STUDIES OF BIOLOGICAL SIGNIFICANCE OF APOPTOSIS UPON WHITE-SPOT SYNDROME VIRUS INFECTION IN PENAEID SHRIMP

ANCHUKORN RIJIRAVANICH

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY (ANATOMY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2007**

Thesis
Entitled

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…….
Miss Anchukorn Rijiravanich
Candidate

…….
Prof. Boonsirm Withyachumnarnkul,
M.D., Ph.D.
Major-Advisor

…….
Prof. Timothy W. Flegel,
Ph.D.
Co-Advisor

…….
Prof. Prasert Sobhon,
Ph.D.
Co-Advisor

…….
Lect. Kanokpan Wongprasert,
Ph.D.
Co-Advisor

…….
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies

…….
Assoc. Prof. Jittipan Chavadej,
Ph.D.
Chair
Doctor of Philosophy Program
in Anatomy
Faculty of Science

Thesis
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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Anatomy)
on
January 17, 2007

…….
Miss Anchukorn Rijiravanich
Candidate

…….
Assoc. Prof. Chaitip Wanichanon,
Ph.D.
Chair

…….
Prof. Boonsirm Withyachumnarnkul,
M.D., Ph.D.
Member

…….
Prof. Prasert Sobhon,
Ph.D.
Member

…….
Prof. Timothy W. Flegel,
Ph.D.
Member

…….
Dr. Craig L. Browdy
Ph.D.
Member

…….
Lect. Kanokpan Wongprasert
Ph.D.
Member

…….
Prof. M.R. Jisnuson Svasti,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

…….
Prof. Amaret Bhumiratana,
Ph.D.
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENT

This thesis study was supported by the Thailand Research Fund (Royal Golden Jubilee Grant No. 4.A.MU/44I.1). First of all I would like to express my sincere gratitude to my major advisor, Prof. Boonsirm Withyachumnarnkul, my co-advisors, Prof. Timothy Flegel, Prof. Prasert Sobhon, Dr. Craig Browdy, and Dr. Kanokpan Wongprasert, for spending their precious time on helping, supporting, suggesting and discussing my study and research. It is your very kindness to let me be one of your supervised students.

I am also grateful for teachers, staffs and students at Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp) and Department of Anatomy, Faculty of Science, Mahidol University. Thanks to Shrimp Culture Research Center, Charoen Pokphand Feedmill Co. Ltd. for continual supplies of virus, Dr. Amornrat Phongdara for *P. merguiensis cap-3* sequence, Dr. Saengchan Senapin for *P. monodon cap-3* sequence, and Medical Scholar Program (MSP), Mahidol University for continual financial support.

I am sincerely appreciated everyone at Hollings Marine Laboratory and South Carolina Department of Natural Resoruces and Medical University of South Carolina. I am grateful for supports, suggestions, help with reagents and revision of manuscript from Dr. Robert Chapman, Dr. Greg Warr, Dr. Paul Gross, Dr. Javier Robalino, Dr. Nuala O'Leary and Bryan Mathis. Appreciation is also offered to Caroline Payne, James Powell, Deliah Arrington and Eleanor Shepard for help with stocking, rearing and challenging shrimp.

Last but not least, without love, caring and understanding from my family, Ph.D. would be just a dream of my life.

Anchukorn Rijiravanich

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ANCHUKORN RIJIRAVANICH 4437254 SCAN/D
Ph.D. (ANATOMY)
THESIS ADVISORS: BOONSIRM WITHYACHUMNARNKUL, M.D.,Ph.D., TIM FLEGEL, Ph.D., PRASERT SOBHON, Ph.D., KANOKPAN WONGPRASERT, Ph.D.

ABSTRACT

Apoptosis has long been observed in viral target organs of white-spot syndrome virus (WSSV)-infected shrimp and its level has been reported to increase as the shrimp approach death. Although apoptosis is purported to be an effective anti-viral defense mechanism in invertebrates, its benefits are controversial with respect to shrimp infected with WSSV. While other studies have focused on verifying apoptosis as a host defense mechanism in 'immune' or viral resistant/tolerant shrimp, observations of viral epizootics in Thai farming practices have led to an interesting proposal that shrimp mortality due to WSSV infection is a result of triggered apoptosis.

This study attempted to investigate if viral triggered apoptosis is protective or detrimental to infected shrimp. First, the mechanism of death protection from WSD by hyperthermia was tested and it has been shown that hyperthermia-induced viral resistance in black tiger shrimp *Penaeus monodon* is not correlated with apoptosis but is instead a result of the inhibition of virus production. Secondly, reverse genetics (e.g. RNAi) has been applied on a caspase-3 homologue (*cap-3*), a key protein in induction of apoptosis, from the American white shrimp *Penaeus* (*Litopenaeus*) *vannamei* and wsv390, ORF390 of WSSV (which has been demonstrated to have an anti-apoptotic activity *in vitro*) to test the earlier proposal. Effects on mortality, apoptosis induction and viral production at low- and high- doses of WSSV were evaluated.

In a high-dose WSSV challenge, both *cap-3* and wsv390 silencing had no significant effect on WSSV-induced mortality, except for a delay in mean time to death, which was attributed to a non-specific anti-viral response induced by injection of dsRNA. An increase in viral yield was shown in *cap-3* RNAi shrimp whereas a decrease was shown in wsv390 RNAi shrimp compared to the viral challenged control and dsRNA treatment control shrimp. A continual increase in apoptosis was detected in hemocytes of *cap-3* dsRNA, viral control and dsRNA control shrimp. wsv390 RNAi, however; displayed a significant boost of apoptosis in the beginning and kept steady at that level along the progression of disease. At a low dose WSSV challenge, *cap-3* but not wsv390 silencing co-related with a lower level of cumulative mortality, relative to silencing of a control gene.

Although more extensive studies will be required to resolve the role of apoptotic response of shrimp to WSSV, this study suggests that *cap-3* controls WSSV replication in an apoptosis-independent manner; wsv390 exerts dual roles on regulating host apoptosis and it is necessary for viral replication but not for severity of infection; and finally, apoptosis may exacerbate rather than decrease mortality in WSSV-challenged shrimp.

KEY WORDS: APOPTOSIS; CASPASE-3; wsv390; dsRNA; WHITE-SPOT SYNDROME VIRUS
90 P.

การศึกษาความสำคัญทางชีวภาพของการตายของเซลล์แบบอะพอพโทสิสต่อการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งกลุ่มพีเนียด (STUDIES OF BIOLOGICAL SIGNIFICANCE OF APOPTOSIS UPON WHITE-SPOT SYNDROME VIRUS INFECTION IN PENAEID SHRIMP)

อัญชุกร ริจิรวนิช 4437254 SCAN/D

ปร.ด. (กายวิภาคศาสตร์)

คณะกรรมการควบคุมวิทยานิพนธ์: บุญเสริม วิทยชำนาญกุล, M.D., Ph.D., ทิม เฟลเกล, Ph.D., ประเสริฐ โศภน, Ph.D., กนกพรรณ วงศ์ประเสริฐ, Ph.D.

บทคัดย่อ

การตายของเซลล์แบบอะพอพโทสิสนั้นถูกตรวจพบในอวัยวะเป้าหมายของการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งและการตายของเซลล์ในลักษณะนี้ก็เพิ่มขึ้นเมื่อกุ้งใกล้ตาย แม้จะเข้าใจกันว่าอะพอพโทสิสเป็นกลไกป้องกันไวรัสที่มีประสิทธิภาพในสัตว์ไม่มีกระดูกสันหลัง ผลดีของอะพอพโทสิสที่เกิดจากการติดเชื้อไวรัสตัวแดงดวงขาวในกุ้งก็ยังเป็นที่ถกเถียงกันอยู่ ในขณะที่การศึกษาอื่น ๆ ได้พยายามที่จะแสดงให้เห็นว่าอะพอพโทสิสเป็นกลไกป้องกันโรคของกุ้งที่ได้รับภูมิคุ้มกันหรือกุ้งที่ทนต่อการติดเชื้อไวรัส การสังเกตการณ์การเกิดโรคไวรัสระบาดในการเพาะเลี้ยงกุ้งในประเทศไทยได้ทำให้เกิดการสันนิษฐานที่ว่าการตายของกุ้งจากไวรัสตัวแดงดวงขาวนั้นเป็นผลมาจากการเกิดการตายของเซลล์แบบอะพอพโทสิส

การศึกษานี้ได้พยายามที่จะตรวจสอบว่าอะพอพโทสิสที่ถูกกระตุ้นโดยไวรัสนั้นช่วยป้องกันกุ้งหรือทำให้เกิดผลร้ายกับกุ้งที่ติดเชื้อไวรัส ในขั้นแรกเป็นการทดสอบหากลไกของการป้องกันการตายของกุ้งจากโรคตัวแดงดวงขาวเมื่อเลี้ยงที่อุณหภูมิสูง และพบว่าการทนต่อโรคของกุ้งกุลาดำที่เลี้ยงที่อุณหภูมิสูงนั้นไม่ได้เกิดจากการตายของเซลล์แบบอะพอพโทสิส แต่น่าจะเป็นผลมาจากการยับยั้งการเพิ่มจำนวนของไวรัส ในขั้นต่อมาเทคนิค RNAi ได้ถูกนำมาใช้เพื่อพิสูจน์สมมุติฐานข้างต้น โดยได้ทำการยับยั้งการแสดงออกของยีน caspase-3 หรือ *cap-3* ในกุ้งขาว ซึ่งยีนนี้เป็นยีนที่เกี่ยวข้องกับการเกิดอะพอพโทสิส และยีน wsv390 ของเชื้อไวรัสตัวแดงดวงขาวที่ได้มีการรายงานว่าสามารถยับยั้งการเกิดอะพอพโทสิสในเซลล์ทดลองได้ หลังจากยับยั้งการแสดงออกของยีนทั้งสองและฉีดเชื้อไวรัสตัวแดงดวงขาวที่ปริมาณสูงหรือต่ำแล้ว จึงได้ทำการตรวจหาผลกระทบต่ออัตราการตายของกุ้ง การเกิดอะพอพโทสิส และการเพิ่มจำนวนของไวรัส

เมื่อฉีดกุ้งด้วยปริมาณไวรัสที่สูง การยับยั้งการแสดงออกของยีน *cap-3* และ wsv390 ไม่ทำให้กุ้งรอดตาย แต่การฉีด dsRNA เข้าไปสามารถที่จะช่วยให้กุ้งตายช้าลงได้เล็กน้อยซึ่งเป็นผลมาจากกลไกการต้านไวรัสแบบไม่จำเพาะเจาะจงจากการฉีด dsRNA ในกุ้งกลุ่มที่ถูกยับยั้งการแสดงออกของยีน *cap-3* นั้นพบว่ามีการเพิ่มขึ้นของปริมาณไวรัสแต่ในกุ้งกลุ่มที่ถูกยับยั้งการแสดงออกของยีน wsv390 กลับมีปริมาณไวรัสลดลงเมื่อเทียบกับกุ้งกลุ่มควบคุม การเกิดอะพอพโทสิสของเซลล์เม็ดเลือดของกุ้งกลุ่มที่ถูกยับยั้งยีน *cap-3* มีการเพิ่มขึ้นเหมือนกับกุ้งกลุ่มควบคุม อย่างไรก็ดี กุ้งที่ถูกยับยั้งยีน wsv390 กลับมีปริมาณเซลล์เม็ดเลือดที่ตายแบบอะพอพโทสิสมากกว่าปกติในตอนต้นและคงอยู่ที่ระดับนี้ในระหว่างที่โรคดำเนินต่อไป เมื่อฉีดกุ้งด้วยปริมาณไวรัสที่น้อย พบว่าการยับยั้งยีน *cap-3* ช่วยทำให้กุ้งรอดตายได้ดีกว่ากุ้งกลุ่มควบคุมอย่างมีนัยสำคัญ แต่การยับยั้งยีน wsv390 ไม่ช่วยให้กุ้งรอดตาย

แม้จะมีการศึกษาอีกมากที่ต้องทำเพื่อพิสูจน์ให้เห็นถึงบทบาทของการตายของเซลล์แบบอะพอพโทสิสในกุ้งที่ติดเชื้อไวรัสตัวแดงดวงขาว การศึกษานี้บ่งชี้ว่ายีน *cap-3* ควบคุมการเพิ่มจำนวนของไวรัสตัวแดงดวงขาวแบบไม่ขึ้นกับการตายของเซลล์แบบอะพอพโทสิส ยีน wsv390 ของไวรัสทำหน้าที่ได้สองหน้าที่ในการควบคุมการเกิดอะพอพโทสิสของเจ้าบ้านและยีนนี้มีความสำคัญกับการเพิ่มจำนวนของไวรัสแต่ไม่จำเป็นสำหรับความรุนแรงของโรค และการตายของเซลล์แบบอะพอพโทสิสนั้นอาจจะให้ผลลบกับกุ้งมากกว่าที่จะช่วยลดอัตราการตายของกุ้งที่ติดเชื้อไวรัสตัวแดงดวงขาว

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LIST OF ABBREVIATIONS

AcMNPV	=	*Autographa californica* nuclear polyhedrosis virus
ADAR	=	RNA-specific adenosine deaminase
AIF	=	apoptosis-inducing factor
ANOVA	=	analysis of variance
Bcl-2	=	B-cell lymphoma 2
bp	=	base pair
CBV	=	Chinese baculovirus
cDNA	=	complementary DNA
CUB	=	C1r-C1s/sea urchin protein Uegf/bone morphogenetic protein 1
CDP	=	CUB domain protein
cRNA	=	complementary RNA
C_T	=	threshold cycle
CTL	=	cytotoxic T lymphocyte
DAPI	=	4',6-Diamidine-2'-phenylindole dihydrochloride
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
dpc	=	day post-challenge
dsRNA	=	double-stranded RNA
EBV	=	Epstein-Barr virus
EDTA	=	ethylenediamine tetra-acetic acid
EF-1α	=	elongation factor 1α
eIF-2α	=	eukaryotic initiation factor 2 alpha
FBS	=	fetal bovine serum

LIST OF ABBREVIATIONS (CONT.)

GTPase	=	guanosine triphosphatase
H&E	=	hematoxylin and eosin
HBV	=	hepatitis B virus
HHNBV	=	hypodermal and hematopoietic necrosis baculovirus
HIV	=	human immunodeficiency virus
hpc	=	hour post-challenge
HSV	=	herpes simplex virus
IAP	=	inhibitor of apoptosis protein
IBDV	=	infectious bursal disease virus
ICE	=	interleukin-1-converting enzyme
IFN	=	interferon
Ig	=	immunoglobulin
IHHNV	=	infectious hypodermal and hematopoietic necrosis virus
ISG	=	interferon-stimulated gene
LHM	=	lobster hemolymph medium
LM	=	light microscopy
LMP-1	=	latent membrane protein 1
miRNA	=	micro RNA
mRNA	=	messenger RNA
NBT/BCIP	=	Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
NF-κB	=	nuclear factor kappa B
NGS	=	normal goat serum
NS	=	nonstructural
OAS	=	2',5'-oligoadenylate synthetase
ORF	=	open reading frame

LIST OF ABBREVIATIONS (CONT.)

PBS	=	phosphate buffer saline
PBST	=	phosphate buffer saline containing 0.05% Tween-20
PCD	=	programmed cell death
PCR	=	polymerase chain reaction
pi	=	post-infection
PKR	=	RNA-dependent protein kinase
PL	=	post-larvae
PmNOB	=	*Penaeus monodon* non-occluded baculovirus
pRb	=	retinoblastoma tumor suppressor protein
PRDV	=	penaeid rod-shaped DNA virus
PSS	=	penaeid shrimp salt solution
RACE	=	rapid amplification of cDNA ends
RNA	=	ribonucleic acid
RNAi	=	RNA interference
RNaseL	=	ribonuclease L
rRNA	=	ribosomal RNA
RT-PCR	=	reverse transcription-polymerase chain reaction
RV-PJ	=	rod-shaped virus of *Penaeus japonicus*
SEMBV	=	systemic ectodermal and mesodermal baculovirus
SF-21	=	*Spodoptera frugiperda* 21
SFV	=	Semliki Forest virus
siRNA	=	short interfering RNA
SPF	=	specific pathogen-free
ssRNA	=	sigle-stranded RNA
SV40	=	simian virus 40

LIST OF ABBREVIATIONS (cont.)

TCTP	=	translationally controlled tumor protein
TEM	=	transmission electron microscopy
T_m	=	melting temperature
TNF	=	tumor necrosis factor
TRAIL	=	TNF-related apoptosis-inducing ligand
TSV	=	Taura syndrome virus
VSV	=	vesicular stomatitis virus
VV	=	vaccinia virus
WSBV	=	white spot baculovirus
WSD	=	white-spot disease
WSSV	=	white-spot syndrome virus
YHV	=	yellow head virus
χ^2	=	chi-square

CHAPTER I
INTRODUCTION

White-spot syndrome virus (WSSV) is the causative agent of white-spot disease (WSD), which is a major cause of mass mortality in shrimp farming industry today. The disease was first reported in China in 1993 (1, 2) in the marine shrimp *Penaeus chinensis* and has spread worldwide to all shrimp species including *Penaeus monodon* and *Penaeus (Litopenaeus) vannamei*, the two most cultured species. Histopathological features of WSSV-infected shrimp at the early stage of infection are nuclear hypertrophy, chromatin margination and apoptosis of cells of ectodermal and mesodermal origins (3-5).

Apoptosis has been shown to play a critical role in vertebrate defense against viral pathogens (6, 7). In vertebrates, viral infection can trigger apoptosis in infected cells and subsequent phagocytosis of apoptotic bodies by macrophages can lead to the mounting of specific immunity (8). Although apoptosis may suppress viral replication in some infected cells, other viruses such as herpes simplex virus type 1 (HSV-1), poliovirus and vesicular stomatitis virus (VSV) can grow significantly in cells undergoing apoptosis (7, 9). Nevertheless, in insects (which lack adaptive immunity), apoptosis is reported to be extremely powerful in limiting viral replication, infectivity, and spread, through mechanisms that involve the premature lysis of infected cells (10, 11). In crustaceans, occurrence of apoptosis upon viral infection has long been observed. Upon WSSV infection, apoptosis has been detected in several viral target tissues of shrimp, and the level of apoptosis seems to increase as WSD progresses towards shrimp death (4, 5, 12, 13). Several studies have investigated the changes in the level of apoptosis-related gene expression in WSSV-infected shrimp. So far, one down-regulated anti-apoptosis factor and several other up-regulated apoptosis factors have been detected (14-16). Despite all these findings, there is still no clear conclusion as to whether apoptosis constitutes an antiviral immune response in shrimp.

Caspases are cysteine proteases that bring about most of the morphological changes that are collectively characterized as apoptosis. Elimination of caspase activities can slow down or even prevent those changes (17). Phongdara *et al.* (15) discovered for the first time the caspase gene, *cap-3*, in the cultured banana shrimp, *Penaeus (Fenneropenaeus) merguiensis*. This newly found shrimp caspase gene is believed to function like human caspase-3 (15), which is one of the key components of the apoptotic process. In mammals, the crucial task of caspase-3 in programmed cell death process (both in the extrinsic and mitochondrial pathways) has been studied with the generation of caspase-3 deficient mice and caspase-3 mutated/deleted cell lines (18-20). Collectively, these findings imply that, if apoptosis plays a role in mortality of shrimp with WSD, then ablating *cap-3* should improve their survival.

Double-stranded RNA (dsRNA), a common intermediate formed during the life cycle of many viruses, is a potent inducer of innate antiviral immunity. It has been shown to cause a strong, sequence-specific antiviral response in shrimp, both *in vivo* and *in vitro*. For example, an *in vitro* study has shown that replication of yellow head virus (YHV) can be significantly restricted by the addition of YHV-gene-targeted dsRNA into penaeid shrimp cell culture (21). In *P. monodon*, *P. vannamei* and *P. chinensis*, administration of virus-specific dsRNA *in vivo* also prevents mortality induced by several viruses, including YHV, WSSV, and Taura syndrome virus (TSV) (21-23). Apart from these sequence-dependent anti-viral phenomena, *in vivo* studies with *P. vannamei* showed that administration of dsRNA evoked limited innate anti-viral immunity in a sequence-independent manner (24), a response that could be overwhelmed by a high-dose viral challenge. In addition to its anti-viral properties, dsRNA injection induces in shrimp a classical RNAi-like effect, characterized by systemic down-regulation of endogenous gene expression in a sequence-specific manner (22).

It has been predicted in the viral accommodation theory that viral triggered apoptosis may be a major cause of mortality and that reduced rates of cell death may allow for attenuation of viral pathogenicity in shrimp (25, 26). Verification of the significance of apoptosis in viral pathogenicity could thus be important for the shrimp farming industry worldwide. In this thesis research, hyperthermia-induced WSSV-resistance and the advantages of the three distinct dsRNA-induced phenomena have

been employed to clarify if apoptosis upon WSSV infection is either beneficial or detrimental to the shrimp.

CHAPTER II
LITERATURE REVIEW

1. White-Spot Syndrome Virus

1.1 History

White-spot syndrome virus (WSSV) has had a great impact on shrimp culture worldwide and it remains a major problem. It causes white-spot disease (WSD) or red body-white patch disease and was first recognized in China in 1993 (1, 2). Thereafter, outbreaks of WSSV spread rapidly throughout the Asian shrimp-farming industry in Japan, Indonesia, Thailand, Malaysia, and India (3, 27). WSSV was initially limited to Asia but was later reported from North- and South-America where it has continued to cause major losses (28). Intensive shrimp cultivation, inadequate sanitation, poor management and worldwide transfer of live shrimp have further aggravated disease incidence and enhanced disease dissemination (29).

Over the last decade, many scientific reports on WSSV have appeared describing the same agent, but using several different names. “Systemic ectodermal and mesodermal baculovirus” (SEMBV) or “*Penaeus monodon* non-occluded baculovirus” (PmNOBII) was described from Thailand in 1994 (27), “Rod-shaped virus of *Penaeus (Marsupenaeus) japonicus*” (RV-PJ), or “Penaeid rod-shaped DNA virus” (PRDV) was described from Japan in 1993 (30), and “hypodermal and hematopoietic necrosis baculovirus” (HHNBV) and “Chinese baculovirus” (CBV) in 1993 from China (31, 32). Then “White spot baculovirus” (WSBV) or PmNOBIII was described from Taiwan in 1995 (1, 33, 34). Lightner (1996) has grouped these viruses in a single white spot syndrome virus (WSSV) complex. Today, the name “White-Spot Syndrome virus (WSSV)” is used by most research groups (25, 35). Although WSSV shares some histopathological features with insect viruses in the family *Baculoviridae*, it does not produce occlusion bodies comprised of proteins that protect

viruses from decay. Based on virion morphology, genomic structure and composition, and phylogenetic analysis, WSSV is finally described to be a member of a new genus *Whispovirus* within a new virus family called *Nimaviridae* (36).

In Thailand, WSSV was first found unexpectedly in late 1993 during the course of experimental infection of *P. monodon* with YHV for virus isolation and purification (27). Since then it has quickly spread to most of the shrimp farming areas in Thailand. Practically, captured broodstock and post-larvae (PL) used to stock rearing ponds are known to carry WSSV. However, mass mortalities usually occur with juvenile shrimp, possibly precipitated by environmental factors (25, 37, 38). Other crustaceans, including crabs, crayfish, copepods and perhaps even aquatic insect larvae are also accepted to be capable of WSSV infection (39-43). For some of these crustaceans, WSSV is not lethal and therefore they can serve as reservoirs for the virus when they are introduced into shrimp ponds along with sea water, making elimination of WSSV from shrimp cultivation very difficult (26). Ponds stocked with latent WSSV-infected (grossly normal but WSSV-PCR positive) post-larvae of *P. monodon* develop into WSSV outbreak ponds in approximately 40-45 days (38) while the incubation period in experimental infections is 2 to 7 days (27, 34).

1.2 Clinical signs of white-spot disease

White-spot diseased shrimp, from both experimental and natural infection, are characterized by a combination of clinical signs. A principal clinical sign is the presence of white spots in the exoskeleton. However, WSSV is not always lethal. Under non-stressful conditions, infected shrimp with white spots may survive to harvest (44). On the other hand, lethargy, a pink to reddish body coloration, gathering of affected shrimp around the edges of ponds at the surface during the day, rapid reduction in food consumption, and then mass mortalities of up to 70 to 100% may occur within a week after the emergence of gross signs. The occurrence of this terminal condition is significantly affected by rearing conditions, handling stress, and temperature stress (3, 44).

