FUNCTIONAL CHARACTERIZATION OF HEAT SHOCK PROTEINS DURING STRESS RESPONSE IN PACIFIC WHITE SHRIMP *Penaeus vannamei* AND BRINE SHRIMP *Artemia franciscana*

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ลักษณะสมบัติเชิงหน้าที่ของฮีตช็อกโปรตีนในการตอบสนองต่อความเครียดในกุ้งขาวแปซิฟิก *Penaeus vannamei* และอาร์ทีเมีย *Artemia franciscana*

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วิศรุต จันทร์ปรุง : ลักษณะสมบัติเชิงหน้าที่ของฮีตช็อกโปรตีนในการตอบสนองต่อความเครียดในกุ้งขาวแปซิฟิก *Penaeus vannamei* และอาร์ทีเมีย *Artemia franciscana*. (FUNCTIONAL CHARACTERIZATION OF HEAT SHOCK PROTEINS DURING STRESS RESPONSE IN PACIFIC WHITE SHRIMP *Penaeus vannamei* AND BRINE SHRIMP *Artemia franciscana*) อ.ที่ปรึกษาหลัก : ศ. ดร.อัญชลี ทัศนาขจร, อ.ที่ปรึกษาร่วม : ศ. ดร.ปีเตอร์ บูชิเออร์,ศ. ดร.กิลเบิร์ต วาน แสตปเพน,ดร.เปรมฤทัย สุพรรณกูล

กลุ่มอาการตับและตับอ่อนตายเฉียบพลันที่เกิดจากการติดเชื้อแบคทีเรียวิบริโอพาราฮีโมไลติคัสสายพันธุ์ที่มีพลาสมิดที่สามารถสร้างท็อกซินได้เป็นสาเหตุสำคัญในการเกิดการตายจำนวนมากในฟาร์มเลี้ยงกุ้งขาวทั่วทวีปเอเชีย ในงานวิจัยนี้พบว่าการกระตุ้นกุ้งด้วยความร้อนที่ไม่ตายแบบต่อเนื่อง (จาก 28 °C ไปยัง 38 °C ครั้งละ 5 นาที ซ้ำเป็นเวลา 7 วัน) สามารถป้องกันการติดเชื้อแบคทีเรียวิบริโอพาราฮีโมไลติคัสได้ โดยมีอัตราการรอดมากกว่า 50% ในขณะกลุ่มที่ไม่ได้ถูกกระตุ้นด้วยความร้อนมีอัตราการรอดเพียง 20 % ผลการศึกษาการแสดงออกของยีนด้วยเทคนิค qRT-PCR พบว่ามีการแสดงออกที่เพิ่มมากขึ้นของยีนฮีตช็อกโปรตีน 70 และ 90 และยีนในระบบภูมิคุ้มกันได้แก่ *LvproPO1* และ *LvCrustin1* ในกุ้งที่กระตุ้นด้วยความร้อน และเมื่อทำการยับยั้งการแสดงออกของยีนฮีตช็อกโปรตีน 70 และ 90 พบว่าความสามารถในการทนต่อการติดเชื้อวิบริโอพาราฮีโมไลติคัสซึ่งก่อให้เกิดโรคลดน้อยลงและยังส่งผลให้แอคติวิตี้ของระบบโพรฟีนอลออกซิเดสลดลงอีกด้วย ในการศึกษาหน้าที่ของโปรตีนฮีตช็อกโปรตีน 70 ผลการทดลองพบว่าการกระตุ้นกุ้งด้วยความร้อนสามารถกระตุ้นการผลิตโปรตีนฮีตช็อกโปรตีน 70 ในเม็ดเลือดกุ้งเพิ่มมากขึ้นเมื่อเทียบกับกลุ่มที่ไม่ถูกกระตุ้นด้วยความร้อน เมื่อฉีดโปรตีนรีคอมบิแนนท์ 70 (1 nmol) เข้าสู่ตัวกุ้งปริมาณ พบว่ายีนในระบบภูมิคุ้มกันของกุ้งหลายตัวได้แก่ *LvMyD88 LvIKKε LvIKKβ LvCrustin1 LvPEN2 LvPEN3 LvproPO1 LvproPO2* และ *LvTG1* มีการแสดงออกเพิ่มมากขึ้นและนอกจากนี้ยังเพิ่มการต้านทานของกุ้งต่อการติดเชื้อแบคทีเรียวิบริโอพาราฮีโมไลติคัสได้ โดยกุ้งมีอัตราการรอดเมื่อติดเชื้อเพิ่มจาก 20% ในกลุ่มควบคุมเป็นมากกว่า 75% ในกลุ่มที่ฉีดด้วยรีคอมบิแนนท์โปรตีน แสดงให้เห็นว่าฮีตช็อกโปรตีน 70 ทำหน้าที่สำคัญในการตอบสนองต่อการติดเชื้อแบคทีเรียโดยการกระตุ้นการทำงานในระบบภูมิคุ้มกันของกุ้ง นอกจากนี้ยังได้ใช้ข้อมูลลำดับนิวคลีโอไทด์จากฐานข้อมูลจีโนมของ *Artemia franciscana* ในการศึกษาและระบุลำดับนิวคลีโอไทด์ ลำดับกรดอะมิโน และ แผนภูมิความสัมพันธ์ทางพันธุกรรมของยีนในกลุ่มฮีตช็อกโปรตีน 70 พบว่าจากฐานข้อมูลฯ สามารถระบุยีนที่แตกต่างกันของยีนในกลุ่มฮีตช็อกโปรตีน 70 ได้จำนวน 4 ยีน ซึ่งประกอบไปด้วย *HSC70 HSC70-5 BIP* และ *HYOU1* และเมื่อศึกษาการแสดงออกของยีนในกลุ่มฮีตช็อกโปรตีน 70 ในตัวอย่างอาร์ทีเมียที่ถูกคัดเลือกสายพันธุ์ให้สามารถทนต่อความร้อนได้ในระยะวัยรุ่น พบว่าระดับการแสดงออกของยีน *HSP70* และ *HSC70* มีการแสดงออกที่สูงขึ้นอย่างมีนัยสำคัญในอาร์ทีเมียกลุ่มที่ถูกคัดเลือกสายพันธุ์ฯ เทียบกับกลุ่มที่เลี้ยงในสภาวะปกติ ยิ่งไปกว่านั้นเมื่อกระตุ้นอาร์ทีเมียระยะนอเพลียสด้วยความร้อนแบบไม่ตาย พบว่ายีน *HSP70 HSC70* และ *HSC70-5* มีการแสดงออกเพิ่มมากขึ้นอย่างมีนัยสำคัญเมื่ออาร์ทีเมียที่ถูกคัดเลือกสายพันธุ์ฯ และถูกกระตุ้นด้วยความร้อนแบบไม่ตายเทียบกับอาร์ทีเมียที่ถูกคัดเลือกสายพันธุ์แต่ไม่ถูกกระตุ้นด้วยความร้อนแบบไม่ตาย ในทางตรงกันข้ามยีน *BIP HYOU1* และ *HSPA4* ไม่พบการแสดงออกเพิ่มมากขึ้นอย่างมีนัยสำคัญ มากไปกว่านี้ได้ทำการค้นหาความแตกต่างเพียงลำดับนิวคลีโอไทด์เดียวในยีน *HSP70* และ *HSC70* พบว่ายีน *HSC70* มีความแตกต่างเพียงลำดับนิวคลีโอไทด์ที่ตำแหน่ง 171 (C171A; N57K) ซึ่งเป็นตำแหน่งบนบริเวณโดเมน ATP-binding ซึ่งน่าจะมีความเป็นไปได้ที่จะเกี่ยวข้องกับการเพิ่มความต้านทานต่อความร้อน และจากการศึกษาลักษณะฟีโนไทป์ในยีสต์ *Saccharomyces cerevisiae* ยืนยันได้ว่ายีสต์ที่มีพลาสมิด HSC70-N57K สามารถทนต่ออุณหภูมิสูงได้มากกว่ายีสต์ที่มีพลาสมิด WT-HSC70 และสืบเนื่องจากการที่ลักษณะฟีโนไทป์ N57K สามารถพบได้ในอาร์ทีเมียที่ถูกคัดเลือกสายพันธุ์ให้ทนความร้อน มากกว่ากลุ่มที่ไม่ถูกคัดเลือกสายพันธุ์ ทำให้ฟีโนไทป์ดังกล่าวสามารถใช้ในการคัดเลือกสายพันธุ์อาร์ทีเมียให้ทนต่อความร้อนได้

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Wisarut Junprung : FUNCTIONAL CHARACTERIZATION OF HEAT SHOCK PROTEINS DURING STRESS RESPONSE IN PACIFIC WHITE SHRIMP *Penaeus vannamei* AND BRINE SHRIMP *Artemia franciscana*. Advisor: Prof. Dr. Anchalee Tassanakajon Co-advisor: Prof. Dr. ir. Peter Bossier,Prof. Dr. Gilbert Van Stappen,Dr. Premruethai Supungul

Acute hepatopancreatic necrosis disease (AHPND) caused by the bacterium *Vibrio parahaemolyticus* carrying toxin produced plasmid (VP_{AHPND}), has led to severe mortalities in farmed penaeid shrimp throughout Asia. Our studies demonstrated that a chronic non-lethal heat shock (chronic-NLHS) could enhance resistance of the Pacific white shrimp *Penaeus vannamei* to VP_{AHPND} infection. Shrimp exposed to chronic-NLHS (28 °C to 38 °C, 5 min for 7 days) had higher survival rate (>50%) than that of the control non-heated shrimp (20%) when they were challenged with VP_{AHPND}. The qRT-PCR analysis revealed that the expression of heat shock proteins, *LvHSP70* and *LvHSP90*, as well as other immune-related genes, *LvproPO1* and *LvCrustin1*, were induced upon exposure of shrimp to chronic NLHS. Moreover, *LvHSP70* and *LvHSP90* gene silencing eradicated the VP_{AHPND} resistance in the chronic-NLHS shrimp and decreased PO activity. Further investigation showed that chronic-NLHS immediately activated the production of *Lv*HSP70 in shrimp hemocytes. Injection of 1.0 nmol recombinant *Lv*HSP70 (r*Lv*HSP70) of injection induced the expression of several immune-related genes (*LvMyD88, LvIKKε*, *LvIKKβ*, *LvCrustin1*, *LvPEN2*, *LvPEN3*, *LvproPO1*, *LvproPO2*, and *LvTG1*) in the shrimp immune system. Interestingly, r*Lv*HSP70 enhanced *P. vannamei* resistance to VP_{AHPND} by increasing survival rate from 20% (control group) to >75%. These results suggested that *Lv*HSP70 plays crucial roles in bacterial defense by activating shrimp immune system. In addition, four novel HSP70 family genes designated as *HSC70*, *HSC70-5*, *BIP*, and *HYOU1* were identified from the draft *Artemia* transcriptome database by sequence analysis that contained the specific conserved domain of HSP70 family gene. Expression analysis revealed that in the juvenile state, the transcript level of *HSP70* and *HSC70* was significantly higher in the selective breeding for induced thermotolerance population (TF12) of *Artemia* relative to a control population (CF12). Moreover, after a NLHS treatment of TF12 at the nauplii state, *HSP70, HSC70*, and *HSC70-5* transcrips were significantly ($P<0.05$) up-regulated. In contrast, the expression of the other members of *Artemia franciscan* HSP70 family showed no significant ($P>0.05$) induction. Furthermore, SNP polymorphisms of *HSP70* and *HSC70* genes were investigated and the result identified a SNP at position 171 in *HSC70* (C171A; N57K) located at ATP-binding domain which might be potentially associated with the increased thermotolerance. The genotype analysis result showed significant ($P<0.01$) difference of this SNP between CF12 and TF12. Moreover, the phenotypic analysis by yeast (*Saccharomyces cerevisiae)* system confirmed that yeast contained the HSC70-N57K plasmid could tolerate high temperature than yeast containing the WT-HSC70 plasmid. As the N57K genotype was more prevalent in TF12 population than that in CF12 suggesting that it can be used in selective breeding for induced thermotolerance in *A. franciscana*.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
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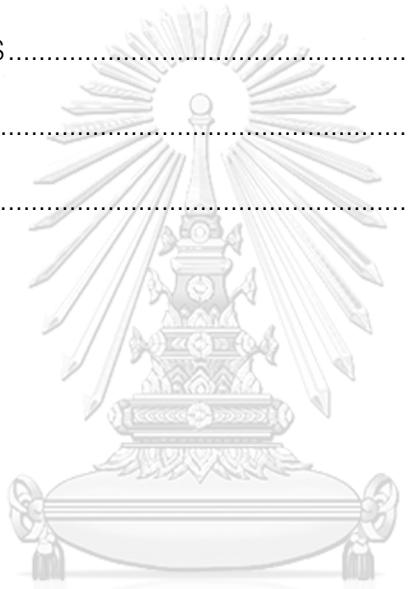

“I hereby declare that this thesis/dissertation has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own. This work has been submitted for a double Ph.D. degree: Ph.D. of Biochemistry and Molecular Biology, Faculty of Science, Chulalongkorn University and Doctor of Applied Biological Sciences: Aquaculture, Faculty of Bioscience Engineering, Ghent University. Copyright is shared by Chulalongkorn University and Ghent University”

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LIST OF ABBREVIATIONS

˚C	Degree celcius
AHPND	Acute Hepatopancreatic Necrosis Disease
ARC	Laboratory of Aquaculture & Artemia Reference Center
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CF12	non-selective breeding population
dsRNA	Double stranded ribonucleic acid
EF1-α	Elongation factor 1 alpha
EMS	Early mortality syndrome
ER	Endoplasmic reticulum
FAO	Food and Agriculture Organization of the United Nations
GFP	Green fluorescence protein
h	Hour
HLS	Hemocyte lysate
HSP	Heat shock protein
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	Kilobase
L	Litre
LHS	Lethal heat shock
Lv	*Litopenaeus vannamei*
LB	Luria-Bertani
μE	Microeinstein
μM	Micromolar

μg	Microgram
μL	Microlitre
M	Molar
M	Meter
mg	Milligram
min	Minute
mL	Mililitre
NLHS	Non-lethal heat shock
O.D.	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pm	*Penaeus monodon*
r	recombinant
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-PCR	Reverse transcription/polymerase chain reaction
SFB	San Francisco Bay *Artemia* strain
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SNP	Single-nucleotide polymorphism
sHSP	small heat shock protein
SSS	saline solution
s	Second
THC	Total hemocyte number

TSB	Tryptic soy broth
WGS	whole-genome sequencing
WSSV	White spot syndrome virus
YHV	Yellow head virus

CHAPTER I

INTRODUCTION, LITERATURE REVIEW & RESEARCH OBJECTIVE

1. Introduction

1.1 Shrimp aquaculture and production

From the past to the present, aquaculture has been increasing considerably to reach higher food demands of the world. Among those aquaculture farming, shrimp has quickly become the most valuable seafood in the world. FAO estimated that the global production of shrimp farming will reach 4.0 million tonnes in 2018 (Figure 1.1A). The major shrimp producers are China, followed by Thailand, Indonesia, India, Vietnam, Brazil, Ecuador, and Bangladesh. However, nearly 75 to 80 percent of the production is originated in Asia-Pacific. Focusing on Thailand, shrimp aquaculture plays an important role in the economy. At present, the white-leg shrimp *Litopenaeus* or *Penaeus vannamei* leads the shrimp farming list in Asia, followed by the black tiger shrimp *Penaeus monodon* (Figure 1.1B). The shrimp farms are located and distributed along the coastal areas especially in the South of Thailand; Suratthani and Nakhonsrithammarat provinces deliver the majority of shrimp production. Moreover, the shrimp farms are also located in the central and eastern provinces such as Phetchaburi, Samutsongkham, Samutsakhorn, Chachoengsao, and Chantaburi. Unfortunately, Thai shrimp production declined nearly 22.7 percent in 2011-2014 (Figure 1.2) because of infectious diseases especially new emerging diseases which have contributed to a major loss of shrimp production in recent years (Lai et al., 2015).

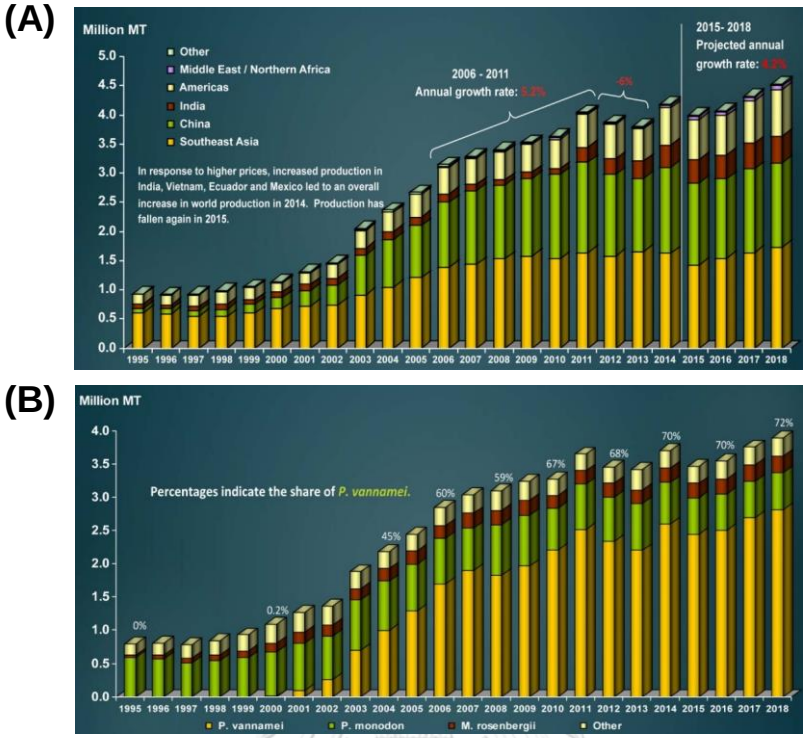

Figure 1.1 Shrimp aquaculture production by (A) world region and (B) in Asia by species during 1995-2018 Sources: FAO (2016) for 1995-2011; FAO (2016) and GOAL (2014) for 2012-2014; GOAL (2016) for 2014-2018 (Anderson et al., Shrimp production review, GOAL 2016.) The mass production (Million Tons) of shrimp indicated by country (A) and shrimp species (B). The data showed the increasing of shrimp production during 1995-2011. After that, the decreasing was showed during a new emerging disease outbreak especially in the white-leg shrimp called “AHPND”.

An emerging disease outbreak called “early mortality syndrome” (EMS) or acute hepatopancreatic necrosis disease (AHPND) (FAO, 2014) has severely damaged the shrimp aquaculture since 2009. This disease typically affects the post larvae within 20-30 days after stocking and causes mass mortalities (up to 100%) in farmed shrimp (De Schryver et al., 2014). The high mortalities occur in both the white-leg shrimp (*P. vannamei*) and the black tiger shrimp (*P. monodon*) (FAO, 2013). Currently, detection methods of AHPND are available using histological examination (Tran et al., 2013) and

molecular diagnostic techniques (Dangtip et al., 2015; Han et al., 2015; Kongrueng et al., 2015). Although the early diagnosis and detection of AHPND make control and prevention of AHPND possible in farmed shrimp, effective measures to control the disease are urgently required.

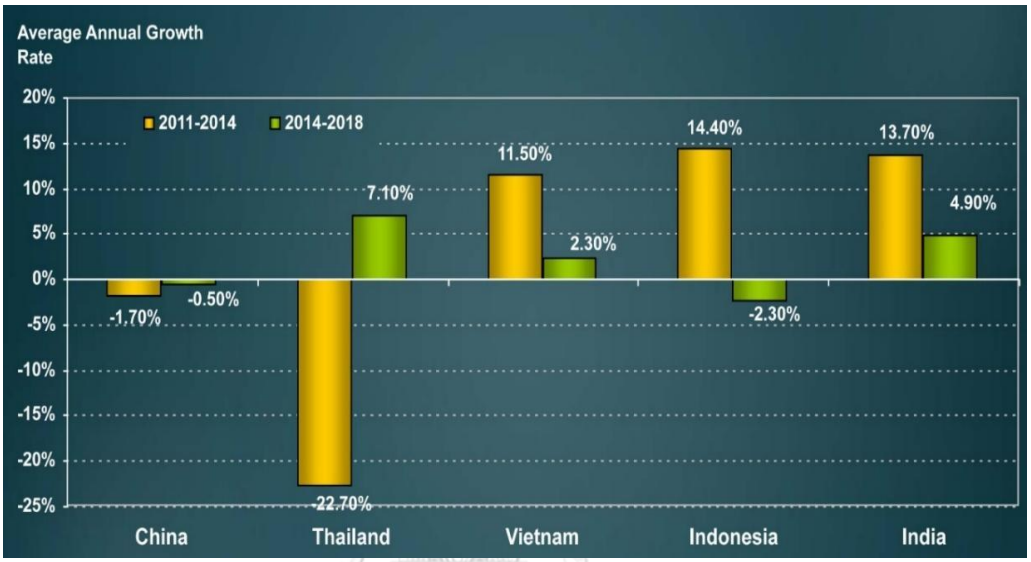

Figure 1.2 Shrimp aquaculture in Asia during 1995-2018 Sources: FAO (2016) for 1995-2011; FAO (2016) and GOAL (2014) for 2012-2014; GOAL (2016) for 2014-2018 (Anderson et al., Shrimp production review, GOAL 2016.) The dramatically decreasing of average annual growth of shrimp production (22.70%) was found in Thailand due to the new emerging disease outbreak "AHPND" during 2011-2014.

1.2 White-leg shrimp (*Litopenaeus vannamei* or *Penaeus vannamei*)

The major aquaculture shrimp contribution has come from *P. vannamei* production. According to Figure 1.1B, the production of shrimp aquaculture in Asia and also in the world have changed from *P. monodon* to *P. vannamei.* In 2000 *P. vannamei* occupied only 0.2 percent of all shrimp species. Interestingly, the production of *P. vannamei* was dramatically increased, occupying now 72% of all crustacean species. *P. vannamei* takes three months to reach a market size between 15 and 25 g, while *P.*

monodon requires at least four months to reach approximately 25 g or larger (Pongtippatee et al., 2018). This is considered to be the major reason for the dramatic shift towards white shrimp production.

Figure 1.3 White-leg shrimp (*Litopenaeus vannamei* or *Penaeus vannamei*) (http://www.fishmarket.ge/en/vanammei-prawn) *P. vannamei* is a penaeid shrimp. Its color is translucent, bluish or olive with dusky bands, reddish-brown on the antennules, but distinguished by white legs. The last abdominal segment has three lateral scar-like ridges.

1.2.1 Habitat and taxonomy

The white-leg shrimp is native to the Eastern Pacific coast of Mexico and in the North, through Central and South America as far South as Tumbes in Peru. This animal likes areas where water temperatures are usually 25 °C throughout the year (Dugassa and Gaetan, 2018).

Based on the taxonomic classification, the white-leg shrimp belong to the phylum Arthropoda, which is defined by having joined appendages and exoskeleton or cuticle that is periodically shed (Bailey-Brock and Moss, 1992). The taxonomic classification of *P. vannamei* is described as below (Dugassa and Gaetan, 2018)

Domain: Eukarya

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Superorder: Eucarida

Order: Decapoda

Suborder: Dendrobranchiata

Superfamily: Penaeoidea

Family: Penaeidae Genus: Penaeus

Species: Penaeus vannamei

1.2.2 Morphology

The external morphology of *P. vannamei* is shown in Figure 1.4. It contains 19 pairs of body segments. The first five pairs of segments are the head part (cephalon part). The next eight pairs of segments are the thorax part and the last six pairs are found in the abdomen. The head and thorax parts are combined to from cephalothorax. The exoskeleton of the cephalothorax functions to covers the gills and it also protects the gill chamber. The abdomen of the white-leg shrimp has six pairs of segments. The swimming legs (pleopods) are located on the first five pairs of the segments of the abdomen. The last pair of the segment is the tail fan. This segment comprises of two pairs of uropods and the telsons which can help a shrimp to jump quickly backward in the case of hazard (Dugassa and Gaetan, 2018).

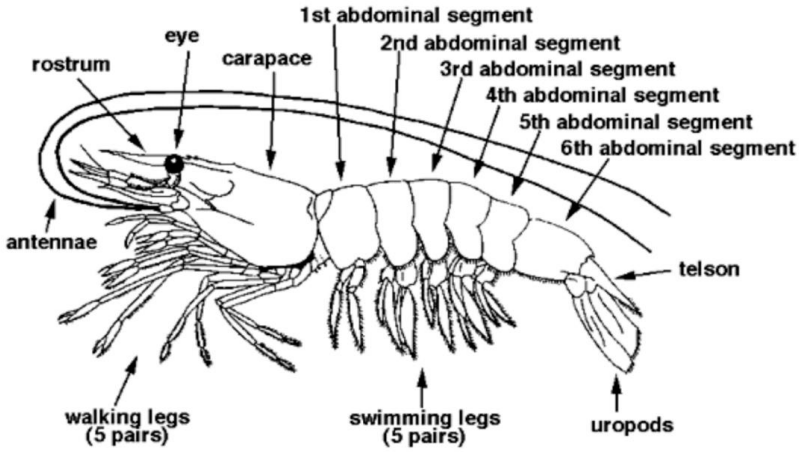

Figure 1.4 The external morphology of *Litopenaeus vannamei* or *Penaeus vannamei* (http://www.dnr.sc.gov/marine/pub/seascience/pdf/shrimpscience) Total of body segments contains 19 pairs. The first five pairs of the segment are the head part (cephalon part). The next eight pairs of the segment are the thorax part and the last six pairs of the segment are found in the abdomen. The head and thorax parts are combined to form the cephalothorax. The exoskeleton of the cephalothorax functions to covers the gills and it also protects the gill chamber. The abdomen of the white-leg shrimp has six pairs of segments. The swimming legs (pleopods) are located on the first five pairs of the segments of the abdomen. The last pair of the segment is the tail fan. This segment comprises two pairs of uropods and the telsons which can help a shrimp to jump quickly backward in the case of hazard.

1.3 Disease in shrimp

Infectious diseases especially new emerging diseases have contributed to a major loss of shrimp production in recent years (Lai et al., 2015). According to the GOAL 2016 survey, the most important issues and challenges in shrimp aquaculture are disease problems (Figure 1.5) that can cause high mortality in shrimp aquaculture and lead to economic losses worldwide. The economically-important pathogen infections are presented and arbitrarily grouped as viral, bacterial, fungal and parasitic diseases

(Ganjoor, 2015). Viral infections are divided further into two groups: RNA viruses and DNA viruses. The infections attributed to DNA viruses are: White Spot Disease (WSD) Disease, *Penaeus stylirostris* densovirus (*Pst*DNV) previously known as Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) Disease, *Penaeus monodon* densovirus (*Pm*DNV) formerly known as Hepatopancreatic Parvo-like Virus (HPV) Disease and *Penaeus monodon* nucleopoly-hedrovirus (*Pemo*NPV) previously known as Monodon Baculovirus (MBV) Disease. The shrimp infections caused by RNA viruses are Yellow Head Virus (YHV) Disease, Taura Syndrome Virus (TSV) Disease, and Infectious Myonecrosis Virus (IMNV) Disease. Fungal disease includes larval mycosis, while parasitic disease consists of the current emerging threat to the shrimp industry and the Hepatopancreatic Microsporidiosis caused by *Enterocytozoon hepatopenaei* (EHP). While, the bacterial diseases, are listed including Luminous Bacterial Disease, Non-luminous Vibrio Infections, and Acute Hepatopancreatic Necrosis Disease (AHPND). The latter is caused by the bacteria *Vibrio parahaemolyticus*.

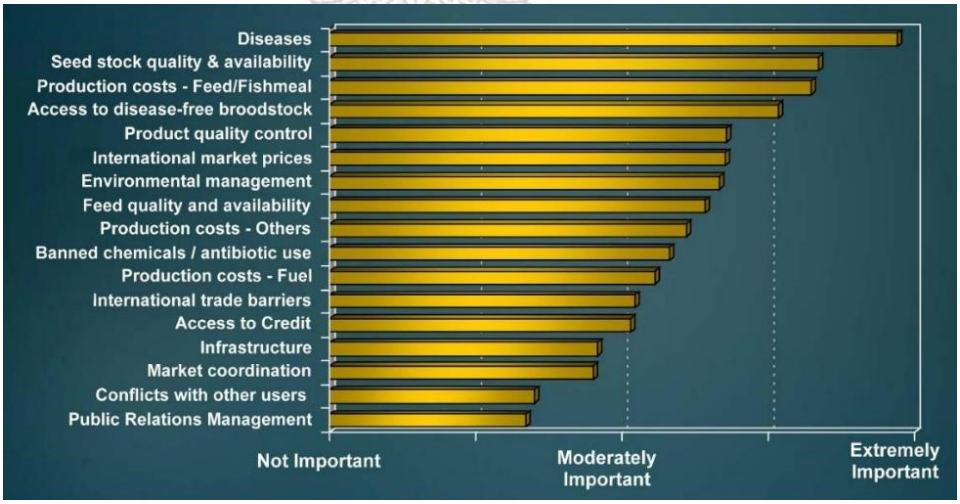

Figure 1.5 GOAL 2016 survey in issues and challenges of shrimp aquaculture in Asia (Modified; Anderson et al., Shrimp production review, GOAL 2016) The 17 issuers were rearranged by survey and indicated in tree level (not important, moderately, and extremely important). The major challenge issues that show the extremely important level in shrimp aquaculture are diseases.

1.3.1 Viral diseases

1.3.1.1 White spot syndrome disease

White spot syndrome disease (WSD) has caused tremendous economic losses to the shrimp aquaculture worldwide since its emergence in the 1990s. This disease caused by the white spot syndrome virus or WSSV belongs to the member of *Nimaviridae* family (Verbruggen et al., 2016). The nucleotide component is a large double-stranded DNA (dsDNA). This virus has a broad hots range such as shrimp, crayfish, lobsters, and crab (Hameed et al., 2003). However, the disease has mainly been a problem in farmed penaeid (family Penaeidae) shrimps. WSSV can infect various shrimp tissues such as antennal grand, cuticular epidermis, gill, muscle, lymphoid organ, nervous tissue, haematopoietic tissue, connective tissue of some organs (Kou et al., 1998). The principal clinical sign is the development of white spots on the carapace (Figure 1.6 A-B) which result from accumulation of calcium in cuticle (Pazir et al., 2012). Moreover, after infection, the infected shrimp show lower food consumption and loss of shell and lethargy. Appendages turn red or pink, typical of the acute phase of infection (Figure 1.6C). 80-100 percent of mortalities are found within 2-10 days after infection (Flegel, 1997).

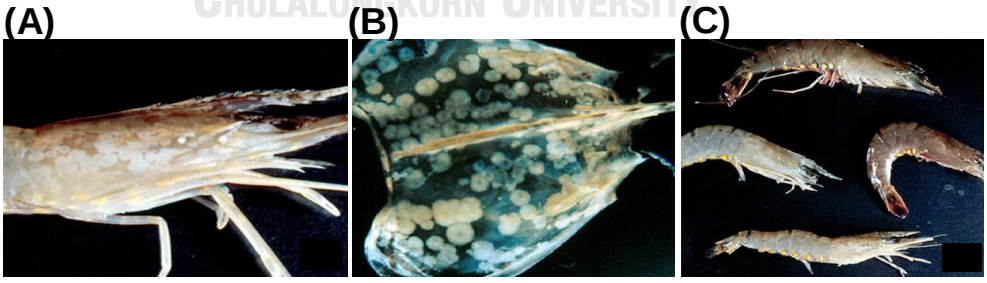

Figure 1.6 The principle clinical sign and the symptoms of White spot syndrome disease in shrimp (Lightner, 1996) The development of white spots on the carapace which result from the accumulation of calcium in cuticle. The infected shrimp show lower food consumption and loss of shell and lethargy. Appendages turn red or pink.

Recently, the mechanisms of WSSV infection and its pathogenesis have been the focus by several research groups (Kalia and Jameel, 2011; Mercer et al., 2010; Sodeik, 2000; Wang et al., 2019; Yang et al., 2019). The key interactions between viral envelope proteins and host cell receptors during WSSV exposure have been revealed. For example, the study in *Marsupenaeus japonicus*, WSSV envelope protein VP466 was demonstrated to be able to bind the host Rab6 protein and increase its GTPase activity both *in vivo* and *in vitro* (Ye et al., 2012). Moreover, the interaction of viral protein WSSV134 (VP36A) and *Pm*HHAP has been identified suggesting that *Pm*HHAP and WSSV134 play a role in the host–pathogen interaction and work concordantly to control apoptosis in WSSV infection (Apitanyasai et al., 2018). In addition, WSSV453 interacts with *Pm*proPPAE2 and interferes with the activation of active *Pm*PPAE2 resulting in anti-melanization activity (Sutthangkul et al., 2017). However, the large body of work and the understanding of WSSV infection and pathogenesis are far from complete. The questions include those focused on mechanisms of endosomal escape, virion assembly, exit from the host cell. Also, the role of miRNA regulation is still unclear. Nevertheless, there is an urgent need for disease mitigation (and prevention). Some of the more promising results include strategies spanning immune system activation, vaccinations, RNAi, and the application of herbal extracts (Bindhu et al., 2014; Huang et al., 2013; Syed Musthaq and Kwang, 2014; Thitamadee et al., 2014).

1.3.1.2 Taura syndrome disease

Taura syndrome virus (TSV) has emerged as a major pathogen of the white-leg shrimp *P. vannamei* aquaculture. The disease was first discovered in Ecuador in 1992, from *P. vannamei* shrimp samples (Bonami et al., 1997)and the virus is globally distributed with the greatest concentration of infections in the Americas and Southeast Asia. The spreading of this virus has caused economic loss (Wertheim et al., 2009). TSV outbreaks in shrimp aquaculture farming can decimate shrimp populations with high

mortality ranging from 40 to 90% (Hasson et al., 1995). TSV is classified as a member of the family *Dicistroviridae* which is a small, non-enveloped, icosahedral virus containing a single-stranded positive-sense RNA (ssRNA) (Bonami et al., 1997). The replication of TSV can be detected in various tissues such as foregut, hindgut, gills, and appendage (Hasson et al., 1999). The infected shrimp shows clinical signs such as pale red body surface and appendages. Tail fan and pleopods are particularly red, the shell is soft and gut empty. Death usually occurs at molting, there are also multiple irregularly shaped and randomly distributed melanised cuticular lesions (Figure 1.7A-C) (Australian Government Department of Agriculture, 2012).

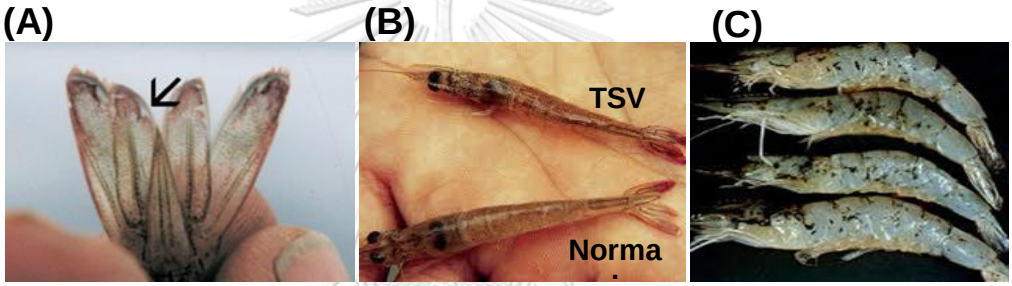

Figure 1.7 The principle clinical sign of Taura syndrome disease in shrimp (Lightner, 1996) The clinical signs are pale red body surface and appendages, particularly red in tail fan and pleopods (arrow), and soft shell and gut empty.

The development of the selective breeding program has been established to select *P. vannamei* for resistance to TSV infection. This selective breeding program resulted in an increase of final pond survival equal to the levels of those before the initial Taura syndrome outbreak (Cock et al., 2009). This established program is most successful in controlling and preventing Taura syndrome disease making TSV no longer a major disease in white-leg shrimp. Nevertheless, the presence of resistance and susceptible TSV lines are of great benefits for the study of disease resistance mechanisms that could lead to the development of shrimp populations with resistance to other pathogens. Transcriptome analysis of hemocytes of shrimp resistance to TSV

revealed the differentially expressed gene sets highlighting several putative genes involved in the immune response activity including pathogen/antigen recognition including such as immune regulator, proPO pathway cascade, antioxidation, and protease. This result provides valuable information on gene functions associated with resistance to TSV in *L. vannamei* (Guo et al., 2013). Interestingly, oligo-microarray analysis identified several genes that were differentially expressed in the TSV resistance and susceptible *L. vannamei* lineages following infection with TSV (Boube et al., 2014). A set of 11 genes were found to be able to correctly classify white shrimp as resistant or susceptible based on gene expression data.

1.3.1.3 Yellow head disease

Yellow head disease is caused by yellow head virus (YHV) which is a major virulent pathogen with ability to infect several penaeid shrimp species such as Black tiger shrimp (*Penaeus monodon*), Kuruma prawn (*Metapenaeus japonicus*), white-leg shrimp (*Penaeus* vannamei), and White banana prawn (*Fenneropenaeus merguiensis*) (Australian Government Department of Agriculture, 2012; Flegel, 1997). The disease outbreaks in penaeid shrimp cause economic loss of millions of US dollars in Thailand (Senapin et al., 2010). The disease was first found in Thailand in 1990 and has caused extensive losses to shrimp farmers in the eastern, and central parts of the country and mostly in southern Thailand. Nowadays, several YHV genotypes were identified. YHV genotype 1 (YHV-1) was first recognized in the 1990s and was the cause of mass mortalities of cultured *P. monodon* in Thailand (Chantanachookin et al., 1993).Gill-associated virus (GAV) is known as YHV genotype 2 (YHV-2). YHV genotypes 3–6 occur commonly in healthy *P. monodon* in Asia, East Africa and Australia (Wijegoonawardane et al., 2008) and recently YHV-3 has been identified in mainland China in 2018 (Chen et al., 2018a). YHV-7 and YHV-8 are, detected in diseased *P. monodon* and *Fenneropenaeus chinensis* in Australia and China in 2012, respectively; however, the role of YHV-7 and YHV-8 remains unknown despite the mortalities observed (Liu et al.,

2014a; Mohr et al., 2015; Zhu et al., 2016). Yellow head disease is named for the yellowish cephalothorax and overall sallow coloration (Boonyaratpalin et al., 1993; Flegel, 1997). The causative agent of YHV is a positive-sense, single-stranded RNA virus in the genus *okavirus*, family *Roniviridae* and order *Nidovirales* with the genome size of 26,622 nucleotides (Sittidilokratna et al., 2008). This disease causes severe damages in lymphoid and gill leading to high shrimp mortality (Flegel, 2006). The gross pathological signs are a pale body and light-yellow coloration of the hepatopancreas and gills leading to yellowing of the cephalothorax and general bleaching of the body (Figure 1.8A-B). Following the infection, the number of affected shrimp is increased up to 50-60% of total population and cumulative mortalities often reach 100% of affected populations within 2-4 days (Australian Government Department of Agriculture, 2012)

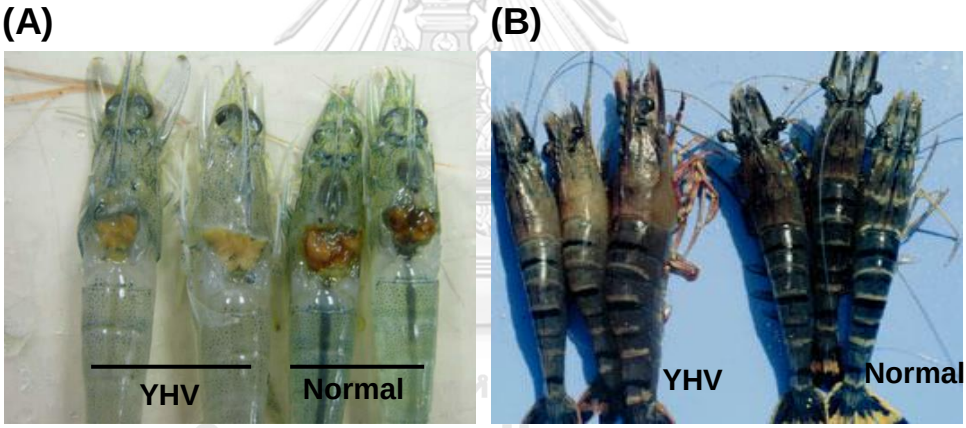

Figure 1.8 The clinical sign of Yellow head disease in shrimp (Amarakoon and Wijegoonawardane, 2017; Flegel, 2006) The gross pathological signs are a pale body and light-yellow coloration of the hepatopancreas and gills leading to yellowing of the cephalothorax and general bleaching of the body.

Various diagnostic methods have been identified and developed for YHV detection such as RT-PCR (Cowley et al., 1999), *in situ* hybridization (Tang et al., 2002), loop-mediated isothermal amplification (RT-LAMP) (Mekata et al., 2006), real-time RT with loop-mediated isothermal amplification (LAMP) (Mekata et al., 2009) and a

combined RT-LAMP and a chromatographic lateral-flow dipstick (LFD) (Khunthong et al., 2013). At present, a comprehensive assessment of host susceptibility to YHV has been revealed including Gulf brown shrimp (*Penaeus aztecus*), Gulf pink shrimp (*P. duorarum*), Kuruma prawn (*P. japonicus*), black tiger shrimp (*P. monodon*), Gulf white shrimp (*P. setiferus*), Pacific blue shrimp (*P. stylirostris*), and white shrimp (*P. vannamei*) (EFSA, 2008).

1.3.2 Bacterial diseases

1.3.2.1 Vibriosis

Vibriosis is one of the major disease problems in shrimp aquaculture. This bacterial disease is responsible for high mortality and economic losses of shrimp culturing worldwide (Begum and Mastan, 2016). The causative agent of this disease is bacteria in a group of Vibrio species which are widely distributed throughout the world. Vibrio-related infections frequently occur in hatcheries, but epizootics also commonly occur in pond-reared shrimp species. Gram-negative bacteria in the family *Vibrionaceae* cause vibriosis. The disease outbreak may occur in case of the environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within shrimp blood (Sizemore and Davis, 1985), or by bacterial penetration of host barriers. The one most important Vibrio spp. is *Vibrio harveyi*, a Gram-negative, motile, rod shape, and luminous bacterium, which is one of the critical etiologic agents of mass mortalities of *P. monodon* larval rearing systems (Chandrakala et al., 2017). The clinical signs are causing the shrimp to be luminescent, mortality ranges from insignificant to 100%, particularly in postlarvae and young juvenile shrimp, and the moribund shrimp appear hypoxic and often come to pond surface or edge, reddish discoloration and show black spots of melanization on the cephalothorax (Figure 1.9A-C).

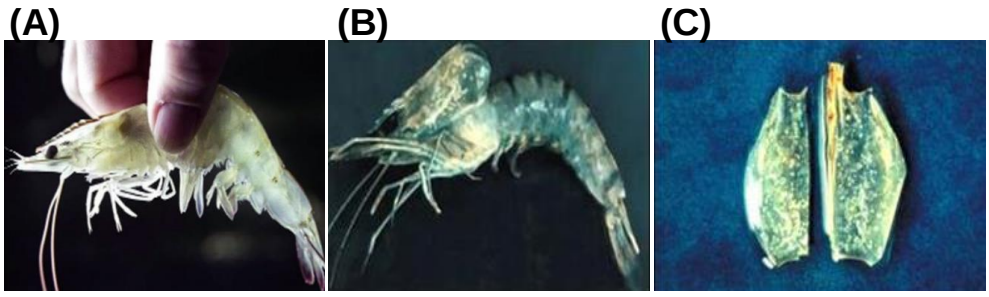

Figure 1.9 The clinical sign of Luminous Vibriosis (BioAqua and Kerala Agricultural University) Shrimp represent the luminescent, reddish discoloration, and black spots of melanization on the cephalothorax.

