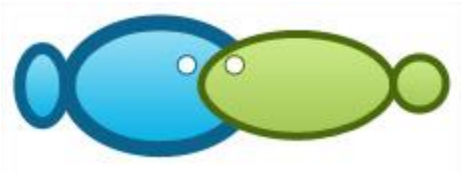

Evaluation of LvCTL3 gene expression encoding C-type lectin from white-leg shrimp (*Litopenaeus vannamei*) infected with AHPND and black gill disease

[1,2]Phuong V. Tran, [3]Vu Q. H. Nguyen, [2]Yen T. H. Pham, [2]Linh Q. Nguyen, [2]Phuoc N. Nguyen

[1] Department of Science, Technology and International relations, Hue University, Hue, Vietnam; [2] University of Agriculture and Forestry, Hue University, Hue, Vietnam; [3] Institute of Biotechnology, Hue University, Hue, Vietnam. Corresponding author: P. N. Nguyen, nguyenngocphuoc@hueuni.edu.vn

Abstract. C-type lectin (CTL) plays an important role in the innate immune responses of white-leg shrimp (*Litopenaeus vannamei*). The upregulation of CTL gene expression occurs when shrimp is infected with bacterial and viral pathogens. The aim of this study was to evaluate the expression of LvCTL3 gene encoding CTL in white-leg shrimp infected with acute hepatopancreatic necrosis disease (AHPND) and black gill disease from Thua Thien Hue Province, Vietnam. Real-time PCR with specific primers targeting LvCTL3 and a reference gene (actin) was used to analyze gene expression in various tissues. The results revealed an increasing trend in the expression of LvCTL3 in tissues such as the intestine, stomach, and hemocytes following AHPND and fungal (black gill disease) infection. Additionally, a strong expression pattern of LvCTL3 was observed in the hepatopancreatics of both AHPND-infected shrimps and black gill diseased shrimps. This finding highlights the significant role of the LvCTL3 gene in the shrimp defense system, as the upregulation of the CTL-encoding gene in key body parts during disease infection suggests its involvement in protection against invading pathogens.

Key Words: *Fusarium solani*, innate immune, penaeid shrimp, *Vibrio parahaemolyticus*, Vietnam.

Introduction. The white-leg shrimp, *Litopenaeus vannamei* (Boone, 1931), is one of the most popular species of cultured penaeid shrimp in Vietnam and is widely cultivated worldwide due to its high commercial value. However, the rapid expansion of *L. vannamei* aquaculture has increased the disease outbreaks, which has become a major challenge for the sustainable development of shrimp culture. In recent years, the acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus* and black gill disease caused by *Fusarium solani* has resulted in significant economic losses to shrimp farmers in Thua Thien Hue province, Vietnam (Phuong et al 2023).

Unlike mammals and other vertebrates, shrimp lack an adaptive immune system, therefore have to mainly rely on the innate immune system, a non-specific defense mechanism that helps prevent infections by pathogenic microorganisms. The innate immunity is activated by the recognition of different microbial cellular components (pathogen-associated molecular patterns, or PAMPs) by the host-associated pattern recognition receptors (PRRs), which triggers different signaling pathways and subsequently leads to different cellular and humoral immune responses (Kulkarni et al 2021). C-type lectins (CTLs), a dominant immune component in shrimp, play a role in innate immune responses such as pattern recognition receptors, agglutination, and contribute to increased antimicrobial activities in crustacean by recognizing and binding to specific CTL domains (Li et al 2022; Luo et al 2023).

C- type lectins (CTLs) are a group of calcium-dependent carbohydrate-binding proteins, mediated through a C-type carbohydrate recognition domain (CRD), and they are now referred to as the lectins that contain the conserved structure of C-type lectin domain

(CTLD) (Li et al 2022). CTLs bind to carbohydrate-based or other ligands to mediate cell adhesion, recognize pathogens, and play important roles in the immune system (Shang-Guan et al 2021). Seven types of lectins are found in shrimp: C-type, L-type, P-type, M-type, fibrinogen-like domain lectins, galectins, and calnexin/calreticulin that are involved in the innate immune system.