1.3 Histo- and cytopathology of shrimp infected with WSSV

Observation of affected shrimp by LM reveals WSSV-infected cells in tissues of ectodermal and mesodermal origin (27, 45, 46) and the histopathology is similar in all susceptible tissues (3). By H&E staining, cells in the early stages of infection show nuclear hypertrophy, nucleolar dissolution, chromatin margination, and transformation of the central area into a homogeneous eosinophilic region separated from the marginated chromatin by an unstained area as a Cowdry A-type inclusion. Later, the inclusion expands to fill the entire nucleus and becomes basophilic meanwhile the cytoplasm becomes less dense and translucent. At the late stage of infection, the nuclear membrane is disrupted. This suggests the possibility that massive death of individual cells from lytic infection induces multifocal necrosis leading to dysfunction of target organs and therefore death (3, 47).

By *in situ* hybridization assay, initial infection sites, the preferred tissues, and disease progression in *P. monodon* were studied further (48). At 16 h post-infection (pi), WSSV-positive cells were initially observed in the stomach, gills, cuticular epidermis, and hepatopancreas. At 22 h pi, the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, midgut, and hindgut were found to be WSSV-positive. The nervous tissue and compound eyes did not show WSSV-positive cells until 40 h pi at which time white spots first appeared in the cuticle. By 52 or 64 h pi, the stomach, gills, cuticular epidermis, lymphoid organ, hematopoietic tissue, and antennal gland were all heavily infected with WSSV and were severely damaged from multifocal necrosis. The organs which were more lightly infected by WSSV included the hepatopancreas, nerve nodes, compound eyes, muscles and connective tissue of the midgut and hindgut. The degree of infection of these organs did not increase and they maintained organ integrity up to the late infection stages, although a few cells had cytopathological signs and were lysed. Eventually, shrimp began to die at 64 h pi. In 1997, *in situ* hybridization and TEM, revealed WSSV in reproductive organs of *P. monodon* for the first time (45). Thus, by *in situ* hybridization, it has been confirmed that WSSV specifically infects tissues of ectodermal and mesodermal origin in shrimp. Histological diagnosis of WSSV infection is best carried out using epithelium of the stomach which, in shrimp, is covered with cuticle and is of ectodermal origin. Other

good tissues to examine for characteristic lesions are epidermal tissues of the cephalothorax and gill tissue (48).

WSSV-PCR was used to study tissue specificity of WSSV in shrimp and crabs (45, 49). Pleopods, gills, stomachs, abdominal muscle tissues, haemolymph, gut, heart, pereiopods, lymphoid organs, epidermis, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, and eye stalks were examined and all were found to support WSSV replication. In very lightly infected specimens (nested PCR positive), WSSV was particularly prevalent in gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, integument, pereiopods, eye stalks, and the hepatopancreas. In heavily infected shrimp (one step PCR positive), many of the tissues that were two-step PCR positive in lightly infected specimens were now heavily infected while others, such as the nerves and hepatopancreatic tubule epithelial cells, were still apparently not targets, even in the patent stage of infection. Furthermore, in very lightly infected shrimp, the lymphoid organ was not a main target for WSSV replication, although it became so in patent specimens.

TEM observations of affected animals showed aggregations of bacilliform viral particles in the hypertrophied nuclei and this corresponded to intranuclear inclusions by LM (1-3, 27, 33, 45). WSSV replication takes place in the nucleus and is first indicated by chromatin margination and nuclear hypertrophy. Viral morphogenesis begins by the formation of membranes *de novo* in the nucleoplasm and by the elaboration of segmented, empty, long tubules. These tubules break into fragments to form naked empty nucleocapsids. After that, a membrane envelopes the capsids, leaving an open extremity. The nucleoproteins, which have a filamentous appearance, enter the capsids through this open end. When the core is completely formed, the envelope narrows at the open end and forms the apical tail of the mature virion.

1.4 Apoptosis in WSSV infection

In addition to histopathological changes described above, apoptosis has been detected, at both molecular and cellular levels, in WSSV target tissues of infected shrimp including gills, subcuticular epithelium, stomach epithelium, integument, hematopoietic tissues, lymphoid organ, and hemocytes. A correlation between severity of infection and percentage of apoptotic cells has also been reported (4, 5). However,

there was no TEM evidence of apoptosis in enlarged nuclei of WSSV-infected subcuticular epidermal cells (5).

2. Responses of Shrimp to WSSV Infection

In general, the shrimp response to viral infection contrasts sharply with that of bacterial infection or fungal infection and with that for viral infections in vertebrates (25). It is commonly stated that shrimp, as an invertebrate, lack the specific immunity of vertebrates and that they rely mostly on innate immunity. Intensive studies at the molecular level have focused on processes involved in shrimp innate immune response to microbial infections. (50-54). A strong cellularly-mediated response usually results in eventual clearance of the invading organisms from tissues and hemolymph. Massive hemocytic aggregation, often leading to encapsulation and granuloma formation, usually occurs in heavy bacterial infection. By contrast, there is still very little knowledge on crustacean defense mechanisms to viral pathogens, compared to studies on responses to bacteria, fungi, and inert materials. From field observations and experiments, Flegel (26) has tried to summarize the general characteristics of shrimp and vertebrate interaction with viral pathogens as shown in Table 1. Viral infections in shrimp usually show no inflammatory response, in spite of considerable tissue damages. Massive hemocytic aggregation and melanization, usually occurring in bacterial infections, have not been detected however migration of hemocytes to the site of viral infection has been observed in the stomach epithelium of *P. monodon* (12). No antibodies are found in the hemolymph. Surviving shrimp always remain infected and they are still infectious when co-cultured with naïve shrimp. In addition, the occurrence of single to multiple persistent viral infections is normal (55).

As the hemocyte is the effector cell of the cellular immune response in shrimp (functioning in the processes of clotting, melanization, phagocytosis, encapsulation, non-self recognition, cytotoxicity and cell-to-cell communication (54)), studies have recently focused on hemocytic reaction to WSSV infection. In general, experimentally WSSV-infected moribund *P. monodon*, *P. japonicus*, *P. chinensis* and *Penaeus (Fenneropenaeus) indicus* showed a significant decline in total hemocyte counts but a significant increase in number of apoptotic hemocytes (5, 12, 56-58). Changes in the

proportion of different hemocyte subpopulations were also found in freshwater crayfish *Pacifastacus leniusculus* (47), the banana prawn *P. merguiensis* (59) and *P. monodon* (12).

Table 1. General characterizations of shrimp and vertebrate responses to viral infection (26)

Arthropods (including shrimp)	Vertebrates
Inflammatory response uncommon or missing	Inflammatory response common
Recovered animals commonly infected	Recovered animals commonly not infected
Recovered animals commonly infectious	Recovered animals commonly not infectious
Tolerance to viruses common	Tolerance to viruses uncommon
No antibodies found in serum	Antibodies found in serum
Multiple active infections common and normal	Multiple active infections uncommon to rare
Copious virion production	Comparatively low virion production

2.1 Apoptosis as a defense mechanism

In an attempt to prevent viral replication, viral dissemination or persistent viral infection of the cell, many of these protective measures actually involve the induction of programmed cell death, or apoptosis. In insects which lack an adaptive immune response, apoptosis has been shown to be an extremely powerful anti-viral response mechanism. By premature lysis of the infected cells, this non-specific defense mechanism leads to a reduction of viral replication, infectivity, and the ability of the virus to spread within the insect host, even if a successful infection is established (9-11). In vertebrates, the capability of apoptosis in limiting viral multiplication, although not as efficient as in insects, is responsible for the elimination of infected cells through the induction of an adaptive arm of immunity involving phagocytosis by macrophages, interferon-induced cytotoxic T lymphocyte (CTL) activation and viral peptide recognition by CTL (9, 60, 61).

A significant occurrence of apoptosis was also detected in withdrawn hemolymph of WSSV-infected shrimp as mentioned earlier. Nuclear fragmentation has been observed by fluorescence microscopy with DAPI-stained hemolymph smears

plus apoptotic bodies have also been seen by transmission electron microscopy in hemocytes in the lacunar spaces of shrimp subcuticular epidermis (5). Furthermore, a report of increased caspase-3 activity by almost six-fold in WSSV-infected shrimp compared with uninfected control shrimp has confirmed the occurrence of the 'programmed cell death' process upon WSSV infection (5). Several studies have investigated the changes in the level of apoptosis-related gene expression in WSSV-infected shrimp. So far, there was only one down-regulated, apoptosis-related gene which is the translationally controlled tumor protein (TCTP, or fortilin) gene of which the coding protein functions as an anti-apoptotic factor in mammals (14). On the other hand, apoptosis-related genes showing an up-regulation include a proteasome 26S subunit (a multicatalytic protease which regulates protein composition during cell apoptosis), Ras-like GTP-binding protein (which is involved in signaling cellular proliferation and apoptosis), QM protein (or ribosomal protein L10, a putative tumor suppressor peptide which is associated with apoptosis), programmed cell death 6-interacting protein (Alix) (an apoptotic factor), and *cap-3* (a shrimp caspase gene) (15, 16). The *cap-3* gene is the first shrimp caspase gene found in the white shrimp *P. merguiensis* (GenBank accession no. **AY839873**) (15). Its 823 bp cDNA represents a protein of 320 deduced amino acids which contain a highly conserved QACRG pentapeptide active site (Fig. 1). Phylogenetic analysis and caspase activity assay using a proluminescent caspase-3/7 substrate suggest that *cap-3* functions as human caspase-3. Caspase sequences in other shrimp species have been discovered following the white shrimp including *P. monodon* (GenBank accession no. **DQ846887**) (Fig. 2) and *P. japonicus* (GenBank accession no. **EF079670**) (Fig. 3) confirming the presence of caspase cascade in shrimp and its involvement in programmed cell death.

The idea of apoptosis as a defense mechanism in arthropods is supported by the findings of anti-apoptosis genes in insect viruses. In response to the evolutionary pressure of apoptosis, viruses have evolved several gene products with the ability to modulate apoptosis within the host (61, 62). On the one hand, it appears that viruses block virally-triggered apoptosis early in infection to prevent premature death of the host cell and so allow massive production of virus progeny or facilitate a persistent infection. On the other hand, it appears that apoptosis induction represents a method by which viruses can induce host cell death as the culmination of lytic infection to pro-

```
1    MSSAVDSARAEAHPKDGCDGGNQSLANTTENRGAEAEEPA  40
41   KDVPWGRTTAYTVVDGLSERYPMNNRPRGAALIFAHSEFK  80
81   NESLRPRPSAAHDAEIASDAFKALGFLPEVFSNLTKDELE  120
121  KTLHEVSKRDHSGSDALVIVFMSHGEVKPRNNMEFVWAKD  160
161  DKIPTKELWINFTAERCASLAGKPKLYFIQACRGLDVDKG  200
201  VNMSRAVRGMAVQTDNIGSTEEYVIPVHADQLVMWASYPG  240
241  FPAFTSNRKGIQGSVFIHYLAENLKNSANISPRPSLSSIL  280
281  LKVSREVAVLYESDIGSRNKYHENKQVPYIHSTLLREIYF  320
```

Figure 1. Deduced amino acid sequence of *P. merguiensis cap-3* gene. Putative cleavage sites are shown as the underlined while QACRG conserved pentapeptide active site is in bold.

```
1    MSDADDSARAEAQPRDGRDGENEGLANTTENRGSEAEEPA  40
41   KNVPWGRPTAYTAVDGLSECYPMNRRPRGAALIFAHSKFD  80
81   NKDLKPRPSAAHDAELVRGAFEALEFQPEVFLDLTRNDLE  120
121  GKLSEVAKRDHSGCDALAIVFMSHGEVKPRNNMEFVWAKD  160
161  DKIPTKELWINFTAERCAGLAGKPKLYFIQACRGPDVDKG  200
201  VNMTRAVRGMAVQTDSIEEYVIPIHADQLVMWASYPGFPA  240
241  FTSQREGIQGSVFIHFLAENLRNYALSSPRPSLSSILLKV  280
281  SREVAVLYESDIDSQNQYHKNKQVPYIHSTLLREIYF     317
```

Figure 2. Deduced amino acid sequence of *P. monodon cap-3* gene. Putative cleavage sites are shown as the underlined while QACRG conserved pentapeptide active site is in bold.

```
1    MDEVIQVTQWLSSITPRKKAKLFFFTTHADAPTPMVGSND  40
41   VDNHEETQLPLATTVALHNFKNIDEFLKKKVNTFYCNNVI  80
81   ENEITKTDYEYLRFYSACKYLDVVPPFLDLEKKSAIQNKF  120
121  EMTDEERDYRLLLDSADLVLEKVCDSLKPAGVSQMWTALR  160
161  KDRNLIRGVKTIEIIGFSVDPNPTGIKECLFFFIVRHLQM  200
201  IKKINRVNTNRLERLIAQCDESSACEEGRTIEFIIDSLRF  240
241  YPTELYPFGFCLIMCVNEGRDGAMQEIEKTMEISRHLGMI  280
281  THTEKNPIKETIERVKEELMKPKYQFYSSFTCWFMSHGDS  320
321  NSVVLADGETIEREWFLTSFPDISTFQLKPKVFFMVSCRG  360
361  ANKFRLEGDEFECQSATCDYYDIGSALIRNRMLDITDSVR  400
401  DISYVQPMMDTLVAYSTIPDHLSGRANTHGSIYVDRACVL  440
441  MSENISRGRSLTEILEHLSQDLHEILQETMDEDRIVYKQA  480
481  CHYDSFFRKTLKLPYTRYPKEDVHSAPQ              508
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Figure 3. Deduced amino acid sequence of *P. japonicus* caspase gene.

mote viral dispersal while evading host inflammatory and other immune responses. Table 2 summarizes examples of viral products which have been found to block/induce apoptosis in animal and insect cells (7). The mechanisms of apoptosis manipulation of these proteins include (as shown in Table 3): modulating TNF and Fas apoptosis signaling pathway (such as E3-10.4K and E3-14.5K of adenoviruses and LMP-1 of EBV), controlling immune activity and cell proliferation through the IFN-mediated apoptotic pathway (ex. vFLIPs of herpesviruses), acting as Bcl-2 protein to regulate cytochrome c/Apaf-1/caspase-9 pathway (such as BHRF1 of EBV and E1B-19K of adenoviruses), suppressing caspases (such as baculovirus inhibitor of apoptosis protein or IAP and p35), manipulating cell cycle (such as large T antigen of simian virus 40 and pX protein of hepatitis B virus), dealing with the oxidative stress feature of apoptosis (such as GPx protein of hepatitis C virus, HIV-2, coxsackievirus B3 and measles virus), functioning as a serine/threonine kinase (such as U_S3 and U_S5 of herpes simplex virus), effecting transcription of the infected cells (such as E2 of paillomaviruses) and targeting endoplasmic reticulum (such as M-T4 of myxomavirus) (see review in 61). However, strategies to overcome the hindering effects of apoptosis include not just a simple biochemical manipulation via viral anti-apoptosis genes but also rapid multiplication of viruses before an effective immune response can be mounted and an establishment of persistent infection (9, 61). WSSV has also been shown to possess an anti-apoptosis gene in its ORF390 (63). ORF390 of WSSV, or wsv390 (GenBank accession no. **NP 477912**), is 966 bp long and encodes an about 35kDa protein which contains two caspase-9 cleavage sites, LLVETDGPS and VKLEHDGSK, and a caspase-3 cleavage site, EEDEVDGVP (Fig. 4). Its activity in blocking apoptosis has been revealed in an insect cell line (63) however there is still no solid *in vivo* data which confirms its function in shrimp WSSV infection.

2.2 Resistance/Tolerance

Evidence of resistant, tolerant or persistent viral infections have been seen in many shrimp species (25, 26, 64). Shrimp species, varieties, and strains that are not susceptible to a viral agent will not become infected upon challenge and will show no evidence of pathology or response to the pathogen. Susceptible shrimp, species, varieties, and strains will become infected upon challenge and may show signs of viral

pathology, at least initially. Subsequent to infection, susceptible shrimp that are resistant to a viral pathogen will show evidence of defense and clearance activities and will eventually become free of the virus and viral pathology. By contrast, susceptible shrimp that are tolerant to a viral pathogen will show evidence of a persistent, transmissible viral infection with or without clearly detectable signs of viral pathology. Generally, resistance or clearance appears to be the case in vertebrates while tolerance appears to be the case in arthropods (26). However, this is not a strict, exceptionless rule.

Table 2. Viral products with apoptosis-inducing or apoptosis-inhibiting activities

Virus	**Viral protein(s) that inhibit apoptosis**	**Virus**	**Viral protein(s) that induce apoptosis**
Adenovirus	E1B-19K, E1B-55K, E3-14.7K, E3-10.4/14.5K	Adenovirus	E1A 12S and 13S proteins, E3, E4
Baculovirus	p35, Inhibitor of Apoptosis (IAP)	Chicken anaemia virus	Apoptin (VP3)
Human cytomegalovirus	IE1, IE2	Human immunodeficiency virus type 1	Tat
Epstein-Barr virus	LMP-1, BHRF1	Parvovirus	NSP
Hepatitis B virus	pX	SV40	Large T antigen
Hepatitis C virus	Core protein	Human papillomavirus	E2, E7
Herpesviruses	γ34.5 gene, vFLIPs		
Human papillomavirus	E6		

Table 3. Mechanisms of apoptosis manipulation by viral products

Mechanisms of Apoptosis Manipulation
IFN pathway mediators
Bcl-2-related proteins
Caspase suppressors
Cell cycle regulators
Oxidative stress regulators
Protein kinases
Transcriptional modifiers
Endoplasmic reticulum-targeting factors