1.3.2.2 Acute Hepatopancreatic Necrosis Disease (AHPND)

Infectious diseases especially new emerging diseases have contributed to a major loss of shrimp production in recent years. Acute Hepatopancreatic Necrosis Disease or AHPND should be considered as a new emerging shrimp disease that has attacked shrimp farms worldwide. This disease was first reported in China (2009) and caused mass mortality, and then was spread in Vietnam (2010), afterward in Malaysia (2011) and Thailand (2012) and finally in Mexico (2013) (Zorriehzahra, 2015) (Figure 1.10A). At first, this disease was named Early Mortality Syndrome (EMS) due to mass mortality within a few days after shrimp post larvae stocking. This disease is an acute disease and affected region in China causing 80% loss of their products due to this catastrophic disease (Lightner, 2012). In Thailand, the shrimp production declined nearly 30-70 percent because of AHPND (FAO, 2014). The disease typically affects the post larvae within 20-30 days after stocking and causes mass mortalities (up to 100%) in farmed shrimp afterward (De Schryver et al., 2014). The high mortalities occur in both the black tiger shrimp (*P. monodon*) and the white-leg shrimp (*P. vannamei*) (FAO, 2013).

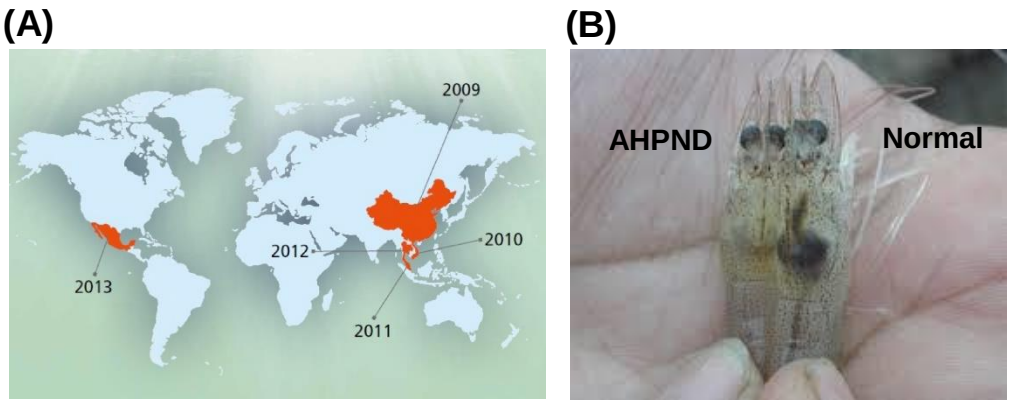

Figure 1.10 (A) Geographical distribution of AHPND in the world (Zorriehzahra, 2015) The disease was first reported in China (2009) and caused mass mortality, and then was spread in Vietnam (2010), afterward in Malaysia (2011) and Thailand (2012) and finally in Mexico (2013) **(B) Gross signs of *Penaeus vannamei* affected by AHPND** (Lightner, 2012) Left-hand site shrimp represented empty stomach, atrophied, pale hepatopancreas, and empty midgut compared to normal shrimp in right-hand site which showed full stomach, large, pigmented hepatopancreas, and full midgut.

The causative agent of AHPND has been identified as a unique strain of bacterium *Vibrio parahaemolyticus* which is a halophilic Gram-negative bacterium. This bacterium is commonly found in estuarine, marine and environment (Tran et al., 2013). However, in the beginning, it was unclear how this opportunistic bacterium had become virulent and capable of causing disease outbreak in shrimp aquaculture. An empty stomach and midgut, and a pale to white atrophied hepatopancreas were observed as clinical symptoms in infected shrimp (Figure 1.10B) (Tran et al., 2013).

Moreover, histological examination of the diseased shrimp further showed that the HP tubule epithelial cells sloughed into the HP tubule lumens (Lightner, 2012; Tran et al., 2013). While, in the initial, acute stage of AHPND, many bacteria could be identified in the stomach, however, at the same time, the apparent bacterial colonies were not observed in the hepatopancreas tube lumens (Gomez-Gil et al., 2014; Soto-Rodriguez et

al., 2015; Tran et al., 2013) (because apparently the symptoms of AHPND are caused by a toxin secreted by the pathogen (Tran et al., 2013). This hypothesis was further supported by reverse gavage bioassays in which introduction of the bacteria-free supernatant of the bacterial culture into healthy shrimp induced typical AHPND symptoms (Sirikharin et al., 2015; Tran et al., 2013). Subsequent investigations focused on isolating AHPND variants (Joshi et al., 2014) and on the comparison of the draft genome sequences between AHPND-causing and non-AHPND-causing strains (Giffin et al., 2014; Han et al., 2015; Yang et al., 2014b). The result of next-generation sequencing (NGS) platform to sequence and compare three virulent (3HP, 5HP, and China) and one non-virulent (S02) *V. parahaemolyticus* strains found that a 69-kb extrachromosomal plasmid was present in all AHPND-causing strains but not in the non-virulent strain. Moreover, the sequence analysis demonstrated that it is a homolog of the insecticidal Photorhabdus insect-related (Pir) binary toxin PirA/PirB (Yang et al., 2014b). The importance of these two toxins to AHPND was confirmed by subsequent studies (Lai et al., 2015; Theethakaew et al., 2017; Tinwongger et al., 2016). At present, they are now referred to as PirA/PirB (PirAvp/PirBvp). The binary toxin $PirAB^{vp}$ have been shown to cause the typical histological symptoms of AHPND in infected shrimps (Lin et al., 2017). Moreover, experimental data indicated that the virulence of *V. parahaemolyticus* AHPND (VP_{AHPND}) depends on the isolates, and the rate of mortality is dose-dependent (Joshi et al., 2014). The clinical signs of AHPND are mass mortality (up to 100%) within 20-30 days after stocking (Zorriehzahra, 2015). Previous report found that the first mortality occurred within 18 h (Tran et al., 2013) and showed the clinical symptoms such as low growth rate, spiral swimming, pale and atrophied hepatopancreas (HP), and an empty stomach (ST) and midgut (MG) (Figure 1.11) (Tran et al., 2013; Zorriehzahra, 2015). Moreover, the histological sections of infected shrimp showed sloughing of hepatopancreas (HP) tubule epithelial cells (Figure1.12) (Tran et al., 2013).

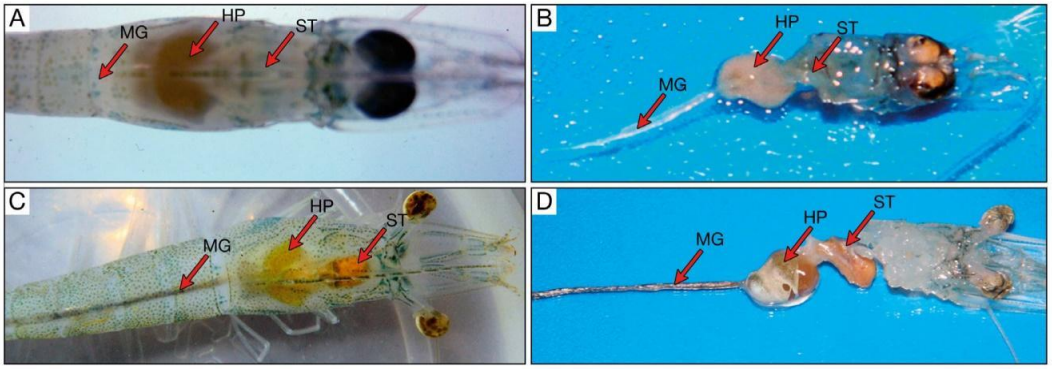

Figure 1.11 Gross signs of AHPND-infected shrimp (Tran et al., 2013) (A, B) normal shrimp and (C, D) infected shrimp Picture A and B showed the gross signs of AHPNS-infected shrimp. Pale, atrophied hepatopancreas (HP), and an empty stomach (ST) and midgut (MG), which was induced by immersion bioassay. Picture C and D showed normal shrimp in the negative control group, showing a normal size HP with dark orange color and a full stomach and midgut. (B) and (D) are dissected individuals from (A) and (C), respectively.

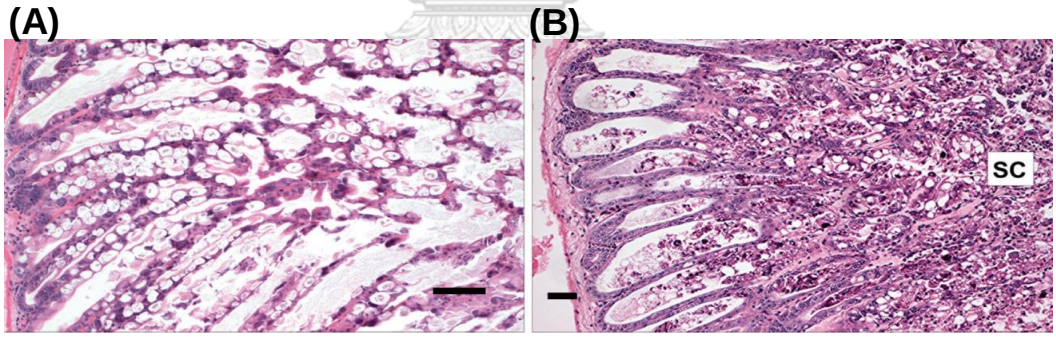

Figure 1.12 Histological sections of shrimp hepatopancreas (Tran et al., 2013) Histological sections from the immersion bioassay showing the AHPND lesions induced by pathogenic bacterial isolates. (A) normal shrimp and (B) infected shrimp, SC indicate acute HP tubule degeneration and significant sloughing (SC) of HP tubule epithelial cells. Picture A showed normal HP tubule structure. (B) Affected HP of shrimp after immersion of VP_{AHPND} showing acute HP tubule degeneration and SC of HP tubule epithelial cells; the pathology progresses from proximal to distal. Scale bars A: 100 μm and B: 50 μm

Several detection methods for AHPND are available using histological examination (Tran et al., 2013) and molecular diagnostic techniques (Dangtip et al., 2015; Han et al., 2015; Kongrueng et al., 2015). Recently, the new method used for detection of VP_{AHPND} was published in 2017 by using monoclonal antibody developed for detection of PirvpA and PirvpB (Wangman et al., 2017). Moreover, several strategies have been proposed to control this disease for example; use probiotics as potential biocontrol agents to improve the survival of *P. vannamei* (Chomwong et al., 2018; Wang et al., 2018). Furthermore, the study of gnotobiotic *Artemia* model revealed that the compound phloroglucinol could protect axenic brine shrimp larvae against *V. parahaemolyticus* (Kumar et al., 2018b). Although, the early diagnosis, the detection of AHPND, and biological control methods make it possible for control and prevention of AHPND in farmed shrimp, effective measures to control the disease are urgently required.

1.4 Shrimp immune system

Shrimp, similar to other invertebrates, lack an adaptive immune system. Instead, they rely on their effective cellular and humoral innate immune system to defend against hostile microorganisms (Bachere et al., 2000). In crustaceans, including shrimps, the major immune reactions occur in the hemolymph, which consists of three different principal types of hemocytes; the hyaline, granular and semi-granular hemocytes (Figure 1.13) (Rowley, 2016). Several immune molecules are produced and stored in the granules of hemocytes before being released into the hemolymph upon the activation by the bacterial and/or fungal cell wall components (Tassanakajon et al., 2013). The innate immunity in shrimp consists of microbial recognition systems, prophenoloxidase (proPO) activating system, clotting system, phagocytosis, encapsulation, and nodule formation, antimicrobial proteins and reactive oxygen compounds (Figure 1.14). The first line of innate immunity is triggered by pathogens and is mediated by the pattern recognition

proteins (PRPs) which are function as the first and essential step to recognize the non-self-material which has gained entrance into the shrimp body. The PRPs can recognize conserved molecules on the surface of invading microorganisms, such as lipopolysaccharide (LPS) and peptidoglycan (PG) from bacteria, laminarin, like β-1,3-glucan structure from fungi, and double-stranded RNA (poly I:C), mimicking viral infection, have been identified as pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). Binding of PRPs to PAMPs triggers a series of immune responses leading to activation of the host defense system.

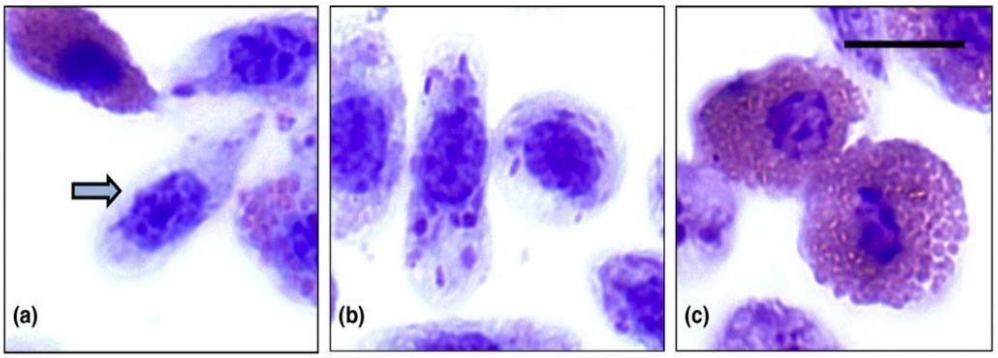

Figure 1.13 Classification of hemocytes in the edible crab, *Cancer pagurus* (Rowley, 2016) (A) Hyaline cells (arrow) with a largely agranular cytoplasm. (B) Semi-granular cells showing granule diversity. (C) Granular cells containing abundant eosinophilic granules. Scale bar: 10 mm

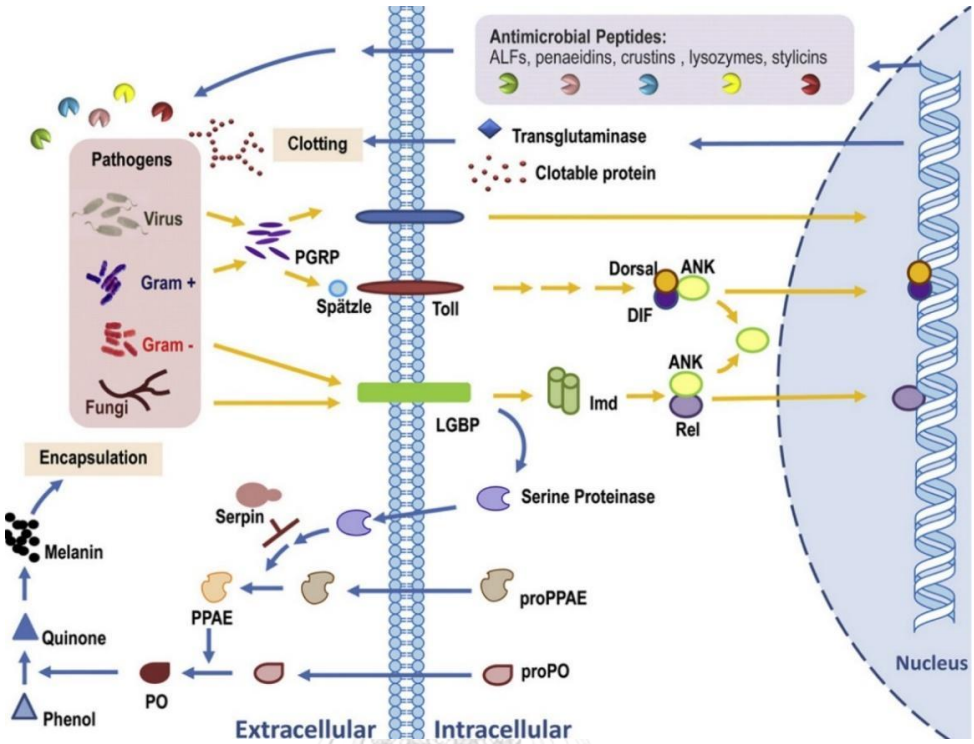

Figure 1.14 A schematic model of shrimp immune system (Tassanakajon et al., 2013). The innate immunity is triggered by the binding of PRRs to invading pathogens infection. Subsequently activates the host defense systems which lead to the activation of the cellular and humoral immune response.

1.4.1 Cellular immune responses

Compared to the knowledge on the humoral immune response, the information on cellular immune response of shrimp is still limited. Phagocytosis has been shown to have crucial roles in shrimp immune system. Phagocytosis is the most common reaction of defense cell mechanisms. By this process, cells (hemocytes) ingest and destroy invading pathogens, foreign particles or modified (aged) cells of the body itself (Secombes, 1996) Moreover, encapsulation and nodule formation (Figure 1.15) are

processed by which several hemocytes cooperate with each other aiming to stop the action of invading organisms, when the host is attacked by either extremely-large particles or numerous tiny particles, to be ingested then destroyed by individual cells (Söderhäll and Cerenius, 1992). The previous study in *P. monodon* hemocyte revealed that the three hemocyte types have various cellular responses to lipopolysaccharide (LPS) stimulation. Granular cells and semi-granular cells are the main cell types for cellular immune responses against LPS (Xian et al., 2017), as reflected by the decrease of granular cells and semi-granular cells after shrimp being injected with LPS. The reduction of these two hemocyte cells might be due to ROS/NO-induced apoptosis. The results suggest that hemocyte apoptosis would eliminate damaged or weak cells and contribute to hemocyte renewal and may be a defending strategy against pathogens (Xian et al., 2016). Apoptosis or programmed cell death is an important cellular process for removing cells that bear sustained genetic damage or that undergo uncontrolled cellular proliferation (Bortner et al., 1995). Apoptosis is also considered as an innate cellular response to inhibit viral replication and eliminate viral-infected cells in eukaryotic organisms (Everett and McFadden, 1999; Koyama et al., 2000). The previous study has found that apoptosis occurred after WSSV infections in shrimp (Leu et al., 2013), indicating that virus infection induces apoptosis in crustaceans (Menze et al., 2010).

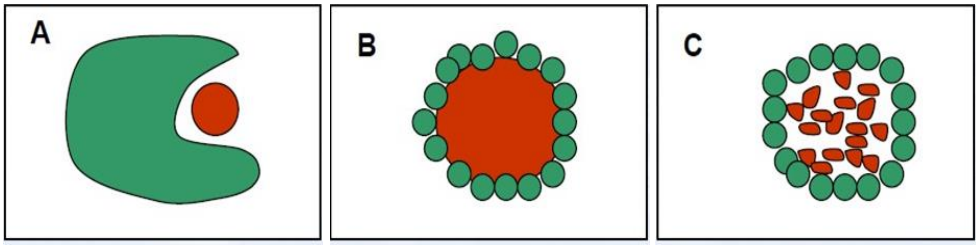

Figure 1.15 Defense cell processes include (Martínez, 2007): (A) Phagocytosis, (B) Encapsulation, and (C) Nodule formation. Hemocytes are represented in green, while the invading organisms appear in red.

1.4.2 Humoral immune responses

The humoral responses that are involved in the synthesis and release of several immune proteins, include antimicrobial peptides (AMPs), proteinase inhibitors, cytokine-like factors, etc. Macromolecules mediate the humoral responses in hemolymph. The important humoral responses are the melanin synthesis by the prophenoloxidase (proPO) system, the blood clotting system, and the generation of circulating antimicrobial peptides (AMPs). Humoral immunity involves innate signaling cascades, including the NF-kB signaling, Toll and Immune Deficiency (IMD) pathways, resulting in the activation of genes involved in host defense (Tassanakajon et al., 2018). Immune defenses are triggered by pathogen-associated molecular patterns (PAMPs) or the viral protein antigens, which are sensed by pattern recognition receptors (PRRs) (Hillyer, 2016) resulting in the activation of NF-kB signaling pathway. The activation triggers the secretion of circulating antimicrobial peptides and other immune proteins to eradicate the infection (Tassanakajon et al., 2018). The two major signaling immune pathways are directly involved in these responses, known as Toll and IIMD pathways.

The Toll pathway plays a key role in the response to Gram-positive bacteria with Lys-type peptidoglycans (PNGs), possibly fungi and some viruses (Tassanakajon et al., 2018). PRPs are recognized by pathogens then the protease cascade is probably induced to finally cleave Pro-Spatzle into active-Spatzle (Shi et al., 2009; Wang et al., 2012). After that, the active-Spatzle binds to the receptor (Toll receptor) then sending the infection signal into the cell. A series of signaling proteins comprising of Myeloid differentiation factor 88 (MyD88) (Wen et al., 2013; Zhang et al., 2012b), Tube (Li et al., 2014b; Wang et al., 2011), Pelle (Li et al., 2014a; Wang et al., 2011) and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (Deepika et al., 2014) relay the signal to the Dorsal-Cactus complex (Huang et al., 2010; Li et al., 2014a; Wen et al.,

2013). Cactus is phosphorylated, then dissociates from Dorsal, after which it is degraded, while the unmasked NF-kB transcription factor Dorsal migrates into the nucleus to regulate the synthesis of cognate AMPs (Tassanakajon et al., 2018).

Besides the Toll pathway, the IMD pathway is initiated on direct interaction of the Gram-negative bacterial PNGs to the transmembrane receptors (Tassanakajon et al., 2018). In contrast with the Toll pathway, the IMD pathway is preferentially triggered by Gram-negative bacteria and some Gram-positive *Bacilli* with the meso-diaminopimelic acid-type (DAP-type) PNGs. The bacteria and some RNA viruses are recognized by the membrane-bound PRPs (Jearaphunt et al., 2015; Udompetcharaporn et al., 2014; Wang and Wang, 2013). The infection signal is sent into the cells and relays via two routes to activate a NF-kB transcription factor Relish. The first route is through the IMD (Feng et al., 2014; Lan et al., 2013) and probably a few signaling proteins. On the other hand, the second route is through the transforming growth factor b-activated kinase 1 (TAK1)/TGF-b activated kinase 1/MAP3K7 binding protein 2 (TAB2) complex and IkB kinase (IKK) complex (Wang et al., 2016; Wang et al., 2015; Wang and Wang, 2013). The ankyrin repeat of Relish, analogous to Cactus, is cleaved off and the activated Relish migrates into the nucleus to regulate the synthesis of AMPs under this pathway (Feng et al., 2014; Huang et al., 2009; Li et al., 2009; Shi et al., 2015; Visetnan et al., 2015).

Moreover, the activation of the prophenoloxidase (proPO) cascade leads to a principal innate immune response in shrimp via the melanization pathway (Amparyup et al., 2013; Tassanakajon et al., 2013). During pathogen exposure, the binding of PRPs to corresponding microbial cell wall components activates the serine proteinase cascade which subsequently activates the final proteinases, called proPO-activating enzymes (PPAEs). After that, the PPAEs, cleave the inactive proPOs to active POs which lead to the initiation of the melanin formation. Inevitably, the activation of the melanization cascade needs to be tightly regulated and locally restricted to prevent damage to the host by controlling the proteinases and inhibiting the proPO system (Amparyup et al., 2013; Charoensapsri et al., 2014). The melanization pathway consists of three major processes which are the pathogen recognition, the proteolytic cascade, and the

activation of proPO enzymes (Amparyup et al., 2013). Currently, several melanization/proPO system-related genes have been identified and functionally characterized in shrimps, whose studies contribute to the knowledge of shrimp humoral immune responses (Tassanakajon et al., 2018).

1.5 Heat shock proteins

The families of heat shock proteins (HSPs) are also known as stress proteins and extrinsic chaperones. They are a group of highly conserved proteins of varying molecular weight which are classified according to their molecular mass in kiloDaltons (kDa). The major HSPs family include HSP110, HSP100, HSP90, HSP70, and HSP60, and the small HSPs (sHSPs), with the latter having a molecular mass less than 40 kDa such as HSP21, and HSP10 (Yik Sung, 2013). HSPs can also be classified according to their synthesis. The first are those commonly expressed constitutively like heat shock cognates (HSCs) while the other are inducible during protein denaturation that can be caused by cell exposure to heavy metals, high temperature, ultraviolet light, inflammation, infection, cellular toxin and other stressors (Roberts et al., 2010). HSPs are well known as molecular chaperones, aiding nascent polypeptide folding and oligomerization, protecting proteins from irreversible denaturation, re-folding or degrading damaged proteins, translocating proteins into membrane-bound cell compartments and contributing to disease resistance. HSPs originally were discovered upon heat shock as chromosomal puffs that were observed in the salivary glands of fruit flies (*Drosophilia*) (Whitley et al., 1999).

The conserved structural domains are observed in HSP70 and HSP90 (Figure 1.16). HSP90 has three structural domains which compose of an N-terminal NBD (green) that may also contain a peptide binding element. The middle segment (yellow) interacts with client proteins and also contributes a loop that catalyzes ATP hydrolysis. The C-

terminal domain (red) is implicated in homodimerization. HSP70 has two major domains: a 44- kDa N-terminal NBD (green) and a 27-kDa C-terminal substrate binding domain (yellow and red). This region comprises the main substrate binding domain (yellow; 18 kDa) that consists of two times four antiparallel -strands and four connecting loops and makes contact with the bound substrate, as well as an α-helical lid region (red; 10kDa). Both molecules have a linker region (blue) that is thought to mediate communication between the major subdomains (Javid et al., 2007).

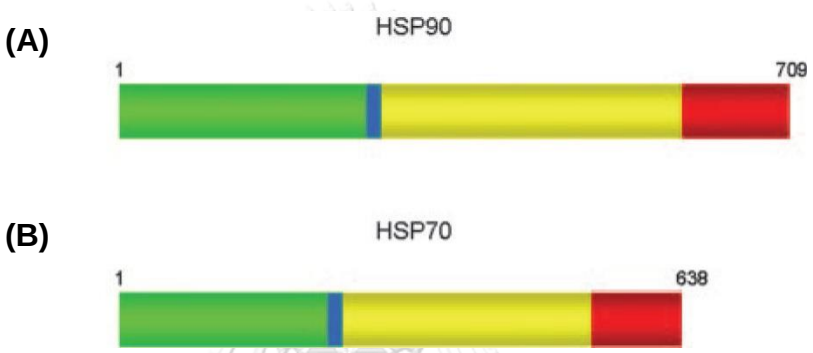

Figure 1.16 Schematic of the domain structures of (A) yeast HSP90 and (B) *E. coli* HSP70 (Javid et al., 2007) HSP90 showed an N-terminal NBD in green that may also contain a peptide binding element. The middle segment, shown in yellow, interacts with client proteins and also contributes a loop that catalyzes ATP hydrolysis. The C-terminal domain shown in red is implicated in homodimerization. HSP70 has two major domains: a 44- kDa N-terminal NBD (green) and a 27-kDa C-terminal substrate binding domain (yellow and red). This region comprises the main substrate binding domain (yellow; 18 kDa) that consists of two times four anti-parallel β-strands and four connecting loops and contact the bound substrate, as well as an α-helical lid region (red; 10kDa). Both molecules have a linker region (blue) that is thought to mediate communication between the major subdomains

The HSP family of main interest for disease control is HSP70 but the sHSPs, HSP90 and HSP60, as well as the co-chaperone HSP40, appear to ameliorate infection by pathogens. The sHSPs provide oligomeric platforms for the ATP-independent binding

of structurally perturbed proteins, preventing their irreversible denaturation when cells are stressed. HSP90, HSP70, and HSP60 are stress induced and they have the ability to protect proteins from irreversible denaturation. However, the major function of these chaperone families is to bind and fold nascent proteins through ATP-driven allosteric rearrangement, although the molecular structure and mechanism of action of each chaperone differ. The HSPs function cooperatively by forming intracellular networks of chaperones, co-chaperones and accessory proteins. sHSP monomers, consisting of a conserved α-crystallin domain flanked by an amino-terminal sequence and a carboxyl-terminal extension (Hilario et al., 2011; McHaourab et al., 2009; Sun and MacRae, 2005), assemble into oligomers (Kennaway et al., 2005). The α-crystallin domain contributes to dimerization of monomers and substrate binding, activities that depend on the amino- and carboxyl- terminal regions for greatest efficiency (Hilario et al., 2011; McHaourab et al., 2009) sHSP oligomers either disassemble or undergo structural rearrangement during stress, increasing surface hydrophobicity and enhancing reaction with substrate proteins (Benesch et al., 2008; Hilario et al., 2011). Proteins released from sHSPs when stress passes either refold spontaneously or with the assistance of an ATP-dependent HSP such as HSP70 (Lee and Vierling, 2000). The primary role of sHSPs during exposure to stress, including infection, is to protect proteins from irreversible denaturation.

Under acute thermal stress in the culture environment, it was found that the white shrimp HSP40 (*LvHSP40*) transcript levels were significantly induced in muscle, gill, and hepatopancreas (Chen et al., 2018b). The expression profiles of four HSP genes (*LvHSP60*, *LvHSP70*, *LvHSC70*, and *LvHSP90*) of *L. vannamei* were significantly induced, and the transcription level of *LvHSP70* was the most sensitive to temperature fluctuations (Qian et al., 2012). Moreover, *HSP60* and *HSP10* genes from *Scrippsiella trochoidea* were rapidly up-regulated upon exposure to both low and high temperatures

(Deng et al., 2018). In pathological situations, such as necrotic cell death, the HSPs can be released into the extracellular environment with cellular proteins to induce autoimmunity by receptor-mediated activation of the innate immune response (Routsias and Tzioufas, 2006). In shrimp, there are evidences that the HSPs are highly expressed in response to pathogen infection. The *Lv*HSP60 protein from *P. vannamei* was significantly up-regulated in the gills, hepatopancreas and hemocytes after challenged with either Gram-positive and Gram-negative bacteria (Zhou et al., 2010). On the contrary, the *PmHSP21* mRNA level was significantly decreased after WSSV infection (Huang et al., 2008). In addition, the transcriptomic results in RNA-seq analysis of the freshwater prawn *Macrobrachium rosenbergii* show up-regulation of the heat shock proteins; HSP21, HSP70, and the small heat shock proteins in response to *V. parahaemolyticus* infection (Rao et al., 2015).

1.6 Heat shock protein 70 family

The HSP70 family is a well-studied group of stress response proteins in prokaryotic and eukaryotic organisms. (Brocchieri et al., 2008). This family is composed of several highly related protein isoforms ranging in molecular weight from 66 to 78 kDa (Tavaria et al., 1996). The members of the HSP70 family genes can vary depending on the organism. The human HSP70 family comprises 13 genes that differ from each other in expression level, subcellular location, and amino acid constitution (Tavaria et al., 1996). However, in general, HSP70 family genes are divided into four groups.

The first group is HSP70, which is usually not or mildly expressed in normal conditions. However, the expression can be rapidly induced when cells are exposed to heat, cold, or other stressors (Liu and Cao, 2018; Ren et al., 2018). The second group is HSC70, which is expressed constitutively in most human tissues. In humans, HSC70

shows 86% identity to HSP70 (Daugaard et al., 2007). According to previous reports, the expression of HSC70 is also induced by heat stress (Wu et al., 2008), The third group is the glucose-regulated protein 78 (GRP78) which is also known as immunoglobulin heavy-chain binding protein (BIP) or heat shock 70 kDa cognate 3. GRP78 is an essential endoplasmic reticulum (ER) chaperone that locates in the ER and plays essential roles in the unfolded protein response (UPR), oxidative stress, ER calcium binding, and the activation of the transmembrane ER stress sensors. GRP78 can be induced by stresses such as salinity, pH, and massive metal exposure. (Daugaard et al., 2007; Li et al., 2018a). The fourth group is GRP75 that is mainly expressed in the mitochondria and it is not induced in response to stress (Daugaard et al., 2007).

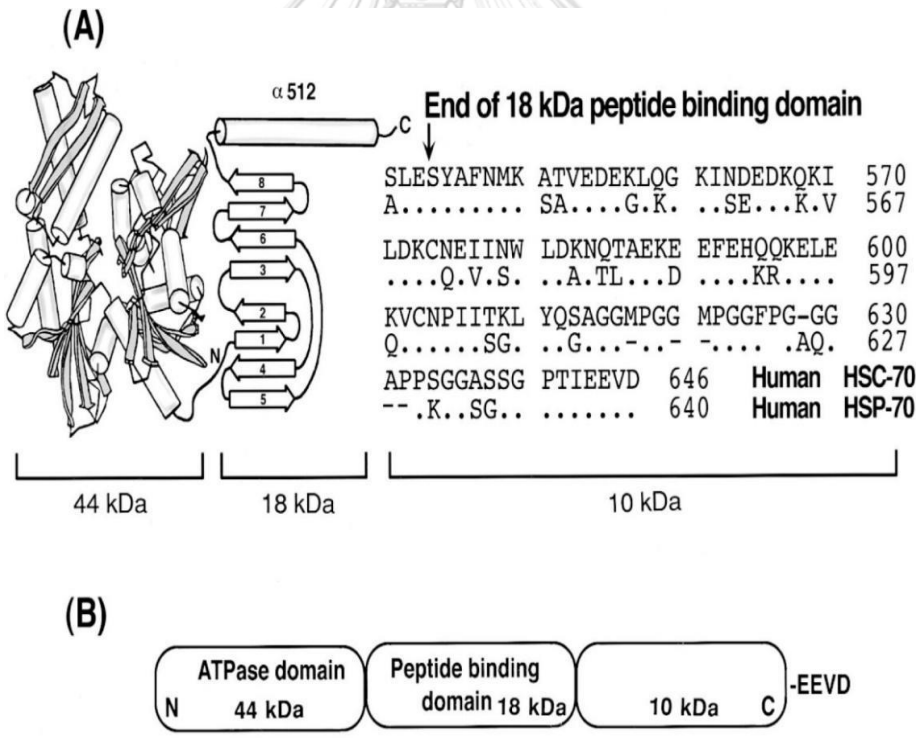

Figure 1.17 Molecular structure of human HSC70 and human HSP70 (Kiang and Tsokos, 1998) (A) 44 kDa fragment (amino acid residues 1–386) at N-terminus contains four domains forming two lobes with a deep cleft in between. 18 kDa fragment (amino acid residues 384–543) contains two 4-stranded antiparallel β-sheets and single a-helix.

10 kDa fragment (amino acid residues 542–646 for HSC-70 and 542–640 for HSP70) at C-terminus conserves EEVD terminal sequence. (B) N-terminal 44 kDa domain is ATPase domain; 18 kDa domain is peptide-binding domain; C-terminal 10 kDa fragment carries highly conserved EEVD terminal sequence, which is present in all eukaryotic HSP70.

The HSP70 family is required for various cellular processes and the response to environmental change and survival (Brodsky and Chiosis, 2006). HSP70s are highly conserved and demonstrated a 60–78% base identity among eukaryotic cells and a 40–60% identity between eukaryotic HSP70 and *E. coli* DnaK (similar to the HSP70) (Bardwell and Craig, 1984; Caplan et al., 1993; Craig and Jacobsen, 1985). Figure 1.17A shows the molecular structure of human heat shock cognate 70 kDa (HSC70), the constitutive form of HSP70 present at low levels in unstressed cells. The 44 kDa fragment (amino acid residues 1–386) from the N-terminus has been characterized by X-ray crystallography, which indicates that there are four domains forming two lobes with a deep cleft in between (Bork et al., 1992). The 18 kDa peptide-binding domain (amino acid residues 384–543) consists of two four-stranded antiparallel β-sheets and a single α-helix, as determined by multidimensional nuclear magnetic resonance (Morshauser et al., 1995). The 10-kDa C-terminus (residues 542–646) has been predicted using the Garnier algorithm to be primarily α-helix, followed by a glycine/proline-rich aperiodic segment next to the highly conserved EEVD terminal sequence (Demand et al., 1998b). These four terminal amino acids are present in all eukaryotic HSP70 which affect the amount of mRNA translated during heat stress (Denisenko and Yarchuk, 1990). Figure 1.17A also shows the molecular structure of human HSP70. The 44 and 18 kDa fragments are the same as those composing HSC70. The 10 kDa C-terminal of HSP70 differs by 26 amino acid residues relative to HSC70 and is 6 amino acids shorter (Leung and Hightower, 1997). Recent biochemical studies

of HSC70 fragments generated using recombinant DNA technology have led to mapping and characterization of the domains (Figure 1.17B). The 44 kDa fragment contains the ATPase domain. The 18 kDa fragment contains the peptide-binding domain that binds unfolded and folded peptides (Wang et al., 1993).

1.7 *Artemia*

1.7.1 Habitat and taxonomy

It is well known that *Artemia* also known as brine shrimp inhabit hypersaline environments (Triantaphyllidis et al., 1998). This animal is found in the natural hypersaline water bodies such as inland salt lakes and pans, coastal lagoons, and salt works at high salinity levels around the world. Moreover, the species can be found under climatological conditions that range from humid and sub-humid to arid and at altitudes from sea level to over 5000 m (Triantaphyllidis et al., 1998; Zhang et al., 2013).

Artemia franciscana is the principal North American species. It has been introduced through either deliberate or inadvertent releases into Europe, Africa, Australia, and Asia. It is the most studied *Artemia* species, that is considered a "superspecies" and exhibits high levels of phenotypic plasticity (Browne and Wanigasekera, 2000).

The taxonomy shows that *Artemia franciscana* belongs to the phylum Arthropoda, subphylum: Crustacea and class: Branchiopoda. According to several reports of Molecular phylogenetic analyses, Branchiopoda is classified into different groups depending on the input data that are used for analysis. However, most studies show that Branchiopoda are clustered close to Hexapods and are considered as a sister lineage with Hexapods (Eyun, 2017; Jondeung et al., 2012; Oakley et al., 2013; Regier et al., 2005; Reumont et al., 2012; Rota-Stabelli et al., 2013)((Figure 1.18).

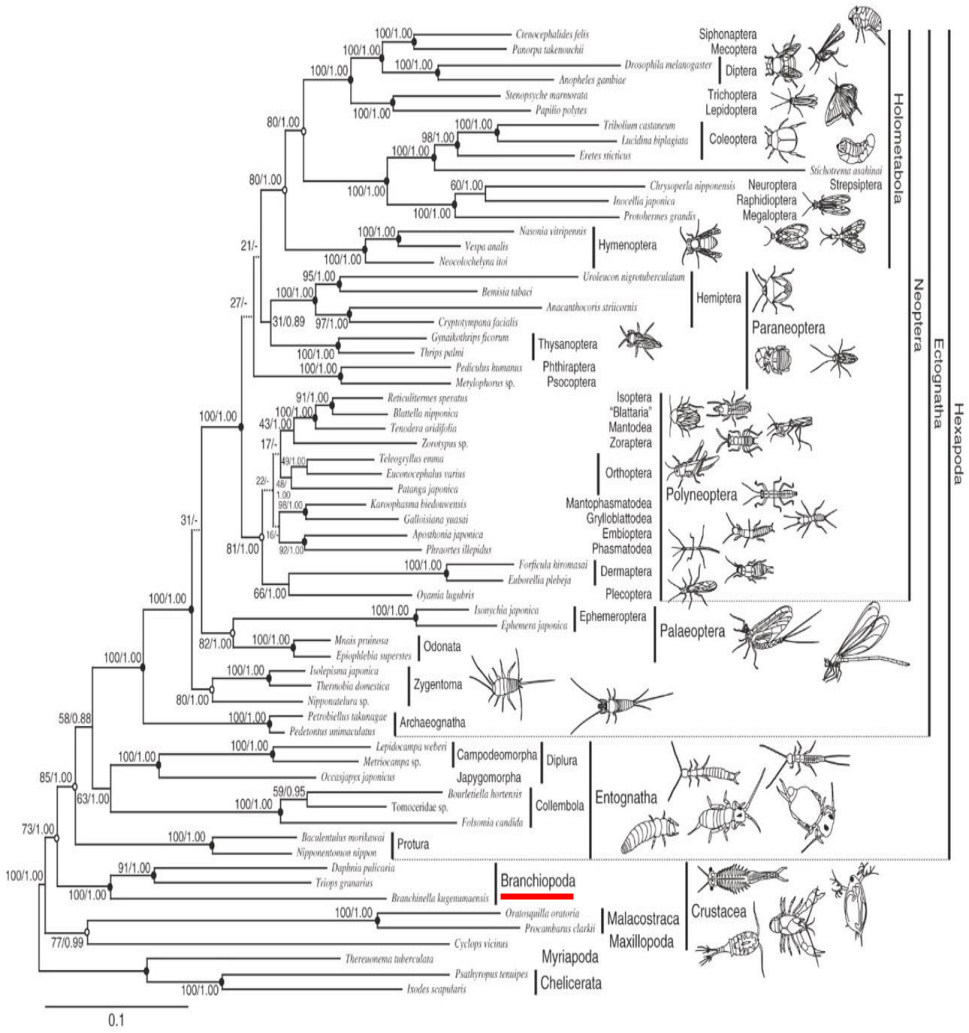

Figure 1.18 The maximum likelihood tree of pancrustaceans inferred from the amino acid sequences (Sasaki et al., 2013) The branch lengths were calculated from the concatenated alignment of the three protein sequences. Bootstrap values and posterior probabilities are shown at nodes. Dot-marked nodes: bootstrap value > 90%, posterior probability = 1.00. Circle-marked nodes: bootstrap value 70%-90%, posterior probability = 1.00 (except node 59). Internal branches drawn as dotted lines: not supported by Bayesian analysis.

The genus *Artemia* is a group of sibling species and superspecies which is identified by the criterion of reproductive isolation. The taxonomist assigned species names to populations with different morphologies, collected at different temperatures

and salinities. Later on, the profusion of names was abandoned, and all brine shrimp was referred to as *Artemia salina* (Linnaeus, 1758). Now different names are assigned to reproductively isolated populations or clusters of populations: *A. tunisiana*, *A. parthenogenetica*, *A. urmiana*, *A. sinica*, *A. persimilis*, *A. tibetiana*, *A. franciscana*, and *Artemia sp.* The taxonomic classification of *A. franciscana* is described as below.

Domain: Eukarya

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Branchiopoda

Order: Anostraca

Family Artemiidae

Genus: *Artemia*

1.7.2 Morphology

Artemia is a typical arthropod with a segmented body to which are attached broad leaf-like appendages that greatly increase the apparent size of the animal. The total length of the animal body is usually about 8-10 mm and 10-12 mm for the adult male and the female, respectively. However, the width of both sexes, including the legs, is about 4 mm. The body of this animal is divided into three parts: head, thorax, and abdomen (Figure 1.19). The head consists of one prostomial and five metameric segments. The thorax is constructed of eleven segments, each provided with a pair of swimming legs, while the abdomen is composed of eight segments. The eighth or last abdominal segment possesses the cercopods, called the furca or telson (Cassel, 1937; Criel, 1999).

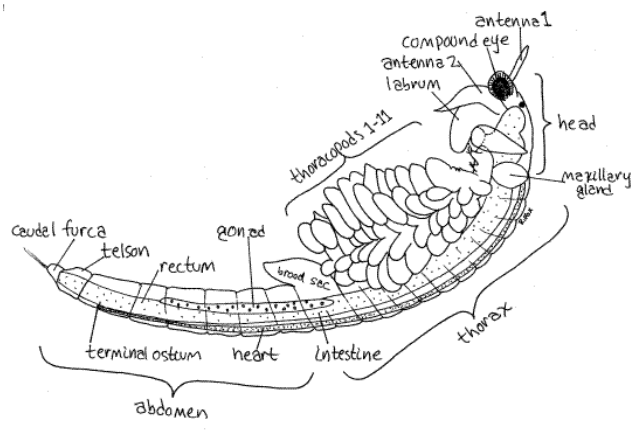

Figure 1.19 A female brine shrimp, *A. franciscana*, viewed from the left (Richard, 2001) The adult body of *Artemia* consists of a head, eleven "thorax" segments, two genital segments, six post-genital segments, and a telson.