In addition, many studies have shown that the expression of shrimp CTL genes increases upon infection with both bacterial and viral pathogens. According to Song et al (2019), a novel CTL gene (LvCTLU) from *L. vannamei* was isolated and plays an important role in microbe agglutination and phagocytosis of *Vibrio parahaemolyticus*, the causative agent of AHPND in white-leg shrimp. In a recent study by Li et al (2022), rLvLec could induce non-specific immune response, including phagocytosis, release of phenoloxidase, hemagglutination and bacteriolysis through cGMP-PKA pathway *in vivo*. Other studies also identified that two genes of CTLs (LvLectin-1 and LvLectin-2), as well as an upregulation of the LvCTL3 gene were highly expressed in white-leg shrimp during *Vibrio anguillarum* challenge (Wang & Wang 2013). According to Li et al (2014), LvCTL3 may play a significant role in the innate immunity of shrimp against bacterial and viral infections. The purified LvCTL3 can agglutinate such as Gram-negative microbe *Vibrio alginolyticus* and *V. parahaemolyticus* and Gram-positive bacteria *Bacillus subtilis* in the presence of calcium ions, but cannot agglutinate Gram-positive bacteria *Streptococcus agalactiae*. In addition, LvCTL3 mRNA can be detected in all tested tissues and recombinant LvCTL3 protein can agglutinate pathogenic Gram-negative and Gram-positive bacteria in shrimp, and significantly reduce the mortality of shrimp infected with *V. parahaemolyticus* and WSSV (Li et al 2014). Currently, studies have consistently shown that upon infection with bacterial or viral pathogens, white-leg shrimp exhibit high expression levels of CTL genes, indicating the role of CTLs in the shrimp's defense mechanism. However, very few studies have reported the expression of the CTL-encoded gene (LvCTL3) in farmed *L. vannamei* when they are infected with fungal pathogens such as *Fusarium solani*. It is important to understand the regulation of CTLs genes expression in shrimps to identify the response of the innate immune system of shrimps and find a new way to protect shrimps from pathogen infections. In this study, we evaluated the expression level of the LvCTL3 gene in response the innate immune system of shrimp toward to two types of pathogens: *F. solani*, fungal pathogen and the bacterium *V. parahaemolyticus*.

Material and Method

Tissue sampling and RNA extraction. 20 white-leg shrimps with an average weight of 7.5±2 g (8.5±0.5 cm) were collected from a commercial shrimp farm (Phong Dien, Thua Thien Hue Province) and transported to the Laboratory of Fish Pathology, Faculty of Fisheries, University of Agriculture and Forestry, Hue University, Hue city, Vietnam. Among these, 5 shrimp showed characteristic signs of black spot in gills, as described by Yao et al (2022), and 5 exhibited typical signs of AHPND infection, as described by Lightner et al (2012). The causative agent of black gill diseases or AHPND in white leg shrimp have been identified as *F. solani* or *V. parahaemolyticus* respectively, according to the study of Phuong et al (2023). Another of 10 non-infected shrimp from another farm were selected as control specimens.

6 tissues (hepatopancreas, intestines, stomach, gill, muscle and hemocytes) of each shrimp were sampled, transferred into Eppendorf tubes, frozen in liquid nitrogen and stored at -40^{0}C for total RNA extraction. Total RNA was extracted using GeneJET RNA Purification Kit (Catalog No. K0731,) according to the manufacturer's instructions. The integrity and purity of RNA were determined as the 260/280 nm absorbance ratio. The first strand cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Catalog No. K1622, ThermoScientific, USA) using 2 µg of total RNA as a template. Then, cDNA was subjected to gene expression analysis.

Real-time quantitative PCR. Quantitative PCR (qPCR) was conducted to evaluate the expression level of LvCTL3 gene. To quantify the gene expression of LvCTL3, specific primers for the reference genes were designed for LvCTL3 and actin (Table 1).

Table 1

Primer sequences used for qRT-PCR

Primer	*Sequences*	*References*
RT-LvCTL3-F	5′-ATGTTCTTCGTGCTCCTGCTGT-3′	Li et al (2014)
RT-LvCTL3-R	5′-GCAGTGGTCGTAAATGTTGTG-3′	
Actin F	5′-GCACAGTAAAGGCGTTGTGA-3′	Tang et al (2021)
Actin R	5′-ACATCTGCTGGAAGGTGGAC-3′	

RT-qPCR was performed using the GoTaq(R) qPCR Master Mix (A6001, Promega, USA). The cycling parameters were 95°C for 2 min, 40 cycles at 95°C for 15 s, 62°C for 1 min. The reactions included 10 µL of GoTaq qPCR Master Mix 2X (Promega, USA), 1 µL/primer (10 pmol), 1 µL of cDNA, and 7 µL of distilled water.