```
1    MCTLKTYKMTTSTEISKNLSDVLSIKATGDWCSNIKTVFS  40
41   PFTEGKGNLPNSLPFTRSPNTTCGSREAANATEHFITVFA  80
81   KDKYERKRVKRTIGFTLDNTKELTPNRYLVADVYSWQEEK  120
121  MVFEGFCVPPGKSGTFVRYSNEDKSFLLADTGRYMKKKYD  160
161  DPENKTSSGGDDDDDDDDDDDNNNVDVYEENDPRNVFEV  200
201  EKDEKYACTFSILVYRAMKKSPPVCRGLLVETDGPSSHPK  240
241  RAPSAFNPFGGSSMLNGYGAGADALEEEDEVDGVPERERI  280
281  TNFALKRGPATGQNFVSVKLEHDGSKADLYNVTCFSKQRG  320
321  V
```

Figure 4. Deduced amino acid sequence of wsv390. Putative cleavage sites are shown as the underlined.

Implications regarding disease tolerance in shrimp were initially revealed by the first widespread, pathogenic, hypodermal and hematopoietic necrosis virus (IHHNV) infection in *Penaeus (Litopenaeus) stylirostris* and *P. vannamei* in the Americas (65) and secondly by the observation of yellow head virus from the second most serious viral epizootic of shrimp occurring in Thailand in the early 1990s (66). Based on these two instances, a prediction was made for a viral epizootic in Thai shrimp ponds that, within 1-2 years from the initial period of a widespread catastrophic outbreak of a new virus, the outcome of viral presence in a pond will change from universally disastrous to innocuous and the prevalence of ponds containing WSSV-infected shrimp of normal appearance will increase (67). In late 1994, occurrence of WSSV outbreaks began in Thailand and the phenomenon was consistent with the previous prediction (38, 68). The mechanism was suggested to be due more to a genetic change in the host rather than a change in the virus or to be via a process of self-recognition during viral exposure in early larval stages. These data have led to the proposed hypothesis of 'viral accommodation', which states that virally-triggered apoptosis is the cause of mortality and that shrimp accommodate viral pathogens as persistent infections through a kind of memory that functions in dampening virally-triggered apoptosis to specifically reduce the severity of disease (26).

One of the phenomena that renders the shrimp resistant/tolerant to WSSV is high culturing temperature. It has been noticed in farming practices in Thailand that in

summer or during hot weather periods, *P. monodon* shrimp are less susceptible to WSD from WSSV infection (69). It has also been found in *P. vannamei* that an increase in temperature from 26°C to 33°C completely inhibited or dramatically reduced mortality of infected shrimp (70, 71). However, after exposure to hyperthermia and switching to normal temperature (27°C) post challenge, shrimp developed WSD and died (71) (Fig. 5). In *P. monodon*, preliminary studies by a private company showed similar results that water temperature of 33°C reduced shrimp mortality from WSSV challenge, compared to shrimp reared at 28-30°C (unpublished data). The shrimp challenged at 33°C became WSSV infected, as detected by PCR. They were therefore tolerant, rather than resistant, to WSSV infection. However, some of the shrimp that were exposed for long duration at high temperature with low doses of WSSV cleared their load of WSSV.

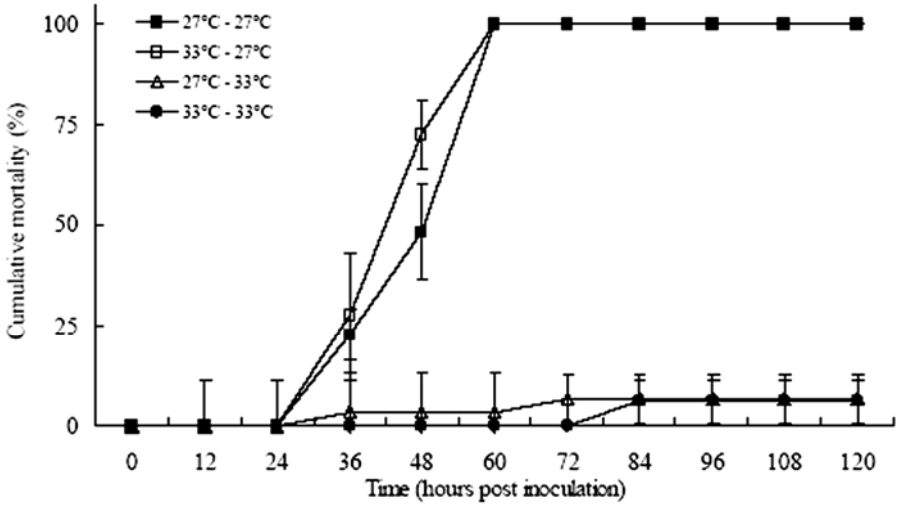

Figure 5. Cumulative percent mortality of *P. vannmei* challenged with WSSV at different culture temperatures. Shrimp reared at high temperature before and after challenge (33-33°C) and shrimp reared at normal temperature before but high temperature after challenge (27-33°C) survived WSD significantly better than shrimp reared at normal temperature before and after challenge (27-27°C) and shrimp reared at high temperature before but normal temperature after challenge (33-27°C).

3. Double-stranded RNA (dsRNA)

dsRNA, a common intermediate generated during the genome replication of positive-strand RNA, dsRNA, or DNA viruses (72), is a potent trigger of RNA silencing or RNA interference (RNAi) and an innate immune response against viral infection in eukaryotic cells (73). Via RNAi, a strongly conserved sequence-specific gene silencing mechanism, dsRNAs are processed into small RNA fragments of 21-25 nucleotides (miRNAs or siRNAs) prior to forming an RNA-induced silencing complex (RISC) which hybridizes with the mRNA of complementary sequences resulting in an mRNA cleavage or translational repression (Fig. 6) (74, 75). This gene silencing machinery functions in two distinct processes, including regulation of cellular gene expression and constitution as an arm of the antiviral network that inhibits incoming viruses by specific degradation of viral products (Fig. 7) (76). Apart from the RNAi-related antiviral pathway, dsRNA associated with viral replication also induces a multitude of innate antiviral responses through the induction of type I interferons (IFNs) (Fig. 8) (73, 77-79). IFNs comprise a family of cytokines expressed in response to viral infection and other insults and regulate a myriad of cellular and systemic responses directed to control viral propagation. Type I IFNs, which include several IFN-α and a single IFN-β type, are directly inducible by virus. Induction of IFN by dsRNA leads to the upregulation of the IFN-stimulated genes (ISGs) which will generate an inflammatory response, shut down protein translation, modify RNA molecules and promote apoptosis. Among the IFN-induced proteins implicated in the antiviral actions of IFNs in virus-infected cells are RNA-dependent protein kinase (PKR), the 2',5'-oligoadenylate synthetase (OAS), the RNA-specific adenosine deaminase (ADAR), and the Mx protein GTPases (see review in 77). PKR, one of the best known IFN-induced proteins, is a cytoplasmic serine-threonine kinase with dsRNA binding motifs. Once activated, PKR induces IFN generation and inhibits protein synthesis by phosphorylating the eukaryotic initiation factor 2 (eIF-2α) therefore leading to inhibition of viral protein expression and limitation of viral replication and pathogenesis. Besides its function as a regulator of protein translation, PKR also exerts antiviral activity through the induction of apoptosis involving eIF-2α phosphorylation and the activation of transcription factors NF-κB, p53 or STATs (80-

83). IFN-induced OAS catalyzes the synthesis of unusual nucleic acid-based 2'-5'-oligoadenylates which then activate a ribonuclease, RNaseL, to nonspecifically degrade single-stranded RNAs (ssRNAs) including mRNA and rRNA in the cytoplasm of the cell, thus limiting replication of the virus. ADAR, an important RNA-editing enzyme, post-transcriptionally modifies RNA by deaminating adenosine to yield inosine, resulting in an alteration of viral and cellular RNA's functional activities and thus affecting biological processes of the infected cells. Mx proteins are GTPases that have been shown to exert antiviral activity from animal model studies which establish that Mx alone is sufficient to block the replication of virus in the absence of any other IFN-α/β-inducible proteins. Its spectrum of antiviral activities and molecular mechanisms are dependent on the specific Mx protein, its subcellular site of localization and the type of challenge virus examined.

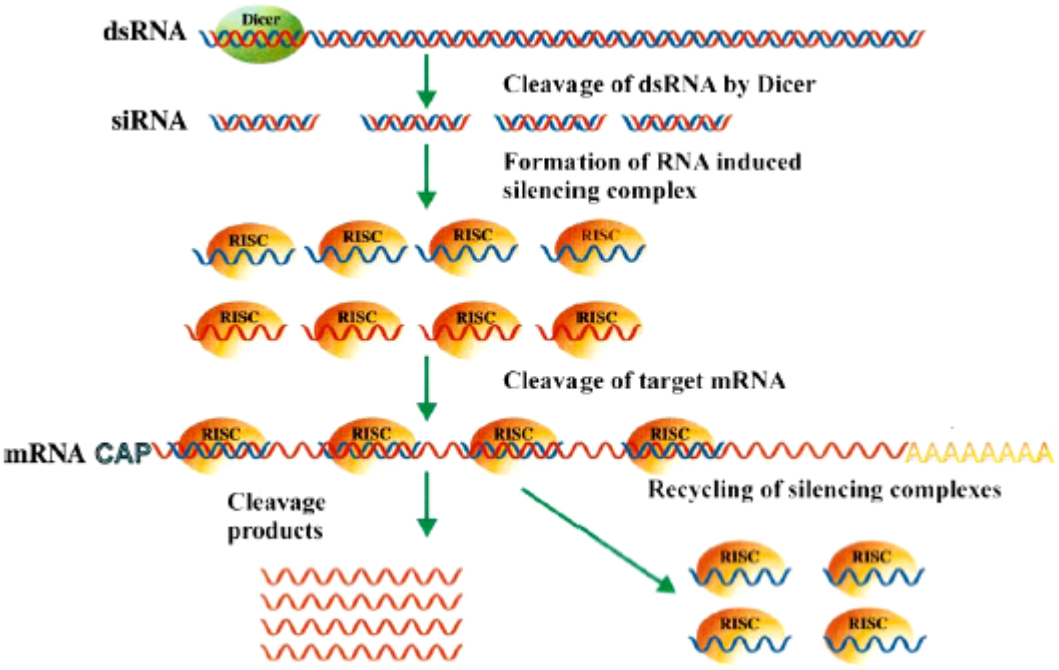

Figure 6. Schematic representation of RNAi machinery (74). Primary dsRNAs are processed by Dicer into small fragments of 21-25 bp (miRNAs or siRNAs) which will then form an RNA-induced silencing complex (RISC). Upon hybridization of RISCs with the target mRNA, cleavage of target mRNA is triggered resulting in a translational repression of that gene.

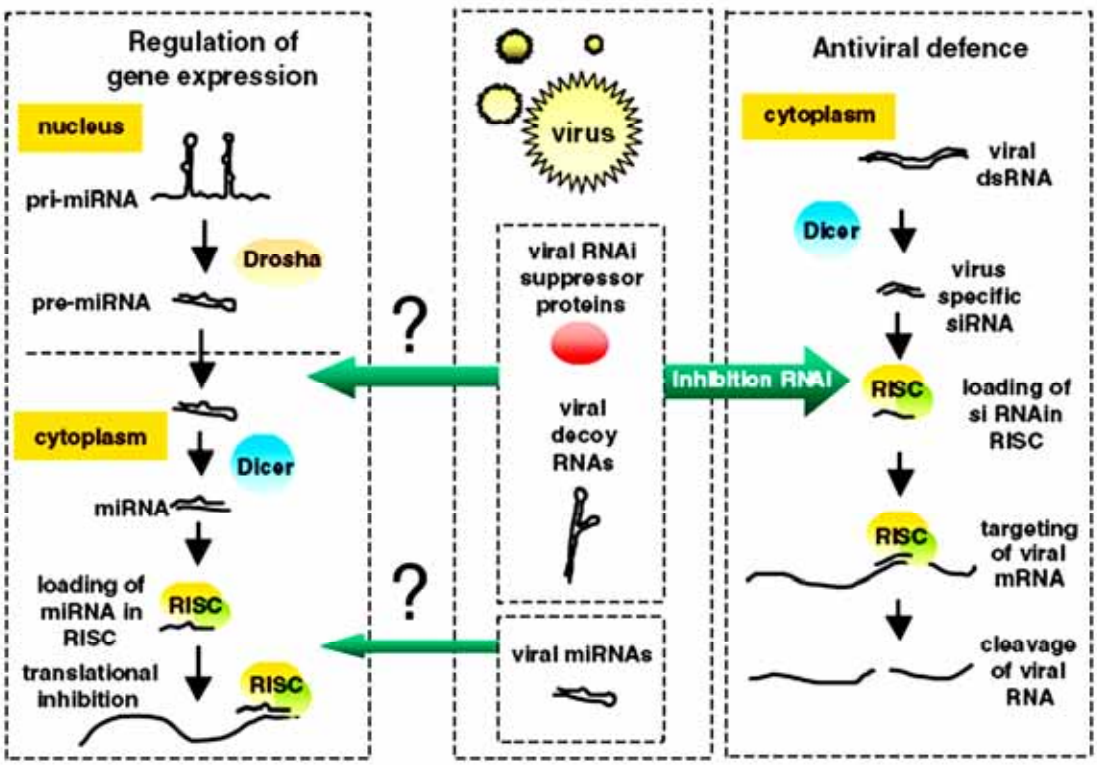

Figure 7. RNAi as mechanisms of gene expression regulation and of antiviral defense (76). Regulation of gene expression is driven by primary miRNAs (pri-miRNA) in the nucleus which is cleaved into pre-miRNA and miRNA by Drosha and Dicer prior to forming RISC, leading to a translation inhibition of the target gene. RNAi-mediated antiviral defense is triggered by presence of viral dsRNA in the cytoplasm. Virus specific siRNA is then generated from cleavage of viral dsRNA by Dicer prior to loading into RISC, resulting in a cleavage of viral RNA.

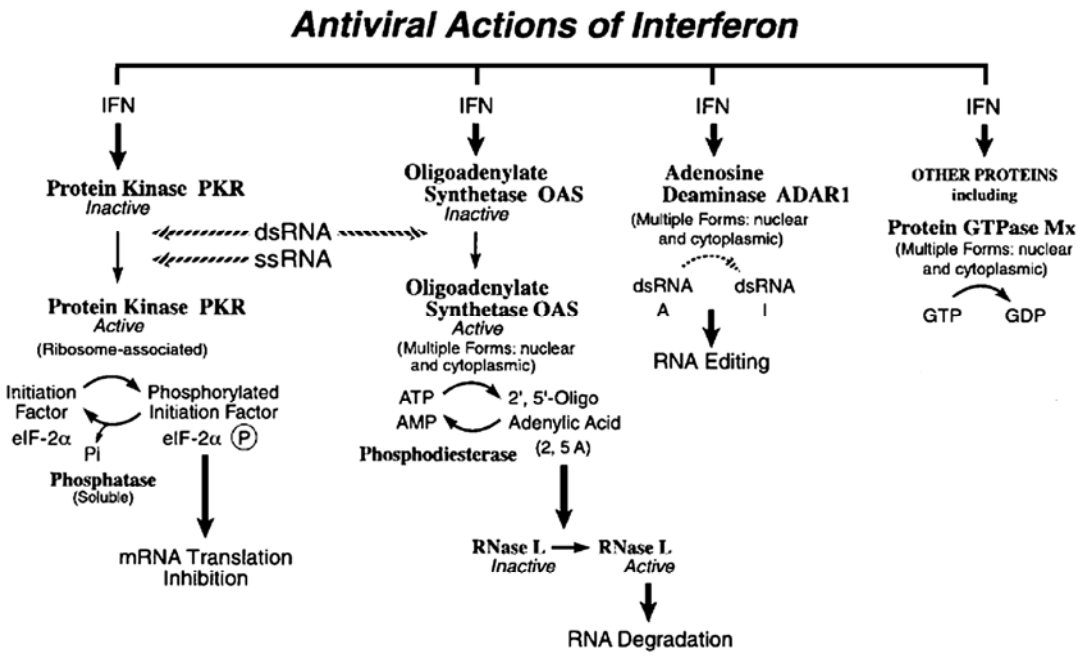

Figure 8. Functions of IFN-inducible proteins which are believed to affect virus multiplication (77). PKR kinase inhibits translation initiation through the phosphorylation of protein synthesis initiation factor eIF-2α. OAS synthetase and RNase L nuclease mediate RNA degradation by the formation of 2',5'-oligoadenylate. ADAR edits dsRNA by deamination of adenosine to yield inosine. Mx protein GTPase family appears to target viral nucleocapsids and inhibit RNA synthesis.

In invertebrates, with penaeid shrimp as a model, three distinct phenomena induced by administration of long dsRNA have recently been revealed both *in vivo* and *in vitro* (84). These include a nonspecific innate immune response against viral infection, an RNAi-mediated specific antiviral immunity and a systemic endogenous shrimp gene silencing. Due to the lack of genes homologous to IFNs or to the major effectors of the IFN response in several fully sequenced invertebrate genomes (85-88), it has been generally accepted that invertebrates have no dsRNA-induced innate immune responses. However, recent evidence showed that injections of dsRNAs or siRNAs of arbitrary sequences partially protected the shrimp from TSV and WSSV infections, suggesting the presence of dsRNA molecular pattern recognition as an innate anti-viral immune response in penaeid shrimp (24, 89). The mechanism of this

broad-spectrum protection in shrimp is presently unknown but it was found to correlate with the inhibition of viral replication as protected shrimp showed less numbers of WSSV infected cells by immunohistochemical staining compared to no dsRNA treated control shrimp (24). Nonetheless, this sequence-independent protection from dsRNA can be overwhelmed by high doses of viral challenge, the dose at which another distinct and more effective anti-viral mechanism has been demonstrated (22). The administration of virus-specific dsRNA prior to or together with high doses of viral challenge induces a potent RNAi-based specific antiviral response that results in highly efficient control of viral disease progression in challenged shrimp (22, 23, 90). This RNAi-related antiviral phenomena has also been noticed in *in vitro* studies of YHV challenged shrimp primary cell culture with YHV nonstructural gene targeting dsRNA treatment confirming the existence of RNAi machinery in shrimp and its function in sequence-specific pathway of antiviral immunity (21). Though these results are not formal proof that shrimp use RNAi as a natural antiviral mechanism against these viruses (84). The last phenomenon found to be triggered by administration of dsRNA is an RNAi-mediated endogenous shrimp gene knockdown (22). By injecting cognate long dsRNA of a shrimp gene, substantial and specific gene silencing can be induced systematically, implying the traveling of dsRNA from the site of injection to distant tissues and the existence of cell surface receptors for dsRNA uptake into the cells (84). This finding opens great possibilities of using reverse genetic approaches to understand gene function in shrimp, especially the immune genes for the sake of disease control in farming practices, although interpretation is still complicated by non-specific induction of antiviral immune response.

CHAPTER III
MATERIALS AND METHODS

Part I: WSSV Infection Bioassay

1. Animals and Experimental Viral Infection

Shrimp (*P. monodon*) weighing 15-20 grams were used for WSSV challenge and buffer injection control. After 2-day acclimation, shrimp were injected with 100 µl of WSSV-containing shrimp extract (1:100 dilution in lobster hemolymph medium, LHM) or plain LHM. At 24 and 48 hrs after challenge, 6 shrimp were collected from each treatment for hemocyte sampling using 26-guage syringes filled with 4% paraformaldehyde in 0.45M NaCl at a ratio of 1:1 for immunohistological studies.

2. Immunohistochemistry and DAPI Staining

Fixed hemocytes were washed 3 times with PBS by centrifugation at 700g (held at 4 ºC) and resuspended in 70% ethanol. Aliquots were let to dry on Histobond® UniMark® microscope slides (Marienfeld) and stored at 4ºC until staining. Prior to staining, cells were rehydrated in PBS for 15 min, permeabilized with 0.1% TRITON X-100 in PBS (containing 10% fetal bovine serum, FBS) for 5 min and washed 3 times with PBS, 5 min each. Non-specific binding was blocked using 10% FBS for 30 min. Cells were then incubated with polyclonal rabbit anti-VP664-1 antibody (91) (gift from Dr. Lo CF), diluted 1:1000 in 10% FBS, at 4ºC overnight. Excess primary antibody was washed with PBST (0.05% Tween-20 in PBS) 5 times for 5 min each, and bound antibody was detected using alkaline phospatase-conjugated goat anti-rabbit IgG antibody (Zymax™ Grade) (ZYMED® Laboratories, Invitrogen immunodetection), 1:200 dilution in 10% FBS. After a 60-min incubation with secondary antibodies, slides were washed 5 times, 5-min each, with PBST and treated with NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) substrate (Roche) for 30 min. Stained cells were imaged

under an Olympus BX51 microscope. WSSV-positive cells developed a purple-blue color and 1000 cells total were counted.

For apoptosis detection, dried cells were rehydrated in PBS for 15 min followed by 15-min incubation with 5 μg/ml DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) solution (Roche) for nuclear stain. Excess DAPI was washed off with methanol and distilled water. Stained slides were mounted using ProLong® Gold antifade reagent (Molecular Probes), dried overnight to let the reagent cure, sealed with clear nail polish and stored at -20°C until imaging with a Nikon ECLIPSE E600 fluorescent microscope. Apoptotic cells characterized by condensed and fragmented nuclei were counted in 1000 cells total.

Part II: Hyperthermia and WSSV Infection Bioassay

1. Animals and Experimental Viral Infection

Shrimp (*P. monodon*) weighing 15-20 grams were reared in separated tanks, 20 animals per tank, for 5 days of acclimation and temperature control (26-28ºC or 32-34ºC.) The temperature was kept under control until the end of the bioassay. One day after the water temperature was stable at the specified level, shrimp were injected intramuscularly with 100 μl of WSSV containing shrimp extract (1:1000 dilution in LHM) or LHM. Mortality was recorded daily over a period of 7 days after viral injection. At 50% cumulative percent mortality, which was 56 hr post-challenge (hpc), and 168 hpc, individual surviving shrimp were taken for hemolymph and pleopod collection. Hemolymph was withdrawn directly into 10% formaldehyde in 0.45M NaCl using 26-gauge syringes for immunofluorescent studies of hemocytes. Pleopods were kept in absolute ethanol until DNA isolation for WSSV detection.

2. DNA Isolation and Detection and Quantification of WSSV Using Real-Time SYBR Green PCR

To isolate DNA, pleopods were removed from absolute ethanol into DNA lysis buffer (SBBU) and mechanically homogenized with 1.5-ml-microtube-size homogenizers followed by incubation with 1 μg/ml Proteinase K (final concentration) at 56ºC, overnight. Aliquots of 50 μl of sample solution were taken for DNA isolation

using Agencourt SprintPrep™ Kits (Agencourt Bioscience). Amount of isolated DNA was measured spectrophotometrically and diluted to be 2.5 ng/µl prior to WSSV detection by real-time SYBR Green PCR (92). Standards were from a 10-fold dilution of WSSV containing genomic DNA which was extracted from pleopods of WSSV-infected, moribund shrimp. SYBR Green PCR amplifications were performed in a GeneAmp 7500 thermocycler coupled with a GeneAmp 5700 sequence detection system (PE Applied Biosystems). The reactions were carried out in a 96-well plate in a 25-µl reaction volume containing 7.1 µl of 2× QuantiTect™ SYBR® Green PCR Master Mix (Qiagen), a 0.24 µM concentration of each forward and reverse primer and 5 ng of isolated DNA. The primers for WSSV (Table 4) were 101-110F, 5'-GAT AAG AGA GGT CGA CAC TAG TAG TGT TAT TGC T-3', and 101-165R, 5'-CCA CTG TGC CAG CTA TTG CA-3'. The primers for β–actin were 178F, 5'-GGT CGG TAT GGG TCA GAA GGA-3', and 228R, 5'-TTG CTT TGG GCC TCA TCA C-3'. The PCR cycle was set to 50°C for 2 min then 95°C for 10 min followed by 40 cycles of 95°C 10 min intervals and 60°C 1 min intervals. Melting curve analysis was performed later at 95°C for 15s, 60°C for 1 min and 95°C for 15s. Samples were run in duplicate and standards were run in triplicate in a separated plate. Data analysis was performed using the relative standard curve method.