1.7.3 Life cycle and sexually reproduction

As mentioned before, *Artemia* is extremely euryhaline. It can even survive for short periods in freshwater, however, it cannot produce a new generation. *Artemia* also can survive at high temperatures ranging from 15 to 55 °C. *Artemia* has two modes of reproduction. Sometimes nauplii (first *Artemia* swimming stage) develop in the ovisac of the mother and is born live. However, when the body of water where adult *Artemia* are living begins to dry up and salinities rise, embryos are encased in a hard capsule, or cyst, so that they are protected and can hatch later when conditions are better. The cyst is 200 to 300 micrometers in diameter, depending on the species of *Artemia*. Its external layer is a hard, dark brown shell (Figure 1.20). Dry conditions can cause the encysted embryo to enter a dormant state, which allows it to withstand complete drying, temperatures up to 90 °C or near absolute zero, high energy radiation, and a variety of organic solvents. The dehydrated cyst can be stored for months or years without loss of hatchability.

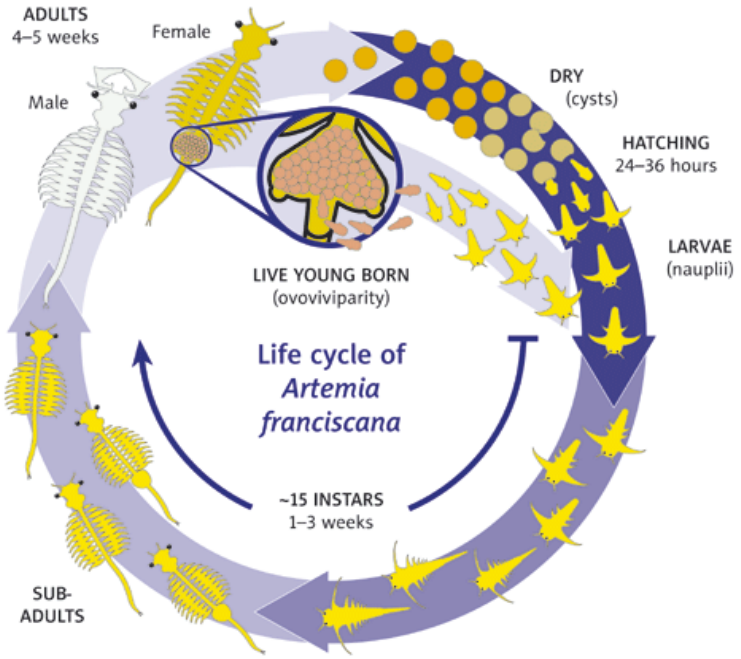

Figure 1.20 The life cycle of sexually reproducing *Artemia* (Ogello et al., 2014) Either cysts or free-swimming larvae (nauplii) are released by the *Artemia* female. They develop into juveniles and eventually adults. Adult males and females are distinguishable by the naked eye: males have two claspers on the head and females have an abdominal ovisac.

The *Artemia* embryo required only water and oxygen for initiating normal development. However, the constant temperature above 25 °C and light near the can increase the hatching rate (basically light is a necessity for hatching). *Artemia* cysts are best stored in a tightly sealed container in a cool, dry environment and, if possible, vacuum packed. Within 15 to 20 hours after being placed in seawater at 28 °C the shell breaks and the pre-nauplius in E-1 stage appears. For the first few hours, the embryo hangs beneath the cyst shell in what is called the umbrella stage. The newly hatched *Artemia* relies on its yolk sac for nutrients because its mouth and anus are not fully developed. The pre-nauplius E-2 stage is then released as a free-swimming nauplius

called an Instar 1 nauplius. In this stage, it is brownish orange because of its yolk reserves. It uses specially modified antennae for locomotion and later for food filtering. Approximately 6 hours after hatching it molts into the second larval stage (Instar II) and starts filter feeding on microalgae, bacteria, and detritus. The *Artemia* nauplius can live on yolk and stored reserves for up to 5 days or through the Instar V stage, but its caloric and protein content diminish during this time (Briski et al., 2008).

1.7.4 Application in aquaculture industry and research

Artemia is commonly used as live feed in most aquaculture activities, especially for larval growth and for more than 85% of reared marine species (FAO, 2010). Typically, the marketed *Artemia* cysts are stored under dehydrated (5-10 percent) and vacuum storage conditions to guarantee the maximal viability. *Artemia* cysts offered on the market can be kept for years and hatched just within 24 hours on demand to produce the living nauplii (Lavens and Sorgeloos, 1996).

In scientific researches, *Artemia* is known as a model organism called “aquatic *Drosophila”* since they comprise short life cycle and fast breeding efforts for aquaculture under laboratory conditions (Gajardo et al., 2001). Moreover, *Artemia* is widely used in comparative toxicity assays for a range of compounds which results are often correlated to humans (Freitas et al., 2011; Molina-Salinas and Said-Fernández, 2006; Rajasree et al., 2011). Brine shrimp *Artemia* represents another model species in several research fields, such as biodiversity, crustacean metabolism, shrimp resistance to *Vibrio* pathogen and interaction between sexual and parthenogenetic populations (Haldar et al., 2011; Munoz et al., 2010). Furthermore, *Artemia* is interesting for studying the evolutionary processes due to several factors thought to be responsible for genetic differentiation and speciation as observed in *Artemia* (Pilla and Beardmore, 1994). *Artemia* has been proven to be an invaluable genetic model for fine-scale studies of

microevolutionary divergence, as shown in *A. franciscana* after introduction in Vietnam for a year (Kappas et al., 2004). According to phylogenetic analysis, brine shrimp are closely related to insect-crustacean branching (Figure 1.18), making them a suitable tool for understanding the evolutionary relationship from crustacean ancestors to origins of insects. Genes underlying the extreme phenotypes of *Artemia* might be the utmost interesting point that makes *Artemia* a promising model for stress response studies.

1.8 Selective breeding in aquaculture

Selective breeding exploits substantial genetic variation that is present for most traits with desirable qualities. Generally, the main target is the growth rate of aquaculture species. Infectious disease is a major constraint for all species of aquaculture farming. However, nowadays disease resistant capacities have been considered as the initial emphasis to improve organism for infectious disease via selective breeding. The majority of farmed fish and shellfish production is based on non-selected stocks. Provided that genetic variance for disease resistance is present in the broodstock, selective breeding can contribute to improved disease resistance, as disease resistance trait have been found to be heritable.

1.8.1 Mass selection

The selective breeding techniques applied to improve resistance of aquaculture species to infectious diseases depend on the structure and technology that is used in the breeding program. According to the highly productive nature of most aquaculture species, and the typically low economic value of juveniles, the simple selective method; mass selection approaches can be applied. The high selection intensity could enable rapid genetic progress for resistance traits (Gjedrem and Baranski, 2009). Mass selection produced a large number of surviving oysters, with 60% higher resistance to

Oyster Herpes Virus compared with controls after four generations of mass selection (Dégremont et al., 2015). Moreover, successful mass selection has been applied to Penaeid shrimps (*P. vannamei*) which can resist Taura Syndrome Virus (Cock et al., 2009). However, this breeding scheme is not often performed, as there is a risk of inbreeding and subsequent inbreeding depression, especially if few families are involved (albeit this has not been widely observed in bivalve mass selection experiments) (Dégremont et al., 2015). Also, during mass selection for disease resistance, broodstock which has been exposed to the disease agent, can present a biosecurity risk to hatcheries particularly, if the pathogen can be vertically transmitted.

1.8.2 Family selection

Most of advanced selective breeding methods for aquaculture species is the use of family selection. Aquaculture species are particularly responsive to this technique due to the possibility of obtaining high numbers of full siblings and other close relatives of the selected candidates for testing (Gjedrem and Baranski, 2009). This technique involves the maintenance of a breeding nucleus with candidate parental broodstock from a high number of diverse genetics of families. Full siblings of these animals can be placed into field conditions or sent for experimental disease challenge testing to obtain family-level data on disease resistance. Accurate tracking of families and pedigree is achieved by tagging or using genetic markers. Advances in genotyping technology, such as the development of high-throughput genotyping for single-nucleotide polymorphism (SNP) multiplexes, have enabled rapid and accurate family assignment (Vandeputte and Haffray, 2014). Family selection of disease resistance has been highly successful for several species and diseases (Bishop and Woolliams, 2014; Yáñez et al., 2014). However, it does suffer some drawbacks, such as the cost of routine disease challenge data collection, and the lack of opportunity to capitalize on half of the genetic

variation (the within-family component). An additional challenge for breeding programs of many aquaculture species is genotype by environment interaction, in which the performance of the selected animals can vary markedly across diverse production environments e.g. in tilapia breeding (Sae-Lim et al., 2016). This results in re-ranking of genotypes across environments and effectively reduces the overall response to selection within a breeding programme (Sae-Lim et al., 2016).

1.8.3 Marker-assisted selection

Marker-assisted selection is one route to building on family selection and gain information of the comparative disease resistance from selected candidates within a full sibling family i.e. the within-family genetic variation (Sonesson, 2007). Marker-assisted selection is based on the principle of quantitative trait loci (QTL) detection to include the interested trait on selecting animals by verifying whether they carry the favorable alleles at the QTL. Mapping of QTL is a major goal for aquaculture genetics and breeding research to yield some successful practical results. A successful example of QTL analyses applied to selective breeding is the case of infectious pancreatic necrosis resistance in Atlantic salmon, which a major QTL explains the majority of the genetic variance for resistance (Houston et al., 2010; Houston et al., 2008; Moen et al., 2009) has been demonstrated as successful means for controlling the disease (Moen et al., 2015). QTL-affecting resistance was selected for other disease examples including the cases of salmonid alphavirus (Gonen et al., 2015), ISAV (Moen et al., 2007), and *Gyrodactylus salaris* (Gilbey et al., 2006) in salmon, lymphocystis disease in Japanese flounder (Fuji et al., 2006), Bonamiosis in the European Flat Oyster (Lallias et al., 2009), and *Flavobacterium psychrophilum* in rainbow trout (Vallejo et al., 2014). However, a single QTL of marker-assisted selection is lacking power in animal breeding, because most economically important traits have a polygenic genetic architecture (Meuwissen et

al., 2013). While recent domestication of aquaculture species may result in an oligogenic architecture for disease resistance traits, it is also important to consider that the effect of a given QTL may differ according to the environment and the genetic background of the population.

1.8.4 Genomic selection

Genomic selection (GS) is the state-of-the-art and modern selective breeding method in aquaculture. In GS, genome-wide markers are used to calculate genomic breeding values without prior knowledge of the underlying QTL affecting the trait of interest (Meuwissen et al., 2001). The premise of GS is that marker effects are estimated in a "training" population, which has been measured for both phenotypes (e.g. disease resistance) and genotypes, and then the developed model is used to generate genomic breeding values on selected candidates via genotypes only. While the initial concept of GS was the detection and utilization of population-wide linkage disequilibrium between genome-wide markers and QTL (Meuwissen et al., 2001), the benefits of genomic selection also include a more accurate estimation of the genetic relationship between any two individuals that could be given by pedigree records alone, particularly within families (Meuwissen et al., 2013). In all aquaculture species studies, the use of GS has resulted in higher prediction accuracy of breeding values than the use of pedigree information alone (Dou et al., 2016; Odegard et al., 2014; Tsai et al., 2015). A prerequisite for genomic selection is a platform to generate high-density SNP marker genotypes a cross populations of animals and SNP arrays, which have been developed for several aquaculture species, including Atlantic salmon (Houston et al., 2014; Yanez et al., 2016)(Houston et al., 2014; Yáñez et al., 2016), rainbow trout (Palti et al., 2015), common carp (Xu et al., 2014), and catfish (Liu et al., 2014b). A major downside of GS is the cost due to the expense of high-density genotyping in large numbers of individuals.

1.9. Literature review

1.9.1 Non-lethal heat shock

Several reports have shown that exposure of aquatic animals to non-lethal heat shock (NLHS) could induce protection against bacterial and viral infections (Norouzitallab et al., 2015, Vega et al., 2006) by induction of heat shock proteins (HSPs) and subsequently activate the immune system. The families of HSPs that function as molecular chaperones, are classified according to their molecular mass in kiloDaltons (kDa) such as HSP110, HSP90, HSP70, HSP60, HSP21, and HSP10. In the pathological situation, such as necrotic cell death, HSPs can be released into the extracellular environment complexes with interact or fragmented cellular protein. These complexes can contribute to the induction of autoimmunity by receptor-mediated activation of the innate immune response (Routsias et al., 2006). In shrimp, it was evidenced that HSPs were highly expressed in response to pathogen infection. *LvHSP60* from *P. vannamei* was significantly up-regulated in the gills, hepatopancreas and haemocytes after both of Gram-positive and Gram-negative bacterial challenges (Zhou et al., 2010). *PmHSP21* mRNA level was significantly decreased after WSSV infection (Huang et al., 2008). In addition, transcriptomics result in RNA-seq analysis of *Macrobrachium rosenbergii* (freshwater prawn) showed up-regulation of heat shock protein; *HSP21*, *HSP70,* and small heat shock proteins in response to *V. parahaemolyticus* (Rao et al., 2015). NLHS of *Artemia franciscana* induces HSP70 expression and protects Artemia larvae against *V. campbellii* and *V. proteolyticus* (Sung et al., 2007; Norouzitallab et al., 2015). Shrimp *Penaeus monodon* exposing to short hyperthermal heat stress have shown HSP70 accumulation and tolerance against gill-associated virus (GAV) (Vega et al., 2006). Priming of Asian green mussel *Perna viridis* with NLHS promoted thermotolerance and increased resistance to *V. alginolyticus* (Aleng et al., 2015). However, NLHS of *P.*

vannamei did not enhance tolerance against *V. harveyi* despite significantly up-regulation of HSP70 (Hong Loc et al., 2013). NLHS also fails to protect platyfish *Xiphophorus maculates* against *Yersinia ruckeri* infection, but protection is enhanced when NLHS is combined with injection of bacterial HSPs, GroEL, and DnaK (Ryckaert et al., 2010). Moreover, some evidence showed that feeding *P. vannamei* with food pellets containing *Escherichia coli* overexpressing the heat shock protein Dnak can enhance the Pacific white shrimp tolerance to *V. harveyi* infection (Sinnasarmy et al., 2015).

Besides the induction of HSP expression, NLHS also induces the expression of several immune-related genes. After induction of white shrimp with NLHS, it was found that *proPO* and hemocyanin mRNA levels were up regulated (Hong Loc et al., 2013). Injection of *P. vannamei* with recombinant DnaK and then challenged with *V. campbelli* have affected the transcription of three immune genes; transglutaminase-1 (TGase-1), prophenoloxidase-2 (proPO-2) and endogenous HSP70 (*LvHSP70*). Likewise, in black tiger shrimp injected with Dnak, a significant increase in proPO expression was observed (Phuoc et al., 2015). The concurrent introduction of heat stress, toleration of bacterial infection and HSPs production, suggesting that the role of HSPs is mediating the effects of heat stress via chaperoning and/or immune activation. Nonetheless, only a limited number of genes affected by heat stress are known, and only little is known about the majority of genes regulated in association with innate immunity.

1.9.2 Heat shock protein 70 (HSP70)

Heat shock proteins (HSPs), as extrinsic chaperones, are a group of highly conserved proteins of varying molecular weight (16–100 kDa) which are produced in all cellular organisms when exposed to cellular stress. Although original work in the fruit fly showed that this response was up-regulated by exposure of cells to heat, it is now recognized that this response is universal to all cells and that other stressors, such as

toxins, protein degradation, hypoxia, and microbial infection can also induce the up-regulation of HSPs (Georgopoulos and Welch, 1993). The best studied HSP in aquatic organisms is HSP70, particularly in terms of thermotolerance. The expression of HSP70 is important in enhancing protection when an organism responds to a specific stress signal following an initial transient (Yeong and MacRae, 2011). For many aquatic crustaceans, HSP70 expression will be induced at temperatures in the 35 to 38°C range. For example, in a previous study, high levels of HSP70 expression were detected in the freshwater giant prawn *Macrobrachium rosenbergii* when exposed to change in temperature from 25 to 35°C for a period of 2 h (Aslah et al., 2018).

Several studies have raised the possibility that HSP70 may be involved in various aspects of the immune system (Kaufmann and Schoel, 1994). Several investigations in shrimp revealed that HSP70s family including HSP70, HSC70, and HSC70-5 were correlated with a different change of gene and protein expression during viral infection (Valentim-Neto et al., 2014; Yan et al., 2010; Yuan et al., 2017). HSP peptides have been shown to promote the production of anti-inflammatory cytokines, indicating immunoregulatory potential of HSP. Therefore, the presence of immune responses to HSP in inflammatory diseases can be seen as an attempt of the immune system to correct the inflammatory condition. HSP70 can modulate inflammatory responses in models of arthritis, colitis and graft rejection (Borges et al., 2012). According to the study of HSP70 in tumor situation, the result indicated that HSP70-peptide might efficiently prime circulating T-cells. Therefore, upregulating HSP70 and causing local necrosis in tumor tissue by hyperthermia offers great potential as a new approach to directly activate the immune system (Milani et al., 2002). Moreover, the study of immunostimulant agent in 60 full-sib families of Abalone *Haliotis rufescens* has been found that high HSP70 levels were observed in abalones subjected to immunostimulation in both the intracellular and extracellular hemolymph fractions.

HSP70 levels in both fractions and at both ages showed low (heritability) h^2 and repeatability, with values that were not significantly different from zero. However, HSP70 induced levels had an additive genetic coefficient of variation CV_A of 13.3–16.2% in young adults and of 2.7–8.1% in pre-harvest adults. Thus, despite its low h^2, the result suggests that HSP70 synthesis in response to an immune challenge in red abalone has the potential to evolve through selection because of its large phenotypic variation and the presence of additive genetic variance, especially in young animals (Brokordt et al., 2015).

1.9.3 Heat shock protein 70 family

During heat stress treatment, the induction of HSP70 family genes (HSP70s) is the main cellular response to protecting them against various stressors. Heat stress can disturb cellular homeostasis in all organisms, potentially resulting in death (Aleng et al., 2015). However, to mitigate this stress, metabolic profiles are changed to faster re-establish metabolite homeostasis by, e.g., restoring cell damage (Malmendal et al., 2006), in which HSP70s play an essential role. Several previous studies have shown that heat stress increased HSP70 gene expression and enhanced brine shrimp (*A. franciscana*) tolerance to *Vibrio campbellii* infection (Norouzitallab et al., 2015). Moreover, this stress also enhanced brine shrimp tolerance to Cd and Zn exposure (Pestana et al., 2016). Besides, a member of heat shock protein 70 family gene; *PmGrp78* was significantly induced after exposure with high salinity, pH and, heavy mental (Li et al., 2018a). Recently, according to transcriptome analyses of *A. fransicana* which was exposed with salt stress, the result showed that stress response genes such as Heat shock 70kDa protein cognate were upregulated (De Vos et al., 2019).

It has been reported that the HSP70 family induces innate immune cells such as dendritic cells, macrophages, and monocytes, to produce several pro-inflammatory

cytokines via certain receptors and a signal transduction pathway in higher eukaryotes (Wallin et al., 2002). HSP70 can bind to pathogen-associated molecular pattern (PAMP) molecules and modulate PAMP-induced Toll-like receptor (TLR) signaling activities crucial for the stimulation of innate immunity and elimination of pathogens (Pockley et al., 2008; Yang et al., 2008). In crustaceans, feeding either heterologous or homologous HSP70 has been found to successfully improve phenoloxidase (PO) activity and protect Artemia from *Vibrio* infection (Baruah et al., 2011). In another study, the injection of recombinant *Escherichia coli* heat shock protein (rDnaK) stimulated the immune system of *L. vannamei* by increasing the expression of proPO-2 and TGase-1 (Hu et al., 2014).

1.9.4 HSP70 family polymorphism

Selective breeding of farmed animals is economically important for quantitative trains and has a high potential for increasing production. The quality of aquaculture production can be improved by understanding of genetic fundamentals of thermal stress especially, associated with HSP70s family. One of the most common form of genetic polymorphism is single nucleotide polymorphisms (SNPs). SNP is an individual nucleotide base difference between two DNA sequence which can be categorized according to nucleotide substitution (Edwards et al., 2007). So far, a certain number of SNPs polymorphism in HSP70 has been reported to be associated with stress tolerance that provides the evidence to understand the mechanism of stress response. For example, the SNP in the coding region of human HSPA1B (1267A>G), and HSPA1L (2437T>C) were associated with heat shock response in human (Singh et al., 2010). Similar in Italian Holstein cows, the presence of SNPs (C/- and G/T) in the 5'-UTR region of inducible Hsp70.1 improved heat stress response and tolerance to heat (Basirico et al., 2011). In aquatic animal, *P. vannamei*, five SNPs were identified in the fragment of HSP70 of TSV-resistant population, however, the only one significant difference of the

allelic frequency of SNP 892 C/T was found (Xiaohan et al., 2008). Moreover, four SNPs that were related with heat tolerance were identified in the promoter sequence of HSP70 in two species of scallops (*Argopecten irradians irradians* and *A. i. concentricus*) (Yang et al., 2014).

1.10 Research objectives

The objectives of this Ph.D. thesis are threefold. Firstly, it was investigated whether a non-lethal heat shock (NLHS) can induce protection against a strain of *V. parahaemolyticus,* which can cause acute hepatopancreatic necrosis disease (AHPND) in white-leg shrimp *P. vannamei*. For that, the expression profiles of HSPs and immune-related genes were examined upon NLHS and *V. parahaemolyticus* infection to check for the association between HSPs expression and stress exposure. Moreover, an RNA interference (RNAi) experiment was conducted to verify the hypothesis that *LvHSPs* are mediating the NLHS-based protection against VP_{AHPND}.

Secondly, with the knowledge of the HSPs function during stress response, and in view of the outcome of the first objective, it was verified to what extent recombinant *P. vannamei* HSP70 (r*Lv*HSP70) would protect against VP_{AHPND} infection. Shrimp survival was observed upon r*Lv*HSP70 injection. Moreover, the expression of some immune-related genes was analyzed to verify how r*Lv*HSP70 can enhance shrimp resistance to VP_{AHPND} infection. It was anticipated that this research would help to understand *Lv*HSP70 function in shrimp during stress, including its role in immune response.

The outcome of objective 1 and 2 indicated that HSP70 play an important role during stress response. Yet HSP70 is not unique, in the sense that the HSP70 family is composed of several members which are highly conserved and may play an important role during stress response. It has been shown that some HSP70 family members are

highly induced upon heat stress. However, the HSP70 family in Pacific white shrimp has not been investigated in great detail, especially their response upon e.g. NLHS and pathogen infection and their role in an immunological response. This lack of knowledge originates in the absence of an annotated genome at the start of this study.

At the Artemia Reference Center at Ghent University a draft genome and transcriptome of *A. franciscana* was available. *Artemia* is used as a model in other research fields, such as crustacean metabolism, biodiversity studies, and *Vibrio* pathogen resistance in shrimps. So, as a third objective, it was tried to identify members of HSP70 family by searching the transcriptomic database of *A. franciscana*. Furthermore, the expression profile of HSP70 family members was determined in two *Artemia* populations, a control population (CF12) that went through 12 generations of isothermal lab culturing conditions and a selected population (TF12) which is composed of survivors, over 12 generations, of an induced thermotolerance treatment. In addition, the expression of the HSP70 members was also examined after NLHS of the CF12 and TF12 populations. Candidate HSP70 family genes that were differentially expressed in the thermotolerant population were further analyzed for polymorphism between CF12 and TF12. The phenotypic effect of different alleles of the HSC70 gene was verified by expression of the different alleles in yeast. Such a polymorphism could be of high relevance for selective breeding in crustacean species.

The research is presented in four chapters:

Chapter II: HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock

Chapter III: *Litopenaeus vannamei* heat shock protein 70 (*LvHSP70*) enhances resistance to a strain of *Vibrio parahaemolyticus*, which can cause acute hepatopancreatic necrosis disease (AHPND), by activating shrimp immunity

Chapter IV: Sequence and expression analysis of HSP70 family genes in *Artemia franciscana*

Chapter V: Genotypic and phenotypic analysis of HSP70 family genes in thermotolerant *Artemia franciscana*

This research aims to provide basic knowledge on the link between heat resistance and disease resistance in shrimp and *Artemia*. The research could contribute to the development of novel strategies to prevent disease outbreak potentially leading to an enhanced sustainability of shrimp aquaculture.

CHAPTER II

HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus* carrying toxin-producing plasmid, has led to severe mortalities in farmed penaeid shrimp throughout Asia. Previous studies reported that non-lethal heat shock (NLHS) could enhance disease tolerance in aquatic animals. Here, we investigate whether the NLHS could enhance the survival of shrimp *Peneaus vannamei* upon challenge with an AHPND-causing strain of *V. Parahaemolyticus* (VP_{AHPND}). Two NLHS conditions, acute and chronic NLHSs, were used. The former abruptly exposed the juveniles shrimp from 28 °C to 38 °C for 30 min only once whereas the latter exposed the shrimp to 38 °C for 5 min every day for 7 days. The treated shrimp were, then, challenged with VP_{AHPND} at day 3, day 7 and day 30 during the recovery time after the treatment. The results showed that the shrimp exposed to either acute or chronic NLHS had higher survival rate (> 50%) than that of the non-heated shrimp control (20%) when they were challenged with VP_{AHPND} at day 3 recovery time. However, only those exposed to chronic NLHS showed the VP_{AHPND} protection at day 7 and day 30 recovery times. Furthermore, the qRT-PCR analysis revealed that the expression of heat shock proteins, *LvHSP70*, *LvHSP90* as well as other immune-related genes, *LvproPO* and *LvCrustin*, were induced upon exposure of shrimp to chronic NLHS. Interestingly, gene silencing of *LvHSP70* and *LvHSP90* eliminated the VP_{AHPND} tolerance in the chronic NLHS shrimp and had decreasing PO activity suggesting that these *LvHSPs* played crucial roles in bacterial defense in shrimp. All together, we show for the first time that the NLHS enhance the shrimp tolerance to VP_{AHPND} infection and this is likely mediated by the induction of *LvHSP70*, *LvHSP90* and subsequent activation of the proPO system.

In this chapter, I investigated whether the NLHS could induce protection against the strain of VP_{AHPND} in the Pacific white-leg shrimp *P. vannamei*. The juveniles shrimp were exposed to two different procedures of heat treatment, acute- and chronic-NLHS, before they were challenged thereafter with VP_{AHPND} at day 3, day 7, or day 30 recovery times. The shrimp survival was observed and the gene expression of *LvHSPs* as well as some other immune- related genes were analyzed. Furthermore, the RNA interference (RNAi) was conducted to confirm the importance of *LvHSPs* in mediating the NLHS protection against VP_{AHPND}.

2.1 Results

2.1.1 Effect of temperature of heat shock treatment on the percent survival of *P. vannamei*

Prior to heat shock treatment, the maximum abrupt temperature that could retain 100% shrimp survival after heat treatment was identified. This temperature is a non-lethal heat shock (NLHS) temperature that was used in further experiments. The animals were separated into four groups of 20 juveniles (2-3 g) shrimp, acclimated at ambient temperature (28 ± 1 °C) and after that, each group was abruptly heat-shocked for 30 min at various temperatures: 37, 38, 39, or 40 °C. Then, the animals were transferred immediately back to 28 °C. The percent survival of treated shrimp was determined at 6 h post heat treatment in all temperature groups. The swimming animal was counted as surviving shrimp. In contrast, the moribund shrimp was defined as a non-surviving shrimp. The result showed that the shrimp could survive 100% up to 38 ºC and mortality occurred at and above 39 ºC with approximately 50% survival (Fig. 2.1). The maximum abrupt temperature of 38 ºC was defined as a NLHS temperature that could retain 100% survival was used for heat shock treatment in subsequent experiments.

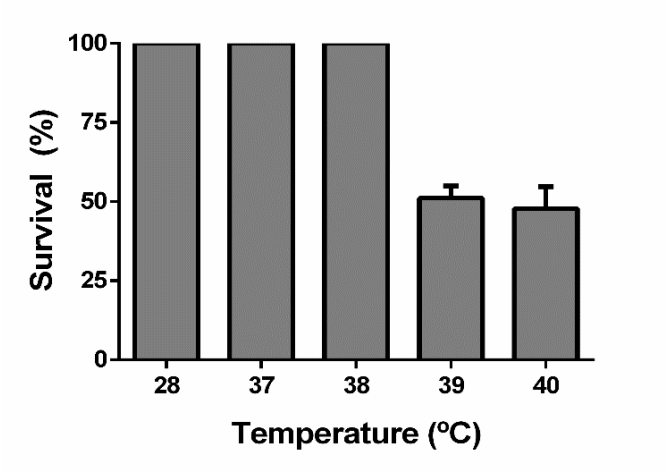

Figure 2.1 Determination of a NLHS treatment temperature in *P. vannamei* Juvenile *P. vannamei* (3-5 g) were acclimated at 28 ± 1 °C, then 20 each were abruptly exposed for 30 min at heat shock temperatures of 37 ± 0.2 ºC, 38 ± 0.2 ºC, 39 ± 0.2 ºC, and 40 ± 0.2 ºC and, then, transferred immediately back to ambient temperature. One control group was kept at 28 ± 1 °C. Animal survival was observed after 6 h post heat treatment. The error bars are the SD values from three replicates.

2.1.2 Effect of NLHS treatment on the protection of *P. vannamei* upon VP_{AHPND} infection

Juvenile shrimp (2-3 g) was separated to 35 L aquarium tank with each 20 individuals. They were immersed in the culture media of VP_{AHPND} at various concentration. It was found that the LD_{50} dose of VP_{AHPND} was 1×10^6 CFU/ mL which was used in the subsequent challenge experiments. Twenty juveniles (2-3 g) *P. vannamei* in each group were exposed to two different NLHS conditions: the acute NLHS condition, 30 min abrupt heating at 38 ºC once and the chronic NLHS condition, 5 min abrupt heating at 38 ºC daily for 7 days. At 3-, 7-, and 30-day recovery times, the animals were challenged by immersion with 1×10^6 CFU/mL of VP_{AHPND} in 35 L aquatic tanks and the survival was observed every 12 h for 120 h. The results showed that exposing the shrimp to chronic heat stress could enhance shrimp survival from approximately 20-30% survival in the non-heatshocked control groups up to 50-60% in

the NLHS groups at all recovery times (Fig. 2.2A, 2.2B, and 2.2C). However, it was found that the acute heat stress could increase shrimp tolerance to bacterial infection only temporary when challenged at day 3 recovery time (Fig. 2.2A). On the other hand, no protection was observed when challenged at longer recovery time (Fig. 2.2B and 2.2C).

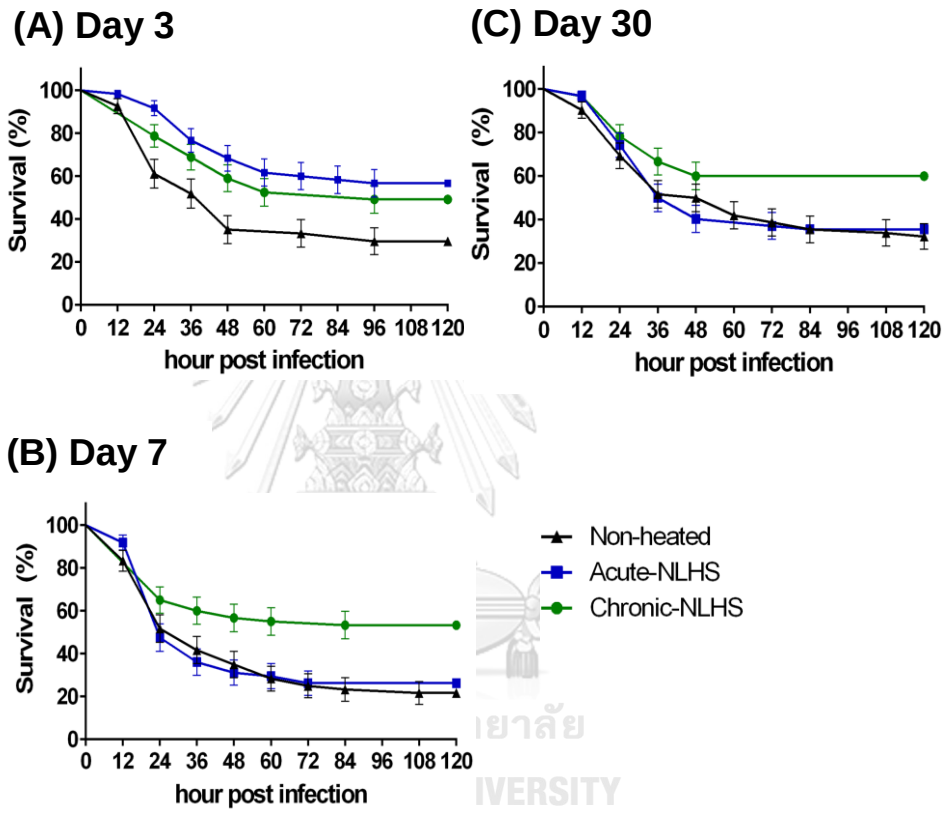

Figure 2.2 Effect of NLHS treatment on shrimp survival to VP_{AHPND} infection. of *P. vannamei*. Juvenile *P. vannamei* acclimated at 28 ± 1 °C were exposed to acute NLHS (abrupt heating at 38 ± 0.2 ºC for 30 min) or chronic NLHS (abrupt heating at 38 ± 0.2 ºC for 5 min daily for 7 days). After completed heating at (A) day 3 (B) day 7 or (C) day 30 recovery times, shrimp were challenged by immersion with 1×10^6 CFU/mL of VP_{AHPND}. Survivors were counted every 12 h for 120 h after challenge. A non-heatshocked group was a control. The error bars are the SD values from three replicates.

2.1.3 Effect of NLHS treatment on the expression level of *LvHSP*s and immune-related genes transcripts in *P. vannamei*

According to the challenge test in 2.1.2, chronic NLHS could enhance shrimp tolerance to VP_{AHPND} infection which was likely associated with the increased expression of heat shock proteins and the induction of shrimp innate immunity. The differential gene expression of *LvHSPs* and immune-related genes were verified by qRT-PCR to determine the transcript expression profiles of *LvHSP*s and some immune-related genes of shrimp exposed to chronic NLHS. Candidate heat shock protein genes: *LvHSP10, LvHSP21*, *LvHSP60, LvHSP70*, and *LvHSP90* were selected for the determination of gene expression after chronic heat treatment at various recovery times point after return to ambient temperature to 120 h.

Temporal gene expression profile of five *LvHSPs* genes in hemocytes of *P. vannamei* after chronic NLHS treatment are shown in Fig. 2.3A. First, *LvHSP10* was not significantly induced under NLHS treatment. However, it was slightly increased at 0 h and 3 h post-NLHS treatment. Moreover, the significant down-regulation was observed at 12 h, 24 h, 48 h, and 72 h post-NLHS treatment. Similarly, the considerable reduction of *LvHSP21* gene expression level was found at 3 h, 6 h, 48 h, 72 h, and 96 h. While the substantial upregulation in *LvHSP21* was not found at all recovery time points. For the expression of *LvHSP60*, a significant increase of gene expression level was not observed, however, slight increase of *LvHSP60* transcript was observed at 0 h and 3 h post NLHS treatment, but significant down-regulation of *LvHSP60* was shown at 24 h and 72 h post-NLHS treatment. In contrast, a dramatic upregulation of *LvHSP70* at immediately recovery time after NLHS at 3 h followed by down-regulation at 12-120 h post-NLHS treatment. Furthermore, the gene expression of *LvHSP90* was dramatically

increased at 0 h, 3 h, and 6 h immediately after post-NLHS treatment and then, followed by down-regulation at 12-120 h post-NLHS treatment.

Interestingly, the expression of two immune-related genes, *LvCrustin* and *LvproPO*, were significantly up-regulated from 0 h to 120 h recovery time. The expression reached the maximum of 12-fold at 48 h recovery time for *LvCrustin* and 14-fold at 72 h recovery time for *LvproPO* (Fig. 2.3B). On the contrary, the relative expression of *LvALF*, *LvA2M*, and *LvKazal* was not induced after the heat shock treatment. The expression of *LvA2M* and *LvKazal* was slightly down-regulated (Fig. 2.3B).

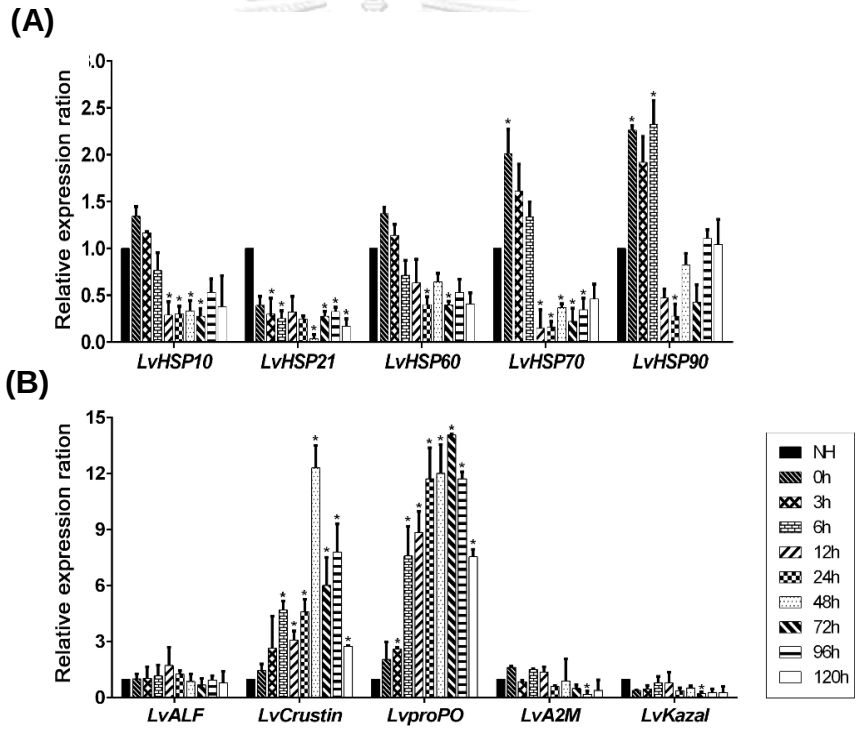

Figure 2.3. Temporal gene expression level of *LvHSPs* and immune-related genes after NLHS treatment. (A) The *LvHSP* mRNAs and (B) immune-related mRNAs were quantified by qRT-PCR at various recovery times after chronic NLHS (0, 3, 6, 12, 24, 48, 72, 96, and 120 h) compared to the control non-heat-shocked shrimp. The EF1α was used as an internal control. The error bars are the SD values from three replicates. Asterisks indicate statistically significant different ratios (>2-fold) compared with the control.

2.1.4 Effect of *LvHSP70* and *LvHSP90* expression level upon VP_{AHPND} infection

As shown that NLHS could induce the transcription level of *LvHSP70* and *LvHSP90* and led to an increase of the percent survival of heat-shocked shrimp after challenged with VP_{AHPND}. To further investigate whether the functions of these two genes are associated with the protection against VP_{AHPND} infection in shrimp or not, the difference in gene expression level of *LvHSP70* and *LvHSP90* were verified in shrimp hemocyte after infected with VP_{AHPND} at various time points. The result revealed that *LvHSP70* mRNA level was dramatically induced upon exposure with both of *V. parahaemolyticus* strains (non-VP_{AHPND} and VP_{AHPND}) at 6 h post-exposure by approximately 5-fold and 12.5-fold, respectively, compared with 0 h post-exposure of non-VP_{AHPND} as control (Fig 2.4A). After that, the expression was decreased to the basal level at 12 h post-exposure and 24 h post-exposure for both strains. However, at 48 h post-exposure, shrimp that were infected with VP_{AHPND} showed increase expression of *LvHSP70* by approximately 5-fold but, the induction was not observed in the non-VP_{AHPND} group (Fig 2.4A). On the other hand, a significant ($P < 0.05$) upregulation of *LvHSP90* expression level was observed at the late phase of exposure (24 h post-exposure) only in the group of shrimp that infected with VP_{AHPND} by approximately 7.5-fold (Fig 2.4B).

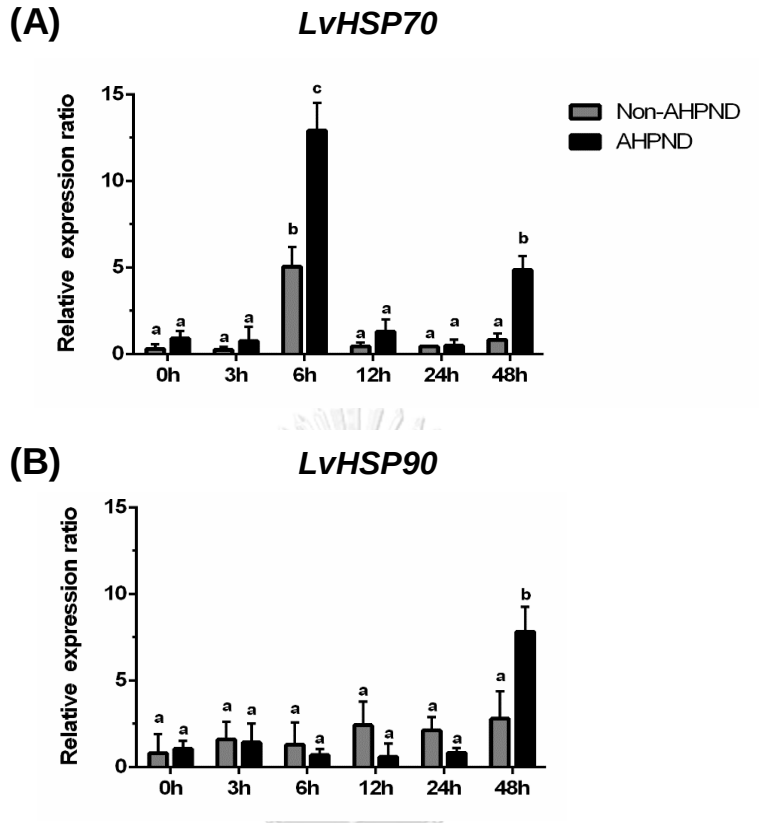

Figure 2.4 Temporal gene expression level of heat shock proteins after *V. parahaemolyticus* infection The expression level of (A) *LvHSP70* and (B) *LvHSP90* was quantified by qRT-PCR at various time points after VP_{AHPND} infection (0, 3, 6, 12, 24, and 48 h) compared to the control ($VP_{non\text{-}AHPND}$). The *EF1α* was used as an internal control. The error bars are the SD values from three replicates. The alphabets denote statistically significant difference between values obtained for VP_{AHPND} and $VP_{non\text{-}AHPND}$ ($P < 0.05$).

2.1.5 Effect of knockdown of *LvHSP70* and *LvHSP90* on shrimp resistance to VP_{AHPND}

2.1.5.1 *LvHSP70* and *LvHSP90* genes expression level after dsRNA injection

To further investigate the function of *LvHSP70* and *LvHSP90* in VP_{AHPND} resistance, RNAi-mediated gene knockdown was performed to knockdown the *LvHSP70* and *LvHSP90* transcripts in the shrimp. The *GFP* dsRNA was used as a control. After 24 h of ds*LvHSP70* and ds*LvHSP90* injections, total RNA was extracted from hemocytes of

knockdown and control *P. vannamei* and then subjected to semi-quantitative RT-PCR. Both *LvHSP70* and *LvHSP90* transcripts were successfully suppressed (Fig. 2.5A) up to nearly 90% (Fig. 2.5B and 2.5C). Complete knockdown (100%) of the *LvHSP70* and *LvHSP90* transcripts was avoided because it resulted in high mortality of shrimp (data not shown).