The process was conducted using the QuantStudio™ 5 Real-Time PCR system (ABI, USA). The amplification curve was examined, and the relative gene expression level was normalized to the reference sample of non-infected shrimp. Eventually, relative expression levels were analyzed by the 2-△△Ct method (Schmittgen & Livak 2008). β-actin (Lv actin) was used as the reference gene for data normalization in the RT-qPCR analysis using the 2^(-ΔΔCt) method.

Statistical analysis. The statistical analysis of the data was carried out by IBM SPSS Statistics 22 software and the values with $p<0.05$ were considered significant using Student's tests with significance at $p<0.05$ (*) and $p<0.01$ (**).

Results

Gene expression level of LvCTL3 in black gill infected shrimp. The LvCTL3 gene was expressed in all tested tissues (hepatopancreas, gill, muscle, intestine, stomach, and hemocyte) of the black gill diseased of *L. vannamei*. Overall, the expression levels of the LvCTL3 varied among the organs (Figure 1).

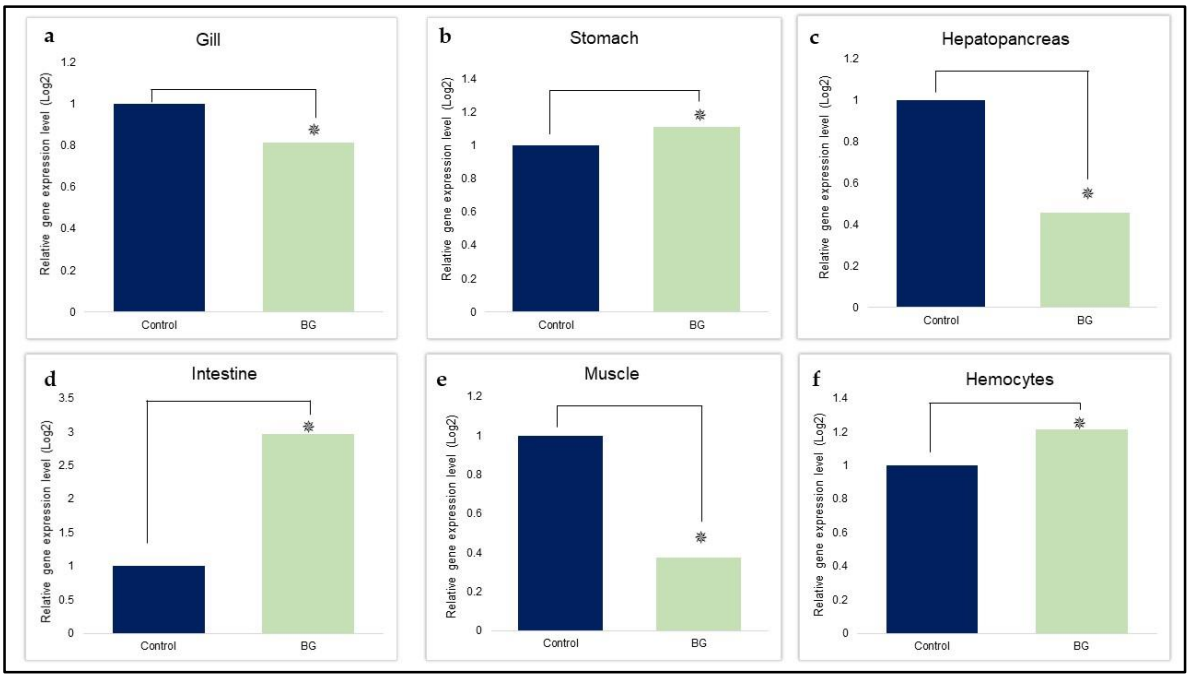

Figure 1. Differential expression of genes in tissues of black gill diseased (*Litopenaeus vannamei*) shrimp; relative gene expression levels were calculated using the Livak method (2ΔΔCT) (n=3); for each organ, the expression level was used as control and set to 1; ❋ - significant differences (t-test, $p<0.05$); BG - black gill infected shrimp.