3. Data Analysis for SYBR Green PCR

At the end of each SYBR Green PCR run, data analysis was performed with the 5700 Sequence Detection System software (SDS version 1.2) and the relative amount of virus was determined using the relative standard curve method. Briefly, the threshold PCR Cycle (C_T) obtained in each sample was verified and inversely calculated back into copy number from the standard curve of the corresponding gene. The average copy number of target gene (WSSV), in individual shrimp, was then divided by the average copy number of control gene (β-actin) to achieve the normalized WSSV value. The average of normalized WSSV values from five individual shrimp of each treatment was used for statistical analysis.

4. Immunofluorescent Staining of Hemocytes

Fixed hemocytes were centrifuged at 700g (while held at 4ºC) for 10 min and washed 3 times with PBS. Cell pellets were then resuspended with 70% ethanol and aliquot were let dry on Histobond® UniMark® microscope slides (Marienfeld). Dried slides were kept at 4ºC until staining. Prior to staining, slides were rehydrated in PBS for 15 min and permeabilized with 0.1% TRITON X-100 in PBS (containing 10% normal goat serum, NGS) for 5 min. Non-specific binding was blocked by 10% NGS 30-min incubation. Anti-VP28 mouse monoclonal IgG 5-5C at 1:1000 final dilution in 10%NGS (93) (gift from Javier Robalino) was used as primary antibody to detect WSSV in hemocytes. After primary antibody incubation at 4ºC overnight, excess antibody was washed 5 times, 5 min each, with PBST followed by Alexa® 594-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Molecular Probes) diluted 1:500 in 10% NGS as secondary antibody. After incubating with secondary antibody for 30 min, slides were washed 5 times, 5 min each, with PBST and nuclear-counterstained with DAPI for 15 min. Excess DAPI was washed with methanol and distilled water. Stained cells were then mounted with ProLong® Gold antifade reagent (Molecular Probes), dried overnight to let the reagent cure, sealed with clear nail polish and stored at -20°C until imaging with a Nikon ECLIPSE E600 fluorescent microscope. WSSV-positive cells displayed the red color of Alexa® 594 while apoptotic cells were characterized as described previously. Both of them were counted as the number of cells in 2000 cells.

Part III: dsRNA Bioassays

1. Animals and Experimental Viral Infection

Shrimp were stocked and challenged as described by Robalino *et al.* (22, 24). Specific pathogen-free (SPF) *P. vannamei* (1 g BW) were stocked individually in 260-ml flasks and acclimatized for 2-3 days before experiments, with 100% water exchange daily (artificial seawater; Marine Environment), and fed approximately half a pellet of commercial feed every day. After acclimatization, 37-43 shrimp each were treated with 5 μg of various dsRNA, a dose determined to be effective for mRNA silencing, or saline (400mM NaCl-10mM Tris-Cl, pH 7.4) by intramuscular injection

into the 2nd abdominal segment 2 days prior to high or low dose WSSV challenge. Control shrimp were injected with SPF shrimp extract at an equivalent dilution. The injection volume of dsRNA and viral extract was 20 µl per shrimp. The dsRNAs used were *cap-3* dsRNA (dsRNA targeting shrimp caspase gene), complement subcomponents C1r-C1s/sea urchin protein Uegf/bone morphogenetic protein 1 (CUB) domain protein (CDP, GenBank accession no. **AY907539**) (22) dsRNA (dsRNA of non-apoptosis related shrimp gene, serving as dsRNA treatment control), wsv390 dsRNA (dsRNA targeting ORF390 of WSSV) and VP19 dsRNA (dsRNA of WSSV envelope protein VP19). High and low doses of WSSV were prepared by diluting WSSV-containing extract 10^6 times and 24×10^6 times, respectively, with saline. The former caused 100% mortality while the latter caused approximately 80 ± 5 % mortality. After WSSV injection, mortality was recorded daily over a period of 10-12 days. Hemocytes were taken by directly withdrawing hemolymph into 26-gauge syringes pre-filled with 10% formalin in penaeid shrimp salt solution (PSS). Hepatopancreas were collected into RNAlater (Ambion) for total RNA isolation. Pleopods were kept in sarcosyl-urea (1% sarcosyl, 8M urea, 20mM sodium phosphate, 1mM EDTA) until DNA isolation. Gills were fixed in Davidson's fixative prior to tissue processing for histological studies.

2. RNA Isolation and cDNA Synthesis

Total RNA isolation of hepatopancreas collected in RNAlater (Ambion) was performed by using an RNeasy Mini Kit (Qiagen). Amounts of isolated RNA were then measured spectrophotometrically. Five µg of each RNA sample was treated with DNase I (Fermentas) and then reverse transcribed into cDNA using PowerScript™ Reverse Transcriptase (BD Biosciences Clontech). Transcribed cDNA was diluted 20 times and 2 µl aliquots were used as templates in PCR and real-time SYBR Green PCR.

3. Isolation of *P. vannamei cap-3* cDNA

Partial sequences of *P. vannamei cap-3* were amplified by RT-PCR from total RNA of normal and WSSV-infected shrimp, using primers that were designed from *P. merguiensis* (GenBank accession no. **AY839873**) (15) and *P. monodon cap-3* genes

(GenBank accession no. **DQ846887**) (a gift from Dr. Saengchan Senapin). The forward primers were CAP1F, 5'-AAG CTT GCT GCT CTC ATC TTC GCT CA-3', and CAP2F, 5'-AAG CTT GTC TAG CCG GCA AAC CTA AG-3', with *Hind III* restriction site at the 5'end. The reverse primers were CAP1R, 5'-TCT AGA TCA GCC GTG AAG TTT ATC CA-3', and CAP2R, 5'-TCT AGA CCA CTT CCC TGC TGA CTT TG-3', with *Xba I* restriction site at the 5'end (Table 4). To obtain the full length *cap-3* cDNA, 5'-RACE (Rapid Amplification of cDNA Ends) and 3'-RACE cDNA libraries (BD SMART™ RACE cDNA Amplification Kit; BD Biosciences Clontech) of hemocytes, hepatopancreas, and gill tissues were used (gifts from Dr. Nuala O'Leary, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC) as templates for PCR with CAP1R and CAP2R reverse primers and CAP1F and CAP2F forward primers. Expected RT-PCR products were gel-purified using Qiaquick Gel Extraction Kit (Qiagen) and cloned into pCR2.1®-TOPO® or pCR®4-TOPO® cloning vectors (Invitrogen). Clones were then transformed into One Shot® TOP10 competent cells (Invitrogen). Positive colonies were tested for the insert by PCR and *EcoR I* restriction enzyme digestion. Plasmids were extracted using Qiaprep Spin Miniprep Kit (Qiagen) or Qiagen Plasmid Maxi Kit (Qiagen) and sequenced on both strands. DNA sequencing was performed using an ABI prism 377 automated sequencer. Finally, the sequence of *P. vannamei cap-3* was confirmed by sequencing the PCR product of the full length cDNA.

4. Construction of dsRNAs

dsRNAs were constructed by the annealing of ssRNAs (single-stranded RNAs) generated by in vitro transcription using recombinant pCR®4-TOPO® vectors (Invitrogen) as templates. Portions of *P. vannamei cap-3* (317-bp) and wsv390 (294-bp) were amplified by PCR from infected and non-infected *P. vannamei* hepatopancreas cDNA using primers CAP1F and CAP1R for *cap-3* and 390-2F, 5'-AAG CTT CGA GGT CGA AAA GGA TGC AA-3' and 390-2R, 5'-TCT AGA ACA AAG TTC TGG CCA GTT GC-3' for wsv390 (Table 4). PCR products were subcloned into pCR®4-TOPO® cloning vectors for ssRNA transcription using T3 and T7 phage RNA polymerases (Promega). DNA templates were degraded following transcription by addition of DNase I (Promega) at a ratio of 1 U/µg of template, 15

min incubation at 37°C and the transcripts were purified by organic solvent extraction (standard method). cRNA strands of T3 polymerized and T7 polymerized were mixed together in the presence of 400mM NaCl-10mM Tris-Cl (pH 7.4) and annealed by incubation at 75°C, 65°C, and room temperature for 15 minutes each. The formation of dsRNA was monitored by determining the size shift in agarose gel electrophoresis, and the concentration of dsRNA was measured spectrophotometrically. CDP and VP19 dsRNAs were as described by Robalino *et al*. (22).

5. Quantification of *cap-3* Expression Using Two-Step Real-Time SYBR Green RT-PCR

Transcribed cDNA samples, diluted to 1:20, were used as templates in real-time SYBR Green PCR to detect *cap-3* expression along with an internal control gene, elongation factor 1α (EF-1α). Standards were prepared from 10-fold dilutions of RT-PCR products which cover real-time RT-PCR amplicons. Primers for constructing a *cap-3* standard which gave an amplicon size of 457 bp, were vCAP-330F: 5'AAG CTT GTT CTT CGA CCT CAC GAA GG3' and vCAP-777R: 5'-CTC GAG GGC CAA GAA GTG GAT GAA GA-3'. Primers for an EF-1α standard construction, giving amplicon size 294 bp, were EF1a-294F: 5'-CAC TGC TCA CAT TGC CTG CA3' and EF1a-294R: 5'TTA CTT CTT CTT GAG GGC CT-3' (Table 4). The SYBR Green RT-PCR assay was carried out in a GeneAmp 7500 thermocycler (PE Applied Biosystems). The amplifications were performed in a 96-well plate in a 25-µl reaction volume containing 7.1 µl of 2× Quantitect^{TM} SYBR$^{®}$ Green Master Mix (Qiagen), 0.24 µM each of forward and reverse primers and 2 µl of 1:20-diluted cDNA. Forward and reverse primers specific to *cap-3* and EF-1α genes were vCAP-432F: 5'-CGG CGA AGT CAA AGC CAG AAA CAA-3', vCAP-497R: 5'-TTG GTG GGA AGC CTA TCG TCT TTG-3', EF1α-25F: 5'-TCG CCG AAC TGC TGA CCA AGA-3' and EF1α-79R: 5'-CCG GCT TCC AGT TCC TTA CC-3' (Table 4). *cap-3* and EF-1α amplicon sizes were 66 and 55 respectively. The thermal profile was 50^{o}C for 2 min and 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. Dissociation stages were performed later at 95°C 15s, 60°C for 1 min and 95°C for 15s to confirm product specificity. Samples were run in triplicate and standards were

run in duplicate for every plate. The relative level of expression was calculated using the relative standard curve quantitation method.

6. DNA Isolation and Detection and Quantification of WSSV Using Real-Time SYBR Green PCR

Pleopods in sarcosyl-urea were incubated at 80°C for 3 days and 50 µl aliquots of sample solution were taken for DNA isolation using Agencourt SprintPrep™ Kits (Agencourt Bioscience). Extracted DNA was diluted 50 times with deionized water and aliquots of 2 µl were used as templates in real-time SYBR Green PCR for WSSV detection as described earlier.

7. Data Analysis for SYBR Green PCR and RT-PCR

At the end of each SYBR Green PCR run, data analysis was performed with the 5700 Sequence Detection System software (SDS version 1.2) and the relative amount of virus was determined using the relative standard curve method as described earlier. For SYBR Green RT-PCR, the relative level of mRNA expression was calculated in the same manner as the SYBR Green PCR for WSSV detection. In this case, the target gene was *cap-3* and the control gene was EF-1α.

8. Immunofluorescent Staining of Gill Tissues and Hemocytes

For histological studies, gill tissues were fixed in Davidson's fixative for 24 hrs and stored in 70% ethanol until tissue processing and hemocytes were fixed by the direct withdrawal of hemolymph into 10% formaldehyde in 0.45M NaCl. Paraffinized tissues were sectioned into 5-µm thickness and attached to Histobond® UniMark® microscope slides (Marienfeld) with an overnight-42°C incubation. Fixed hemocytes were let stand on Histobond® UniMark® microscope slides (Marienfeld) for 2 hours to allow the attachment of cells to the slides. Remaining liquid was poured off and dried slides were kept at 4°C until staining.

Immunofluorescent staining of hemocytes was performed as described in part II. For gill tissues, slides were deparaffinized through xylene and a series of diluted alcohol prior to equilibrating with PBS for 15 min. Tissues were permeabilized with 0.2% TRITON X-100 in PBS for 15 min. Non-specific binding was blocked by 30-

min incubation with blocking solution containing 5% fetal bovine serum (FBS) and 0.5% bovine serum albumin (BSA) in PBS. Anti-vp28 monoclonal IgG 5-5C, 1:1000 final dilution in blocking solution, was used as primary antibody to detect WSSV. After primary antibody incubation at 4°C overnight, excess antibody was washed 5 times, 5 min each, with PBST followed by Alexa® 594-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Molecular Probes) diluted 1:500 in blocking solution as secondary antibody. After incubating with secondary antibody for 30 min, slides were washed 5 times, 5 min each, with PBST and counterstained nuclei by incubating with DAPI solution for 15 min. After washing excess DAPI in methanol and distilled water, stained slides were mounted with ProLong® Gold antifade reagent (Molecular Probes), dried overnight to let the reagent cure, sealed with clear nail polish and stored at -20°C until imaging with Olympus BX51 fluorescent microscope.

9. Statistical Analysis

All the numerical data are expressed as mean ± standard deviation, and comparisons between groups were analyzed by Chi-square test (in the case of mortality in bioassay experiments), or by one-way ANOVA (in any other cases).

Table 4. List of primers

Target gene	Name	Type of primers	Sequence
WSSV	101-110F	forward	5′-GAT AAG AGA GGT CGA CAC TAG TAG TGT TAT TGC T-3′
	101-165R	reverse	5′-CCA CTG TGC CAG CTA TTG CA-3′
wsv390	390-2F	forward	5′-AAG CTT CGA GGT CGA AAA GGA TGC AA-3′
	390-2R	reverse	5′-TCT AGA ACA AAG TTC TGG CCA GTT GC-3′
β–actin	178F	forward	5′-GGT CGG TAT GGG TCA GAA GGA-3′
	228R	reverse	5′-TTG CTT TGG GCC TCA TCA C-3′
cap-3	CAP1F	forward	5′-AAG CTT GCT GCT CTC ATC TTC GCT CA-3′
	CAP2F	forward	5′-AAG CTT GTC TAG CCG GCA AAC CTA AG-3′
	CAP1R	reverse	5′-TCT AGA TCA GCC GTG AAG TTT ATC CA-3′
	CAP2R	reverse	5′-TCT AGA CCA CTT CCC TGC TGA CTT TG-3′
	vCAP-330F	forward	5′-AAG CTT GTT CTT CGA CCT CAC GAA GG-3′
	vCAP-777R	reverse	5′-CTC GAG GGC CAA GAA GTG GAT GAA GA-3′
	vCAP-432F	forward	5′-CGG CGA AGT CAA AGC CAG AAA CAA-3′
	vCAP-497R	reverse	5′-TTG GTG GGA AGC CTA TCG TCT TTG-3′
EF-1α	EF1a-294F	forward	5′-CAC TGC TCA CAT TGC CTG CA-3′
	EF1a-294R	reverse	5′-TTA CTT CTT CTT GAG GGC CT-3′
	EF1α-25F	forward	5′-TCG CCG AAC TGC TGA CCA AGA-3′
	EF1α-79R	reverse	5′-CCG GCT TCC AGT TCC TTA CC-3′

CHAPTER IV
RESULTS

1. Apoptotic Response of Hemocytes to WSSV Infection

Upon WSSV infection, the number of apoptotic hemocytes and WSSV-infected cells were counted in DAPI-stained hemocyte samples and in immunohistochemically-stained hemocyte samples (Fig. 9), respectively, collected from 6 individual shrimp at 1 and 2 days post-challenge (dpc). Table 5 shows the number of apoptotic hemocytes and WSSV-positive hemocytes in 1000 counted cells. At 1 dpc (Fig. 10), WSSV-infected shrimp had 11.167 ± 15.791 WSSV-positive cells and 0.667 ± 1.033 apoptotic cells. At 2 dpc, the number of WSSV-positive cells and apoptotic cells drastically increased to 126.667 ± 72.613 and 88.833 ± 29.634, which were 11.343 ± 6.503 and 133.25 ± 44.451-fold higher than the ones in 1 dpc, respectively. This result confirms that viral replication increases as WSD progresses towards shrimp death and the level of apoptosis occurring upon infection can be correlated with the amount of viral load.

2. Hyperthermia Inhibits WSSV Replication in Shrimp

2.1 Shrimp mortality upon infection

Cumulative percent mortality of shrimp upon WSSV challenge at 26-28°C and 32-34°C rearing temperature is shown in Fig. 11. At 26-28°C, infected shrimp reached 50% mortality within 56 hrs post-challenge (hpc) and 100% in 120 hpc while challenged shrimp, reared at 32-34°C, gradually died and had only 15% and 25% mortality at 56 and 120 hpc. At the end of the experiment (168 hpc), the latter group had 40% mortality which is significantly lower than the mortality of the former group ($p<0.05$ by χ^2-test). Buffer-injected shrimp which were reared at 26-28°C and 32-34°C