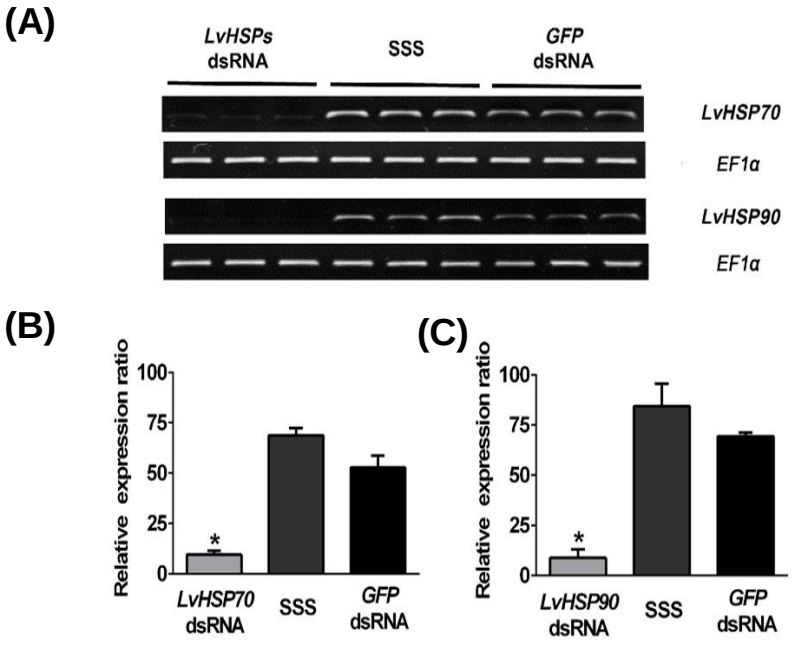

Figure 2.5 Expression of ***LvHSP70*** and ***LvHSP90*** transcripts in dsRNA injected ***P. vannamei***. Juvenile shrimp were injected with either 5 μg/g shrimp of GFP dsRNA or 5 μg/g shrimp of *LvHSP70* dsRNA or 1 μg/g shrimp of *LvHSP90* dsRNA. The gene knockdown efficiency of *LvHSP70* and *LvHSP90* was analyzed by (A) Semi-quantitative RT-PCR and (B, C) the relative expression analysis. Juvenile shrimp injected with saline solution (SSS) and GFP dsRNA act as controls. This experiment was done in triplicate. The error bars are the SD values from the three replicates. Asterisk indicates statistically significant different ratio compared to SSS and GFP dsRNA injection ($P < 0.05$).

2.1.5.2 The percent survival of *P. vannamei* after VP_{AHPND} infection

Gene knockdown of *LvHSP70* and *LvHSP90* was done to investigate the function involved in shrimp resistance to VP_{AHPND}. After the chronic NLHS treatment experiment, *P. vannamei* were intra-muscularly injected with ds*LvHSP70* ords*LvHSP90* or ds*GFP* at day two recovery time. Twenty-four hours after dsRNA injection, the animals were challenged with $VP_{non\text{-}AHPND}$ or VP_{AHPND} by immersion method and the mortality was observed every 12 h post-exposure till 120 h post-exposure. The swimming animal was counted as a surviving shrimp. While the moribund shrimp was defined as a dead shrimp. The cumulative mortality was higher in the group of *LvHSP70*- and *LvHSP90*-knockdown shrimp as compared with the control group ~~that~~ injected with *GFP* dsRNA. Insignificant mortality of knockdown shrimp was shown in $VP_{non\text{-}AHPND}$ infection suggesting the crucial role of both *LvHSPs* in NLHS-induced VP_{AHPND} tolerance (Fig. 2.6).

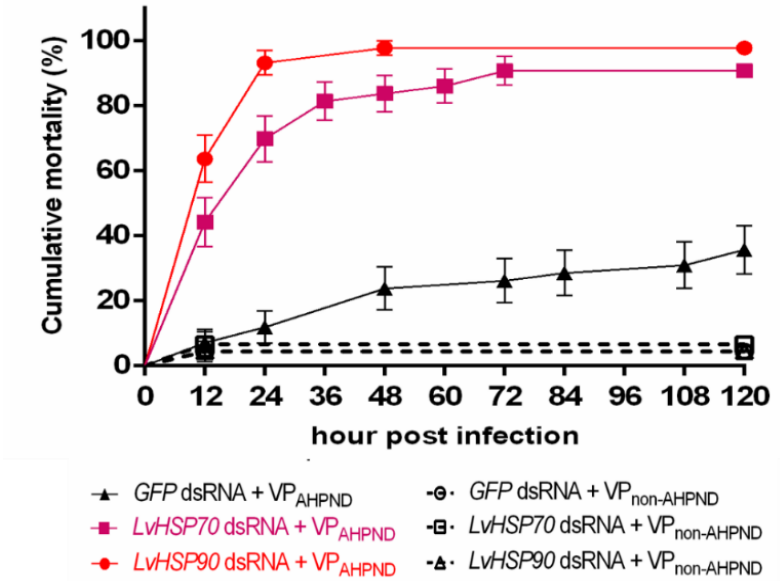

Figure 2.6 Effect of gene knockdown of *LvHSP70* and *LvHSP90* on cumulative mortality of *P. vannamei* followed VP_{AHPND} infection Fifteen shrimps exposed to chronic-NLHS followed by dsRNA injection were exposed to $VP_{non\text{-}AHPND}$ or VP_{AHPND}. The cumulative mortality was observed every 12 till 120 h post-exposure. This experiment was done in triplicate. The error bars are the SD values from the three replicates.

2.1.5.3 Bacterial counts in stomach of *P. vannamei* infected with VP_{AHPND}

Bacterial counts in the stomach of *LvHSP70*- and *LvHSP90*- knockdown shrimp after VP_{AHPND} infection was determined to confirm the infection. The amount of VP_{AHPND} in shrimp stomach was detected by total plate dot counting compared with that in the control shrimp injected with ds*GFP*. The results revealed that the VP_{AHPND} concentration (CFU/mL) was much higher in the stomach of *LvHSP70 and LvHSP90* knockdown shrimp, when compared with the control shrimp, injected with SSS or dsGFP (Fig. 2.7). Moreover, the result showed that without NLHS treatment, *P. vannamei* got the highest VP_{AHPND} infection as expected when compared to those exposed to chronic NLHS (SSS and *GFP* dsRNA injected groups).

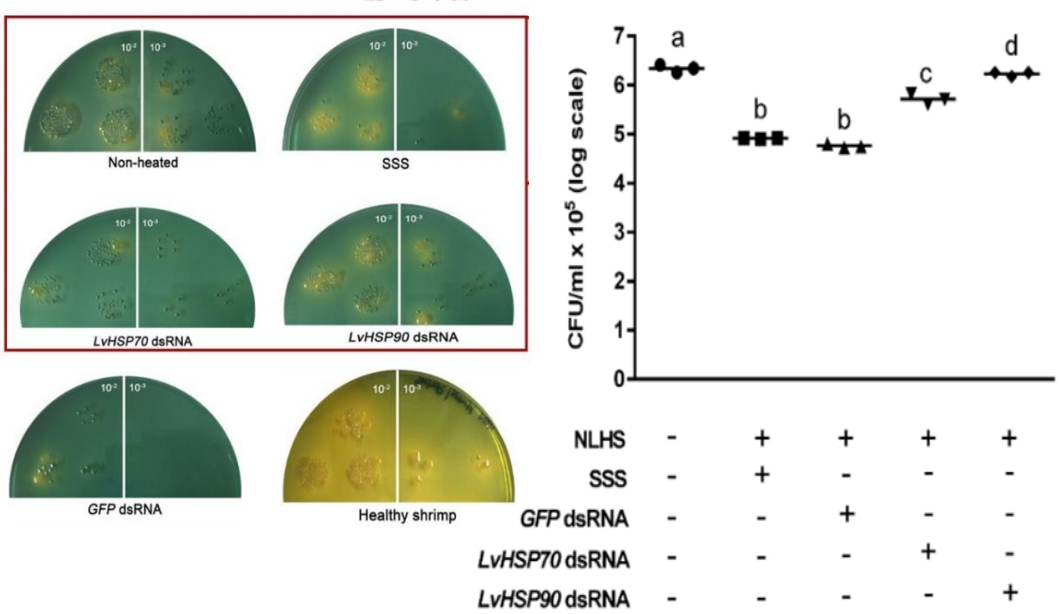

Figure 2.7 Bacterial counting in the stomach of *LvHSP70*- and *LvHSP90*- knockdown *P. vannamei* infected with VP_{AHPND}. Bacteria counting was performed by using TCBS spot assays. Three groups of three individual shrimp, each was exposed to chronic NLHS and injected with 5 µg/g shrimp of GFP dsRNA or5 µg/g shrimp of *LvHSP70* dsRNA or 1 µg/g shrimp of *LvHSP90* dsRNA. After 24 h post dsRNA injection, shrimp were immersed in the culture media of 1×10^6 CFU/mL VP_{AHPND}. After 24 h post-exposure,

stomach was individually collected to determine the amount of VP_{AHPND} by dotting on TCBS agar. After incubating overnight, the total numbers of viable green colonies (CFUs) were evaluated. NLHS shrimp injected with SSS or GFP dsRNA were used as controls. The alphabets denote the statistically significant difference between values obtained for NH and NLHS injected with dsRNA ($P < 0.05$).

Moreover, the AP4 nested PCR method was also performed to confirm the high amount of VP_{AHPND} infection in the *LvHSP70* and *LvHSP90* knockdown shrimp. Genomic DNA (50 ng/µl) extracted from the stomach tissue was used as a template for PCR. The results showed a lower level of single amplicon band of 230 bp from the 2nd (nested) PCR step in shrimp that were injected with SSS and *GFP* dsRNA (Fig. 2.8) indicating a low level of VP_{AHPND} target in the template DNA. However, the *LvHSP70*- and *LvHSP90*-knockdown shrimp and non-heatshocked shrimp showed higher band intensity of 230 bp product with additional amplicon bands (Fig.2.8) similar to the positive control of VP_{AHPND} cultured overnight indicating a high level of VP_{AHPND} target in the template DNA. Therefore, *Lv*HSP70 and *Lv*HSP90 are essential for resistance to VP_{AHPND} infection.

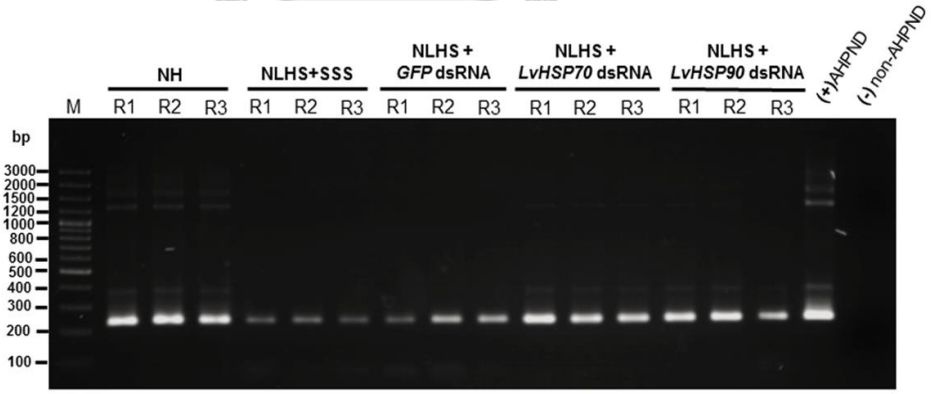

Figure 2.8 The PCR detection of VP_{AHPND} in stomach of *LvHSP70*-, *LvHSP90*-knockdown shrimp Genomic DNA was extracted and subjected to AP4 nested PCR detection was performed and analyzed by 1.8% agarose gel electrophoresis. $VP_{non\text{-}AHPND}$ and VP_{AHPND} cultures were used as a negative and positive control, respectively. NLHS shrimp injected with SSS or GFP dsRNA were used as controls.

2.1.5.4 The PO activity of *LvHSP70*- and *LvHSP90*- knockdown shrimp after VP_{AHPND} infection

Additionally, shrimp exposed to NLHS and subsequently injected with ds*LvHSP70* and ds*LvHSP90* followed by VP_{AHPND} infection were subjected to determine the hemolymph PO activity. A significant decrease in PO activity was observed in the *LvHSP70*- and *LvHSP90*- knockdown shrimp compared with the control shrimp injected with SSS and *GFP* dsRNA (Fig. 3.9). Thus, the gene knockdown of *LvHSP70* and *LvHSP90* could affect melanization in shrimp upon bacterial infection. Moreover, NLHS shrimp that were injected with SSS and *GFP* dsRNA showed a higher PO activity than the non-heat-shocked shrimp (Fig. 2.9) suggested that the NLHS could induce the proPO activating-system in shrimp.

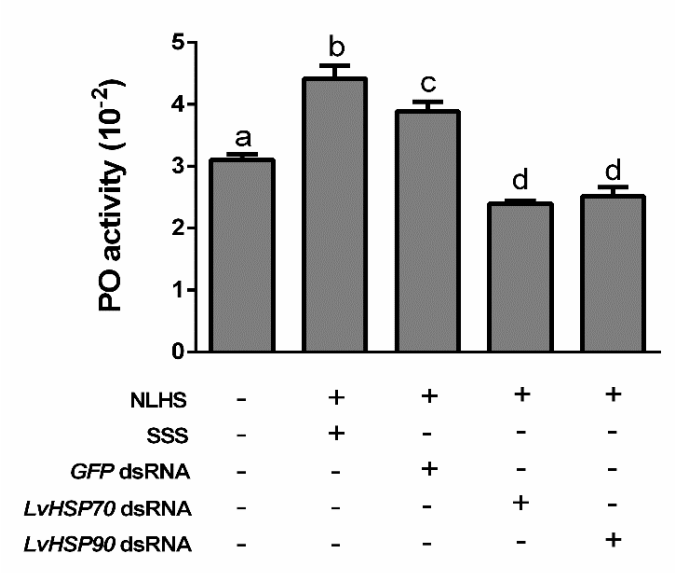

Figure 2.9 Reduction of hemolymph PO activity in *LvHSP*- knockdown shrimp challenged with VP_{AHPND}. Juvenile *P. vannamei* (2-3 g) exposed to NLHS were injected with 5 µg/g shrimp of GFP dsRNA or 5 µg/g shrimp of *LvHSP70* dsRNA or 1 µg/g shrimp of *LvHSP90* dsRNA, followed by immersion with 1×10^6 CFU/mL VP_{AHPND}. After 24 h post-exposure, the hemolymph was withdrawn, and 200 µg of hemolymph proteins were separately mixed with the PO substrate (3 mg/µl L-DOPA). The PO activity was defined as ΔA_{490}/mg protein/min. The experiment was performed in triplicate of three pooled

hemolymph samples, each sample from three shrimp. The data are shown as the means ± SD and the alphabets indicate the statistically significant difference between values obtained from NH and NLHS injected with dsRNA ($P < 0.05$).

2.2 Discussion

In this study both acute (a single NLHS) and chronic (daily NLHS for 7 days) heat treatments were applied to investigate the effect of NLHS on disease resistance. The result showed that chronic-heat stress could induce shrimp resistance to VP_{AHPND} from 3-days up to 30-days in the recovery time with high percent survival compared to the isothermic control group. On the other hand, acute heat stress also can induce shrimp resistance to bacterial infection but only for a short period, namely up to 3-day recovery after the NLHS. In previous studies including the study described by Loc et al. (2013), NLHS treatment of *P. vannamei* by acute NLHS of 38 ºC for 30 min could not enhance shrimp tolerance to *V. harveyi* infection(Loc et al., 2013). In the Rex rabbit (*Oryctolagus cuniculus*), exposed to chronic heat stress at 36 ºC from an ambient temperature of 26 ºC, heat stress genes and proteins (HSP60, HSC70, HSP70, and HSP90) were highly upregulated after a chronic NLHS. Interestingly, the upregulation was sustained up to nine weeks into the recovery of the NLHS treatment (Pei et al., 2012). All these findings indicate that heat shock proteins production coincides with enhanced resistance to disease in animals.

Under chronic-heat treatment, significant induction of *LvHSP70* and *LvHSP90* was found. Similarly, Loc et al. (2013) reported that when *P. vanamei* was exposed to NLHS, the expression of *LvHSP70* was significantly ($P<0.05$) increased compared to non-heat-treated shrimp (Loc et al., 2013). *P. monodon* showed highly up-regulation of *PmHSP21*, *PmHSP70*, and *PmHSP90* in the hepatopancreas within the first hours after the heat exposure (Rungrassamee et al., 2010). Moreover, Wang and team found that

HSPP90 mRNA expression was significantly (*P<0.01*) upregulated after high-temperature treatment (35 ºC for 1 h) relative to the control (15 ºC) in the Triangle Sail Mussel (*Hyriopsis cumingii*) (Wang et al., 2017).

In addition, the *Lv*HSP70 protein which was detected in both cytoplasm and nucleus of hemocyte cells was immediately induced at 0 h recovery time after chronic-NLHS. The up-regulation of *Lv*HSP70 protein in hemocytes after exposure to heat stress might relate with the up-regulation of transcriptome levels leading to the translation process of this protein. Several reports in *A. franciscana* found that after NLHS (38 °C for 30 min) the HSP70 protein production was upregulated (Norouzitallab et al., 2015; Pestana et al., 2016; Yik Sung et al., 2007). Moreover, in *Perna viridis* (Asian Green Mussel), *Pv*Hsp70-2 was induced in all tissues examined when mussels were heated at 38 °C for 30 min followed by 6 h recovery (Aleng et al., 2015). Recently, it was shown that abrupt heat shock at 38 °C for 30 min induced the protein expression level of *Lv*HSP70 up to a later time point (8 h recovery) (Loc et al., 2013). These differences in temporal induction pattern are most probably due to the varying conditions used for heat shock treatment across these different studies. In addition, by using immunofluorescence, we clearly show the high induction of *Lv*HSP70 at the protein level in shrimp haemocyte that was exposed to NLHS conditions, as compared to the non-heatshocked shrimp. Therefore, NLHS was able to induce the expression of *LvHSP70* at both the transcriptional and translational levels. In contrast, the *LvHSP21* was down-regulated after chronic heat stress while the previous experiments in *P. monodon* showed a constitutive expression of *PmHSP21* but inducible immediately after acute heat stress (Huang et al., 2008; Rungrassamee et al., 2010). We speculate that this may be due to the difference in heat stress conditions.

The early upregulation of *LvHSPs* within a few hours after heat stress suggests that they may function as a regulator of other genes. The previous report found that heat

stress could induce synthesis of sHSPs and HSP90 leading to enhancement of the *Caenorhabditis elegans* immunity to pathogenic *Pseudomonas aeruginosa* (Singh and Aballay, 2006). The mechanism might involve heat shock transcription factor-1 and the associated DAF2/DAF-16 pathway which regulates aging and immunity in nematodes (Singh and Aballay, 2006). Here, under chronic-heat stress we observed a dramatic increase of mRNA transcription levels of *LvproPO* and *LvCrustin*. Similar to the previous report in *P. monodon* that the transcript levels of *crustinPm5*, *crustinPm1*, and *crustin-likePm* in epipodite were upregulated upon heat treatment (Vatanavicharn et al., 2009) and in *P. vannamei* that the mRNAs encoding proPO increased significantly after NLHS (Loc et al., 2013). Moreover, injection of recombinant Dnak followed by *V. campbellii* challenge of *P. vannamei* also affected transcription of proPO-2 (Phuoc et al., 2016).

Furthermore, several reports revealed that *HSP70* and *HSP90* were induced after pathogen infection especially bacterial infection. For example, in the swimming crab (*Portunus trituberculatus*) a significant ($P<0.01$) induction of *PtHSP70 was* demonstrated after infection with *Vibrio alginolyticus*, which is the main causative agent of disease-causing massive mortality in *P. trituberculatus* (Cui et al., 2010). Moreover, the transcription of *LvHSP70* was also clearly ($P<0.05$) upregulated in hemocytes after infection with the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *V. alginolyticus* (Zhou et al., 2010). Similar to this study, the significant ($P<0.05$) upregulation of *LvHSP70* was found after VP_{AHPND} infection. On the other hand, the mRNA level of *LvHSP90* was significantly ($P<0.05$) upregulated post-VP_{AHPND} infection. Likewise, in *Megalobrama amblycephala,* expression of HSP90 was upregulated after *Aeromonas hydrophila* infection. Moreover, *HcHSP90* mRNA expression in the hemolymph of Triangle Sail Mussel (*Hyriopsis. cumingii*) was upregulated and reached a peak level post *A. hydrophila* infection (Wang et al., 2017).

The gene expression analysis results revealed that HSP70 and also HSP90 might play an important role in stress response. So, RNAi interference of *LvHSP70* and *LvHSP90*. following the challenge of the knockdown shrimp with VP_{AHPND}, was preform and resulted in an increase in shrimp mortality which was corresponding with the increase of bacterial counts and high levels of VP_{AHPND} infection detected by plate dot counting and AP4 PCR diagnosis method. Previously, an increased expression of HSP70 in *A. franciscana* also coincided with a reduced bacterial load in *V. campbellii* challenge (Sung et al., 2008). Besides, HSP90 also enhanced the *C. elegans* immunity to pathogenic *Pseudomonas aeruginosa* (Singh and Aballay, 2006). Moreover, Iryani et al., 2017 found that knockdown of HSP70 by RNAi reduces the tolerance of *A. franciscana* nauplii to heat stress and *V. campbellii* infection (Iryani et al., 2017). These two results indicated that HSP70 and HSP90 play an important role in shrimp defense against bacterial exposure. Moreover, closely related to previous work that pretreated *Artemia* with phenolic compound phloroglucinol that could induce HSP70 production and enhance the protection of *A. franciscana* against *V. parahaemolyticus* infection. The result indicated that increased survival in *Artemia* pretreated with phloroglucinol was most likely due to a significant increase in HSP70 production (Kumar et al., 2018b). However, the study of *HSP90* and the protection against VP_{AHPND} is firstly verified in this study. The results indicated that chronic-NLHS could enhance protection of shrimp against VP_{AHPND} and there might be because of the induction of *LvHSPs* especially *Lv*HSP70 and *Lv*HSP90 that directly or indirectly enhance shrimp immunity via several immune pathways.

2.3 Materials and methods

2.3.1 Shrimp samples

Juvenile *P. vannamei* (2-3 g body weight) and sub-adult *P. vannamei* (10-15 g body weight) were kindly provided by the Marine Shrimp Broodstock Research Center II (MSBRC-2), Charoen Pokphand Foods PCL in Phetchaburi province, Thailand. The animals were acclimated under constant aeration (> 5 ppm) at the ambient temperature of 28 ± 1 °C and 20 parts per thousand (ppt) of salinity for one week before use. During acclimation, shrimp were fed thrice daily with shrimp feed.

2.3.2 Determination of the NLHS temperature for *P. vannamei*

To determine the appropriate NLHS temperature, four groups of twenty *P. vannamei* (2-3 g), acclimated at 28 ± 1 °C, were abruptly exposed to heat shock temperatures at 37 ºC, 38 ºC, 39 ºC, or 40 ºC in heat shock treatment tank (100 L) for 30 min. Shrimp were, then, transferred immediately after heat shock back to ambient temperature for recovery and shrimp survival was observed at 6 h recovery time by counting the live animals. Shrimp raised at 28 ± 1 °C were used as the control. The percent survival was calculated as $N_t/N_0 \times 100$ where N_0 and N_t are initial and final numbers of live shrimp, respectively. Experiments were done in triplicate.

2.3.3 Chronic and acute NLHS of *P. Vannamei*

Juvenile *P. vannamei* (2-3 g body weight) acclimated at 28 ± 1 °C for one week were separated into two groups for two heat shock treatment conditions. In acute NLHS group, shrimp was transferred from 28 ± 1 ºC to 38 ± 0.2 ºC and incubated for 30 min once whereas those in chronic NLHS group were subjected to 5 min heating at 38 ± 0.2 ºC daily for 7 days. The aeration in both conditions was controlled by maintaining the dissolved oxygen (DO) at high ppm (10 ppm). After heat exposure, they were

transferred immediately back to ambient temperature for recovery before sampling for differential gene expression, challenge test and gene-specific knockdown. Shrimp in the control group were non-heatshocked juvenile shrimp. The details experimental design is shown schematically in Fig. 2.10

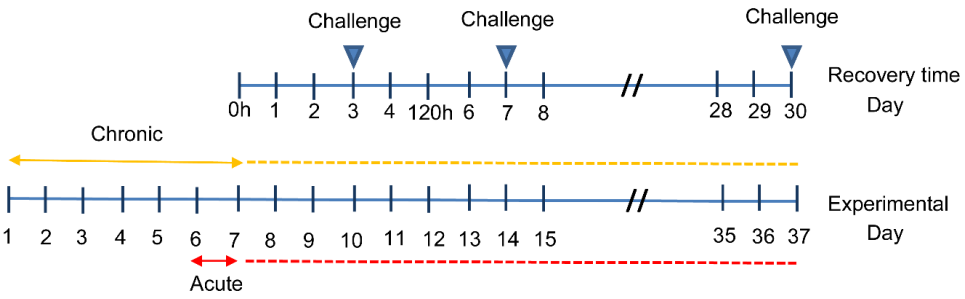

Figure 2.10 Schematic of heat treatment and challenge experiments

The chronic NLHS, shrimp were abruptly heated for 5 min every day for 7 days while in acute NLHS, shrimp was heated once for 30 min. After that, shrimp were recovered at ambient temperature. After heat treatment, the recovery time started immediately when shrimp were transferred to ambient temperature (0 h recovery time). The challenge experiments of both groups were conducted in the same period at day 3, day 7 and day 30 in the recovery period.

2.3.4 Bacterial culture and immersion dose of *V. parahaemolyticus*

2.3.4.1 *V. parahaemolyticus* (AHPND and non-AHPND culture)

Bacterial isolates of AHPND strain (VP_{AHPND}); Chanthaburi2, and non-AHPND strain ($VP_{non\text{-}AHPND}$); Rayong1, used in the study were checked for their virulence by immersion and examination of shrimp hepatopancreas using histological method (Supungul et al., 2017). The bacteria were grown overnight at 30 ºC in tryptic soy agar plates (TSA) (Becton, Dickson, and Company) supplemented with 1.5% NaCl. After that, one colony was picked, inoculated into tryptic soy broth (TSB) containing 1.5% NaCl

and cultured to stationary phase by incubation 24 h with shaking at 150 rpm at 30 ºC. The cell density was determined with a spectrophotometer at 600 nm. The OD_{600} of 1.0 was equivalent to approximately 1×10^9 CFU/mL according to the total plate count method.

2.3.4.2 LD_{50} identification of VP_{AHPND}

Twenty juveniles reared at 28 ± 1 ºC were incubated in separate aquaria containing 35 L of filtered seawater with the VP_{AHPND} at the final concentration of 1×10^5, 5×10^5, 1×10^6, and 5×10^6 CFU/mL. Survival was observed every 12 h after challenge for 120 h by counting the swimming animals. Each treatment was performed in triplicate and the percentage of survival was calculated as above — the lowest concentration of VP_{AHPND} causing 50% mortality after 48 h post-exposure was used for further experiments.

2.3.5 *V. parahaemolyticus* protection after NLHS

Twenty juveniles from each group (6 groups) were treated under chronic NLHS. At 3-day-, 7-day-, and 30-day recovery times, they were, then, challenged with 1×10^6 CFU/mL of VP_{AHPND} and $VP_{non\text{-}AHPND}$. The survival was observed every 12 h for 120 h as described in 2.5.2. Moribund shrimp were counted as dead shrimp. Each treatment was performed in triplicate. Non-heat juveniles were used as a control. The detail of the challenged test is shown schematically in Fig. 2.10

2.3.6 Temporal gene expression analysis by qRT-PCR after chronic NLHS

2.3.6.1 Hemocyte collection after NLHS treatment

The hemolymph was collected from nine shrimp (2-3 g body weight) each at various time points: 0, 3, 6, 12, 24, 48, 72, 96, and 120 h, after completed chronic NLHS

and at 0 h for non-heat group as control using 10% (w/v) sodium citrate as an anticoagulant. Hemolymphs from three individual shrimp were pooled. Hemocytes were collected by centrifugation at 800 × g for 10 min at 4°C. The supernatant was removed and hemocytes pellet was used for RNA extraction step.

2.3.6.2 Total RNA extraction and First-strand cDNA synthesis

Total RNA was extracted from the pooled hemocytes from three individuals in each time point using the TRI Reagent (Molecular Research Center) according to the manufacturer's protocol. Briefly, hemocyte was homogenized in TRI Reagent, then incubated 30 min on ice after that 200 µl of chloroform was added. After centrifugation at 12,000 × g for 15 min at 4 °C, the RNA-containing aqueous (upper) phase was transferred to a new tube and precipitated with 1 volume of isopropanol. Next, the sample was centrifuged at 12,000 × g for 15 min at 4 °C, the pellet was washed with 1 mL of 75% (v/v) ethanol in diethyl pyrocarbonate (DEPC)-treated water and centrifuged again. The RNA pellet was air-dried and dissolved in an appropriate volume of DECP-treated water. The contaminating genomic DNA was eliminated by treatment with DNase I (RNase-free) (New England BioLabs). The purity and quantity of the RNA were determined by 2 % agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific), respectively. The first strand cDNA was synthesized from 500 ng of total RNA using a cDNA synthesis kit (Thermo Scientific). The mixture of RNA, Oligo (dT), and reverse transcriptase was incubated at a temperature in a series of 25 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min, respectively, then the samples were stored at -20 °C for further investigation.

2.3.6.3 Quantitative RT-PCR analysis of gene expression

The qRT-PCR was performed in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using QPCR Green Master Mix Fluorescein (Biotechrabbit) with

forward and reverse primers, specific for *LvHSP* genes (*LvHSP10*, *LvHSP21*, *LvHSP60*, *LvHSP70*, and *LvHSP90*) and other immune-related genes (*LvproPO*, *LvA2M*, *LvCrustin*, *LvALF*, and *LvKazal*) (Table 2.1). The housekeeping gene, *EF1α*, was used as an internal control. The cycling parameters started with an initial activation at 95 ºC for 60 s followed by 40 cycles of denaturation 95 ºC for 15 s, and extension 72 ºC for 30 s. The fluorescent signal intensities were recorded at the end of each cycle. Melting curve analysis was performed from 55 to 95 ºC with continuous fluorescent reading every 0.5 ºC increments to confirm that only one product was amplified. The cycle threshold (Ct) values and fold difference in quantity for each *LvHSP* and other immune-related genes were recorded by CFX Manager Software (Bio-Rad). Relatively to the expression of *EF1α* transcripts, the relative expression was calculated by the $2^{-\Delta\Delta_{Ct}}$ method (Livak and Schmittgen, 2001). The amplification was done in triplicate for each sample. The results are presented as the relative expression ratio, which were normalized to *EF1α* gene transcript levels in the same sample. The statistical significance of any differences was tested by using a one-way ANOVA followed by the Duncan's new multiple range test using the SPSS software.

2.3.7 Temporal gene expression analysis by qRT-PCR upon VP_{AHPND} infection

2.3.7.1 *V. parahaemolyticus* AHPND and non-AHPND stains challenged

Two groups of 90 juveniles (2-3 g body weight) each which were separated into 30 juveniles in each aquaria tank containing 35 L of filtered seawater, were immersed with final concentration 1×10^6 CFU/mL of VP_{AHPND} and $VP_{non\text{-}AHPND}$ respectively.

2.3.7.2 Hemocyte collection after VP_{AHPND} infection

Shrimp hemolymph was collected from nine shrimp each at various time points: 0, 3, 6, 12, and 24 h, after VP_{AHPND} infection. On the other hand, the hemolymph was collected from the control group infected with $VP_{non\text{-}AHPND}$ at the same time point of VP_{AHPND} infection. Then hemolymph was collected at similar time points as mentioned above. Hemolymph was collected using 10% (w/v) sodium citrate as an anticoagulant. Then, the three hemolymph were pooled. The hemocytes were collected by centrifugation at 800 × g for 10 min at 4°C. The supernatant was removed and hemocytes pellet was used for RNA extraction.

2.3.7.3 Analysis of *LvHSP70* and *LvHSP90* transcription levels

Total RNA was extracted from the hemocytes using the TRI Reagent (Molecular Research Center) and the contaminating genomic DNA was eliminated by treatment with DNase I (RNase-free) (New England BioLabs). The first strand cDNA was synthesized from 500 ng of total RNA using a cDNA synthesis kit (Thermo Scientific). Quantitative Real-time RT-PCR was performed in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using QPCR Green Master Mix Fluorescein (New England BioLabs) with forward and reverse primers, specific for *LvHSP70* and *LvHSP90*. The housekeeping gene, *EF1α*, was used as an internal control (Table 2.1). The cycling parameters started with an initial activation at 95 ºC for 60 s followed by 40 cycles of denaturation 95 ºC for 15 s, and extension 72 ºC for 30 s. The amplification was done in triplicate for each sample. The results are presented as the relative expression ratio, which were normalized to *EF1α* gene transcript levels in the same sample by using the $2^{-\Delta\Delta_{Ct}}$ method. The statistical significance of any differences was tested by using a one-way ANOVA followed by Duncan's new multiple range test using the SPSS software.

Table 2.1 Nucleotide sequence of the primers used that design for gene expression analysis and RNAi technique.

Primer name	Sequence (5'-3')	Purpose
LvHSP10-F	TCGACCGTGTGCTGGTCCAA	qRT-PCR
LvHSP10-R	GTGGTGCCAGCATCAGTTCT	
LvHSP21-F	CACGAGGAGAAGTCTGACAAC	qRT-PCR
LvHSP21-R	GAGAGCGAGGACTTGATGAG	
LvHSP60-F	TGCCAACAACACCAACGAAG	qRT-PCR
LvHSP60-R	GCCAACATAACTCCACGCCT	
LvHSP70-F	CTCCTGCGTGGGTGTGTT	RT-PCR, qRT-PCR
LvHSP70-R	GCGGCGTCACCAATCAGA	
LvHSP90-F	TGGGCTTCTACTCCGCCTACC	RT-PCR, qRT-PCR
LvHSP90-R	ACGGTGAAAGAGCCTCCAGCA	
LvALF-F	GTTATCACGCCCTTTTCTAC	qRT-PCR
LvALF-R	GAAGAATGACCTGTCCAAC	
LvA2M-F	GCACGTAATCAAGATCCG	qRT-PCR
LvA2M-R	CCCATCTCATTAGCACAAAC	
LvCrustin-F	GCTGGCCTCGATAAGTGTTG	qRT-PCR
LvCrustin-R	CATCGGTCGTTCTTCAGATG	

LvKazal-F	CGACAAGACCTGCAATGG	qRT-PCR
LvKazal-R	AAACAGCTGCCTGGAAAC	
LvproPO-F	CGGTGACAAAGTTCCTCTTC	qRT-PCR
LvproPO-R	GCAGGTCGCCGTAGTAAG	
ds*LvHSP70*-F	CGAAGGACGCTGGACACATCT	RNAi
ds*LvHSP70*-R	GGACGATGTCGTGGATCTG	
T7ds*LvHSP70*-F	TAATACGACTCACTATAGGCGAAGGACGCTGG ACACATCT	RNAi
T7ds*LvHSP70*-R	TAATACGACTCACTATAGGGGACGATGTCGTG GATCTG	
ds*LvHSP90*-F	AGACCGAGTACCTGGAGGAA	RNAi
ds*LvHSP90*-R	GACGCACGTACAGCTTGATCT	
T7ds*LvHSP90*-F	TAATACGACTCACTATAGGAGACCGAGTACCTG GAGGAA	RNAi
T7ds*LvHSP90*-R	TAATACGACTCACTATAGGGACGCACGTACAG CTTGATCT	
ds*GFP*-F	ATGGTGAGCAAGGGCGAGGA	RNAi
ds*GFP*-R	TTACTTGTACAGCTCGTCCA	
T7ds*GFP*-F	TAATACGACTCACTATAGGATGGTGAGCAAGG GCGAGGA	RNAi

T7ds*GFP*-R	TAATACGACTCACTATAGGTTACTTGTACAGCTC GTCCA	
EF1α-F	CTTGATTGCCACACTGCTCAC	RT-PCR, qRT-PCR
EF1α -R	TCTCCACGCACATAGGCTTG	

2.3.8 *In vivo* gene knockdown of *LvHSP70* and *LvHSP90*

2.3.8.1 Preparation of double-stranded RNA (dsRNAs)

Double-stranded RNAs (dsRNAs) of *LvHSP70*, *LvHSP90*, and *GFP* genes were generated using *in vitro* T7 RiboMAX™ Express Large-Scale RNA Production System (Promega). For each gene, two PCR reactions were set up using the gene-specific primers flanked by a T7 promoter sequence at the 5' ends (Table 2.1) to produce the sense and anti-sense strands DNA templates separately. To produce dsRNA, 1.5 mg of each gel purified PCR product was used as a template for *in vitro* transcription by overnight incubation. After that, the equal amounts of sense and anti-sense single-stranded RNA were mixed and incubated at 70 °C for 10 min, and slowly cooled down at room temperature to form dsRNA. The remaining DNA templates were eliminated with RNase-free DNase I (Promega) at 37 °C for 30 min then purified by standard phenol-chloroform extraction. The quality of dsRNAs was verified by agarose gel electrophoresis with UV visualization and quantified by using NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.3.8.2 *In vivo* gene knockdown of *LvHSP70* and *LvHSP90* mediated by RNA interference

Three healthy individual juveniles (2-3 g body weight) in each group were injected with gene-specific dsRNAs in 25 µl of 150 mM saline solution (SSS) (5 µg/g shrimp ds*LvHSP70* or 1 µg/g shrimp ds*LvHSP90* or 5 µg/g shrimp ds*GFP*) or SSS. The dsRNA was intramuscularly injected into the third abdominal segment. After 24 post dsRNA injection (three shrimp per group), the hemolymph was drawn using 10% (w/v) sodium citrate as an anticoagulant and the hemocytes were separated by centrifugation at 800 × g for 10 min at 4 ºC. Total RNAs and cDNAs were prepared as described in 2.3.6.2 to test the gene knockdown efficiency.

2.3.8.3 Semi-quantitative RT-PCR analysis of gene expression in knockdown shrimp

The efficiency of *LvHSP70* and *LvHSP90* transcript knockdown was determined by semi-quantitative RT-PCR, which was performed by using *LvHSP70* and *LvHSP90* gene-specific primers (Table 2.1). A fragment of *EF1α* was also amplified and used as an internal control. The cDNA from each RNA sample was used as the template for the PCR amplification. The total amount of PCR reaction was 25 µl reaction consisting of 1 µl of 10-fold diluted cDNA as a template, 2.5 mM of dNTP, 5 µM of each gene-specific primer set and 1 unit of RBC Taq DNA polymerase (RBC Bioscience). The PCR conditions were 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and subsequently, a final 72 °C for 5 min, except only 26 cycles were performed for the *EF1α*. The PCR products were analyzed by 2.0% (w/v) agarose gel electrophoresis and visualized by UV-transillumination. The results are presented as the relative expression ratio, which was normalized to *EF1α* gene transcript levels in the same sample. The statistical significance of any differences was tested by using a one-way ANOVA followed by Duncan's new multiple range test using the SPSS software.

2.3.9 Effect of *LvHSP70* and *LvHSP90* knockdown shrimp

2.3.9.1 Cumulative mortality after VP_{AHPND} challenged

Shrimp (2-3 g body weight) were first subjected to chronic NLHS as described in 2.3.3 After completing the chronic NLHS and a further acclimatization at 28 °C for two days, they were divided into three groups of 15 shrimp each. Two groups were injected with gene-specific dsRNA in SSS (5 µg/g shrimp of ds*LvHSP70* and 1 µg/g shrimp of ds*LvHSP90* respectively) and another group was injected with 5 µg/g shrimp of *GFP* dsRNA in SSS serving as the control. 24 hours post-injection (hpi) (72 h recovery time), these shrimps were challenged with 1 $\times 10^6$ CFU/mL of either strain of *V. parahaemolyticus* (VP_{AHPND} or $VP_{non\text{-}AHPND}$) by immersion as previously described. The cumulative mortality of each shrimp group was recorded every 12 h until 120 h post exposure to the bacterial. This experiment was performed in triplicate.

2.3.9.2 Bacteria counting and semi RT-PCR detection of VP_{AHPND}

Shrimp (2-3 g body weight) were treated by chronic- NLHS as described in 2.2.3 and allowed to recover for 48 h. Two groups of three shrimp were injected with *LvHSP70* and *LvHSP90* dsRNAs, and GFP (control) respectively. Twenty-four hours after dsRNA injection, they were immersed in water containing VP_{AHPND} as described in 2.2.5. Twenty-four-hour post-exposure, shrimp stomachs were individually collected and homogenized in 0.85% SSS. The homogenized tissue was serially diluted and dotted onto a thiosulfate citrate bile salts sucrose agar plates (TCBS) (Becton, Dickson and Company) with three replicates. The number of bacterial green colonies as CFU/mL was then counted after incubation at 30 ºC for 18 h. Healthy shrimp stomachs were collected and the number of bacteria determined as a negative control.

Besides bacterial counting, the homogenized stomachs, also used in bacterial counting experiment, were subjected for genomic DNA extraction by using Genomic

DNA Mini Kit (Tissue) (Geneaid Biotech). The DNA concentrations were determined using NanoDrop 2000 Spectrophotometer (Thermo Scientific) and adjusted to a concentration of 50 ng/µl with elution buffer (Geneaid Biotech) for PCR detection of VP_{AHPND}. The AP4 nested PCR method was used for approximate quantification of VP_{AHPND} as previously reported (Dangtip et al., 2015). Both of VP_{AHPND} and $VP_{non\text{-}AHPND}$ cells were used for positive and negative control, respectively. The PCR products were analyzed by 1.0% (w/v) agarose gel electrophoresis and visualized by UV-transillumination.

2.3.9.3 Hemolymph PO activity assay

The PO activity in the gene silenced shrimp was determined in the hemolymph of chronic-NLHS treatment shrimp injected with dsRNA and non-heated shrimp. The hemolymph PO activity, expressed as the amount of dopachrome formation from the L-3,4-dihydroxyphenylalanine substrate, was measured as previously reported (Amparyup et al., 2009). Briefly, total hemolymph proteins of 200 mg in 85 µl of 10 mM Tris-HCl pH 8.0 were mixed with 15 µl freshly prepared 3mg/mL L-DOPA in water (Fluka). The absorbance at 490 nm was monitored for 30 min by using Molecular devices Spectramax 250 Microplate Reader. The PO activity was reported as ΔA_{490}/mg protein/min. The experiment was performed in triplicate of three pooled hemolymph samples, and each sample was from three shrimp.