In the black gill disease of shrimp, the expression of LvCTL3 increased in the intestine, hemocyte, and stomach. The highest expression was observed in the intestine, which was approximately three times higher than that in the control group. In the stomach and hemocyte, the expression of LvCTL3 increased by approximately 1.1 and 1.21 times, respectively, compared to the control group. However, in gills, muscle, and hepatopancreas, the expression of the gene tended to decrease and was lower than that in the control samples. The expression of the LvCTL3 gene were 0.81, 0.37, and 0.45 times compared to their controls in the gill, muscle, and hepatopancreas, respectively (Figure 2).

The LvCTL3 gene expression levels were compared between the control group and black gill diseased shrimp. The expression level was significantly increased in the intestine, stomach, and hemocytes, indicating the role of LvCTL3 in the immune system of infected shrimp.

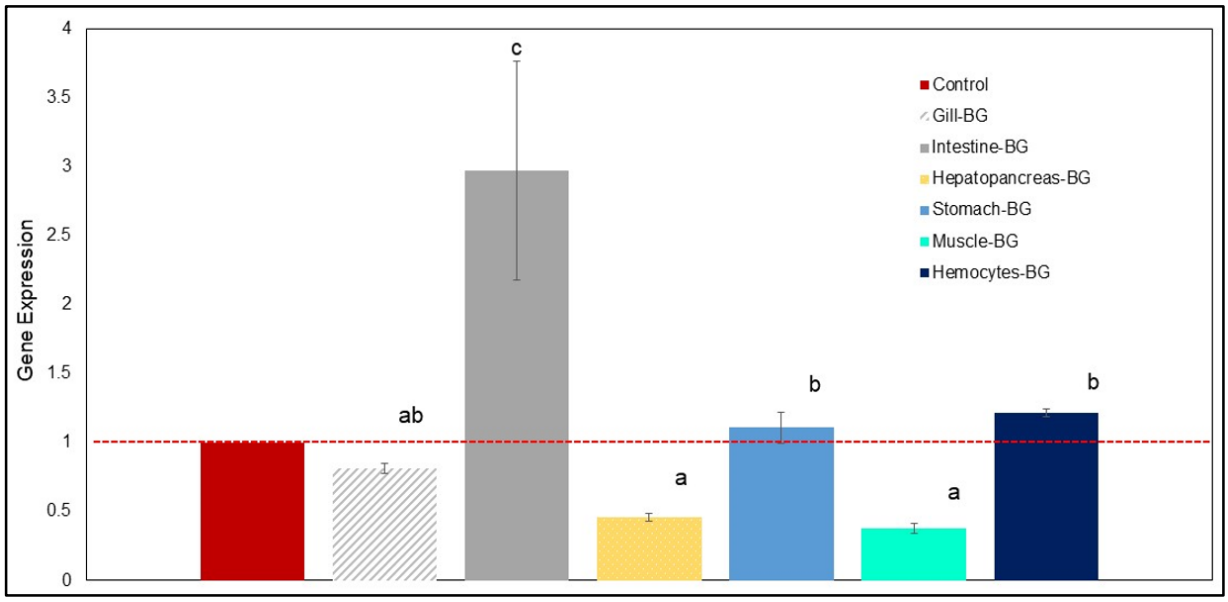

Figure 2. Tissue distribution of LvCTL3 gene during black gill (BG) infection of shrimp (*Litopenaeus vannamei*).

Expression of LvCTL3 gene in AHPND infected shrimp. In AHPND infected shrimp, the expression of LvCTL3 gene was significantly increased in the hepatopancreas, showing more than a 28 times increase compared to the control group ($p<0.05$; Figures 3 and 4). Conversely, the expression of LvCTL3 decreased in other tissues, including the gills, intestine, hemocytes, stomach, and muscle, during the AHPND infection. The gills of shrimp showed a slight down-regulation of LvCTL3, approximately 0.73-fold compared with the control (Figure 3a). Similarly, the expression of LvCTL3 in the intestine and hemocyte of the AHPND-infected shrimp was 0.6 to 0.63 times lower compared to the control (Figure 3b, 3f). In the stomach and muscle, the expression levels of LvCTL3 were significantly lower than those in the non-infected shrimp, by approximately 0.31 and 0.18 times, respectively ($p<0.05$; Figure 3d).