both showed no mortality until the end of the experiment. This confirms the previous findings that hyperthermia reduces shrimp mortality upon WSSV infection.

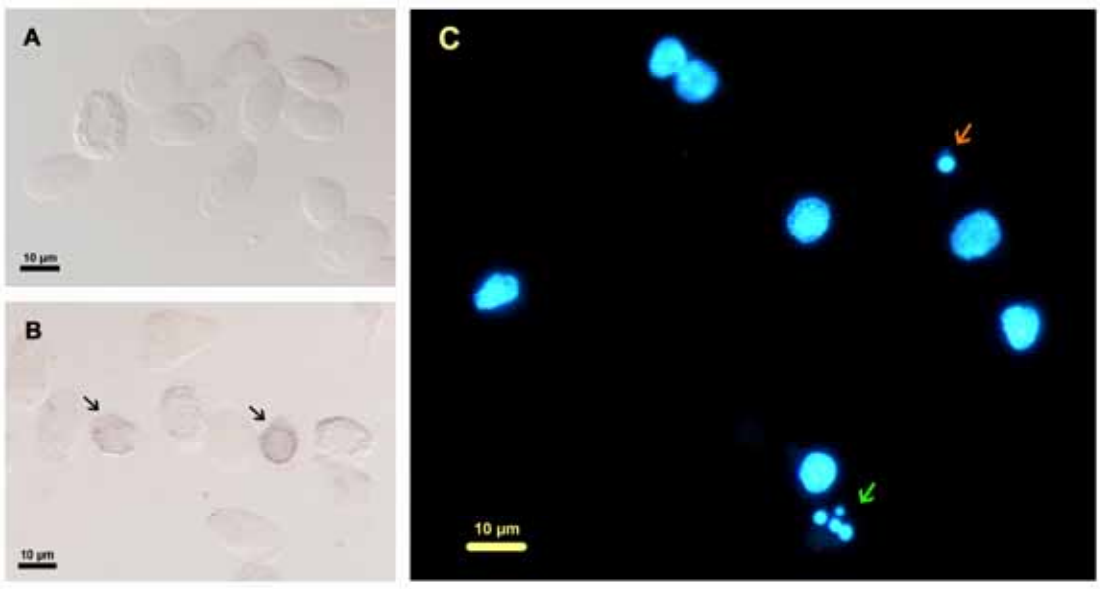

Figure 9. WSSV-positive hemocytes and apoptotic hemocytes of *P. monodon* 2 days post-challenge with WSSV. Hemocytes were immunohistologically-stained with anti-VP664-1 antibody for WSSV detection. (A) Hemocytes from buffer-injected control shrimp. (B) Hemocytes from WSSV-challenged shrimp. WSSV-positive cells displayed blue-purple coloration of NBT/BCIP substrate development (arrow). (C) Apoptotic hemocytes from WSSV-challenged shrimp were revealed by DAPI nuclear stain. Condensed nucleus (orange arrow) and fragmented nucleus (green arrow) are indicated.

Table 5. The number of apoptotic hemocytes and WSSV-positive hemocytes from individual *P. monodon* challenged with WSSV.

Group	Number of WSSV-positive cells (in 1000 cells)	Number of apoptotic cells (in 1000 cells)
1 dpc	1	0
	2	0
	42	0
	12	0
	9	2
	1	2
Mean ± SD	11.167 ± 15.791	0.667 ± 1.033
2 dpc	180	106
	13	98
	200	37
	92	120
	181	73
	94	99
Mean ± SD	126.667 ± 72.613	88.833 ± 29.634

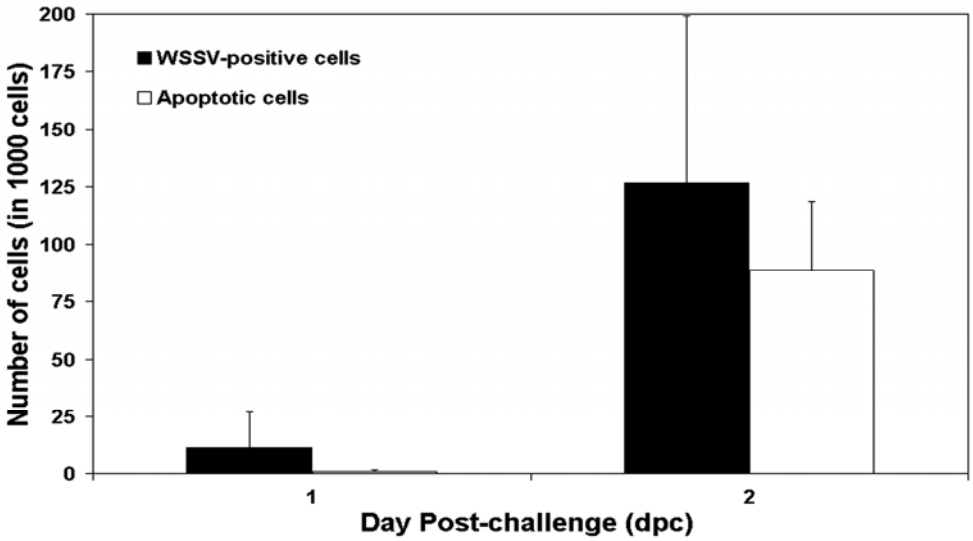

Figure 10. Number of apoptotic hemocytes and WSSV-positive hemocytes of *P. monodon* challenged with WSSV. A significant increase was observed in both the number of apoptotic cells and the number of WSSV-positive cells from 1 dpc to 2 dpc.

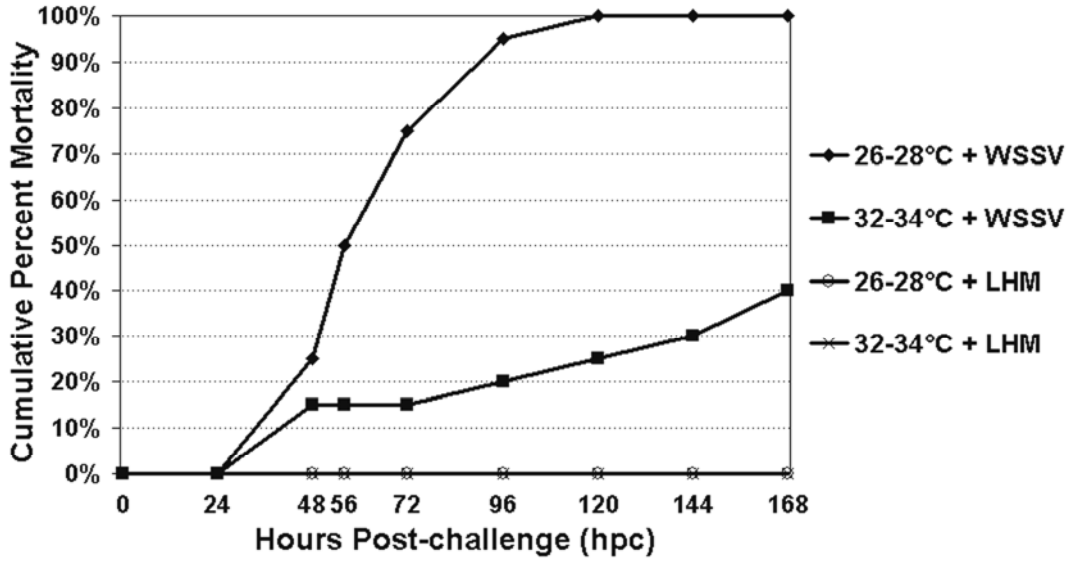

Figure 11. Cumulative percent motality of *P. monodon* reared at 26-28°C or 32-34°C and challenged with WSSV. Hyperthermia reduced shrimp mortality following WSSV infection. At 50% mortality of shrimp reared at 26-28°C (56 hpc), shrimp reared at 32-34°C had only 15% mortality. The former reached 100% mortality within 120 hpc while 60% of the latter survived WSD at 168 hpc.

2.2 Amount of WSSV load

WSSV load was measured in individual collected shrimp by real-time SYBR Green PCR, including 10 of 56 hpc-WSSV-challenged shrimp at 26-28°C, 10 of 56 hpc-buffer-injected shrimp at 26-28°C, 17 of 56 hpc-WSSV-challenged shrimp at 32-34°C, 10 of 56 hpc-buffer-injected shrimp at 32-34°C, 10 of 120 hpc-buffer-injected shrimp at 26-28°C, 12 of 120 hpc-WSSV-challenged shrimp at 32-34°C and 9 of 120 hpc-buffer-injected shrimp at 32-34°C. For each sample, the amplification of WSSV-specific and β-actin-specific products was confirmed by examining the amplification plots and the corresponding dissociation curves. A dissociation curve with a single peak at the expected melting temperature (T_m = 74°C for WSSV and T_m = 78°C for β-actin) indicates exclusive amplification of WSSV and β-actin targets (Fig. 12). The virus was detected only in the virus-injected shrimp, not in the buffer-injected shrimp.

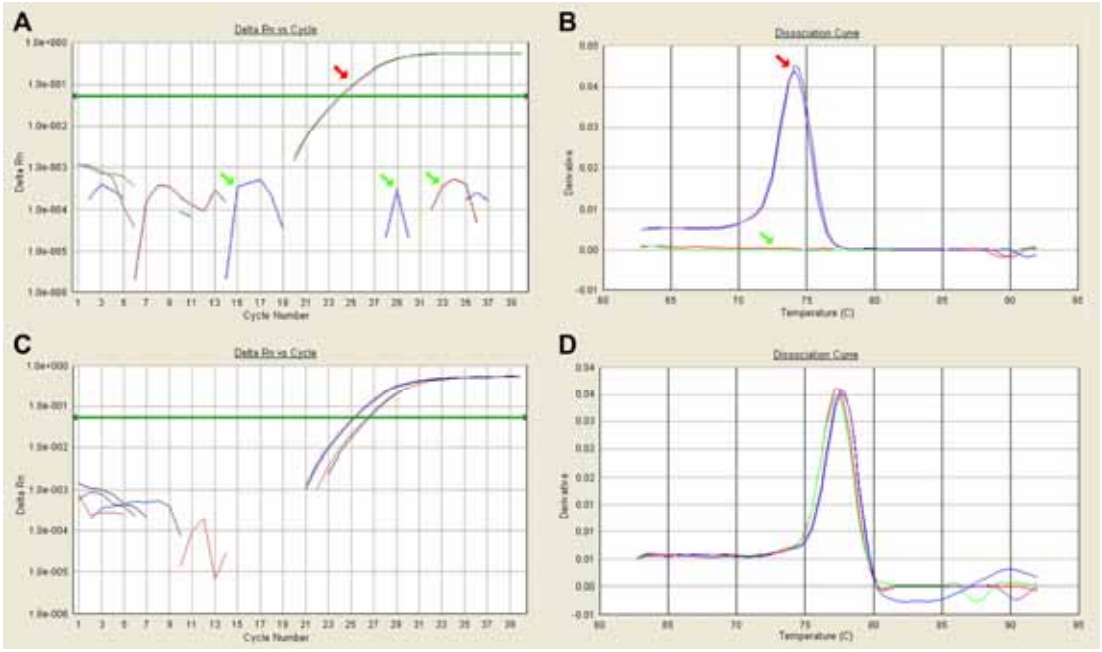

Figure 12. Amplification plots and dissociation curves of WSSV and β-actin targets by real-time SYBR Green PCR. (A) Amplification plots of WSSV detection. WSSV-positive shrimp showed amplification of WSSV target (red arrow) while WSSV-negative shrimp showed no amplification signal (green arrow). (B) Dissociation curve of WSSV detection. A single peak of dissociation curve at T_m = 74°C indicates specificity of the WSSV target PCR product (red arrow). Straight line confirms no WSSV amplification in WSSV-negative shrimp (green arrow). (C) Amplification plots of β-actin detection. Both WSSV-positive and WSSV-negative shrimp showed amplification of β-actin gene. (D) β-actin PCR product displayed a single peak of dissociation curve at T_m = 78°C.

The normalized WSSV value of each individual infected shrimp which indicates the amount of viral load is shown in Table 6 and Fig 13, 14. At 56 hpc, all of the 10 surviving shrimp reared at 26-28°C were detected WSSV positive (100%) with a mean normalized WSSV amount of 0.023324854 ± 0.018754567, whereas only 7 out of 17 surviving shrimp reared at 32-34°C were WSSV positive (41.2%) and its mean normalized WSSV amount was 3.5193E-06 ± 4.29022E-06. The amount of virus in shrimp reared at 32-34°C, 56 hpc was 6627.697 ± 5329.062-fold lower than that of the shrimp reared at 26-28°C. At 168 hpc, the percent number of WSSV-positive shrimp

out of WSSV-challenged shrimp reared at 32-34°C decreased to 16.7% (2 out of 12 surviving shrimp) with a mean normalized WSSV amount of 3.31101E-06 ± 2.48609E-06. This indicates that prevention of mortality from WSD by hyperthermia is related to inhibition of viral replication.

Table 6. Number of WSSV-positive surviving *P. monodon* challenged at 26-28°C or 32-34°C by real-time SYBR Green PCR detection and the average normalized WSSV value of individual shrimp. The normalized value was obtained by dividing averaged copy number of WSSV with averaged copy number of β-actin gene.

Group	No. of WSSV detected samples/No. of collected samples		Normalized WSSV value
26-28°C 56 hpc	10/10 (100%)		0.002845945
			0.020561111
			0.046605691
			0.036831252
			0.017779831
			0.053224944
			0.035394539
			0.01507892
			0.004091546
			0.000834762
		Mean ± SD	0.023324854 ± 0.018754567
32-34°C 56 hpc	7/17 (41.2%)		8.59072E-07
			9.45147E-07
			2.31834E-06
			3.43939E-06
			1.75968E-06
			1.30396E-05
			2.27385E-06
		Mean ± SD	3.5193E-06 ± 4.29022E-06
32-34°C 168 hpc	2/12 (16.7%)		1.55308E-06
			5.06894E-06
		Mean ± SD	3.31101E-06 ± 2.48609E-06

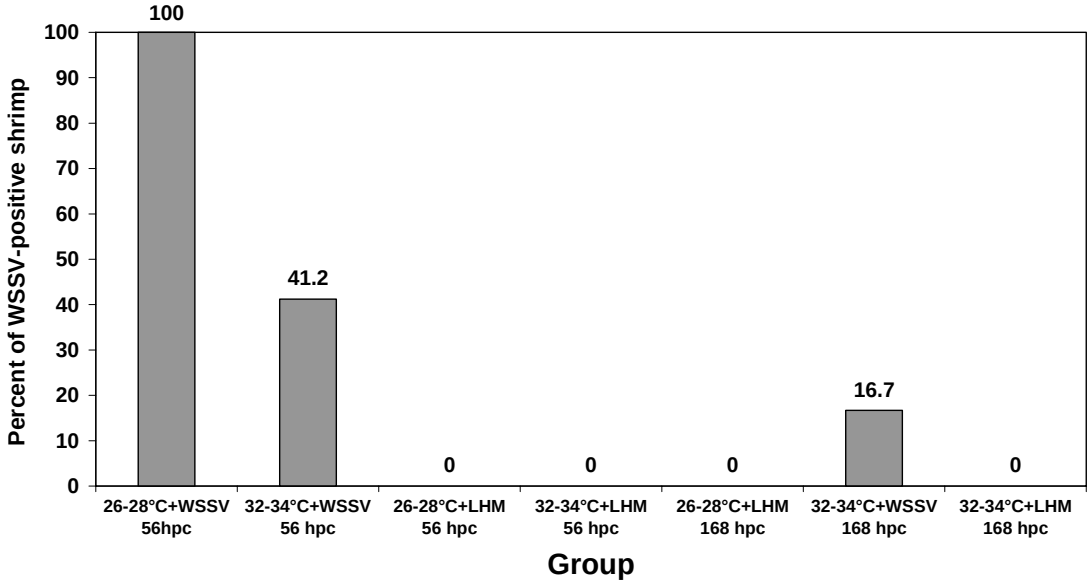

Figure 13. Percent of WSSV-positive *P. monodon* surviving challenge at 26-28°C or 32-34°C by real-time SYBR Green PCR detection.

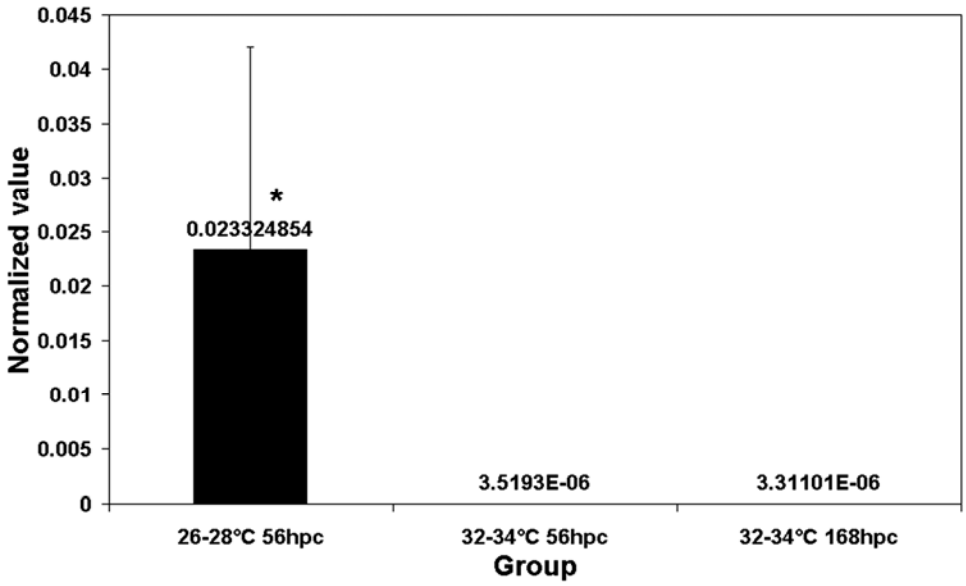

Figure 14. Normalized WSSV value for *P. monodon* challenged at 26-28°C or 32-34°C. Amount of WSSV was examined by real-time SYBR Green PCR using pleopod DNA of individual shrimp. The normalized value was obtained by dividing averaged copy number of WSSV with averaged copy number of β-actin gene. Amount of virus in shrimp reared at 26-28°C was significantly higher than that of shrimp reared at 32-34°C (*) ($p<0.05$).

2.3 Detection of WSSV and Apoptosis by Immunofluorescence

By immunofluorescent staining of hemocytes using antibody against WSSV envelope protein and nuclear counterstaining with DAPI, the amount of WSSV-positive cells and apoptotic cells were counted as shown in Table 7 and Fig. 15, 16. The number of WSSV-positive or apoptotic cells was counted out of 2000 cells. Virally-challenged shrimp reared at 26-28°C had numbers of WSSV positive cells, apoptotic cells and apoptotic WSSV-positive cells at 4805.9 ± 2330.14, 194.37 ± 178.15 and 294.1 ± 221.08 -fold higher (respectively) than the ones of virally-challenged shrimp reared at 32-34°C. None of the latter group showed WSSV positive cells by immunofluorescence and this corresponded with the very small amount of viral load and the percent number of WSSV-positive shrimp detected by real-time SYBR Green PCR. The number of apoptotic cells of shrimp reared at 32-34°C was not different from those of buffer-injected shrimp at both 56 and 168 hpc. This may suggest that prevention of WSD by hyperthermia is not caused by apoptosis of the host cells.

Table 7. Number of WSSV-positive hemocytes, number of apoptotic hemocytes, and number of apoptotic WSSV-positive hemocytes from surviving *P. monodon* reared at 26-28°C or 32-34°C with WSSV challenge

Group		No. of WSSV-positive cells (in 2000 cells)		No. of apoptotic cells (in 2000 cells)		No. of apoptotic WSSV-positive cells (in 2000 cells)	
		Mean	SD	Mean	SD	Mean	SD
26-28°C + WSSV	56 hpc	282.7	137.07	205.8	188.63	17.3	13.005
32-34°C + WSSV	56 hpc	0.0588	0.2425	1.0588	1.5996	0.0588	0.2425
26-28°C + LHM	56 hpc	0	0	0.5	0.8498	0	0
32-34°C + LHM	56 hpc	0	0	0.7	1.567	0	0
32-34°C + WSSV	168 hpc	0	0	1	1.1832	0	0
32-34°C + LHM	168 hpc	0	0	1.4444	2.555	0	0
26-28°C + LHM	168 hpc	0	0	2.2	1.8738	0	0

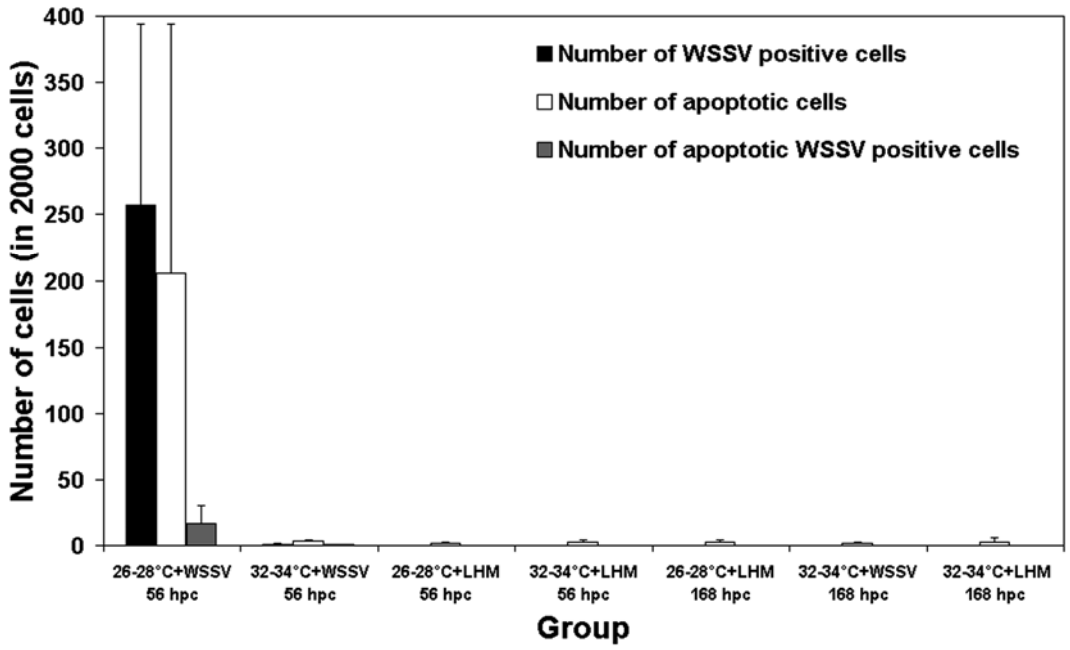

Figure 15. Number of WSSV-positive hemocytes, number of apoptotic hemocytes, and number of apoptotic WSSV-positive hemocytes from surviving *P. monodon* reared at 26-28°C or 32-34°C with WSSV challenge.

3. Full Length Sequence of *P. vannamei cap-3*

By 5' and 3'-RACE, 951 bp of *P. vannamei cap-3* cDNA full-length sequence was obtained (GenBank accession no. **DQ988351**) resulting in a deduced protein of 316 amino acid residues (Fig. 17). BLASTN results showed 82% identity while BLASTX results showed 76% identity, 86% positives to *P. merguiensis* caspase. The deduced amino acid sequence was searched for motifs against the Prosite database using the ScanProsite tool (http://br.expasy.org/tools/scanprosite/) and 2 hits by profile and 1 hit by pattern were found. The 2 profile hits were the caspase family p20 domain profile (from amino acid residue 67-196, score 35.245) (PROSITE accession no. **PS50208**) and the caspase family p10 domain profile (from amino acid residue 236-316, score 12.413) (PROSITE accession no. **PS50207**). The single pattern hit was the caspase family cysteine active site (PROSITE accession no. **PS01122**), KPKLYFIQA CRG, from amino acid residue 183-194. QACRG is a conserved pentapeptide active site motif for caspase 1 to 7. Upon activation, caspase is cleaved into small and large subunits with the predicted cleavage sites DGRDG, PEVDK and VQTDS (analysis

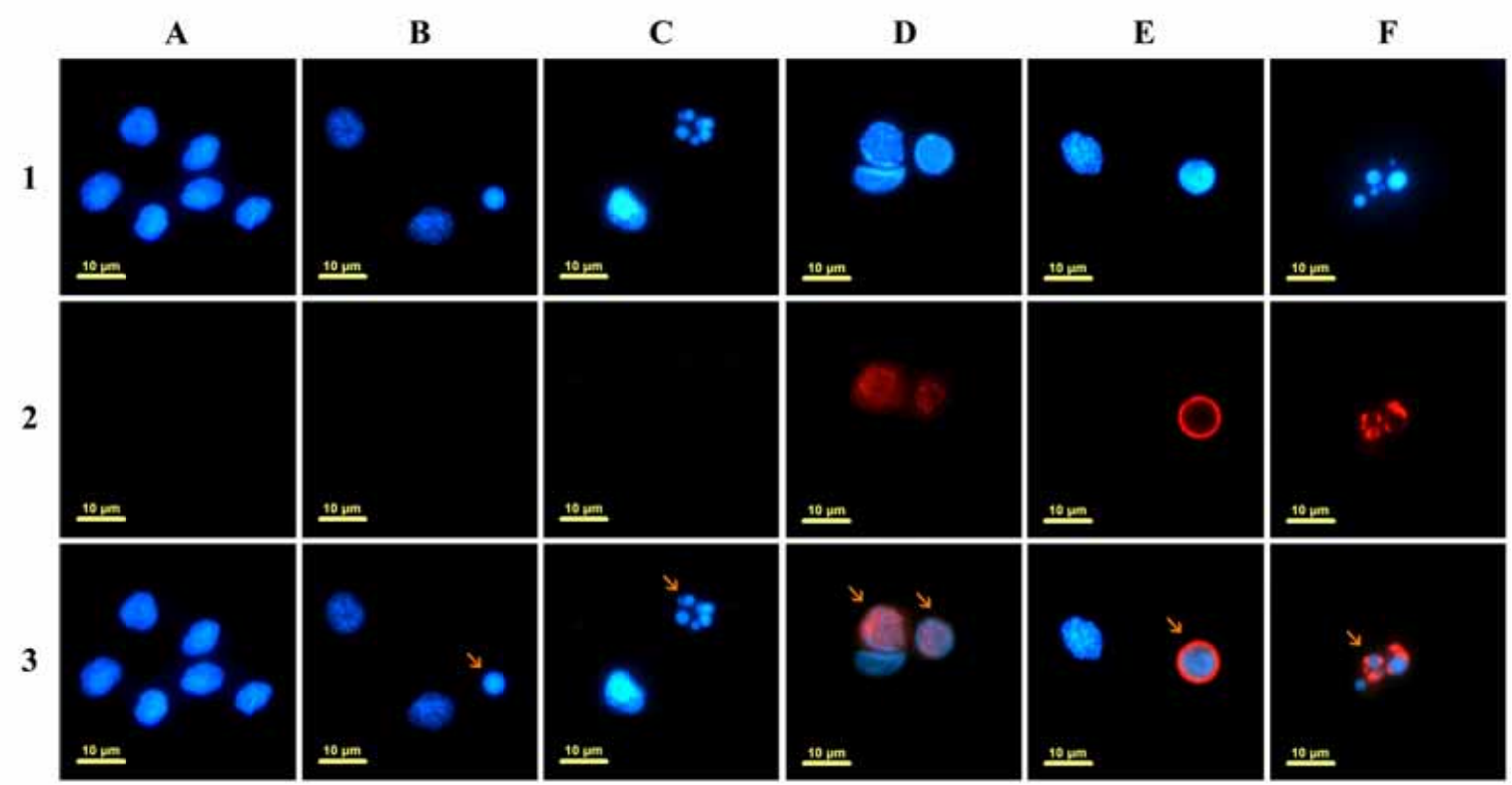

Figure 16. Apoptotic hemocytes, WSSV-positive hemocytes and apoptotic WSSV-positive hemocytes. 2 laser excitation lines of DAPI (1) and Alexa® 594 dye (2) were used. Merged images from 2 channels are shown in 3. (A) Immunofluorescent stained hemocytes of the non-challenged shrimp. No WSSV was detected using anti-VP28 antibody. WSSV-infected shrimp showed apoptotic cells characterized by condensed nucleus (B) or fragmented nucleus (C), WSSV-positive cells of which the viruses accumulate in the nucleus (D) or in the cytoplasm (E), and apoptotic WSSV-positive cells (F).

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  1  atgagcagcgccggcgatgcagcgagagccgaggcccagcgggcggatgggcgcgacggc   60
      M  S  S  A  G  D  A  A  R  A  E  A  Q  R  A  D  G  R  D  G
 61  gggaacaaaggcactgccagcacgacggagaaccggggcaagagggcggaggaatatgcg  120
      G  N  K  G  T  A  S  T  T  E  N  R  G  K  R  A  E  E  Y  A
121  cagaacgcgatttgtgggcgtcccaccgcacacacggtcgtggacggactcagcgagcgt  180
      Q  N  A  I  C  G  R  P  T  A  H  T  V  V  D  G  L  S  E  R
181  tacccaatgagccgtcggccccggggcgctgcgctcatcttcgcgcactccgagttcgag  240
      Y  P  M  S  R  R  P  R  G  A  A  L  I  F  A  H  S  E  F  E
241  gataaagctctcaaaccgcgacccagtgccgcccacgacgccgaaatcgcgagagccgcc  300
      D  K  A  L  K  P  R  P  S  A  A  H  D  A  E  I  A  R  A  A
301  ttcgaggccctcgacttccaacctgaagtgttcttcgacctcacgaaggacgagctcgtg  360
      F  E  A  L  D  F  Q  P  E  V  F  F  D  L  T  K  D  E  L  V
361  aggaaactccgcgaagtttctcagcgcgaccacagcggctgcgacgcccttgccgtagtg  420
      R  K  L  R  E  V  S  Q  R  D  H  S  G  C  D  A  L  A  V  V
421  ttcatgagccacggcgaagtcaaagccagaaacaacatggagttagtttgcgccaaagac  480
      F  M  S  H  G  E  V  K  A  R  N  N  M  E  L  V  C  A  K  D
481  gataggcttcccaccaaggaactgtggataaacttcacggctgagcggtgcccgggactc  540
      D  R  L  P  T  K  E  L  W  I  N  F  T  A  E  R  C  P  G  L
541  gccggcaaacctaagctgtacttcattcaggcttgcagaggccccgaggtggacaaaggc  600
      A  G  K  P  K  L  Y  F  I  Q  A  C  R  G  P  E  V  D  K  G
601  gtgagaatgactcggcccgcgtttggcataccggttcagacggacagcatacaggaggac  660
      V  R  M  T  R  P  A  F  G  I  P  V  Q  T  D  S  I  Q  E  D
661  tatgttattcccctccatgctgatcagcttgttatgtgggcttcctacccgggctttccg  720
      Y  V  I  P  L  H  A  D  Q  L  V  M  W  A  S  Y  P  G  F  P
721  gccttcacgagtgaacgtaaagggatccaaggaagtgtcttcatccacttcttggccgag  780
      A  F  T  S  E  R  K  G  I  Q  G  S  V  F  I  H  F  L  A  E
781  aacctcaagaactgtgccagtacatctccacgactgtcttccattcttctcaaagtcacc  840
      N  L  K  N  C  A  S  T  S  P  R  L  S  S  I  L  L  K  V  T
841  agggaagtagcagtcctctacgagagcgaaattgattctcaaaacccatatcacaaaaac  900
      R  E  V  A  V  L  Y  E  S  E  I  D  S  Q  N  P  Y  H  K  N
901  aaacaagttccttacatccactcgacactcctccgtgaaatttacttctaa          951
      K  Q  V  P  Y  I  H  S  T  L  L  R  E  I  Y  F  -
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Figure 17. Full length sequence of *P. vannamei cap-3* cDNA (951 bp) and deduced protein (316 amino acid). Putative cleavage sites are shown as the underlined while QACRG conserved pentapeptide active site is in grey color.

was made by GraBCas software: http://www.uniklinik-saarland.de/med_fak/humangenetik/software/index.html). SEIDS was shown to be a cleavage site of caspase 3, 4, 6, 7, 8 and 9 with medium to high stringency and thus is presented here although C-terminal cleavage of caspase has never been reported.

4. Effect of Down-Regulation of Shrimp Caspase and wsv390 upon WSSV Infection

4.1 Effectiveness of *cap-3* and wsv390 gene silencing

Via an RNAi-like mechanism, the shrimp caspase gene and wsv390 were silenced in shrimp by injection of their cognate long dsRNAs prior to low- or high-dose WSSV challenge. Three shrimp injected with normal saline and three shrimp injected with *cap-3* dsRNA were assayed for *cap-3* versus β-actin gene expression in their hepatopancreas 2 days after injection of dsRNA (Fig. 18a). Results by RT-PCR showed detectable *cap-3* expression in the saline-injected, but not in the *cap-3* dsRNA-injected animals, confirming that *cap-3* expression was silenced by *cap-3* dsRNA.

For wsv390 knock-down, three saline-injected WSSV-challenged shrimp and three wsv390 dsRNA-injected WSSV-challenged shrimp were assayed 3 days after WSSV injection by RT-PCR for wsv390 expression in their hepatopancreas versus VP28 expression (Fig. 18b). Results showed that administration of wsv390 dsRNA was able to silence the expression of wsv390 to some extent. The expression of VP28 (and VP19, data not shown) was also negatively affected. This suggests that wsv390 dsRNA treatment not only down-regulates the expression of wsv390 gene but also the expression of other viral proteins leading to the question of its influence on viral multiplication.

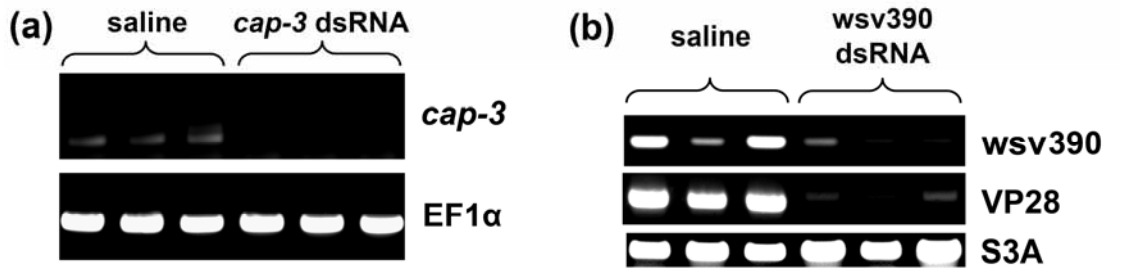

Figure 18. Silencing of (a) *P. vannamei cap-3* and (b) wsv390. The shrimp were injected either with 5 μg of *cap-3* dsRNA, wsv390 dsRNA or saline. For determination of *cap-3* expression, two days after dsRNA injections, RT-PCR was performed to assay expression of *cap-3* and reference gene elongation factor-1α (EF-1α) using total RNA from the shrimp hepatopancreas. For determination of wsv390 expression, 2 days after dsRNA injections, WSSV or negative shrimp extract were injected. RT-PCR was performed to assay wsv390 expression in reference to VP28 and 40S ribosomal protein S3A expression using total RNA of 3 dpc shrimp hepatopancreas. PCR products were run on 1.5% agarose gel and stained with ethidium bromide. Each lane represents cDNA from an individual shrimp.

4.2 Effects of *cap-3* and wsv390 genes silencing in the presence of low dose WSSV challenge

4.2.1 Shrimp mortality upon infection

In the low dose WSSV challenge system, innate immunity is triggered by administration of any dsRNA which, in this experiment, was represented by CDP dsRNA. Final mortality for the WSSV-injected control group (no dsRNA treatment) was 79.1%, while that for the CDP dsRNA group (52.4%), wsv390 dsRNA group (39.5%) and *cap-3* dsRNA group (27.3%) were significantly lower ($p<0.05$ by χ^2-test) (Fig. 19). The difference in mortality between the CDP dsRNA group and *cap-3* dsRNA group was also significant ($p<0.05$ by χ^2-test) but not between the CDP dsRNA group and wsv390 dsRNA group, and wsv390 dsRNA group and *cap-3* dsRNA group ($p>0.05$ by χ^2-test). This indicates that silencing of *cap-3* affords partial protection from the lethality of WSSV infection at this low viral dose when compared

to silencing of the control gene (CDP). Nevertheless, administration of wsv390 dsRNA is not able to aggravate the severity of infection.

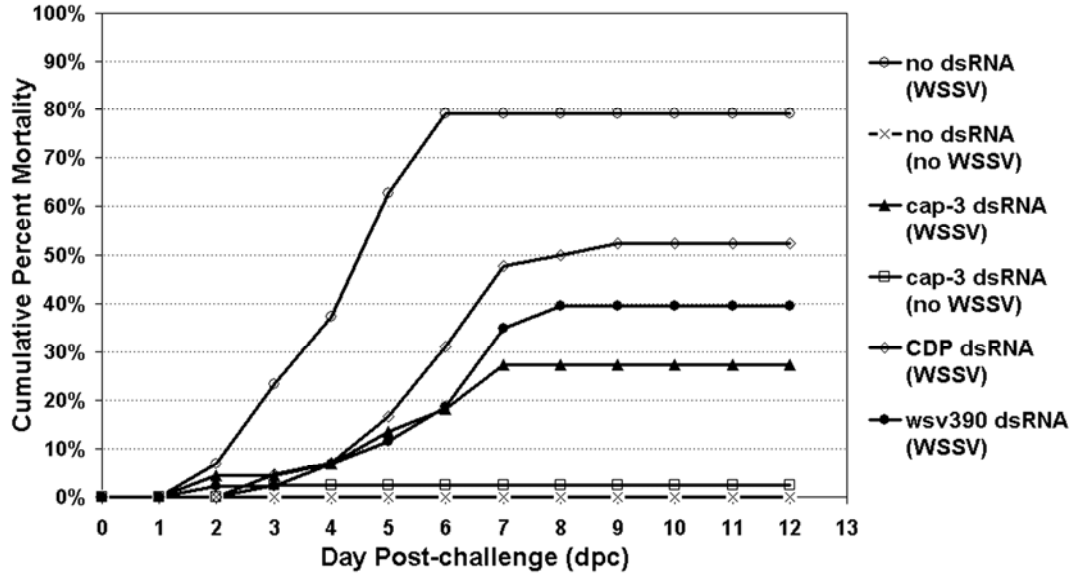

Figure 19. Cumulative percent mortality of *P. vannamei* challenged with a low dose of WSSV. *cap-3* dsRNA, CDP dsRNA, wsv390 dsRNA or saline were injected 2 days prior to viral injection. The *cap-3* dsRNA group had significantly lower mortality than the no dsRNA and the CDP dsRNA groups. The wsv390 dsRNA group had no significant difference of mortality from both the CDP dsRNA group and the *cap-3* dsRNA group.

