecular mechanisms, often ending in highly specialized biochemical reactions between the microbe and its target cells. As a result, bacteria manipulate diverse host cellular processes for the benefit of the microbe but not for the host (Keyser et al 2008). In many cases, bacterial virulence factors require complicated secretion systems allowing the toxins or adhesins to be presented in the right context and at the accurate time on the bacterial cell (Wilson et al 2019). Interestingly, type 3 secretion system (T3SS) is known to be a potent virulence mechanism that is involved in invasion, proliferation, and causing disease. T3SS is shared by a broad spectrum of pathogenic Gram-negative bacteria that interact with the host by injecting effector proteins into the cytosol of host cells (Keyser et al 2008). It has been reported that in *V. harveyi*, T3SS is regulated by quorum sensing (Pena et al 2019), so probably, in the triple quorum sensing mutant JMH634L the T3SS is inactivated and no cellular damage can be induced by JMH634L exposure.

Apparently, expression of the *lgbp* gene is sustained in the challenge with a more virulent strain (except at 6h). Early upon BB120L exposure, *proPO* expression is upregulated relative to JMH634L, which might be logical for a more virulent strain. Yet other effector genes such as *ftn* and *pxn* are down-regulated later on. Hence a dynamic differential regulation is displayed here. *Mip* and *spn* are upregulated later on, which might be needed to regulate for instance phenoloxidase activity induced by the virulent strain. For the cases JMH634L and JMH634NL, both strains are non-pathogenic to *Artemia* in the challenge test although some differences in *in vitro* virulence factors were noticed between these strains (Hong et al 2016). Most of the tested genes (except the *spn* gene) exhibited no significant difference in gene expression between these treatment groups at either time point. This might be the consequence of the fact that both strains are non-pathogenic and might not be able to inflict cellular damage or they are subject to host phagocytosis without further negative consequences.

It has not been possible yet to demonstrate that non-luminescent strains are capable of switching back to luminescent strains, neither *in vitro* nor *in vivo*. Yet this may be happening as the BB120L strains produce more virulent factors which could be beneficial in the infection process. Since BB120L and BB120NL are not significantly different in virulence towards *Artemia* (Hong et al 2016), such a back switch from non-luminescent to luminescent could be assumed on that basis. It is difficult to imagine what the effect could be of such a back-switch at the level of the host. One can assume that there would be little difference in host response or merely a delay in response because of a slower infection process. This could explain why the BB120L strain induces a more rapid response only in the beginning and little difference in expression level is found later upon exposure. Confirmation that a back-switch *in vivo* is present needs to be addressed.

Conclusions. Exposure to the virulent or non-virulent luminescent and non-luminescent wild-type BB120 and its quorum sensing mutant significantly altered the host defense response in terms of immune-related gene expression in *Artemia*. Results of this study provide evidence that alternation of in vitro virulence factors such as luminescence and the enzyme activities caused by phenotypic variation might contribute to the pathogen-induced processes and host-pathogen interaction, by which bacteria can modulate host defense reaction according to the pathogenic invader. Virulent strains or strains producing more virulent factors induce *proPO* expression only early on upon exposure while *spn*/*mip* are upregulated later on, probably to regulate phenoloxidase activity. The *lbgp* gene was overexpressed within the experimental time frame. This is unexpected because most of the test effector genes are down-regulated towards the end of the experiment. The difference in gene expression dynamics of the BB120L and B120NL strains leads to the assumption that the non-luminescent strains may switch back to the luminescent strains after infection, but that remains speculative. However, the study primarily focuses on gene expression changes. Functional validation of the observed changes in gene expression (e.g., through protein expression analysis or functional assays) needs more study.

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