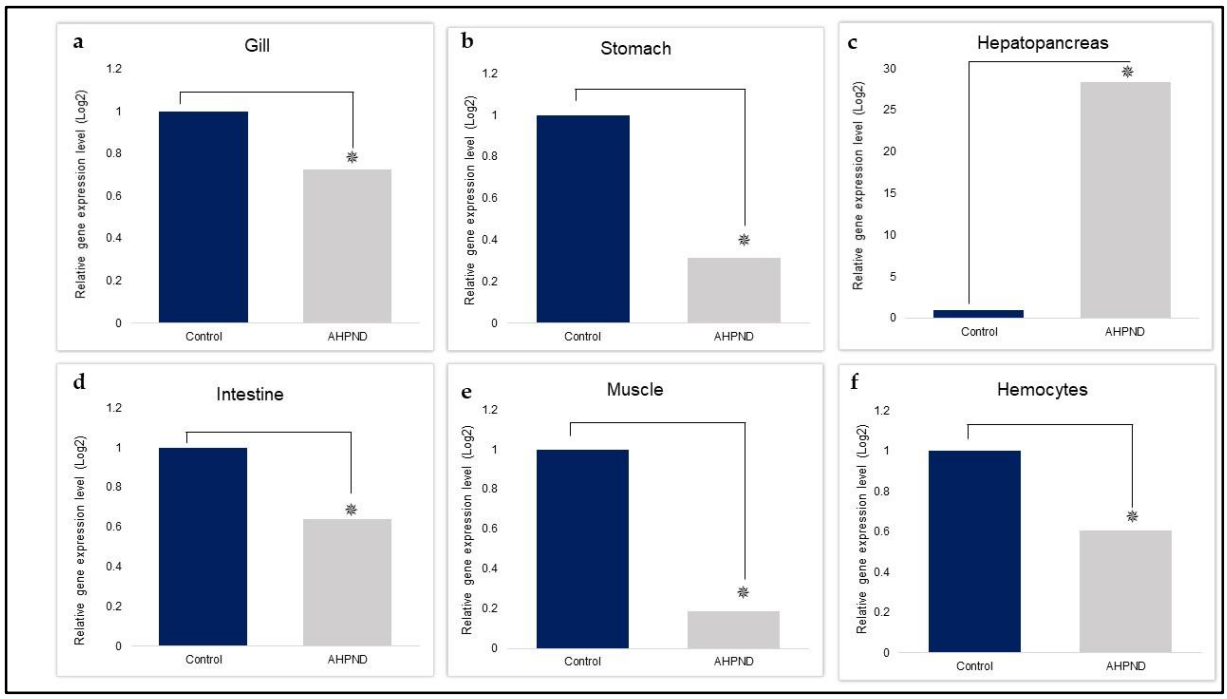

Figure 3. Gene expression of LvCTL3 in different tissues of AHPND infected *Litopenaeus vannamei*; for each organ, the expression level was used as control and set to 1; ❋ - significant differences (t-test; $p<0.05$); AHPND - acute hepatopancreatic necrosis disease.