CHAPTER III

Litopenaeus vannamei heat shock protein 70 (LvHSP70) enhances resistance to a strain of *Vibrio parahaemolyticus*, which can cause acute hepatopancreatic necrosis disease (AHPND), by activating shrimp immunity

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Abstract

Heat shock protein 70 (HSP70) acts as a molecular chaperone and a stress protein, but also plays important roles in innate and adaptive immune responses. Previous studies have reported that non-lethal heat shock (NLHS) could enhance the resistance of Pacific white shrimp *Litopenaeus vannamei* to a specific stain of *Vibrio parahaemolyticus*, which carried a toxin-producing plasmid (VP_{AHPND}), via the induction of *LvHSP70* transcription. Here, we further investigated the specific function of *Lv*HSP70 in shrimp immunity. The upregulation of *Lv*HSP70 at the protein level was detected during recovery time after NLHS treatment, using both western blot analysis and immunofluorescence microscopy. We found that NLHS immediately activated the production of *Lv*HSP70 in shrimp hemocytes and that such induction was observed in all three types of hemocytes: hyaline; granular and semi-granular cells. Furthermore, the role of *Lv*HSP70 in bacterial defense was investigated using the heterologous expression of recombinant *Lv*HSP70 (r*Lv*HSP70) in *Escherichia coli*. Shrimp receiving r*Lv*HSP70 by injection showed an increased survival rate (75%) to VP_{AHPND} infection compared to just 20% survival in the control group injected with bovine serum albumin (BSA). We also demonstrated that the injected r*Lv*HSP70 accumulated in shrimp hemocytes and was detected in the intracellular space of hemocyte cells leading to the induced expression (*P<0.05*) of several immune-related genes (*LvMyD88*, *LvIKK*β, *LvIKKε*, *LvCrustin I*, *LvPEN2*, *LvPEN3*, *LvproPO1*, *LvproPO2,* and *LvTG1*). Collectively, these results suggest that *Lv*HSP70 plays a crucial role in bacterial defense by activating the shrimp immune system.

In this chapter, I further investigated the induction of *Lv*HSP70 in shrimp hemocytes upon NLHS treatment at the protein level and studied the protective effect of injecting recombinant *P. vannamei* HSP70 (r*Lv*HSP70) against VP_{AHPND} infection. Shrimp survival was observed, and the expression of some immune-related genes was analyzed. We found that r*Lv*HSP70 could enhance shrimp resistance to VP_{AHPND} infection by inducing shrimp immunity.

3.1 Results

3.1.1 The detection of endogenous *Lv*HSP70 in shrimp hemocyte cells

The functional study of *Lv*HSP70 at the protein levels was performed to gain more understanding on the involvement of *Lv*HSP70 in shrimp immunity. First, the presence of endogenous *Lv*HSP70 in *P. vannamei* hemocytes were demonstrated using western blot analysis with a specific antibody of HSP70. SDS-PAGE separated total protein from *P. vannamei* hemocyte lysate supernatant and cell-free plasma. Then, *Lv*HSP70 was detected using anti-human Hsp70 antibody. The detected band of endogenous *Lv*HSP70 was found only in hemocyte lysate supernatant fraction but not in plasma (Fig. 3.1A and 3.1B). To further investigate which types of shrimp hemocyte cells produce *Lv*HSP70, immunofluorescence detection by specific HSP70 antibody and confocal microscopy were conducted. Nuclei were stained blue with TO-PRO-3 iodide dye while *Lv*HSP70 was visualized green by Alexa Fluor 488 dye. *Lv*HSP70 was expressed in all three types of shrimp hemocytes (hyaline, granular and semi-granular) and the protein was detected in both the cytoplasm and nucleus (Fig. 3.1C)

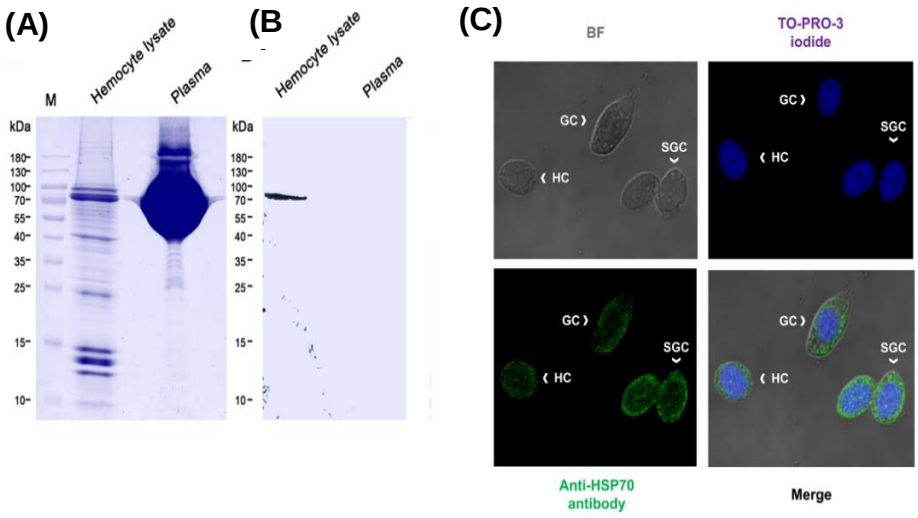

Figure 3.1 Detection of endogenous *Lv*HSP70 in shrimp hemocytes (A) Total protein from hemocyte lysate (30 µg) and plasma (100 µg) was extracted from healthy shrimp *P. vannamei* and resolved by SDS-PAGE. (B) Immunoblotting using anti-human HSP70 antibody. 'M' represents molecular mass standards in kDa. (C) The presence of *Lv*HSP70 in different types of shrimp hemocytes (HC: hyaline; GC: granular; SGC: semi-granular) were analyzed by confocal microscopy. *Lv*HSP70 was detected by an anti-human HSP70 antibody (shown in green) while nuclei are shown in blue. 'BF' indicates the bright field

3.1.2 Effect of NLHS treatment on the expression of *Lv*HSP70 protein

The differential protein expression of *Lv*HSP70 after NLHS treatment was performed. Shrimp hemocytes were collected for hemocyte lysate supernatant preparation at various recovery time points (0, 6, 12 and 24 h). Protein expression was then detected by western blot analysis using anti-human HSP70 antibody. As shown in Fig. 3.2A and 3.2B, *Lv*HSP70 (approximately 70 kDa) was immediately expressed at high levels after NSLHS treatment (0 h recovery time) compared with non-heatshocked controls. Furthermore, according to Fig 3.2C, the immunofluorescence technique and confocal microscopy also detected the higher signal intensity of Alexa Fluor 568 (green

color) in hemocytes after NLHS treatment compared to the non-heatshocked control group.

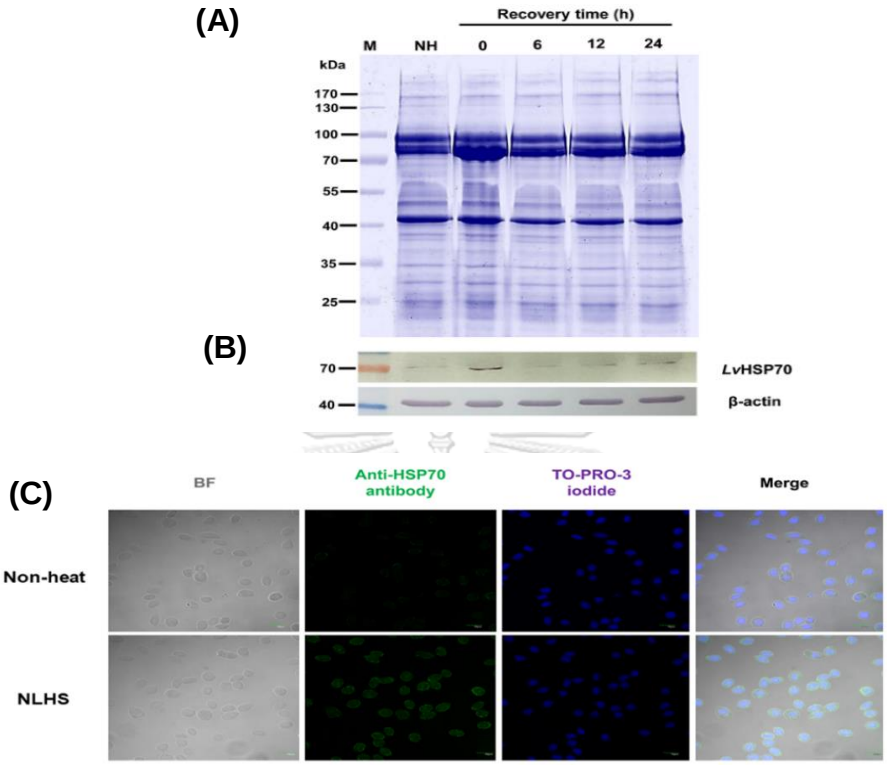

Figure 3.2 The protein expression of *Lv*HSP70 in non-heatshocked and NLHS-treated *P. vannamei* hemocytes (A) Total protein from hemocyte lysate (30 µg) collected at various recovery time points after NLHS, and in a non-heat-shocked (NH) group, were separated by SDS-PAGE. (B) The immunoblotting detection of *Lv*HSP70 using anti-human HSP70 antibody. β-actin was used as internal control. (C) Hemocytes from a non-heat-shocked group and a NLHS group (0 h recovery time) were stained with anti-human HSP70 (green) and nuclei with TO-PRO3 iodide (blue). The fluorescent signal was detected by confocal microscopy. 'BF' indicates a bright field.

3.1.3 Recombinant protein expression of *Lv*HSP70 in *E. coli*

To further characterize the function of *Lv*HSP70 in shrimp immune system during VP_{AHPND} infection, we produced a recombinant *Lv*HSP70 (r*Lv*HSP70) protein in *E. coli*

cells. The open reading frame (ORF) of *LvHSP70* was amplified by PCR, cloned into the pET22b expression vector and then transformed into an *E. coli* strain, BL21(DE3) codon plus. The construct was designed to produce the r*Lv*HSP70 protein with a 6xHis-Tag at the amino terminus. The production of r*Lv*HSP70 was highly produced and had increased in both insoluble inclusion bodies and soluble fraction after induction of IPTG at 2 h, 4 h till 6 h as shown in SDS PAGE in Fig. 3.3A and western immunoblotting in Fig 3.3B. To purify the protein, the soluble fraction at 6 h after induction with IPTG was selected then was subjected to purification using a Ni–NTA column. The r*Lv*HSP70 protein was eluted with 50 mM imidazole following by 300 mM imidazole. As shown in Fig. 3.4, a single step of purification yielded a purified r*Lv*HSP70, approximately 70 kDa in size. The identity of the r*Lv*HSP70 was confirmed by western immunoblotting using an anti-His antibody.

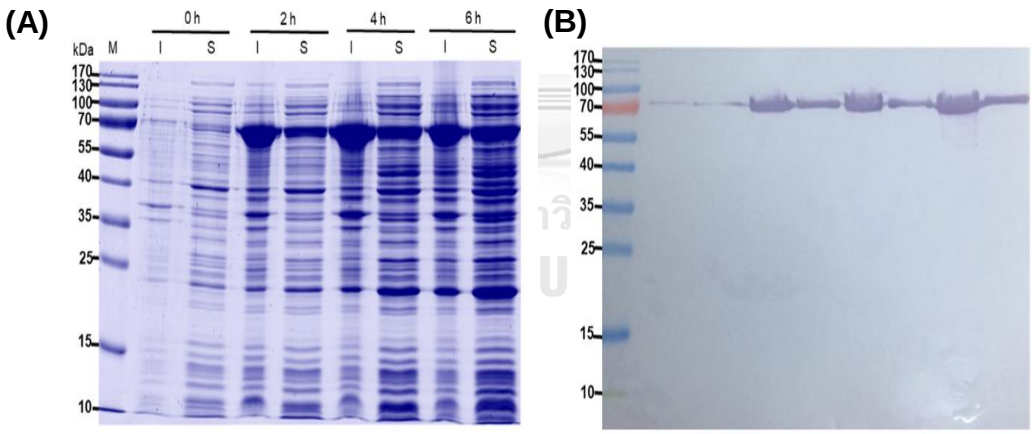

Figure 3.3 SDS-PAGE and Western blot analysis of r*Lv*HSP70 after induced with IPTG
(A) 12.5% SDS-PAGE gel (B) western immunoblotting were used to separate and confirm the production of r*Lv*HSP70 after induced with IPTG at various time points (0 h, 2 h, 4 h, and 6h). Lane 'M' represents the molecular weight protein marker in kDa, 'I' indicates insoluble inclusion bodies and 'S' indicates the soluble fraction. The r*Lv*HSP70 concentrate fraction was confirmed by western blot analysis using an anti-His tag.

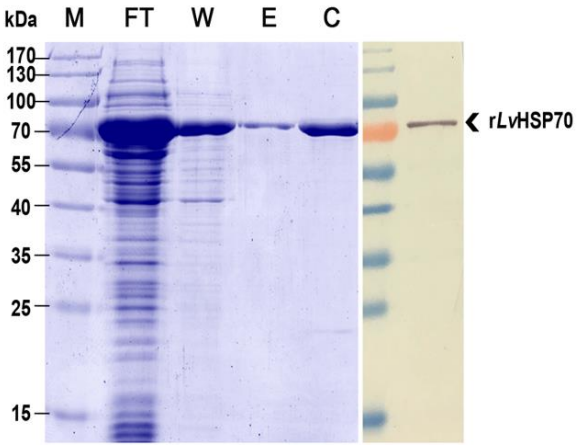

Figure 3.4 Western blot analysis of r*Lv*HSP70 after purification by Ni^{2+} affinity chromatography A 12.5% SDS-PAGE gel was used to separate r*Lv*HSP70v, which was then purified by Ni-NTA chromatography. Lane 'M' represents the molecular weight protein marker in kDa, 'FT' indicates the flow-through fraction, 'W' indicates the wash fraction, 'E' indicates the elution fraction and 'C' indicates the concentrate fraction. The r*Lv*HSP70 concentrate fraction was confirmed by western blot analysis using an anti-His tag.

3.1.4 Effect of r*Lv*HSP70 injection on the percent survival of VP_{AHPND}-infected *P.* vannamei

The direct effect of *Lv*HSP70 on the induction of resistance to bacteria was confirmed by injection of the recombinant protein, followed by VP_{AHPND} infection and determination of the percent survival of the animals. Shrimp (3-5 g body weight) were injected with 1.0 nmol of r*Lv*HSP70 and then challenged with 1×10^6 CFU/mL of VP_{AHPND} by immersion; the percent survival was then observed every 12 h for 5 days (120 h). The results showed that shrimp receiving r*Lv*HSP70 had a higher percent survival rate following to VP_{AHPND} infection (up to 75%) as compared to shrimp injected with a BSA, which is a control group (20% survival; Fig.3.5). Shrimp injected with PBS showed 100 % survival rates (data not shown).

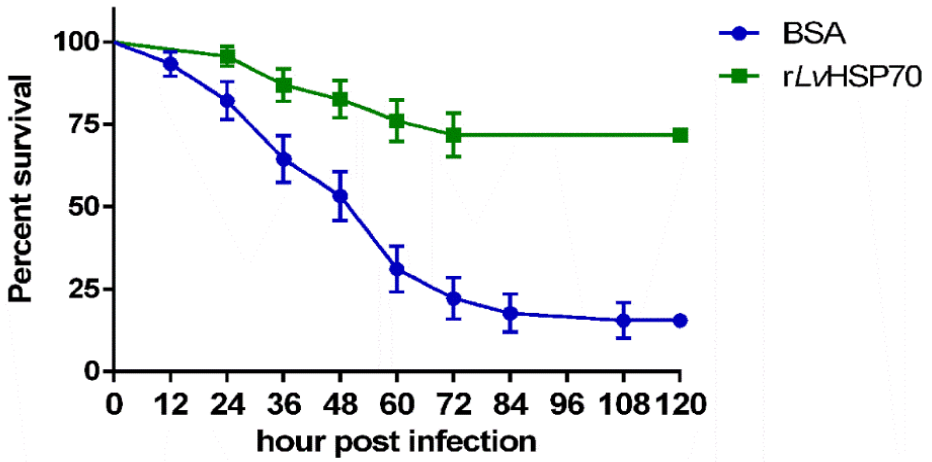

Figure 3.5 Percent survival of *P. vannamei* challenged with VP_{AHPND} Twenty juvenile shrimp were injected with 1.0 nmol r*Lv*HSP70 (green line) and 1.0 nmol BSA (blue line). Six hours post-injection, shrimp were immersed with $1x10^6$ CFU/mL of VP_{AHPND}. The proportion of shrimp surviving was observed every 12 h post-exposure until 120 h post-exposure. This experiment was performed in triplicate. Error bars represent standard deviation from the three replicates.

3.1.5 Detection of r*Lv*HSP70 in shrimp hemocytes after intramuscular injection

To understand how the injection of r*Lv*HSP70 enhances shrimp survival after infection with VP_{AHPND}, we investigated the localization of r*Lv*HSP70 in shrimp hemocytes after intramuscular injection by immunofluorescence and confocal microscopy technique. r*Lv*HSP70 injection was detected by using His tag residue that specific with anti-His Tag antibody. The recombinant protein produced by the empty vector of pET43b, containing the NUS Tag was used as the control protein. The results showed that at 0.5 hpi, no red signal of r*Lv*HSP70-His Tag was detected. However, the signals of r*Lv*HSP70-His Tag were detected starting at 3 h after r*Lv*HSP70 injection. Moreover, a clear signal detection was shown at 24 h post injection with r*Lv*HSP70. The results suggested that r*Lv*HSP70 could enter into hemocyte cells, both in the cytoplasm and

nucleus. On the other hand, the control group, which was injected with 70kDa Nus tag protein, was unable to enter the cell as no signal was detected (Fig. 3.6).

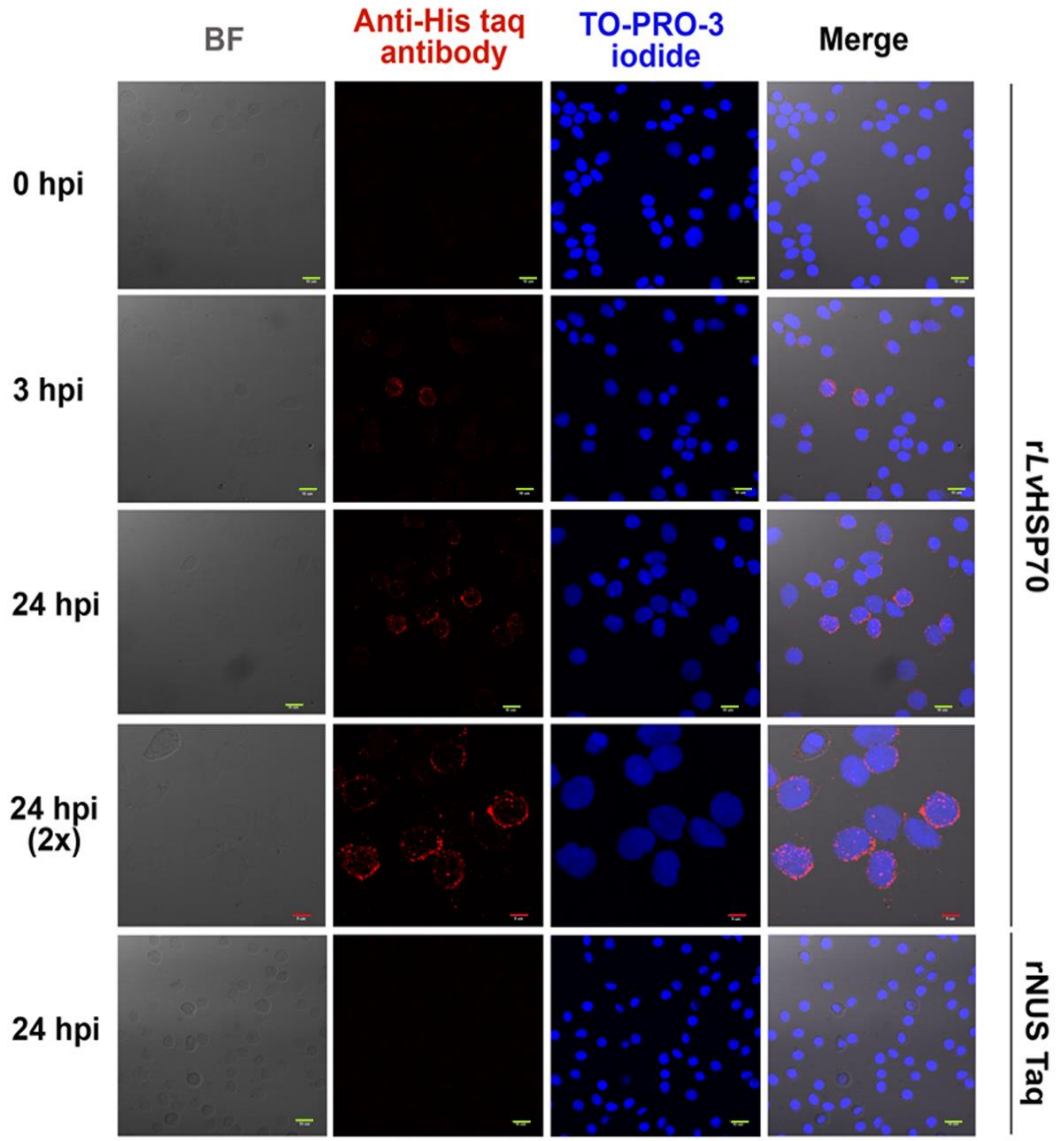

Figure 3.6 Localization of r*Lv*HSP70 by immunofluorescence spectroscopy. The detection of r*Lv*HSP70 in hemocyte cells at 0h, 3h and 24h post-injection by immunofluorescence using an anti-His tag antibody conjugated with Alexa Fluor 568 (shown in red). The control group was injected with rNUS tag protein. The red and yellow scale indicate 5 and 10 µm respectively.

3.1.6 Effect of r*Lv*HSP70 injection on the expression of immune-related genes in shrimp hemocyte

qRT-PCR was performed to determine changes in the mRNA expression level of several immune-related genes such as the signaling pathway: Toll and IMD pathway, prophenoloxidase activating pathway, the downstream regulation: antimicrobial peptide and blood clotting system. These genes were selected based on their immune function and were classified into four groups: genes involved in the NF-κB signal transduction pathway (*LvMyD88*, *LvIKKε* and *LvIKK*β); antimicrobial peptide genes (*LvCrustin I, Crustin-like Lv*, *LvPEN2, LvPEN3, LvPEN4)*; critical genes in the prophenoloxidase-activating (proPO) system (*LvproPO1, LvproPO2*) and transglutaminase (*LvTG1)*.

The purified r*Lv*HSP70 (1.0 nmol) was injected into shrimp muscle (3-5 g body weight). Then, hemocytes were collected to verify gene expression at 0.5, 3, 6, 12, 24 and 48 hpi. The results showed that at 6 hpi, the expression of *LvMyD88* was significantly up-regulated ($P<0.05$) at 6 until 24 hpi with the highest induction (by 6-fold) at 6 hpi (Fig. 3.7) while that of IKK genes was significantly upregulated ($P<0.05$) by 2.5-fold at 6 hpi and decreased to basal levels at 12 and 24 hpi (Fig. 3.7). Furthermore, the antimicrobial peptide genes *LvCrustin I*, *LvPEN2* and *LvPEN3* were significantly induced ($P<0.05$) at 6, 12 and 3 hpi, respectively, particularly *LvCrustin I*, which was influenced by approximately 4-fold (Fig. 3.7). On the other hand, the expression of *Crustin-like Lv* and *LvPEN4* were not induced by r*Lv*HSP70 (Fig. 3.7). proPO genes also showed significant upregulation ($P<0.05$). *LvproPO1* expression was increased by 2.5-fold at 3 hpi while *LvproPO2* expression was induced by 3.5-fold at 6-hpi (Fig. 3.8). The expression of *LvTG1* was also significantly upregulated ($P<0.05$) by 2.5-fold at 24 hpi and then returned to basal levels at 48 hpi (Fig. 3.8).

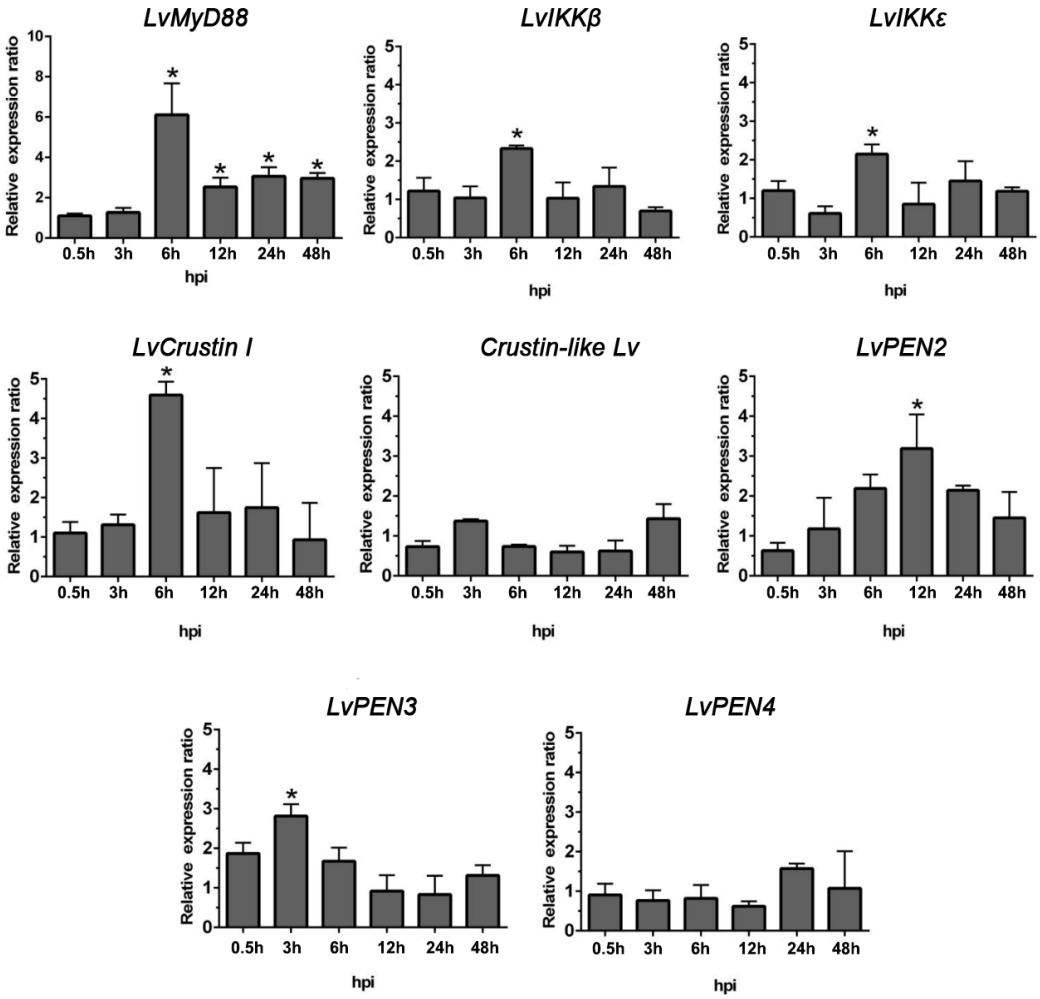

Figure 3.7 Expression analysis of genes in the NF-κB signal transduction pathways and antimicrobial peptides after r*Lv*HSP70 injection Relative gene expression was quantified by qRT-PCR at 0.5, 3, 6, 12, 24 and 48 h after injection with 1.0 nmol r*Lv*HSP70 and compared to shrimp injected with 1.0 nmol of BSA as a control. Relative expression was normalized with BSA expression. *EF1α* was used as an internal control. Error bars represent standard deviation from the three replicates. Asterisks indicate statistically significant different ratios compared with the control (*P<0.05*).

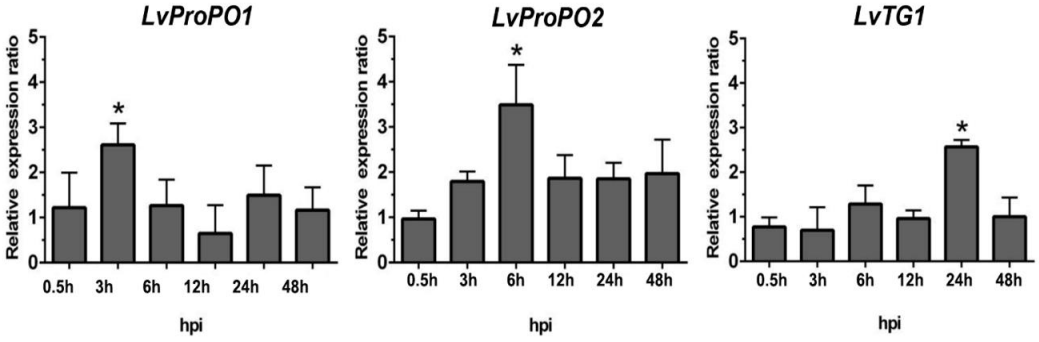

Figure 3.8 Expression analysis of immune-related genes following r*Lv*HSP70 injection The expression levels of *LvProPO1, LvProPO2* and *LvTGase* were quantified by qRT-PCR at 0.5, 3, 6, 12, 24 and 48 h after injection with 1 nmol r*Lv*HSP70 and compared to shrimp injected with 1 nmol BSA as a control. Relative expression was normalized with BSA expression. *EF1α* was used as an internal control. Error bars represent standard deviation from the three replicates. Asterisks indicate statistically significant different ratios compared with the control (*P<0.05*).

3.2 Discussion

Previous reports have shown that NLHS treatment can help aquatic animals such as *Artermia* and Asian green mussel to tolerate pathogens (Norouzitallab et al., 2015; Aleng et al., 2015). Previous work in *P. vannamai*, we successfuly showed, for the first time, that NLHS treatment (28 ± 1 ºC to 38 ± 0.2 ºC and incubated for 5 min daily for 7 days) enhanced the tolerance of shrimps to VP_{AHPND} infection. This was mediated by the induction of *LvHSP70* expression, as well as the expression of certain key immune genes (Junprung et al., 2017). Nevertheless, the role of *Lv*HSP70 in bacterial defense has not yet been directly investigated. Here, we further characterized the function of *Lv*HSP70 in shrimp immunity. First, we showed that *Lv*HSP70 was detected in hemocyte cells but not in plasma, and that the protein was localized in both the cytoplasm and

nucleus of all three types of shrimp hemocytes. This expression was related to the function of hemocytes in crustaceans which are known to play a critical role in immunity and hemostasis (Rowley, 2016).

Abrupt heat shock during NLHS treatment enhanced shrimp tolerance to VP_{AHPND} up to 30 days recovery and also induced the level of *LvHSP70* gene expression in hemocytes during the early recovery phase, i.e. 0, 3 and 6 h (Junprung et al., 2017). At the protein level, we showed that *Lv*HSP70 was immediately induced at 0 h recovery time. However, a previous study of *L. vannamei* showed that abrupt heat shock at 38 °C for 30 min induced the protein expression level of *Lv*HSP70 to a later time point (8 h recovery) (Loc et al., 2013). Moreover, in *Perna viridis* (Asian Green Mussel), *Pv*Hsp70-2 was induced in all tissues examined when mussels were heated at 38 °C for 30 min followed by 6 h recovery (Aleng et al., 2015). These differences in the time of induction were most probably due to the varying conditions used for heat shock treatment across these different studies. In addition, by using immunofluorescence, we clearly showed the high induction of *Lv*HSP70 at the protein level in shrimp that were exposed to NLHS conditions, as compared to the non-heated shrimp. Therefore, NLHS was able to induce the expression of *LvHSP70* at both the transcriptional (Junprung et al., 2017) and translational levels.

Our previous study showed that exposing shrimp to NLHS enhanced the shrimp's tolerance to a unique strain of the bacterium *V. parahaemolyticus*, which is a causative agent of AHPND. However, the increased levels of survival were affected by the induction of *LvHSP70*, *LvHSP90, LvCrustin I* and *LvproPO1* (Junprung et al., 2017). In this experiment, r*Lv*HSP70 was used to specifically investigate protein function in the immune defense against VP_{ANPHD} infection. Purified r*Lv*HSP70 was injected into the abdominal muscle of shrimps which were then challenged with VP_{AHPND}. Our results clearly showed that r*Lv*HSP70 enhanced shrimp resistance to VP_{AHPND}. A previous report

also showed that HSP70s, derived from either prokaryotic (*E. coli*) or eukaryotic (Artemia) sources, generated protective immunity in the crustacean Artemia against *V. campbellii* infection (Baruah et al., 2011). Similarly, when the shrimp *L. vannamei* was injected with either recombinant DnaK (bacteria HSP), or a chemically synthetic 50 amino acid peptide fragment which represented part of the peptide binding domain of DnaK (DnaK442–491), the shrimp showed activation of the immune response but no enhancement in survival rate after bacterial infection (Hu et al., 2014).

In this study, we showed that r*Lv*HSP70 entered into the intracellular space of hemocyte cells and was localized in both the cytoplasm and nucleus, while also accumulating in the plasma membrane. A previous report from neuronal cells showed that HSP70 might be released and enter the bloodstream under many pathological conditions (Calderwood et al., 2007). Moreover, several reports found that HSP70, upon binding to the Toll-like receptor (TLR) on the plasma membrane, activated intracellular signal transduction and transcription pathways, including the NF-kB and interferon response factor pathways (Asea et al., 2002; Vabulas et al., 2001) suggesting that *Lv*HSP70 functions as an activating protein that induces parts of the immune pathway, such as the Toll-like receptor pathway. Moreover, HSP70 has also been shown to bind to other pathogen-associated molecular patterns (PAMPs) such as endotoxin LPS and LPS associated molecules, via the protein-binding domain (Basu et al., 2002).

Furthermore, we observed that the expression of *LvMyD88*, a representative initiation molecule of the Toll pathway was induced upon the injection of r*Lv*HSP70. Supported by the previous study that *LvMyD88* expression was highly produced in shrimp hemocytes after being challenged with *V. parahaemolyticus* and *Staphyloccocus aureus* (Zhang et al., 2012a). Another study showed that HSP70-induced proinflammatory cytokine production was mediated via the MyD88/IRAK/NF-B signal transduction pathway (Asea et al., 2002). Besides, IKKs, which mediate the

phosphorylation of IkB and represent a point of convergence for most of the signal transduction pathways that lead to NF-kB activation. The degradation of IkBa results in the nuclear translocation of activated NF-kB proteins that finally induced the regulation of the antimicrobial peptides investigated. So *LvIKKε* and *LvIKK*β were selected to study the effect of r*Lv*HSP70 injection. The fact that the expression of both *LvIKKε* and *LvIKK*β were significantly upregulated indicated that r*Lv*HSP70 induced the shrimp immune system via different signal transduction pathways. In human monocytes, HSP70 possesses potent cytokine activity and can bind with high affinity to the plasma membrane, eliciting a rapid intracellular Ca^{2+} flux that activates IkB kinase (IKK) activity (Asea et al., 2002). These signaling pathways have been shown to be involved in the synthesis of several immune molecules, including antimicrobial peptides (AMPs) (Tassanakajon et al., 2018). The up-regulation of signaling molecules both in Toll and IMD pathways were in accordance with our present results which showed that the expression of shrimp AMPs (*LvCrustin I*, *LvPEN2* and *LvPEN3*) were significantly upregulated after the injection of r*Lv*HSP70. In the shrimp, *P. monodon*, some of these AMPs genes, such as *CrustinPms*, have been shown to be regulated upon the induction of *PmHSP70* and heat stress via the heat shock protein element (HSE) which was located in the promoter region (Vatanavicharn et al., 2009). Recently, our group reported that crustin p was differentially expressed in *L. vannamei* when challenged with VP_{AHPND} at 48 hpi compared with 0 hpi (Maralit et al., 2018) suggesting that HSP70 might activate a signaling pathway resulting in the increased expression of AMPs and subsequently enhanced the survival rate of *P. vannamei* infected with VP_{AHPND}. The previous report found that genomic structure of *crustinPm5* gene contains a putative heat-shock regulatory element (HSE) at the 5´ upstream region suggesting that this gene is regulated upon heat stress but no direct evidence has been shown (Vatanavicharn et al., 2009).

In crustaceans, TGase has been shown to be involved in the blood coagulation system, which is the first line of defense and an integral part of the overall invertebrate immune system (Maningas et al., 2008). TGase is released from hemocytes in response to tissue damage caused by stress or the encroachment of microbial pathogens; it then catalyzes polymerization of the plasma clotting protein to achieve clotting (Maningas et al., 2008; Wang et al., 2001). Moreover, the knockdown of *TGase* in shrimps resulted in increased mortality when challenged with white spot virus and *Vibrio penaeidida* (Maningas et al., 2008). Additionally, knocking down the TGase gene in *Pacifastacus leniusculu*s led to morphological changes in hemocytes (Lin et al., 2008). This finding indicates that TGase exerts the effect upon hematopoiesis in addition to coagulation. In our present results, we observe that r*Lv*HS70 strongly induce *LvTGase* expression at 24 hpi. Further studies will now be needed to unravel how the apparent function of *Lv*TGase in hematopoiesis and coagulation can be reconciled with its activation by *Lv*HSP70.

The prophenoloxidase (proPO) activating system, also known as the melanization pathway, is an essential part of the shrimp immune system and has been proven to be an essential immune defense mechanism against several pathogens, including bacteria and viruses (Amparyup et al., 2013; L and K, 2004; Söderhäll and Häll, 1984). Our present results showed that the two proPO genes (*LvproPO1* and *LvproPO2)* were also significantly induced upon r*Lv*HSP70 injection. Similarly, accumulating evidence showed that the level of mRNA encoding proPOs was significantly increased following both acute NLHS and chronic NLHS (Georgopoulos and Welch, 1993; Loc et al., 2013). Moreover, the injection of recombinant Dnak, followed by *V. campbellii* challenge, also affected the transcription of proPO-2 in the shrimp *P. vannamei* (Phuoc et al., 2016).

The role of HSP70 as an activator of the proPO system was confirmed by knocking down the transcription of HSPs. Hemolymph PO activity of *LvHSP70*- and

LvHSP90- knockdown shrimp was significantly decreased. Since the proPO-activating system plays an essential role in the bacterial defense in shrimp (Amparyup et al., 2009), gene knockdown of *LvHSP70* and *LvHSP90* may affect the resistance to VP_{AHPND} infection by subsequent reduction of PO activity. This study demonstrated that the upregulation of *LvHSP70* and *LvHSP90*, *LvproPO*, and *Lvcrustin* after chronic heat stress enhanced the white shrimp resistance to VP_{AHPND} and the knockdown of *LvHSPs* results in high cumulative mortality. This finding is in line with the report that feeding *Artemia franciscana* with either *Artemia* HSP70 or the *E. coli* HSP70 followed by *V. campbellii* challenge enhanced the proPO system at both mRNA and protein activity levels and led to the high survival rate of *Artemia* (Baruah et al., 2011). Moreover, previous studies have reported the correlation between the proPO system and AMP synthesis (Amparyup et al., 2013; Fagutao et al., 2009; Roh et al., 2009). Gene knockdown of shrimp proPOs resulted in a reduction of AMP gene expression (Amparyup et al., 2012) which might be related to the cross-talk of the proPO system and the toll pathways which regulates the AMP synthesis (Fagutao et al., 2009; Roh et al., 2009). Thus, the upregulation of immune genes, *LvproPO*, and *LvCrustin*, after chronic NLHS may likely be correlated and together play a crucial role in bacterial defense leading to shrimp resistance to VP_{AHPND}.

3.3 Materials and methods

3.3.1 Detection of *Lv*HSP70 in shrimp hemocyte

3.3.1.1 Hemocyte lysate and cell-free plasma preparation

Hemocyte lysate was prepared to determine the localization of *Lv*HSP70 expression. Hemolymph was collected from five healthy *P. vannamei* (10-15 g body weight) using 10% (w/v) sodium citrate as an anticoagulant. The hemocytes and plasma

were separated by centrifugation at 800 x g for 10 min at 4 °C, and the supernatant was collected as cell-free plasma then kept on ice. Hemocytes were collected and washed twice with 1X phosphate buffer saline, pH. 7.4 (1XPBS, pH 7.4) (0.137 M NaCl, 0.0027 M KCL, 0.01 M Na_2HPO_4, and 0.0018 M KH_2PO_4), the hemocytes were resuspended with 100 µl of 1X PBS, pH 7.4. The hemocyte cells were then lysed by sonication. After that, the solution was separated by centrifugation at 12,000 x g for 10 min at 4 °C and total protein was measured using Bradford reagent (Bio-Rad, USA).

3.3.1.2 SDS-PAGE and western blot analysis

Thirty micrograms of hemocytes lysate and 100 µg of plasma protein were mixed with loading dye, heated at 105°C for 5 min and loaded onto a 12.5% (w/v) sodium dodecyl sulfate (SDS)- polyacrylamide gel. Electrophoresis was performed at 25 A for 1 h. First Gel was stained with Coomassie blue. For western blot analysis, the proteins from the SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, USA). Membranes were incubated with blocking solution (5% skimmed milk in washing buffer (1XPBS, pH 7.4 containing 0.2% (v/v) Tween-20)) for 30 min at room temperature (25°C) to which a 1:5000 dilution of mouse monoclonal human antibody specific to HSP70 (Thermo Fisher, USA) was added. Membranes were washed 3 times for 15 min with washing buffer before incubation with 1:3000 diluted HRP conjugated goat anti-mouse IgG (Jackson Immune Research). The membranes were re-washed 3 times for 15 min with washing buffer and HSP70 was detected using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific).

3.3.1.3 Immunofluorescence detection of *Lv*HSP70

The presence of *Lv*HSP70 in hemocytes was detected using a mouse monoclonal antibody specific to human Hsp70 (C92F3A-5) (Merck, Germany). The hemolymph from healthy *P. vannamei* (10-15 g body weight) was collected from five non-heat-shocked specimens and five specimens immediately after chronic-NLHS treatment and fixed in 4% paraformaldehyde at a 1:1 ratio. Hemocytes were separated by centrifugation and resuspended in 1XPBS, pH 7.4. The cells were then counted by hemocytometer and coated (110 cells/slide) onto poly-L-lysine slides (Thermo Scientific, USA). Then, cells were washed with 1XPBS, pH 7.4 three times, permeabilized by 1% Triton X-100 in 1XPBS, pH 7.4 for 5 min at room temperature and washed three more times. The cells were blocked with 10% fetal bovine serum (FBS) in 1XPBS, pH 7.4 at room temperature for 1 h and then probed with a 1:200 dilution of a monoclonal antibody specific to human Hsp70 at room temperature for 2 h. The negative control was incubated with 1% FBS in 1XPBS, pH 7.4. The slides were then washed and probed with secondary antibody, a 1:1000 dilution of a goat anti-mouse antibody conjugated with Alexa Fluor 488 (Thermo Fisher, USA), in 1XPBS, pH 7.4 containing 1% FBS at room temperature for 1 h. Cells were also stained for nuclear DNA by incubating with 1:1500 TO-PRO®-3 iodide (Thermo Fisher, USA) before mounting with medium Prolong Gold antifade reagent (Thermo Fisher, USA). Fluorescent staining was then observed under FV1000 confocal laser scanning microscope (Olympus, USA).

3.3.2 Analysis of *Lv*HSP70 protein expression level after NLHS

Changes in the protein expression of *Lv*HSP70 after chronic non-lethal heat shock (chronic-NLHS) treatment, was determined by western blot analysis. Hemolymph was collected from five juvenile shrimp at various recovery time points after chronic-

NLHS (0, 6, 12 and 24 h) and a non-heat control group, using 10% (w/v) sodium citrate as an anticoagulant. Hemocytes were separated by centrifugation and then washed twice with 1 mL of 1XPBS, pH 7.4. After cell lysis, 30 µg of the hemocyte lysate was separated by SDS-PAGE and the expression of *Lv*HSP70 determined by western blotting using mouse monoclonal human antibody specific to HSP70 (Thermo Fisher, USA) as described in 3.3.1.2. Besides, the hemolymph was collected from five juvenile shrimp at various recovery time points after chronic-NLHS (0, 6, 12 and 24 h) and a non-heat control group. The hemocytes were separated and then fixed in 4% paraformaldehyde at a 1:1 ratio. After that, immunofluorescence microscopy was used to detect the expression of *Lv*HSP70 as described in 3.3.1.3.