4.3 Effects of *cap-3* and wsv390 genes silencing in the presence of high dose WSSV challenge

4.3.1 Shrimp mortality upon infection

To study *cap-3* and wsv390 gene silencing upon WSSV infection, a high-dose WSSV challenge which can overwhelm the effect of innate antiviral immunity was tested. As shown in Fig. 20, the cumulative percent mortality after WSSV injection of all the virus-injected groups, except VP19 dsRNA group, reached 100% within 7 dpc. Chi-square analysis showed that the cumulative percent mortality of the CDP dsRNA group, a dsRNA treatment control, and *cap-3* dsRNA group 2, 3

and 5 dpc was significantly different from that of the viral control group ($p<0.05$). On day 4 post-challenge, the *cap-3* dsRNA group, but not the CDP dsRNA group, was significantly different from the viral control group ($p<0.05$). There was no significant difference between CDP dsRNA and *cap-3* dsRNA cumulative percent mortality on any dpc ($p>0.05$). In virally-challenged groups which reached 100% mortality, the mean 'time to death' was calculated by averaging days to death of every animal in the group and was statistically tested by one-way ANOVA (Table 8 and Fig. 21). The viral infection control group had a significantly shorter mean time to death (4.075 ± 1.309 days) from both the CDP knockdown group (4.892 ± 1.173 days) and *cap-3* knockdown group (5.175 ± 1.107 days) ($p<0.05$). But the mean time to death of the *cap-3* knockdown group was not significantly different from that of the CDP knockdown group ($p>0.05$). For the wsv390 dsRNA group, its mean time to death (4.25 ± 1.296 days) was not significantly different from those of the viral infection control group and of the CDP dsRNA group ($p>0.05$) but was significantly shorter than that of the *cap-3* dsRNA group. This result suggests that at high doses of WSSV, suppression of *cap-3* expression has no specific effect on shrimp mortality, but that the injection of dsRNA (regardless of sequence) slows down the onset of mortality, as previously shown (21, 24). However, effects of *cap-3* and wsv390 knockdowns do exist in a reverse manner.

Table 8. Mean time to death of *P. vannamei* challenged with a high dose of WSSV

Group	Time to Death	
	Mean	**SD**
no dsRNA	4.075	1.309
cap-3 dsRNA	5.175	1.107
CDP dsRNA	4.892	1.173
wsv390 dsRNA	4.250	1.296

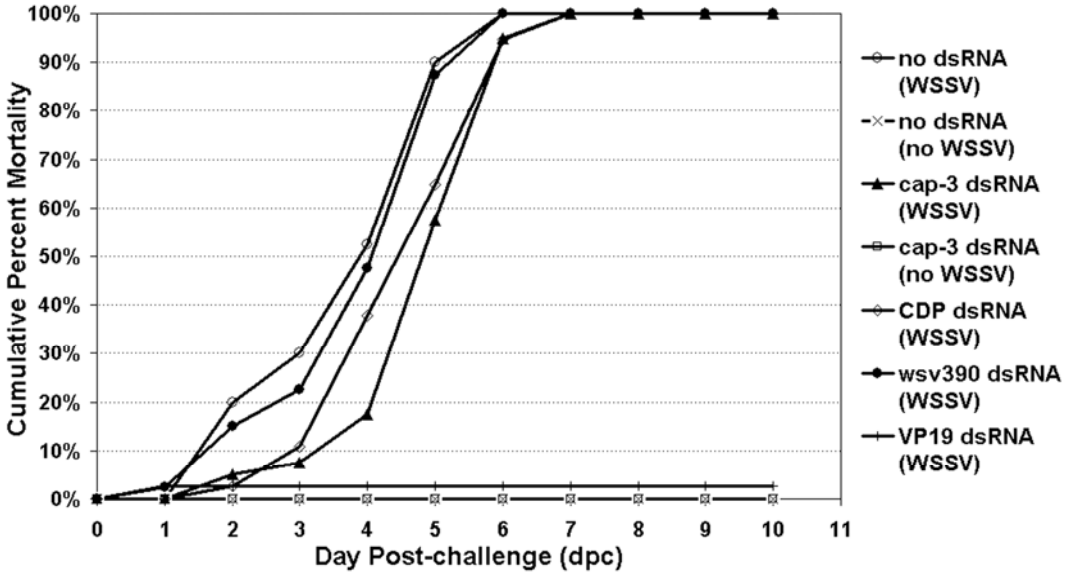

Figure 20. Cumulative percent mortality of *P. vannamei* challenged with a high dose of WSSV. Shrimp injected with saline (no dsRNA), *cap-3* dsRNA, wsv390 dsRNA or CDP dsRNA 2 days prior to WSSV challenge reached 100% mortality within 7 dpc while negative control groups (VP19 dsRNA injection and non-challenged saline injection) had cumulative percent mortality less than 5%.

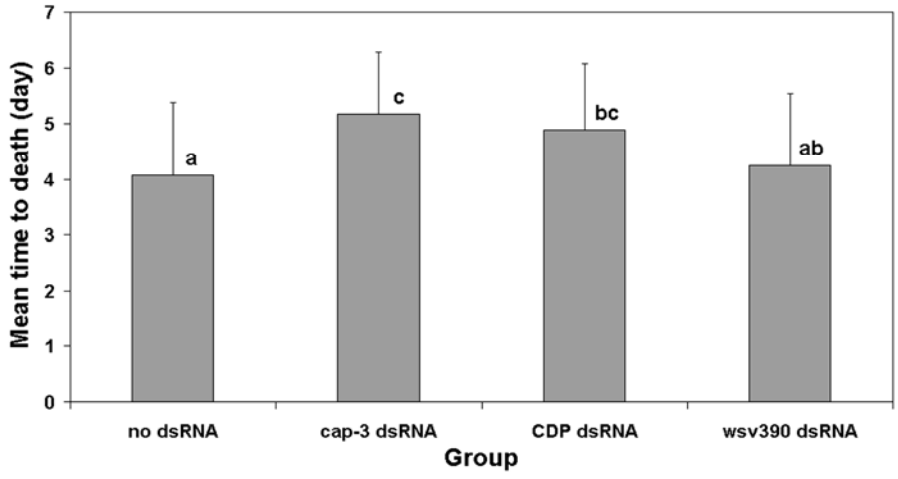

Figure 21. Mean time to death of *P. vannamei* challenged with a high dose of WSSV. The positive control group (no dsRNA-WSSV-injected group) showed a significantly shorter time than the *cap-3* dsRNA and CDP dsRNA groups but were

similar to the wsv390 dsRNA group. Same letter indicates no significant difference ($p>0.05$) while different letter indicates a significant difference ($p<0.05$).

4.3.2 Expression of *cap-3* after high-dose WSSV challenge

The relative level of mRNA expression of the *cap-3* gene was measured by SYBR Green RT-PCR of the hepatopancreas tissues of 4-5 individual shrimp, collected at 3, 4 and 5 dpc, from each group. The amplification specificity for *cap-3* and the corresponding internal control, EF-1α, was determined for each sample by analyzing the amplification plots and the dissociation curves. The amplification profiles and dissociation curves of the *cap-3* and EF-1α genes of representative healthy and WSSV-infected samples are shown in Fig. 22. A dissociation curve showing a single peak at a melting temperature (77°C for *cap-3* and 79°C for EF-1α) expected for that amplicon suggests specific amplification.

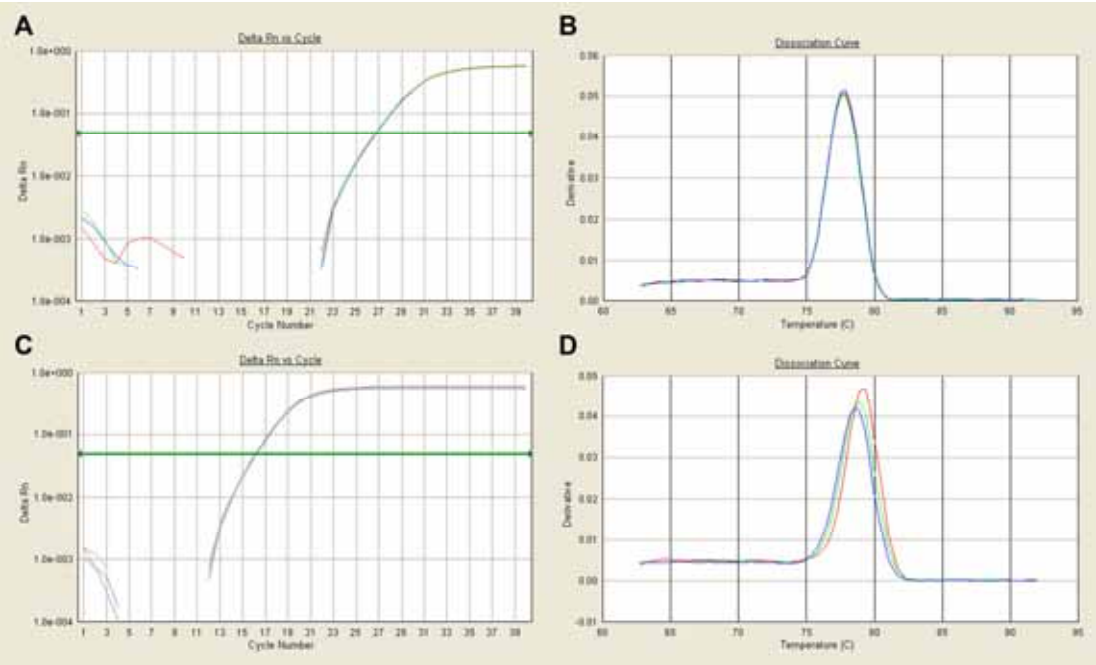

Figure 22. Amplification plots and dissociation curves of *P. vannamei cap-3* and EF-1α targets by real-time SYBR Green RT-PCR. *cap-3* amplifications of representative WSSV-positive and WSSV-negative shrimp (A) in reference to EF-1α (C). Specificity of *cap-3* and EF-1α PCR products is confirmed by dissociation curves of a single peak at T_m = 77°C (B) and 79°C (D), respectively.

The normalized *cap-3* value of individual shrimp collected and average normalized *cap-3* value of each group on 3, 4 and 5 dpc are shown in Table 9 and Fig. 23. Statistical analysis revealed that there was no significant difference in *cap-3* expression among any groups on 3 and 5 dpc ($p>0.05$). On day 4 post-challenge, the levels of *cap-3* expression of no virus (no dsRNA) group and *cap-3* dsRNA (WSSV) treated group were significantly different ($p<0.05$) but not among any other groups. The magnitude of difference in the former relative to the latter was 3.6 ± 2.02 fold. When expression of *cap-3* was compared within each group of 3, 4 and 5 dpc, no significant difference was observed in any group ($p>0.05$). This unchanging expression of *cap-3* in the hepatopancreas may imply a lack of change in apoptosis in the hepatopancreas of all groups as the experiment goes on.

4.3.3 Amount of WSSV load after high-dose WSSV challenge

In invertebrates, it has been suggested that apoptosis is a mechanism by which the host cell prematurely terminates viral infection. If this is true, then inhibition of apoptosis would allow infection to go to completion, thus increasing the total virus production, but inhibition of anti-apoptosis would promote more apoptosis to occur, thus reducing the number of replicating viruses. To determine if knockdown of *cap-3* and wsv390 has any effects on viral production, WSSV load was measured in 4-5 individual shrimp from each group, collected at 3, 4 and 5 dpc by real-time SYBR Green PCR (Table 10). The virus was detected only in the virus-injected samples, not in the SPF shrimp extract-injected samples. For each sample, the amplification of WSSV-specific product was confirmed by examining the amplification plots and the corresponding dissociation curves as described earlier.

As shown in Fig. 24a, the mean normalized WSSV values of the virally-challenged (no dsRNA) control group, CDP dsRNA group and wsv390 dsRNA group were not significantly different from 3 to 5 dpc ($p>0.05$). However, the significant increase of 13.8 ± 2.8 fold was observed in the *cap-3* dsRNA group between 3 and 5 dpc ($p<0.05$). At 4 dpc of this group, there were shrimp having high WSSV loads similar to ones at 5 dpc and shrimp having low WSSV loads like ones at 3 dpc. This high standard deviation implies that viruses multiply in a very different rate on this day. When statistical comparison was made among groups within each 3, 4

and 5 dpc (Fig. 24b), there was a significant difference on 3 and 5 dpc ($p<0.05$) but not on 4 dpc ($p>0.05$). At 3 dpc, the amount of virus in the WSSV-challenged control

Table 9. Normalized *cap-3* value of *P. vannamei* challenged with a high dose of WSSV. The normalized value was obtained by dividing averaged *cap-3* copy number with averaged EF-1α copy number. At 3, 4 and 5 dpc, *cap-3* expression was examined by two-step real-time SYBR Green RT-PCR using total RNA from hepatopancreas of 4-5 individual shrimp from each group.

	3 dpc	4 dpc	5 dpc
no dsRNA (WSSV)	0.007320627	0.007741689	0.012328958
	0.045702318	0.019509329	0.016275516
	0.023291732	0.023390606	0.012283909
	0.05510596	0.026262768	0.014612851
		0.036241752	0.01938371
Mean ± SD	0.032855159 ± 0.021630373	0.022629229 ± 0.010373713	0.014976989 ± 0.002979207
no dsRNA (no WSSV)	0.008247056	0.014424794	0.018485924
	0.031108554	0.010245673	0.017289418
	0.04227825	0.034518656	0.015993427
	0.055789956	0.43453629	0.022267806
		0.047462702	0.046961431
Mean ± SD	0.034355954 ± 0.020119637	0.030021091 ± 0.016875889	0.024199601 ± 0.012938033
***cap-3* dsRNA (WSSV)**	0.00262727	0.004194317	0.00447296
	0.003057922	0.005871335	0.009434553
	0.010868417	0.011445175	0.011038765
	0.01714027	0.005014841	0.009099822
	0.018133107	0.015074199	0.019908834
Mean ± SD	0.010365397 ± 0.007412271	0.008319973 ± 0.004725654	0.010790987 ± 0.005652741
***cap-3* dsRNA (no WSSV)**	0.01107285	0.00947411	0.011130724
	0.006604764	0.010481798	0.011382133
	0.031086186	0.022133611	0.008793933
	0.032862426	0.014842383	0.026807427
	0.033081996	0.011500907	0.010882183
Mean ± SD	0.022941641 ± 0.012993689	0.013686562 ± 0.005135195	0.013799280 ± 0.007344022
CDP dsRNA (WSSV)	0.006020916	0.006007476	0.003768871
	0.00817236	0.007059405	0.015931545
	0.032018507	0.012920562	0.006777266
	0.028357451	0.017730951	0.017608218
	0.034746376	0.022778179	0.02896757
Mean ± SD	0.021863122 ± 0.013690315	0.013299315 ± 0.007101745	0.014610694 ± 0.009946826
wsv390 dsRNA (WSSV)	0.012405074	0.008912091	0.018799368
	0.008870127	0.007822103	0.017635675
	0.046755298	0.015849155	0.011962612
	0.037785104	0.011968946	0.013770789
	0.064935306	0.031252209	0.026674889
Mean ± SD	0.034150182 ± 0.023620734	0.015160901 ± 0.009519691	0.0177686677 ± 0.005702941

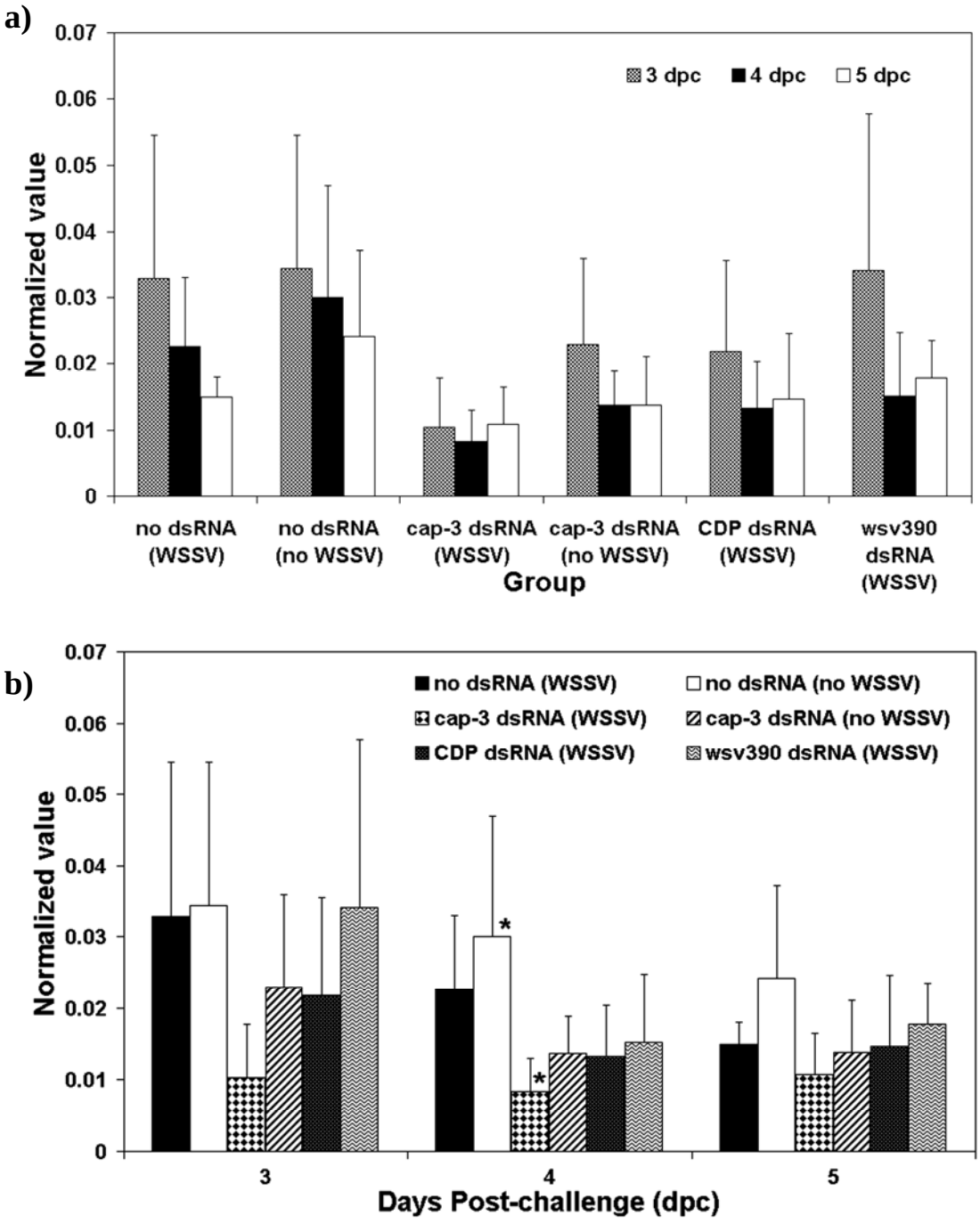

Figure 23. Normalized *cap-3* value of *P. vannamei* challenged with a high dose of WSSV. The normalized value was obtained by dividing averaged *cap-3* copy number with averaged EF-1α copy number. No significant difference was found within each group among 3, 4 and 5 dpc (a). Only no dsRNA (no WSSV) group on 4 dpc expressed significantly higher level of *cap-3* than *cap-3* dsRNA (WSSV) group (*) (b).

Table 10. Normalized WSSV value of *P. vannamei* challenged with a high dose of WSSV. The normalized value was obtained by dividing averaged WSSV copy number with averaged β–actin copy number. At 3, 4 and 5 dpc, amount of WSSV load was examined by real-time SYBR Green PCR using pleopod DNA of 5 individual shrimp from each group. All the non-challenged groups were detected WSSV-negative by real-time SYBR Green PCR.

	3 dpc	4 dpc	5 dpc
no dsRNA (WSSV)	0.026126632	0.010956692	0.018415442
	0.025259136	0.032115057	0.009417977
	0.015715894	0.003396581	0.015183251
	0.014626348	0.019849471	0.015798239
	0.021255735	0.02204252	0.018440837
Mean ± SD	0.020596749 ± 0.005296701	0.017672064 ± 0.010969590	0.015451149 ± 0.003684992
***cap-3* dsRNA (WSSV)**	0.002558351	0.010905257	0.045057824
	0.005803753	0.001135377	0.042148702
	0.00510014	0.001786568	0.061449048
	0.003209387	0.097234278	0.060910606
	0.003470473	0.061033963	0.068375444
Mean ± SD	0.004028421 ± 0.001364149	0.034419089 ± 0.042952552	0.055588325 ± 0.011376512
CDP dsRNA (WSSV)	0.013654524	0.016027936	0.01634578
	0.023759736	0.014573807	0.014732225
	0.011621384	0.005513141	0.006623488
	0.015038451	0.018737879	0.018440806
	0.01234817	0.020307386	0.006413249
Mean ± SD	0.015284453 ± 0.004913945	0.015032030 ± 0.005774378	0.012511110 ± 0.005626885
wsv390 dsRNA (WSSV)	0.002730389	0.007180567	0.000263183
	0.000667774	0.000155564	0.000271698
	0.000378445	0.000521922	0.000228169
	0.006480965	0.000112659	0.000396872
	0.008135575	0.001442551	0.001129578
Mean ± SD	0.003678630 ± 0.003484719	0.001882653 ± 0.003009522	0.000457900 ± 0.000380867

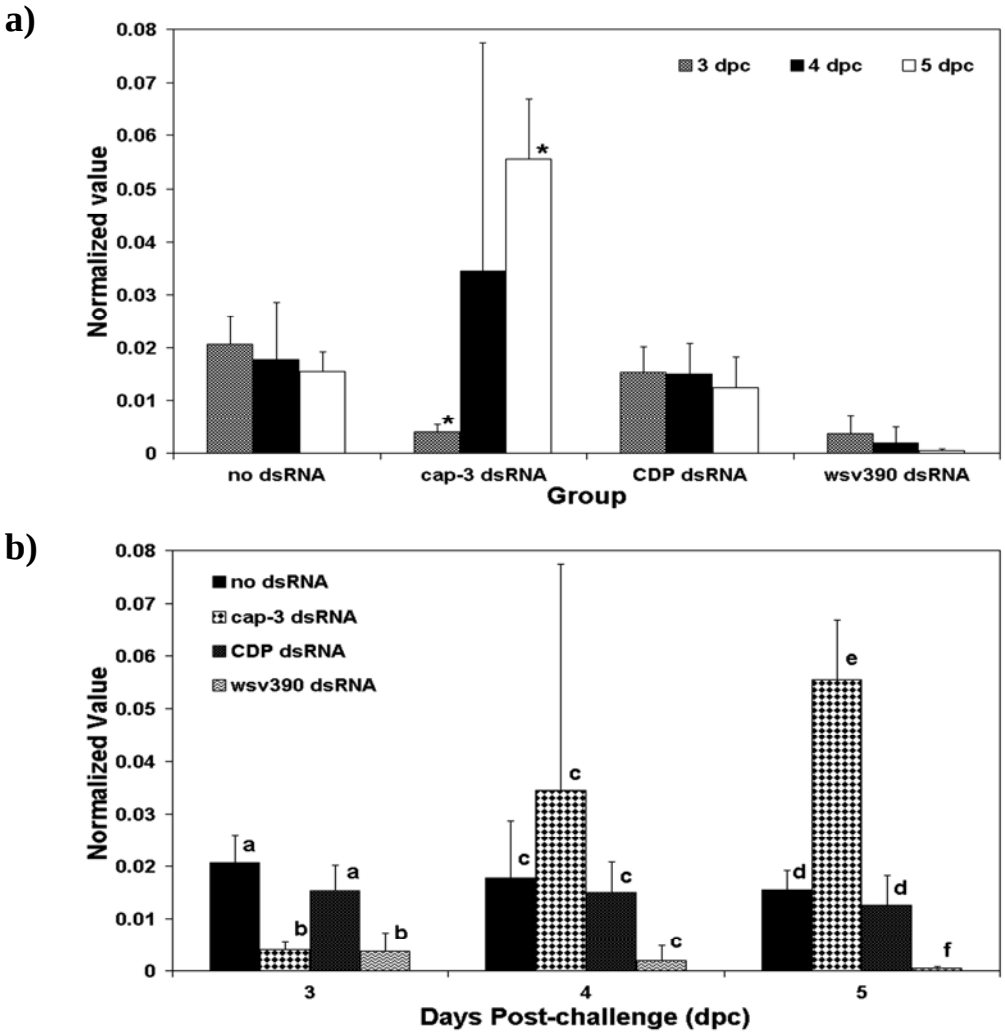

Figure 24. Normalized WSSV value of *P. vannamei* challenged with a high dose of WSSV. The normalized value was obtained by dividing averaged WSSV copy number with averaged β–actin copy number. (a) Only *cap-3* dsRNA group showed significantly different amount of viral load among 3, 4 and 5 dpc (*). (b) On 3 dpc, amount of virus in the viral control group was similar to the one in CDP dsRNA group while the one of *cap-3* dsRNA was similar to the one of wsv390 dsRNA group. The former two contained significantly greater amount of virus than the latter two. On 4 dpc, no significant difference of virus yields was observed among all groups. On 5 dpc, amount of virus of the viral control group was similar to the one of the CDP dsRNA group. However, the one of the *cap-3* dsRNA group was significantly greater than any other groups and the one of the wsv390 dsRNA group was significantly lower than any other groups. Same letter indicates no significant difference ($p>0.05$) while different letter indicates a significant difference ($p<0.05$).

group was not significantly different from that in CDP dsRNA treated group while that in the *cap-3* dsRNA group was not significantly different from that in the wsv390 dsRNA group ($p>0.05$). The former two were significantly higher than the latter two for at least 2.57 to 7.04 times. On 4 dpc, although no significant difference was observed ($p>0.05$), if *cap-3* dsRNA group was taken out of the analysis, the amount of virus of wsv390 dsRNA group would be significantly lesser than that of the viral infection control group for 9.39 ± 5.83 times and that of the CDP dsRNA group for 7.98 ± 3.07 times ($p<0.05$). On 5 dpc, the viral load of the WSSV infected control group and CDP dsRNA group was not significantly different ($p>0.05$) but both of them were significantly different from *cap-3* dsRNA and wsv390 dsRNA groups ($p<0.05$). Mean normalized WSSV value of the *cap-3* dsRNA group was 3.6 ± 0.74, 4.44 ± 0.91 and 121.4 ± 24.84 times higher than those of the viral control, CDP dsRNA and wsv390 dsRNA groups, respectively. The latter had 33.74 ± 8.05 and 27.32 ± 12.29 times lower mean normalized WSSV value than the no dsRNA and CDP dsRNA groups, respectively. As the amount of viral load in dsRNA treatment control and viral challenged control is not significantly different while the former had lesser mean time to death, this suggests that the non-specific innate immunity induced by administration of any dsRNA is not attributed merely by the inhibition of viral multiplication. This is, however, not a definite conclusion and still needs a confirmation from innate immune studies using low dose WSSV challenge system. The dramatically significant increase in viral replication by *cap-3* RNAi, compared to the virally-challenged control group, demonstrates that *cap-3* controls WSSV replication in *P. vannamei*. For wsv390 RNAi, the restriction of viral replication without prevention of shrimp death implies that wsv390 may be necessary for the replication of the virus but not for the severity of infection.

4.3.4 Detection of WSSV and apoptosis after high-dose WSSV challenge

To verify if *cap-3* silencing can inhibit apoptosis and wsv390 silencing can promote apoptosis upon WSSV infection, the level of apoptosis was examined by counting the number of apoptotic cells in gill tissues and hemocytes. Samples collected from 5 individual shrimp of each group on 3, 4 and 5 dpc were stained with

anti-WSSV envelope protein antibody and nuclear-counterstained with DAPI. The number of apoptotic cells was counted out of 3000 cells in gill tissues and out of 1000 cells in hemocytes.

In hemocytes (Table 11 and Fig. 25, 26), a significant rise ($p<0.05$) of apoptosis induced by WSSV challenge was first observed on 4 dpc that was roughly 5-10 fold higher than buffer-injected control shrimp and it increased to 13-26 fold on 5 dpc. The numbers of apoptotic hemocytes of the virally-challenged control group and of the dsRNA treatment control group were not significantly different between 3 and 4 dpc ($p>0.05$) but were significantly increased for 2.49 ± 0.83 and 2.53 ± 0.5 times, respectively, from 4 dpc to 5 dpc ($p<0.05$). The *cap-3* dsRNA group showed no significant difference in the number of apoptotic hemocytes from the WSSV control group on 3 dpc, from all other virus injected groups on 4 dpc, and from the virus control group and CDP dsRNA group on 5 dpc ($p>0.05$). It exhibited a significantly higher number of apoptotic hemocytes at a total of 10.35 ± 2.59 times from 3 to 5 dpc (4.93 ± 0.98 times from 3 to 4 dpc and 2.1 ± 0.52 times from 4 to 5 dpc) ($p<0.05$). The apoptotic level of hemocytes of wsv390 dsRNA-treated shrimp was significantly higher than that of any other groups on 3 dpc ($p<0.05$) but it was steady at this level until 5 dpc on which day it carried a significant lower number of apoptotic hemocytes than any other virus injected groups ($p<0.05$). The difference, however, was only approximately 1.5 fold. Altogether, these findings suggest that suppression of *cap-3* alone cannot inhibit apoptosis of hemocytes induced by high-dose WSSV challenge. In addition, its level of apoptosis seems to be correspondent with mortality and amount of virus implying that the ability of *cap-3* to restrict virus replication is apoptosis-independent. This is contrary to the virally-challenged control shrimp and dsRNA treatment control shrimp for which the rate of viral replication does not change with the progression of WSD while mortality and level of apoptosis emerge in a stepwise manner, suggesting that, in general, WSSV-induced apoptosis occurs in an amplification manner with a constant amount of viral triggering. Injections of a viral apoptosis inhibitor dsRNA (wsv390), which cannot protect the shrimp from WSD (in contrast to other viral gene targeting dsRNA treatments), induces a significant increase of apoptosis at first then limits it to that extent along the progression of infection, like-