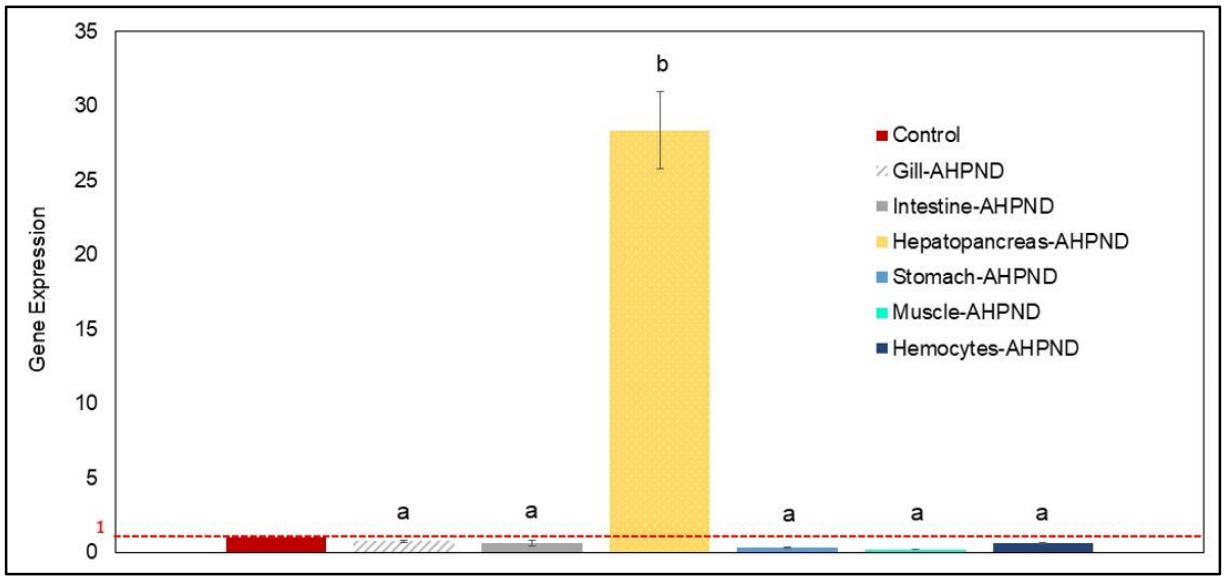

Figure 4. Tissue distribution of LvCTL3 gene during AHPND infection of shrimp (*Litopenaeus vannamei*).

Discussion. Various types of CTL with structural and functional diversity may constitute a recognition network against invading bacterial and viral pathogens and play essential roles in the defense system of shrimp (Li & Xiang 2013; Viana et al 2022). In this study, we found that the expression level of LvCTL3 was significantly higher in the hepatopancreas of *L. vannamei* infected with *V. parahaemolyticus*. Meanwhile, the expression level of LvCTL3 showed a tendency to increase in the intestine, stomach, and hemocytes when infected with *F. solani*, causing black gill symptoms in shrimp. This suggests that LvCTL3 plays a role in the innate responses against bacterial invasions.

These findings are consistent with those of previous reports on the distribution of CTL genes in shrimp. In recent articles, multiple forms of CTL have been identified in insects and crustaceans, particularly in shrimp (Drickamer & Taylor 1993; Li et al 2014; Matos et al 2018). According to Li & Xiang (2013) and Viana et al (2022), there is a group of 17 types of C-type lectins mainly expressed in the hepatopancreas and hemocytes of white leg shrimp (Lv- type lectin), and other three LVT-type lectins expressed in hemocytes, hepatopancreas, and gills. FcLec1 and FcLec2 of shrimp *Fenneropenaeus chinensis* and LvCTL1 of shrimp *L. vannamei* were predominantly expressed in the gills, hepatopancreas, stomach, and intestine. Unlike other shrimp lectins, FcLec3 and FcLec5 of *F. chinensis*, PmAV and PmLT of *P. monodon*, and LvLT of *L. vannamei* were only detectable in the hepatopancreas. Similarly, LvCTL4 exhibits high expression levels in multiple organs, including the gills, intestine, epithelium, and hepatopancreas, with the hepatopancreas showing the highest expression and the gills exhibiting the lowest expression (Li et al 2015). The expression of LvCTL4 in gills was upregulated in response to *V. parahaemolyticus* challenge (Li et al 2015).