3.3.3 Expression of recombinant *Lv*HSP70

Two gene-specific primers, *Nde*I r*Lv*HSP70-F with 5' *Nde*I site and *Not*I r*Lv*HSP70-R, with 5' *Not*I site (Table 2.2), were designed in order to amplify the mature *LvHSP70* gene. The amplified mature gene was then cloned into the pET-22b vector. The recombinant plasmid, pET22b- *LvHSP70,* was then transformed into a BL21(DE3) *E. coli* strain and recombinant *Lv*HSP70 (r*Lv*HSP70) was expressed with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Thermo Fisher, USA) and cultured to stationary phase by incubation 6 h with shaking at 250 rpm at 37 ºC. The cells were harvested at 6 h post induction by centrifugation at 10,000 × g for 10 min at 4 °C. The soluble fraction was collected, and sonication was performed. The soluble protein fraction was purified by using a Ni-NTA affinity column (GE Healthcare). The purified r*Lv*HSP70 was then dialyzed against 20 mM Tris-HCl pH 8.0 and then concentrated. The recombinant protein was analyzed as described in 3.3.1.2 with some modification. Briefly, The SDS-PAGE (12.5% (w/v) acrylamide) was performed followed by coomassie brilliant blue

staining then was confirmed the purification efficiency using western blot approach with anti-His tag antibody (1:5000; Thermo Scientific, USA) as primary antibody and goat anti-mouse IgG-AP conjugate (1:10,000; Jackson Immune Research, USA) as secondary antibody. The positive bands were detected using NBT/BCIP solution. This purified protein was used to study the *in vivo* effect of r*Lv*HSP70 injection in shrimp.

3.3.4 Effect of r*Lv*HSP70 upon VP_{AHPND} infection

Forty juvenile shrimp (2-3 g body weight) were separated into two groups twenty shrimp each; the experiment group was injected with 1.0 nmole of purified r*Lv*HSP70 while, the control group was injected with 1.0 nmole BSA. After six hours post-injection, shrimp were challenged with LD_{50} dose (1×10^6 CFU/mL) of VP_{AHPND} by immersion in aquaria containing 35 L. Shrimp survival was observed every 12 hours for a total of 120 hours. Moribund shrimp were counted as being dead. This experiment was performed in triplicate.

3.3.5 Detection of r*Lv*HSP70 in the intracellular space of shrimp hemocytes

Ten juvenile shrimp (10-15 g body weight) were injected with 1.0 nmole of r*Lv*HSP70, while the control group shrimp was injected with 1.0 nmole of 70 kDa recombinant proteins containing the Nus Tag sequence expressed with the pET-43.1 (rNus) which is the protein taq that locate on pET43. After that shrimp hemolymph was collected at 0, 3 and 24 hpi, immediately fixed with 4% paraformaldehyde and hemocytes were collected by centrifugation. Immunofluorescence detection of r*Lv*HSP70 and rNus was carried out according to the method described in section 3.3.1.3, but with some modifications. r*Lv*HSp70 and rNus were detected by a 1:200 dilution of monoclonal antibody specific for His taq (Thermo Scientific, USA) at room

temperature for 2 h. The secondary antibody and the detection method were as described in 2.11.3 but using a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 568 (Thermo Fisher, USA). Fluorescent staining was then evaluated using FV1000 confocal laser scanning microscope (Olympus).

3.3.6 Temporal gene expression analysis after r*Lv*HSP70 injection as determined by qRT-PCR

Shrimp (2-3 g body weight) were injected with either 1.0 nmole of purified r*Lv*HSP70 or 1.0 nmole of BSA for the control group. Then, the hemolymph was collected individually from nine juveniles at each time point (0.5, 3, 6, 12, 24, and 48 hpi) using 10% (w/v) sodium citrate as an anticoagulant. Hemocytes were separated for total RNA extraction. Then, cDNA was synthesized from three pooled RNA samples for each time point (3 samples from 9 shrimps). cDNA samples were then used to investigate the expression levels of *LvMyD88*, *LvIKK*β, *LvIKKε*, *LvCrustin I*, *Crustin-like Lv, LvPEN2*, *LvPEN3*, *LvPEN4, LvproPO1*, *LvproPO2,* and *LvTG1.* qRT-PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) using SsoFast™ EvaGreen® Supermixes (Bio-Rad, USA) with forward and reverse primers that were specific for immune-related genes (Table 3.1). The housekeeping gene, *EF1α*, was used as an internal control. The cycling parameters started with initial activation of 95ºC for 5 min followed by 40 cycles of 95ºC for 30 s, gene-specific annealing temperatures for 45 s and 72ºC for 30 s. Fluorescent signal intensities were recorded at the end of each cycle. Melting curve analysis was then performed from 55 to 95ºC with continuous fluorescent reading in 0.5ºC increments to confirm that only one product was amplified. The cycle threshold (Ct) values and fold difference for each immune-related gene were recorded using CFX Manager Software (Bio-Rad). Expression was calculated by the

$2^{-\Delta\Delta_{Ct}}$ method relative to the expression of *EF1α* transcripts. Amplification was performed in triplicate for each time point. The statistical significance of any differences was tested by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test using statistical package for the social sciences (SPSS) software (IBM, USA).

Table 3.1 Nucleotide sequence of the primers designed for temporal gene expression analysis.

Primer name	Sequence (5'-3')	Purpose
*Nde*I r*Lv*HSP70-F	ATTACATATGGCAAAGGCACCTGCTGT	Recombinant protein
*Not*I r*Lv*HSP70-R	ATTAGCGGCCGCATCGACCTCCTCGATGGTG	
LvPEN2-F	TCGTGGTCTGCCTGGTCTT	qRT-PCR
LvPEN2-R	CAGGTCTGAACGGTGGCCTTC	
LvPEN3-F	CACCCTTCGTGAGACCTTTG	qRT-PCR
LvPEN3-R	AATATCCCTTTCCCACGTGAC	
LvPEN4-F	ATGCTACGGAATTCCCTCCT	qRT-PCR
LvPEN4-R	ATCCTTGCAACGCATAGACC	
LvIKKβ -F	ACCACACTTTCCACCTTTGG	qRT-PCR
LvIKKβ -R	TCCCGATGAAGGAAGAACAC	
LvIKKε-F	TTGGCTTCTTTCCAGGACAC	qRT-PCR
LvIKKε-R	TTTTATGGCTGCCAGGAGTC	
LvproPO1-F	AACTCCATTCCGTCCGTCTG	qRT-PCR
LvproPO1-R	GGCTTCGCTCTGGTTAGGAT	
LvproPO2-F	CTCAGCGTGAACTCGCCTTA	qRT-PCR
LvproPO2-R	CCTGCTCAGTGTACGGTCT	

LvMyD88-F	GCTGTTCCACCGCCATTT	qRT-PCR
LvMyD88-R	GCATCATAGTGCTGTAGTCCAAGA	
LvTG1-F	GAGCTTCAAGATCGAGGATCGA	qRT-PCR
LvTG1-R	GCTGGTGTTCGTAGCGGTTATC	
LvCrustin I-F	GCTGGCCTCGATAAGTGTTG	qRT-PCR
LvCrustin I-R	CATCGGTCGTTCTTCAGATG	
Crustin like Lv-F	GTAGTATTAGCCGTTGTCGCC	qRT-PCR
Crustin like Lv-R	AAAGACGCCACCTACTCCAG	
EF1α-F	CTTGATTGCCACACTGCTCAC	qRT-PCR
EF1α-R	TCTCCACGCACATAGGCTTG	

CHAPTER IV

Sequence and expression analysis of HSP70 family genes in *Artemia franciscana*

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Abstract

Thus far, only one gene from the heat shock protein 70 (*HSP70*) family has been identified in *Artemia franciscana*. Here, we used the draft *Artemia* transcriptome database to search for other genes in the HSP70 family. Four novel HSP70 genes were identified and designated *heat shock cognate 70* (*HSC70*), *heat shock 70 kDa cognate 5* (*HSC70-5*), *Immunoglobulin heavy-chain binding protein (BIP)*, and *hypoxia up-regulated protein 1* (*HYOU1*). For each of these genes, we obtained nucleotide and deduced amino acid sequences, and reconstructed a phylogenetic tree. Expression analysis revealed that in the juvenile state, the transcription of *HSP70* and *HSC70* was significantly ($P>0.05$) higher in a population of *A. franciscana* selectively bred for increased induced thermotolerance (TF12) relative to a control population (CF12). Following non-lethal heat shock treatment at the nauplius stage, transcription of *HSP70*, *HSC70*, and *HSC70-5* were significantly ($P<0.05$) up-regulated in TF12. In contrast, transcription of the other HSP70 family members in *A. franciscana* (*BIP*, *HYOU1*, and *HSPA4*) showed no significant ($P>0.05$) induction. Gene expression analysis demonstrated that not all members of the HSP70 family are involved in the response to heat stress and selection and that especially altered expression of HSC70 plays a role in a population selected for increased thermotolerance.

In this chapter, novel members of the HSP70 family were identified from a draft *Artemia* genome database (ARC Lab, Ghent University) using bioinformatic tools. Orthology prediction methods analyzed the complete open reading frame (ORF) for each gene identified. Gene sequences were then subjected to phylogenetic analysis. Next, we investigated the expression of the identified HSP70 members in two populations of *Artemia*: a control population (CF12) that experienced 12 generations of isothermal laboratory culture conditions and a selected population (TF12) featuring the survivors of over 12 generations of an induced thermotolerance treatment. In addition, the expression of the newly identified HSP70 members was also examined following non-lethal heat shock (NLHS) in both CF12 and TF12 populations. Our results showed that members of the HSP70 family respond differentially at the transcriptional level to "thermal" selection or to NLHS, or to both.

4.1 Results

4.1.1 Sequence identification and phylogenetic analysis of the HSP70 family in *Artemia*

To understand the role of HSP70 family genes in thermal stress, the HSP70 members were identified and characterized in *A. franciscana* by searching against an *Artemia* transcriptome database. The partial cDNA sequence of HSP70s was then confirmed by PCR amplification using juvenile *Artemia* (and sequencing. Sequence analysis revealed four members of HSP70 family with a complete ORF containing putative conserved HSP70 domains which are *heat shock cognate 70* (*HSC70*), *heat shock 70 kDa cognate 5* (*HSC70-5*), *heat shock 70 kDa cognate 3* (*HSC70-3*)/*BIP*/*Grp78*, and *hypoxia up-regulated protein 1* (*HYOU1*). Moreover, a partial sequence of HSPA4 is also reported below.

4.1.1.1 Heat shock cognate 70 (HSC70)

The complete ORF of *HSC70* is 1953 bp (accession number: MH992632) encoding a putative protein of 650 amino acids (Fig. 4.1A). This sequence contains a typical ATP-binding domain and a substrate-binding domain of the HSP70 family (Fig. 4.1B). Moreover, the deduced amino acid sequence in *HSC70* includes the three signatures of the HSP70 family genes including IDLGTTYS (residues 9-16), LIFDLGGGTFDVSIL (residues 196-210), and IVLVGGSTRIPKVQK (residues 334-348) predicted by ExPASy web. Moreover, a series of unique repeat GG[X]P motifs and a cytosolic HSP70–specific motif, EEVD (residues 647-650) was found at the C-terminus of HSC70 (Fig. 4.1A). The protein has a molecular mass of 70.89 kDa and theoretical isoelectric point (pI) of 5.38 predicted by the ExPASy web tools. A phylogenetic tree was constructed based on the deduced amino acid sequence of complete ORF HSC70's. The phylogenetic tree composed of three main classes namely insects, crustaceans, and mammalian (Fig. 4.5), revealed that *A. franciscana HSC70* exhibited the highest similarity to HSC70 of *Daphnia magna* (A0A0P5G8T5) with 88% identity.

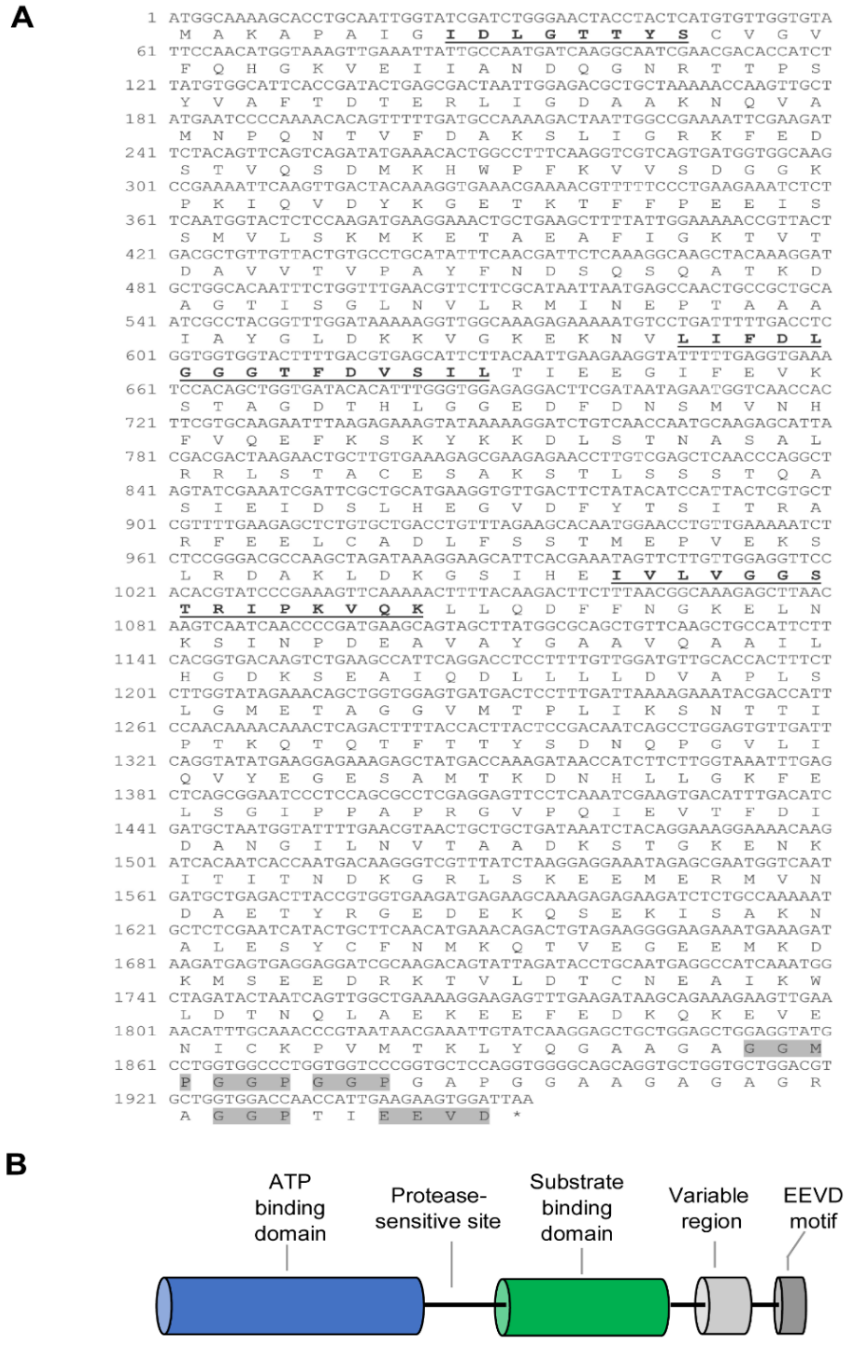

Figure 4.1 Schematic diagram and domain analysis representing the complete ORF of *A. franciscana HSC70* (A) Nucleotide and deduced amino acid sequences and (B) cartoon showing a linear representation of HSC70. Three HSP70 protein family signatures are labeled with the bold letter and underlined. The C-terminal region containing tetrapeptide GG[X]P and an EEVD-motif is labeled with a grey background.

4.1.1.2 Heat shock 70 kDa cognate 5 (HSC70-5)

The complete ORF of *HSC70-5* is 2028 bp (accession number: MH992633) which translates to a putative protein of 675 amino acids (Fig. 4.2A). The deduced amino acid sequence contains a typical ATP-binding domain and substrate-binding domain (Fig. 4.2B). In addition, it includes three signatures of HSP70 family genes; IDLGTTNS (residues 50-57), VYDLGGGTFDISVL (residues 235-248), and VLLVGGMTRMPKVQE (residues 376-390) (Fig. 4.2A) as predicted by ExPASy web tools. The protein has a molecular mass of 73.30 kDa and theoretical pI of 5.57 predicted by the ExPASy web. According to Fig. 5, a phylogenetic tree constructed based on the deduced amino acid sequence of *HSC70-5* complete ORF showed two different classes: insects and crustaceans. *Artemia franciscana HSC70-5* was closely related to insect amino acid sequences (Fig. 4.5), with the highest similarity to *HSC70-5* of *Onthophagus taurus* (XP_022919865.1) by sharing 82% identity.

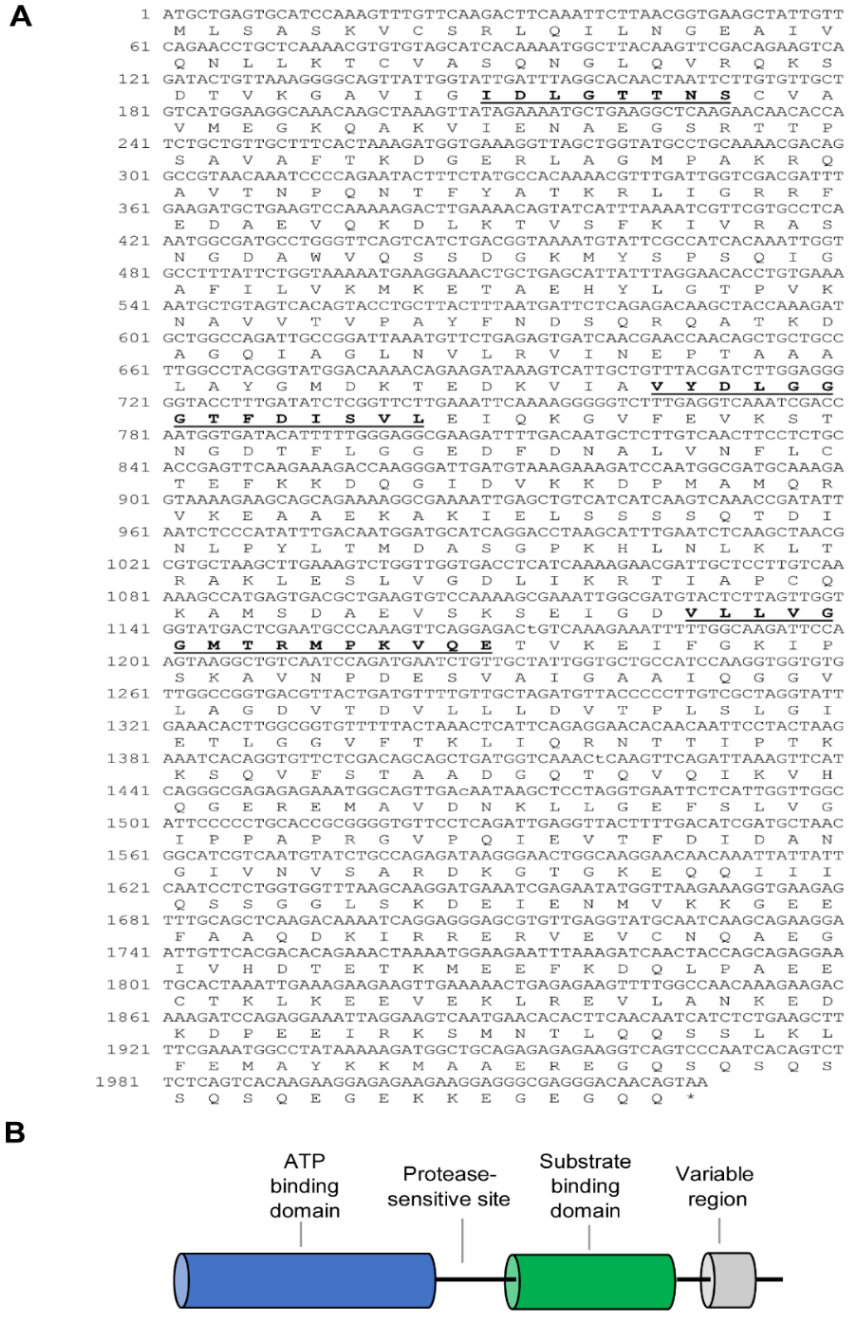

Figure 4.2 Schematic diagram and domain analysis representing the complete ORF of *A. franciscana HSC70-5* (A) Nucleotide and deduced amino acid sequences and (B) cartoon showing a linear representation of HSC70. Three HSP70 protein family signatures are labeled with the bold letter and underlined.

4.1.1.3 Immunoglobulin heavy-chain binding protein (BIP)

The complete ORF of *A. franciscana BIP* (accession number: MH992635) is 1956 bp encoding a protein of 651 amino acids (Fig 4.3A). This protein (Fig. 4.3B) contains an ATP-binding domain and a substrate-binding domain. The signal peptide for secretion into ER, 1MKILVLLSLLAVAFA15, is located at the N-terminus. The deduced amino acid sequence of *A. franciscana BIP* contains the following conserved signatures: IDLGTTYS (residues 30-37), VFDLGGGTFDVSLL (residues 218-231), and IVLVGGSTRIPKIQQ (residues 355-369). Moreover, the ATP/GTP-binding site motif A (P-loop) found at the residues 153-160 (AEAYLEKK) (Fig 3A). The C-terminus of BIP has a putative ER retention tetrapeptide, KDEL at residues 648-651 predicted by ExPASy web tools (Fig. 4.3A and 4.3B). The protein has a molecular mass of 72.16 kDa and the theoretical pI of 5.12 predicted by the ExPASy web. A phylogenetic tree of two different classes of insecta and crustaceans shown in Fig. 4.5. It revealed that *A. franciscana BIP* exhibited the highest similarity to HSC70-3 of *D. magna* with 87% identity.

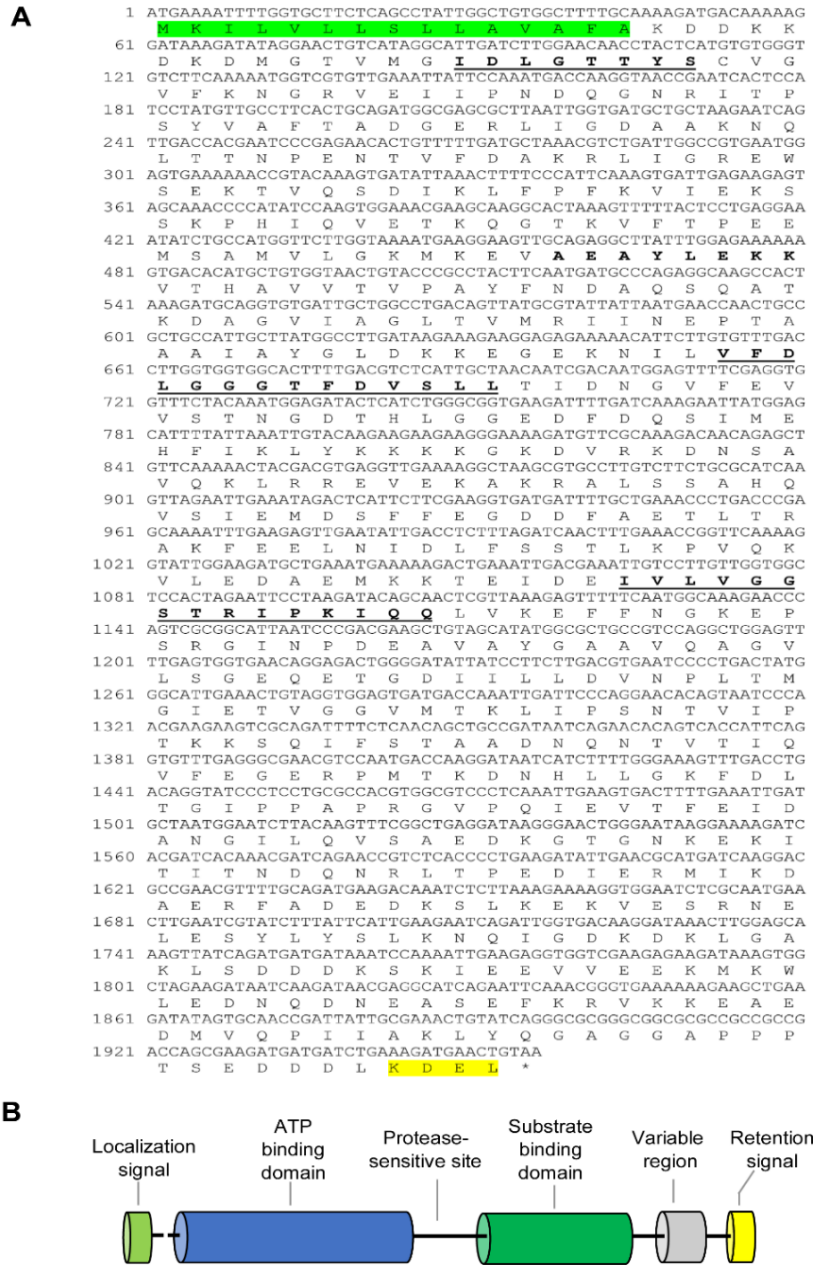

Figure 4.3 Schematic diagram and domain analysis representing the complete ORF of *A. franciscana BIP* (A) Nucleotide and deduced amino acid sequences and (B) cartoon showing a linear representation of HSC70. The signaling peptide is labeled with a green background. Three HSP70 protein family signatures are marked with the bold letter and underlined. The ATP/GTP-binding site motif A (P-loop) is showed with a bold letter. The C-terminal region containing a KDEL-motif is labeled with a yellow background representing the endoplasmic reticulum retention sequence.

4.1.1.4 Hypoxia up-regulated protein 1 (HYOU1)

The complete ORF of *HYOU1* (accession number: 992634) is 2649 bp in length, encoding a putative protein with 882 amino acids (Fig. 4.4A). This protein contains putative conserved domains of the HSP70 superfamily, namely an ATP-binding and a substrate binding domain (Fig 4.4B). The signal peptide for secretion into ER, 1MKILVLLSLLAVAFA15, is located at the N-terminus. One signature motif of HSP70 family genes found at residue 368-382; IILVGGNTRMPAVQA (Fig. 4.4A). The protein has a molecular mass of 100 kDa and theoretical pI of 5.23 predicted by the ExPASy web. A phylogenetic tree was separated into two different classes including Insecta and crustaceans. *A. franciscana HYOU1* had the highest sequence similarity to crustacean protein: *Artemia sinica* with 95% identity (Fig. 4.5).

4.1.1.5 Heat shock 70 kDa protein 4 (HSPA4)

The partial cDNA sequence of HSPA4 is 1269 bp (accession number: MK036505). The blast result showed that the partial cDNA of HSPA4 was similar to HSPA4 isoform X2 in *Bombus terrestris* with the highest alignment score and 62% identity. The alignment of the amino acid sequence and HSPA4 isoform X2 shared the highest homology at the N-terminus of HSPA4 *B. terrestris*. The partial HSPA4 protein sequence contains a part of the ATP binding domain at the N-terminus. One signature motif of HSP70 family genes was found at residues 339-353; VEIVGGSTRIPAVKT.

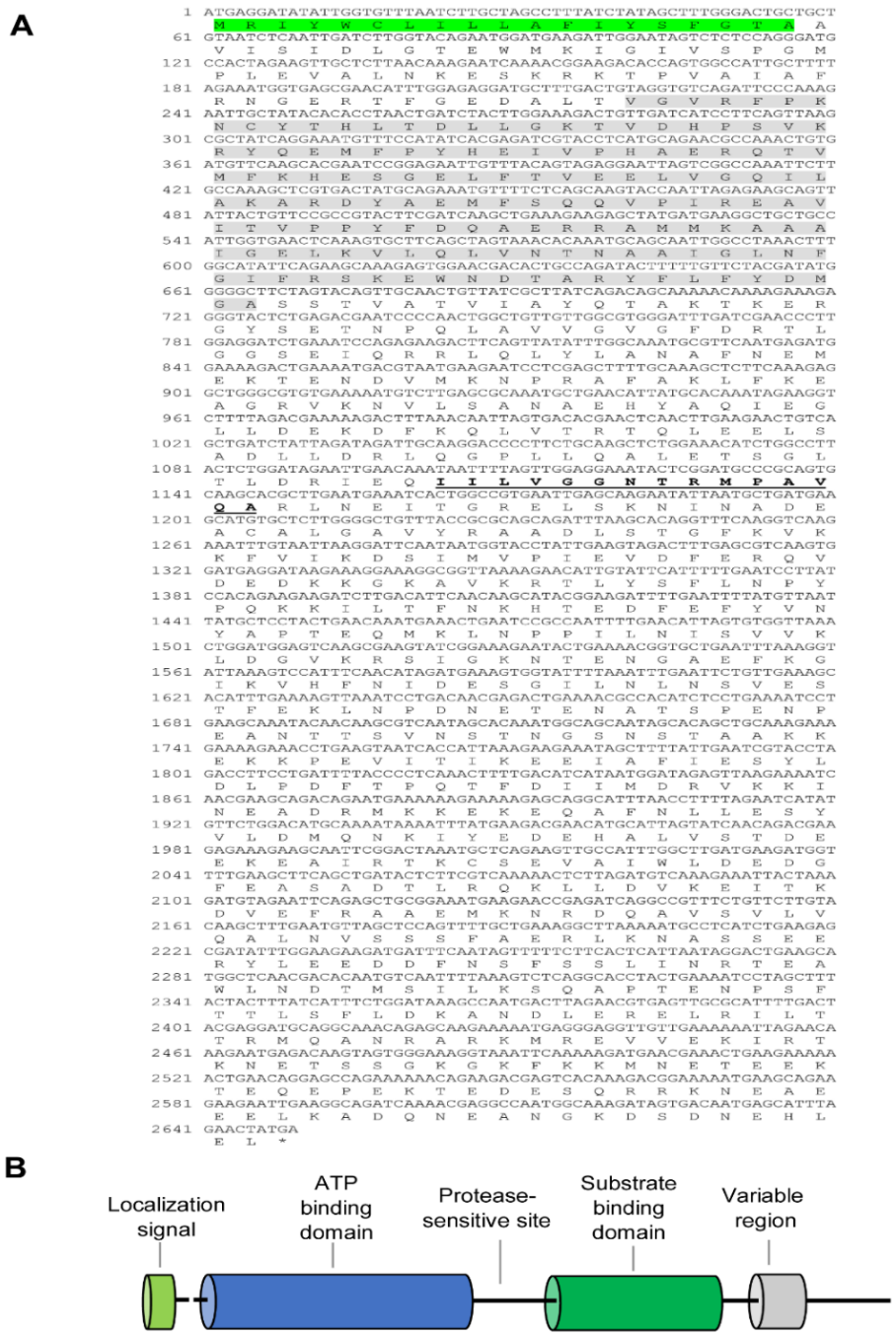

Figure 4.4 Schematic diagram and domain analysis representing the complete ORF of *A. franciscana HYOU1* (A) Nucleotide and deduced amino acid sequences and (B) cartoon showing a linear representation of HSC70. The signaling peptide is labeled with a green background. One HSP70 protein family signatures are marked with the bold letter and underlined. HYOU1-like_NBD domain is highlighted with the grey background.

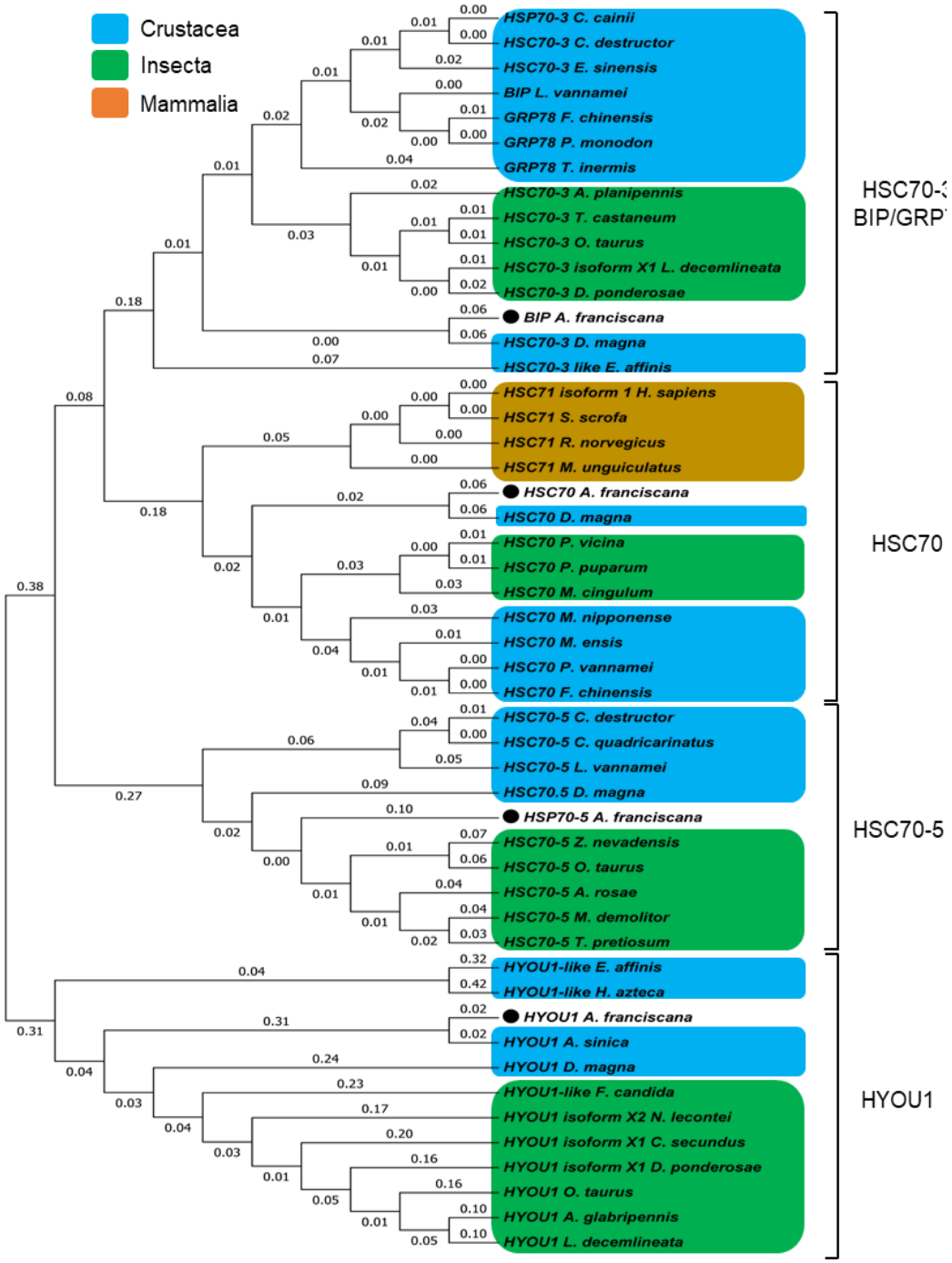

Figure 4.5 Phylogenetic tree analysis of HSP70 family members in *A. franciscana* The neighboring phylogenetic tree of HSP70 family members based on their amino acid sequence. Phylogenetic tree analysis of the partial or complete ORF amino acid sequences of five novel HSP70 family genes were aligned with ClustalW and constructed the phylogenetic tree by the neighbor-joining method using the MEGA 7.0

software. The GeneBank numbers were listed as follows: **HSC70-3/BIP/GRP78** (*Cherax cainii*: AKB96213.1; *Cherax destructor*: AKB96212.1; *Eriocheir sinensis*: AHA61465.1; *Litopenaeus vannamei*: AFQ62791.1.); *Fenneropenaeus chinensis*: ABM92447.1; *Penaeus monodon*: ARW29625.1; *Thysanoessa inermis*: ARN17957.1; *Agrilus planipennis*: XP_018330023.1; *Tribolium castaneum*: XP_008200986.2; *Onthophagus Taurus*: XP_022900037.1 *Leptinotarsa decemlineata*: XP_023015278.1;; *Dendroctonus ponderosae*: XP_019762356.1; *Artemia franciscana*: MH992635; *Daphnia magna*: A0A0N8CDY8 ; *Eurytemora affinis*: XP_023346701.1), **HSC70** (*Homo sapiens*: NP_006588.1; *Sus scrofa*: NP_001230836.1; *Rattus norvegicus*: NP_077327.1; *Meriones unguiculatus*: XP_021498722.1; *Artemia franciscana*: MH992633; *Daphnia magna*: A0A0P5G8T5; *Polyrhachis vicina*: AGF33487.1; *Pteromalus puparum*: ACA53150.1; *Macrocentrus cingulum*: ACD84943.1; *Macrobrachium nipponense*: ABG45886.1; *Metapenaeus ensis*: ABF20530.1; *Litopenaeus vannamei*: ABP01681.1; *Fenneropenaeus chinensis*: AAW71958.1), **HSC70-5** (*Cherax destructor*: AKB96210.1; *Cherax quadricarinatus*: AKB96209.1; *Litopenaeus vannamei*: ANJ04741.1; *Daphnia magna*: KZS16423.1; *Artemia franciscana*: MH992632; *Zootermopsis nevadensis*: XP_021939160.1; *Onthophagus taurus*: XP_022919865.1; *Athalia rosae*: XP_012264348.1; *Microplitis demolito*: XP_008560903.1; *Trichogramma pretiosum*: XP_014221706.1), and **HYOU1** (*Hyalella Azteca*: XP_018009810.1; *Artemia franciscana*: 992634; *Artemia sinica*: AKG51639.1; *Daphnia magna*: A0A0P5LA98; *Eurytemora affinis*: XP_023323737.1; *Folsomia candida*: XP_021945992.1; *Neodiprion lecontei*: XP_015511534.1; *Cryptotermes secundus*: XP_023720786.1; *Anoplophora glabripennis*: XP_018567874.1; *Leptinotarsa decemlineata*: XP_023015278.1; *Dendroctonus ponderosae*: XP_019762356.1; *Onthophagus Taurus*: XP_022900037.1)

4.1.2 Expression analysis of HSP70s family genes in CF12 and TF12 juveniles

The relative gene expression of the identified HSP70 family genes was examined in the juvenile TF12 and CF12 *Artemia* and analysed by qRT-PCR using specific primers

for six HSP70 family genes including *HSP70*, *HSC70*, *HSC70-5*, *BIP*, *HYOU1*, and *HSPA4*, and one chaperone protein, *protein disulphide isomerase* (*PDI*). The relative gene expression of six HSP70 family genes and one chaperone protein is shown in Fig 6. Significant higher expression of *HSP70* was observed in TF12 (*P<0.05*) as compared to that in CF12. Moreover, a 3-fold higher expression of *HSC70* was found in TF12 relative to CF12 (*P<0.01*). On the other hand, *HSC70-5*, *BIP*, *HYOU 1*, and *HSPA4* did not show a significantly different gene expression between CF12 and TF12. In addition, the relative expression of the control chaperon gene *PDI* did not show a significant difference either. In summary, these results suggested that the selection for induced thermotolerance in *Artemia* constitutively increased HSP70 expression but more explicitly HSC70 expression (Fig. 4.6).

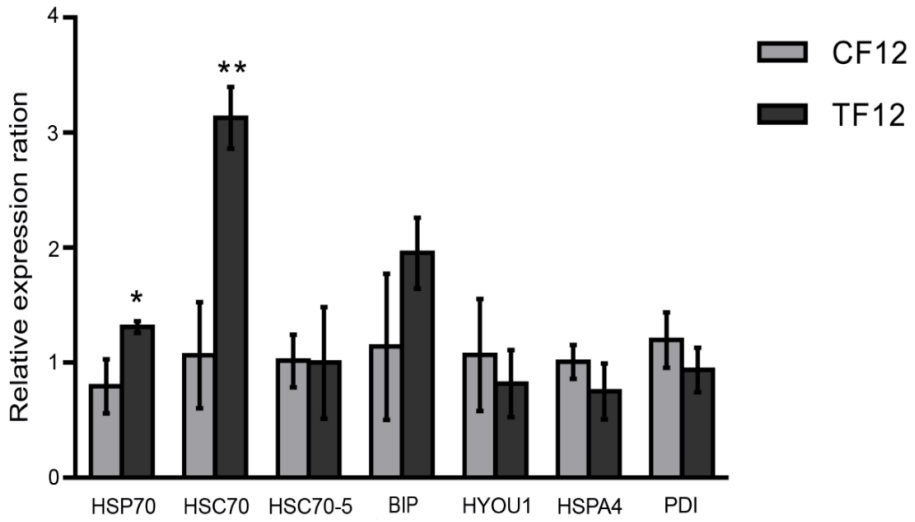

Figure 4.6 The thermal tolerance selection breeding program induced the expression of HSP70 and HSC70 genes in *A. franciscana*. The relative gene's expression of HSP70s family was quantified by qRT-PCR with juvenile *Artemia* between TF12 (selective breeding for induced thermotolerance) compared to the control (CF12; non- selective breeding for induced thermotolerance). The EF1α was used as an internal control. The error bars are the SD values from three replicates. Asterisks indicate statistically significant different ratios compared with the control (* indicates *P<0.05* and ** indicates *P<0.01*).

4.1.3 Expression of HSP70 family genes in CF12 and TF12 nauplii after a NLHS

In the next experiment, it was verified to what extent selected animals would respond differentially to a non-lethal heat shock (NLHS) relative to non-selected animals (Fig 4.7).

In the control population (C12), it found that *HSC70-5* and *BiP* (3 h post-NLHS) and *HSP70* expression (6 h post-NLHS*)* were significantly upregulated. In contrast, no significantly different expression of *HSC70*, *HYOU1,* and *HSPA4* and *PDI* was observed at both time points post-NLHS.

In the TF12 population, NLHS modulated significantly *HSP70*, *HSC70*, *HSC70-5* expression. At 3 and 6 h post-NLHS, *HSP70* and *HSC70* expression were induced by approximately 3-fold and 2-fold higher, respectively. Moreover, *HSC70-5* was slightly up-regulated at 3 and 6 h post-NLHS. Finally, NLHS did not affect *BIP*, *HYOU1*, *HPA4* and *PDI* expression.

A 2-way ANOVA revealed an interaction between NLHS and selection for induced thermotolerance for HSP70 (both at 3 and 6 h post-NLHS) and for HSC70 and HSC70-5 (at 6 h post-NLHS). For HSC70-5 the interaction was antagonistic, while in the other 2 cases it was synergistic. These observations indicate that the selection for induced thermotolerance positively interferes in a NLHS (at least for HSP70 and HSC70). It is remarkable to observe that HSC70 is not responsive to a NLHS in the control population but sensitive to NLHS in TF12. In contrast, for *BIP*, *HYOU1*, *HSPA4,* and *PDI*, the effect of NLHS was not dependent on selective breeding for induced thermotolerance (Fig 4.7).