Table 11. Number of apoptotic hemocytes of *P. vannamei* challenged with a high dose of WSSV (number of cells in 1000 total counted cells). At 3, 4 and 5 dpc, amount of apoptotic cells was counted in DAPI-stained hemocytes of 5 individual shrimp from each group.

	3 dpc	4 dpc	5 dpc
no dsRNA (WSSV)	42	72	445
	49	116	309
	48	188	208
	59	60	242
	49	141	230
Mean ± SD	49.4 ± 6.107	115.4 ± 52.152	286.8 ± 96.129
no dsRNA (no WSSV)	19	10	9
	20	18	13
	4	7	9
	11	8	9
	12	14	34
Mean ± SD	13.2 ± 6.535	11.4 ± 4.561	14.8 ± 10.872
***cap-3* dsRNA (WSSV)**	30	168	368
	24	166	262
	69	104	352
	15	164	439
	22	187	235
Mean ± SD	32 ± 21.366	157.8 ± 31.452	331.2 ± 82.841
***cap-3* dsRNA (no WSSV)**	12	19	18
	1	27	12
	14	11	15
	60	16	29
	18	21	30
Mean ± SD	11.25 ± 7.274	18.8 ± 5.933	20.8 ± 8.228
CDP dsRNA (WSSV)	43	110	340
	137	153	304
	85	92	338
	43	88	242
	97	127	216
Mean ± SD	81 ± 39.674	114 ± 26.768	288 ± 56.480
wsv390 dsRNA (WSSV)	166	112	180
	144	120	138
	106	112	156
	111	95	147
	188	99	194
Mean ± SD	143 ± 35.171	107.6 ± 10.310	163 ± 0.005702941

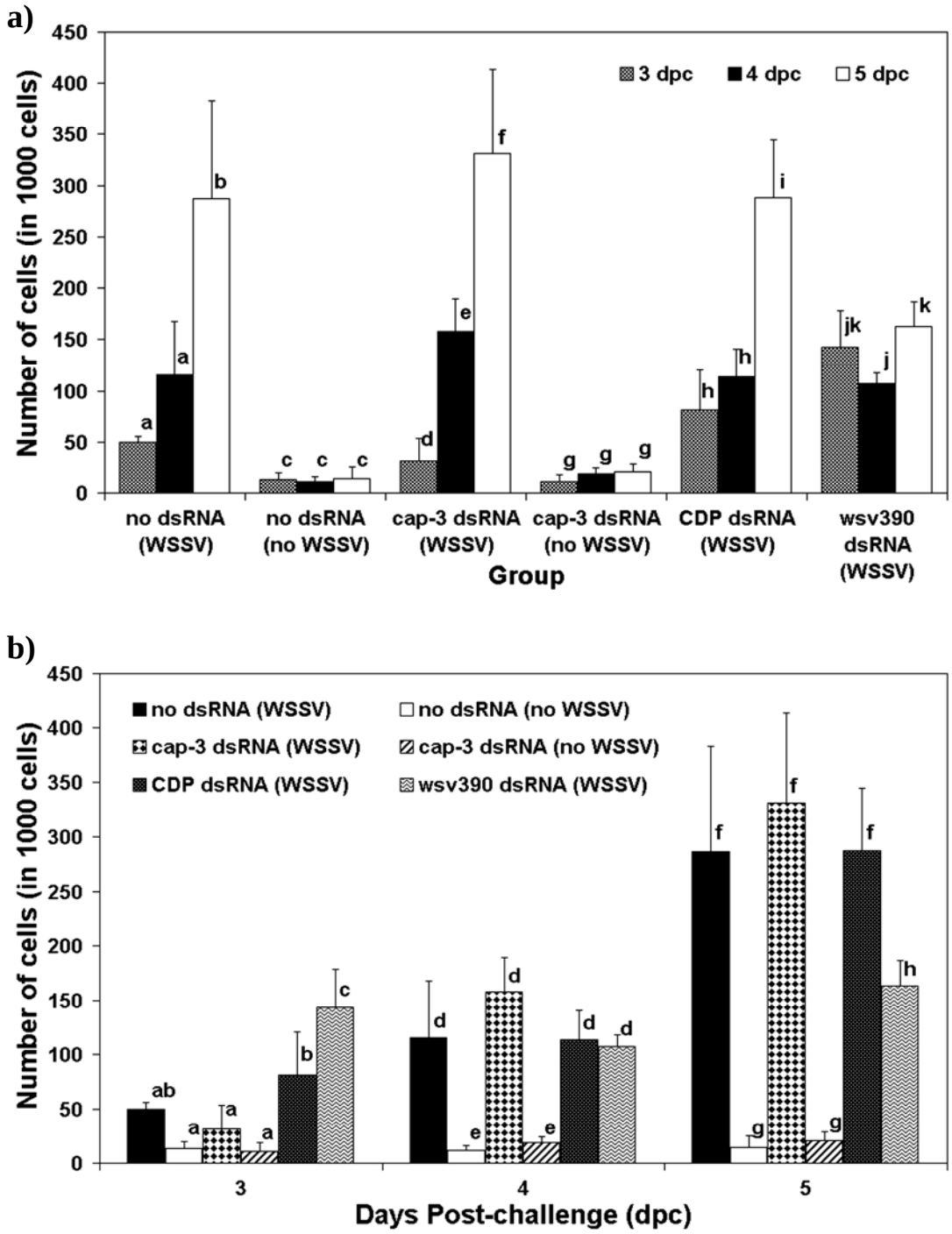

Figure 25. Number of apoptotic hemocytes of *P. vannamei* challenged with a high dose of WSSV (number of cells in 1000 total counted cells). Same letter indicates no significant difference ($p>0.05$) while different letter indicates a significant difference ($p<0.05$). (a) The viral control group, *cap-3* dsRNA group and CDP dsRNA group showed dramatically increasing number of apoptotic hemocytes from 3 to 5 dpc while

Figure 25 (cont.). only slight changes were observed in wsv390 dsRNA group. Both no dsRNA (no WSSV) group and *cap-3* dsRNA (no WSSV) group had few apoptotic hemocytes on all 3 days. (b) On 3 dpc, wsv390 dsRNA shrimp exhibited highest number of apoptotic hemocytes than any other groups. CDP dsRNA group had a significant difference of apoptotic hemocytes from the *cap-3* dsRNA group but not from the viral control group. Both the viral control group and *cap-3* dsRNA (WSSV) group had an indistinguishable number of apoptotic hemocytes from the non-challenged groups. On 4 dpc, all viral challenged groups displayed a similar number of apoptotic hemocytes. On 5 dpc, the number of apoptotic hemocytes was similar between viral control group, *cap-3* dsRNA (WSSV) group and the CDP dsRNA group. Only wsv390 dsRNA group had a significantly lower number of apoptotic hemocytes than the other viral challenged groups.

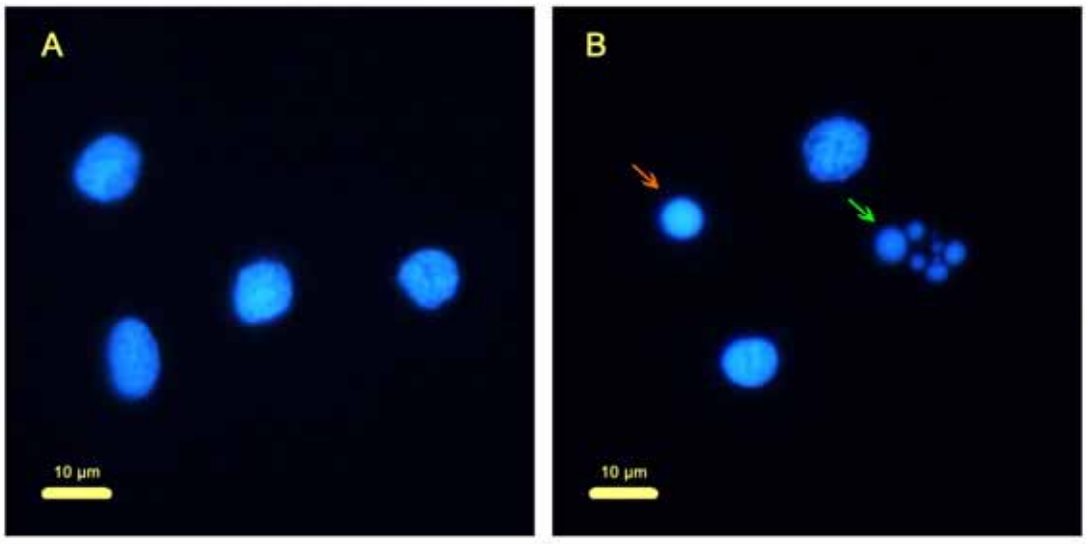

Figure 26. Hemocytes from non-challenged control shrimp (A) and apoptotic hemocytes from *cap-3* dsRNA treated (WSSV challenged) shrimp (B). Condensed nucleus (orange arrow) and fragmented nucleus (green arrow) are indicated.

wise with the constant amount of viral load. This significant increase of apoptosis at first confirms the function of wsv390 in inhibition of apoptosis *in vivo,* especially during the early stages of infection, and this is, as shown by the viral load data, for the virus itself to proliferate. However, the steadiness of the level of apoptosis implicates that wsv390 might somehow function in induction of apoptosis at late stages of infection and thus is involved in the amplification process of apoptosis as WSD progresses. Moreover, although *cap-3* suppression caused a marked enhancement and wsv390 repression caused a marked reduction of WSSV replication, they had virtually the same killing effect on the shrimp as dsRNA treatment control, implicating that shrimp mortality from WSD is not necessarily to be correlated with the viral load.

In gill tissues (Table 12 and Fig. 27, 28), the number of apoptotic cells ranged into the double digits in 3000 total counted cells only while the number of apoptotic hemocytes increased up to 400 out of 1000 total counted cells. A significant increase was observed from 3 dpc to 5 dpc in *cap-3* dsRNA (WSSV), CDP dsRNA and wsv390 dsRNA groups ($p<0.05$) but not in the viral injection control group ($p>0.05$). The number of apoptotic cells in gill tissues of the latter group was significantly higher than that of the wsv390 dsRNA group on 3 dpc and those of the other virally-challenged groups on 4 dpc ($p<0.05$) but, on 5 dpc, it was not significantly different from any other WSSV injected groups ($p>0.05$). *cap-3* dsRNA, CDP dsRNA and wsv390 dsRNA groups showed no significant difference of the number of apoptotic cells on each 3, 4 and 5 dpc ($p>0.05$). This data of apoptosis in gill tissues were not in the same tendency as the data of apoptosis of hemocytes, implying that gill tissue is not involved in the host defense response upon WSSV infection although it is one of the major target organs of WSSV.

Table 12. Number of apoptotic cells in gill tissues of *P. vannamei* challenged with a high dose of WSSV (number of cells in 3000 total counted cells). At 3, 4 and 5 dpc, amount of apoptotic cells was counted in DAPI-stained gill section of 4-5 individual shrimp from each group.

	3 dpc	4 dpc	5 dpc
no dsRNA (WSSV)	12	30	18
	23	16	21
	12	16	16
	15	33	23
	14	39	
Mean ± SD	15.2 ± 4.55	26.8 ± 10.38	19.5 ± 3.11
no dsRNA (no WSSV)	0	0	0
	0	2	0
	0	2	0
	0	1	0
	0	1	0
Mean ± SD	0	1.2 ± 0.84	0
***cap-3* dsRNA (WSSV)**	9	11	23
	13	8	23
	10	15	16
	8	13	18
	9	13	17
Mean ± SD	9.8 ± 1.92	12 ± 2.65	19.4 ± 3.36
***cap-3* dsRNA (no WSSV)**	1	0	1
	1	1	0
	0	1	0
	0	1	0
	2	0	2
Mean ± SD	0.8 ± 0.84	0.6 ± 0.55	0.6 ± 0.89
CDP dsRNA (WSSV)	10	7	31
	7	17	20
	10	23	30
	16	21	20
	19	18	20
Mean ± SD	12.4 ± 4.93	17.2 ± 6.18	24.2 ± 5.76
wsv390 dsRNA (WSSV)	11	18	22
	4	13	14
	8	14	25
	9	18	18
	8	18	19
Mean ± SD	8 ± 2.55	16.2 ± 2.49	19.6 ± 4.16

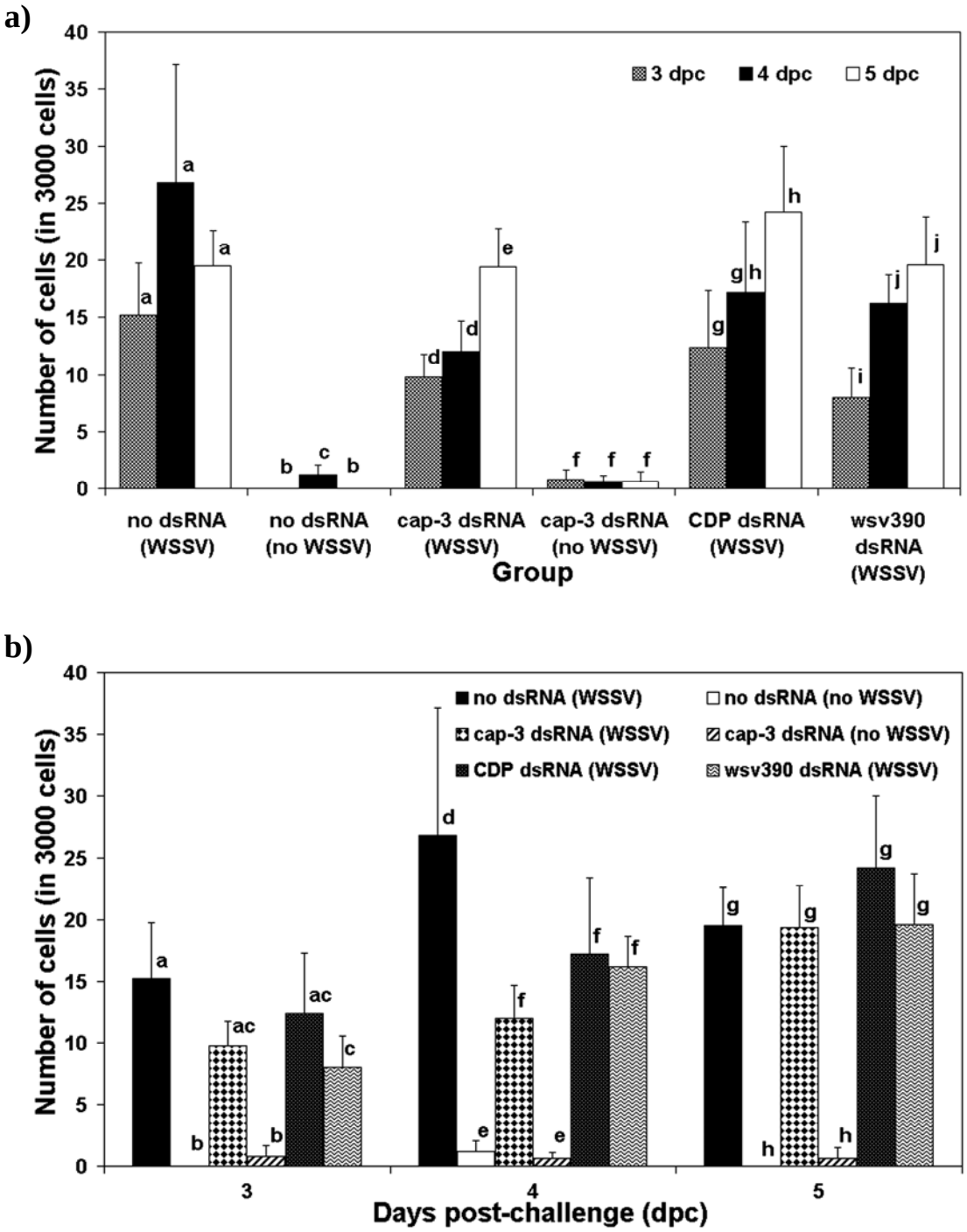

Figure 27. Number of apoptotic cells in gill tissues of *P. vannamei* challenged with a high dose of WSSV (number of cells in 3000 total counted cells). Same letter indicates no significant difference ($p>0.05$) while different letter indicates a significant difference ($p<0.05$). (a) No significant difference of number of apoptotic cells was observed in viral control group, no dsRNA (no WSSV) group, and *cap-3* dsRNA (no

Figure 27 (cont.). WSSV) group while *cap-3* dsRNA (WSSV) group, CDP dsRNA group, and wsv390 dsRNA group had an increase in number of apoptotic cells from 3 to 5 dpc. (b) On 3 dpc, among WSSV challenged shrimp, only the viral control group had a significant greater number of apoptotic cells than wsv390 dsRNA group. On 4 dpc, the number of apoptotic cells was similar among *cap-3* dsRNA (WSSV), CDP dsRNA and wsv390 dsRNA groups but all these three were significantly lower than the one in the viral control group. On 5 dpc, all of the viral challenged groups were similar in number of apoptotic cells.

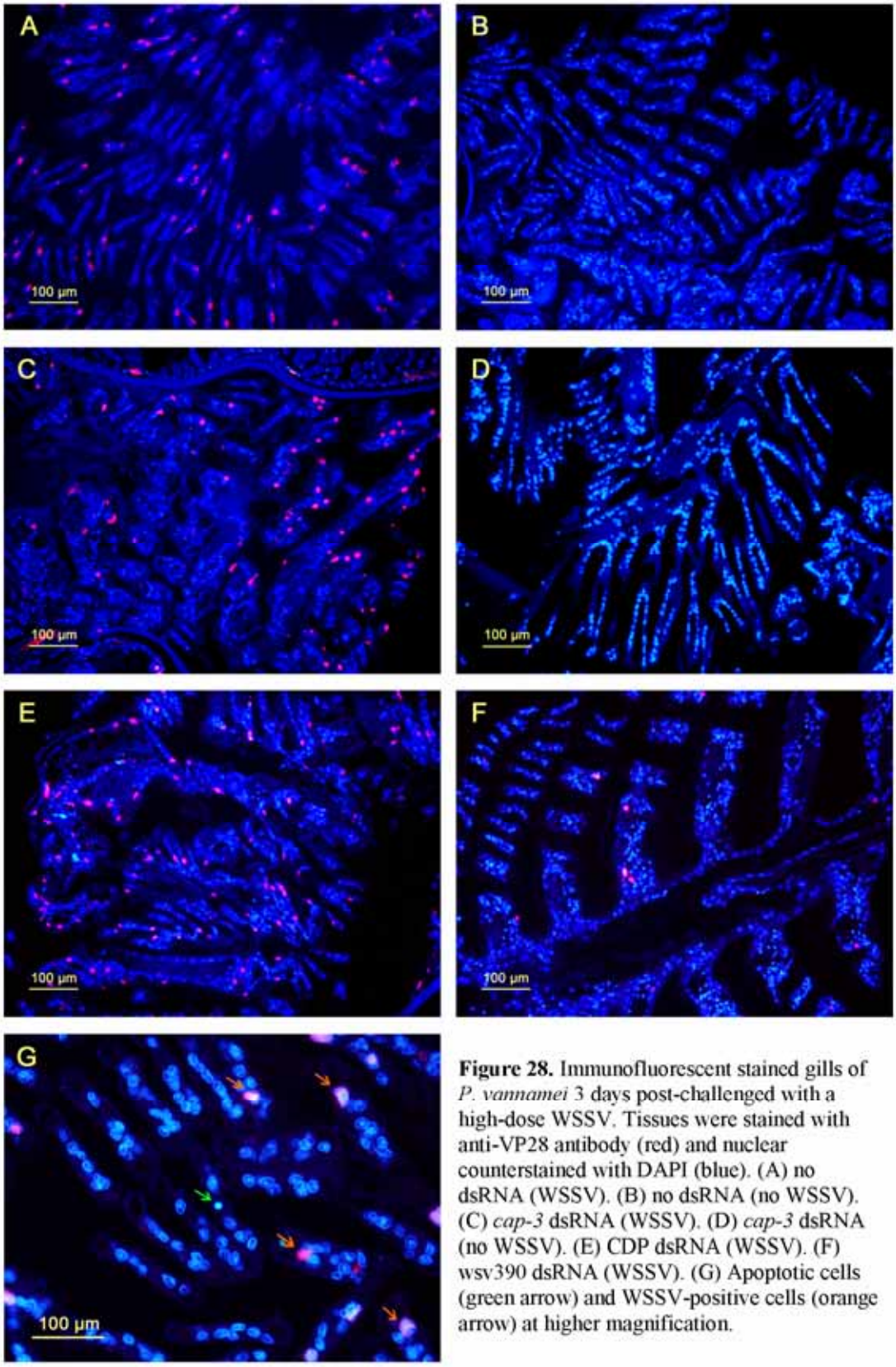

Figure 28. Immunofluorescent stained gills of *P. vannamei* 3 days post-challenged with a high-dose WSSV. Tissues were stained with anti-VP28 antibody (red) and nuclear counterstained with DAPI (blue). (A) no dsRNA (WSSV). (B) no dsRNA (no WSSV). (C) *cap-3* dsRNA (WSSV). (D) *cap-3* dsRNA (no WSSV). (E) CDP dsRNA (WSSV). (F) wsv390 dsRNA (WSSV). (G) Apoptotic cells (green arrow) and WSSV-positive cells (orange arrow) at higher magnification.

CHAPTER V
DISCUSSION

Due to the lack of specific immune responses, it has been proposed that invertebrates need a highly effective programmed cell death mechanism to control invading viruses (9). Nevertheless, two diametrically opposed hypotheses of the role of apoptosis in viral infections have been proposed: apoptosis as a host antiviral defense mechanism versus apoptosis as a pathogen-mediated mechanism to enhance viral replication, induce immune dysregulation, and promote persistent infection. Shrimp manifest apoptosis in response to WSSV infection although they also possess some limited, specific or 'quasi' immune response for protection by pre-exposure to either living organisms or vaccine-like reagents (94-97). Additionally, increasing levels of apoptosis have been shown to be correlated with increasing severity of infection and progression towards death (5). Studies of hemocytes from light and heavily infected shrimp in the first part of this dissertation also confirmed the situation by displaying a marked increase in number of apoptotic hemocytes as well as in number of WSSV containing hemocytes. At the molecular level, it has been reported that virally-challenged, moribund shrimp express significantly higher levels of caspase that is typically activated in the early stages of apoptosis (15), but lower levels of TCTP that is related to a mammalian anti-apoptosis factor (14). This corresponds to a report that WSSV-resistant shrimp showed a remarkable up-regulation of the TCTP gene (98) in haemocytes. Further, WSSV-resistant kuruma shrimp *P. japonicus* or 'immunized' shrimp (shrimp that have survived an initial WSSV challenge) display a significantly lower incidence of apoptosis than naïve shrimp, suggesting that apoptosis was not a major reason for their ability to survive a WSSV super-challenge (13, 95). The weight of the information seems to indicate that apoptosis is promoted in shrimp dying of a viral challenge but suppressed in survivors.

A counter view has been proposed to the effect that antiviral apoptosis is the reason for hyperthermal resistance of the Pacific white shrimp *P. vannamei* to WSSV (99). At high rearing temperatures (32-34°C), these shrimp survived WSSV infection significantly better than shrimp reared at lower temperatures (26-28°C) (70). This was also applied in giant tiger prawn *P. monodon*. However, the degree of apoptosis of the former group was significantly lower than the latter group and was similar to the unchallenged control group in *P. monodon*. Quantitation of WSSV by real-time PCR revealed that about half of the hyperthermally-reared shrimp were WSSV negative and that the amount of virus in surviving shrimp was significantly lower than that of the temperature control group. In *P. vannamei* and crayfish *Procambarus clarkii*, hyperthermia has also been reported to reduce viral load, suggesting either direct or indirect negative effects of high temperature on viral replication (100, 101). In addition, it was found that none of the apoptosis-related genes were differentially expressed in hyperthermia-induced WSSV resistant *P. vannamei* by suppression-subtractive hybridization assay but genes of the virus itself (102). Altogether, this implicates that hyperthermia protects shrimp from WSSV infection by inhibition of viral replication rather than antiviral immune mechanisms such as apoptosis. All of these findings suggest the possibility that virus-induced apoptosis in shrimp is pathogenic rather than preventive as stated in the previously proposed concept of 'viral accommodation' (26).

The first discovery of caspase gene in the white shrimp *P. merguiensis* (15) has paved the way for studies of mechanism of apoptosis upon viral infection at the molecular level. In this study, in order to investigate the roles of apoptosis upon WSSV infection in penaeid shrimp, a reverse-genetic technique (e.g. RNAi) has been applied on an apoptosis-inducing gene of shrimp, *cap-3*, and an anti-apoptosis factor of WSSV, wsv390, in Pacific white shrimp, *P. vannamei* following Robalino *et al.* (22, 24). *cap-3* full length sequence of *P. vannamei* was identified and long dsRNA specific to *cap-3* and wsv390 were made accordingly. These dsRNAs were used to silence their specific gene target *in vivo* under high- and low-dose of WSSV challenge and thus revealing possible biological significances of apoptosis in WSSV infected shrimp.

In the high dose WSSV challenge system, intact functions of *cap-3* were revealed. According to Robalino *et al.* (22, 24), at high doses of viral challenge, the effect of innate immunity is overwhelmed by the amount of virus thus unwinding an approach to study the gene function in response to viral infection. In contrast to partial protection in the low dose experiment, *cap-3* RNAi cannot prevent shrimp mortality under high doses of WSSV challenge. This suggests that effect of *cap-3* gene on shrimp defense against WSSV infection is visible only if the viral dose is below the silencing event's threshold of effectiveness. Although prevention of mortality is absent in this situation, similarity in the level of apoptosis in hemocytes to the viral control shrimp and dsRNA treatment control shrimp versus an enhancement of WSSV yield implicates the capability of apoptosis-independent viral replication control of the *cap-3* gene (Fig. 29). Similar cases are reports on restriction of HIV, adenovirus, Semliki Forest virus (SFV) and vaccinia virus (VV) replications by inhibition of apoptosis. z-VAD-fmk, a pro-apoptotic interleukin-1-converting enzyme (ICE)-like protease inhibitor, and E1B 19K protein, a potent apoptosis inhibitor of adenovirus, blocked apoptosis of CD4+ T cell induced by HIV resulted in significantly enhanced virus production and established persistent infection (103, 104). z-VAD-fmk inhibited apoptosis in adenovirus infection significantly increased infectious virus yield and attenuated virus release (105). Ability of *ced-3*, *ced-4*, *ced-9* and *egl-1* on controlling VV replication in *Caenorhabditis elegans* has also been shown. Strong loss-of-function mutation (*ced-3*, *ced-4* and *egl-1*) and gain-of-function mutation (*ced-9*) of these programmed cell death (PCD) genes blocked most, if not all, apoptosis during *C. elegans* development resulting in an increase of VV load. This, furthermore, has been shown to be unlikely to be caused by the extra live cells (106). The finding of which is most consistent with *cap-3* RNAi is in the study of action of bcl-2, an antiapoptotic proto-oncogene, on SFV infected rat prostatic adenocarcinoma cell line AT3 (107). AT3 overexpressing Bcl-2 (AT3Bcl-2) cells slowed down SFV A7 production but allowed steadily increased virus yield and a continual decrease in living cells with time. At 24 hpc, AT3Bcl-2 gave a similar number of living cells to that of the AT3Neo control culture but a thousand-fold lower viral load. While cell death was continually occurring, extracellular virus continued to accumulate in the supernatant of infected AT3Bcl-2 cells, reaching levels similar to those in infected AT3Neo cells by 48 hpc

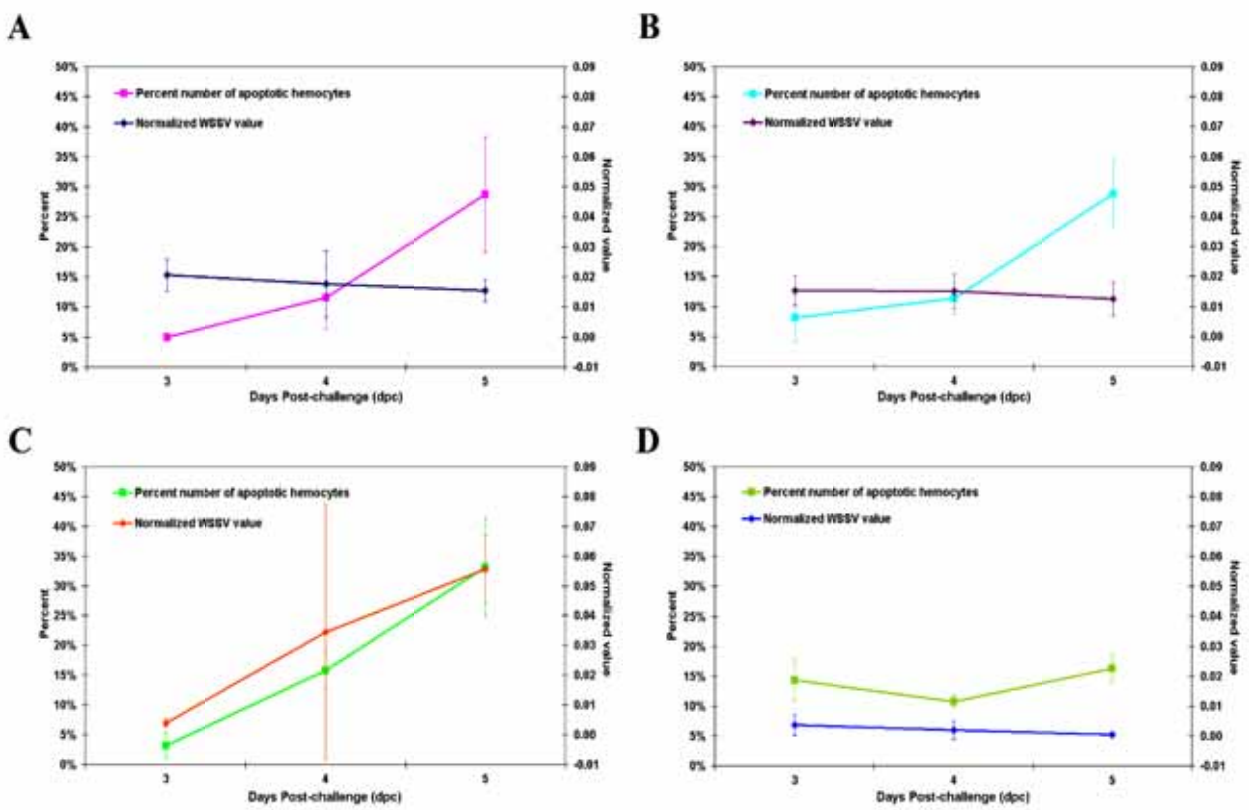

Figure 29. Correlations between percent number of apoptotic hemocytes and the viral load of *P. vannamei* challenged with a high dose of WSSV. Shrimp were treated with saline (A), CDP dsRNA (B), *cap-3* dsRNA (C) or wsv390 dsRNA (D) 2 days prior to a high-dose WSSV challenge. Hemocytes and pleopods were collected on 3, 4 and 5 dpc. Hemocytes were stained with DAPI and percent number of apoptotic cells was calculated. The viral load was measured in pleopod DNA using real-time SYBR Green PCR.