Immune response is essential for protecting organisms from invading pathogens. Like other invertebrates, shrimps have humoral and cellular immune responses when exposed to bacteria or PAMPs. These proteins play crucial roles in the identification and neutralization of pathogens. AHPND is a severe disease that affects shrimps. It is characterized by the atrophy of the hepatopancreas, accompanied by unique histopathological changes during the acute phase of the disease. Therefore, the high expression level of LvCTL3 in the hepatopancreas of shrimp infected with AHPND may indicate the activation of the immune system to fight the disease. It is possible that LvCTL3 is involved in the recognition of PAMPs, leading to the activation of the immune system. In a report by Viana et al (2022), the lectins described for *L. vannamei* are divided into C-type, L-type, and galectin, being mainly expressed in the hepatopancreas and hemocytes. They are involved in several immune response pathways such as phagocytosis, hemocyte recruitment, prophenoloxidase activation, and gene regulation. After synthesis in the hepatopancreas, these CTLs are released into the blood, captured by hemocytes, and stored within granular cells (Junkunlo et al 2012; Luo et al 2023). Most lectins associated with the immune response in shrimp showed specific expression or exhibited the highest expression levels in the hepatopancreas, such as LvLT, LvCTL1, and LvCTL5 (Ma et al 2007; Zhao et al 2009; Luo et al 2019; Luo et al 2023). According to Li et al (2022), this is the first time to reveal the immune function of a NLR like gene in crustaceans. Knockdown of LvNLRPL1 accelerated the proliferation of *Vibrio* in the hepatopancreas and increased the mortality rate of shrimps after *Vibrio* infection. Further research is necessary to determine the role of LvCTL3 in the immune response to pathogenic *Vibrio* spp. causing AHPND.

According to Runsaeng et al (2015), a new C-type lectin, designated FmLC1, was cloned from the hepatopancreas of the banana shrimp (*Fenneropenaeus merguiensis*). FmLC1 mRNA was less abundant in the hepatopancreas of shrimp, whereas it was mainly expressed in the stomach and intestine. Additionally, the expression of FmLC in the intestine was higher in the stomach and the hepatopancreas when the banana shrimp was challenged with *V. harveyi* or WSSV. This study evaluated the expression levels of the LvCTL3 gene between the control and AHPND infected shrimp. The LvCTL3 gene exhibited high expression level in the hepatopancreas, indicating its role in the immune system of AHPND infected shrimps. Therefore, further studies of the interaction between LvCTL3 and AHPND are required to provide new insights into the immune system and defense mechanisms of crustaceans, and specifically white-leg shrimp, as well as their potential infectious agents.

Currently, the understanding of the mechanisms of C-type lectins on fungal infection in shrimp is limited. A report by Shiokawa et al (2017) revealed that CRLs are expressed in myeloid cells and play a central role in host defense against fungal infections by coordinating the innate immunity. Upon ligand binding, CLRs stimulate cellular responses by inducing the production of cytokines and reactive oxygen components via the Syk/CARD9 signaling pathway, leading to fungal elimination. CTLs are pattern recognition receptors (PRR) that recognize bacteria leading to the clearance of pathogens through various mechanisms such as agglutination, prohenoloxidase activation, phagocytosis, cell

adhesion, nodulation of hemocytes or direct killing of bacteria (Wang & Wang 2013; Shi et al 2014; Hou et al 2015; Wang et al 2020; Praparatana et al 2022; Zhang et al 2023). LdlrCTL could be involved in the immune response, exhibits agglutination activity against bacteria *V. parahaemolyticus* and fungi *Aspergillus niger,* and could potentiate the phagocytosis of hemocytes (Liang et al 2019).

Conclusions. Gene expression analysis of black gill disease in shrimp indicated that LvCTL3 was present at a higher level in the intestine, stomach and hemocytes. On the other hand, the hepatopancreas of AHPND infected shrimp exhibited a significantly higher expression of LvCTL3 compared to other tissues.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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Authors:
Phuong Vinh Tran, Department of Science, Technology and International relations, Hue University, 03 Le Loi St., 49000 Hue City, Vietnam, e-mail: tvphuong@hueuni.edu.vn
Vu Quang Hoang Nguyen, Institute of Biotechnology, Hue University, Provincial Road No.10, 49000 Hue City, Vietnam, e-mail: nguyenquanghoangvu@hueuni.edu.vn
Yen Thi Hai Pham, University of Agriculture and Forestry, Hue University, 102 Phung Hung St., 49000 Hue City, Vietnam, e-mail: phamthihaiyen@hueuni.edu.vn
Linh Quang Nguyen, University of Agriculture and Forestry, Hue University, 102 Phung Hung St., 49000 Hue City, Vietnam, e-mail: nguyenquanglinh@hueuni.edu.vn
Phuoc Ngoc Nguyen, University of Agriculture and Forestry, Hue University, 102 Phung Hung St., 49000 Hue City, Vietnam, e-mail: nguyenngocphuoc@hueuni.edu.vn

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