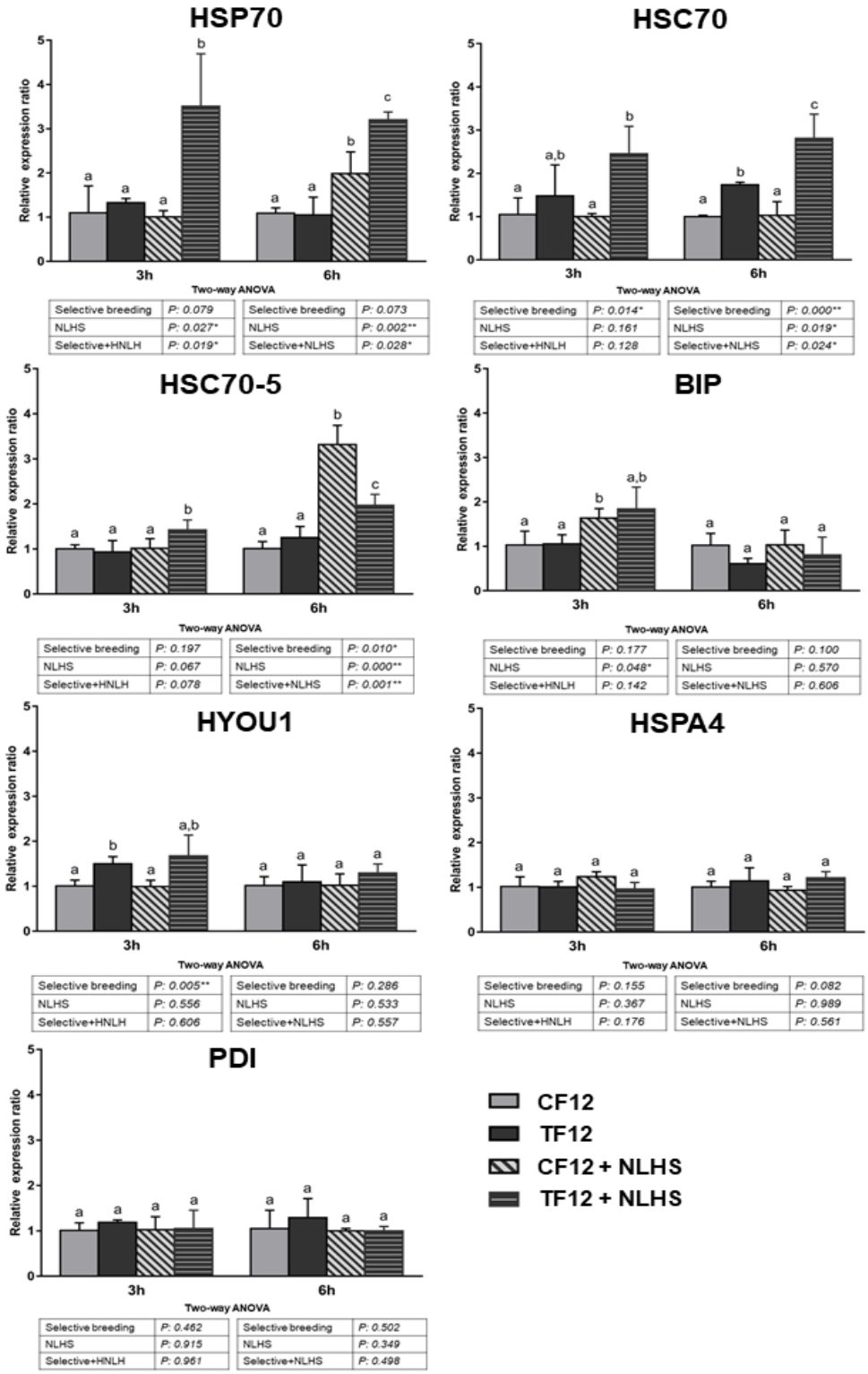

Figure 4.7 HSP70s family genes expression level after NLHS treatment of nauplii CF12 and TF12. Four groups of swimming nauplii; CT12 (non-selective breeding for induced thermotolerance animals), TF12 (selective breeding for induced thermotolerance

animals) were treated with NLHS (CF12+NLHS and TF12+NLHS) and then animals were collected after 3 and 6 h recovery time to determine relative gene expression of HSP70s family genes; *HSP70, HSC70, HSC70-5, BIP, HYOU1*, and *HSPA4* and *PDI* control gene by using qRT-PCR. The EF1α was used as an internal control. The error bars are the SD values from three replicates. Significant differences are indicated as the group of alphabets and are accepted at $P < 0.05$. Two-way ANOVA was performed to check the relation between selective breeding for induced thermotolerance and NLHS factors. Asterisks indicate statistically significant different ratios (* indicates $P<0.05$ and ** indicates $P<0.01$).

4.2 Discussion

Only one HSP70 family gene, *HSP70* (accession number: AF427596), has been identified and reported so far in *A. franciscana*. The *A. franciscana HSP70* mRNA containing 2081 bp was published by Chen and MacRae (2001). In this study, we identified novel HSP70 family genes in *A. franciscana*, namely *HSC70*, *HSC70-5*, *BIP*, and *HYOU 1.*

From the sequence analysis, HSC70 showed high sequence similarity to HSC70 of various organisms. Furthermore, the tetrapeptide GG[X]P and the cytosolic HSC70–specific motif EEVD at the C-terminus were identified in the HSC70 protein sequence. Demand et al. (1998) pointed out that cytosolic eukaryotic HSC70 possess GG[X]P repeats and the EEVD motif at the carboxy terminus whereas other HSP70 family members lack such structural elements (Demand et al., 1998a). The function of the tetrapeptide GG[X]P remains elusive. On the other hand, the EEVD motif is involved in the intramolecular regulation of *HSC70* as a previous report found that the deletion of EEVD motif affected the ATPase activity and the ability to interact with substrates (Demand et al., 1998a; Freeman et al., 1995). The phylogenetic tree revealed that *A.*

franciscana HSC70 was closely related to insect, crustacean and mammalian *HSC70s*, especially *Daphnia magna* (88% identity). Gene expression analysis showed that no significant up-regulation of *HSC70* was observed after NLHS of CF12 animals which suggest constitutive expression and impregnability to stress. These results are congruent with experiments in humans, where this gene was expressed constitutively in most tissues (Dworniczak and Mirault, 1987). Moreover, the level of *HSC70* mRNA in *Tetranychus urticae* (two-spotted spider mite) was not significantly changed by heat and cold shock treatments (Shim et al., 2006) and also in corn earworm (*Helicoverpa zea*), the *HSC70* expression was not induced by heat and cold stress treatments (Zhang and Denlinger, 2010). These results suggest that HSC70 is cytosolic and constitutively expressed. Moreover, some studies reported that HSC70 was translocated to the nucleus upon NLHS (Brocchieri et al., 2008). In addition, *HSC70-5* exhibited high similarity to HSC70-5 of the beetle *Ointhophagus taurus* (82% identity) and was shown to be induced by heat stress similar to the recently discovered *Penaeus vannamei HSC70-5* that could also be induced by thermal stress (Yuan et al., 2017). In S2 cells, *Lv*HSC70-5 has been localized in mitochondria where it functions as the regulator of mitochondrial morphology and cellular homeostasis (Banerjee and Chinthapalli, 2014; Yuan et al., 2017). Furthermore, *BIP* was identified in this study which was very close to the heat shock protein cognate 3 of *Chelax destructor* (88% identity). *BIP* was reported to function in the ER lumen and can be induced by environment stresses (Li et al., 2018b; Luan et al., 2009). Besides, *HYOU1* also was identified. This protein was grouped into group III of the human HSP70 family which was divided into two subgroups: a 105/110 kDa protein and a 170 kDa protein. *HYOU1* coding for the 170 kDa protein (GRP170) was found in animals and plants but seems to be absent in other lineages including fungi (Brocchieri et al., 2008). *HYOU1* is an ER-resident chaperone protein, member of the heat shock protein and ER stress protein families. This gene

locates in the ER via the induction of ER stress (Brocchieri et al., 2008; Giffin et al., 2014).

It appeared that some *Artemia* HSP70 members are closely related to their corresponding HSP70s in crustaceans. On the other hand, some were closely related to their corresponding HSP70s in insects (Hexapoda), maybe because both groups are classified into the same phylum Arthropoda. *Artemia franciscana* is classified into the phylum: Arthropoda, subphylum: Crustacea, and class: Branchiopoda. Phylogenetic clustering showed that HSC70, BIP, and HYOU1 in *Artemia* are closely related to those of Crustacea, more specifically the class Malacostraca, consistent with results obtained by Regier et al. (2010). The alignment of 45 kB DNA sequences from 62 single-copy nuclear protein-coding genes from 75 arthropod species generated a family tree of arthropods in which Branchiopoda cluster together with Multicrustacean (Copepod, Malacostraca, and Thecostraca) in a clade named Vericrustacean (Copepod, Malacostraca,Thecostraca, and Branchiopoda) (Regier et al., 2010). It suggested that *Artemia* (Branchiopoda) is classified into a similar clade with Malacostraca (shrimp, crab etc.). On the other hand, *Artemia* HSC70-5 is closely related to insect HSC70-5 (Hexapoda). This means that *Artemia* (Branchiopoda) might also be closely associated with insects, especially the Hexapoda subphylum. This observation is supported by several recent studies (Eyun, 2017; Oakley et al., 2013; Regier et al., 2005; Reumont et al., 2012; Rota-Stabelli et al., 2013). The studies by Oakley et al. (2013) and Eyun (2017) are based on the use of new transcriptomes and a new morphological matrix (including fossils), but also existing expressed sequence tags, mitochondrial genome, nuclear genome, ribosomal DNA data, and 24 nuclear protein-coding genes to generate new insights into Pancrustacean phylogeny. The results revealed that Branchiopoda are considered to belong to the clade Allotriocaride similar to Hexapoda explaining why some crustacean genes are closely related to insects (Hexapoda). However, according

to several phylogenetic analyses on Arthropods (Eyun, 2017; Jondeung et al., 2012; Oakley et al., 2013; Regier et al., 2005; Regier et al., 2010; Reumont et al., 2012; Rota-Stabelli et al., 2013), we found the different classification results of Branchiopoda (*Artemia*) with other class in phylum Arthropods was depending on the cluster of input data. For example, the phylogenetic result based on HSC70-5 (mitochondrial HSP) indicated a close relationship with Insects, consistent with Oakley results, where such a result was obtained by including the mitochondrial genome in their analysis (Oakley et al., 2013).

Many studies have indicated that a non-lethal heat shock (NLHS) (but also other stresses) can induce the expression and production of HSP70, such as in *Artemia*, *P. vannamei* , *P. monodon*, and *Perna viridis* (Aleng et al., 2015; Junprung et al., 2017; Norouzitallab et al., 2015; Rungrassamee et al., 2010). However, only the expression of one gene, namely HSP70 has been verified. In our study, the effect of a NLHS was examined not only for HSP70 but also for *HSC70*, *HSC70-5*, *BIP*, *HYOU1*, and *HSPA4*. The results showed that *HSP70*, *HSC70-5*, and *BIP* were significantly ($P<0.05$) induced after NLHS (see Fig 3.24). HSC70-5 has been identified before to be induced by heat stress in *P. vannamei* (but also by WSSV virus infection) (Yuan et al., 2017). As a member of the HSP70 family, HSC70-5/S70P is localized in the mitochondria and confers thermal tolerance by preventing protein aggregation, but also regulates mitochondrial morphology and cellular homeostasis (Banerjee and Chinthapalli, 2014). Similarly, *Lv*HSC70-5 was also localized in the mitochondria and was up-regulated by both heat and cold shock treatments. Therefore, *Lv*HSC70-5 likely works as a protein chaperone to engage in *L. vannamei* tolerance to thermal stress. Aquatic invertebrates are mainly dependent on HSP70s, as observed in the genome of the oyster *Crassostrea gigas*, which encodes for >88 HSP70s (compared to 17 in humans), which are involved in cellular protection against heat or other stresses (Zhang et al., 2012a). In oyster, it has

been described that a HSP70 family member located in the ER are heat shock inducible (Zhang et al., 2012a). Moreover, an experiment on thermal acclimation in *D. melanogaster* demonstrated that the up-regulation of transcriptional activity and the change in protein abundance of HSP70 were observed in 31 °C-acclimated (heat acclimation) flies when compared with 25 °C-acclimated flies. A significantly higher survival rate ($P<0.001$) was shown in heat-acclimated flies exposed to acute high temperature, suggesting that HSP70 plays a more important role in the acquisition of thermotolerance during acclimation (Colinet et al., 2013). The previous study and this report suggest that HSP70 family members either located in the cytosol (HSP70), mitochondria (HSC70-5) and ER (BIP) are heat shock inducible.

In the TF12 *Artemia* population, obtained after 12 generations of selective breeding for induced thermotolerance, significantly higher expression for *HSP70* and *HSC70* was observed relative to the control population (CF12), suggesting that this is linked to the increasing selective pressure applied over the 12 generations. This result is similar to the selective breeding for induced thermotolerance in sunflower where thermotolerant lines showed enhanced expression of heat shock proteins. The expression of HSP 18.1, HSP 90 and HSP 104 was induced upon heat-induction (Kumar et al., 1999). However, in our study, we determined for the first time the expression pattern profile of different members of HSP70 family genes in a line selectively breed for induced thermotolerance. Moreover, our results showed that selection can influence the NLHS response: selection for induced thermotolerance significantly increased ($P<0.05$) the induction of *HSP70*, *HSC70* and *HSC70-5* in TF12 after NLHS. On the other hand, HYOU1 and HSPA4 did not show significantly up-regulation after NLHS, for both CF12 and TF12 populations.

In summary, this study identified novel genes from the HSP70 family in *Artemia* and highlighted their probable functional association with heat stress. *HSP70*, *HSC70*

and *HSC70-5* exhibit responses to heat stress and might represent a potential biomarker for selective breeding for the induced thermotolerance of *A. franciscana*. Taken together, these results suggest that those HSP70 family genes possibly contribute to the process of thermal tolerance in *A. franciscana*. HSC70, generally accepted to be expressed constitutively, displays increased expression after selection for induced thermotolerance and becomes responsive to a non-lethal heat shock is such population.

4.3 Materials and methods

4.3.1 Identification of the HSP70 genes family in *Artemia*

4.3.1.1 Experimental animals

Artemia cysts originating from the San Francisco Bay (SFB, ARC1768) were hatched under the axenic system beginning with a decapsulation step (Baruah et al., 2015). In short, the cysts were hydrated in 10 mL sterile distilled water for 1h with aeration. After hydration, adding 330 μL 32% w/v NaOH and 5 mL 14% w/v NaOCl. The decapsulation step was terminated by adding 5 mL 10 g/L autoclaved $Na_2S_2O_3$. Next, the decapsulated cysts were then abundantly washed with 35 g/L sterile artificial seawater (Aquarium Systems) and were suspended in 50 mL Falcon™ tubes containing 30 mL sterile artificial seawater. Then, the decapsulated cysts were incubated on a rotor for 28 h at 28 °C with constant illumination of approximately 27 μE/m2/s. Both decapsulation and hatching procedures were performed under a laminar flow hood and using autoclaved materials (121 °C for 20 min) for preserving axenic conditions. After 28 h, swimming nauplii were transferred to 1-L glass bottles containing 800 mL sterile artificial seawater and fed with live algae *Tetraselmis suecica* for 18 days until juvenile state.

4.3.1.2 Identification of the HSP70 family in the *Artemia* transcriptome database

To identify HSP70 gene family genes, reference genes from other species phylogenetically as close as possible to *Artemia*, containing putative conserved HSP70 domain (Table 2.3) were blasted against an *Artemia*_transcriptomic database (ARC Laboratory, Ghent University). The matching nucleotide sequences were used to design specific primers (Table 4.1). RNA was extracted from juvenile *Artemia* which was described in 4.3.1.1 using RNeasy Plus Mini Kit (Qiagen). The purity and quantity of the RNA were determined by 2 % agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific), respectively. The first strand cDNA was synthesized from 1 µg of total RNA using the RevertAid™ Hminus First-strand cDNA synthesis kit (Fermentas). Then, the cDNA was used for PCR amplification in a total volume of 50 µl containing 1.25 unit of DreamTaq DNA Polymerase (Thermo Scientific), 1x DreamTaq Buffer, 0.2 mM each dNTP, 0.5 µM of each primer. After an initial denaturation step at 94 °C for 2 min, the PCR was done by 30 cycles at 94 °C for 30 s, gene-specific annealing temperatures (Table 4.2) for 30 s and 72 °C for 3 min, finishing the reaction at 72 °C for 10 min. The PCR product was purified by using Wizard® SV Gel and PCR Clean-Up System (Promega). The purity was checked by agarose gel electrophoresis. The concentration was measured with NanoDrop 2000 spectrophotometer before sending for sequencing with an automated sequencer (Sanger sequencing) by a commercial service (LGC Genomics).

Table 4.1 The reference HSP70 family genes from other species

Gene / Species	Accession Number
HSP70 (*Danaus plexippus*)	EHJ73892.1
HSP70 (*Daphnia magna*)	EU514494.1
HSP70 (*Artemia sinica*)	KF683905.1
HSP70 (*Bombyx mori*)	BAF69068.1
HSP70 (*Tigriopus kingsejongensis*)	APH81352.1
HSP70 (*Cotesia chilonis*)	AKA09521.1
HSP70 (*Orius sauteri*)	AIK01869.1
BIP (*Litopenaeus vannamei*)	AFQ62791.1
GRP78 *(Aphis gossypii*)	AKO69637.1
BIP (*Litopenaeus vannamei*)	AFQ62791.1
HSC70 (*Plutella xylostella*)	AFC38439.1
HSC70 (*Metapenaeus ensis*)	ABF20530.1
HSC70 (*Cotesia chilonis*)	AKA09522.1

4.3.1.3 Sequence analysis

The complete ORF sequences of HSP70 family were blasted using BLASTX (https://blast.ncbi.nlm.nih.gov) to identify the conserved domains and the most homologous protein with the following parameters: database: non-redundant protein sequences (nr), organism: all (default) and algorithm: blastp (protein-protein BLAST). After that, phylogenetic analysis of each HSP70 family candidate gene was performed. Sequence alignments using ClustalW were done with amino acid sequences of the candidate HSP70 family genes based on the full-length deduced amino acid sequences of the presumed orthologous genes in typical species with the highest scores in blastp. The ClustalW alignments were done with two alignment method: pairwise alignment (10,

0.1) and multiple alignments (10, 0.2). This numbers in parentheses represent gap opening penalty and gap extension penalty respectively. The delay divergent cut-off was 30%. Then, the phylogenetic tree was constructed by the neighbor-joining (NJ) algorithm using the MEGA 7.0 software. Bootstrap sampling was reiterated 1000 times. The conserved domain was analyzed by ExPASy web (https://prosite.expasy.org/). Moreover, the protein molecular mass and theoretical isoelectric point (PI) were predicted by the ExPASy web.

Table 4.2 Nucleotide sequence of the primers designed for *Artemia* HSP70 family.

Primer name	Sequence (5'-3')	Purpose	Annealing (°C)
HSC70-F	GCATTCTTACAATTGAAGAAGGTAT	RT-PCR	55
HSC70-R	ATCGGGGTAGCAAGAACAATCCATTT		
HSC70-5-F	ATGCTGAGTGCATCCAAAGTTTGTT	RT-PCR	55
HSC70-5-R	ACTCCTTCTTCTCTCCTTCTTGT		
BIP/GRP78-F	TTAAACGAAATAAACCAATCAGAATT	RT-PCR	60
BIP/GRP78-R	ATCGGTTGCACTATATCTTCAGCT		
HYOU1-F	ATAGGCTCCTGTCACCGAAACTGAT	RT-PCR	57
HYOU1-R	AGGGAAAATACATGGTCTTGGTTT		
HSPA4-F	AAATCGTTCTACAGCAGCAATAGTTGTCT	RT-PCR	60
HSPA4-R	CCATTATCAAGTCCAGAGGGATCCTGC		
exHSC70-F	ACTTACTCCGACAATCAGCC	qRT-PCR	60
exHSC70-R	CAAATGTCACTTCGATTTGAGGA		
exHSC70-5-F	GAAGGCTCAAGAACAACACC	qRT-PCR	60
exHSC70-5-R	TTCAAATCGTCGACCAATCAAA		
exBIP/GRP78-F	GTGGCTCCACTAGAATTCCTAA	qRT-PCR	60
exBIP/GRP78-R	GTTCACCACTCAAAACTCCAG		
exHYOU1-F	CTAGAAGTTGCTCTTAACAAAGA	qRT-	60

exHYOU1-R	AGATCAGTTAGGTGTGTATAGCAAT	PCR	
exHSPA4-F	TGATTTTGGACAATCGGCTTTAC	qRT-PCR	60
exHSPA4-R	CGATATTTGGTCTTAAATTCTCCGA		
exHSP70-F	CGATAAAGGCCGTCTCTCCA	qRT-PCR	60
exHSP70-R	CAGCTTCAGGTAACTTGTCCTTG		
exPDI-F	AGCGTTCATTGAAGATAATGAAGT	qRT-PCR	60
exPDI-R	CATCACTAACGTCATGATCTGC		
EF1α-F	TCGACAAGAGAACCATTGAAAA	qRT-PCR	60
EF1α -R	ACGCTCAGCTTTAAGTTTGTCC		

4.3.2 Differential gene expression of TF12 and CF12 populations

4.3.2.1 TF12 and CF12 populations

In this experiment, we used two *Artemia* populations developed and selectively bred by ARC Laboratory, Ghent University. Cysts of *A. franciscana* from San Francisco Bay (SFB, ARC1767) were hatched non-axenically. After that, swimming nauplii were collected and divided into two groups. The first group was exposed to a non-lethal heat shock (NLHS) at 37 °C for 30 min and then subsequently transferred to 28 °C for a 5 h recovery. These animals were subsequently exposed to a lethal heat shock (LHS) at 41 °C for 10 min. After that, the nauplii were transferred back to 28 °C and survivors were collected (on average 1%; called TF population). On the other hand, the second group was grown isothermally, and this group is referred to as the CF population. The animals from the TF or CF populations were divided equally into 5 tanks containing approximately 40 L of 35 g/L synthetic sea water with continuous aeration and illumination of approximately 27 μE/m2/s of 16 h per day. The animals were fed with a variety of feed which are brown and green microalgae, dried algae, live *T. suecica,* and *Artemia* enrichment media. In detail, we used different algae pastes; Nannochloropsis

(Nanno 3600), Pavlova (Pavlova 1800), Isochrysis (1800), and Tetraselmis from Reed Mariculture company, Freeze dried algae; Phytobloom freeze dried Nannochloropis (Necton), Enrichment product (Bernaqua), and live algae; *T. suecica* (cultured at ARC Laboratory). The animals were fed at every alternate day with a mixture of either *Nannochloropsis* and *Isochrysis* or *Tetraselmis* and Pavlova. Feeding was performed through automatic feeding several times a day and also during the night. During this time, the diluted algae pastes were maintained at 4 °C and continuous aeration was provided. The animals were always supplemented with dried algae mix and a small amount of live *Tetraselmis*. Dried and live algae were added manually only once a day to each tank. The animals also supplemented them with enrichment product. Feeding was done after cleaning. Tanks were cleaned and water was changed daily. As explained in Fig. 4.8, the parental generation animals were grown to adult and the produced nauplii (F1 generation) were collected, then treated with a similar procedure as the parental generation as described above with the exception that the lethal heat shock was more severe every generation. LHS conditions are listed in Table 4.3. Cysts produced by every generation were collected as well. The production of CF12 and TF12 animals took about one year (on average one month per generation). Cysts of CF12 and TF12 generations were available at Ghent University.

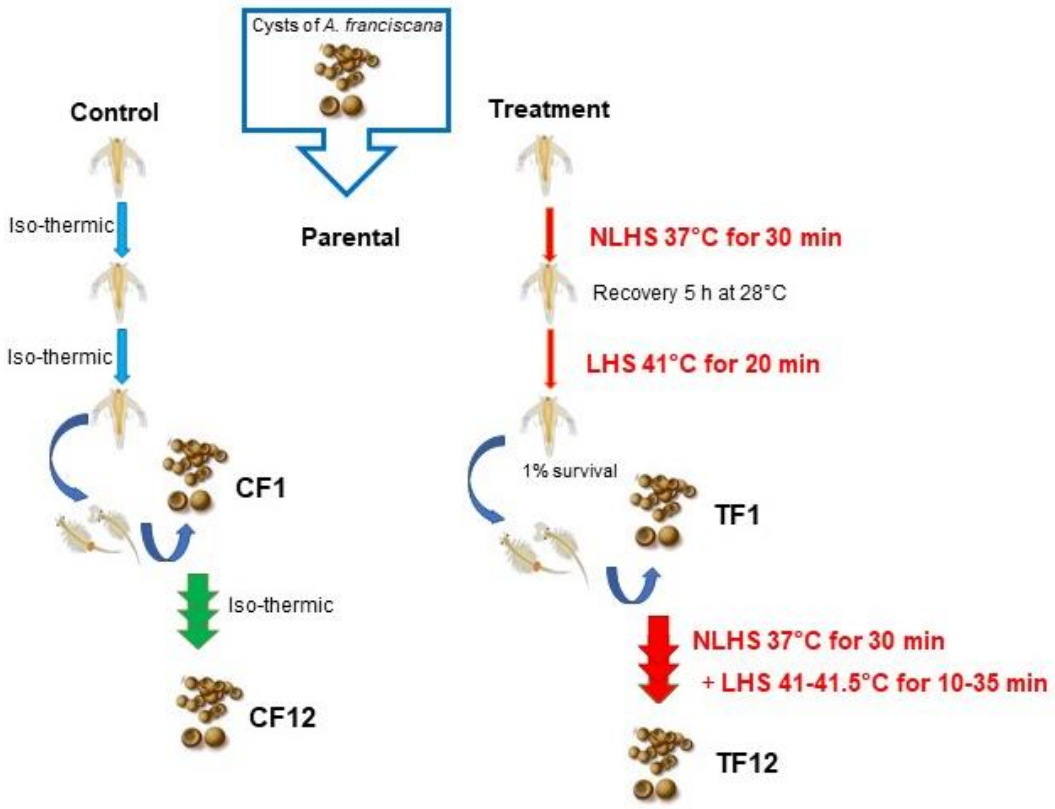

Figure 4.8 Schematic of the production of CF12 and TF12 Upon hatching, the F0 progeny (Parental) were divided into two groups. One group was exposed to NLHS (37 °C for 30 min) followed by 5 h recovery period at 28 °C and the second LHS at 41 °C for another 20 min (TF0). The other group was grown isothermally at 28 °C (CF0). After that, the parental (F0) females from the treatment (TF0) and control (CF0) groups produced their next generation larvae i.e., TF1 and CF1, respectively. The F1 larvae from both groups were further exposed to the heat treatment as mention in Table 2.5 until they produced the cysts of F12 (TF12 and CF12).

Table 4.3 Protocol for the production of the TF12 population, surviving increasingly severe lethal heat shock after being exposed to non-lethal heat shock

Generation	*Heat shock procedure*	
	NLHS	LHS
Parental	30 min. 37 °C – 5 h recovery – 20 min. 41 °C	
F1	30 min. 37 °C – 5 h recovery – 10 min. 41 °C	
F2	30 min. 37 °C – 5 h recovery – 13 min. 41 °C	
F3	30 min. 37 °C – 5 h recovery – 15 min. 41 °C	
F4	30 min. 37 °C – 5 h recovery – 18 min. 41 °C	
F5	30 min. 37 °C – 5 h recovery – 20 min. 41 °C	
F6	30 min. 37 °C – 5 h recovery – 20 min. 41 °C	
F7	30 min. 37 °C – 5 h recovery – 20 min. 41 °C	
F8	30 min. 37 °C – 5 h recovery – 25 min. 41 °C	
F9	30 min. 37 °C – 5 h recovery – 30 min. 41.5 °C	
F10	30 min. 37 °C – 5 h recovery – 30 min. 41.5 °C	
F11	30 min. 37 °C – 5 h recovery – 35 min. 41.5 °C	

4.3.2.2 Axenic hatching of populations TF12 and CF12

TF12 and CF12 cysts were hatched under axenic conditions by starting with the decapsulation step as described in 4.3.2.1. After 28 h, swimming nauplii were selected, then transferred to the new 50 mL Falcon™ tubes containing 30 mL sterile 35 g/L artificial seawater and cultured on a rotor incubated for 28 h at 28 °C with constant illumination of approximately 27 μE/m2/s. After that, the swimming nauplii were

separated into two groups: one was used to study gene expression under NLHS treatment (4.3.2.3). Another one was fed with a variety of feed, such as brown and green microalgae, dried algae, live *T. suecica,* and *Artemia* enrichment media. The animals were grown for 18 days after hatching to the juvenile stage and used for gene expression analysis (2.18.4).

4.3.2.3 Non-lethal heat shock treatment and animal sampling

Swimming nauplii (approximately 600 animals) both of TF12 and CF12 were divided into two groups and each group was transferred to a 50 mL glass tube containing 30 mL sterile 35 g/L artificial seawater in three biological replicates. Three circulating water baths (Lauda) were set at the respective temperatures of 60 °C for immediate temperature increase (temperature boosts), at 37 °C for a NLHS incubation and at 28 °C for recovery at ambient temperature. CF12 and TF12 nauplii were exposed to a NLHS by immediate incubation at temperature boost to increase the inside tube temperature to 37 °C, then transferred to the NLHS bath to maintain 37 °C for 30 min. Finally, they were transferred back to ambient temperature for recovery. CF12 and TF12 nauplii incubated at 28 °C acted as a control. The treated animals were passed through an autoclaved filter, and samples were collected after 3 h and 6 h recovery in each biological replicate tube, and then stored at 80 °C until they were used for differential gene expression analysis (4.3.2.4).

4.3.2.4 Gene expression analysis by qRT-PCR

To examine the HSPs gene family expression level in juvenile populations of CT12 and TF12 (see 4.3.2.2) and the effect of NLHS on thermal tolerance of selectively bred *Artemia* nauplii from 4.3.2.3, total RNA was extracted from juvenile and nauplii samples using RNeasy Plus Mini Kit (Qiagen). The purity and quantity of the RNA were

determined by 2 % agarose gel electrophoresis and NanoDrop 2000 spectrophotometer (Thermo Scientific), respectively. The first strand cDNA was synthesized from 1 µg of total RNA using the RevertAid™ Hminus First-strand cDNA synthesis kit (Fermentas)

The qRT-PCR was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) with specific primers, for HSP70s family genes; HSP70, HSC70, HSC70-5, BIP/GRP78, HYOU1 and HSA4 and Protein disulfide isomerase (PDI), a chaperone activity gene, as a control. The housekeeping gene, *EF1α* was used as an internal control (Table 4.2). The cycling parameters started with an initial activation at 95 ºC for 5 min followed by 40 cycles of 95 ºC for 15 s, 60 ºC for 30 s, and 72 ºC for 30 s. The fluorescent signal intensities were recorded at the end of each cycle. Melting curve analysis was performed from 55 ºC to 95 ºC with continuous fluorescent reading every 0.5 ºC increments to confirm that only specific product was amplified. The cycle threshold (Ct) values and fold difference in quantity for each gene were recorded by StepOne™ Software (Applied Biosystems). Relatively to the expression of *EF1α* transcripts, the relative expression was calculated by the $2^{-\Delta\Delta_{Ct}}$ method (Livak and Schmittgen, 2001). The amplification was done in triplicate for each sample. The data were analyzed using a two-way ANOVA (independent parameters; selection and NLHS) followed by Duncan's new multiple range test using the software SPSS.

CHAPTER V

Genotypic and Phenotypic analysis of HSP70 family genes in thermotolerant *Artemia franciscana*

Wisarut Junprung, Anchalee Tassanakajon, Premruethai Supungul, Gilbert Van Stappen, and Peter Bossier

Manuscript in preparation

Abstract

In a previous study, expression analysis revealed that in the juvenile state, the transcript level of *HSP70* and *HSC70* was significantly higher after selective breeding for induced thermotolerance population (TF12) of *Artemia* relative to a control population (CF12). Moreover, after a NLHS treatment of TF12 at the nauplii state, *HSP70*, *HSC70*, and *HSC70-5* transcripts were significantly ($P<0.05$) up-regulated. The result indicated that HSP70 and HSC70 might be associated with selective breeding for induced thermotolerance in *Artemia*. So, SNP polymorphisms of *HSP70* and *HSC70* genes were investigated and the result identified a SNP at position 171 in *HSC70* (C171A; N57K) located in ATP-binding domain which might be potentially associated with the increased thermotolerance. The genotypic analysis result showed a significant ($P<0.01$) difference of the frequency of this SNP between CF12 and TF12. Moreover, the phenotypic analysis in yeast expression (*S. cerevisiae)* system confirmed that yeast containing a HSC70-N57K plasmid could tolerate a higher temperature than yeast containing a WT-HSC70 plasmid. As the N57K genotype was more prevalent in the TF12 population than in the CF12 population, it suggests that this SNP can be used in selective breeding for induced thermotolerance in *A. franciscana*.

In this chapter, the genotype frequencies at the loci in the coding gene of *A. franciscana HSP70* and *HSC70* were investigated in two populations; TF12 and CF12. Moreover, a differential phenotype was demonstrated in yeast by expressing HSC70 recombinantly using two different protocols for verifying thermotolerance in yeast.

5.1 Results

5.1.1 SNP identification of *HSP70* and *HSC70*

In view of the significantly higher expression level of *HSP70* and *HSC70* in the population selectively bred for induced thermotolerance, SNP polymorphisms in *HSP70* and *HSC70* was investigated which might be potentially associated with the increased thermotolerance. The complete ORF of *HSP70* and *HSP70* was amplified from whole *Artemia* cDNAs of the 100 pooled samples of CF12 and TF12 nauplii and analyzed by the Chromas DNA sequencing software (comparing TF12 and CF12). Sequence analysis revealed double peaks at the nucleotide position 171 in TF12 of HSC70 compared to one peak in CF12 (Fig. 5.1) However, such a difference was not found in HSP70 (data not shown). The SNP polymorphism in the cDNA of HSC70 at position 171 is located in the ATP-binding domain and it is heterozygous (C/A) in TF12, but, homozygous of C/C in CF12 (Fig. 5.1). Amino acid sequence analysis showed that this SNP was a non-synonymous polymorphism which changed Asparagine (AAC) to Lysine (AAA). Moreover, the group of amino acid was changed from a polar side chain to an electrically charged side chain (negative charged). To further confirm the specificity of the SNP, HSC70 variation across 15 species including *A. franciscana* was verified by using Clustal tool. The result showed that all species share a similar amino acid at the SNP position, being Asparagine (N) (Fig. 5.2).

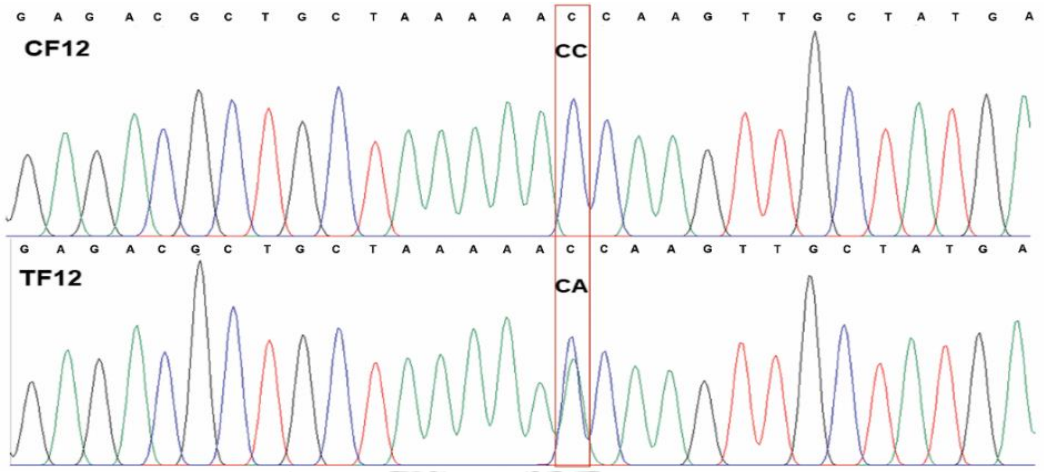

Figure 5.1 Segments of the cDNA sequence electropherograms of HSC70 from the CF12 and TF12 populations. Two peaks were observed in the electropherograms of heterozygotes as shown in red line box on TF12 sample. Genotype is labeled on electropherograms.

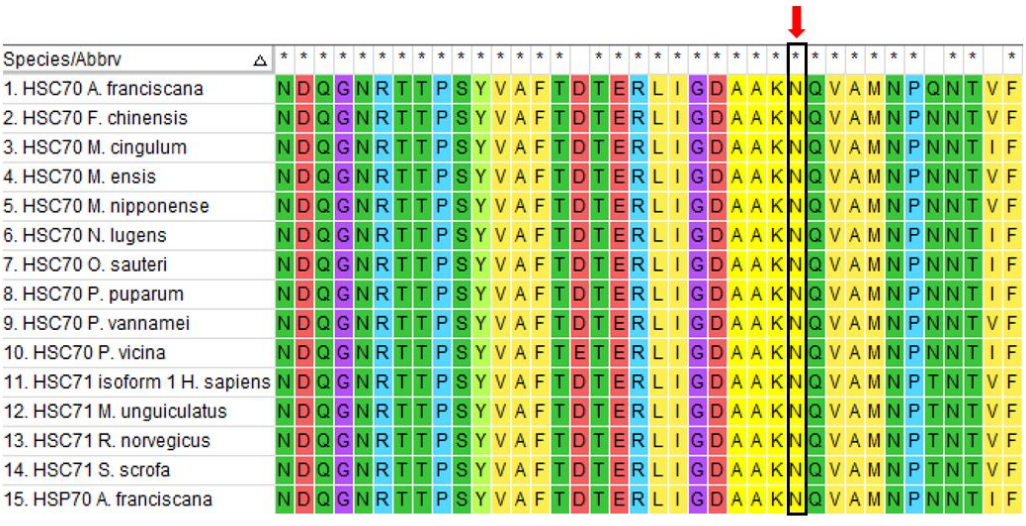

Figure 5.2 The variation of the HSC70 deduced amino acid sequence at the polymorphic position in various species The amino acid position of the SNP is shown in a black box highlighted with a red arrow.

5.1.2 Verification of the *HSC70* SNP and the association with thermotolerance of *A. franciscana*

Approximately 100 individual cysts of TF12 and CF 12 populations were used to identify the genotype of HSC70 at the ATP binding site at the individual level. After genomic DNA extraction of individual *Artemia* cysts, real-time PCR was used to identify the genotype using two allele-specific forward primers derived from the SNP of the ATP binding domain. The size of PCR product was approximately 100 bp as shown on agarose gel electrophoresis representing each genotype. Three different patterns representing genotype C/C, C/A, and A/A were shown in Fig. 5.3A. Cyst number 1 was preferentially amplified with the forward-C primer, but, not with the forward-A primer resulting in a C/C homozygous genotype (Fig. 5.3A). In contrast, cyst number 3 was preferentially amplified with the forward-A primer, but not with the forward-C primer representing an A/A homozygous genotype (Fig. 5.3A). Moreover, cyst number 2 could be amplified with both C and A primers represented a C/A heterozygous genotype (Fig. 5.3A). Examples of eight individual cysts with different genotypes are shown in Fig. 5.3B. The frequencies of different genotypes in 103 individual *Artemia* cysts of the CF12 population were C/C: 72.8%, C/A: 19.4% and A/A: 7.80%. On the other hand, the genotype frequencies of 98 individual *Artemia* cysts of the TF12 population were C/C: 27.5%, C/A: 67.4% and A/A: 4.10% (Table 1). χ^2 test showed a significant difference at N57K site when comparing the CF12 and TF12 population (χ^2 =49.2, *P*=0.000) (Table 5.1).

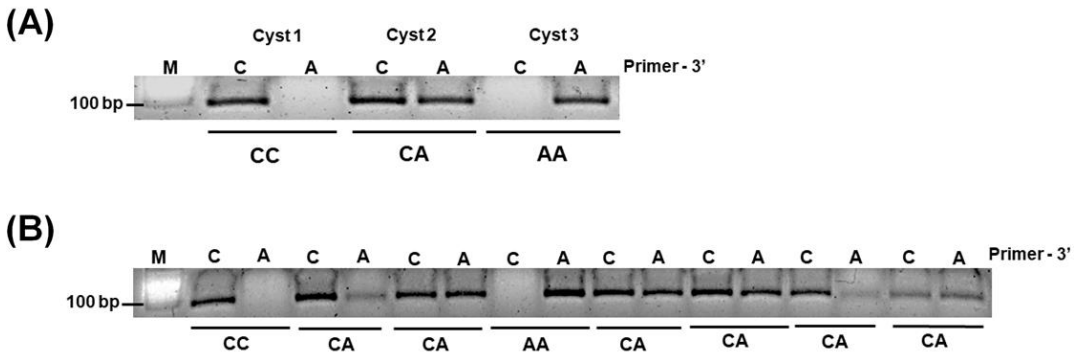

Figure 5.3 Agarose gel electrophoresis for SNP detection. (A) The PCR product pattern for SNP detection by using "C" and "A" primers detecting three possible genotypes (C/C, C/A and A/A). (B) The SNP detection result of eight individual cysts of TF12.

Table 5.1 Genotype frequencies at the SNP position 171 of HSC70 cDNA in CF12 and TF12 populations.

Gene	Position	Domain Position	Ref nt	Var nt	Ref aa	Var aa	Synonymous/ Non Synonymous
HSC70	171	ATP-binding	C	A	N	K	Non Synonymous

171C>A	CF12	TF12	χ^2	*p* value
n	103	98	49.2	0.00 *
Homozygous (CC)	**75** (72.8%)	**27** (27.5%)		
Heterozygous (CA)	**20** (19.4%)	**67** (68.4%)		
Homozygous variant (AA)	**8** (7.80%)	**4** (4.10%)		

*Statistically significant (*P*<0.01)

5.1.3 Phenotypic analysis of the *A. franciscana* HSC70 N57K allelic variant by expression in yeast.

To determine the effect of the SNP (C171A; N57K) on yeast cell growth during heat stress, plasmids were constructed expressing both allelic variants, namely "wild-

type" HSC70 or "N57K" variant of HSC70. The plasmids are transformed into two *S. cerevisiae* strains, namely WT *S. cerevisiae* and an isogenic yeast strain in which 2 HSP70 genes are deleted (Δ ssa1 Δ ssa2). This strain has been described to be heat sensitive (Baxter and Craig, 1998). The production of *Artemia* HSP70 in yeast *S. cerevisiae* was confirmed by western blot analysis. The specific 70 kDa band of *Artemia* HSP70 was found in both yeast strains producing WT HSC70 and the N57K variant (Fig 5.4).

The four strains, namely of WT yeast either transformed with wild type HSC70 or HSC70- N57K, or the Δ ssa1 Δ ssa2 yeast transformed with wild type HSC70 or HSC70-N57K were then serially diluted onto selective medium agar supplemented with galactose for induction. The plates were incubated at various temperatures, namely 28 ºC, 34 ºC, 37 ºC, and 40 ºC for 5 days. As evident from the data presented in Fig. 5.5, the N57K of HSC70 enhanced yeast cell growth at 37 ºC compared with WT HSC70. The enhancement was observed in both yeast strains. Moreover, at 34 ºC only in the Δ ssa1 Δ ssa2 *S. cerevisiae* strain HSC70-N57K showed increased growth. On the other hand, the substantial difference in yeast cell growth was not observed at 28 ºC in both yeast strains. Whereas, at 40 ºC, all yeast cells could not grow.

Moreover, the HSC70-N57K phenotype was confirmed by incubating the four strains under a different temperature regime. For that, the four strains were exposed to a lethal-heat shock (LHS) (40 ºC) for various time intervals, namely 1, 2, 3, and 6 h. After that, the yeast culture plates were transferred to ambient temperature (28 ºC) for 5 days to observe growth. The results revealed that especially after 3 and 6 h exposure to 40 °C, the HSC70-N57K genotype could sustain growth. The effect was more pronounced in the yeast in which ssa1 and ssa2 are deleted (Fig. 5.6).