and exceeding this level by 72 hpc. Altogether, these findings prove the capability of PCD-genes to limit viral replication as an evolutionarily conserved host defense mechanism against virus infection. Nevertheless, potent inhibition of apoptosis has been established in all these studies whereas *cap-3* RNAi, in high-dose WSSV infection, was shown to be unable to inhibit apoptosis. This suggests that shrimp *cap-3* alone is enough to control WSSV replication, although the mechanism is not yet known, and that apoptosis is not necessary for this viral inhibition capability of *cap-3*. The much lower extent of apoptosis in gill tissues compared to that in hemocytes supports this suggestion.

Next on the list is the level of *cap-3* expression when *cap-3* RNAi was shown to be ineffective in inhibiting apoptosis but still affecting virus production. The unchanged expression of *cap-3* in the hepatopancreas of shrimp at 3, 4 and 5 dpc implies no changes in expression level of apoptosis gene in the hepatopancreas of all groups as disease progressed. Studies of tissue tropism of WSSV infected shrimp by *in situ* hybridization (48) and PCR (45, 49) have revealed that the hepatopancreas is only lightly infected along the course of WSD and its organ integrity is maintained up to the late stage of infection. Besides, there has never been report on evidence of apoptosis in shrimp hepatopancreas beforehand (4, 5). This indicates that the hepatopancreas is not one of the main targets of WSSV. However, it might be that the level of expression of apoptosis genes is altered earlier in infection or as generally known, caspase function is driven by the activation of pro-caspase in the cytoplasm rather than an upregulation of gene expression.

Results from high dose challenge have sparked interest in seeing if *cap-3* RNAi would augment protection over the non-specific immune response induced by arbitrary dsRNA. The focuses were thus sharpened on the low dose experiment. In a low dose WSSV challenge system, significant protection against mortality by *cap-3* RNAi suggests that virally-triggered apoptosis may affect shrimp negatively and cause more mortality to WSSV infected shrimp. As the mechanism behind this is not yet known and evidence of occurrence of apoptosis together with the viral load data in the low dose experiment is still lacking, the conclusion is presently uncertain. If *cap-3* silencing proceeds by similar mechanisms in both low-dose and high-dose challenges, it may be inferred that suppression of *cap-3* expression alone is not sufficient to inhibit

apoptosis; death of shrimp from WSD is not due to apoptosis but is correlated with the function of *cap-3* gene; and *cap-3* limits viral replication. Conversely, if apoptosis can be inhibited by *cap-3* RNAi under low dose WSSV challenge, this will indicate that the assumption made above is correct and it will strongly support the earlier proposal that apoptosis is the cause of mortality upon viral infection (26). Nonetheless, even if the latter case is revealed, questions remain. If apoptosis is detrimental to the virally-infected shrimp, why was inhibition of apoptosis by *cap-3* dsRNA unable to protect all of the shrimp? It is possible (1) that *cap-3* was incompletely knocked down or (2) that there are other caspases functioning in the shrimp apoptotic pathway and their action can compensate for loss of *cap-3* (e.g. caspase 7), (3) that even if caspases are inhibited, mitochondrial outer membrane permeabilization is sufficient to kill the cells, (4) that necrosis might occur in addition to apoptosis at the culmination of a lytic infection, (5) that the process of viral genome replication itself, even at the basal levels, is cytotoxic enough to kill the infected host, (6) or that there are other unknown factors contributing to pathogenicity of the virus apart from apoptosis.

Studies of caspase 3, 7 and double-knockout mice have demonstrated that caspase 3 and 7 have some overlapping but also some distinct roles in apoptosis. Caspase 3 controls DNA fragmentation and morphological changes of apoptosis while caspase 7 appears to be more important to the loss of cell viability. Both, however, are critical for mitochondrially-mediated apoptosis as they control the loss of mitochondrial transmembrane potential and apoptosis inducing factor (AIF) release. They may also serve for the promotion of further cytochrome *c* release to amplify the initial death signal (18). Nonetheless, the apoptotic programme is much more than just caspases, and in many cell types, activation of the apoptotic programme inevitably confers death, both through caspase-dependent and caspase-independent pathways, but always with a necrotic morphotype (17, 108). Additionally, considering that there are overlap and crosstalk between the pathways for apoptosis and necrosis (6), massive necrosis might take place during the transformation of infection into the lytic stage and bring about the failure of organs and eventual death. Cytotoxicity in the course of viral genome replication is another possible cause of death which has been suggested in VV infected *C. elegans* instead of virus-triggered apoptosis (106). Finally, the situation is complicated by the fact that environmental conditions (e.g., pH of the water and the

level of nitrate/nitrite) and physiological conditions of the shrimp (e.g., molting stage) also have important impacts on the outcome (i.e., death or survival) of a viral infection.

Comparable to the gene silencing by RNAi, viral functions can also be silenced by dsRNA molecules specific to the viral genes. This mechanism is considered to be an evolutionarily conserved defense mechanism against viruses and foreign nucleic acids and is present in every eukaryote. Inhibition of viruses by RNAi has been described in most if not all RNA and DNA viruses (109). Mechanism of inhibition in DNA viruses is due to silencing of viral functions through viral mRNA cleavage, similarly to the activity of RNAi on cellular functions whereas in RNA viruses, RNAi is also able to cleave the viral genome (74). Recently, RNAi-directed specific antiviral immune response against WSSV and YHV has also been discovered in shrimp both *in vitro* and *in vivo* (21, 22, 90). Administration of dsRNA specific to WSSV envelope protein and YHV non-structural protein efficiently helped protect shrimp from mortality upon infection by inhibition of viral multiplication. While WSSV envelope protein RNAi (e.g. VP28, VP19) effectively prevents shrimp mortality for up to 100%, YHV structural protein RNAi has been shown to be less potent than non-structural protein RNAi.

Although wsv390 has been shown to play an anti-apoptosis role *in vitro* (63), its knockdown under low dose WSSV challenge did not result in significantly better protection than dsRNA control treatment. Under high dose, there was no dissimilarity in mortality with the viral control group, suggesting that virulence of WSSV is independent of wsv390 function. The mutant of baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (lacking a functional anti-apoptotic p35 gene) induced apoptotic programmed cell death in insect cell line *Spodoptera frugiperda* 21 (SF-21), leading to a significant decrease in viral progeny production compared with the wild-type virus. *In vivo* analysis revealed that this p35 mutant required approximately 1,000-fold higher amount of budded virus than wild-type virus to yield 50% lethality in *S. frugiperda* larvae (110). By contrast, wsv390 silenced WSSV exhibited virulence indistinguishable from that of the control groups, even though wsv390 is necessary for viral replication. wsv390 RNAi reduced viral yields upon high dose WSSV challenge. Nonetheless, according to the level of apoptosis in gills,

this was not directly due to death of early infected cells. In gill tissues that displayed a much lower incidence of apoptosis than hemocytes, WSSV detection by anti-VP28 antibody revealed small numbers of positive cells that correlated with the real-time PCR quantitation results, indicating that different tissues respond differently to WSSV. Apoptosis in both the viral control group and the dsRNA control group increased with time post-challenge whereas wsv390 RNAi caused a significant boost in the beginning but were absent of the time course amplification manner afterwards (Fig. 29). This incidence suggests for the first time the dual functionalities of wsv390 in WSSV infected shrimp: as an apoptotic inhibitor in the early stage and an apoptotic inducer at late stages of infection. Similarities have been shown previously in HIV trans-activating protein Tat and vpr protein, large T antigen of simian virus 40 (SV40), pX protein of hepatitis B virus (HBV), and 17-kD nonstructural (NS) protein of infectious bursal disease virus (IBDV). Both Tat and vpr have been reported to exert two distinctly opposite influences on apoptotic mechanism in HIV infection (111, 112). Studies indicated that Tat induces apoptosis involving CD95 (APO-1/Fas) ligand, TNF-α, NF-κB, caspase, CD178, and bcl-2 and bax (113-118). Converse reports have shown that protein Tat is able to increase resistance of Jurkat T cell to different apoptotic stimuli including acute HIV-1 infection (119) and that its anti-apoptosis activity is correlated with an upregulation of bcl-2 expression (120, 121). Concentration of extracellular Tat plays part in determining its action on apoptosis: high concentration (nM-μM) induces apoptosis and low concentration (pM) inhibits apoptosis in both Jurkat cells and primary peripheral blood mononuclear cells (121). Additionally, a study of McCloskey *et al.* (111) explained that addition of exogenous Tat induced apoptosis in HIV-uninfected T cells whereas endogenously expressed Tat in Tat-transfected cells provided a protection from activation-induced apoptosis. Exogenously expressed Tat, however, has also been demonstrated to inhibit the TNF-related apoptosis-inducing ligand (TRAIL)-mediated death pathway (122). Likewise is vpr protein which displays anti-apoptotic activity at early time post-infection (24-48 hrs) and promotes spontaneous apoptosis especially of the productively HIV-infected cells later (8 days onwards) (112, 123-126). The large T antigen of SV40 and pX protein of HBV regulates host apoptosis by acting as modulators of the cell cycle, which are comprised almost equally of apoptotic inhibitors and inducers (61). By

means of binding to the retinoblastoma tumor suppressor protein (pRb), large T antigen can suppress caspase-1-induced apoptosis in a p53-dependent pathway (127) or promote apoptosis by increasing the transcriptional activity of E2F which will then trigger p73 expression (128, 129). For pX protein of HBV, its controlled expression regulates p53-dependent apoptosis: an increase in production of pX induces apoptosis while reciprocally a decline inhibits apoptosis (130-132). Upon IBDV infection, the accumulation of NS protein within the host plasma membrane induces cell lysis and apoptosis (133, 134) however NS knockout mutant virus by reverse genetics revealed that NS has an anti-apoptotic function at the early stage of infection (135). These findings facilitate in the speculation of possible mechanisms of function shifting of wsv390 from apoptosis prevention in the early stage to apoptosis activation in the late stage of WSSV infection that might be involving the concentration or level of expression of wsv390 along WSD, cell cycle arrest, and the source of wsv390 protein (extracellular or intracellular). In a study concerning an adenovirus vector that proliferates in human cancer cell lines and sensitizes them to TNF-α-mediated apoptosis, it was found that while apoptosis during viral DNA replication compromised virus production, apoptosis after virion assembly enabled its release from infected cells and dissemination (136). Viral particles were also detected within the apoptotic bodies or associated with them, allowing phagocytosis by neighboring cells as a potential means for viral spread. This later induction of apoptosis also supported the spreading of virus *in vivo* in subcutaneous tumors or liver metasteses. Collectively, all these findings support the idea that the same viral protein can cause opposite effects on apoptosis regulation. This represents a useful strategy developed by the virus to manipulate the turnover of host cells for its own advantage, thus ensuring viral survival, replication and spread.

In summary, apoptosis can have two contradictory roles in the pathogenicity of viral infection – suppression or enhancement – depending on the situation. Generally, virally-induced apoptosis suppresses pathogenicity by taking a principal role in controlling viral replication in the early stage of infection, limiting inflammatory reactions at the site of infection, and inducing specific immunity. By contrast, apoptosis enhances HIV-1 pathogenicity when massive cell death is significantly augmented in indispensable organs, signaling the onset of disease (6, 7, 9). This might

be manipulated by the viral products that can induce apoptosis at late infection stages for the sake of dissemination of the virus itself. Although this thesis study adds to the accumulating molecular knowledge related to death from viral infections in shrimp, the complexity of interacting molecular pathways and environmental and physiological factors outlined above suggests that much more work is needed to determine the precise role of apoptosis in viral pathogenesis.

CHAPTER VI
CONCLUSION

1. In general, apoptosis triggered upon WSSV infection in viral target tissues of shrimp increases steadily as WSD progresses however the amount of virus production only increases to a limited number and the rate of replication is kept at that level as WSD progresses.
2. The level of occurrence of apoptosis is diverse among tissues. Hemocytes display higher apoptotic response than gills. The hepatopancreas is not a good site for apoptosis observation upon WSSV infection.
3. Hyperthermia protects shrimp from WSD by inhibition of WSSV replication and this is not related to apoptosis.
4. Severity of WSD is not determined by the amount of virus.
5. *P. vannamei* also possesses a caspase gene which is 82% identical to the one of *P. merguiensis* although its action in the apoptosis pathway is not yet known.
6. Upon WSSV infection in shrimp, *cap-3* controls virus replication in an apoptosis-independent manner.
7. *cap-3* silencing alone cannot inhibit apoptosis at high doses of WSSV challenge.
8. Functions of *cap-3* affect shrimp negatively and probably intensify severity of WSD.
9. wsv390 is necessary for WSSV replication but not for virulence of infection.
10. wsv390 of WSSV has dual functionalities in regulating host apoptosis: inhibiting apoptosis in early stages of infection and inducing apoptosis at late stages of infection.

CHAPTER VII
RECOMMENDATION

As partial protection from *cap-3* RNAi has been demonstrated when shrimp were challenged with low doses of WSSV, it will be very valuable and worthwhile to look into the molecular level of what happens here. In addition, more control group should be added in this case (e.g. non-specific dsRNA such as duck Igυ dsRNA) to confirm the result. The study of other caspases and apoptosis-related genes of shrimp will allow better inhibition of apoptosis and more information might be obtained from this. Furthermore, *in vitro* RNAi studies are required to evaluate the molecular interaction of *cap-3* on the limitation of virus replication and of wsv390 on controlling host apoptosis. All these will lead to a better understanding of the role of apoptosis upon WSSV infection in penaeid shrimp and a deeper understanding of host-virus interaction in marine invertebrates.

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BIOGRAPHY

NAME	Ms. Anchukorn Rijiravanich
DATE OF BIRTH	29 September 1979
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTION ATTENDED	Mahidol University, 1997-2000: Bachelor of Science (Medical Science) Mahidol University, 2001-2007: Doctor of Philosophy (Anatomy)
SCHOLARSHIPS	Medical Scholars Program, Mahidol University (2000-2001) Royal Golden Jubilee (RGJ), Thailand Research Fund (TRF) (2001-2007)
HOME ADDRESS	2100/71 Nrathivasrajnakrin 22, Chongnonsri, Yannawa, Bangkok 10120, Thailand