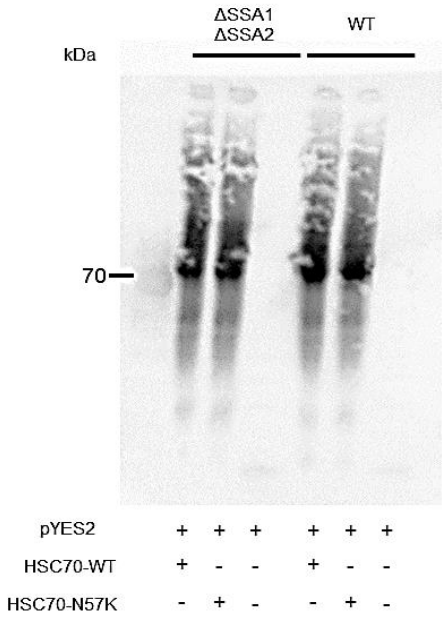

Figure 5.4 Western blot detection of rHSC70 production in yeast *Artemia* HSC70 protein production in each condition was detected by western blot analysis using anti-His tag antibody as the primary antibody and secondary HRP conjugated goat anti-mouse IgG.

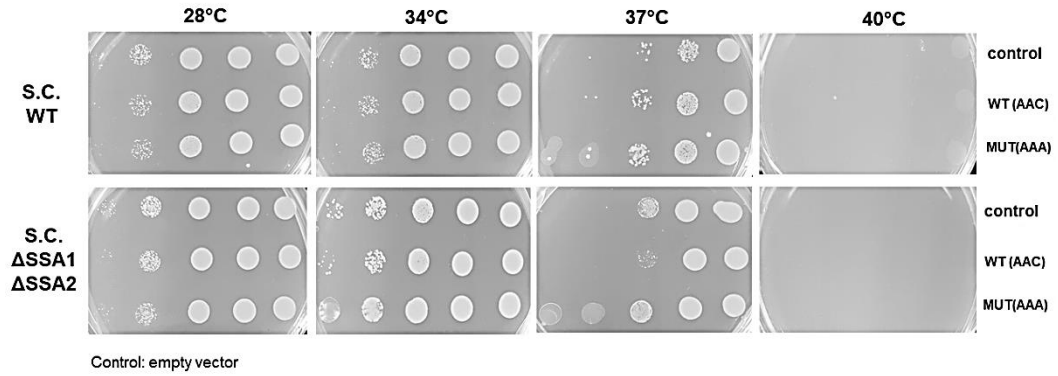

Figure 5.5 The growth of yeast expressing WT-HSC70 and HSC70-N57K at various temperatures A series of 5-fold serial dilution of WT-HSC70 and HSC70-N57K in wild type yeast and the isogenic deletion strain (Δ ssa1 Δ ssa2) of *S. cerevisiae* were spotted on the induction medium agar. Plates are incubated at 28 ºC, 34 ºC, 37 ºC, and 40 ºC, and growth was observed after 5 days incubation.

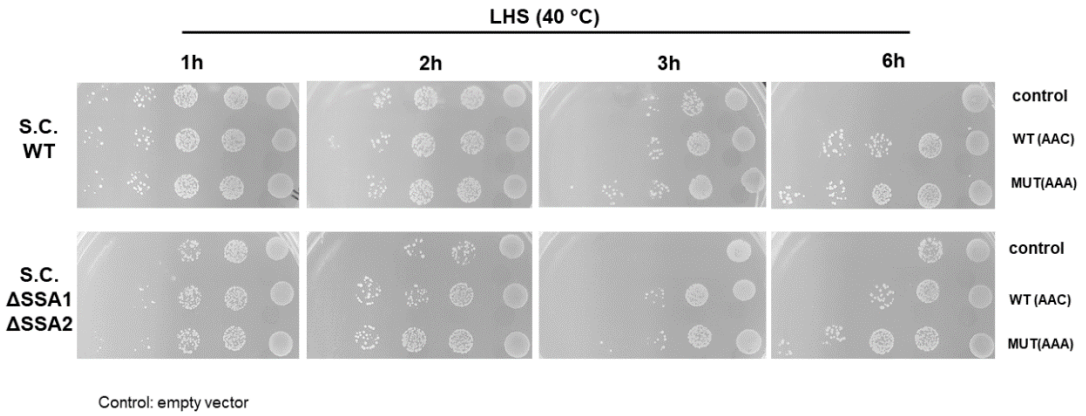

Figure 5.6 The growth of yeast expressing the WT-HSC70 and HSC70-N57K subsequent to a lethal-heat shock during various time intervals A series of 5-fold serial dilution of WT-HSC70 and HSC70-N57K in wild type yeast and its isogenic strain (Δ ssa1 Δ ssa2) were spotted on induction medium agar. The agar plates were incubated at LHS (40 ºC) during various time intervals (1h, 2h, 3h, and 6h). After that, plates were incubated at 28 ºC and cell growth was observed after 5 days incubation.

5.2 Discussion

The difference between the two populations (CF12 and TF12) with respect to induced thermotolerance might be caused by polymorphism including insertion, deletion, and SNP in those genes (either in coding or non-coding regions). The main regulation at the transcription level might depend on the interaction between the transcription factor and their putative elements at the promoter region of HSP70 genes (Yang et al., 2014a). An evolution experiment was conducted to verify that selection acts on *Drosophila* HSP70 expression and inducible thermotolerance (Bettencourt et al., 1999). The result illustrated that both the kinetics and magnitude of HSP70 expression and inducible thermotolerance in more than 20 years of laboratory evolution at different rearing temperatures yielded evolved differences among *Drosophila* populations (Bettencourt et al., 1999). One candidate gene was 87A7 HSP70 cluster, showing

variation in populations (Bettencourt et al., 1999). Two polymorphisms (insertion and deletion) at the promoter region of HSP70 were investigated and suggested that natural selection imposed by temperature and thermal variability might affect HSP70 allele frequencies (Bettencourt et al., 2002). Moreover, Yang et al. (2014) found that heat tolerance of two species of Scallops was associated with the polymorphism in the promoter sequence of HSP70 (Yang et al., 2014a). Beside the promoter region, the previous study also found that the polymorphism especially SNP could be identified in coding region and was called coding-region SNPs or cSNPs. Cargill et al., (1999) described a systematic survey of SNPs in the coding regions of human genes. The identification of SNPs in 106 genes (not included HSP) relevant to cardiovascular disease, endocrinology, and neuropsychiatry were screened in an average of 114 independent alleles using two independent screening methods. The result found 560 SNPs, including 392 cSNPs divided roughly equally between those causing synonymous and non-synonymous changes. The cSNPs most likely influence disease and alter the amino acid sequence of the encoded protein (Cargill et al., 1999). Moreover, cattle populations were screened for polymorphisms in the leptin gene and five cSNPs were found in the regions containing the coding sequences that might associate with feed intake and fat-related traits (Lagonigro et al., 2003). In addition, Nikbin et al. (2014) identified the novel SNPs in HSP70 coding region and their association with semen quality traits on goats. The result found two synonymous SNPs and these two SNPs might affect semen quality (Nikbin et al., 2014).

So, we assumed that several organisms might tolerate heat stress because the increased transcription level leads to an increase in protein expression level. Moreover, the SNPs of coding gene are interesting because some of them, called non-synonymous SNPs introduce amino acid polymorphisms into their encoded proteins causing structural change of the protein (Chasman and Adams, 2001). Whole-transcriptome or

transcriptome sequencing approached are powerful bioinformatic research tools which can explain these non-homogenous HSP70 expression trends. Though there was some inconsistency in previous studies, the regulation of gene expression and the confirmation of the protein structure of the HSP70 and HSC70 were believed to play essential roles in heat stress response and are believed to be related to selective breeding for induced thermotolerance.

In this study, the DNA sequences of complete ORFs of *A. fanciscana HSP70* and *HSC70* in TF12 and CF12 were verified. The result identified a single nucleotide polymorphism that classified into non-synonymous polymorphism in HSC70 of TF12, yet no SNP was found in HSP70. This polymorphism was at the position 171C⟶A of the cDNA which located at the ATP-binding domain and changed asparagine to lysine at amino acid position 57. However, in CF12 population only 171C not 171A was found. As mentioned, the SNP polymorphism is located in the conserved domains of HSC70 that is an ATP-binding domain. This domain has been reported to be essential for the inhibition of the release of the second mitochondria-derived activator of caspase (Smac) and apoptosis in C2C12 cells (Jiang et al., 2009). It has been demonstrated that HSP70 overexpression markedly inhibited the release of Smac and prevented the activation of caspases-9, caspases-3, and apoptosis in C2C12 cells under H_2O_2 treatment. According to co-immunoprecipitation, the result revealed no direct interaction between HSP70 and Smac. However, the mutation analysis showed that the ATP-binding domain of HSP70, rather than the peptide-binding domain, was essential for these observed HSP functions (Jiang et al., 2009). Not only HSP70 gene, but the effect of SNP of ATP-binding domain was also found in Rho, a cellular protein required for certain transcription termination events in *E. coli*. The SNP which changed phenylalanine to cysteine mutation at residue 232 in the ATP-binding domain of Rho was observed. This mutation thus appeared to uncouple the protein's ATPase activity from its helicase

enzyme function, so Rho can no longer harness available energy for use in subsequent reactions (Pereira and Platt, 1995). All results suggested that the ATP-binding domain is an essential domain that prevents cell damage and provides energy during the cell stress response. SNPs in that domain might change the dynamics of ATP metabolisms, although this needs to be verified.

To verify the hypothesis that this SNP in the ATP binding domain is associated with heat tolerance, the allele and genotype frequencies at locus in the coding gene of *A. franciscana HSC70* was verified. The result showed significantly (P<0.01) different genotypes ratio's (WT-HSC70 and HSC70-N57K) between two populations (TF12 and CF12). Moreover, the phenotypic analysis result showed that yeast (*S. cerevisiae*) that contained HSC70-N57K plasmid could tolerate higher temperature than yeast containing WT-HSC70 plasmid. Taken together, the shift in genotypic ratio's in the two populations and the increased thermotolerance in the TF12 *Artemia* population and the in the yeast expressing HSC70-N57K, suggest that this SNP is linked to increased thermotolerance. This strong association between genotype and phenotype makes the HSC70-N57K SNP a potential marker for the selection of *A. franciscana* for induced thermotolerance and maybe other crustaceans. Unraveling the biochemical consequences of such a mutation is important for understanding the fundamentals of the genetic adaptation processes and evolution in organisms (Loewe and Hill, 2010).

5.3 Materials and methods

5.3.1 Single nucleotide polymorphism (SNP) analysis

5.3.1.1 *HSP70* and *HSC70* SNP polymorphism identification in *A. franciscana*

The stored cysts of CF12 and TF12 were hatched under the axenic system as described in 4.3.2.1 to obtain nauplii. After that, total RNA extraction was performed with

100 pooled nauplii in each population. Then the cDNA was synthesized from 1 µg of total RNA. cDNA of TF12 and CF12 were used to amplify HSP70 and HSC70 by the full-length ORF primers (Table 2.6). Next, the PCR products were purified before sending for sequencing. The different genotypes of HSP70 and HSC70 in both populations were determined by the segments of the cDNA sequencing electropherograms results using the Chromas DNA sequencing software. Then the SNP position was identified and compared with other species using Clustal Omega.

5.3.1.2 SNP polymorphisms screening of two *A. franciscana* populations

The SNP was scored in approximately 100 individual cysts of both populations. The stored CT12 and TF12 cysts were washed with sterile distilled water, then each individual cyst was separated into 1.5 microcentrifuge tube, one cyst per tube. DNA was extracted by using the DNA extraction kit (Wizard® SV Genomic DNA Purification System, Promega) with some modification. In short, the individual cyst was incubated with sterile distilled water at room temperature overnight (16-18 h). After that, water was eliminated then followed by adding the digestion solution (nuclei lysis solution, 0.5M EDTA (pH.8.0), proteinase K (20 mg/mL) and RNase A solution) and incubated overnight at 55 °C in the water bath (Grant Instruments). Next day, genomic DNA was extracted following the standard protocol (Promega). A sequence-specific PCR amplification technique was used with two allele-specific forward primers which differ at the 3'-end of the primer, matching either to the wild-type template or N57K variant of HSC70 template (Table 5.2). The real-time PCR was developed to identify the target sequence which is preferentially amplified or poorly amplified. Each genomic DNA sample from individual cysts was amplified by using qRT-PCR StepOnePlus™ Real-Time PCR System (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) with forward and reverse primers, specific for SNP polymorphism

(Table 5.2) that was identified in 2.19.1. The cycling parameters started with initial activation at 95 ºC for 5 min followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 45 s and 72 ºC for 30 s. The fluorescent signal intensities were recorded at the end of each cycle. Melting curve analysis was performed from 55 ºC to 95 ºC with continuous fluorescent reading every 0.5 ºC increments to confirm that the product was amplified. After that, agarose gel electrophoresis was performed to confirm the presence of an amplicon depending on the primer pair used. The PCR fragment amplifications from individual cysts both of CF12 and TF12 different genotypes were analyzed. The Chi-squared test (χ^2 test) was employed for testing significance of allele frequencies and genotype frequencies by using IBM SPSS software. The differences were considered statistically significant $P<0.05$.

Table 5.2 Nucleotide sequence of the primers designed for genotype and phynotype investigation.

Primer name	Sequence (5'-3')	Purpose	Annealing (°C)
SNP-WT HSC70-F (AAC)	GGAGACGCTGCTAAGAAC	PCR-SNP detection	60
SNP- N57K HSC70-F (AAA)	GGAGACGCTGCTAAGAAA		
SNP-HSC70-R	TGGAATTCAAAGCAGAGCCT		
NotI-HSC70-F	GCGGCCGCATGGCAAAAGCACCT GCAATTGG	PCR-vector construction	58
R-HSC70-XbaI	ATCCACTTCTTCAATGGTTGGTCTC TAGA		

5.3.2 Phenotype analysis by yeast cell growth and thermotolerance selection

5.3.2.1 Vector construction

The full-length ORF HSC70 was amplified from the cDNA of 100 pooled samples of TF12. The PCR products were checked by agarose gel electrophoresis. After PCR purification step, purified PCR products were cloned into pGEM cloning vector (Promega), then transformed into the *E. coli* competent cells (JM109). The Blue-white screening was performed to select for white colonies that contained inserted HSC70 fragment. To separate the WT HSC70 and the N57K variant of HSC70 genotype, colony PCR was performed. All white colonies were picked and dissolved in sterile distilled water and used as PCR template. Each genotype was selected with two allele-specific forward primers (Table 5.2) in a total volume of 50 µl containing 1.25 unit of DreamTaq DNA Polymerase (Thermo Scientific), 1x DreamTaq Buffer, 0.2 mM each dNTPs, 0.5 µM of each primer. After an initial denaturation step at 94 °C for 2 min, the PCR was done by 30 cycles at 94 °C for 30 s, gene-specific annealing temperatures (Table 5.2) for 30 s and 72 °C for 3 min, finishing the reaction at 72 °C for 10 min. After that, both of WT HSC70 and N57K variant of HSC70 PCR products were purified and then sent for sequencing to confirm the SNP polymorphism at position 177 of the nucleotide sequence. Next, the DNA fragments containing the HSC70 full-length ORF both of WT HSC70 and N57K variant of HSC70 were amplified with the full-length ORF primer conjugated with restriction enzyme site (*Not*I-F and R-*Xba*I) (Table 5.2). The DNA fragments were produced by PCR. They were then inserted into the *Not*I-*Xba*I sites of the *Saccharomyces cerevisiae* yeast expression vector which is pYES2/CT (Invitrogen). The resulting vectors were named pYES2-HSC70-WT and pYES2-HSC70- N57K.

5.3.2.2 Protein expression and thermotolerance selection

The constructed vectors were again sequenced to confirm the whole sequence including the N57K variant position. After that, pYES2-HSC70-WT, pYES2-HSC70- N57K and the control (empty vector of pTES2/CT) were transformed by using S.c. EasyComp™ Transformation kit (Invitrogen) to *S. cerevisiae* isogenic wild-type strain (SL314-A1, MATa leu2-3, 112 trp1-1 ura 3-11, 15 leu2Δcan1-100 ade2-1) and the isogenic deletion of ssa1Δ ssa2Δ strain (SL314-A1, MATa trp1-1 ura3-1 his3-11,15 leu2Δcan1-100 ade2-1 ssa1::HIS3 ssa2::LEU2). These were sourced from the Susan Lindquist lab and kindly provided by Elizabeth Craig (University of Wisconsin, Madison, WI). A single colony was inoculated into 15 mL of the appropriate SC selective medium (6.7 g yeast nitrogen base with amino acid (Sigma), 0.1 g leucine (Sigma), 0.1 g lysine (Sigma), 0.1 g uracil (Sigma), 0.1 g adenine (Fluka) and 2% glucose (VMR chemicals)). The culture was incubated overnight at 28 ºC on shaking incubator. After that, the OD_{600} of the overnight cultures were determined and then adjusted with induction medium (SC selective medium without glucose, 20% galactose (Alfa Aesar) until OD_{600} was 0.4 in total 50 mL. The overnight adjustment cultures were pelleted and then resuspended with 50 mL induction medium and cultured at 28 ºC for 24 h on shaking incubator. After that, the OD_{600} of the overnight cultures were determined again and adjusted to 1.0 in total of 2 mL. The adjustment cultures were used for serial dilution. A series of 5-fold serial dilution was made and spotted on the induction medium agar. The thermotolerance verification was performed in two ways. In a first approach, the medium agars were incubated at various temperatures (28 ºC, 34 ºC, 37 ºC, and 40 ºC) for 5 days. In a second approach, the medium agars were incubated at a high temperature (40 ºC) for different time periods (1h, 2h, 3h, and 6h). After that, the plates were transferred to 28 ºC for 5 days. The yeast survival (as scored by regrowth) was

observed at day-5 after incubation. The production of each recombinase HSC70 protein was detected by western blot analysis using anti-His tag antibody (1:1000; Thermo Scientific) as primary antibody and secondary 1:3000 diluted HRP conjugated goat anti-mouse IgG (Jackson Immune Research).

CHAPTER VI

GENERAL DISCUSSION, CONCLUSSION & FUTURE PERSPECTIVE

6.1 General discussion

Acute hepatopancreatic necrosis disease (AHPND) caused by the bacterium *Vibrio parahaemolyticus* carrying toxin producing plasmid (VP_{AHPND}), has led to severe mortalities in farmed penaeid shrimp worldwide. The causative agent of AHPND has been reported as a unique strain of the bacterium *V. parahaemolyticus* (Joshi et al., 2014). The VP_{AHPND} colonizes at shrimp stomach and produces soluble toxins that enter the hepatopancreas causing cell sloughing and finally shrimp mortality (Sirikharin et al., 2015). Nowadays, several VP_{AHPND} draft genome sequences have been reported and they revealed that this VP_{AHPND} strain has a pVA1-like plasmid carrying *pirAvp* and *pirBvp* genes that can produce the toxins. Several publications reveal sequencing results of many strains of VP_{AHPND} that were isolated and identified in several countries around the world (Devadas et al., 2018; Kumar et al., 2018a). AHPND toxicity requires the production of both toxins to kill the shrimp while there is no evidence that either *pirAvp* or *pirBvp* alone can cause AHPND pathology at realistically natural concentrations (Prachumwat et al., 2019). Many isolates of *V. parahaemolyticus* containing toxins have been reported for example three bacterial strains (1D, 3HP, and 5HP) from a shrimp farm in Thailand that was experiencing massive death within 35 days after stocking (Joshi et al., 2014). Owing to the widespread infection of VP_{AHPND}, the effective treatment for AHPND is urgently required to counteract the high mortality rate of the cultured shrimp.

Several reports have shown that a non-lethal heat shock (NLHS) treatment could enhance resistance to infections in aquatic animals. For example, *Artemia* (*A. franciscana*) which were treated at NLHS temperature of 37 ºC for 30 min, displayed an enhanced tolerance against *V. campbellii* and *V. proteolyticus* infections (Norouzitallab et al., 2015; Yik Sung et al., 2007). Moreover, a NLHS of 38 ºC for 30 min could also

enhance resistance to *V. alginolyticus* infection in Asian green mussel (*Perna viridis*) (Aleng et al., 2015). According to Loc et al. (2013), a NLHS imposed on *P. vannamei* similar to the one described for Asian green mussel could not enhance shrimp tolerance to *V. harveyi* infection (Loc et al., 2013). In this study, we first embarked on a search for a heat treatment that could enhance resistance to bacterial infection in white shrimp. First, the maximum temperature that shrimp could sustain while still displaying 100% survival (NLHS) after heat treatment was verified. Temperatures ranging from 36 °C to 40 °C were used to treat the shrimp for 30 min. Based on this experiment a NLHS on *P. vannamei* was defined as an abrupt transfer from ambient temperature to 38 ± 0.2 ºC for 30 min.

In this study, I presented here for the first time that the NLHS conditions could induce protection against VP_{AHPND} in the Pacific white shrimp *P. vannamei* (Fig 2.2). The gene expression analysis after chronic-NLHS demonstrated that the *LvHSP70*, *LvHSP90*, *LvCrustin*, and *LvproPO* are highly up-regulated suggesting that they might play an important role in bacterial defense (Fig 2.3). Moreover, *LvHSP70* was significantly increased after exposure to VP_{AHPND} (Fig. 2.4). The *LvHSP70*- and *LvHSP90*-silenced shrimp do no longer show bacterial protection induced by the NLHS (Fig. 2.6-2.9) implying that the *Lv*HSP70 and *Lv*HSP90 are the heat stress responsive proteins that mediate the immunity of shrimp against VP_{AHPND} infection.

Heat shock proteins (HSPs) are a family of highly conserved proteins. Their expression responds to environmental stress (Roberts et al., 2010). Most HSPs are generally stress-inducible as they play a particularly essential role in cells that are exposed to stressful conditions. Initially, these molecules were generally thought to occur intracellularly. However, some HSPs are secreted to the cell exterior particularly in response to stress. For this reason, they are generally regarded as danger signaling

biomarkers. In this way, they activate the immune system to react to prevailing adverse cellular conditions (Zininga et al., 2018). For instance, heat stress is a major stress that profoundly affects a cell. The effect of heat stress can disturb cellular homeostasis in all organism, potentially resulting in their death (Aleng et al., 2015). However, to mitigate this stress, metabolite profiles are changed to faster re-establish metabolite homeostasis by, e.g., restoring cell damage (Malmendal et al., 2006). Several reports indicated that HSP induction after heat stress leads to cell protection by activating cellular defense mechanisms such as induced reactive oxygen species (ROS) and catalase (CAT) activity in gills and promoted tolerance against environmental stress (Han et al., 2019; Liu et al., 2018). Moreover, heat shock proteins such as HSP60, HSP70, and, HSP90 are induced under heat stress to protect the host against pathogens and they are thought to mediate humoral and cellular innate immune responses (Borchel et al., 2018; Yik Sung and MacRae, 2013; Zhang et al., 2018)

Understanding the role of HSP70 in the immune system could lead to disease control in aquatic organisms. However, the role of HSP70 in the immune system of aquatic animal is poorly defined although several studies are available on *HSP70* gene expression following bacterial and viral infections. Moreover, HSP70 is a set of family genes that is composed of several genes and each gene has different function and response. Most studies have focused on HSP70. The understanding of the functions of the other members in the HSP70 family could explain how the organism tolerates heat stress and how they contributed to disease resistance. In this study, we used the model crustacean organism, *A. franciscana*, to identify and investigate gene expression of HSP70 family genes in selective breeding program for induced thermotolerance of *A. franciscana*. In addition, a SNP was identified in a HSP70 family member that is apparently contributing to stress resistance.

In the present study, I further investigated the induction of *Lv*HSP70 in shrimp hemocytes upon NLHS treatment at the protein level and found that LvHSP70 was significantly expressed after NLHS (Fig. 3.2). Moreover, the studies of the protective effect of injecting recombinant *L. vannamei* HSP70 (r*Lv*HSP70) against VP_{AHPND} infection was shown. Shrimp survival was observed (Fig. 3.5), and the expression of some immune-related genes was analyzed (Fig 3.7, 3.8). I found that r*Lv*HSP70 could enhance shrimp resistance to VP_{AHPND} infection by inducing shrimp immunity.

During this study, *P. vannamei* genomic data were not available yet. However, *Artemia* draft genome from ARC Lab, Ghent University is available. So, this database was used to identify members of HSP70 family in *Artemia*. Thus far, only one gene of the HSP70 family, namely, HSP70 has been described in *Artemia franciscana* (Kellogg, 1906) (accession number: AF427596). Acknowledging that in all eukaryotes this family has multiple members (Tavaria et al., 1996; Werner-Washburne and Craig, 1989), it is particularly interesting to investigate this family of genes in stress tolerant organism as *Artemia*.

In this study, novel members of the HSP70 family were identified from a draft *Artemia* genome database using bioinformatic tools. Four full-lengths and one partial ORF (Fig. 4.1-4.4) were found. Orthology prediction methods analyzed the complete open reading frame (ORF) for each gene identified. Gene sequences were then subjected to phylogenetic analysis and found that they were classified closely to insect and crustacean. This result was supported by several studies about phylogenetic tree analysis in Pancrustacean (Reumont et al., 2012). Moreover, the result of the expression of the identified HSP70 members in two populations of *Artemia*: a control population (CF12) that experienced 12 generations of isothermal laboratory culture conditions and a selected population (TF12) featuring the survivors of over 12 generations of an induced thermotolerance treatment showed significant up-regulation of HSP70 and

HSC70 in TF12 (Fig. 4.6). In addition, the expression of the newly identified HSP70 members was also examined following non-lethal heat shock (NLHS) and found that HSP70, HSC70-5 and HSC70 were significant induced by heat stress (Fig. 4.7). This result indicated that members of the HSP70 family respond differentially at the transcriptional level to “thermal” selection or to NLHS, or to both. Furthermore, the phenotype and genotype of HSP70 and HSC70 were analysed by gene expression analysis (chapter 4). The different responses of HSP genes between two species located in the different living environments or selective breeding programs with the thermal selection might be caused by the adaptation mechanisms to protect the cell from heat damage. Highly expression of HSP70 at that time is possible for protecting the cell from heat stress.The regulation at the transcription level is mainly depended on the interaction between the transcription factor and their putative trans-actings elements in the promoter regions of HSP70 genes (Zhao et al., 2013). Polymorphism in the promoter sequence was reported in several genes, for example, Yang and team found that heat tolerance of two species of Scallops was associated with the polymorphism in the HSP70 promoter sequence (Yang et al., 2014). However, not only promoter sequence that can obseve SNP, coding SNPs are also interesting, in part, because some of them, termed non-synonymous SNPs, introduce amino acid polymorphisms into their encoded proteins (Chasman and Adams, 2001).

In this study, the sequencing result of full-length ORF of *A. fanciscana HSC70* in TF12 has identified a single nucleotide polymorphism that was classified into non-synonymous polymorphism (Fig.5.1 and Table 5.1). This polymorphism was at the position C171A of the cDNA that located at the ATP-binding domain and changed asparagine to lysine at amino acid position N57K. Together, the phenotype analysis result that the expressed HSC70 recombinant protein in yeast (*S. cerevisiae*) containing MUT (AA) allele showed tolerance to high temperature than that in WT (CC) allele (Fig.

5.5, 5.6). As the 171-AA genotype was more prevalent in the heat-resistant population than in the heat-sensitive population, it was suggested to be a potential marker associated with resistance to heat stress. The characterization of such mutation might provide important insight into understanding the fundamentals of the genetic adaptation processes and evolution in organisms (Loewe et al., 2010).

6.2 Conclusion

In this study, we showed that chronic-NLHS could enhance the resistance of the white-leg shrimp *Penaeus vannamei* to AHPND-causing strain of Vibrio parahaemolyticus (VP_{AHPND}) and the gene expression analysis revealed that *LvHSP70* and *LvHSP90*, as well as other immune-related genes, *LvproPO1* and *LvCrustin1*, were induced under exposure to this heat stress treatment. *LvHSP70* and *LvHSP90* gene knockdown eradicated the VP_{AHPND} resistance in the chronic-NLHS treated shrimp and decreased PO activity suggesting the involvement of these proteins in bacterial resistance of *P. vannamei*.

The function of *Lv*HSP70 was further studied and it was found that endogenous *Lv*HSP70 observed in all three types of shrimp hemocytes (hyaline, granular, and semi-granular cells) was immediately activated after chronic NLHS treatment. Furthermore, *P. vannamei* receiving recombinant *Lv*HSP70 (r*Lv*HSP70) had enhanced resistance to AHPND infection as a survival rate of shrimp was increased from 20% (control group) up to >75%. The survival was associated with the up-regulation of several shrimp immune-related genes (*LvMyD88, LvIKKε*, *LvIKK*β, *LvCrustin1*, *LvPEN2*, *LvPEN3*, *LvproPO1*, *LvproPO2*, and *LvTG1*).

The further study of HSP70 family members in crustacean was carried out by using a draft *Artemia* transcriptome database. Four novel HSP70 family genes classified as *HSC70*, *HSC70-5*, *BIP*, and *HYOU1* were identified. The transcript levels of *HSP70* and *HSC70* were significantly (P<0.05) higher by selective breeding for induced

thermotolerance population (TF12) of *Artemia* relative to a control population (CF12). Moreover, *HSP70*, *HSC70*, and *HSC70-5* transcripts were significantly (P<0.05) up-regulated after a NLHS treatment of TF12 at the nauplii state. On the other hand, the expression of *BIP*, *HYOU1,* and *HSPA4* showed no significant (P<0.05) induction.

The different expression level of *HSC70* triggered the search for (non-synonymous) SNPs. A SNP was found at the nucleotide position 171 (C171A; N57K) in the HSC70 gene which located in the ATP-binding domain. Moreover, the genotype analysis result showed a significant frequency (P<0.01) difference of C171A; N57K between CF12 and TF12. The relevance of this differential frequency in TF12 and CF12 was confirmed by phenotypic analysis in yeast (*S. cerevisiae*) . The result showed that yeast containing a HSC70-N57K plasmid could tolerate a higher temperature than yeast containing WT-HSC70 plasmid.

The knowledge of HSP70 in white-leg shrimp and HSP70 family in *Artemia* clearly demonstrate the crucial role of this protein family in heat tolerance and disease resistance in crustaceans.

6.3 Future perspective

Acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus* carrying toxin-producing plasmid, has led to severe mortalities in both farmed white shrimps and black tiger shrimps worldwide. Although methods for early diagnosis and detection of AHPND including histological examinations and molecular diagnostics have been implemented to reduce the loss from the disease outbreaks, more effective measures are urgently required for disease prevention and control. In this study, I demonstrated that the exposure of Pacific white-leg shrimp *P. vannamei* to chronic-NLHS could enhance the resistance to VP_{AHPND} infection, corroborating with several previous reports in aquatic organisms such as mussels (Aleng et al., 2015), fish (Ryckaert et al., 2010), and *Artemia* (Norouzitallab et al., 2015; Yik Sung et al., 2007). In

application to shrimp farming, I suggest that shrimp farmers can practically use this heat stress technique in hatcheries where post-larval shrimp can be transferred from ambient temperature to chronic-NLHS (28 ºC to 38 ºC, 5 min for 7 days). Following the NLHS treatment, the shrimps should be allowed to recover at least 3 days before releasing into the grow-out ponds. As I found that chronic-NLHS could enhance shrimp resistance to AHPND till 30 days recovery time, this treatment could, therefore, protect shrimp from post-larval stage into sub-adult stage, the latter harbouring probably a better developed immune system. Additionally, the heat shock treatment of both NLHS and LHS can be applied for selective breeding of shrimps for induced thermotolerance similar to the selected population of TF12 *Artemia* reported in this study. Selective breeding for induced thermotolerance can also induce the expression of genes in the HSP70 family that leads to the activation of innate immune systems and enhancing disease resistance.

Moreover, I found that the resistance of shrimps to AHPND was related to the upregulation of HSP70 gene and protein expression (Chapter 2 and 3). These results together with several previous studies (Aleng et al., 2015; Norouzitallab et al., 2015; Yik Sung et al., 2007) indicated that animals with high level of HSP70 can resist bacterial infection. Besides inducing HSP70 with NLHS, more practical methods of feeding the animals with HSP70-inducing compounds have been considered as potential disease control and health management strategies in aquaculture. The compound Tex-OE®, a patented extract from the skin of the prickly pear fruit, *Opuntia ficus indica*, was reported to enhance the production of Hsp70 in fish and shrimp tissues without any adverse effect, protecting the animals against various abiotic stressors (Niu et al., 2014). Recently, the phenolic compound phloroglucinol was reported to induce the production of HSP70 and it served as an effective inducing compound against AHPND in *A. franciscana* (Kumar et al., 2018b). Considering these findings, the HSP70-inducing compounds can be mixed with animal feed, to enhance bacterial resistance.

Nevertheless, supplying feed additives at the appropriate stages and amounts are critical and should be optimized for the effectiveness and practical use in shrimp farming.

Besides the application in shrimp farming, advances in the molecular mechanisms will delineate the importance of HSP70 in the activation of the innate immune system. For example, the study of HSP70 gene knockdown (Chapter 2) and also the injection of r*Lv*HSP70 (Chapter 3) indicated that HSP70 could inhibit the growth of bacteria and activate several immune-related signaling pathways such as the proPO activating system and the Toll signaling pathway. Previous study in other Eukaryotes found that HSP70 could induce pro-inflammatory cytokine production by macrophages in human (Gao and Tsan, 2003). Moreover, HSP70 also could induce CCR5-mediated calcium signaling in dendritic cells in human (Bendz et al., 2008). Furthermore, RNA seq and protein-protein interaction (PPI) networks in white-leg shrimp demonstrated that HSPA8 or HSC70 were suggested as the top three hub regulation-genes in response to acute high-pH stress (Huang et al., 2018). This means that HSC70 play an important role in stress response. However, the molecular mechanism regulating shrimp immunity by HSP70 is poorly understood. In the future, the interaction of HSP70 with other proteins in the innate immune system should be further investigated to provide insights in the role of HSPs in immune modulation as previously been reported in various organisms (see a review by (Zininga et al., 2018)).

Moreover, this study provides information regarding the members of HSP70 family in crustacean by using *Artemia* as a model. Eukaryotes express more than one gene encoding HSP70 proteins. For instance, human express 13 HSP70 genes (Tavaria et al., 1996) whereas the yeast *Saccharomyces cerevisiae* consist of eight putative HSP70 (Werner-Washburne and Craig, 1989). Here, I identified four novel HSP70 genes classified as *HSC70*, *HSC70-5*, *BIP*, and *HYOU1*, in addition, to the HSP70 which has

been previously reported (Chapter 4). Gene expression analysis of HSP70 members in *Artemia* population selected for induced thermotolerance revealed the possible function of the genes in thermotolerance and disease resistance (Chapter 4). This knowledge can enhance our understanding of the function of each member in the HSP70 family. SNP identification can explain the relationship between the HSP70 family and selective breeding for induced thermotolerance as I found a SNP that is statistically (P>0.01) associated with the selective trait for induced thermotolerance in *Artemia* (Chapter 5). Thus, it is possible to use this SNP as a DNA marker for selection of thermotolerance as well as pathogen resistant animals but whether or not this SNP can be used to identify disease resistant shrimp broodstock in aquaculture farming need to be further verified. Together with the recently published Penaeid shrimp genome (Zhang et al., 2019) and several transcriptome data from RNA sequencing will lead to exponential progress in genome-wide association studies (GWAS), similar to the time that the human genome was released. GWAS can provide an important avenue for undertaking an unbiased evaluation of the association between common genetic variants and disease resistance by the identification of millions of SNPs (Witte, 2010). As I have mentioned, SNPs (with verified phenotypic effects) can potentially act as a biomarker in selective breeding or screening. In that respect, recent release of the whole genome sequence of *L. vannamei* will contribute to the potential of GWAS application in shrimp aquaculture.

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CURRICULUM VITAE

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Personal information

Date & place of birth	28/04/1990, Bangkok, Thailand
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Education

2010 - 2013	**B.Sc. (Biochemistry)** Department of Biochemistry, Faculty of Science, Chulalongkorn University
2013 - 2019	**Ph.D. (Biochemistry and Molecular biology)**

Department of Biochemistry, Faculty of Science, Chulalongkorn University

2018 -2019 **Ph.D. (Applied Biological Science: Aquaculture)**

Department of Animal Science and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University

Research experiences

2012 – 2015 Topic: DNA marker associate with resistance shrimp in *Penaeus vannamei*

2015 – present Topic: Heat shock proteins (*Lv*HSPs) from *Penaeus vannamei* and theirs role in protection against shrimp pathogens

2017 – present Topic: *Artemia* Genome and transcriptome, Genetic breeding and thermotolerante

2018 – present Topic: Biomarker associate with resistance shrimp in *Penaeus vannamei*

Activity experiences

2017-2018 President of Thai Student Association in Belgium

Chairman of the 7th Thai Student Academic Conference (TSAC) in Europe

2015 Assistance organization at SEE Thailand science camp

Assistance organization at ASEAN science camp 2015

2014 Assistance organization at ASEAN science camp 2014

Head of Biochemistry student club, Department of Biochemistry, Faculty of Science, Chulalongkorn University

2013 Participant of Chula-AYFN ASEAN science camp 2013

2012	Trainee of Central institute of forensic science, Thailand
2008 - 2009	Head of student committee, Student committee of high school in Phetchaburi province
	Head of student committee, Student committee of Prommanusorn Phetchaburi School

Scholarship

- Royal Golden Jubilee (RGJ) Ph.D. Program form Thailand Research Fund
- The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund)

Skills

Research technical skills

Molecular biology : Cloning and expression analysis, Genomic DNA isolation, RNA isolation, RT-PCR analysis, Real-Time PCR analysis, Electrophoresis, Western blotting, Genetic Engineering, Recombinant protein expression in bacterial (*E. coli*) and Yeats (*Saccharomyces cerevisiae*) expression systems, RNA interference (RNAi), Genome walking, 5' and 3' RACE, Yeast two hybrid, Co-immunoprecipitation (Co-IP), and Transcriptomics (RNA seq)

Biochemistry and Microbiology : Affinity Chromatography, Spectroscopy, Centrifugation, Enzyme kinetic analysis, Protein expression, and Microbiology technique

Bioinformatics : Sequence analysis using the software tools, open source bioinformatics software, and web services in bioinformatics.

Aquaculture : Shrimp cultural and Artemai cultural

Computer program skills

Good working of computers using Microsoft Office.

Good working knowledge of computers using spreadsheets and data bases.

Good working of graphic design using Adobe Photoshop.

Good working of website design

Language skills

English : Very good for speaking, writing, and reading

Awards

2019	Winning of FUCOBI Foundation's 'Johnnie Castro Montealegre Student Award
2017	Outstanding oral presentation, RGJ-Ph.D. Congress 19, TRF
2016	Outstanding oral presentation, The 5th International Biochemistry and Molecular Biology Conference, Thailand
2013	Good oral presentation, The Hitachi Trophy 2009, The Science Forum 2013, Chulalongkorn University, Thailand

Oral Presentations

Junprung W., Supungul P., Tassanakajon A., *Litopenaeus vannamei heat shock proteins (Hsps) enhances resistance to acute hepatopancreatic necrosis disease (ahpnd), by activating shrimp immunity,* Aquaculture 2019, New Orleans, Louisiana USA

Junprung W., Supungul P., Tassanakajon A., Heat shock proteins play roles in protection against AHPND- causing strain of *Vibrio parahaemolyticus* by induction of shrimp immune system. RGJ-Ph.D. Congress 18, Bangkok, Thailand (2017)

Junprung W., Supungul P., Tassanakajon A., Heat shock proteins play roles in protection against AHPND-causing strain of *Vibrio parahaemolyticus* by induction of shrimp immune system, Asia Pacific Marine Biotechnology Conferences (APMBC) 2017 Conference, The University of Hawaii, Honolulu, USA (2017)

Junprung W., Supungul P., Tassanakajon A., Shrimp *Penaeus vannamei* tolerance to AHPND-causing stain of *Vibrio parahaemolyticus* by non-lethal heat shock is mediated by HSP70 and HSP90, The 21th Biological Sciences Graduate Congress (BSGC), University of Malaya, Kuala Lumpur, Malaysia (2016)

Junprung W., Supungul P., Tassanakajon A., HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of Vibrio parahaemolyticus after non-lethal heat shock, The 4th Thailand Research Fund (TRF) Senior Scholar Group Annual Meeting 2016 (Prof.Dr. Anchalee Tassanakajon), Bangkok, Thailand (2016)

Junprung W., Supungul P., Tassanakajon A., Heat shock protein (*Lv*HSP) from *Penaeus vannamei* and their roles in protection against *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND), The 5th International Biochemistry and Molecular Biology Conference, Thailand (2016)

Junprung W., Supungul P., Tassanakajon A., DNA marker associate with TSV resistance shrimp (*Penease vannamei*), The Science Forum 2013, Chulalongkorn University, Thailand (2013)

Poster Presentation

Junprung W., Supungul P., Tassanakajon A., *Litopenaeus vannamei heat shock protein 70 (Lvhsp70) enhances resistance to acute hepatopancreatic necrosis disease (ahpnd), by activating shrimp immunity,* Aquaculture 2019, New Orleans, Louisiana USA

Publications

Junprung W., De Vos S., Tassanakajon T., Supungul P., Van Stappen G., Bossier P., HSC70 SNP and the association with selective breeding for induced thermotolerance in *Artemia franciscana*. **Manuscript in preparation**

Junprung W., De Vos S., Tassanakajon T., Norouzitallab P., Viet Nguyen D., Van Stappen G., Bossier P., Sequence and gene expression analysis of the HSP70 family in *Artemia franciscana*. **Submitted**

Junprung W., Supungul P., Tassanakajon A. *Litopenaeus vannamei* heat shock protein 70 (*Lv*HSP70) enhances resistance to a strain of *Vibrio parahaemolyticus*, which can cause acute hepatopancreatic necrosis disease (AHPND), by activating shrimp immunity. Developmental and Comparative Immunology. 90 138:146 (2019)

Sornchuer P., **Junprung W.**, Yingsunthonwattana W., Tassanakajon A. (2017) Heat shock factor 1 regulates heat shock proteins and immune-related genes in *Penaeus monodon* under thermal stress. Developmental and Comparative Immunology. 88 19:27 (2018)

Junprung W., Supungul P., Tassanakajon A., HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock. Fish & Shellfish Immunology. 60 237:246 (2017)

Supungul P., Jaree P., Somboonwiwat K., **Junprung W.**, Proespraiwong P., Mavichak R., Tassanakajon A., A potential application of shrimp antilipopolysaccharide factor in disease control in aquaculture. Aquaculture Research. 1-13. (2015)

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REFERENCES

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causing strain of Vibrio parahaemolyticus after non-lethal heat shock. Fish & Shellfish Immunology. 60 237:246 (2017)

Supungul P., Jaree P., Somboonwiwat K., Junprung W., Proespraiwong P., Mavichak R.,Tassanakajon A. A potential application of shrimp antilipopolysaccharide factor in disease control in aquaculture. Aquaculture Research. 1-13. (2015)

AWARD RECEIVED

Winning FUCOBI Foundation's 'Johnnie Castro Montealegre Student Award' (2019)

Outstanding oral presentation, RGJ-Ph.D. Congress 19, TRF (2017)

Outstanding oral presentation, The 5th International Biochemistry and Molecular Biology Conference, Thailand (2016)

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